

Nottingham Trent University

The Gut Microbiota in the Pathophysiology of
Hyperpnoea-Induced Bronchoconstriction:
Investigating the Effects of Short and Long-Term
Prebiotic Supplementation.

By

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Abstract

In adults with allergic asthma, clinical and inflammatory parameters have been associated with features of gut microbial dysbiosis. This has led researchers to investigate whether the severity of asthma can be attenuated through gut-microbiota mediated nutritional interventions, such as prebiotics. Strikingly, following 3-weeks of supplementation with a galactooligosaccharide-based prebiotic, clinically relevant, participant perceivable improvements have been observed in adults with moderate-severe exercise-induced bronchoconstriction. However, given that gut bacterial composition has not been assessed in adults with EIB, it remains to be determined whether prebiotics attenuate the severity of EIB via the gut microbiota. Furthermore, the minimum, clinically important dose required to attenuate the severity of EIB remains to be determined. To address these questions, the current thesis investigated whether adults with EIB displayed features of gut microbial dysbiosis, and whether short-term and long-term supplementation with a low dose galactooligosaccharide-based prebiotic attenuated the severity of EIB.

The first experimental chapter (Chapter IV) investigated whether adults with moderate EIB displayed features of gut microbial dysbiosis through fluorescent *in situ* hybridisation (FISH). A total of seven ($n = 7$) adults with EIB, and seven ($n = 7$) controls took part. Participants provided a faecal sample to assess gut bacterial composition, a blood sample to assess markers of systemic inflammation, and completed the EVH protocol to determine the presence and severity of EIB. Despite significant increases in the percentage decrease in FEV₁ post-EVH (median, IQR: 38, [26-41] vs. 6, [4-7%]; $p = 0.002$), and resting blood eosinophils (8 ± 2 vs. $4 \pm 2\%$; $p = 0.008$), no features of gut microbial dysbiosis were reported in adults with moderate EIB across any bacterial groups when compared to controls ($p > 0.05$). It is possible the current method used to profile the gut microbiota lacked the required sensitivity to detect features of gut microbial dysbiosis. In contrast to the current method, previous research has used metagenomics-based approaches to profile the gut microbiota of adults with allergic asthma to the species and strain levels, identifying features of gut microbial dysbiosis. This study highlights the importance of using techniques capable of profiling the gut microbiota to the appropriate phylogenetic depth.

The second experimental chapter (Chapter V) investigated whether short-term (acute) supplementation with a single low dose galactooligosaccharide-based prebiotic (3.1g; HOST-DM059), attenuated the severity of EIB and markers of systemic inflammation. A total of eight ($n = 8$) adults with mild EIB took part. Following a double-blind, placebo-controlled, crossover design, participants completed two experimental trials. During each experimental trial, participants underwent baseline assessments of pulmonary function, and provided a blood sample to assess markers of systemic inflammation. A single dose of prebiotic or placebo was then administered prior to a 4-hour ingestion period. Following the ingestion period, participants repeated assessments of pulmonary function and provided a second blood sample before completing the EVH protocol. A third blood sample was obtained one-hour after the EVH protocol. When compared to placebo, short-term supplementation with a low dose galactooligosaccharide-based prebiotic did not attenuate the severity of EIB (21 ± 7 vs. $18 \pm 7\%$ decrease in FEV₁ post-EVH; $p = 0.092$), or markers of systemic inflammation (2.1 ± 1.3 vs. $1.8 \pm 0.7\%$ blood eosinophils measured one-hour post EVH; $p > 0.05$).

The third experimental chapter (Chapter VI) investigated whether long-term supplementation with a low dose galactooligosaccharide-based prebiotic attenuated the severity of EIB (4-weeks, $3.1\text{g}/\text{day}^{-1}$; HOST-DM059). A total of nine ($n = 9$) adults with EIB, and eight ($n = 8$) controls took part. Following a double-blind, placebo-controlled, crossover design, participants completed a 10-week nutritional intervention, consisting of two, 4-week supplementation phases each separated by a 2-week washout period. During each experimental trial, participants provided a resting blood sample to assess markers of systemic inflammation and completed the EVH protocol. In the EIB group, long-term supplementation with a low dose galactooligosaccharide-based prebiotic did not attenuate the severity of EIB (22 ± 12 vs. $22 \pm 10\%$ decrease in FEV₁ post-EVH; $p > 0.05$), or markers of systemic inflammation (3 ± 2 vs. $2 \pm 1\%$ resting blood eosinophils; $p > 0.05$). Furthermore, no changes were observed in the proportion of regulatory T cell subsets in adults with EIB following prebiotic supplementation, including Type-1 IL-10 producing T_{REG} cells (1.5 vs. 1.4% of CD3⁺ CD4⁺ T Cells; $p > 0.05$), or naturally derived CD25⁺ FoxP3⁺ T_{REG} cells (6.5 vs. 6.4% of CD3⁺ CD4⁺ T Cells; $p > 0.05$).

The current thesis provides novel contributions by suggesting that a minimum threshold may exist for prebiotic supplementation to induce clinically relevant, participant perceivable improvements in the severity of EIB. Future research should investigate the effects of baseline gut bacterial composition, EIB severity, and habitual dietary fibre intake on the responsiveness to prebiotic supplementation.

Key Words: Gut Microbiota, Asthma, Exercise/Hyperpnoea-Induced Bronchoconstriction, Prebiotic Supplementation, Galactooligosaccharides, Pulmonary Function, Eosinophils, Regulatory T Cells, T-Helper Type-2 (T_H2) Cells

List of Abbreviations

EIB: Exercise-Induced Bronchoconstriction

HIB: Hyperpnoea-Induced Bronchoconstriction

EVH: Eucapnic Voluntary Hyperpnoea

FEV₁: Forced Expiratory Volume in One Second

FVC: Forced Vital Capacity

PEF: Peak Expiratory Flow Rate

FEF_{25%-75%}: Mean Forced Expiratory Flow Between 25% and 75% of FVC

MVV: Maximal Voluntary Ventilation

SET: Sport or Environment Specific Whole-Body Exercise Challenge Test

GOS: Galactooligosaccharide

B-GOS[®]: Bimuno[®] Galactooligosaccharide

FOS: Fructooligosaccharide

T_{REG}: Regulatory T Cells

iT_{REG}: Induced Regulatory T Cells

nT_{REG}: Natural Regulatory T Cells

iT_{R1}: Type-1 IL-10 Producing Regulatory T Cells

FoxP3: Fork-Head Box Protein 3

T_{H2}: T-Helper Type-2 Cells

cT_{H2}: Conventional T-Helper Type-2 Cells

peT_{H2}: Pathogenic Effector T-Helper Type-2 Cells

pT_{H2}MEM: Pathogenic T-Helper Type-2 Memory Cells

GATA-3: Trans-Acting T-Cell Specific Transcription Factor 3

T_{H1}: T-Helper Type-1 Cells

T-bet: T-Box Transcription Factor 21

T_H17: T-Helper Type-17 Cells

ROR γ t: Retinoic Acid-Related Orphan Receptor Gamma T

PGD₂: Prostaglandin D₂

hPGDS: Haematopoietic Prostaglandin D Synthase

CRTH2: Chemoattractant Receptor-Homologous Molecule Expressed on T_H2 Cells

CTLA-4: Cytotoxic T-Lymphocyte Antigen-4

iEOS: Inflammatory Eosinophils

rEOS: Lung-Resident Eosinophils

COX-2: Cyclooxygenase-2

TGF- β 1: Transforming Growth Factor Beta-1

TGFR1: Transforming Growth Factor Beta Receptor I

IL: Interleukin

TSLP: Thymic Stromal Lymphopoietin

NKA: Neurokinin-A

EGF: Epithelial Growth Factor

ZO: Zonula Occludens

MUC5AC: Mucin 5AC

MC_{T/CPA3}: TPSAB1⁺ CPA3⁺ CMA1⁻ Mast Cells

TPSAB1: Tryptase

CPA3: Carboxypeptidase A3

CMA1: Chymase 1

DC: Dendritic Cell

pDC: Plasmacytoid Dendritic Cell

cDC: Conventional Dendritic Cell

CCL: C-C Motif Chemokine Ligand

CCR: C-C Motif Chemokine Receptor

CXCR: C-X-C Motif Chemokine Receptor

NOS: Nitric Oxide

iNOS: Inducible Nitric Oxide Synthase

IFN- α : Interferon Alpha

IFN- γ : Interferon Gamma

TLR: Toll-Like Receptor

IgE: Immunoglobulin E

GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor

HDAC: Histone Deacetylase

HAT: Histone Acetyltransferase

sPLA₂-X: Secreted Phospholipase A₂ Group X

MAPK: Mitogen Activated Protein Kinase

TNF- α : Tumor Necrosis Factor Alpha

ILC: Innate Lymphoid Cell

SIRT: Silent Information Regulator Genes

CD: Cluster of Differentiation

cAMP: Cyclic Adenosine 5'-Monophosphate

GPR: G-Protein Coupled Receptor

NRP-1: Neuropilin-1

GITR: Glucocorticoid-Induced Tumor Necrosis Factor Receptor

OVA: Ovalbumin

HDM: House Dust Mite

SCIT: Subcutaneous Immunotherapy

TSA: Trichostatin-A

STAT6: Signal Transducer and Activator of Transcription 6

NF- κ B: Nuclear Factor Kappa B

MHC-II: Major Histocompatibility Complex II

EGID: Eosinophilic Gastrointestinal Disease

CMBC: Cord-Blood Derived Mononuclear Cells

PBMC: Peripheral Blood Mononuclear Cells

GBD: Global Burden of Disease

DALY: Disability Adjusted Life Years

NHS: National Health Service

U.K.: United Kingdom

IOC-MC: International Olympic Committee Medical Commission

JTFPP: Joint Task Force on Practice Parameters in Exercise-Induced Bronchoconstriction

E. coli: Escherichia coli

COPSAC₂₀₀₀: Copenhagen Prospective Studies on Asthma in Childhood 2000 Birth Cohort

COPSAC₂₀₁₀: Copenhagen Prospective Studies on Asthma in Childhood 2010 Birth Cohort

RSV: Respiratory Syncytial Virus

MIP-2 α : Macrophage Inflammatory Protein 2 Alpha

HDC: Histidine Decarboxylase

ICOS: Inducible T Cell Costimulator

mMCP-1: Mucosal Mast Cell Protease-1

CFU: Colony Forming Units

USFDA: United States Food and Drug Administration

ACQ-7[©]: The Asthma Control Questionnaire-7

ACT[®]: Asthma Control Test

AQLQ[®]: Asthma Quality of Life Questionnaire

BASES: The British Association of Sport and Exercise Sciences

CCG: Clinical Commissioning Group

DPA: Data Protection Act

GDPR: General Data Protection Regulation

REACH: Register of Asthma Research Volunteers Database

AUKCAR: The Asthma UK Centre for Applied Research

BMI: Body Mass Index

NSAIDs: Non-Steroidal Anti-Inflammatory Drugs

COPD: Chronic Obstructive Pulmonary Disease

DRV: Dietary Reference Value

PBS: Phosphate Buffered Saline

PFA: Paraformaldehyde

FISH: Fluorescent *in situ* Hybridisation

RNA: Ribonucleic Acid

EDTA: Ethylenediaminetetraacetic Acid

RCF: Relative Centrifugal Force

RBCs: Red Blood Cells

FCS: Fetal Calf Serum

DMSO: Dimethyl Sulfoxide

FACS: Fluorescence Activated Cell Sorting

MFVLS: Maximal Flow-Volume Loops

ATS: American Thoracic Society

ERS: European Respiratory Society

TLC: Total Lung Capacity

\dot{V}_E : Minute Ventilation

BCa: Bias Corrected Accelerated

CI's: Confidence Intervals

EILO: Exercise-Induced Laryngeal Obstruction

WADA: World Anti-Doping Agency

TUE: Therapeutic Use Exemption

LBP: Lipopolysaccharide Binding Protein

NMR: Nuclear Magnetic Resonance

Dedication

To my father, James Lester. You set this journey in motion long before I walked the path. All my life you have taught me so many values and attributes that have been essential to undertaking my PhD. You have been my biggest support, and greatest motivation, sacrificing so much to see me succeed. I have cherished every opportunity to repay you the only way I can, by trying to make you proud of what we have achieved. I just hope you can be here to share in the celebrations when the journey comes to an end.

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Preface

Unless otherwise indicated by reference to published resources, the work presented in the current thesis is that of the author, and has not been previously submitted for another degree to this or any other University.

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Chapter I

Introduction

Asthma is the second most prevalent chronic respiratory disease in the world, affecting ~ 3.6% of the global population and equating to 0.9% of global morbidity and mortality (GBD Chronic Respiratory Disease Collaborators, 2020). The United Kingdom has the highest prevalence of asthma in the world, with treatment costing the NHS ~ one billion pounds per year (GBD Chronic Respiratory Disease Collaborators, 2020; Mukherjee et al., 2016). Despite enormous expenditure on healthcare services, the prevalence of asthma remains at epidemic levels, with ~ 1,160 people dying every year in the U.K. (GBD Chronic Respiratory Disease Collaborators, 2017; Mukherjee et al., 2016). Exercise-induced bronchoconstriction is a subtype of asthma underpinned by type-2 (eosinophilic) inflammation. Estimates of EIB in athletic populations range from 9% to 71.4% (Dickinson et al., 2006A; Sue-Chu et al., 2010), compared to ~ 22% in the general population (Aggarwal et al., 2018; Brummel et al., 2009; Weiler et al., 2016).

The rapid increase in asthma prevalence has been associated with the hygiene hypothesis (Liu, 2015). Various factors associated with Westernised society prevent adequate exposure to the microbes required to develop optimal immune responses (Debarry et al., 2007; Gerhold et al., 2006; Liu, 2015; Reibman et al., 2008; Riedler et al., 2001; Sbihi et al., 2019; Stein et al., 2016; Stockholm et al., 2018; Thorburn et al., 2015; Wolsk et al., 2016). Fetal immune responses are naturally skewed towards type-2 inflammatory responses, highlighting the importance of early life exposures in reprogramming inflammatory responses (Folsgaard et al., 2012; Prescott et al., 1998). However, factors such as delivery via caesarean section (Clemente et al., 2012; Stockholm et al., 2016; Sevelsted et al., 2016; van Nimwegen et al., 2011), exposure to antibiotics (Arpaia et al., 2013; Arrieta et al., 2015; Cait et al., 2018; Sbihi et al., 2019; Stensballe et al., 2013; Stockholm et al., 2016), or deficiencies in dietary fibre intake help to sustain the development of type-2 inflammation (Dahl, 2006; Folkerts et al., 2018; Huang et al., 2017; McLoughlin et al., 2019; Mukherjee et al., 2016; Singanayagam et al., 2017; Smits et al., 2016; Tan et al., 2014; 2017; Thorburn et al., 2015).

As a result of these factors, recent evidence suggests that perturbations to the gut microbiota may play a critical role in the pathophysiology of asthma. Adults with

allergic asthma display features of gut microbial dysbiosis that are associated with clinical and inflammatory parameters (Barcik et al., 2018; Begley et al., 2018; Hevia et al., 2016; Okba et al., 2018). This has led researchers to investigate whether the severity of asthma can be attenuated through gut microbiota-mediated nutritional interventions, such as prebiotics (Gourbeyre et al., 2011; Huang et al., 2017; Thorburn et al., 2015; Verheijden et al., 2015A; 2015B; 2018; Williams et al., 2016). Recent evidence suggests that both short-term and long-term supplementation with synbiotics or prebiotics can attenuate the severity of asthma and exercise-induced bronchoconstriction in adults (Halnes et al., 2017; Williams et al., 2016). Strikingly, just 4 hours after consuming a synbiotic meal, Halnes et al. (2017), reported a significant increase in FEV₁ in adults with asthma. In addition, following 3-weeks of supplementation with a galactooligosaccharide-based prebiotic (B-GOS®), Williams et al. (2016), reported clinically relevant, participant perceivable improvements in the severity of EIB that were similar in magnitude to that observed following pharmaceutical treatments (Kippelen et al., 2010; Rundell et al., 2005; Santanello et al., 1999; Simpson et al., 2016; Verheijden et al., 2015A; 2018). However, given that gut bacterial composition has not been assessed in adults with EIB, it remains to be determined whether prebiotics attenuate the severity of EIB via the gut microbiota, and what the minimum, clinically important dose required might be to elicit positive changes. This question has become more relevant in recent years as companies begin to develop prebiotic supplements with less impurities to explore whether this leads to further increases in efficacy. However, research to date has only focused on earlier versions of prebiotic supplements with a lower proportion coming from the active ingredient (i.e. 55% galactooligosaccharide; Williams et al., 2016). Whether increasing the proportion of active ingredient delivered leads to further improvements in immune or pulmonary function has yet to be explored.

A potential mechanism by which prebiotic supplementation may attenuate the severity of EIB is through regulatory T cells (T_{REG}). T_{REG} cells orchestrate the tolerogenic immune response to attenuate type-2 inflammation (Bohm et al., 2015; Hong et al., 2018; Maazi et al., 2018; Van der Veecken et al., 2016). Murine models have demonstrated that galactooligosaccharides attenuate the severity of airway eosinophilia and hyperresponsiveness through T_{REG} cell dependent mechanisms (Verheijden et al., 2015A; 2015B; 2018). However, the role of T_{REG} cells in mediating galactooligosaccharide induced improvements in the severity of EIB remain unexplored in adults, representing an exciting area for future research.

The importance of developing novel complimentary therapies for EIB extends beyond concerns associated with the hygiene hypothesis. Alarming, only around half of adults take their medication as prescribed (Cohen et al., 2009; Williams et al., 2004). Poor adherence increases the risk of exacerbations, and the progression towards severe refractory asthma (GBD Chronic Respiratory Disease Collaborators, 2017; Engelkes et al., 2015; Hekking et al., 2015; Ivanova et al., 2012; Mukherjee et al., 2016; Williams et al., 2004). Severe refractory asthma is particularly difficult to treat due to the transition from a type-2 (eosinophilic) to a non-type-2 (neutrophilic) phenotype (Peters et al., 2020). Despite only comprising ~ 3.6% of the total asthma population (Hekking et al., 2015), severe refractory asthma accounts for a disproportionate amount of exacerbations and healthcare associated expenditure (Ivanova et al., 2012; Jackson et al., 2020; Peters et al., 2020). On average, patients with severe refractory asthma experience ~ 1 - 4 exacerbations per year, causing a 48% increase in healthcare associated expenditure (Brinkman et al., 2019A; 2020; Ivanova et al., 2012; Jackson et al., 2020; Peters et al., 2020; Schatz et al., 2014). These observations highlight the importance of preventing the progression of asthma, especially considering the increased risk of adverse events ~~possible safety concerns~~ associated with patients initially prescribed high dose inhaled corticosteroids (Chipps et al., 2020), and the development of tolerance to short-acting β_2 -agonists following repeated use (Hancox et al., 2002; Weiler et al., 2016; Williams et al., 2020).

Interestingly, the development of tolerance to short-acting β_2 -agonists is not only a concern for severe asthma populations, but also exercising individuals including elite athletes. Traditionally, pharmaceutical treatments have been used to provide short-term protection against increases in the severity of EIB around training and competition (Kippelen et al., 2010; Rundell et al., 2005; Simpson et al., 2016). However, questions remain regarding the ethical use of certain short-term pharmaceutical treatments. The most common pharmaceutical treatment used to protect against increases in the severity of EIB is salbutamol.

Although salbutamol is routinely used by athletes with a Therapeutic Use Exemption (TUE), the magnitude of bronchoprotection may be lost following repeated administration, a concept known as tachyphylaxis (Hancox et al., 2002; Williams et al., 2020). The loss of bronchoprotection places athletes at an increased risk of exacerbations, highlighting the importance of maintaining responsiveness to

pharmaceutical treatments by avoiding excessive use (Hancox et al., 2002; Williams et al., 2020). One approach to sparing the use of pharmaceutical treatments could be ~~the use of novel~~ nutritional therapies such as omega-3 supplementation that, when administered in low doses, have been shown to significantly attenuate the severity of EIB by 25% (Williams et al., 2017). More recently however, research has begun to demonstrate a possible link between the gut microbiota and asthma (Hevia et al., 2016), and the potential increased therapeutic efficacy of prebiotic supplementation compared to omega-3 (40% vs. 25% attenuation in the severity of EIB; Williams et al., 2016). However, the use of prebiotics in attenuating the severity of EIB remains largely unexplored.

As a result, the current thesis will investigate whether adults with EIB display features of gut microbial dysbiosis, and whether short-term and long-term supplementation with a low dose galactooligosaccharide-based prebiotic attenuates the severity of EIB.

To address these questions, the following hypotheses were evaluated:

Chapter IV (Study One)

- It was hypothesised that adults with hyperpnoea (exercise)-induced bronchoconstriction would display features of gut microbial dysbiosis, specifically decreases in the number of *Bifidobacteria* and *Lactobacilli*.

Chapter V (Study Two)

- Short term supplementation with a single low dose galactooligosaccharide-based prebiotic of increased purity (3.1g, HOST-DM059; 85% galactooligosaccharides) would attenuate the severity of HIB and markers of systemic inflammation.

Chapter VI (Study Three)

- Long term supplementation with a low dose galactooligosaccharide-based prebiotic (4-weeks of 3.1g/day⁻¹, HOST-DM059; 85% galactooligosaccharides) would attenuate the severity of HIB and markers of systemic inflammation, specifically by increasing the suppressive capacity of T_{REG} cells.

Organisation of the Thesis

Chapter II will provide a critical review of the literature, exploring the role of gut microbial dysbiosis on the pathophysiology of hyperpnoea-induced bronchoconstriction, and investigating the effects of short and long-term prebiotic supplementation. Chapter III, the general methods, will outline the common methods used throughout several experimental chapters. Chapter IV will explore the role of gut microbial dysbiosis in the pathophysiology of hyperpnoea-induced bronchoconstriction. Chapter V will investigate the effects of short-term prebiotic supplementation on the severity of hyperpnoea-induced bronchoconstriction, and markers of systemic inflammation. Chapter VI will investigate the effects of long-term prebiotic supplementation on the severity of hyperpnoea-induced bronchoconstriction, and markers of systemic inflammation, with a particular focus on the function of regulatory T cells. Chapter VII, the discussion, will examine the key findings from the experimental chapters, outline the overall conclusions drawn, and suggest practical applications regarding the future use of prebiotic supplementation in hyperpnoea-induced bronchoconstriction, outlining critical factors that need to be considered when designing and conducting research.

Chapter II

Literature Review

2.1 Defining Asthma & Exercise-Induced Bronchoconstriction

2.1.1 Asthma

Asthma is a chronic syndrome of the airways that causes reversible airway inflammation, hyperresponsiveness and remodeling (Kearley et al., 2005; Maazi et al., 2018; Pavord et al., 2017; Saglani & Lloyd, 2015; Silkoff et al., 2019). Asthma is a heterogenous disorder classified into various subtypes, of which ~ 50% are driven by type-2 (T_H2) inflammation (Wenzel, 2016).

Asthma is typically diagnosed by a physician based on the subjective assessment of symptoms, such as coughing, wheezing, chest tightness, and breathlessness (dyspnoea), considering how severe these symptoms are, and how much they limit daily activities (Dickinson et al., 2011; GBD Chronic Respiratory Disease Collaborators, 2020; Juniper et al., 1999; Rundell et al., 2001; Teeter & Bleecker, 1998). Subjective assessments are less robust than objective assessments, this increases the risk of misdiagnosis or under diagnosis as subjective assessments are not based on objective, pathophysiological mechanisms (Aggarwal et al., 2018; Dickinson et al., 2011; Juniper et al., 2004; Pavord et al., 2017; Wenzel, 2016).

Based on subjective assessments, clinicians tend to underestimate or overestimate improvements in asthma control, particularly when interpreting minimally important clinical improvements (Boulet et al., 2002; Juniper et al., 2004). The use of subjective assessments for diagnosing exercise-induced bronchoconstriction (EIB) is particularly problematic as anecdotal evidence suggests that those who regularly engage in strenuous exercise attribute symptoms such as coughing and chest tightness to the duration or intensity of exercise as opposed to any underlying pathology (Dickinson et al., 2011). Up to 73% of athletes diagnosed with EIB via objective protocols were unaware they suffered from EIB (Dickinson et al., 2011), demonstrating the lack of awareness of EIB in athletic populations. Consequently, subjective diagnoses have hindered the accurate classification of asthma subtypes, and the development of targeted therapies (Heir & Oseid, 1994; Parsons et al., 2007; Pavord et al., 2017; Weiler et al., 2016; Wenzel, 2016). To address this issue, asthma subtypes are now being classified based on observable characteristics and

objective pathways (Altman et al., 2019; Pavord et al., 2017; Silkoff et al., 2019; Wenzel, 2016). By avoiding broad disease labels, and adopting terminology specific to objective pathways, targeted therapies for individual subtypes can be developed (Pavord et al., 2017).

Although objective assessments are becoming more prevalent, the historic lack of these protocols when classifying EIB has led to inaccurate classifications of what researchers referred to as exercise-induced “asthma”, or exercise-induced “bronchospasm” (Weiler et al., 2016). These terms are inaccurate, outdated, and based on non-specific, self-reported respiratory symptoms, as opposed to objective pathways (Weiler et al., 2016). Such misclassifications hinder current understandings of the pathophysiology of EIB, further preventing the development of targeted therapies (Weiler et al., 2016).

2.1.2 Exercise-Induced Bronchoconstriction (EIB)

Although EIB is a subtype of asthma, people with and without asthma can suffer from EIB (Aggarwal et al., 2018; Weiler et al., 2016). Estimates of EIB in athletic populations range from 9% to 71.4% (Dickinson et al., 2006A; Sue-Chu et al., 2010), compared to ~ 22% in the general population (Brummel et al., 2009). Exercise-induced bronchoconstriction is driven by type-2 inflammation (Altman et al., 2019; Anderson, 2011; Anderson and Kippelen, 2013; Hallstrand et al., 2013), characterised by a temporary narrowing of the lower airways that occurs most commonly immediately after high-intensity aerobic exercise (Altman et al., 2019; Molphy et al., 2014; Parsons et al., 2007; Price et al., 2014; Simpson et al., 2016; Weiler et al., 2016). Exercise-induced bronchoconstriction typically develops ~ 2-5 minutes after exercise, and peaks within ~ 5-10 minutes after exercise (Aggarwal et al., 2018; Mannix et al., 2003; Dickinson et al., 2011; Molphy et al., 2014). Objective protocols for assessing EIB have greatly enhanced current understandings of the underlying pathophysiology by allowing key stimuli such as airway dehydration (hyperosmolarity) to be assessed (Anderson et al., 2001; Dickinson et al., 2006A; Molphy et al., 2014).

2.1.3 Hyperpnoea-Induced Bronchoconstriction (HIB)

Hyperpnoea-Induced Bronchoconstriction (HIB) is an objective, surrogate assessment of EIB assessed via eucapnic voluntary hyperpnoea (EVH; Anderson et al., 2001; Williams et al., 2015). Eucapnic voluntary hyperpnoea induces the

airway hyperosmolarity experienced during exercise by asking participants to inhale a dry gas mixture whilst maintaining high levels of ventilation (Anderson et al., 2001; Anderson & Kippelen, 2013; Dickinson et al., 2006A; Mannix et al., 2003). For the current programme of work, HIB refers to adults with or without a subjective (physicians) diagnosis of asthma, who demonstrated objective evidence of EIB in response to the EVH protocol (Anderson et al., 2001, Anderson & Kippelen, 2013; Williams et al., 2015).

2.2 The Prevalence, Morbidity, Mortality and Economic Impact of Asthma

2.2.1 Chronic Respiratory Diseases

As of 2017, the global prevalence of chronic respiratory diseases was ~ 7.1% of the total population, equating to 4.5% of morbidity and 7% of mortality (GBD Chronic Respiratory Disease Collaborators, 2020). Chronic respiratory diseases are the third highest cause of global mortality behind cardiovascular diseases and neoplasms (GBD Chronic Respiratory Disease Collaborators, 2020).

From 1990-2017, the prevalence of chronic respiratory diseases has increased dramatically by 39.8% (390 vs. 545 million people worldwide; GBD Chronic Respiratory Disease Collaborators, 2020). Over this timeframe, morbidity increased by 13.3% (99,103,908 vs. 112,316,763 disability adjusted life years (DALYs), whereas mortality increased by 18% (3,317,205 vs. 3,914,196 deaths per year; GBD Chronic Respiratory Disease Collaborators, 2020). The most prevalent risk factor for chronic respiratory diseases in males is smoking, whereas exposure to air pollution from solid fuels is the most prevalent risk factor in females (GBD Chronic Respiratory Disease Collaborators, 2020).

2.2.2 Asthma

2.2.2.1 The Global Prevalence, Morbidity, Mortality and Economic Impact

Behind COPD, asthma is the second most prevalent chronic respiratory disease in the world (GBD Chronic Respiratory Disease Collaborators, 2020). Around 235 million people suffer with asthma worldwide, equating to 0.9% of global morbidity and mortality (GBD Chronic Respiratory Disease Collaborators, 2020). Global morbidity for asthma has been estimated at ~ 26.2 million DALYs (GBD Chronic Respiratory Disease Collaborators, 2017). Although Mortality decreased by 58.8% from 1990 to 2015, potentially due to factors such as the increased availability of

medications like inhaled corticosteroids (GBD Chronic Respiratory Disease Collaborators, 2017; GINA, 2016). Despite this decrease in mortality, ~ 400,000 people still die every year due to asthma (GBD Chronic Respiratory Disease Collaborators, 2017).

From 1990-2017 the global prevalence of asthma decreased by 0.3% (3.9 vs. 3.6%; GBD Chronic Respiratory Disease Collaborators, 2020). From 10 years of age and onwards, asthma is more prevalent in females compared to males (3.8 vs. 3.3%; GBD Chronic Respiratory Disease Collaborators, 2020; Hekking et al., 2015; Peters et al., 2020), equating to a 0.2% increase in DALYs (1.02 vs. 0.82%; GBD Chronic Respiratory Disease Collaborators, 2020). A lower socioeconomic status has been associated with an increased risk of mortality (GBD Chronic Respiratory Disease Collaborators, 2017), yet mortality rates are similar between females and males (7 vs. 6 deaths per 100,000; GBD Chronic Respiratory Disease Collaborators, 2020). Smoking is a key risk factor for asthma, especially in males (GBD Chronic Respiratory Disease Collaborators, 2020). Around 20% of people with asthma smoke worldwide (Rabe et al., 2004). Despite these estimates, only 16.5% of DALYs have been attributed to smoking or occupational irritants (GBD Chronic Respiratory Disease Collaborators, 2017), highlighting the range of lifestyle and environmental factors that drive the development of asthma.

2.2.2.2 The United Kingdom: Asthma Prevalence, Morbidity, Mortality and Economic Impact

The United Kingdom has the highest prevalence of asthma in the world (GBD Chronic Respiratory Disease Collaborators, 2020). In 2012, ~ 4.3 million people were diagnosed with asthma in the U.K, of which 3.3 million lived in England (Mukherjee et al., 2016). The widespread prevalence of asthma has placed a huge financial burden on the National Health Service (NHS; Mukherjee et al., 2016). In the U.K, just under one billion pounds per year is spent treating asthma (£964,905,000), of which 82.8% is incurred by NHS England (£798,780,000; Mukherjee et al., 2016). Of these costs, 69.1% are attributed to preventive medications (£666,445,000), of which 82.9% is incurred by NHS England (£552,415,000; Mukherjee et al., 2016). Despite the widespread prescription of preventive medications, asthma attacks remain common in patients with moderate (32.4%) and severe asthma (43.7%; Ivanova et al., 2012; Mukherjee et al., 2016). Patients with severe refractory asthma experience ~ 1 - 4 exacerbations per year

(Brinkman et al., 2019A; 2020; Ivanova et al., 2012; Jackson et al., 2020; Peters et al., 2020; Schatz et al., 2014), equating to a 48.1% increase in healthcare associated expenditure (Ivanova et al., 2012).

In the U.K, ~ 1,160 people die every year due to asthma, of which 84.7% occur in England ($n = 982$; Mukherjee et al., 2016). From 2001-2017, a 2.5% decrease in mortality was reported in England and Wales (Shaw et al., 2019). In adults under 75, a 53% decrease in mortality was reported, whereas in adults over 75, a 50% increase was reported (Shaw et al., 2019). This was attributed to inaccuracies in confirming the cause of death in over 75's, as opposed to a true increase in mortality (Shaw et al., 2019). Importantly, ~ two thirds of these deaths could have been prevented by reducing exacerbations, increasing medication adherence, and improving inhaler technique (GBD Chronic Respiratory Disease Collaborators, 2017; Hekking et al., 2015). The huge financial burden, and preventable deaths attributed to asthma, highlight the urgent, unmet need to develop targeted therapies with potential curative capacities and improved adherence outcomes (GBD Chronic Respiratory Disease Collaborators, 2017; 2020; Mukherjee et al., 2016; Pavord et al., 2017; Peters et al., 2020; Silkoff et al., 2019).

2.2.3 The Prevalence of Exercise-Induced Asthma and Exercise-Induced Bronchoconstriction

2.2.3.1 Exercise-Induced Asthma

Exercise-induced asthma is typically diagnosed based on self-reported symptoms, whereas EIB is diagnosed based on objective assessments. Based on self-reported symptoms, the prevalence of asthma has been estimated at 5% in the general population (Heir & Oseid, 1994). Higher prevalence rates for exercise-induced asthma have been reported in elite athletes, especially in those with more years' experience competing at the elite level (Heir & Oseid, 1994; Kennedy et al., 2019; Weiler et al., 2016). Heir and Oseid, (1994), reported a 14.4% prevalence rate of exercise-induced asthma in elite cross-country skiers, whereas Mannix et al. (1999), reported a 41% prevalence rate in figure skaters. The prevalence rate in elite cross-country skiers was primarily attributed to athletes ≥ 24 years old (Heir and Oseid, 1994), suggesting that asthma may be an occupational hazard of elite performance due to the cumulative exposure to high levels of ventilation. Furthermore, the increased prevalence in figure skaters (Mannix et al., 1999), highlights the potential

influence of triggers within specific sporting environments, such as the emissions from ice cleaning machines.

In the general population, asthma typically originates in childhood (Holgate, 2012), whereas in elite cross-country skiers, asthma can develop during adolescence or early adulthood (Heir & Oseid, 1994). Asthma that originates in childhood is typically allergic in nature (Wenzel, 2016), whereas asthma that develops later in life can be attributed to specific environmental factors (Holgate, 2012). Therefore, the increased prevalence of asthma among elite cross-country skiers (Heir and Oseid, 1994), and figure skaters (Mannix et al., 1999), could be due to progressively conditioning larger volumes of cold, dry air, during training and competition (Dickinson et al., 2006A; Molphy et al., 2014; Wenzel, 2016). The later age of onset, and specific environmental factors, may represent a subtype of EIB specific to elite athletes that is underpinned by distinct objective pathways (Wenzel, 2016).

Prevalence estimates based on subjective assessments should be viewed with caution (Sue-Chu et al., 2010; Vakali et al., 2017). Resting assessments of pulmonary function (Mannix et al., 2003), and self-reported respiratory symptoms cannot distinguish between adults with and without an objective diagnosis of EIB (Dickinson et al., 2011; Rundell et al., 2001), nor do they contribute to a robust diagnostic algorithm (Altman et al., 2019; Brummel et al., 2009; Molphy et al., 2014; Parsons et al., 2007; Pedersen et al., 2008; Sue-Chu et al., 2010; Teeter & Bleecker, 1998; Vakali et al., 2017; Weiler et al., 2016). Subjective assessments of EIB are associated with increased rates of false-positive diagnoses (Stadelmann et al., 2011; Parsons et al., 2007; Pedersen et al., 2008; Sue-Chu et al., 2010), highlighting the importance of using objective assessments to establish more accurate prevalence estimates.

The limitations of subjective assessments are highlighted by the similarity in self-reported respiratory symptoms between varsity college athletes with and without an objective diagnosis of EIB (34.9 vs. 36.5%; Parsons et al., 2007). Similar trends have been observed in elite athletes (Pedersen et al., 2008; Stadelmann et al., 2011). Based on self-reported respiratory symptoms, the prevalence of EIB in winter sport athletes has been estimated at 39% (Rundell et al., 2001). In contrast, the prevalence decreased to 26% when based on objective, sport-specific exercise challenge tests (Rundell et al., 2001). Of the athletes with an objective diagnosis of EIB, only 44% had a subjective diagnosis (Rundell et al., 2001). Despite these

discrepancies, athletes without an objective diagnosis of EIB reported experiencing an equal to or greater number of respiratory symptoms post-exercise (Kennedy et al., 2019; Rundell et al., 2001). In elite adolescent swimmers, the prevalence of EIB has been estimated at 83% when based on self-reported respiratory symptoms (Stadelmann et al., 2011). In contrast, the prevalence decreased to 65% when based on objective assessment via EVH (Stadelmann et al., 2011). These comparisons highlight the non-specific nature of self-reported respiratory symptoms (Heir and Oseid, 1994; Parsons et al., 2007; Rundell et al., 2001), and the increased rate of false-positive diagnoses (Stadelmann et al., 2011), emphasising the importance of using standardised, objective assessments when diagnosing EIB (Molphy et al., 2014; Parsons et al., 2007).

2.2.3.2 Exercise-Induced Bronchoconstriction

Current guidelines advocate the use of standardised objective assessments, such as EVH, when diagnosing EIB (Anderson & Kippelen, 2013; Dickinson et al., 2006A; 2011; Pedersen et al., 2008; Sue-Chu et al., 2010; Williams et al., 2015). The International Olympic Committee Medical Commission (IOC-MC), and the Joint Task Force on Practice Parameters in EIB (JTFPP), advocate the use of EVH to diagnose EIB in athletic populations (Hull et al., 2016; Weiler et al., 2016). The most robust assessment of EIB could be obtained by completing multiple objective assessments, such as assessing the response to methacholine, adenosine 5-monophosphate, house dust mite, mannitol, EVH, and whole-body exercise-based protocols, respectively (Vakali et al., 2017; van de Pol et al., 2011). In reality, this approach is not feasible given the time, specialist personnel, equipment, and costs required to complete multiple assessments.

When compared to the indirect challenge of EVH, direct methacholine challenge demonstrates a higher rate of false-positive diagnoses in elite cross-country skiers (Sue-Chu et al., 2010), potentially indicating different pathophysiological mechanisms of EIB in winter sport athletes, highlighting the importance of selecting the appropriate bronchoprovocation technique(s) for the population being investigated. In addition, methacholine, adenosine 5-monophosphate, house dust mite and mannitol-based assessments require medical personnel to administer the test, whereas sport or environment specific whole-body exercise-based protocols are difficult to standardise and compare across sports and cohorts (Rundell et al., 2001).

Based on EVH, the prevalence of EIB has been estimated at 22% in the general population (Brummel et al., 2009). In athletic populations, the prevalence of EIB increases from recreational to elite athletes (Dickinson et al., 2006A; 2011; Molphy et al., 2014; Weiler et al., 2016), especially in high-intensity aerobic sports (Kennedy et al., 2019; Weiler et al., 2016). In recreational athletes, the prevalence of EIB is 13.2% (Molphy et al., 2014). In varsity college athletes, the prevalence increases to 19% (Parsons et al., 2007), whereas in elite athletes, the prevalence ranges from 9-71% (Dickinson et al., 2006A; 2011; Pedersen et al., 2008; Sue-Chu et al., 2010).

In elite athletes, some of the highest prevalence rates have been observed in winter sports such as biathlon and short track speed skating (Dickinson et al., 2006A; 2011; Sue-Chu et al., 2010). This has led researchers to develop winter sport-specific, field-based assessments of EIB, with the view that this would lead to more accurate assessments. Some argue that the EVH protocol lacks ecological validity for winter sport athletes as it does not simulate cold atmospheric temperatures (Kennedy et al., 2019). Typically, the EVH protocol is conducted in a laboratory under ambient conditions. Kennedy et al. (2019), reported a higher prevalence of EIB following a sport-specific cold air-based test when compared to the EVH protocol (44 vs. 31%). In contrast, Dickinson et al. (2006A), reported a much lower prevalence of EIB following a sport-specific cold air-based test when compared to the EVH protocol (21 vs. 71%). Sport specific field-based assessments of EIB vary in their duration, intensity, and environmental conditions, making accurate comparisons between and within sports difficult. In the assessments conducted by Dickinson et al. (2006A), the relative humidity of the air inspired was higher during sport-specific exercise challenge tests for speed skaters (35%), and biathletes (31-34%) compared to the EVH protocol (> 2%). In contrast, the temperature of the air inspired during the EVH protocol (19.1°C), was higher than those used for the sport-specific tests conducted for speed skating (8°C), and biathlon (1-2°C), making it difficult to directly compare the relative contribution of either factor in triggering EIB. Bolger et al. (2011), directly compared the difference in EIB severity between cold and warm air-based bronchoprovocation tests, demonstrating a complete attenuation of EIB following the inhalation of warm, moist air (Bolger et al. 2011). These results demonstrate that dehydration of the airways, achieved via inhaling cold air, dry air, or both, are important triggers of EIB.

High levels of ventilation have also been shown to cause EIB via hyperosmolarity of the airway surface liquid (Dickinson et al., 2006A). Although high levels of ventilation can contribute to EIB (Dickinson et al., 2006A), Brummel et al. (2009), reported similar prevalence rates of EIB in patients who were and were not able to maintain their minimum ventilatory target during the EVH protocol (26 vs. 33% testing positive for EIB), demonstrating that inhaling dry air, even at moderate ventilation levels, can trigger EIB (Brummel et al., 2009; Dickinson et al., 2006A). As the EVH protocol has been optimised to deliver the most potent hyperosmotic stimulus (Anderson et al., 2001; Anderson and Kippelen, 2013; Williams et al., 2015), it is clear why this protocol is advocated by the IOC-MC and JTFPP in EIB (Hull et al., 2016; Weiler et al., 2016). However, that is not to say that the EVH protocol is considered the Gold Standard method of diagnosing EIB (Hull et al., 2016).

The absence of a Gold Standard method for diagnosing EIB in athletic populations has led to inaccurate and inconsistent prevalence estimates (Brummel et al., 2009; Hull et al., 2016; Parsons et al., 2007; Weiler et al., 2016). Use of the EVH protocol remains limited (Boulet et al., 2002; Brummel et al., 2009), inappropriate in severe populations, and impractical in some settings due to the costs associated with testing (Stadelmann et al., 2011; Weiler et al., 2016). Furthermore, the discomfort experienced during the EVH protocol regarding the dry gas mixture (Brummel et al., 2009), may limit compliance and widespread administration, especially in longitudinal assessments or intervention-based trials. Nevertheless, the EVH protocol is safe, quick, easy to conduct (Mannix et al., 2003), and highly reproducible (Williams et al., 2015). Efforts to enable more widespread access to standardised objective assessments will be essential to obtaining more accurate prevalence estimates.

A comparison of the prevalence of exercise-induced asthma and exercise-induced bronchoconstriction is outlined in Tables 2.0 and 2.1 below. Exercise-induced asthma was assessed based on self-reported respiratory systems, whereas EIB was diagnosed via objective protocols. The prevalence rates for the general population are included as a reference category.

Table 2.0 The prevalence of exercise-induced asthma non-athletic and athletic populations.

Exercise-Induced Asthma		
Reference	Population	Prevalence (%)
Heir and Oseid, (1994)	General population	5
Heir and Oseid, (1994)	Elite cross-country skiers	14.5
Parsons et al., (2007)	Varsity college athletes	36.5
Rundell et al., (2001)	Winter sport athletes	39
Mannix et al., (1999)	Figure skaters	41
Stadelmann et al., (2011)	Elite adolescent swimmers	83

Table 2.1 The prevalence of exercise-induced bronchoconstriction in non-athletic and athletic populations.

Exercise-Induced Bronchoconstriction		
Reference	Population	Prevalence (%)
Brummel et al., (2009) ^{EVH}	General population	22
Sue-Chu et al., (2010) ^{EVH}	Cross-country skiers	9
Molphy et al., (2014) ^{EVH}	Recreational athletes	13.2
Parsons et al., (2007) ^{EVH}	Varsity college athletes	19
Rundell et al., (2001) ^{SET}	Winter sport athletes	26
Kennedy et al., (2019) ^{EVH}	Winter sport athletes	31
Pedersen et al., (2008) ^{EVH}	Elite swimmers	31.3
Dickinson et al., (2011) ^{EVH}	Elite athletes	34
Stadelmann et al., (2011) ^{EVH}	Elite adolescent swimmers	65
Dickinson et al., (2006A) ^{EVH}	Elite short-track speed skaters	71.4

EVH eucapnic voluntary hyperpnoea, *SET* sport or environment specific whole-body exercise-based challenge test.

Fatal exercise-associated asthma exacerbations remain an infrequent, yet largely preventable occurrence, attributed to inadequate asthma control (Becker et al., 2004; DiDario & Becker, 2005). From the United States, a total of 61 fatalities were reported across recreational and elite athletes between 1993-2000 (Becker et al., 2004), equating to a death rate of 5 per year. Recreational and elite athletes made up a similar proportional of total fatalities (43 vs. 57% of total fatal exercise-

associated asthma exacerbations; Becker et al., 2004). In 81% of cases, individuals were less than 21 years old, with ~ 50% aged between 13 and 17 (Becker et al., 2004). In these age groups objective assessments of EIB are less common. Furthermore, education regarding the severity of EIB, consequences of poor medication adherence, and implications of inadequate inhaler technique may be limited. Taken together, the high prevalence of EIB across sports and competition levels, combined with the preventable deaths attributed to EIB, reinforce the urgent, unmet need to develop targeted therapies (Pavord et al., 2017). Before such therapies can be developed, a clearer understanding of the pathophysiology of EIB must be obtained.

2.3 The Pathophysiology of Exercise-Induced Bronchoconstriction

2.3.1 The Osmotic Hypothesis

The three pillars of EIB include airway inflammation, hyperresponsiveness (bronchoconstriction), and remodeling, respectively (Maazi et al., 2018; McBrien & Menzies-Gow, 2017; Pavord et al., 2017; Saglani & Lloyd, 2015). Two complementary theories have been proposed to explain the causes of EIB known as the osmotic and thermal hypotheses (Anderson, 2011; Couto et al., 2018; Kippelen et al., 2018; Strauss et al., 1978; Weiler et al., 2016). The primary theory of EIB is considered to be the osmotic hypothesis, as osmotic stimuli are the most potent triggers of airway inflammation and bronchoconstriction (Aggarwal et al., 2018; Brummel et al., 2009; Dickinson et al., 2006A; Weiler et al., 2016).

The osmotic hypothesis states that the ventilatory demands sustained during constant load, high-intensity aerobic exercise, overwhelms the airways ability to condition and humidify (moisten) the inspired air (Anderson & Kippelen, 2013; Kippelen et al., 2018; Molphy et al., 2014; Weiler et al., 2016). The first 10-12 generations of the airways are lined with an airway surface liquid known as periciliary fluid that conditions the inspired air (Anderson & Kippelen, 2013; Kippelen et al., 2018). The volume of the airway surface liquid is regulated by the absorption of Na⁺ ions, and secretion of Cl⁻ ions, from the bronchial epithelium (Tarran, 2005). As outlined in Figure 2.0, during high-intensity exercise, the switch from nasal to mouth breathing accelerates the evaporative loss of airway surface liquid, creating a hyperosmotic state in the airways (Anderson, 2011; Anderson & Kippelen, 2013; Hallstrand et al., 2012; Mannix et al., 2003; Weiler et al., 2016).

The amount of airway surface liquid lost from the airways correlates with EIB severity (Hallstrand et al., 2012). Shearing forces created via the biphasic movement of air over the bronchial epithelium causes the shedding of epithelial cells into the airway lumen (Hallstrand et al., 2012; Tarran, 2005). This decreases the airway diameter and increases the permeability to pro-inflammatory mediators (Earl et al., 2015). In some circumstances, airway remodelling develops as a result of repeated inflammation and hyperresponsiveness (Foster et al., 2017), yet in other circumstances airway remodelling occurs independently (Sagani & Lloyd, 2015).

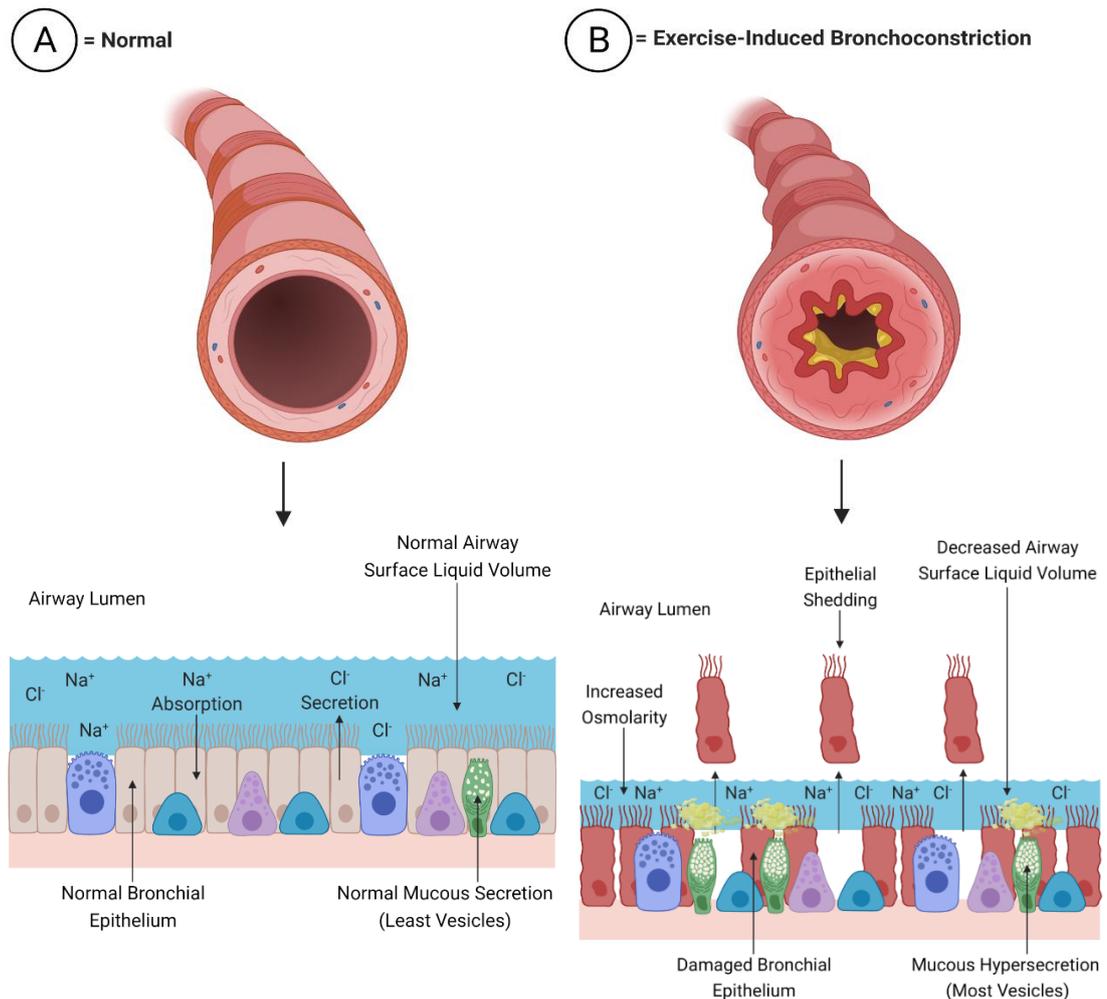


Figure 2.0 Changes in airway physiology between healthy individuals **(A)**, and those with EIB **(B)**. Created with www.BioRender.com. *EIB* exercise-induced bronchoconstriction.

Airway dehydration, shrinkage of bronchial epithelial cells, and mechanical damage stimulate the release of interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP) from bronchial epithelial cells (Couto et al., 2018; Lai et al., 2014). Interleukin-33 is released in response to osmotic stimuli (Lai et al., 2014), whereas TSLP is released in response to mechanical damage. Following the release of these pro-inflammatory epithelial mediators, mast cells, eosinophils and t-helper type-2 (T_H2) cells infiltrate the airways and release a broad range of pro-inflammatory factors, including arachidonic acid, cysteinyl leukotrienes, prostaglandins, histamine and tryptase (Aggarwal et al., 2018; Anderson, 2011; Anderson & Kippelen, 2013; Hallstrand et al., 2012; 2013; Kippelen et al., 2018; Lloyd & Hawrylowicz, 2009; McLoughlin & Mills, 2011; Weiler et al., 2016). Even at low concentrations, cysteinyl leukotrienes are potent drivers of bronchoconstriction (Weiler et al., 2016).

Epithelial sensory nerves detect changes in airway osmolarity (Hallstrand et al., 2012; Weiler et al., 2016). Cysteinyl leukotrienes decrease the activation threshold of sensory nerves, stimulating the release of neurokinin-A (NKA; Hallstrand et al., 2007; 2012), causing the increased production of thicker, more viscous mucous (Hallstrand et al., 2012). Specifically, epithelial growth factor (EGF) increases the secretion of MUC5AC by goblet cells (Hallstrand et al., 2007), causing a decrease in airway diameter (McBrien & Menzies-Gow, 2017; Thorburn & Hansbro, 2010). As a result, the airway smooth muscle becomes hyperresponsive and more permeable to pro-inflammatory mediators triggering bronchoconstriction (Earl et al., 2015; Weiler et al., 2016).

2.3.1.1 Bronchial Epithelial Cells

Following the mechanisms outlined above, bronchial epithelial cells play an important early role in driving airway inflammation (Saglani & Lloyd, 2015). Even at rest, impairments to the integrity of bronchial epithelial cell tight junctions are present in people with asthma (Wawrzyniak et al., 2017). As outlined in Figure 2.1, this is characterised by the decreased expression of junctional molecules, such as occludin, claudin-4, and zonula occludens-1 (ZO-1); as well as adaptor proteins, gap and adherens junctions, including plactoglobin, nectin-1, nectin-2, and connexin-26, respectively (Wawrzyniak et al., 2017). These impairments have been attributed to the upregulation of histone deacetylase (HDAC) enzymes, such as HDAC9 (Sugita et al., 2018; Wawrzyniak et al., 2017). In contrast, inhibiting the activity of HDAC9 restores bronchial epithelial integrity by increasing the expression of junctional

molecules (Wawrzyniak et al., 2017), suggesting epigenetic modifications could be attributed to regulating histone acetyltransferases (HAT; Wawrzyniak et al., 2017).

As well as being a protective barrier (Sugita et al., 2018), the bronchial epithelium plays a critical role in the development of immune cells (Lai et al., 2014). In EIB, the increased production of IL-33 drives the development of pro-inflammatory mediators such as cysteinyl leukotrienes (Lai et al., 2014). The production of cysteinyl leukotrienes is mediated by the exogenous and endogenous production of secreted phospholipase A₂ group X (sPLA₂-X; Figure 2.2; Hallstrand et al., 2012; 2016). As outlined by Hallstrand et al. (2012; 2016), sPLA₂-X is exogenously produced by bronchial epithelial cells, and endogenously produced by eosinophils. Furthermore, TNF- α and IL-1 β also drive the production of cysteinyl leukotrienes (Hallstrand et al., 2016).

Inhibiting the activity of HDACs is essential to restoring bronchial epithelial integrity (Wawrzyniak et al., 2017), and attenuating airway eosinophilia (Hallstrand et al., 2016). The importance of HDAC inhibition extends beyond bronchial epithelial cells to fork-head box protein 3 (FoxP3⁺) regulatory T cells (T_{REG}; Arpaia et al., 2013), and pathogenic T_H2 memory cells (pT_H2_{MEM}; Endo et al., 2015). Interestingly, dietary fibre-based interventions such as prebiotics inhibit the activity of HDACs (Koh et al., 2016), yet investigations remain limited in adults with EIB (Halnes et al., 2017; McLoughlin et al., 2019; Williams et al., 2016).

2.3.1.2 Innate Lymphoid Cells

At the gastrointestinal and respiratory mucosa, group 3 innate lymphoid cells (ILC3s) maintain the structure and integrity of epithelial cells, helping to regulate the translocation of pro-inflammatory mediators between mucosal surfaces (Klose & Artis, 2016; McLoughlin & Mills, 2011; Sugita et al., 2018; Vieira & Pretorius, 2010). In contrast, the production of IL-33 by bronchial epithelial cells causes ILC2s to drive the differentiation of T_H2 cells via IL-13 dependent mechanisms (Halim et al., 2014; Maazi et al., 2018; Sugita et al., 2018). Prostaglandin D₂ (PGD₂) is essential to the activation of ILC2s, the production of IL-5, and the expression of both CD25 and GATA-3, respectively (Maric et al., 2018). The production of IL-5 via PGD₂ is partly mediated by cyclooxygenase-2 (COX-2), haematopoietic prostaglandin D synthase (hPGDS), and chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2), respectively (Maric et al., 2018).

As outlined in an IL-33-based model of airway inflammation, ILC2s decrease bronchial epithelial integrity and increase permeability by producing IL-13 (Halim et al., 2014; Sugita et al., 2018). Neutralising IL-13 restores bronchial epithelial integrity (Sugita et al., 2018), whereas removing ILC2s prevents airway inflammation. These results highlight the critical role of ILC2s in driving bronchial epithelial leakiness and airway inflammation. In addition, ILC2s also play an essential role in driving the differentiation of T_H2 cells (Halim et al., 2014). Naïve CD4⁺ T cells do not express receptors for IL-33, TSLP, or IL-25 (Halim et al., 2014). Therefore, the differentiation of T_H2 cells cannot be directly stimulated via bronchial epithelial cytokines (Halim et al., 2014). The gap between bronchial epithelial cytokines and dendritic cells is bridged by ILC2s, helping to drive the differentiation of T_H2 cells (Halim et al., 2014). Without ILC2s, the differentiation of CD4⁺ GATA-3⁺ T_H2 cells is significantly down regulated (Halim et al., 2014), attenuating airway eosinophilia and mucous hypersecretion.

2.3.1.3 Mast Cells

Mast cells densely populate the airways of people with EIB and asthma (Altman et al., 2019; Carroll et al., 2002; Lai et al., 2014). In EIB, mast cells translocate from the submucosa to the epithelium, driving airway inflammation by increasing the expression of IL-4, IL-5, and IL-13 (Figure 2.4; Altman et al., 2019; Earl et al., 2015; Lai et al., 2014). Specifically, tryptase (TPSAB1⁺), carboxypeptidase A3 (CPA3⁺), chymase-1 (CMA1⁻) mast cells (TPSAB1⁺ CPA3⁺ CMA1⁻ MC_{T/CPA3} mast cells) drive EIB (Altman et al., 2019, Lai et al., 2014). The production of TSLP and IL-33 by bronchial epithelial cells causes mast cells to translocate from the submucosa to the bronchial epithelium, where they differentiate into MC_{T/CPA3} mast cells (Altman et al., 2019; Couto et al., 2018; Lai et al., 2014). Once activated, MC_{T/CPA3} mast cells degranulate, producing cysteinyl leukotrienes via immunoglobulin E (IgE) dependent mechanisms (Lai et al., 2014), and IL-5/IL-13 via IL-33 dependent mechanisms, respectively (Altman et al., 2019). In a feed-forward loop, IL-33 primes MC_{T/CPA3} mast cells (Altman et al., 2019; Lai et al., 2014). In return, MC_{T/CPA3} mast cells increase the production of IL-33, sustaining the production of IL-5 and IL-13 required to drive airway inflammation (Altman et al., 2019).

2.3.1.4 Dendritic Cells

2.3.1.4.1 Plasmacytoid Dendritic Cells (pDCs)

Dendritic cells mediate type-2 inflammation through innate and adaptive pathways (Lambrecht & Hammad, 2010). Specifically, plasmacytoid dendritic cells (pDCs) support the tolerogenic immune response at mucosal surfaces, preventing airway inflammation (Lombardi et al., 2012). Through innate pathways, type-2 inflammation is driven by ILC2s (Halim et al., 2014; Maazi et al., 2018; Sugita et al., 2018). Plasmacytoid dendritic cells mediate ILC2 driven type-2 inflammation by producing interferon alpha (IFN- α , Figure 2.5; Maazi et al., 2018). This attenuates the proliferation and survival of ILC2s, as well as the production of IL-5 and IL-13 (Maazi et al., 2018).

2.3.1.4.2 CD8 α ⁺ β ⁺ Plasmacytoid Dendritic Cells (CD8 α ⁺ β ⁺ pDCs)

Through the adaptive immune system, dendritic cells attenuate type-2 responses by acting as antigen presenting cells in the development of induced T_{REG} cells (iT_{REG}; Gourbeyre et al., 2011; Hansel et al., 2013; Holgate, 2012; Lombardi et al., 2012; Male et al., 2013; Murdoch & Lloyd, 2010). The suppressive capacity of certain T_{REG} cell subsets is mediated by the expression of FoxP3, an anti-inflammatory transcription factor (Sakaguchi et al., 2013). Specifically, CD8 α ⁺ β ⁺ pDCs prevent the development of airway hyperresponsiveness by driving the proliferation of CD4⁺ CD25⁺ FoxP3⁺ nT_{REG} cells by producing retinoic acid and transforming growth factor beta (TGF- β , Figure 2.6; Fantini et al., 2004; Lombardi et al., 2012). As outlined by Lombardi et al. (2012), TGF- β increases the expression of genes associated with retinoic acid in CD8 α ⁺ β ⁺ pDCs. Inducing the proliferation of FoxP3⁺ T_{REG} cells via CD8 α ⁺ β ⁺ pDCs prevents the proliferation of T_H2 cells and the production of IL-4/IL-13 (Lombardi et al., 2012).

2.3.1.5 Eosinophils

Eosinophils play a critical role in driving type-2 inflammation via IL-3, IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF) dependent mechanisms (McBrien and Menzies-Gow, 2017), accounting for ~ 50% of pulmonary leukocyte infiltration (Matsuda et al., 2018; 2019). In addition, eosinophils drive airway hyperresponsiveness and mucous hypersecretion (McBrien & Menzies-Gow, 2017). The magnitude of airway eosinophilia is underpinned by the broad range of pro-inflammatory pathways activated by eosinophils (McBrien &

Menzies-Gow, 2017). Eosinophils contain a broad range of pro-inflammatory mediators, such as cysteinyl leukotrienes (Hallstrand et al., 2012; Weiler et al., 2016), interacting with innate and adaptive arms of the immune system via an extensive network of cell surface receptors to pro-inflammatory mediators, including IL-33, TSLP, TGF- β , IL-5, IL-13, and IgE, respectively (McBrien & Menzies-Gow, 2017).

Eosinophils are recruited to the airways and lung tissues through direct and indirect mechanisms. Through direct mechanisms, eosinophils are recruited by conventional dendritic cells type-1 (cDC1s) via the increased secretion of C-C motif chemokine ligand 17 (CCL17) and CCL22 upon allergen exposure (Figure 2.1; Januskevicius et al., 2020; Yi et al., 2018). In addition, DC2s increase the secretion of CCL17 and CCL22 by producing nitric oxide via inducible nitric oxide synthase (iNOS; Yi et al., 2018). Through indirect mechanisms, chemoreceptors such as C-C motif chemokine receptor 3 (CCR3), and CCR1 recruit eosinophils to the lungs (McBrien & Menzies-Gow, 2017; Ying et al., 1999).

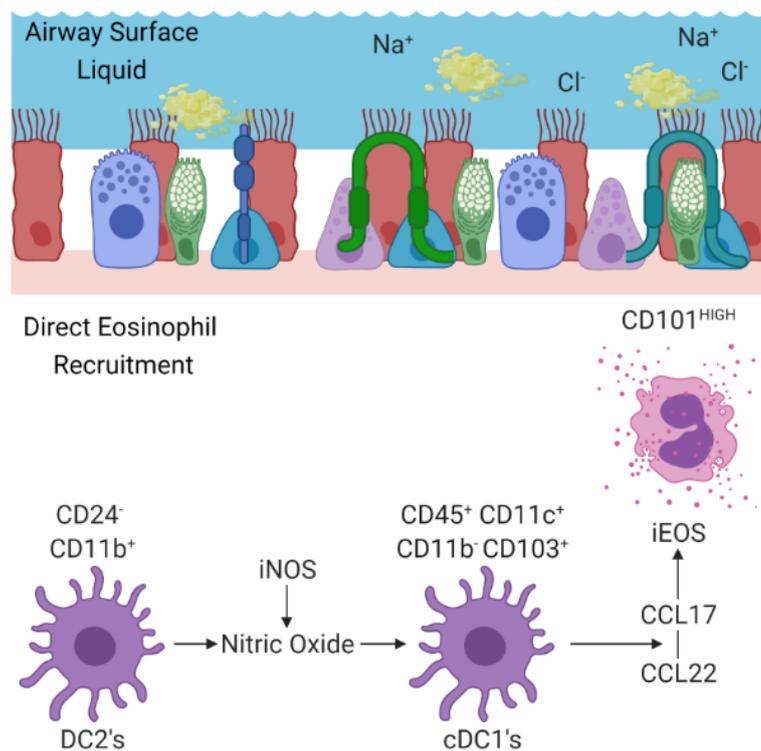


Figure 2.1 cDC1s recruit inflammatory eosinophils to the airways via the increased secretion of CCL17 and CCL22, mediated by nitric oxide (Yi et al., 2018). Created with www.BioRender.com. cDC1 conventional dendritic cell type-1, iEOS inflammatory eosinophil.

Eosinophils create the opportune environment to drive airway inflammation by making the airways more acidic (Kostikas et al., 2002; Kottyan et al., 2009). This is highlighted by the decrease in airway pH of adults with asthma compared to healthy individuals (Kostikas et al., 2002; Kottyan et al., 2009). Eosinophils drive airway inflammation by increasing the adenylate cyclase mediated production of intracellular cyclic adenosine 5'-monophosphate (cAMP) via g-protein coupled receptor 65 (GPR65) dependent mechanisms (Kottyan et al., 2009). In more acidic environments, GPR65⁺ eosinophils are more capable of degranulation and more resistant to apoptosis (Kottyan et al., 2009), increasing the severity of type-2 inflammation.

2.3.1.5.1 Inflammatory Eosinophils (iEOS) and Lung-Resident Eosinophils (rEOS)

Eosinophils can be classified into two subtypes, type-2 inflammatory eosinophils (CD101⁺ iEOS), or lung-resident eosinophils (rEOS; Januskevicius et al., 2020). Inflammatory eosinophils are the most abundant subtype found in the blood of people with moderate asthma (Januskevicius et al., 2020). In addition, higher numbers of activated eosinophils (and basophils) reside in the submucosa of people with asthma who demonstrate severe airway hyperresponsiveness (Ying et al., 1999). Following bronchial allergen challenge, inflammatory eosinophils demonstrate increased viability in people with moderate asthma, as well as greater adherence to airway smooth muscle cells (Januskevicius et al., 2020).

2.3.1.6 Regulatory T Cells

In healthy individuals, T_{REG} cells orchestrate the tolerogenic immune response to attenuate type-2 inflammation (Bohm et al., 2015; Hong et al., 2018; Maazi et al., 2018; Van der Veeken et al., 2016). However, exposure to pro-inflammatory environments alters the expression of chemokine receptors in both regulatory and effector T cells (Bromley et al., 2008; Thomas et al., 2007; Vijayanand et al., 2010). A reduction in the number and/or suppressive capacity of T_{REG} cells has been observed in people with asthma, causing overactive type-2 immune responses (Chen et al., 2018; Hansel et al., 2013; Hartl et al., 2007; Holgate, 2012; Hong et al., 2018; Josefowicz et al., 2012; Lotvall et al., 2012; Ostroukhova et al., 2004; Thorburn & Hansbro, 2010; Verheijden et al., 2015B; 2016; Warrington et al., 2011).

When compared to healthy individuals, T_{REG} cells from people with asthma are more prone to apoptosis, and less able to proliferate, due to the decreased expression of Ki-67 (Chen et al., 2018; Lotvall et al., 2012). Impairments in suppressive capacity have been attributed to the decreased expression of neuropilin-1 (NRP-1), cytotoxic T-lymphocyte antigen-4 (CTLA-4), and glucocorticoid-induced tumor necrosis factor receptor (GITR), respectively (Chen et al., 2018). Alarming, T_{REG} cells can adopt a pathogenic T_{H2} phenotype (Chen et al., 2017), by decreasing their expression of CTLA-4 and IL-10, and increasing their expression of IL-4, IL-5, IL-13, and GATA-3, respectively (Chen et al., 2018). When compared to healthy individuals, the expression of FoxP3 is increased in T_{REG} cells from people with asthma (Chen et al., 2018; Lotvall et al., 2012), potentially reflecting increased attempts to inhibit type-2 inflammation. In contrast, the relative expression of FoxP3 is decreased compared to GATA-3, supporting the adoption of a T_{H2} phenotype (Chen et al., 2018).

T_{REG} cells can be split into various subtypes depending on whether they are induced (peripheral) or natural (thymically derived) in origin (Bohm et al., 2015). Type-1 IL-10 producing T_{REG} cells are induced (CD39⁺ CD4⁺ IL-10⁺ iT_{R1} cells; Bohm et al., 2015; Holgate, 2012; Matsuda et al., 2017; 2018; 2019), whereas FoxP3⁺ T_{REG} cells can be induced (iT_{REG}; CD4⁺ CD25⁻ FoxP3⁺) or natural in origin (nT_{REG}; CD4⁺ CD25⁺ FoxP3⁺), depending on the location of antigen exposure (Bohm et al., 2015; Fantini et al., 2004; Khumalo et al., 2020; Lombardi et al., 2012; Ostroukhova et al., 2004; Polansky et al., 2010; Sakaguchi et al., 2013). IL-10⁺ iT_{R1} cells, FoxP3⁺ iT_{REG} cells, and FoxP3⁺ nT_{REG} cells play critical, yet divergent roles in attenuating type-2 inflammation (Bohm et al., 2015; Hong et al., 2018; Kearley et al., 2005; 2008; Matsuda et al., 2017; 2018; 2019).

2.3.1.6.1 IL-10⁺ T_{R1} Cells

As outlined in an OVA-based model of allergic asthma, IL-10⁺ iT_{R1} cells can attenuate established airway inflammation and hyperresponsiveness (Kearley et al., 2005; 2008), and prevent the development of airway remodelling (Matsuda et al., 2017; 2018; 2019). Interleukin-10 decreases the recruitment of T_{H2} cells, as well as the production of IL-5, IL-13, and TGF-β1 (Kearley et al., 2005; 2008; Matsuda et al., 2017; 2019), reducing the severity of airway eosinophilia and mucous hypersecretion (Figure 2.2). However, their ability to reverse established airway remodelling remains to be determined (Kearley et al., 2008; Matsuda et al., 2017). Following challenge with OVA, the chemokines required to transfer and retain iT_{R1} cells in the lungs are decreased (CCL22, CCL17, and CCL1, respectively; Kearley et al., 2008). Despite these observations, IL-10⁺ iT_{R1} cells are still more abundant in the lungs following antigen exposure than FoxP3⁺ T_{REG} cells (Matsuda et al., 2019), suggesting a direct role of IL-10⁺ iT_{R1} cells in attenuating type-2 inflammation.

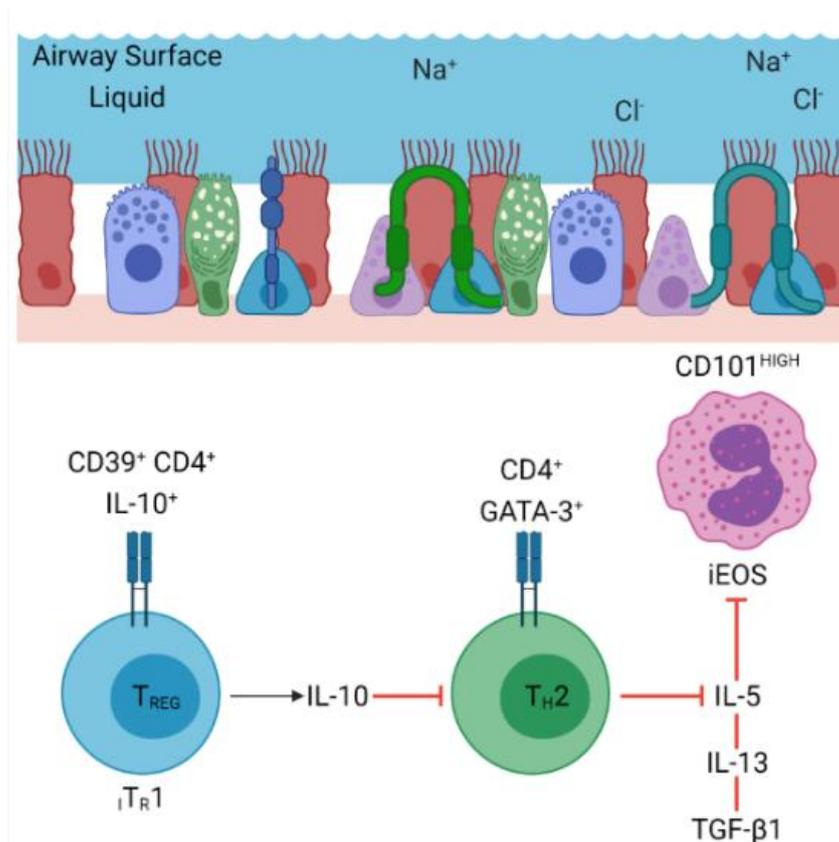


Figure 2.2 IL-10⁺ iT_{R1} cells attenuate airway eosinophilia by decreasing the recruitment of T_{H2} cells, and the production of pro-inflammatory mediators (Matsuda et al., 2017; 2019). Created with www.BioRender.com. iT_{R1} induced type-1 regulatory T cells. Red flat-headed arrows indicate the inhibition of inflammation.

Only ~ 6.5% of IL-10⁺ iT_R1 cells co-express FoxP3 (Matsuda et al., 2019), suggesting that FoxP3⁺ T_{REG} cells play a limited role in directly attenuating type-2 inflammation. As outlined by Johnson et al. (2018), IL-10⁺ iT_R1 cells rely on FoxP3⁺ T_{REG} cells to maintain their suppressive capacity. In an OVA-based model of subcutaneous immunotherapy (SCIT; Matsuda et al., 2018), IL-10⁺ iT_R1 cells proliferated in response to challenge with OVA, whereas FoxP3⁺ T_{REG} cells did not. Less than 5% of IL-10⁺ iT_R1 cells expressed FoxP3 following SCIT and challenge with OVA (Matsuda et al., 2018), providing further support for the direct role of IL-10⁺ iT_R1 cells in attenuating type-2 inflammation.

2.3.1.6.2 FoxP3⁺ T_{REG} Cells

Under inflammatory conditions, the H4 and H3K9 FoxP3 promoter regions become less accessible due to the increased activity of histone deacetylases (Thorburn et al., 2015; Zhang et al., 2012), leading to FoxP3 expression being lost (Khumalo et al., 2020; Matsuda et al., 2018; 2019; Polansky et al., 2010). Importantly, the predominant T_{REG} cell subset responsible for attenuating type-2 inflammation may depend on the inflammatory stimuli being investigated (Matsuda et al., 2018), as well as the amount of antigen encountered, and the pulmonary compartment(s) investigated. Matsuda et al. (2018), administered SCIT prior to challenge with OVA. As a result, their findings are more relevant to preventing the development of asthma, as opposed to attenuating established asthma (Bohm et al., 2015). In contrast, the reality of current treatment involves attenuating established asthma, as opposed to protecting against its development (GBD Chronic Respiratory Disease Collaborators, 2020; Mukherjee et al., 2016; Pavord et al., 2017). Therefore, researchers must consider whether their results intend to inform the future prevention of asthma, or the current attenuation of asthma, by investigating the efficacy of new therapies in acute or chronic asthma models (Bohm et al., 2015; Kearley et al., 2005; 2008).

By establishing chronic asthma before administering SCIT, Bohm et al. (2015), demonstrated the divergent roles of FoxP3⁺ nT_{REG} cells and IL-10⁺ iT_{REG} cells in attenuating established type-2 inflammation. Without FoxP3⁺ nT_{REG} cells, IL-10⁺ iT_R1 cells were less able to proliferate (Bohm et al., 2015). Furthermore, without IL-10, airway inflammation, hyperresponsiveness, and remodelling were not attenuated following SCIT (Bohm et al., 2015). These results suggest that FoxP3⁺ nT_{REG} cells partly mediate the proliferation of IL-10⁺ iT_R1 cells (Bohm et al., 2015), and that IL-

10 plays a critical role in directly attenuating type-2 inflammation. The role of FoxP3⁺ T_{REG} cells in directly attenuating type-2 inflammation is less clear (Matsuda et al., 2019), due to their decreased numbers and rate of proliferation upon exposure to relevant challenges.

The ability of T_{REG} cells to maintain FoxP3 expression under inflammatory conditions is essential to sustaining their suppressive capacity (Lombardi et al., 2012; Sakaguchi et al., 2013), and the development of IL-10⁺ iT_{R1} cells (Bohm et al., 2015). The loss of suppressive capacity is driven by the activation of FoxP3⁺ T_{REG} cells by IL-33 (Chen et al., 2017). Lung derived FoxP3⁺ iT_{REG} cells express the IL-33 receptor ST2 (Chen et al., 2017). When exposed to IL-33, the number of FoxP3⁺ T_{REG} cells expressing ST2 significantly increases, leading to the adoption of a T_{H2} phenotype (Figure 2.3; Chen et al., 2017; 2018). Consequently, the production of TGF-β decreases, and the expression of GATA-3 and ST2 increases (Chen et al., 2017). As a result, this increases the production of IL-5 and IL-13 (Chen et al., 2017), and the inability to suppress responder T cells from proliferating into T_{H2} cells. Khumalo et al. (2020), proposed that the suppressive capacity of FoxP3⁺ iT_{REG} cells can be sustained by maintaining the expression of IL-4 receptor alpha (IL-4Rα).

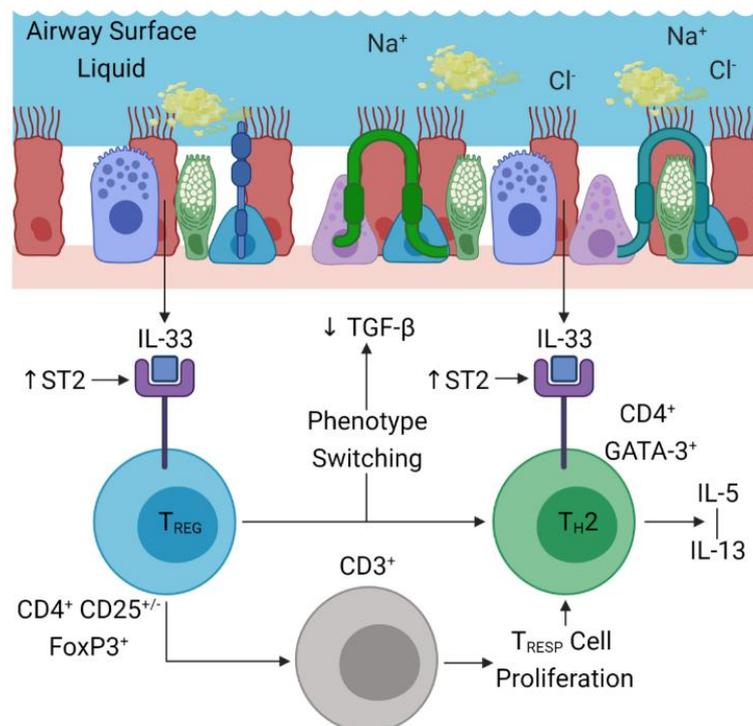


Figure 2.3 IL-33 causes regulatory T cells to adopt a T_{H2} phenotype, increasing the production of pro-inflammatory mediators (Chen et al., 2017; 2018). Created with www.BioRender.com. T_{RESP} responder T cell.

mediastinal lymph nodes can maintain the expression of FoxP3 following the deletion of IL-4R α , a significant reduction is observed in lung derived iT_{REG} cells (Khumalo et al., 2020). These results suggest that FoxP3⁺ iT_{REG} cells maintain their suppressive capacity in the mediastinal lymph nodes, but lose their suppressive capacity upon transfer to the lungs (Hong et al., 2018; Khumalo et al., 2020). The inability of lung derived FoxP3⁺ iT_{REG} cells to maintain their suppressive capacity may be attributed to the increased proliferation of T_{H2} cells in this compartment. In the mediastinal lymph nodes, the increased production of IL-10 and IFN- γ attenuates the proliferation of T_{H2} cells (Khumalo et al., 2020). In the lungs, without sufficient IL-10 and IFN- γ , the decreased suppressive capacity of FoxP3⁺ iT_{REG} cells drives airway eosinophilia, airway hyperresponsiveness, and mucous hypersecretion (Khumalo et al., 2020). In response to SCIT, Bohm et al. (2015), observed a decrease in the number of T_{H2} cells, and the production of IL-5 in the mediastinal lymph nodes, but not in the lungs. These results suggest that the pro-inflammatory environment of the lungs impairs the ability of FoxP3⁺ iT_{REG} cells to attenuate type-2 inflammation. For FoxP3⁺ iT_{REG} cells to attenuate type-2 inflammation, the actions of positive regulators such as TGF- β 1 must exceed those of negative regulators such as IL-5 (Ostroukhova et al., 2004).

Induced T_{REG} cells acquire the expression of TGF- β 1 and FoxP3 following tolerance to OVA (Chen et al., 2018; Ostroukhova et al., 2004), enhancing their ability to attenuate type-2 inflammation. As outlined by Ostroukhova et al. (2004), TGF- β 1 is a marker of FoxP3⁺ iT_{REG} cell viability that helps attenuate type-2 inflammation by maintaining FoxP3 expression (Fantini et al., 2004; Ostroukhova et al., 2004). In addition, TGF- β 1 drives the chitotriosidase-1 (Chit1) dependent differentiation of lung derived FoxP3⁺ iT_{REG} cells (Figure 2.5; Hong et al., 2018). Chitotriosidase-1 is primarily expressed by alveolar macrophages, and to a lesser extent by bronchial epithelial cells (Hong et al., 2018). As part of a protective mechanism, the expression of Chit1 increases in response to OVA (Hong et al., 2018). Mice deficient in Chit1 (Chit1^{-/-}) demonstrate significant increases in the recruitment of T_{H2} cells, the production of IL-4, IL-5, and IL-13, and the magnitude of airway eosinophilia (Hong et al., 2018). In addition, the production of TGF- β 1 and IL-10 significantly decreases (Hong et al., 2018), preventing the differentiation of FoxP3⁺ iT_{REG} cells. Without TGF- β 1, Chit1 is unable to drive the differentiation and proliferation of FoxP3⁺ iT_{REG} cells (Hong et al., 2018), increasing the proliferation of T_{H2} cells (Ostroukhova et al., 2004). Conversely, following co-culture with TGF- β 1 and Chit1,

the differentiation and proliferation of FoxP3⁺ iT_{REG} cells increases (Fantini et al., 2004; Hong et al., 2018). Chitotriosidase-1 increases the expression of TGF-β receptor I (TGFRI) mediated by TGF-β1 in naïve T cells, driving the differentiation of FoxP3⁺ iT_{REG} cells (Fantini et al., 2004; Hong et al., 2018). In turn, FoxP3 sustains the signalling of TGF-β1 mediated by Smad3/4 by downregulating Smad7 (Fantini et al., 2004). These results demonstrate a lung specific mechanism for the synergistic effects of TGF-β1 and Chit1 in driving the differentiation and proliferation of FoxP3⁺ iT_{REG} cells, primarily by maintaining the expression of TGFRI in naïve T cells (Fantini et al., 2004; Hong et al., 2018; Ostroukhova et al., 2004).

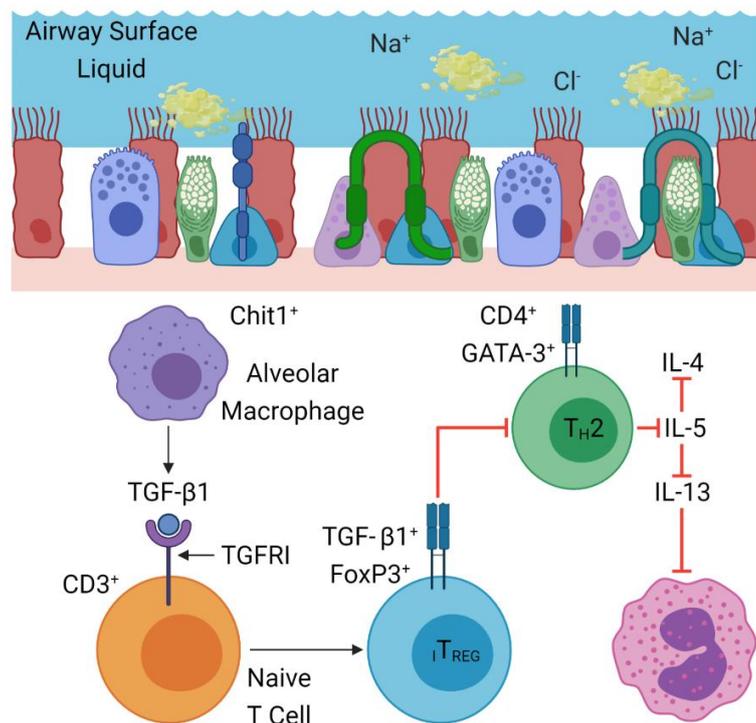


Figure 2.5 TGF-β1 drives the chitotriosidase (Chit1) dependent differentiation of induced FoxP3⁺ regulatory T cells (Hong et al., 2018). Created with www.BioRender.com. *TGFR1* transforming growth factor beta receptor 1. Red flat-headed arrows indicate the inhibition of inflammation.

In contrast to the anti-inflammatory roles outlined above, TGF-β1 also plays pro-inflammatory roles in EIB (Hong et al., 2018; Yi et al., 2018). Specifically, TGF-β1 can drive airway hyperresponsiveness (Ojiaku et al., 2018), and induce tolerance to short-acting β₂-agonists (Ojiaku et al., 2019). Interestingly, TGF-β1 can drive airway hyperresponsiveness independently, or exacerbate histamine induced airway hyperresponsiveness (Ojiaku et al., 2018). Histamine causes bronchoconstriction by binding to GPRs on the airway smooth muscle membrane (Ojiaku et al., 2018).

Pre-treating airway smooth muscle cells with TGF- β 1 causes a significant reduction in airway diameter following histamine exposure (Ojiaku et al., 2018). In addition, TGF- β 1 increases the stiffness of airway smooth muscle cells during contraction (Ojiaku et al., 2018), making them more resistant to relaxation via short-acting β 2-agonists (Ojiaku et al., 2019). Whether the impairment of airway smooth muscle cell relaxation caused by TGF- β 1 occurs through GPR dependent mechanisms has yet to be investigated (Ojiaku et al., 2019), but remains an exciting therapeutic prospect for exercise-induced bronchoconstriction, given that a nutritional intervention in the form of a synbiotic has been shown to modulate the expression and activity of pulmonary GPRs (Halnes et al., 2017).

From an epigenetic perspective, attenuating the activity of HDAC6 increases FoxP3 protein content and acetylation status in T_{REG} cells (Beier et al., 2012), whereas inhibiting HDAC9 increases the suppressive capacity of FoxP3⁺ T_{REG} cells (Beier et al., 2012; Hou et al., 2016). As outlined by Hou et al. (2016), HDAC9 has been associated with an increase in the severity of asthma, but inhibiting HDAC9 via Trichostatin-A (TSA) upregulates the expression of FoxP3 in T_{REG} cells by increasing the production of TGF- β . This leads to a significant decrease in the production of IL-4 and the magnitude of airway eosinophilia (Hou et al., 2016).

The involvement of T_{REG} cell subsets in exercise-induced bronchoconstriction has yet to be investigated. The inability to accurately characterise T_{REG} cell subsets in exercise-induced bronchoconstriction has been driven by the lack of lineage specific markers (Matsuda et al., 2019; Sakaguchi et al., 2013). To improve the characterisation of T_{REG} cells in exercise-induced bronchoconstriction the number of lineage specific markers must be increased by assessing the response to hyperosmotic stimuli (Sakaguchi et al., 2013). Understanding the phenotypic and functional changes that occur in response to hyperosmotic stimuli will enable the development of targeted therapies capable of manipulating the plasticity of T_{REG} cells via epigenetic mechanisms (Van der Veecken et al., 2016).

2.3.1.7 T-Helper Type 2 (T_H2) Cells

Highly differentiated T_H2 cells drive airway inflammation by producing IL-4, IL-5, and IL-13 (Endo et al., 2015; Mitson-Salazar et al., 2016; Thorburn & Hansbro, 2010). However, T_H2 cells are not the only T cells capable of adopting a T_H2 phenotype (Chen et al., 2017; 2018). Pathogenic T_{REG} cells adopt a T_H2 phenotype, enabling the production of IL-4, IL-5, and IL-13 (Chen et al., 2017; 2018). The phenotypic and

functional crossover between T cell subsets has been attributed to the plasticity of T cell lineages (Lotvall et al., 2012). Activated T cells are capable of co-expressing multiple, master regulatory transcription factors, traditionally thought to represent terminally differentiated subsets (Lotvall et al., 2012). For example, activated T cells can co-express T-Bet (T_{H1}), GATA-3 (T_{H2}), FoxP3 (T_{REG}), and ROR γ T (T_{H17}), respectively (Lotvall et al., 2012), causing great difficulty in characterising the role of T cell subsets in type-2 inflammation.

IL-10 directly attenuates the differentiation, survival, and pro-inflammatory capacity of T_{H2} cells by driving apoptosis via the upregulation of granzyme B (Coomes et al., 2017). As outlined in an HDM-based model of allergic asthma, IL-10R α ^{-/-} granzyme B^{-/-} mice demonstrate a significant increase in the proliferation of T_{H2} cells, the production of IL-5 and IL-13, airway eosinophilia, and MUC5AC mediated mucous hypersecretion, respectively (Coomes et al., 2017). In contrast, the adoptive transfer of IL-10^{+/+} T_{H2} cells attenuates type-2 inflammation (Coomes et al., 2017).

At rest, the number of FoxP3⁺ T_{REG} cells, and GATA-3⁺ T_{H2} cells, do not differ between people with high and low eosinophilic asthma, or healthy individuals (Lotvall et al., 2012). Importantly however, following stimulation, the number of T cells co-expressing FoxP3 and GATA-3 increases in people with high eosinophilic asthma (Lotvall et al., 2012). The increased number of GATA-3⁺ T cells in people with asthma has been associated with a decrease in percentage predicted forced expiratory volume in one second (FEV₁; Chen et al., 2018), and increase in airway hyperresponsiveness (Lotvall et al., 2012). It remains to be determined whether T cells that co-express multiple transcription factors are yet to differentiate, or are terminally differentiated cells switching between subsets (Lotvall et al., 2012). Based on these observations, people with severe eosinophilic asthma may have an increased number of pathogenic T_{REG} cells (Chen et al., 2017; Lotvall et al., 2012), which may explain the increased capacity of FoxP3⁺ GATA-3⁺ T cells to produce type-2 cytokines. Furthermore, depending on the markers used to characterise T_{REG} cells, the increased numbers reported in people with severe eosinophilic asthma may be misinterpreted as tolerogenic or pathogenic, with substantial implications for the interpretation of mechanisms.

Eosinophils mediate the recruitment of T_{H2} cells to the lungs (Yi et al., 2018). This occurs through the increased secretion of CCL17 and CCL22 by cDC1s (Yi et al., 2018), supported by the DC2-dependent production of nitric oxide. In addition, IL-4

and TNF- α augment the eosinophil mediated generation of CCL17 and CCL22 by activating STAT6 and NF- κ B, respectively (Liu et al., 2007), increasing the recruitment of CCR4⁺ T_H2 cells (Vijayanand et al., 2010). Eosinophils further support the activation and priming of T_H2 cells by acting as antigen presenting cells via MHC-II (McBrien & Menzies-Gow, 2017).

2.3.1.7.1 Pathogenic T_H2 Memory Cells (pT_H2_{MEM})

The broad role of T_H2 cells in type-2 inflammation is well established. However, research is beginning to outline the specific role of T_H2 cell subsets in type-2 inflammation. Type-2 inflammation is maintained by pathogenic T_H2 memory cells (pT_H2_{MEM}) through IL-33 dependent mechanisms (Figure 2.6; Endo et al., 2015). As outlined in a murine model of chronic rhinosinusitis, pT_H2_{MEM} cells acquire the ability to drive airway eosinophilia and produce IL-5 forty-five days after being challenged with OVA, highlighting their longevity (Endo et al., 2015). In addition, IL-33 generates pT_H2_{MEM} cells by increasing the expression of ST2, sustaining cellular viability and preventing proliferation into effector T cells (Endo et al., 2015). Via epigenetic mechanisms, IL-33 induces remodelling of the *Il5* (IL-5) chromatin structure via p38-MAPK (Endo et al., 2015). The decreased activity of histone acetyltransferases enables pT_H2_{MEM} cells to express and produce IL-5 (Endo et al., 2015), driving airway eosinophilia and hyperresponsiveness.

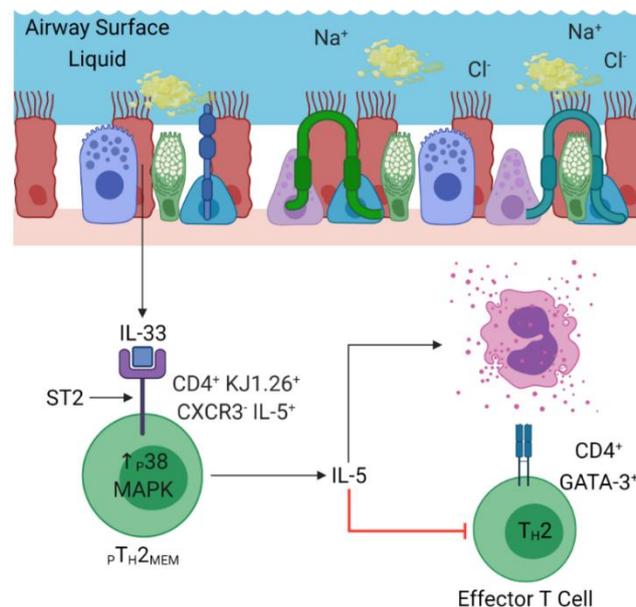


Figure 2.6 Pathogenic T_H2 memory cells (pT_H2_{MEM}) drive airway eosinophilia through IL-33 dependent mechanisms (Endo et al., 2015). Created with www.BioRender.com. Red flat-headed arrows indicate the inhibition of inflammation.

Without the IL-33 receptor (ST2), pT_{H2MEM} cells are unable to drive airway eosinophilia, hyperresponsiveness, mucous hypersecretion, or airway remodelling (Endo et al., 2015). Accordingly, the proliferation of lung-derived pT_{H2MEM} cells is attenuated in IL-33^{-/-} mice challenged with OVA (Endo et al., 2015), reducing the magnitude of airway eosinophilia and the production of IL-5. By inhibiting the IL-33 mediated phosphorylation of p38 via the pharmacological inhibitor SB203580 (Endo et al., 2015), IL-33 was unable to induce the expression of ST2, or the production of IL-5 by pT_{H2MEM} cells (Endo et al., 2015). In addition, SB203580 reversed remodelling of the *I*/5 chromatin structure (Endo et al., 2015). These observations, verified in human CD45RO⁺ CD4⁺ T_{MEM} cells (Endo et al., 2015), demonstrate that IL-33 plays a critical role in prolonging type-2 inflammation via the induction of pT_{H2MEM} cells.

2.3.1.7.2 Pathogenic Effector Memory T_{H2} Cells (peT_{H2})

When compared to conventional T_{H2} cells (cT_{H2}), pathogenic effector memory T_{H2} cells (peT_{H2}) demonstrate an enhanced ability to drive type-2 inflammation (Figure 2.7; Mitson-Salazar et al., 2016). When compared to healthy individuals, the number of peT_{H2} cells are increased in people with allergic asthma (Mitson-Salazar et al., 2016). In contrast, the number of cT_{H2} cells do not differ between healthy individuals and those with allergic asthma (Mitson-Salazar et al., 2016), suggesting that cT_{H2} cells are not the key subset involved in the pathophysiology of exercise-induced bronchoconstriction. As outlined by Mitson-Salazar et al. (2016), peT_{H2} cells highly express CD161. This facilitates the expression of hPGDS and the ability to produce PGD₂ (Mitson-Salazar et al., 2016). Specifically, CD161^{HIGH} hPGDS⁺ peT_{H2} cells express (and produce) more IL-5 and IL-13 than cT_{H2} cells (Mitson-Salazar et al., 2016), and are more responsiveness to stimulation by IL-25, IL-33, and TSLP due to the increased expression of cell surface receptors. The increased expression of hPGDS correlates with the increased expression of IL-5 and the increased magnitude of blood eosinophilia (Mitson-Salazar et al., 2016). The expression of ST2, and the increased responsiveness to IL-33, may augment the differentiation of peT_{H2} cells via ILC2 dependent mechanisms (Halim et al., 2014; Maazi et al., 2018; Mitson-Salazar et al., 2016; Sugita et al., 2018). As peT_{H2} cells are the only T cells that express hPGDS (Mitson-Salazar et al., 2016), hPGDS is an ideal candidate marker to identify and investigate the phenotypic and functional characteristics of peT_{H2} cells in exercise-induced bronchoconstriction.

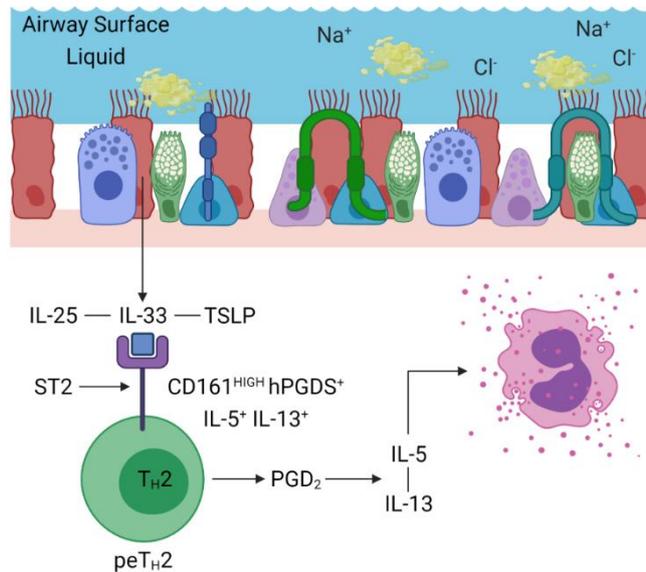


Figure 2.7 Pathogenic effector memory T-helper type-2 cells (peTH₂) drive airway eosinophilia through the increased expression of CD161 and hPGDS, and the increased production of PGD₂ (Mitson-Salazar et al., 2016). Created with www.BioRender.com. *hPGDS* haematopoietic prostaglandin D synthase.

Inhibiting the production of PGD₂ is essential to attenuating type-2 inflammation. As outlined by Maric et al. (2018), PGD₂ is a potent activator of ILC2s. Once activated, ILC2s acquire the ability to express hPGDS and produce PGD₂ (Maric et al., 2018). Blocking hPGDS via CRTH2 antagonists abolishes the activation of ILC2s (Maric et al., 2018), inhibiting their ability to produce PGD₂. As peTH₂ cells also express CRTH2, and produce PGD₂ (Mitson-Salazar et al., 2016), it is plausible to speculate that CRTH2 antagonists could attenuate the severity of exercise-induced bronchoconstriction by inhibiting the activation and pro-inflammatory capacity of peTH₂ cells.

Furthermore, peTH₂ cells demonstrate an increased ability to migrate to and populate sites of inflammation (Mitson-Salazar et al., 2016). In eosinophilic gastrointestinal disease (EGID), peTH₂ cells are more abundant in the gut (Mitson-Salazar et al., 2016). Blood derived peTH₂ cells express the gut homing receptors $\alpha_4\beta_7$ and CCR3 (Mitson-Salazar et al., 2016), enhancing their ability to migrate to the gut via CCL11 dependent mechanisms. Given the role of peTH₂ cells across eosinophilic inflammatory disorders (Mitson-Salazar et al., 2016), it is possible that peTH₂ cells may be more abundant in the lungs of people with exercise-induced bronchoconstriction, and express pulmonary homing receptors when circulating in the blood. The potential dual role of peTH₂ cells in gut and lung-derived eosinophilic

inflammation could represent an important contributor to the gut-lung microbial axis in asthma (Budden et al., 2017; Fujimura & Lynch, 2015; Huang & Boushey, 2014; Kozik & Huang, 2019; Mitson-Salazar et al., 2016; Singanayagam et al., 2017). Intriguingly, the role of $\text{peT}_{\text{H}2}$ cells remains unexplored in exercise-induced bronchoconstriction, let alone the effects of prebiotic supplementation on this subset.

The cascade of events outlined in this section, demonstrating how inflammation is caused during EIB, is well documented. However, emerging research in animal models and other phenotypes of asthma suggest that these pathophysiological mechanisms may be partly mediated by the role of the gut microbiota in regulating host immune function (Begley et al., 2018), and that nutritional interventions such as probiotics, prebiotics, or synbiotics may attenuate various aspects of type-2 inflammation via the gut microbiota by addressing features of gut microbial dysbiosis (Arrieta et al., 2015; Thorburn et al., 2015).

2.4 The Gut Microbiota

2.4.1 Defining the Gut Microbiota and Microbiome

The gut microbiota is the collection of microorganisms that colonise the gastrointestinal tract, whereas the gut microbiome refers to the genes encoded by the microbiota that interact with and modulate host immune function (Almonacid et al., 2017; Clemente et al., 2012; Fujimura & Lynch, 2015; Gibson & Roberfroid, 1999; Sbihi et al., 2019; Sender et al., 2016A; Ursell et al., 2012). The gut microbiota is primarily made up of bacteria that have a symbiotic, neutral, or pathogenic relationship with the host (Grigg & Sonnenberg, 2017; Sender et al., 2016A). Bacteria are classified into 5 major phyla, before being sub-classified at the domain, phylum, class, order, family, genus, species, and strain levels, respectively (Figure 2.8; Barcik et al., 2019; Geva-Zatorsky et al., 2017; Han et al., 2012).

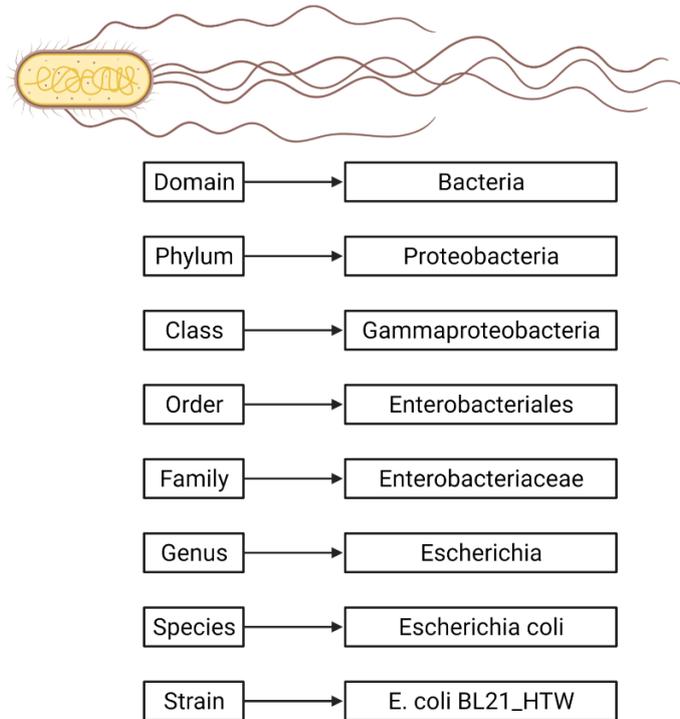


Figure 2.8 A representative example of bacterial classification using *Escherichia coli* (adapted from Han et al., 2012). Created with www.BioRender.com.

The gut microbiota includes other microorganisms such as eukaryotes, archaea, viruses, and fungi (Sender et al., 2016A). Separate terms are used to describe the viral and fungal components of the gut microbiota, including the gut “virome”, and gut “mycobiome”, respectively. For the current programme of work, the terms gut “microbiota”, and “microbiome”, refer only to the bacterial component of the gut microbiota, unless otherwise specified.

2.4.2 Lifestyle & Environmental Factors

In accordance with the hygiene hypothesis (Liu, 2015), various lifestyle and environmental factors associated with Westernised society have been linked to the development of asthma through gut microbial dysbiosis (Arrieta et al., 2015; Halnes et al., 2017; Sbihi et al., 2019). Gut microbial dysbiosis is defined as an imbalance or deficiency in the number or functional capacity of the symbiotic bacteria required to positively modulate host immune function. These lifestyle and environmental factors are classified as macro or micro-level exposures, depending on whether they impact the development of asthma at the population level or the individual level (Sbihi et al., 2019). Macro-level exposures such as second-hand smoke impact a larger number of people, typically with a less severe effect on asthma outcomes (Sbihi et al., 2019). In contrast, micro-level exposures such as caesarean delivery

(Stokholm et al., 2016; Sevelsted et al., 2016; van Nimwegen et al., 2011), impact a smaller number of people with much greater adverse effects on asthma outcomes (Sbihi et al., 2019).

The timing, duration, combination, magnitude, and frequency of macro and micro-level exposures varies by individual, making it difficult to quantify the effect of isolated exposures at the individual level (Sbihi et al., 2019). Research has primarily investigated the effects of macro and micro-level exposures in isolation, without accounting for the confounding effects of other macro or micro-level exposures (Sbihi et al., 2019). As a result, isolated investigations of macro or micro-level exposures should be viewed with caution, especially regarding the proposed relative risk of certain exposures on the development or progression of asthma (Sbihi et al., 2019). Of particular importance to asthma in adults, exposures that occur during pregnancy, early childhood, and adolescence, may only manifest as features of gut microbial dysbiosis and asthma later in life (Clemente et al., 2012; Liu, 2015; Sears et al., 2003). These observations demonstrate the importance of understanding all macro and micro-level exposures that may contribute to the development and treatment of asthma in adulthood (Sbihi et al., 2019).

2.4.2.1 Macro-Level Exposures

Macro-level exposures associated with the development of asthma include urbanisation and social deprivation (Malik et al., 2012; Rodriguez et al., 2019), air pollution and second-hand smoke (He et al., 2020; Khreis et al., 2017; Malik et al., 2012), dampness and mould (Sharpe et al., 2015). An overview of the macro-level exposures associated with the development and progression of asthma is provided in Figure 2.9 below.

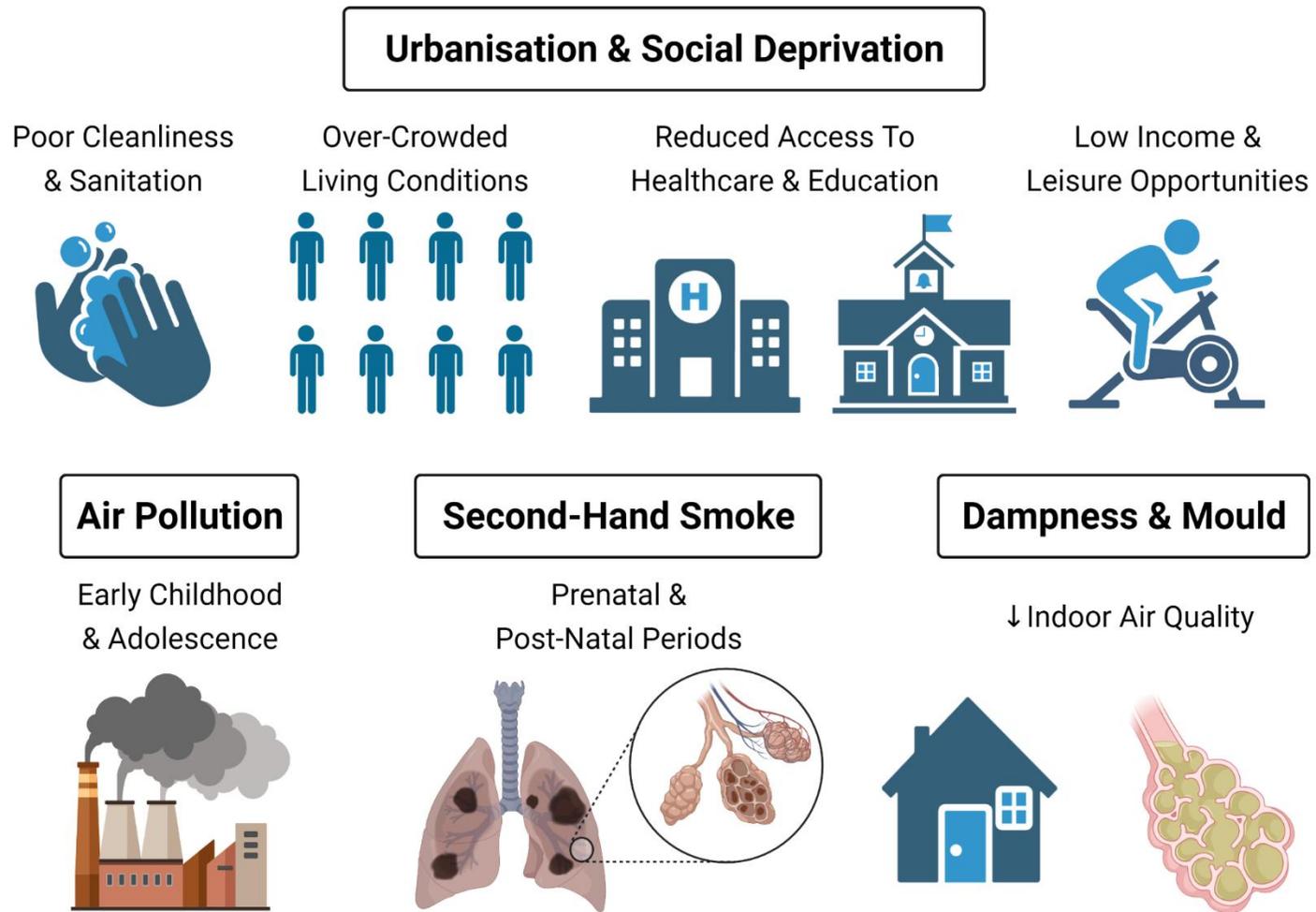


Figure 2.9 Macro-level exposures associated with the development and progression of asthma (Feddema & Claassen, 2020; He et al., 2020; Khreis et al., 2017; Malik et al., 2012; Rodriguez et al., 2019; Sbihi et al., 2019; Sharpe et al., 2015). Created with www.BioRender.com.

2.4.2.2 Micro-Level Exposures

A broad range of micro-level exposures have been associated with the development and progression of asthma, including a maternal history of asthma (Sears et al., 2002; Stockholm et al., 2018), reduced maternal and infant exposure to microbes (Debarry et al., 2007; Gerhold et al., 2006; Reibman et al., 2008; Riedler et al., 2001; Stockholm et al., 2018; Thorburn et al., 2015; Wolsk et al., 2016), birth order (Kragh et al., 2016), skewing of fetal immune responses (Folsgaard et al., 2012; Prescott et al., 1998), mode of birth (Clemente et al., 2012; Stockholm et al., 2016; Sevelsted et al., 2016; van Nimwegen et al., 2011), premature delivery and low birth weight (Sonnenschein-van der Voort et al., 2014), infant feeding regimen (Sears et al., 2002; Tachimoto et al., 2020), rate of weight gain after birth (Sonnenschein-van der Voort et al., 2014), place of delivery (van Nimwegen et al., 2011), vaccination practices (Thorburn et al., 2010), season of birth (Thysen et al., 2016), intrapartum or infant exposure to antibiotics (Arpaia et al., 2013; Arrieta et al., 2015; Cait et al., 2018; Sbihi et al., 2019; Stensballe et al., 2013; Stockholm et al., 2016), dog exposure at birth (Bufford et al., 2008), decreases in family size (Ball et al., 2000; Grigg and Sonnenberg, 2017; Stein et al., 2016; Stockholm et al., 2018; Thorburn et al., 2015; Wolsk et al., 2016), low attendance at day care or nursery (Kramer et al., 1999), exposure to viral lower respiratory tract infections (Feddema and Claassen, 2020; Sigurs et al., 2010), and (of particular importance to the current work) decreases in dietary fibre intake (Dahl, 2006; Folkerts et al., 2018; Huang et al., 2017; McLoughlin et al., 2019; Mukherjee et al., 2016; Singanayagam et al., 2017; Smits et al., 2016; Tan et al., 2014; 2017; Thorburn et al., 2015).

2.4.2.2.1 Habitual Dietary Fibre Intake

Dietary fibre provides an essential source of nutrients for the gut microbiota (Holscher, 2017; Huang et al., 2017). Sufficient dietary fibre intake speeds up retention time (Macfarlane et al., 1998), preventing colonisation of the gastrointestinal tract by pathogenic bacteria. As outlined in an HDM-based model of asthma, administering a high fibre or high acetate diet to pregnant mice prevented the development of asthma in adult offspring (Thorburn et al., 2015). Follow up investigations in non-asthmatic humans demonstrated that sufficient dietary fibre intake during the latter stages of pregnancy was associated with a reduction in the frequency of cough and wheeze in offspring during the first year of life (Thorburn et

al., 2015). This was attributed to increased concentrations of acetate in the mother's serum during pregnancy (Thorburn et al., 2015).

Comparisons of dietary strategies between those who follow ancestral (paleolithic) practices, and those who live in Westernised society, have highlighted the alarming magnitude of insufficient dietary fibre intake characteristic of Westernised society (De Filippo et al., 2010; Schnorr et al., 2014; Smits et al., 2016). When compared to children from Burkina Faso, children from Italy consume much less dietary fibre (De Filippo et al., 2010). Natural wholegrains, legumes, and vegetables were staple foods in the diets of children from Burkina Faso (De Filippo et al., 2010), whereas children from Italy consumed excessive amounts of animal protein, sugar, and starch at the expense of dietary fibre (Folkerts et al., 2018). The increased intake of dietary fibre in children from Burkina Faso was associated with an increase in the number of gastrointestinal *Bifidobacteria*, whereas pathogenic microbes were higher in faecal samples of children from Italy (De Filippo et al., 2010). The decrease in symbiotic gut bacteria reported in Westernised individuals could lead to greater colonisation of the gastrointestinal tract by pathogenic bacteria (Smits et al., 2016).

Comparisons between the Hadza from Tanzania, who follow a hunter-gatherer lifestyle, and those from Westernised society, support the differences observed by De Filippo et al. (2010), demonstrating an increase in gut bacterial diversity in the Hadza when compared to Westernised individuals (Schnorr et al., 2014). It is important to note, however, that different gut microbes serve different purposes depending on the cultural practices of their host, and the surrounding environment (De Filippo & Tuohy, 2014; Schnorr et al., 2014). For example, colonisation of the gastrointestinal tract by *Bifidobacteria* is not a universal signature of a healthy gut microbiota. Some differences are to be expected between those who follow ancestral practices, and those who follow Westernised culture. For example, no *Bifidobacteria* were observed in faecal samples obtained from the Hadza (Schnorr et al., 2014). These results suggest that *Bifidobacteria* are not essential to maintaining immune function in the Hadza, as opposed to any features of gut microbial dysbiosis. Despite the expected differences between ancestral and Westernised cultures, the deficiencies observed in those from Westernised society extended beyond expected cultural or environmental differences, highlighting features of gut microbial dysbiosis that could be related to aberrant immune function.

Decreases in habitual dietary fibre intake have been implicated in the development of gut microbial dysbiosis and asthma (De Filippo et al., 2010; Folkerts et al., 2018; Huang et al., 2017; Liu, 2015; Schnorr et al., 2014; Singanayagam et al., 2017; Trompette et al., 2014; Wood et al., 2015). When compared to healthy controls, adults with severe asthma consume less dietary fibre, have worse pulmonary function, and increased levels of systemic and airway inflammation (Berthon et al., 2013; Wood et al., 2015). Decreases in dietary fibre intake have been associated with decreases in percentage predicted FEV₁, as well as increases in exhaled nitric oxide and airway eosinophilia, respectively (Berthon et al., 2013; Wood et al., 2015). Taken together, the decreased intake of anti-inflammatory nutrients, such as dietary fibre (Berthon et al., 2013), and the increased intake of pro-inflammatory nutrients, such as saturated fat (De Filippo et al., 2010; Wood et al., 2015), lead people with severe asthma to consume a more pro-inflammatory diet.

No decreases in dietary fibre intake have been reported in adults with mild or moderate asthma (Berthon et al., 2013). A potential explanation could be a severity specific relationship between decreases in dietary fibre intake and increases in asthma severity. Alternatively, the absence of association could be due to the validity of dietary assessment methods. The protective effect of dietary fibre reported by Berthon et al. (2013), was based on retrospective data from a validated food frequency questionnaire. More robust methods of assessing dietary fibre intake, such as prospective quantitative monitoring, using software capable of classifying different sources of dietary fibre, could provide further clarity regarding the role of dietary fibre intake in mild-moderate asthma.

2.4.2.2.1.1 Short-Chain Fatty Acids

The fermentation of dietary fibre by the gut microbiota produces anti-inflammatory metabolites called short-chain fatty acids, specifically acetate, propionate and butyrate (Arpaia et al., 2013; Berthon et al., 2013; Holscher, 2017; Macia et al., 2014; Macfarlane et al., 1998; Maslowski et al., 2009; Sbihi et al., 2019; Tan et al., 2014; Thorburn et al., 2015; Trompette et al., 2014). Short-chain fatty acids are produced in the proximal colon (Macfarlane et al., 1998), and play critical roles in sustaining immune function throughout the body.

2.4.2.2.1.2 Gastrointestinal Immune Function

In the gastrointestinal tract, acetate and propionate increase the abundance of colonic T_{REG} cells (Arpaia et al., 2013), whereas butyrate maintains the integrity of epithelial cells by providing a key source of energy to colonocytes (McLoughlin et al., 2019). Specifically, butyrate activates GPR43 and GPR109A on epithelial cells, sustaining mucosal barrier integrity (Holscher, 2017; Macia et al., 2015; Tan et al., 2014; 2017). Maintaining gastrointestinal epithelial integrity protects against damage caused by type-2 inflammation (Josefowicz et al., 2012). Once damaged, the gastrointestinal epithelium becomes more permeable, enabling certain gut bacteria to translocate into the bloodstream (O'Dwyer et al., 2016). Importantly, bacteria that were considered symbiotic whilst in the gastrointestinal tract can be identified as pathogenic upon translocation into the bloodstream (Gourbeyre et al., 2011; Sbihi et al., 2019). Toll like receptors acting as pattern recognition receptors label the bacteria as pathogenic (Gourbeyre et al., 2011; Sbihi et al., 2019). Foreign elements such as lipopolysaccharide in the bacterial cell wall (Ding et al., 2018), and flagellin are identified by dendritic cells as microbe associated molecular patterns, stimulating innate and adaptive immune responses via M-cell dependent mechanisms (Clemente et al., 2012; Hansel et al., 2013; Holgate, 2012; Male et al., 2013; Murdoch & Lloyd, 2010; Sbihi et al., 2019; Tuohy & Scott, 2014). These immune responses have the adverse effect of neutralising symbiotic bacteria in the bloodstream via opsonisation and phagocytosis (Gourbeyre et al., 2011; Male et al., 2013; Warrington et al., 2011). The danger posed to symbiotic microbes by translocating into the bloodstream, and their subsequent inability to support immune function, highlights the importance of maintaining gastrointestinal epithelial integrity through sufficient dietary fibre intake (Budden et al., 2016; Gourbeyre et al., 2011; Huang & Boushey, 2014; Josefowicz et al., 2012; Vieira & Pretorius, 2010).

2.4.2.2.1.3 Systemic & Pulmonary Immune Function

In addition to their effects on immune function in the gastrointestinal tract, short-chain fatty acids also modulate systemic and pulmonary immune function (Sbihi et al., 2019; Schroeder & Backhed, 2016). Specifically, short-chain fatty acids drive the development of anti-inflammatory immune cells (Arpaia et al., 2013; Trompette et al., 2014), and attenuate the production of pro-inflammatory mediators (Cait et al., 2018; Koh et al., 2016; Thorburn et al., 2015).

Histone acetyltransferases (HAT) are the predominant inhibitors of histone deacetylases (HDAC; Alhamwe et al., 2018). Inhibiting the activity of HDAC9 increases the suppressive capacity of FoxP3⁺ T_{REG} cells by enhancing the binding of acetylated FoxP3 to the IL-2 promoter region (Alhamwe et al., 2018; Arpaia et al., 2013; Beier et al., 2012; Hou et al., 2016; Tan et al., 2014; Thorburn et al., 2015). The activity of HATs is increased by short-chain fatty acids via communication with GPRs (Koh et al., 2016). Sputum HDAC9 expression decreases following inulin consumption (McLoughlin et al., 2019), demonstrating the ability of prebiotics to modulate the activity of HDACs via short-chain fatty acids. Given that GPRs are expressed by immune cells, such as eosinophils (Kottyan et al., 2009), dendritic cells (Trompette et al., 2014), and T_{REG} cells (Tan et al., 2017), these observations highlight a potential mechanism of interaction between gut bacterial metabolites and host immune cells.

In systemic circulation, acetate is found in the highest concentrations (Holscher, 2017; Macia et al., 2014; McLoughlin et al., 2019; Tan et al., 2014; 2017; Thorburn et al., 2015; Trompette et al., 2014). Acetate attenuates the development of asthma by binding to GPR43 on immune cells (Macia et al., 2015; Maslowski et al., 2009; Sbihi et al., 2019). In the absence of GPR43, short-chain fatty acids lose their ability to attenuate the development of asthma (Maslowski et al., 2009), leading to an increase in airway inflammation and eosinophil peroxidase activity.

Acetate also attenuates airway inflammation by inhibiting the activity of HDAC9 via FoxP3⁺ T_{REG} cells (Thorburn et al., 2015), increasing acetylation of the H4 and H3K9 FoxP3 promoter regions, respectively. Increased acetylation of the FoxP3 promoter regions increases the expression of FoxP3, increasing the number and suppressive capacity of peripheral lymph node-derived T_{REG} cells (Thorburn et al., 2015). The acetate-induced inhibition of HDAC9 significantly decreased systemic and pulmonary eosinophilia, mucous hypersecretion, and airway hyperresponsiveness (Thorburn et al., 2015). In the absence of HDAC9, mice were unable to develop asthma (Thorburn et al., 2015), demonstrating the importance of HDAC9 in driving the development of asthma. In contrast, in the absence of T_{REG} cells, acetate was unable to attenuate the development of asthma (Thorburn et al., 2015), demonstrating the importance of T_{REG} cells in acetate-mediated immunosuppression. Given the positive correlation reported between sputum eosinophilia and HDAC9 expression (McLoughlin et al., 2019), and the role of

inhibiting HDAC9 in maintaining bronchial epithelial integrity (Wawrzyniak et al., 2017), and FoxP3⁺ T_{REG} cell suppressive capacity (Beier et al., 2012; Hou et al., 2016), HDAC9 is a key therapeutic target for prebiotic supplementation. Interestingly, the effects of galactooligosaccharide-based prebiotic supplementation on HDAC9 remain to be determined, representing an exciting area of future research.

Butyrate and to a lesser extent propionate induce the differentiation of new iT_{REG} cells by increasing the expression of FoxP3 through TGF- β and dendritic cell dependent mechanisms (Arpaia et al., 2013). Butyrate and propionate also preserve the suppressive capacity of established iT_{REG} cells by maintaining the expression of FoxP3 (Arpaia et al., 2013). Inhibiting the activity of HDAC increased acetylation of the FoxP3 protein, subsequently increasing the expression of FoxP3 in iT_{REG} cells (Arpaia et al., 2013). In addition, butyrate also plays a role in suppressing airway eosinophilia, inhibiting the production of TNF- α (Ni et al., 2010), decreasing mucous hypersecretion, and reducing the number of IL-4 producing CD4⁺ T cells (Cait et al., 2018). Butyrate decreases the activation of dendritic cells by increasing the expression of CD80 and CD86, preventing dendritic cells from translocating to the mediastinal lymph nodes where they drive airway inflammation (Cait et al., 2018).

As outlined in an HDM-based model of asthma (Trompette et al., 2014), a high fibre diet or direct feeding of propionate in the drinking water decreased the severity of asthma. Through GPR41-dependent mechanisms, propionate promoted the maturation of CD11b^{HIGH} CD40^{LOW} PD-L2^{LOW} CD86^{LOW} DCs in the lung draining lymph nodes, which had an impaired ability to drive type-2 inflammation (Trompette et al., 2014). This led to a significant decrease in airway eosinophilia, mucous hypersecretion, and airway hyperresponsiveness, respectively (Trompette et al., 2014). In addition, a high fibre diet, or direct feeding with propionate, significantly increased the gastrointestinal abundance of *Bifidobacteria*, as well as serum concentrations of acetate and propionate (Trompette et al., 2014). Conversely, short-chain fatty acids could not be detected in the lungs (Trompette et al., 2014), suggesting that the severity of asthma was attenuated through improvements in systemic immune function.

2.4.3 The Impact of Early Life Factors on Asthma in Adulthood

As outlined by a longitudinal investigation (Sears et al., 2003), various factors associated with the hygiene hypothesis have been linked to the persistence of asthma from childhood to early adulthood, as well as the relapse of asthma during adulthood. Sears et al. (2003), prospectively monitored the persistence of asthma from childhood to early adulthood, conducting objective assessments of pulmonary function and airway hyperresponsiveness at 9, 11, 13, 15, and 21 years of age. Assessments of bronchodilator reversibility were also conducted at 18 and 26 years of age (Sears et al., 2003). The persistence of asthma into adulthood was associated with increased sensitisation to house dust mite, airway hyperresponsiveness between the ages of 9 and 21, being female, and smoking at 21 years of age (Sears et al., 2003), whereas relapse was associated with increased sensitisation to house dust mite, airway hyperresponsiveness between the ages of 9 and 21, and being diagnosed with wheeze during early life.

From childhood to early adulthood, the greatest impairments in pulmonary function were observed in those with persistent wheezing who had ever been prescribed inhaled corticosteroids, and had reported airway hyperresponsiveness on 3 or more occasions (Sears et al., 2003). Importantly, the rate of change in pulmonary function from childhood to early adulthood was similar in participants who did and did not have persistent asthma (Sears et al., 2003). The key difference was the significantly lower starting point for pulmonary function of children with persistent asthma (Sears et al., 2003). These results highlight the importance of controlling for early life exposures during childhood to prevent the persistence of asthma into adulthood.

Although various factors increase the risk of asthma in adulthood, the consumption of dietary fibre decreases the risk of asthma in adulthood (Thorburn et al., 2015). It should be noted, however, that the protective effects of dietary fibre were observed when administered prior to the development of asthma (Thorburn et al., 2015). Further research is required to investigate the effects of dietary fibre on the severity of established asthma. Encouragingly, administering acetate to adult mice reduced HDAC activity, potentially HDAC9 (Thorburn et al., 2015), suggesting that immunological reprogramming induced by dietary fibre during adulthood could increase the suppressive capacity of FoxP3⁺ T_{REG} cells (Beier et al., 2012; Hou et al., 2016). An overview of the early life micro-level exposures associated with the development of asthma is provided in Figures 2.10 and 2.11 below.

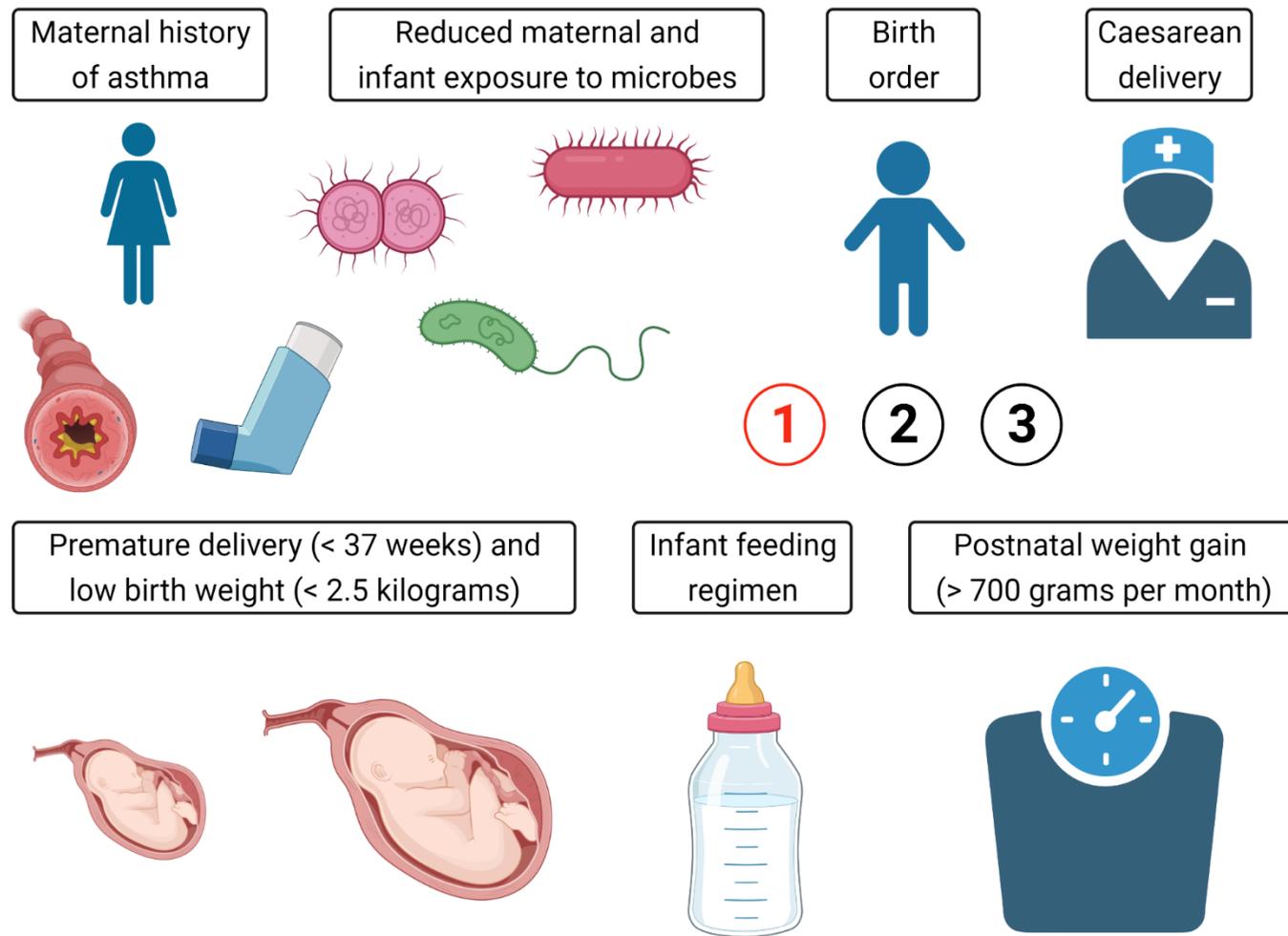


Figure 2.10 Early life micro-level exposures associated with the development of asthma (Part I, Clemente et al., 2012; Debarry et al., 2007; Gerhold et al., 2006; Folsgaard et al., 2012; Kragh et al., 2016; Prescott et al., 1998; Reibman et al., 2008; Riedler et al., 2001; Sears et al., 2002; Sonnenschein-van der Voort et al., 2014; Stockholm et al., 2016; 2018; Sevelsted et al., 2016; Tachimoto et al., 2020; Thorburn et al., 2015; van Nimwegen et al., 2011; Wolsk et al., 2016). Created with www.BioRender.com.

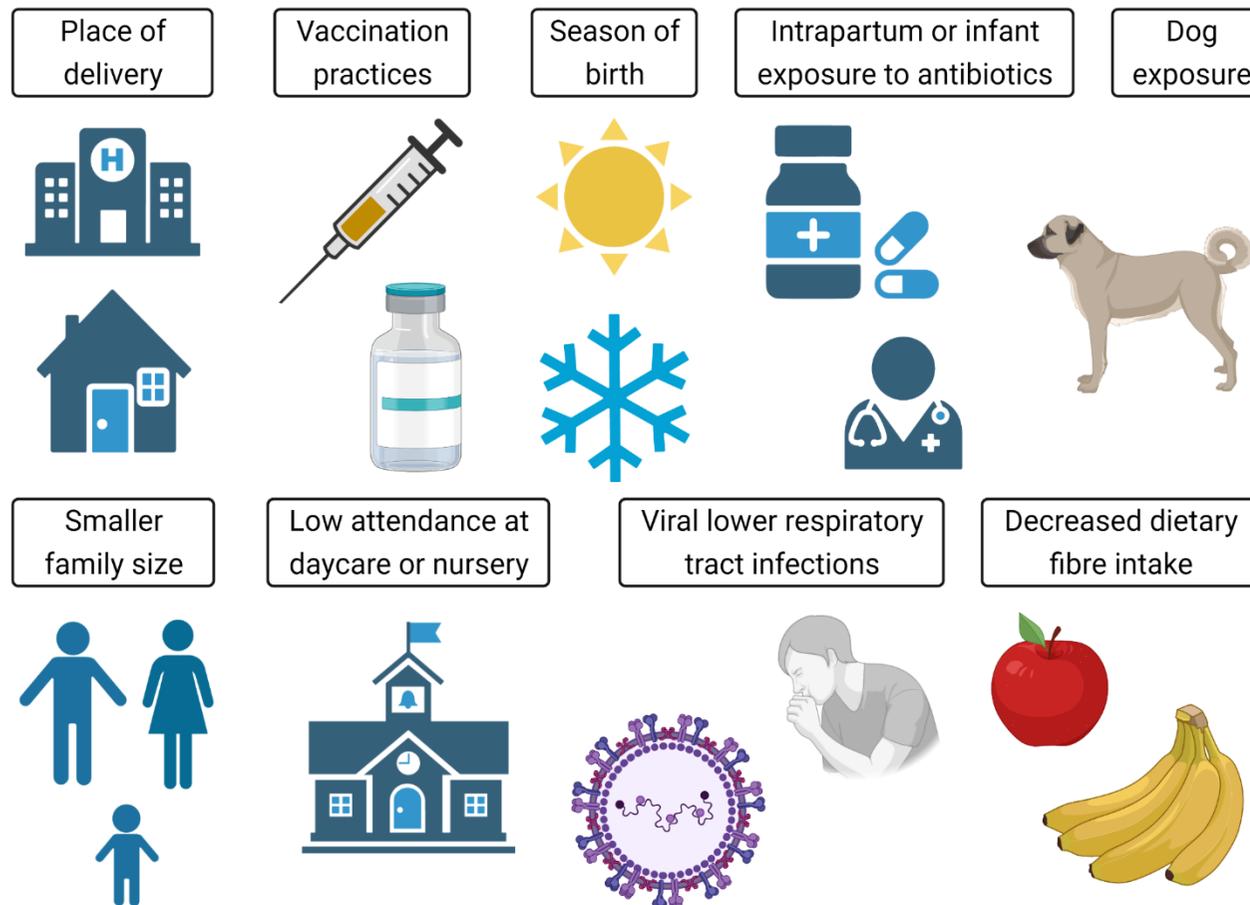


Figure 2.11 Early life micro-level exposures associated with the development of asthma (Part II, Arpaia et al., 2013; Arrieta et al., 2015; Ball et al., 2000; Bufford et al., 2008; Cait et al., 2018; Dahl, 2006; Grigg & Sonnenburg, 2017; Feddema & Claassen, 2020; Folkerts et al., 2018; Huang et al., 2017; Kramer et al., 1999; McLoughlin et al., 2019; Singanayagam et al., 2017; Sbihi et al., 2019; Sigurs et al., 2010; Smits et al., 2016; Stein et al., 2016; Stensballe et al., 2013; Stockholm et al., 2016; 2018; Tan et al., 2014; 2017; Thorburn et al., 2010; 2015; Thysen et al., 2016; van Nimwegen et al., 2011; Wolsk et al., 2016). Created with www.BioRender.com.

2.4.4 Gut Microbial Dysbiosis & Asthma

2.4.4.1 The Gut Microbiota in Healthy Individuals

The vast majority of immune development occurs in the gastrointestinal tract in partnership with the gut microbiota (Grigg & Sonnenberg, 2017). However, the importance of the gut microbiota to human health has long been inferred based on the proposed magnitude by which bacterial cells outnumber human cells (Luckey, 1972). According to an estimate by Luckey, (1972), bacterial cells outnumber human cells by a ratio of ~ 10:1 (Sender et al., 2016A; 2016B). This estimate was never intended to be used as empirical evidence, but was widely adopted nonetheless (Sender et al., 2016B). More recently, empirical evidence has discounted this notion and proposed a revised ratio of ~ 1.3:1 bacterial to human cells (Sender et al., 2016A).

For a 70-kg male, the total number of bacteria that colonise the internal and external surfaces of the body is ~ $3.8\text{-}3.9 \times 10^{13}$ cells, equating to ~ 0.3% of total body mass (Sender et al., 2016A). In contrast, the total number of human cells is ~ 3.0×10^{13} , of which ~ 84% are non-nucleated red blood cells (Sender et al., 2016A; 2016B). The vast majority of these bacteria reside in the gastrointestinal tract, specifically the colon (10^{14} cells; Sender et al., 2016A; 2016B). Lower numbers are found in the stomach (10^7), upper small intestine (duodenum and jejunum; 10^7), and lower small intestine (ileum), respectively (10^{11} ; Sender et al., 2016A; 2016B). When compared to the colon, the stomach, upper small intestine, and lower small intestine make a negligible contribution to the total bacterial content of the gastrointestinal tract (Sender et al., 2016A; 2016B).

From a bacterial perspective, this ratio is determined by colonic volume and bacterial density (Sender et al., 2016A). From a human perspective, this ratio is determined by blood volume and haematocrit percentage (Sender et al., 2016A). In contrast to the estimate proposed by Luckey, (1972), bacterial cells could in fact be outnumbered by human cells following a single void of the bowels, given that ~ one third of colonic bacteria is excreted in faeces (Sender et al., 2016B). This could lead to bacterial cells being outnumbered by human cells on a daily basis (Sender et al., 2016B), depending on the frequency of bowel movements and rate of bacterial recolonisation.

As a result, the revised ratio proposed by Sender et al. (2016A), dispels long-standing myths inferring the importance of the gut microbiota to human health based on numerical superiority (Luckey, 1972; Sender et al., 2016A; 2016B). Importantly however, the fact that bacterial cells do not outnumber human cells by a factor of 10:1 does not diminish their importance to human health (Sender et al., 2016A; 2016B), but instead realigns the origins of importance towards their powerful immunomodulatory capacities.

When analysing the gut microbiota as a whole, the broad range of bacteria make it difficult to identify those with immunomodulatory properties. To address this issue, Geva-Zatorsky et al. (2017), investigated the immunomodulatory properties of 53 bacterial species, comprising 62 bacterial strains, using a series of monocolonisation based experiments in gnotobiotic germ-free mice. The bacteria with immunomodulatory properties relevant to the pathophysiology of exercise-induced bronchoconstriction are outlined in Table 2.2 below.

Table 2.2 The immunomodulatory properties of bacteria with relevance to the pathophysiology of exercise-induced bronchoconstriction (Geva-Zatorsky et al., 2017).

Bacteria	Immunomodulatory Properties
<i>Bacteroides dorei</i>	Upregulated the production of IL-22 by ILCs in the colon and small intestine.
<i>Bifidobacterium longum</i>	Upregulated the production of IL-22 by ILCs in the colon and small intestine. Increased the number of T _H 1 cells in the colon.
<i>Acinetobacter Iwoffii</i> , <i>Clostridium sordellii</i> and <i>Veillonella</i>	Downregulated the production of IL-22 by ILCs in the colon and small intestine.
<i>Bacteroides vulgatus</i>	Increased the expression of CD11c in CD11b ⁺ CD11c ⁺ plasmacytoid dendritic cells in the colon. Increased the relative abundance of CD103 ⁺ dendritic cells in the colon. Increased the relative abundance of FoxP3 ⁺ regulatory T cells in the colon.
<i>Fusobacterium varium</i>	Upregulated the transcription of genes responsible for arachidonic acid metabolism. Decreased the number of CD4 ⁺ T cells in the colon.
<i>Coprobacillus</i>	Increased the number of IL-10 producing CD4 ⁺ T cells in the small intestine.
<i>Segmented filamentous bacteria</i>	Increased the number of T _H 17 cells in the colon and small intestine.

IL interleukin, *ILCs* innate lymphoid cells, *T_H* T helper cell subtype, *FoxP3* fork-head box protein 3.

These results demonstrate the ability of bacteria to adopt anti-inflammatory or pro-inflammatory properties. Interestingly, species and strains from the same phylum or genera did not demonstrate the same immunomodulatory effects (Geva-Zatorsky et al., 2017), highlighting the divergent mechanisms by which individual bacteria modulate host immune function. As outlined in Table 2.2, various members of the gut microbiota may be capable of modulating immune cells involved in the pathophysiology of exercise-induced bronchoconstriction, including ILCs (Halim et al., 2014; Maric et al., 2018; Sugita et al., 2018), pDCs (Lombardi et al., 2012; Maazi et al., 2018), IL-10 producing CD4⁺ T cells (Bohm et al., 2015; Faith et al., 2012; Matsuda et al., 2017; 2018; 2019), and FoxP3⁺ T_{REG} cells, respectively (Fantini et al., 2004; Khumalo et al., 2020; Lombardi et al., 2012; Ostroukhova et al., 2004; Polansky et al., 2010; Sakaguchi et al., 2013). In addition to the immunomodulatory effects of certain bacteria in the gastrointestinal tract (Geva-Zatorsky et al., 2017), certain bacteria are capable of modulating systemic immune function.

Following monocolonisation with *Segmented filamentous bacteria*, *Clostridium ramosum*, and *Bacteroides thetaiotaomicron*, Geva-Zatorsky et al. (2017), reported an increase in the relative abundance of FoxP3⁺ T_{REG} cells in the systemic lymphoid organs. These observations provide an example of what has been termed the “Gut-Lung Axis” in asthma (Huang & Boushey, 2014; Trompette et al., 2014). The Gut-Lung Axis proposes a bidirectional, reciprocal relationship between the gastrointestinal and pulmonary systems, driven by interactions between the gut bacteria, their metabolites, and components of host immune function (Budden et al., 2016; Huang & Boushey, 2014; Johnson et al., 2018; Schroeder & Backhed, 2016; Trompette et al., 2014; Vieira & Pretorius, 2010; Vital et al., 2015). This relationship is supported by the anatomical and structural similarities between the gastrointestinal and respiratory systems, enabling the translocation of bacteria (O’Dwyer et al., 2016), their metabolites (Holscher, 2017; Koh et al., 2016; Macia et al., 2014; Monda et al., 2017; Tan et al., 2014; 2017; Trompette et al., 2014), and components of host immune function, respectively (McLoughlin & Mills, 2011; Vieira & Pretorius, 2010). In healthy individuals, interactions between the gut microbiota and host immune system lead to the development of immunological tolerance (Garn et al., 2021; Konieczna et al., 2012; Lathrop et al., 2011), whereas in asthma, these interactions lead to overactive and disproportionate pro-inflammatory responses.

By outlining the role of certain bacteria in modulating host immune function (Geva-Zatorsky et al., 2017), healthy reference ranges have been established for 28 gut bacteria that are positively or negatively associated with gastrointestinal and metabolic disorders (Almonacid et al., 2017). Such information has greatly enhanced the clinical utility of microbial analyses in the diagnosis, treatment, and monitoring of these disorders (Almonacid et al., 2017). However, some of these reference ranges demonstrate substantial variation, such as *Prevotella* and *Bifidobacteria* (Almonacid et al., 2017), causing issues for researchers and clinicians when attempting to identify features of gut microbial dysbiosis. Assessing longitudinal changes in gut bacterial composition will enable greater confidence in identifying features of gut microbial dysbiosis (Almonacid et al., 2017; Flores et al., 2014).

Perhaps unsurprisingly, bacteria that colonise the airways of people with asthma have received more attention than bacteria that colonise the gut (Huang & Boushey, 2014). The airways are not sterile, even in healthy individuals (Hilty et al., 2010). Clinically important differences in the lung microbiota have been observed between adults with and without asthma (Beck et al., 2012; Goleva et al., 2013; Hilty et al., 2010; Huang et al., 2015). Some bacteria are even capable of modulating the responsiveness to inhaled corticosteroids (Goleva et al., 2013). Both symbiotic and pathogenic bacteria reside in the airways attempting to modulate immune function (Goleva et al., 2013; Huang et al., 2015; Larsen et al., 2014; 2015). Symbiotic bacteria such as *Prevotella melaninogenica*, *Prevotella nanceiensis*, and *Prevotella salivae* attenuate airway inflammation by decreasing the production of TNF- α by lymphocytes (Larsen et al., 2015), as well as the production of TNF- α and TSLP by lung tissue cells. In contrast, pathogenic bacteria such as *Haemophilus influenzae B*, *non-typeable Haemophilus influenzae*, and *Moraxella catarrhalis* augment airway inflammation by increasing the production of IL-1 β , CCL20, and macrophage inflammatory protein 2 alpha (MIP-2 α ; Larsen et al., 2015), causing significant damage to the bronchioles and alveoli. The expression of sPLA₂-X by bronchial epithelial cells is increased by IL-13, and a combination of TNF- α and IL-1 β (Hallstrand et al., 2016). In addition, TNF- α and IL-1 β also drive the production of cysteinyl leukotrienes (Hallstrand et al., 2016), outlining potential mechanisms by which pathogenic airway bacteria may drive the pathophysiology of asthma.

2.4.4.2 The Gut Microbiota in Asthma

Despite establishing healthy references ranges for gut bacteria in gastrointestinal and metabolic disorders (Almonacid et al., 2017), knowledge of gut bacteria that are positively or negatively associated with asthma remains limited, especially in adults (Begley et al., 2018; Hevia et al., 2016; Okba et al., 2018).

Features of gut microbial dysbiosis have been reported in adults with asthma (Hevia et al., 2016; Okba et al., 2018). At the genus level, Hevia et al. (2016), reported an increase in *Bifidobacterium* and *Faecalibacterium*, whereas at the species level, an increase in *Bifidobacterium adolescentis* was observed. In contrast, at the species level, *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium bifidum* were increased in healthy individuals (Hevia et al., 2016). Importantly, the increase in *Bifidobacterium adolescentis* observed in adults with asthma was restricted to those with shorter-term diagnoses, typically less than 11 years (Hevia et al., 2016). Adults with diagnoses greater than 11 years experienced a decrease in *Bifidobacterium adolescentis* (Hevia et al., 2016). Whether the decrease in *Bifidobacterium adolescentis* was related to the progression of asthma, age-related changes, or both, remains to be determined (Vital et al., 2015). What is clear, is that individuals with long-term asthma may be at greater risk of gut microbial dysbiosis, and increases in asthma severity, as outlined by the increase in total IgE observed in patients with long-term allergic asthma (Hevia et al., 2016).

Okba et al. (2018), went on to report an increase in *Lactobacilli*, and the density of *Escherichia coli*, in adults with atopic asthma. The increase in *Lactobacilli* was attributed to males with atopic asthma (Okba et al., 2018). Current evidence does not support the existence of sex-dependent differences in the gut bacterial composition of adults with asthma. Therefore, it appears that limitations in the methods used to quantify the gut microbiota by Okba et al. (2018), may have influenced their results. Hevia et al. (2016), used culture independent techniques to quantify the gut microbiota, whereas Okba et al. (2018), used semi-quantitative stool cultures. Certain bacteria are more capable of growth under culture-based conditions due to more preferential growth mediums (Barcik et al., 2016; Stokholm et al., 2016). In fact, most bacteria cannot currently be cultured *in vitro*, providing a bias assessment of gut bacterial composition (Barcik et al., 2016; Stokholm et al., 2016). In addition, the time between sample collection and analysis can compromise the reliability of bacterial cultures (Stokholm et al., 2016). Of the 14 bacterial genera

analysed, Okba et al. (2018), demonstrated no growth in 8 genera across all participants, both with and without asthma. Given the limitations of current approaches, culture-based assessments of gut bacterial composition should be viewed with caution until suitable growth mediums are available.

Regardless of the method used to quantify the gut microbiota, a common limitation of these studies was that assessments were based on a single time point. Without longitudinal profiling it can be unclear whether features of gut microbial dysbiosis are related to asthma, or due to variation caused by other factors, such as diurnal effects (Sender et al., 2016A), temporal parameters (Flores et al., 2014), or environmental exposures, respectively (Feddema & Claassen, 2020; He et al., 2020; Khreis et al., 2017; Malik et al., 2012; Rodriguez et al., 2019; Sbihi et al., 2019; Sharpe et al., 2015). By profiling the same participants over time, research has begun to investigate associations between features of gut microbial dysbiosis, clinical and inflammatory parameters of asthma (Begley et al., 2018).

In adults with mild-moderate asthma, features of gut microbial dysbiosis have been associated with decreases in pulmonary function, and increased sensitisation to aeroallergens (Begley et al., 2018). Decreases in FEV₁ were associated with a lower *Bacteroidetes:Firmicutes* ratio, as well as decreases in *Bacteroides* and *Enterobacteriaceae* (Begley et al., 2018; Vital et al., 2015). In contrast, an increase in *Bifidobacterium* and *Lachnospiraceae* was observed (Begley et al., 2018). The increase in *Bifidobacterium* was attributed to obese individuals with asthma (Begley et al., 2018). Participants were classified into 3 sub-clusters depending on different features of gut microbial dysbiosis (Begley et al., 2018). Across all clusters, features of gut microbial dysbiosis were associated with bronchial hyperresponsiveness, bronchodilator reversibility, and/or daily fruit intake (Begley et al., 2018). Within each cluster, different features of gut microbial dysbiosis were associated with varying degrees of impairment in pulmonary function (Begley et al., 2018). The fact that gut bacterial composition remained stable over time provides further confidence in the identification of genuine features of gut microbial dysbiosis in asthma (Begley et al., 2018).

The role of gut microbial dysbiosis in asthma could be explained by the increase in histamine secreting bacteria (Barcik et al., 2016). Histamine is a potent pro-inflammatory mediator of bronchoconstriction and type-2 inflammation (Ojiaku et al., 2018). Barcik et al. (2016), reported an increase in histamine associated microbial

gene expression (histidine decarboxylase; HDC) in non-obese adults with asthma (HDC^{HIGH}) compared to obese adults with asthma (HDC^{LOW}). The prominent histamine secreting microbial strains belonged to *Morganella morganii*, *Escherichia coli*, and *Lactobacillus vaginalis* (Barcik et al., 2016). *Morganella morganii* was significantly elevated in the HDC^{HIGH} asthma group, especially in those with more severe asthma. The HDC^{HIGH} group demonstrated significant impairments in FEV₁ compared to their HDC^{LOW} counterparts (Barcik et al., 2016). The authors speculated that increased levels of systemic histamine in the HDC^{HIGH} asthma group may predispose individuals to more frequent and severe exacerbations (Barcik et al., 2016).

However, more recent research demonstrated a protective effect of microbial histamine secretion on pulmonary type-2 inflammation (Barcik et al., 2019). Following gavage with a strain of *Escherichia coli* genetically modified with *Morganella morganii* to produce histamine (*E. coli* BL21_HTW), a significant reduction in airway eosinophilia and type-2 cytokines was observed in response to OVA sensitisation and challenge (Barcik et al., 2019). The ability of *E. coli* BL21_HTW to protect against type-2 inflammation was attributed to the increased expression of CD80 and PDL2, and decreased expression of PDL1 in CD11c⁻F4/80⁺ MHCII⁺ macrophages (Barcik et al., 2019), as well as an increase in the number of IFN- γ producing lymphocytes.

In mice unable to produce histamine (HDC^{-/-}), the protective effects of *E. coli* BL21_HTW on airway eosinophilia were lost, due to an increase in histamine degrading enzymes, and decrease in histamine receptors, respectively (Barcik et al., 2019). Counterintuitively, these results suggest that promoting gastrointestinal colonisation with histamine secreting bacteria could protect against the development and progression of asthma. The important distinction to be made is the origin of histamine production. Microbial histamine appears to exert anti-inflammatory effects (Barcik et al., 2019), whereas human derived histamine exerts pro-inflammatory effects (Ojiaku et al., 2018). Aided by the Disbiome Database (Janssens et al., 2018), researchers will be able to identify and submit asthma specific features of gut microbial dysbiosis, potentially leading to the quicker development of targeted probiotic or prebiotic therapies (Reid et al., 2017). An overview of the features of gut microbial dysbiosis reported in adults with asthma is outlined in Table 2.3 below.

Table 2.3 Features of gut microbial dysbiosis reported in adults with asthma.

Reference	Method	Bacteria
Hevia et al., (2016)	Culture independent, metagenomics-based analyses of faecal samples obtained from adults with mild-moderate allergic asthma ($n = 21$).	At the genus level, a significant increase in <i>Bifidobacterium</i> and <i>Faecalibacterium</i> . At the species level, a significant decrease in <i>Bifidobacterium adolescentis</i> was observed specifically in adults with long-term allergic asthma.
Okba et al., (2018)	Semi-quantitative stool cultures of faecal samples obtained from adults with allergic asthma ($n = 80$).	At the genus level, a significant increase in <i>Lactobacilli</i> . At the species level, a significant increase in the density of <i>E. coli</i> .
Begley et al., (2018)	Culture independent, metagenomics-based analyses of faecal samples obtained from adults with mild-moderate asthma ($n = 24$).	A lower <i>Bacteroidetes:Firmicutes</i> ratio. At the genus level, a decrease in <i>Bacteroides</i> and <i>Enterobacteriaceae</i> . At the species level, an increase in <i>Bifidobacterium</i> (in obese individuals) and <i>Lachnospiraceae</i> .
Barcik et al., (2016)	Culture independent, metagenomics-based analyses of faecal samples obtained from adults with mild, moderate and severe asthma ($n = 74$).	At the species level, a significant increase in <i>Morganella morganii</i> was observed in non-obese adults with HDC ^{HIGH} asthma, especially in those with more severe asthma.

E. coli Escherichia coli, HDC^{HIGH} refers to non-obese adults with asthma who demonstrated an increase in histamine associated microbial gene expression (histidine decarboxylase). Begley et al. (2018), were the only ones to assess the within-subject stability of gut bacterial composition by assessing changes in gut bacterial composition from faecal samples collected 4-weeks apart.

2.4.5 Gut Microbiota-Based Nutritional Interventions in Asthma

2.4.5.1 Probiotics

Given the features of gut microbial dysbiosis outlined above (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016; Okba et al., 2018), researchers have investigated whether resolving these features leads to improvements in systemic and pulmonary immune function. One potential method is the use of probiotics. Probiotic supplementation is defined as the ingestion of live bacteria that, when consumed in sufficient quantities (i.e. a defined dose of probiotics measured in colony forming units such as 10^9 CFU's), positively modulates host immune function (Gourbeyre et al., 2011; Huang et al., 2017). Probiotic supplementation aims to ensure that symbiotic bacteria are present in the gastrointestinal tract in sufficient quantities to positively modulate immune function (Arrieta et al., 2015; Davis et al., 2011).

As outlined in a chronic OVA-based model of asthma, probiotic supplementation with *Bifidobacterium breve* M-16V administered 1 hour prior to OVA challenge at a dose of 10^9 CFU per day, three times a week from days 22 – 55 attenuated the severity of inflammation by a comparable magnitude to that observed following budesonide therapy (Sagar et al., 2014A). For example, when compared to the control group who received no treatment, mice administered budesonide therapy reported a significant reduction in the total number of inflammatory cells in bronchoalveolar lavage fluid ($155.25 \times 10^4 \pm 12.58$ vs. $83.70 \times 10^4 \pm 13.66$; $p < 0.0001$), with the magnitude of attenuation even greater following treatment with *Bifidobacterium breve* M-16V ($73.50 \times 10^4 \pm 6.42$; $p < 0.0001$). *Bifidobacterium breve* M-16V attenuated the severity of airway eosinophilia, mast cell degranulation, T cell activation, and airway remodeling, respectively (Sagar et al., 2014A). These observations could be attributed to the decrease in pulmonary TH2 transcription factor expression (GATA-3) caused by increases in pulmonary FoxP3⁺ iTREG cells, and the production of IL-10 (Sagar et al., 2014A), as well as increases in the number of systemic FoxP3⁺ iTREG cells. These results demonstrate the critical role of *Bifidobacteria* in attenuating the severity of asthma by enhancing the suppressive capacity of systemic and pulmonary TREG cells, especially in response to bronchoprovocation (Sagar et al., 2014A).

More recently, the therapeutic efficacy of probiotic supplementation has been demonstrated in children at risk of developing asthma (Arrieta et al., 2015).

Administering 50µl of faecal slurry containing 10µl of a multi-strain probiotic, designed to selectively target asthma specific features of gut microbial dysbiosis (*Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* at a dose of one colony per culture), on days 0, 3, 7, and 14 attenuated the development of asthma in mice inoculated with the gut microbiota of children at risk of developing asthma (Arrieta et al., 2015). During the first 100 days of life, children at risk of developing asthma demonstrate features of gut microbial dysbiosis characterised by decreases in the relative abundance of *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia*, as well as reductions in faecal acetate concentrations, respectively (Arrieta et al., 2015). As outlined in an OVA-based model of asthma, administering a multi-strain probiotic targeted towards the asthma specific features of gut microbial dysbiosis, significantly attenuated the development of airway inflammation in adult murine offspring by reducing the total number of inflammatory cells and lymphocytes ($p < 0.05$) as well as neutrophils ($p < 0.0001$; Arrieta et al., 2015). The attenuation of airway inflammation was primarily attributed to the ability of *Lachnospira* to significantly colonise the gastrointestinal tract of adult murine offspring (Arrieta et al., 2015).

In healthy adults, probiotic supplementation with *Bifidobacterium infantis* 35624 for 8-weeks at a dose of 1×10^9 live bacteria per day significantly increased the number of CD4⁺ CD25⁺ ICOS⁺ FoxP3⁺ iT_{REG} cells ($8.14 \pm 0.25\%$ vs. $9.19 \pm 0.34\%$; $p = 0.003$), and IL-10⁺ iT_{R1} cells via mDC and pDC-dependent mechanisms ($p < 0.001$) (Konieczna et al., 2012). Furthermore, CD8 α ⁺ β ⁺ pDCs prevent the development of airway hyperresponsiveness by driving the proliferation of CD4⁺ CD25⁺ FoxP3⁺ nT_{REG} cells by producing retinoic acid and TGF- β (Figure 2.6; Fantini et al., 2004; Lombardi et al., 2012). Inducing the proliferation of FoxP3⁺ T_{REG} cells prevents the proliferation of T_{H2} cells and the production of IL-4 and IL-13, respectively (Lombardi et al., 2012). The observation that probiotic supplementation with based on stimulating the *Bifidobacterium* genus (Konieczna et al., 2012), demonstrates the clinical utility of exploring further *Bifidobacteria* based nutritional interventions such as prebiotics. An overview of the immunomodulatory properties of probiotic supplementation in the pathophysiology of asthma and exercise-induced bronchoconstriction is outlined in Table 2.4 below.

Table 2.4 The immunomodulatory properties of probiotic supplementation in the pathophysiology of asthma and exercise-induced bronchoconstriction.

Reference	Intervention	Immunomodulatory Properties
Sagar et al., (2014A)	Probiotic supplementation with <i>Bifidobacterium breve</i> M-16V in a chronic OVA-based model of asthma.	Attenuated the severity of airway eosinophilia, mast cell degranulation, T cell activation and airway remodeling by a comparable magnitude to budesonide therapy. Decreased the pulmonary expression of GATA-3. Increased the number of systemic and pulmonary FoxP3 ⁺ iT _{REG} cells, and the pulmonary production of IL-10.
Arrieta et al., (2015)	Probiotic supplementation with <i>Lachnospira</i> , <i>Veillonella</i> , <i>Faecalibacterium</i> , and <i>Rothia</i> in an OVA-based model of asthma. Mice were inoculated with the faecal microbiota of children at risk of developing asthma.	Attenuated the development of airway inflammation in adult murine offspring. The attenuation of airway inflammation was primarily attributed to the ability of <i>Lachnospira</i> to significantly colonise the gastrointestinal tract of adult murine offspring.
Konieczna et al., (2012)	Probiotic supplementation with <i>Bifidobacterium infantis</i> 35624 in healthy adults (<i>n</i> = 10).	Increased the number of CD4 ⁺ CD25 ⁺ ICOS ⁺ FoxP3 ⁺ iT _{REG} cells, and IL-10 ⁺ iT _{R1} cells via plasmacytoid and myeloid dendritic cell dependent mechanisms.

OVA ovalbumin, *FoxP3* fork-head box protein 3, *IL-10* interleukin-10, *iT_{REG}* induced regulatory T cell that expresses FoxP3, *iT_{R1}* induced regulatory T cell that produces IL-10. In Sagar et al. (2014A), probiotic supplementation was administered after asthma has been induced, assessing the effect on established asthma as opposed to the development of asthma. Budesonide was used as a reference pharmaceutical treatment for asthma.

2.4.5.2 Prebiotics

Prebiotics are defined as:

“Substrates that are selectively utilised by host microorganisms, conferring a health benefit” (Gibson et al., 2017).

Prebiotics differ from probiotics as they provide a source of energy for existing bacteria in the gut (Verheijden et al., 2015A), whereas probiotics introduce new live bacteria into the gut (Gourbeyre et al., 2011; Huang et al., 2017). Of particular importance to the pathophysiology of asthma and exercise-induced bronchoconstriction, the health benefits of prebiotics extend beyond the gastrointestinal system to the pulmonary system by modulating systemic immune function (Davis et al., 2011; Gibson et al., 2017; Holscher, 2017; Huang et al., 2017; Verheijden et al., 2015A; Vulevic et al., 2015). Galactooligosaccharides are a type of prebiotic demonstrated to exert anti-inflammatory effects in people with and without asthma (Davis et al., 2011; Depeint et al., 2008; McLoughlin et al., 2017; 2019; So et al., 2018; Verheijden et al., 2015A; 2015B; 2016; 2018; Williams et al., 2016).

In healthy individuals, galactooligosaccharide supplementation leads to significant increases in *Bifidobacteria* and *Lactobacilli*, increasing the production of butyrate (So et al., 2018). The positive effects of galactooligosaccharides in healthy individuals is not surprising given the decrease in dietary fibre intake observed across Westernised society (De Filippo et al., 2010; Schnorr et al., 2014). Nonetheless, understanding the response to galactooligosaccharide supplementation in healthy individuals is essential to accurately interpreting the response in people with asthma and exercise-induced bronchoconstriction. Given that significant reductions in dietary fibre intake have been observed in people with severe asthma (Berthon et al., 2013; Wood et al., 2015), galactooligosaccharide supplementation may offer greater therapeutic benefits (McLoughlin et al., 2017). When compared to other prebiotics such as polysaccharides or resistant starch, galactooligosaccharides attenuate systemic inflammatory markers such as C-reactive protein (CRP) to a greater extent (McLoughlin et al., 2017), suggesting that they may be more effective in attenuating the severity of asthma and exercise-induced bronchoconstriction (Williams et al., 2016).

As outlined in an HDM-based model of asthma, galactooligosaccharide supplementation attenuated the magnitude of airway eosinophilia when administered at a dose of either 1% w/w ($p < 0.01$), or 2.5% w/w GOS ($p < 0.05$) for 28 days from -14 to +14 days when Vivinal® GOS syrup with approximately 59% galactooligosaccharides, 21% lactose, 19% glucose, and 1% galactose was administered (Verheijden et al., 2018). This was comparable in magnitude to the attenuation observed following budesonide therapy alone ($p < 0.001$; Verheijden et al., 2015A; 2018; Zhang et al., 2016). Strikingly, when administered as a combination therapy, galactooligosaccharide supplementation and budesonide therapy attenuated the magnitude of airway eosinophilia ($p < 0.0001$), and the severity of airway hyperresponsiveness ($p < 0.001$), by a comparable magnitude to that observed in the control cohort (Verheijden et al., 2018). In addition, the combination of galactooligosaccharides and budesonide significantly decreased the degranulation of mast cells, as well as the production of CCL17, CCL22, IL-13, and IL-33, respectively (Verheijden et al., 2015A; 2018). Attenuating the severity of asthma could be attributed to the significant increase in CD4⁺ CD25⁺ FoxP3⁺ T_{REG} cells observed following galactooligosaccharide supplementation and budesonide therapy ($p < 0.05$; Verheijden et al., 2018). Given that high dose galactooligosaccharide supplementation was more effective than low dose galactooligosaccharide supplementation in reducing the pulmonary recruitment of T_{H2} cells (2.5% w/w vs. 1% w/w), the results of Verheijden et al. (2018), propose a dose-response relationship between galactooligosaccharide supplementation and the severity of asthma.

It is possible that CCL17, CCL22, IL-13, and IL-33 play critical roles in the pathophysiology of exercise-induced bronchoconstriction. For example, CCL17 and CCL22 recruit eosinophils to the airways via cDC1-dependent mechanisms (Januskevicius et al., 2020; Yi et al., 2018), induce the differentiation of T_{H2} cells in the mediastinal lymph nodes (Verheijden et al., 2018), and prevent apoptosis of iTR1 cells upon translocation to the lungs (Kearley et al., 2008). In addition, IL-13 upregulates the transcription of HDACs (Wawrzyniak et al., 2017), increases the expression of sPLA₂-X by bronchial epithelial cells (Hallstrand et al., 2012; 2016), augments the production of cysteinyl leukotrienes (Hallstrand et al., 2012; 2016), enables ILC2s to decrease bronchial epithelial integrity (Halim et al., 2014), triggers the translocation of dendritic cells to the mediastinal lymph nodes (Halim et al., 2014), facilitates the differentiation of T_{H2} cells (Halim et al., 2014; Maazi et al.,

2018; Sugita et al., 2018), enhances the magnitude of airway eosinophilia (Maazi et al., 2018; Sugita et al., 2018), and drives the differentiation of pathogenic T_{REG} cells (Chen et al., 2017; 2018). Finally, IL-33 decreases bronchial epithelial integrity (Halim et al., 2014; Sugita et al., 2018), activates pDCs (Maazi et al., 2018), augments the production of cysteinyl leukotrienes (Lai et al., 2014), triggers ILC2s to produce IL-5 and IL-13 (Maric et al., 2018), drives the differentiation of p_eT_H2 cells (Halim et al., 2014; Maazi et al., 2018; Mitson-Salazar et al., 2016; Sugita et al., 2018), enables MC_{T/CPA3} mast cells to accumulate in the airways (Altman et al., 2019; Couto et al., 2018; Lai et al., 2014), causes FoxP3⁺ T_{REG} cells to lose their suppressive capacity (Chen et al., 2017; 2018), and sustains inflammatory responses via p_TH₂MEM cells (Endo et al., 2015). Therefore, modulating these pro-inflammatory mediators via galactooligosaccharide supplementation could have important implications for exercise-induced bronchoconstriction.

Verheijden et al. (2015B), demonstrated that the ability of galactooligosaccharides to suppress type-2 inflammation was mediated by CD25⁺ FoxP3⁺ iT_{REG} cells. In the absence of FoxP3⁺ iT_{REG} cells, galactooligosaccharides administered at a dose of 1% w/w for 28 days from -14 to +14 days containing Vivinal[®] GOS syrup with approximately 59% galactooligosaccharides, 21% lactose, 19% glucose, and 1% galactose are unable to prevent certain hallmark features of type-2 inflammation, including the production of IL-33, the activation and expansion of GATA-3⁺ T_H2 cells, the magnitude of airway eosinophilia ($p < 0.0001$), and the secretion of IL-4 and IL-13, respectively (Verheijden et al., 2015B). In accordance with the effects of *Bifidobacterium infantis* 35624 on the proliferation of CD4⁺ CD25⁺ ICOS⁺ FoxP3⁺ iT_{REG} cells, and IL-10⁺ iTr1 cells (Konieczna et al., 2012), stimulating the development of *Bifidobacteria* through galactooligosaccharide supplementation also demonstrates immense therapeutic potential in attenuating the severity of asthma and exercise-induced bronchoconstriction by increasing the suppressive capacity of T_{REG} cells (Davis et al., 2011; De Preter et al., 2008; Depeint et al., 2008; Verheijden et al., 2015B).

Despite the critical role of FoxP3⁺ iT_{REG} cells in mediating the galactooligosaccharide-dependent attenuation of type-2 inflammation (Verheijden et al., 2015B; 2018), these interactions remain to be explored in humans. It is also important to note that galactooligosaccharides can attenuate the adverse effects of certain pharmaceutical treatments. Following allergen challenge, budesonide is

associated with an inability to attenuate the production of IL-33 (Verheijden et al., 2015A), as well as a decrease in the relative abundance of FoxP3⁺ T_{REG} cells, both of which can be resolved through galactooligosaccharide supplementation (Verheijden et al., 2015B; 2018).

The benefits of galactooligosaccharide supplementation have also been reported in humans with exercise-induced bronchoconstriction (Williams et al., 2016). Following 3-weeks of prebiotic supplementation with Bimuno[®] galactooligosaccharide at a dose of 5.5 g/day⁻¹ (B-GOS[®]), clinically relevant, participant perceivable improvements in the severity of HIB were observed (Santanello et al., 1999; Williams et al., 2016). The percentage decrease in FEV₁ post-EVH was significantly attenuated by ~ 41%, a comparable magnitude to that observed following pharmaceutical treatments (39-60%; Kippelen et al., 2010; Rundell et al., 2005; Simpson et al., 2016; Verheijden et al., 2015A; 2018; Williams et al., 2016). These improvements were partly attributed to the reduction of TNF- α at rest (2.68 ± 0.98 vs. 2.18 ± 0.59 pg/ml; $p = 0.040$), and the complete attenuation of the 29% increase observed post-EVH prior to supplementation ($p = 0.002$; Williams et al., 2016). In accordance with Verheijden et al. (2015A; 2018), Williams et al. (2016), also reported a significant decrease in CCL17 concentrations (at rest) following supplementation with B-GOS[®]. Although not investigated by Williams et al. (2016), the similar mechanisms of action observed between Verheijden et al. (2015A; 2018), and Williams et al. (2016), suggest that T_{REG} cells may mediate the protective effects of galactooligosaccharide supplementation in humans, representing an exciting area for future research.

The prebiotic administered by Williams et al. (2016), was developed from the β -galactosidase enzyme isolated from *Bifidobacterium bifidum* NCIMB 41171 (Depeint et al., 2008). As a result, B-GOS[®] is highly selective in stimulating the growth and development of certain species of *Bifidobacteria* (Depeint et al., 2008; Vulevic et al., 2015). However, Davis et al. (2011), have suggested that as yet unclassified species of *Bifidobacteria* may be more capable of utilising galactooligosaccharides. In humans, Davis et al. (2011), observed a significant increase in *Bifidobacteria* at the phylum, family, genus, and species levels, following a minimum dose of 10.0 g-day⁻¹. At the species level, significant increases were observed when compared to 0.0 g-day⁻¹ in the relative abundance of *Bifidobacterium adolescentis* (0.34 ± 0.89 vs. $1.03 \pm 1.55\%$; $p < 0.05$),

Bifidobacterium longum (0.09 ± 0.23 vs. $0.33 \pm 0.85\%$; $p < 0.05$), and *Bifidobacterium catenulatum* (0.27 ± 0.88 vs. $0.91 \pm 2.08\%$; $p < 0.01$), as well as 3 as yet unclassified species of *Bifidobacteria* (Davis et al., 2011). Remarkably, the greatest bifidogenic response to galactooligosaccharide supplementation was observed in two of the unclassified species of *Bifidobacteria*, specifically *Bifidobacterium* subspecies II (0.60 ± 1.53 vs. $2.00 \pm 3.45\%$; $p < 0.05$), and *Bifidobacterium* subspecies III (0.78 ± 2.19 vs. $2.50 \pm 4.55\%$; $p < 0.05$; Davis et al., 2011). The response to galactooligosaccharide supplementation remained stable over a 12-week period (Davis et al., 2011). These results suggest that during intervention-based trials up to 12-weeks long, improvements in the clinical or inflammatory parameters of exercise-induced bronchoconstriction may be attributed to immunomodulation, as opposed to methodological limitations.

Williams et al. (2016), administered 5.5 g/day^{-1} of B-GOS[®] for 3-weeks to adults with moderate HIB. Interestingly, Davis et al. (2011), only observed a consistent bifidogenic response to galactooligosaccharide supplementation following a minimum dose of 10 g/day^{-1} . Although gut bacterial composition was not assessed by Williams et al. (2016), the results of Davis et al. (2011), suggest that increases in *Bifidobacteria* may have underpinned the immunomodulatory effects observed by Williams et al. (2016). As a result, it is plausible to speculate that supplementation with B-GOS[®] may have resolved exercise-induced bronchoconstriction specific features of gut microbial dysbiosis. However, the gut bacterial composition of adults with exercise-induced bronchoconstriction has not been profiled, meaning further research is required before such assumptions can be substantiated.

In contrast to the results of Davis et al. (2011), Depeint et al. (2008), reported a significant increase in *Bifidobacteria* following 3.6 g/day^{-1} of supplementation with B-GOS[®] for 7-days in healthy individuals. This could be partly attributed to the increased potency and selectivity of B-GOS[®] (Depeint et al., 2008), requiring a smaller dose to induce similar benefits. Taken together, these results suggest that further research is required to establish the minimal, clinically important dose and duration of galactooligosaccharide supplementation required to attenuate the severity of exercise-induced bronchoconstriction. It should be noted, however, that Davis et al. (2011), observed significant inter-individual variation in the bifidogenic response to galactooligosaccharide supplementation. This could not be explained by baseline differences in gut bacterial composition alone (Davis et al., 2011),

highlighting the need to expand current understandings of the factors influencing individual responses to galactooligosaccharides (Healey et al., 2016; 2018; Swinton et al., 2018).

Through increases in the gastrointestinal content of *Bifidobacteria* following galactooligosaccharide supplementation (Davis et al., 2011), the reduction in the severity of exercise-induced bronchoconstriction observed by Williams et al. (2016), could be attributed to the anti-inflammatory effects of short-chain fatty acids on systemic T_{REG} cells (Thorburn et al., 2015). Short-chain fatty acids are anti-inflammatory metabolites produced by the fermentation of prebiotics (Berthon et al., 2013; Koh et al., 2017; Macfarlane et al., 1998; Maslowski et al., 2009; McLoughlin et al., 2017). They support symbiotic microbes in colonising the gastrointestinal tract by optimising colonic pH (McLoughlin et al., 2017). Given that short-chain fatty acids such as acetate can transfer from the gut into the blood, they have been implicated in positively modulating systemic and pulmonary immune function in asthma (Koh et al., 2016; McLoughlin & Mills, 2011; O'Dwyer et al., 2016; Schroeder & Backhed, 2016; Tan et al., 2014; 2017; Vieira & Pretorius, 2010). An overview of the immunomodulatory properties of galactooligosaccharide-based prebiotic supplementation in the pathophysiology of asthma and exercise-induced bronchoconstriction is outlined in Table 2.5 below.

Table 2.5 The immunomodulatory properties of galactooligosaccharide-based prebiotic supplementation in the pathophysiology of asthma and exercise-induced bronchoconstriction.

Reference	Intervention	Immunomodulatory Properties
So et al., (2018)	Prebiotic supplementation with galactooligosaccharides in healthy individuals ($n = 2099$).	Significant increases in <i>Bifidobacteria</i> and <i>Lactobacilli</i> were observed, significantly increasing the production of butyrate.
Verheijden et al., (2018)	Prebiotic supplementation with Vivinal [®] galactooligosaccharide syrup (Vivinal [®] GOS) in an HDM-based model of asthma.	Significantly attenuated the magnitude of airway eosinophilia, and the production of mMCP-1, by a comparable magnitude to that observed following budesonide therapy.
Verheijden et al., (2018)	Prebiotic supplementation with low and high dose Vivinal [®] GOS, combined with budesonide, in an HDM-based model of asthma.	Significantly attenuated the magnitude of airway eosinophilia, and the severity of airway hyperresponsiveness, by a comparable magnitude to that observed in the control cohort. Significantly decreased the degranulation of mast cells, as well as the production of CCL17, CCL22, IL-13, and IL-33, respectively. Significantly increased the number of FoxP3 ⁺ iT _{REG} cells. High dose Vivinal [®] GOS was more effective in reducing the recruitment of T _H 2 cells to the airways.
Verheijden et al., (2015B)	Prebiotic supplementation with Vivinal [®] GOS in an HDM-based model of asthma.	Significantly attenuated type-2 inflammation by increasing the number of FoxP3 ⁺ iT _{REG} cells.
Williams et al., (2016)	3-weeks of prebiotic supplementation with Bimuno [®]	Significantly attenuated the percentage decrease in FEV ₁ post-EVH by ~ 41%, a comparable magnitude to that observed

galactooligosaccharide powder following pharmaceutical treatments (39-60%; Kippelen et al., (B-GOS®) in adults with 2010; Rundell et al., 2005; Simpson et al., 2016).
moderate hyperpnoea-induced bronchoconstriction ($n = 10$). Completely abolished the production of TNF- α , both at rest, and in response to EVH.
Significantly attenuated the production of CCL17 at rest.

HDM house dust mite, *mMCP-1* mucosal mast cell protease-1, *CCL* CC chemokine ligand, *IL* interleukin, *FoxP3* fork-head box protein 3, *iT_{REG}* induced regulatory T cell, *FEV₁* forced expiratory volume in one second, *EVH* eucapnic voluntary hyperpnoea, *TNF- α* tumor necrosis factor alpha.

2.4.5.3 Synbiotics

The individual benefits of probiotic supplementation (Arrieta et al., 2015; Konieczna et al., 2012; Sagar et al., 2014A), and prebiotic supplementation (Thorburn et al., 2015; Verheijden et al., 2015A; 2015B; 2016; 2018; Williams et al., 2016), have led researchers to question whether synergistic effects could be obtained by combining the two together.

Synbiotics are defined as a combination of probiotics and prebiotics. van de Pol et al. (2011), were amongst the first to investigate the effects of synbiotics in adults with asthma. A synbiotic mixture containing Immunofortis® *Bifidobacterium breve* M-16V (10^{10} CFU), 90% (7.2 grams) short-chain galactooligosaccharides, and 10% (0.8 grams) long-chain fructooligosaccharides was administered twice daily for 4 weeks (van de Pol et al., 2011). When compared to baseline, a significant increase in airway eosinophilia was still observed in response to challenge with HDM following synbiotic supplementation (7.0 ± 2.1 vs. $9.5 \pm 1.7 \times 10^4$ cells per gram of sputum; $p < 0.001$; van de Pol et al., 2011), although serum IL-5 was significantly attenuated 24-hours post challenge. The lack of bronchoprotection demonstrated by the absence of airway eosinophilia being attenuated mirrored the lack of change in airway hyperresponsiveness. A significant improvement was observed in daily peak expiratory flow (PEF) measurements (van de Pol et al., 2011), but this could not be explained by changes in immune function. Given that no improvements were observed in objective diagnostic parameters, such as FEV₁ (Miller et al., 2005), the increase in PEF could be partly attributed to a learning effect of conducting daily measurements. Furthermore, as this was a parallel group design, the effect of expectancy regarding beliefs about being assigned the synbiotic intervention may have influenced daily PEF measurements.

Gastrointestinal issues such as diarrhoea and obstipation were reported following synbiotic supplementation (van de Pol et al., 2011). This could be attributed to the dose of galactooligosaccharide administered. As part of the synbiotic intervention, participants were administered 14.4 g/day^{-1} of galactooligosaccharide, split into two equal doses morning and night (van de Pol et al., 2011). Material safety data sheets indicate that consuming more than 15 grams of galactooligosaccharides at once may lead to gastrointestinal issues. As a result, it is likely that van de Pol et al. (2011), were close to the upper tolerable limit. Interestingly, gastrointestinal symptoms were more common in the placebo group than the synbiotic group (van

de Pol et al., 2011), further highlighting the adverse effects of expectancy in parallel group designs.

Taking into consideration the high dose of galactooligosaccharide administered, the absence of improvements in FEV₁ and airway inflammation were surprising. For example, Williams et al. (2016), observed clinically relevant improvements in FEV₁ and markers of airway inflammation following 3-weeks of supplementation with 5.5 g/day⁻¹ of B-GOS[®]. In contrast, van de Pol et al. (2011), observed no improvements in FEV₁ or airway inflammation despite administering 14.4 g/day⁻¹ of galactooligosaccharides alongside 1.6 g/day⁻¹ of fructooligosaccharides, and 10¹⁰ colony forming units (CFU) of *Bifidobacterium breve* M-16V for 4-weeks. The participants recruited by Williams et al. (2016), were of a moderate severity, whereas those recruited by van de Pol et al. (2011), were of a mild severity. In addition, van de Pol et al. (2011), controlled for the confounding effect of pollen by arranging participation of pollen-sensitised participants outside the pollen season, whereas Williams et al. (2016), implemented no such controls. To some extent, these factors could have affected the response to galactooligosaccharide supplementation, but do not explain the stark contrast in findings.

No prospective assessments of adherence were carried out by van de Pol et al. (2011). Compliance to the intervention was defined as completing at least 2 out of the 4 weeks of the intervention (van de Pol et al., 2011). As outlined by the United States Food and Drug Administration (USFDA), the 50% compliance rate adopted by van de Pol et al. (2011), is much lower than the minimum 80% compliance rate typically adopted in clinical trials. When combined with the increased incidence of gastrointestinal issues, it is possible that poor adherence to the synbiotic intervention led to adverse effects on the primary outcomes.

Furthermore, van de Pol et al. (2011), asked participants to avoid taking their inhaled corticosteroids from 4-weeks before the study until the study was completed. Before conducting objective assessments of asthma or exercise-induced bronchoconstriction, it is best practice to ask participants to temporarily avoid taking their medication so that a true representation of severity can be obtained (Anderson & Kippelen, 2013; Haines et al., 2017; Hull et al., 2015; Simpson et al., 2016; Weiler et al., 2016; Williams et al., 2015; 2016). As outlined in Table 3.2, participants are typically asked to avoid taking inhaled corticosteroids 12 hours before conducting objective assessments, not for 4-weeks before the study starts, and throughout the

intervention period. Taken together, the results of van de Pol et al. (2011), highlight the issues associated with parallel group designs, the need to prospectively monitor (and stringently assess) adherence, and the adverse effects associated with administering high doses.

Sagar et al. (2014B), provided more conclusive evidence for the protective effect of *Bifidobacterium breve* M-16V, administered as part of a synbiotic following the same protocol as Sagar et al. (2014A), on the severity of asthma. In accordance with their previous investigation of *Bifidobacterium breve* M-16V as a probiotic (Sagar et al., 2014A), Sagar et al. (2014B), demonstrated that combining *Bifidobacterium breve* M-16V with non-digestible oligosaccharides increased the suppressive capacity of systemic and pulmonary FoxP3⁺ T_{REG} cells, as evidenced by an increase in the ratio of cells expressing FoxP3 (T_{REG} cells) to those expressing GATA-3 (T_H2 cells) between the control and synbiotic conditions (0.41 vs. 1.20; $p < 0.05$). This led to a significant reduction in the magnitude of airway eosinophilia ($p < 0.05$), mast cell degranulation, T cell activation, and airway remodeling, respectively (de Kivit et al., 2012; Sagar et al., 2014B). *Bifidobacterium breve* M-16V was administered alongside a combination of short-chain fructooligosaccharides, long-chain fructooligosaccharides, and pectin derived acidic oligosaccharides. Given the highly selective ability of galactooligosaccharides to stimulate the growth and development of *Bifidobacteria* (Davis et al., 2011; Depeint et al., 2008), combining *Bifidobacterium breve* M-16V with galactooligosaccharides might enhance the therapeutic efficacy, especially considering the critical role of galactooligosaccharides in maintaining the suppressive capacity of T_{REG} cells (Verheijden et al., 2015B; 2016).

Verheijden et al. (2016), also reported a significant reduction in the magnitude of airway eosinophilia, and the production of IL-4 and IL-5 following fructooligosaccharide, but not galactooligosaccharide-based synbiotic supplementation alongside *Bifidobacterium breve* M16-V (2×10^9 CFU). The proportion of the active prebiotic ingredient was lower in the galactooligosaccharide (59%), compared to fructooligosaccharide-based intervention (100%, and 95% of inulin and oligofructose for the long-chain, and short-chain fructooligosaccharide-based supplements, respectively; Verheijden et al., 2016). This may explain the less potent anti-inflammatory effects observed following galactooligosaccharide supplementation. The development of supplements with increased

galactooligosaccharide content (Grimaldi et al., 2016), will enable such questions to be explored.

Traditionally, research has investigated the effects of long-term supplementation with probiotics (Arrieta et al., 2015; Konieczna et al., 2012; Sagar et al., 2014A), prebiotics (Davis et al., 2011; So et al., 2018; Thorburn et al., 2015; Verheijden et al., 2015A; 2015B; 2016; 2018; Williams et al., 2016), or synbiotics (Sagar et al., 2014B; van de Pol et al., 2011; Verheijden et al., 2015B). However, research has begun to demonstrate that short-term supplementation with synbiotics attenuates the severity of asthma.

Halnes et al. (2017), investigated the effects of short-term supplementation with a single synbiotic meal on the severity of asthma. The multi-strain probiotic component included *Bifidobacterium lactis* Bb12, *Lactobacillus acidophilus* LA5, and *Lactobacillus rhamnosus* GG at doses of $\geq 10^8$ CFU's, whilst the prebiotic component consisted of 3.5 g of inulin (Halnes et al., 2017). Just 4 hours after consuming the synbiotic meal a significant increase in FEV₁ was observed (Halnes et al., 2017). Within the synbiotic group, this was attributed to a significant decrease in markers of airway inflammation from baseline, such as sputum neutrophils (-67.5 [-158.0, -6.4] change 4 hours post ingestion; $p = 0.033$; Halnes et al., 2017), whereas between the synbiotic and control groups, this was attributed to a significant increase in the expression of GPR41 (5.15 [3.96, 22.9] vs. 0.41 [0.31, 2.37]; $p = 0.027$), and GPR43, respectively (6.26 [1.66, 13.5] vs. 0.31 [0.07, 0.67] fold change 4 hours post ingestion; $p = 0.007$; Halnes et al., 2017).

The significant differences in GPR41 and GPR43 expression between the synbiotic and control group were based on the fold-change observed within each group from baseline to post supplementation (Halnes et al., 2017). However, the baseline expression of GPR41 and GPR43 was much lower in the synbiotic group compared to the control group (Halnes et al., 2017). Baseline differences in clinical characteristics, airway inflammatory markers, and pulmonary function were assessed, but not differences in sputum GPR41 and GPR43 expression. As a result, it is possible that the absence of assessing baseline differences in GPR41 and GPR43 expression may invalidate the conclusions drawn by Halnes et al. (2017).

Furthermore, only 59% of participants in the synbiotic group demonstrated objective confirmation of asthma (Halnes et al., 2017), suggesting that nearly half may not have had active asthma. These observations further highlight the issues associated

with using self-reported physician diagnoses as part of inclusion criteria. In addition, given that Halnes et al. (2017), investigated the effects of inulin using a parallel group design, the short-term effects of galactooligosaccharides remain to be determined in more robust cross-over based approaches.

In theory, synbiotic supplementation could lead to greater therapeutic benefits than probiotics or prebiotic alone, yet this is not reflected in practice, at least in humans (van de Pol et al., 2011). There is currently a lack of understanding regarding the individual mechanisms by which probiotics and prebiotics attenuate the severity of asthma in humans, as well as the factors affecting individual responses (Healey et al., 2016; 2018; Swinton et al., 2018). As a result, further research is required to investigate the mechanisms by which prebiotic supplementation attenuates the severity of asthma, and to understand what factors affect individual responses. An overview of the immunomodulatory properties of synbiotic supplementation in the pathophysiology of asthma is outlined in Table 2.6 below.

Table 2.6 The immunomodulatory properties of synbiotic supplementation in the pathophysiology of asthma.

Reference	Intervention	Immunomodulatory Properties
van de Pol et al., (2011)	Synbiotic supplementation with <i>Bifidobacterium breve</i> M-16V administered alongside a combination of 14.4 g/day ⁻¹ of galactooligosaccharides, and 1.6 g/day ⁻¹ of fructooligosaccharides, for 4-weeks in adults with mild-allergic asthma challenged with HDM (<i>n</i> = 12).	FEV ₁ , airway hyperresponsiveness, and markers of airway inflammation were not attenuated. A significant decrease in serum IL-5 was observed 24 hours after challenge with HDM. A significant increase in PEF was observed following synbiotic supplementation.
Sagar et al., (2014B)	Synbiotic supplementation with <i>Bifidobacterium breve</i> M-16V administered at a dose of 10 ⁹ CFU per day alongside a combination of short-chain fructooligosaccharides, long-chain fructooligosaccharides, and pectin derived acidic oligosaccharides three times per week one hour prior to challenge from days 22 - 55 in a chronic OVA-based model of asthma.	A significant increase in the suppressive capacity of systemic and pulmonary FoxP3 ⁺ iT _{REG} cells. A significant decrease in the magnitude of airway eosinophilia, mast cell degranulation, T cell activation, and airway remodeling.
Verheijden et al., (2016)	Synbiotic supplementation with <i>Bifidobacterium breve</i> M-16V (2 × 10 ⁹ CFU) combined with either a 9:1 mixture of Vivinal [®] GOS to long-chain FOS, or a 1:1 mixture of	A significant reduction in the magnitude of airway eosinophilia, and the production of IL-4 and IL-5 was observed following fructooligosaccharide, but not galactooligosaccharide-based synbiotic supplementation.

short-chain FOS to long-chain FOS in an HDM-based model of asthma.

Synbiotic supplementation was administered 2 weeks prior to sensitisation and continued for a further 2 weeks during the experiment.

Halnes et al., (2017)

Synbiotic supplementation with a multi-strain probiotic including $\geq 10^8$ CFU of *Bifidobacterium lactis* Bb12, *Lactobacillus acidophilus* LA5, and *Lactobacillus rhamnosus* GG, with 3.5 grams of inulin as the prebiotic component.

Administered as a single, synbiotic meal in adults with stable asthma challenged with hypertonic saline ($n = 17$).

A significant increase in FEV₁ was observed in the synbiotic group post supplementation.

Within the synbiotic group, a significant decrease in markers of airway inflammation was observed post supplementation.

Between the synbiotic and control group, a significant increase in the expression of GPR41 and GPR43 was observed post supplementation.

FEV₁ forced expiratory volume in one second, IL interleukin, HDM house dust mite, PEF peak expiratory flow, OVA ovalbumin, FoxP3 fork-head box protein 3, iT_{REG} induced regulatory T cell, CFU colony forming units, GOS galactooligosaccharide, FOS fructooligosaccharide, GPR g-protein coupled receptor.

2.5 Summary

Asthma is the second most prevalent chronic respiratory disease in the world, affecting ~ 3.6% of the global population and equating to 0.9% of global morbidity and mortality (GBD Chronic Respiratory Disease Collaborators, 2020). The United Kingdom has the highest prevalence of asthma in the world, with treatment costing the NHS ~ one billion pounds per year (GBD Chronic Respiratory Disease Collaborators, 2020; Mukherjee et al., 2016). Despite enormous expenditure on healthcare services, the prevalence of asthma remains at epidemic levels, with ~ 1,160 people dying every year (GBD Chronic Respiratory Disease Collaborators, 2017; Mukherjee et al., 2016).

Alarmingly, only around half of adults take their medication on the days advised, with some taking a lower dose than prescribed (Cohen et al., 2009; Williams et al., 2004). Poor adherence increases the risk of exacerbations and progression towards severe refractory asthma (GBD Chronic Respiratory Disease Collaborators, 2017; Engelkes et al., 2015; Hekking et al., 2015; Ivanova et al., 2012; Mukherjee et al., 2016; Williams et al., 2004). Severe refractory asthma is particularly difficult to treat due to the transition from a type-2 (eosinophilic) to a non-type-2 (neutrophilic) phenotype (Peters et al., 2020). Despite only comprising ~ 3.6% of the total asthma population (Hekking et al., 2015), severe refractory asthma accounts for a disproportionate amount of exacerbations and healthcare associated expenditure (Ivanova et al., 2012; Jackson et al., 2020; Peters et al., 2020). On average, patients with severe refractory asthma experience ~ 1 - 4 exacerbations per year (Brinkman et al., 2019A; 2020; Ivanova et al., 2012; Jackson et al., 2020; Peters et al., 2020; Schatz et al., 2014), causing a 48% increase in healthcare associated expenditure.

These observations highlight the importance of preventing the progression of asthma. Given the possible safety concerns associated with high dose inhaled corticosteroids (Chipps et al., 2020), and the development of tolerance associated with repeated use of short-acting β_2 -agonists (Hancox et al., 2002; Weiler et al., 2016; Williams et al., 2020), novel complimentary therapies are required to limit the adverse side-effects of high dose therapies (Chipps et al., 2020; Dickinson et al., 2018), maintain responsiveness to rescue medications (Hancox et al., 2002; Weiler et al., 2016; Williams et al., 2020), and provide greater therapeutic benefits than current treatments alone (Verheijden et al., 2018).

The increase in asthma prevalence over recent decades has been associated with the hygiene hypothesis (Liu, 2015). Various factors associated with Westernised society prevent the adequate exposure to microbes required to develop optimal immune responses (Debarry et al., 2007; Gerhold et al., 2006; Liu, 2015; Reibman et al., 2008; Riedler et al., 2001; Sbihi et al., 2019; Stein et al., 2016; Stockholm et al., 2018; Thorburn et al., 2015; Wolsk et al., 2016). Fetal immune responses are naturally skewed towards type-2 inflammatory responses, highlighting the importance of early life exposures in the reprogramming of type-1 inflammatory responses (Folsgaard et al., 2012; Prescott et al., 1998). However, factors such as delivery via caesarean section (Clemente et al., 2012; Stockholm et al., 2016; Sevelsted et al., 2016; van Nimwegen et al., 2011), exposure to antibiotics (Arpaia et al., 2013; Arrieta et al., 2015; Cait et al., 2018; Sbihi et al., 2019; Stensballe et al., 2013; Stockholm et al., 2016), or deficiencies in dietary fibre intake (Dahl, 2006; Folkerts et al., 2018; Huang et al., 2017; McLoughlin et al., 2019; Mukherjee et al., 2016; Singanayagam et al., 2017; Smits et al., 2016; Tan et al., 2014; 2017; Thorburn et al., 2015), sustain the development of type-2 inflammatory responses that underpin asthma.

Recent evidence suggests that perturbations to the gut microbiota, as a result of these factors, play a critical role in the pathophysiology of asthma. Adults with asthma display features of gut microbial dysbiosis (Barcik et al., 2018; Hevia et al., 2016; Okba et al., 2018), that are associated with clinical and inflammatory parameters of asthma (Begley et al., 2018). Importantly, attenuating the features of gut microbial dysbiosis through prebiotic supplementation protects against the development and progression of asthma (Gourbeyre et al., 2011; Huang et al., 2017; Thorburn et al., 2015; Verheijden et al., 2015A; 2015B; 2018; Williams et al., 2016).

Exercise-induced bronchoconstriction is a subtype of asthma underpinned by type-2 (eosinophilic) inflammatory responses. Estimates of EIB in athletic populations range from 9% to 71.4% (Dickinson et al., 2006A; Sue-Chu et al., 2010), compared to ~ 22% in the general population (Aggarwal et al., 2018; Brummel et al., 2009; Weiler et al., 2016). Recent evidence suggests that both short and long-term supplementation with synbiotics or prebiotics can attenuate the severity of asthma and exercise-induced bronchoconstriction (Haines et al., 2017; Williams et al., 2016). Strikingly, just 4 hours after consuming a synbiotic meal, Haines et al. (2017), reported a significant increase in FEV₁ in adults with asthma, whereas Williams et

al. (2016), reported clinically relevant, participant perceivable improvements in airway hyperresponsiveness in adults with hyperpnoea-induced bronchoconstriction following 3-weeks of galactooligosaccharide-based prebiotic supplementation (Santanello et al., 1999; Williams et al., 2016). However, it is currently unknown whether adults with hyperpnoea-induced bronchoconstriction display features of gut microbial dysbiosis, or how galactooligosaccharides modulate immune function.

In mice, the ability of T_{REG} cells to attenuate the magnitude of eosinophilia and airway hyperresponsiveness was identified as a critical mechanism of galactooligosaccharide-induced improvements in asthma (Verheijden et al., 2015A; 2015B; 2018). However, the ability of T_{REG} cells to attenuate eosinophilia and airway hyperresponsiveness via galactooligosaccharides remains unexplored in adults with hyperpnoea-induced bronchoconstriction, representing an exciting area for future research.

As a result, the aims of the current programme of work are:

- To explore whether adults with hyperpnoea-induced bronchoconstriction display features of gut microbial dysbiosis.

And:

- To investigate the effects of short-term and long-term galactooligosaccharide-supplementation on markers of systemic inflammation and pulmonary function in adults with hyperpnoea-induced bronchoconstriction.

Chapter III

General Methods

3.1 Introduction

This chapter provides a detailed overview of the general procedures used throughout this programme of work. This chapter (Chapter III) is split into eight sections. The first section (Section 3.2) summarises the ethical approval processes. The second section (Section 3.3) outlines the participant recruitment methods. The third section (Section 3.4) details the perceptual measures used to monitor perceptions of asthma control and medication adherence. The fourth section (Section 3.5) illustrates the techniques used to assess gut bacterial composition. The fifth section (Section 3.6) explains the procedures used to assess blood-based markers of systemic inflammation. The sixth section (Section 3.7) describes the protocols used to assess hyperpnoea-induced bronchoconstriction. The seventh section (Section 3.8) covers the prebiotic and placebo supplements administered as part of the short-term (Chapter V), and long-term (Chapter VI) prebiotic supplementation studies, respectively. Finally, the eighth section (Section 3.9) elucidates the protocols used for statistical analysis. This thesis consists of three studies outlined in Chapters IV, V, and VI, respectively.

3.2 Ethical Approval

All studies (Chapters IV – VI) were conducted in accordance with the World Medical Association Declaration of Helsinki (World Medical Association, 2013), and the British Association of Sport and Exercise Sciences (BASES) Code of Conduct (British Association of Sport and Exercise Sciences, 2017).

For all studies (Chapters IV – VI), ethical approval was obtained from Nottingham Trent Universities Human Research Ethics Committee (approval numbers: 418, 512 and 484, respectively). For study 3 (Chapter VI), ethical approval was also obtained from the NHS East Midlands Nottingham 2 Research Ethics Committee (approval number: 17/EM/0369), and Health Research Authority (HRA).

For study 3, approval was sought from the NHS Research Ethics Committee and HRA for two reasons. Firstly, Nottingham Trent University does not have a license from the Human Tissue Authority to store biological samples classed as “relevant”

material on campus. For study 3, “relevant” material refers to peripheral blood mononuclear cells (PBMCs). In accordance with the Human Tissue Authority Code of Practice for Research, clauses 69, 78, and appendix B, institutions that do not hold a license from the Human Tissue Authority may collect, store, and analyse biological samples classed as “relevant” material, for specific research projects, if approval is provided by a “recognised” Research Ethics Committee, such as an NHS Research Ethics Committee.

Secondly, NHS ethical approval was sought to recruit participants with asthma from GP practices within Nottingham City’s Clinical Commissioning Group (CCG). There were ~ 70-80 GP practices within Nottingham City’s CCG, creating a wider population of potentially eligible participants than what could be recruited from Nottingham Trent University alone. A research invitation letter was sent to each GP practice to see if they would like to assist with participant recruitment (Appendix A). The GP practices were provided with copies of the participant recruitment poster to display in the waiting area (Appendix C), and copies of the participant information sheet to provide to patients who were interested in taking part (Appendix E). The participant recruitment poster included contact information for the chief investigator from Nottingham Trent University in case patients wanted to discuss taking part.

3.2.1 Written Informed Consent

For all studies, potential participants were provided with a copy of the participant information sheet and given at least 48 hours to review the information. Potential participants were then provided with the opportunity to discuss any questions they had about taking part with the chief investigator either in person or over the phone. For all studies, written informed consent was obtained prior to any procedures taking place (British Association of Sport and Exercise Sciences, 2013). In accordance with Good Clinical Practice, for study 3, written informed consent was reassessed during each visit throughout the nutritional intervention (Appendix F). For all studies, participants were informed they could withdraw their participation at any point without having to provide a reason. However, if participation was withdrawn after a manuscript had been submitted for publication, individual data could not be withdrawn (British Association of Sport and Exercise Sciences, 2013). Upon withdrawing consent, prior to the submission of manuscripts for publication, the participants paper-based and electronic data were destroyed.

3.2.2 Data Collection & Storage

For all studies, data was collected and stored in accordance with the Data Protection Act (DPA) 2018, and the General Data Protection Regulation (GDPR) 2018. For studies one and two (Chapters IV and V), pseudonymised codes were applied to paper-based and electronic source data obtained from participants to maintain confidentiality (British Association of Sport and Exercise Sciences, 2013). Data obtained in paper copy format, such as written informed consent forms and health screen and history questionnaires (Appendix K), were kept in a locked filing cabinet in the chief investigator's office. Electronic data was stored on password protected institutional user accounts, and only shared with members of the research team via institutional e-mail accounts.

For study three (Chapter VI), in accordance with Good Clinical Practice and the Caldicott Principles, pseudonymised codes were applied to paper-based and electronic source data obtained from participants to remove identifiable information and maintain confidentiality. Data obtained in paper copy format that included identifiable information, such as written informed consent forms, were stored in a folder labelled as "sensitive", with "restricted access", and kept in a locked laboratory separate to pseudonymised source data collected in paper copy format to minimise patient identification. To protect against loss or damage, all pseudonymised source data collected in paper copy format was immediately repopulated into an electronic database. All electronic data was stored on a secure online server that could only be accessed by members of the research team (McLoughlin et al., 2019).

For all studies, in accordance with Nottingham Trent Universities Data Protection and Retention Policy, paper-based and electronic data was stored for up to 5 years. If the data was published within this timeframe, it was destroyed upon publication.

3.3 Participant Recruitment

3.3.1 Internal Recruitment Methods

For all studies, participants were recruited from Nottingham Trent University using opportunistic sampling. For studies one and three (Chapters IV and VI), participants with and without asthma were recruited from Nottingham Trent University. For study two (Chapter V) participants with asthma were recruited from Nottingham Trent University. No control group was included in this study. Participant recruitment

posters were distributed across campus, and recruitment stands were set up during Welcome Week, distributing leaflets to potentially eligible participants (Appendix D).

3.3.2 External Recruitment Methods

For study three, a range of external recruitment methods were used to recruit participants with asthma. Firstly, an application was made to screen the Register of Asthma Research Volunteers (REACH) database, developed by the Asthma UK Centre for Applied Research (AUKCAR; Nwaru et al., 2016), against the studies eligibility criteria. The database included people diagnosed with asthma throughout the United Kingdom who wished to take part in research (Nwaru et al., 2016). A database administrator assessed potential participants within the East Midlands against the studies eligibility criteria. Secondly, advertisements were placed in Asthma UK's research and policy bulletin, a newsletter distributed to a volunteer cohort of ~ 150 individuals. Finally, as outlined above, participant recruitment was carried out from GP practices within Nottingham City's CCG. A research invitation letter was sent to each GP practice to see if they would like to assist with participant recruitment (Appendix A). The GP practices were provided with copies of the participant recruitment poster to display in the waiting area (Appendix C), and copies of the participant information sheet to provide to patients who were interested in taking part (Appendix E). The participant recruitment poster included contact information for the chief investigator from Nottingham Trent University in case patients wanted to discuss taking part.

3.3.3 Eligibility Criteria

3.3.3.1 Inclusion Criteria

To be eligible to take part, participants had to be (Lotvall et al., 2012):

- 18-50 years of age at the date of their first visit.
- Have a body mass index (BMI) of 18.5-25 kg/m².
- Be physically active (completing 3 or more exercise sessions a week lasting at least 45 minutes each).
- Be a non-smoker.
- Have asthma defined as Steps 1, 2, or 3 based on British Thoracic Society guidelines.

- Have a current prescription from their GP if diagnosed with asthma for a short-acting β_2 -agonist.
- Must in the researcher's opinion, be able and willing to follow all trial requirements.

3.3.3.2 Exclusion Criteria

Participants were unable to take part if any of the following criteria applied (McLoughlin et al., 2019):

- Have asthma defined as Steps 4 or 5 based on British Thoracic Society guidelines.
- Do not have a current prescription from their GP for a short-acting β_2 -agonist.
- Regularly consume omega-3 fatty acid supplements, and/or have high levels of food-based omega-3 consumption (e.g., more than 1-2 portions of oily fish a week, such as salmon or mackerel).
- Take a daily dose of aspirin or other non-steroidal anti-inflammatory drugs such as ibuprofen.
- Have consumed prebiotics and/or probiotics (supplements), drugs that affect gastrointestinal mobility, or laxatives in the 4 weeks before signing the consent form.
- Are currently taking a daily dose of anti-histamine, which could not be temporarily avoided for 72 hours before each testing session without exacerbation of symptoms.
- Unable to temporarily avoid taking asthma medication(s) before study visits without an exacerbation of symptoms, e.g., short-acting β_2 -agonists (8 hours before), and standard inhaled corticosteroids (12 hours before).
- Have a vegetarian or vegan diet.
- Have previously been diagnosed with chronic obstructive pulmonary disease (COPD), emphysema, chronic bronchitis, or similar respiratory illness.
- Have ever been hospitalised due to asthma (e.g., intensive care unit).
- Have received treatment with oral corticosteroids/been admitted to hospital during the past 12 months for your asthma.
- Asthma medication increased/stepped-up during participation (e.g., from Step 1 to Step 2, Step 2 to Step 3 etc.).
- Have a history of heart failure, pulmonary hypertension, embolism, or other pulmonary heart disease.

- Have a history of recurrent chest infections.
- Currently smoke.
- Are pregnant, planning to become pregnant during the study, or lactating.
- Had an acute infection in the last four weeks, and/or major operation in the past four months.
- Have a history of gastrointestinal drug reaction.
- Have taken antibiotics in the past 3 months.
- Have a history or current evidence of gastrointestinal disease (e.g., chronic constipation, diarrhoea, irritable bowel syndrome, Crohn's disease).
- Have recently taken part in other research projects.
- Are or believe they may be lactose intolerant.
- Regularly take antioxidant supplements.

Standard multivitamin and mineral supplements were acceptable, as long as the product label stated the recommended Dietary Reference Values (DRV's). If a single supplement (e.g., Vitamin C), was more than the recommended daily DRV's, this was checked with the chief investigator.

3.4 Perceptual Measures

3.4.1 The Asthma Control Questionnaire-7 (ACQ-7[®])

The asthma control questionnaire-7 (ACQ-7[®]) was used to assess self-reported participant perceptions of asthma control over the preceding week (Appendix H; Juniper et al., 1999; 2006). The ACQ-7[®] is a validated questionnaire that can discriminate between stable and unstable asthma, and detect clinically important changes in participant perceptions of asthma control (Juniper et al., 1999). The ACQ-7[®] is more informative than the Asthma Control Test (ACT[®]), due to the inclusion of pulmonary function measurements when assessing asthma control, and less onerous than the Asthma Quality of Life Questionnaire (AQLQ[®]; Boulet et al., 2002; Juniper et al., 1999).

At rest, the severity of seven parameters was scored on a 7-point scale, ranging from 0 indicating "least severe", to 6 indicating "most severe" (Juniper et al., 1999; 2006). Participants provided ratings for the first 6 parameters, whereas the seventh parameter (percentage predicted FEV₁) was rated by the researcher. These first 6 parameters included statements assessing night-time awakening by symptoms, symptom severity upon waking, activity limitation due to symptoms, shortness of

breath, wheeze, and the use of short-acting β_2 -agonists, respectively (Juniper et al., 1999). The seventh parameter, percentage-predicted FEV₁, was measured via spirometry (Miller et al., 2005). The asthma control score was calculated by taking the average of the sum of all seven parameters (Juniper et al., 1999; 2006). The lowest average score that could be obtained was 0, and the highest average score that could be obtained was 6. A score of ≤ 0.75 indicated stable asthma, whereas a score of ≥ 1.50 indicated unstable asthma (Juniper et al., 2006).

The ACQ-7[®] was used during studies one and three (Chapters IV and VI), before the EVH protocol was carried out. Participants became accustomed to the ACQ-7[®] during the familiarisation trial. During study one, the ACQ-7[®] was administered during the experimental trial. During study three, the ACQ-7[®] was administered at rest every week throughout the 10-week intervention, including during each experimental trial and the washout period, respectively.

3.4.2 The Medication Adherence Report Scale for Asthma (MARS-A[®])

The Medication Adherence Report Scale for Asthma (MARS-A[®]) was used to monitor self-reported adherence to inhaled corticosteroids over the preceding week (Appendix I, Cohen et al., 2009). The MARS-A[®] has been validated against electronic methods of adherence monitoring (Cohen et al., 2009). However, some degree of over-reporting is expected with self-reported measures (Haynes et al., 2002), regarding the number of days when inhaled corticosteroids are used, and the number of times when prescribed doses are taken (Cohen et al., 2009). The MARS-A[®] is an easy to administer questionnaire that is more cost effective than electronic methods of adherence monitoring (Cohen et al., 2009).

At rest, participants were asked to rate the accuracy of ten statements on a 5-point scale, ranging from 1 indicating “always”, to 5 indicating “never” (Cohen et al., 2009). The ten statements included “I only use my medication when I need it”, “I only use it when I feel breathless”, “I decide to miss out a dose”, “I try to avoid taking it”, “I forget to take it”, “I alter the dose”, “I stop taking it for a while”, “I use it as a reserve, if my other treatment doesn’t work”, “I use it before doing something which might make me breathless”, and “I take it less than instructed” (Cohen et al., 2009). The adherence score was calculated by taking the average of the sum of all ten parameters (Cohen et al., 2009). The lowest average score that could be obtained

was 1, and the highest average score that could be obtained was 5. A score ≥ 4.5 indicated high adherence (Cohen et al., 2009).

The MARS-A[®] was used during study three (Chapter VI). Participants became accustomed to the MARS-A[®] during the familiarisation trial. During study three, the MARS-A[®] was administered at rest every week throughout the 10-week intervention, including during each experimental trial and the washout period, respectively. In accordance with Haynes et al. (2002), the MARS-A[®] was modified to obtain quantitative information on the number of medication doses missed over the preceding week. Additional questions were added to assess the number of medication doses missed (Haynes et al., 2002), as well as the number of nutritional sachets missed over the preceding week (during supplementation periods).

3.5 Gut Bacterial Analysis

3.5.1 Faecal Sample Collection

During the experimental trial of study one (Chapter IV), faecal samples were obtained to assess the gut bacterial composition of adults with and without HIB. The methods used to collect, process and analyse faecal samples were developed in house at the University of Reading (Vulevic et al., 2015). Sample collection at processing was carried out at Nottingham Trent University, whereas sample analysis was conducted at the University of Reading.

On the morning of the experimental trial, faecal samples were collected using a Fecotainer[®] collection kit (Fecotainer[®], 460869, DaklaPack[®], London, United Kingdom). Faecal samples were collected up to 2 hours before the experimental trial to minimise the adverse effects of prolonged exposure to an aerobic environment on gut bacterial composition. Participants were instructed on how to use the Fecotainer[®] during the familiarisation trial. As outlined in Figure 3.0, the Fecotainer[®] is a collapsible collection pot that is positioned under the toilet seat, making sample collection easier and less prone to contamination. The researcher set up the Fecotainer[®] in a designated toilet on campus. Participants were instructed to provide their faecal sample, and seal the Fecotainer[®] by applying the twistable lid before using the bathroom for any other purposes, such as urination, washing or drying their hands. This was to avoid contaminating the faecal sample with urine, water from flushing the toilet, or detergents used for hand washing. Once collected, the Fecotainer[®] was immediately sealed in a biohazard waste bag to further

minimise air contact, exposure, and contamination. As soon as was practical around conducting the other measurements involved in the experimental trial, the faecal sample was transferred to a containment level two laboratory at Nottingham Trent University for processing. In all cases, faecal samples were processed and stored at -20°C within 4 hours of collection.

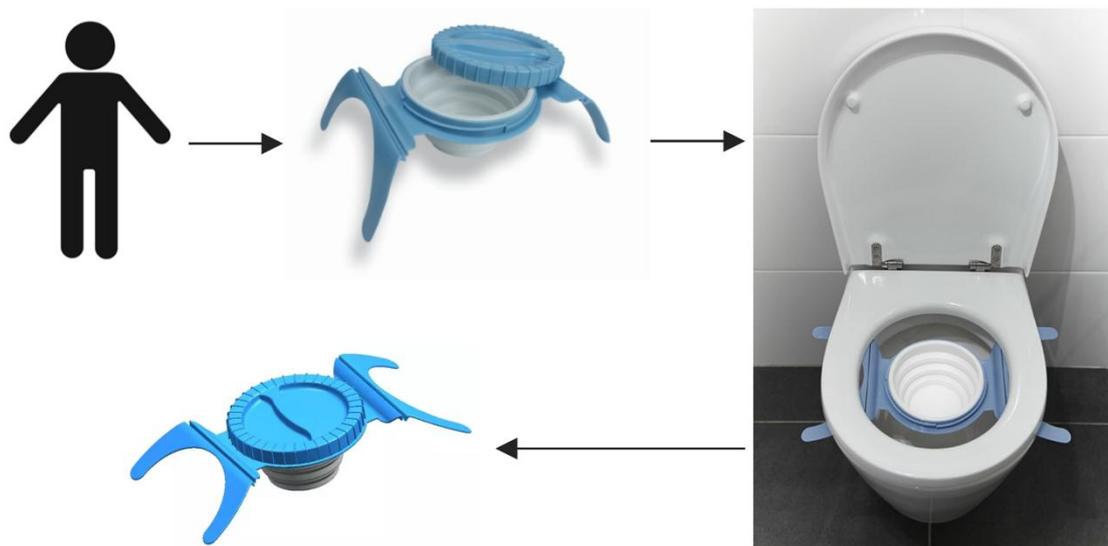


Figure 3.0 An overview of the method used to collect faecal samples using the Fecotainer® collection kit. Created with www.BioRender.com.

3.5.2 Faecal Sample Processing

3.5.2.1 Faecal Sample Dilution

Faecal samples were processed in a containment level two laboratory at Nottingham Trent University. As outlined in Figure 3.1, the first step involved assessing sample dilution by measuring the wet weight and dry weight of faecal samples. A sterile wooden spatula was used to extract ~ 1-gram aliquot from the Fecotainer®. The 1-gram aliquot obtained straight from the Fecotainer® was used as a reference for faecal sample wet weight. To determine faecal sample dry weight, the 1-gram aliquot was dehydrated in an oven preheated to 80° C for 1 hour. The difference in weight after dehydration indicated sample dilution. This was measured to identify factors that could impair the bacterial concentration of the sample, such as diarrhoea, constipation, or obstipation. If gastrointestinal disorders were evident, faecal samples were excluded from analysis.

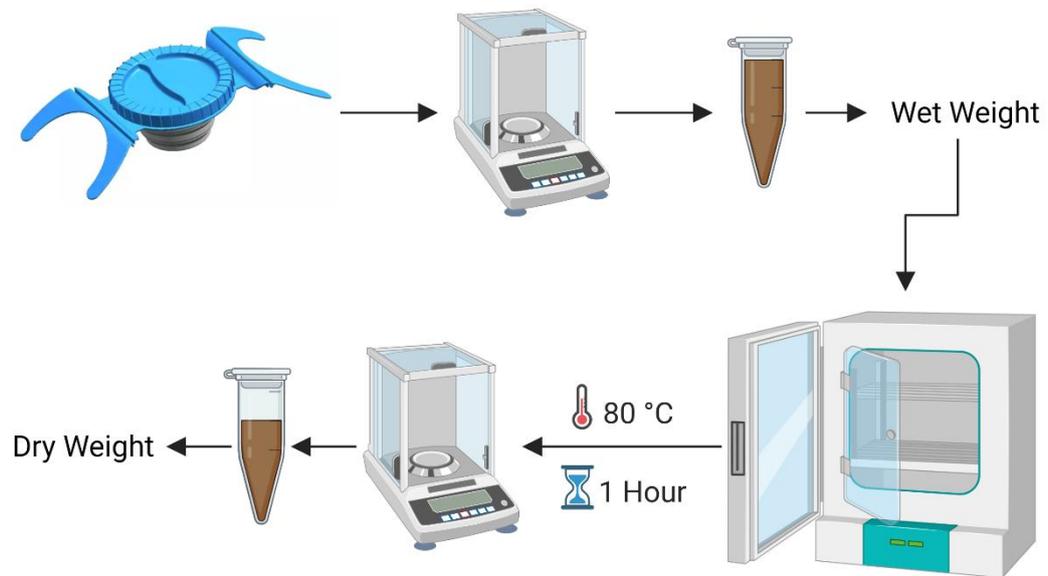


Figure 3.1 The protocol used to assess faecal sample dilution by measuring the difference between wet weight and dry weight. Created with www.BioRender.com.

3.5.2.2 Faecal Sample Fixation

As outlined in Figure 3.2, a 10% faecal slurry was prepared in a sterile 50mL tube (Sarstedt, 65.547.254, Numbrecht, Germany), containing ~ 20g of faecal sample and 90% 1 × phosphate buffered saline (PBS). Once prepared, the faecal slurry was transferred into a square-bottomed stomacher bag (Seward, BA6041, United Kingdom), and mixed in a stomacher at moderate speed for 120 seconds to homogenise the solution (Stomacher®, Seward, 400 Circulator, United Kingdom). After mixing, the sample was transferred into a sterile 50mL tube containing ~ 5mL of glass beads (Merck Millipore, 104017, United Kingdom). The sample was mixed using a vortex for ~ 30-60 seconds to separate the fibre (bottom) from the bacteria (top, Vortex Mixer, Z3121851, Labnet International, New Jersey, USA).

Following separation using the vortex, 5 × 1mL aliquots of the bacterial sample were transferred, by pipetting from the top of the mixture, into sterile 2mL eppendorf tubes (Sigma-Aldrich, BR780546, United Kingdom). The aliquots were then centrifuged at 214 rcf for 3 minutes at room temperature (Sartorius Micro-Centrifuge, MIKR0200, United Kingdom). 375µL aliquots of the bacterial sample were added to sterile 2mL eppendorf tubes containing 1125µL of chilled 4% paraformaldehyde (PFA; Sigma-Aldrich, 101227996, United Kingdom). The samples were inverted once, and incubated at 4°C for 4-8 hours.

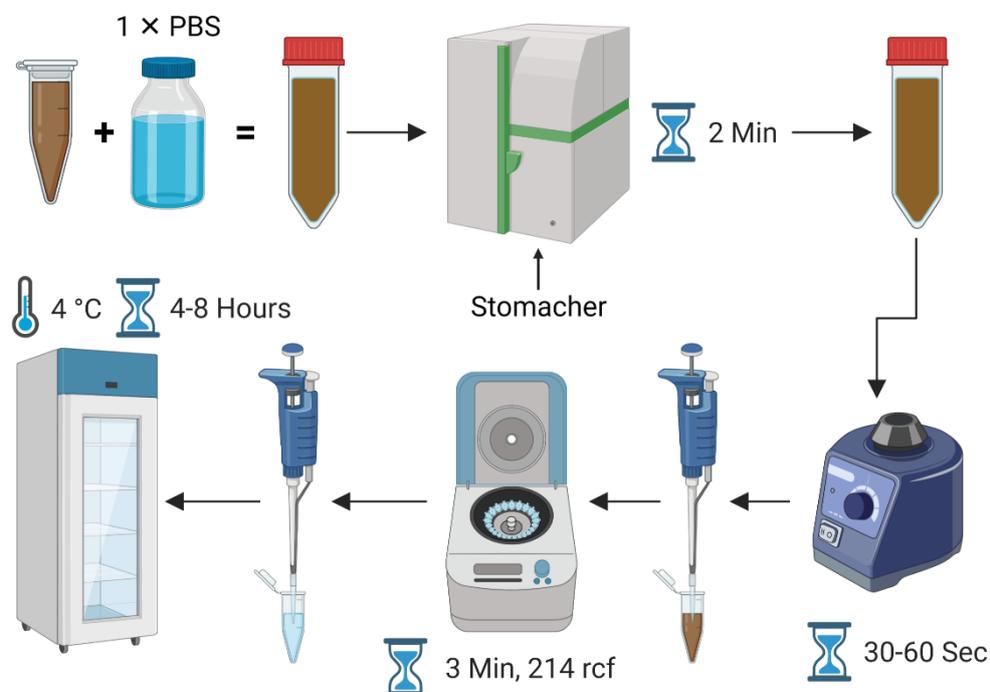


Figure 3.2 The protocol used to fix faecal samples ready for analysis (Part I). Created with www.BioRender.com.

Following incubation, the samples were centrifuged at 16060 rcf for 5 minutes at room temperature to separate the crude faecal water (supernatant) from the bacterial pellet. As outlined in Figure 3.3, the bacterial pellet was washed and resuspended in 1mL of 1 x PBS to remove the 4% PFA solution and prevent further permeability of the bacterial cell wall. Samples were then centrifuged at 16060 rcf for 5 minutes at room temperature. The supernatant was discarded, and the bacterial pellet resuspended in 1mL of 1 x PBS. Washing was repeated twice more. After the final wash, residual liquid was taken from the tube, with care taken to avoid disrupting the pellet. Any residual liquid was removed by drying on tissue paper, and pipetting around the inside of the eppendorf without disturbing the bacterial pellet. The bacterial pellet was then resuspended in 150µL of 1 x PBS. Finally, 150µL of ethanol was added as a cryoprotectant. Samples were mixed using a vortex and stored at -20°C until transport to the University of Reading for analysis.

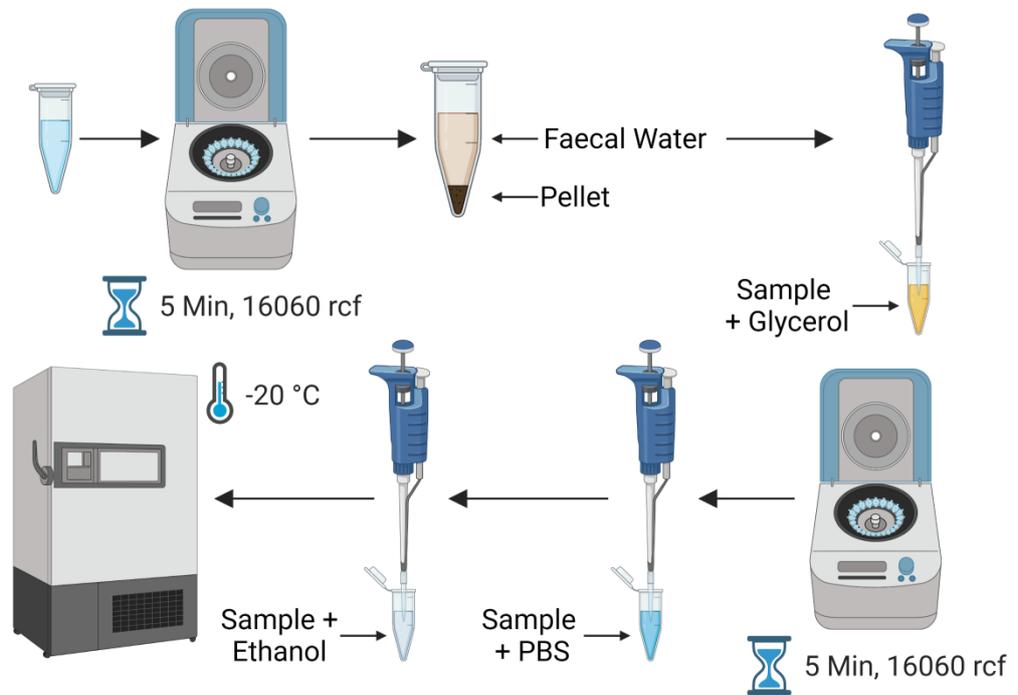


Figure 3.3 The protocol used to fix faecal samples ready for analysis (Part II). Created with www.BioRender.com.

3.5.2.3 Faecal Sample Analysis

Faecal sample analysis was conducted at the University of Reading via fluorescent *in situ* hybridisation (FISH) based flow cytometry. Fluorescent *in situ* hybridisation is a culture independent technique used to assess absolute counts of selected bacteria. During FISH-based flow cytometry, the 16S ribosomal RNA gene (16S rRNA) is targeted due to its ubiquitous expression and preservation of both conserved and variable regions (Gibson & Roberfroid, 1999; Osborn & Smith, 2005). The 16S rRNA gene has hypervariable regions, making it ideal for distinguishing between microbial groups (Gibson & Roberfroid, 1999; Osborn & Smith, 2005).

In accordance with the protocol of Poveda et al. (2020), samples were defrosted and mixed using a vortex for 10 seconds. 75 μ L of the sample was added to a 1.5mL eppendorf tube containing 500 μ L of 1 \times PBS, mixed using a vortex, and centrifuged at 11,337 \times g for 3 minutes. Following supernatant removal, 100 μ L of Tris-EDTA buffer (1mg/mL) was added to the tube. The sample was mixed and incubated in the dark for 10 minutes. Following incubation, the sample was mixed using a vortex, and centrifuged at 11,337 \times g for 3 minutes. Following supernatant removal, the pellet was resuspended in 500 μ L of 1 \times PBS, mixed using a vortex, and centrifuged at 11,337 \times g for 3 minutes.

Following supernatant removal, the pellet was resuspended in 150µL of hybridisation buffer, mixed using a vortex, and centrifuged at 11,337 × g for 3 minutes. Following supernatant removal, the pellet was resuspended in 1mL of hybridisation buffer. 50µL of the sample was transferred into a fresh eppendorf tube before adding 4µL of the oligonucleotide probe solutions (50ng/µL). Samples were mixed using a vortex and incubated overnight at 36°C. Following hybridisation, 125µL of hybridisation buffer was added, the samples mixed using a vortex, and centrifuged at 11,337 × g for 3 minutes. Following supernatant removal, the pellet was resuspended in 175µL of wash buffer, mixed using a vortex, and incubated for 30 minutes at 35°C in the dark. Following incubation, samples were centrifuged at 11,337 × g for 3 minutes. Following supernatant removal, 300µL of 1 × PBS was added, the samples mixed using a vortex, and stored at 4°C in the dark until analysis.

For the current analysis, flow cytometry was used to assess absolute counts of key bacterial groups (Wagner et al., 2003). The fluorescent oligonucleotide probes used to assess key bacterial groups are outlined in Table 3.0 below. Absolute counts for key bacterial groups were determined by comparison to a domain specific probe (EUB338; Amann, Fuchs & Behrens, 2001; Gibson & Roberfroid, 1999; Osborn & Smith, 2005; Wagner et al., 2003). A two colour BD Accuri™ C6 flow cytometer and Accuri CFlow Sampler software was used for analysis (BD Biosciences, Brussels). An overview of the protocol used to analyse gut bacterial samples is outlined in Figure 3.4 below. Data is presented as log₁₀ cells per gram of faeces dry weight (log₁₀ cells/g).

Table 3.0 The key bacteria, and associated oligonucleotide probes, used to assess gut bacterial composition via FISH-based flow cytometry.

Key Bacteria	Probe
Total Bacteria	EUB338
<i>Bifidobacterium</i>	BIF164
<i>Lactobacillus-Enterococcus</i> group	LAB158
<i>Bacteroides-Prevotella</i> group	BAC303
<i>Eubacterium rectale-Clostridium coccoides</i> group	EREC482
<i>Roseburia-E rectale</i> group	RREC584
<i>Atopobium-Coriobacterium</i> group	ATO291
<i>Clostridial cluster IX</i>	PROP853
<i>Faecalibacterium prausnitzii</i>	FPRAU645
<i>Desulfovibrio</i>	DSV687
<i>Clostridium histolyticum-Clostridium perfringens</i> group	CHIS150

FISH fluorescent *in situ* hybridisation.

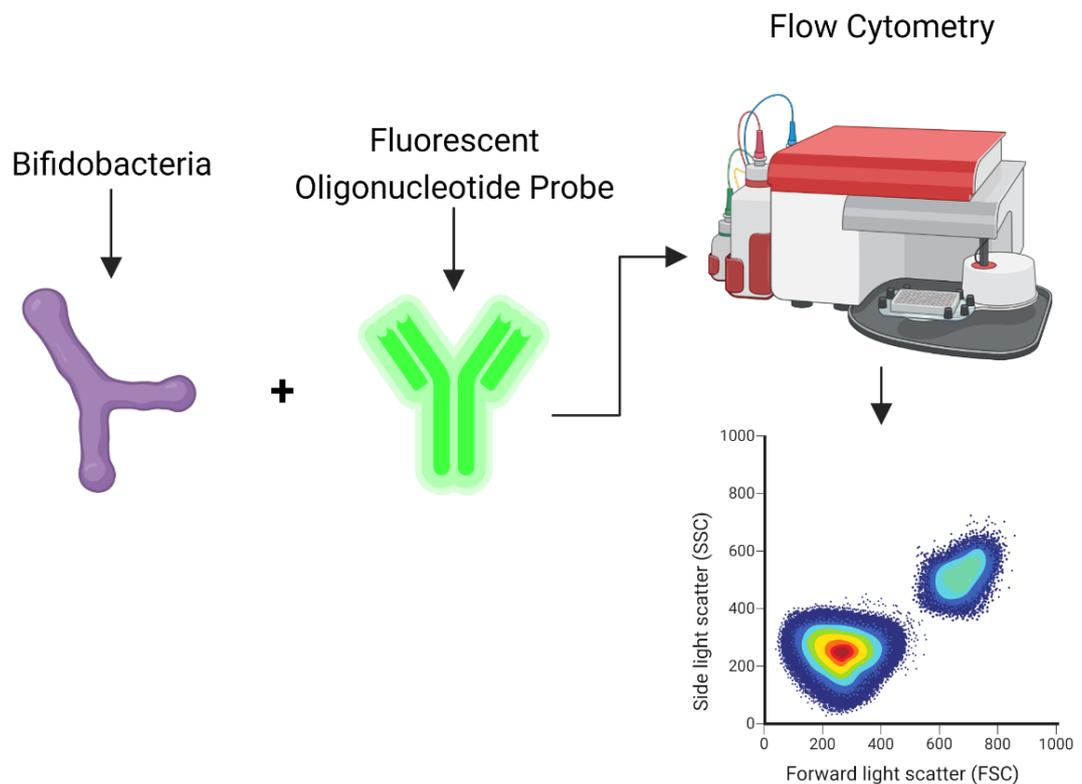


Figure 3.4 Using *Bifidobacterium* as an example, key bacteria from faecal samples were analysed using fluorescent *in situ* hybridisation-based flow cytometry. Created with www.BioRender.com.

3.6 Markers of Systemic Inflammation

3.6.1 Blood Sample Collection

During the experimental trials of all studies (Chapters IV – VI), blood samples were obtained through venepuncture by a trained phlebotomist following sterile protocols (Brooks, 2014; Calfee & Farr, 2002; World Health Organisation, 2010). Written informed consent was obtained prior to all procedures (Appendix G). Following palpation and site selection (Buowari, 2013; World Health Organisation, 2010), venepuncture was conducted on a vein in the antecubital fossa region of the arm. Blood samples were obtained in a closed system using a 21-gauge butterfly needle connected to a pre-attached holder via a luer adapter (BD Vacutainer® Safety-Lok™ Blood Collection Set, 368657, BD Biosciences, New Jersey, USA). Samples were gently inverted 8 times, and placed on a mechanical rotating mixer at room temperature for ~ 30-60 minutes until analysis.

3.6.2 White Blood Cell Analysis

During the experimental trials of all studies (Chapters IV – VI), white blood cell subsets were quantified in fresh whole blood samples using an automated haematology analyser (Sysmex, XS-1000i™, United States). At rest, a 4mL blood sample was collected into a vacutainer tube containing ethylenediaminetetraacetic acid (EDTA, BD Vacutainer®, 367839, BD Biosciences, New Jersey, USA). Blood samples were analysed for absolute counts of total white blood cells, eosinophils and basophils ($\times 10^9/L$), as well as the relative abundance (%) of eosinophils and basophils, respectively. Following collection, blood samples were inverted 8 times, and placed on a mechanical rotating mixer at room temperature until analysis.

In accordance with the manufacturer's instructions, quality control checks were conducted on the morning of each experimental trial (E-Check XS, Sysmex, 211155, United States). This involved running a quality control sample of blood through the analyser that resembled human blood as close as possible. The QC sample was provided by the analyser manufacturer (Sysmex) and was classed as "Level 2", meaning it covered the normal range of concentrations of the parameters being analysed. The quality control sample is accompanied by a CD that contains data for the lower and upper limits of the parameters being analysed. The CD was initially installed via the computer. The quality control sample was then ran once each day a sample needed to be analysed (i.e. running the QC sample first thing in the

morning before a day of data collection). As the QC sample is assessed, the analyser will indicate if any parameters are outside of the expected range. The QC samples had a shelf life of approximately 4-12 weeks (depending on when they were purchased), so these were replaced regularly to ensure the QC analysis was accurate.

3.6.3 Peripheral Blood Mononuclear Cells (PBMC) Analysis

During study three (Chapter VI), peripheral blood mononuclear cells (PBMCs) were isolated from fresh whole blood (30mL) using density gradient centrifugation (Lotvall et al., 2012; Sugita et al., 2018; van de Pol et al., 2011). Blood samples were collected at rest during each experimental trial. The methods used to collect, process and analyse PBMCs were developed in house at Nottingham Trent University by The John van Geest Cancer Research Centre (Chen et al., 2018; Kearley et al., 2005; 2008; Konieczna et al., 2012).

3.6.3.1 PBMC Isolation

At rest, a 30mL blood sample was collected into 3 × 10mL vacutainer tubes containing lithium heparin (BD Vacutainer®, 367526, BD Biosciences, New Jersey, USA; van de Pol et al., 2011). Following collection, samples were inverted 8 times, and placed on a mechanical rotating mixer at room temperature until processing. Samples were transferred to a containment level two laboratory. Sterile protocols were followed throughout the isolation and storage of PBMCs. As outlined in Figure 3.5 below, 15mL of blood was decanted into 2 × sterile 50mL tubes (Sarstedt, 65.547.254, Numbrecht, Germany). Once decanted, 15mL of sterile 1 × PBS was added to each tube (Lonza, 14.10 CHF, Switzerland; Maazi et al., 2018). The blood/PBS mixture from each 50mL tube was then decanted into a sterile 50mL LeucoSep tube containing a partially permeable membrane (Greiner Bio-One, G163290, Austria), filled with 15mL of lymphocyte separation medium (Ficoll-Paque™ Plus Separation Medium, 17-1440-03, GE Healthcare, United Kingdom; Chen et al., 2018; Lotvall et al., 2012; Sugita et al., 2018). The samples were centrifuged at 800 rcf for 30 minutes at 20°C with no brake to isolate the PBMCs from the other blood components. After centrifugation, the plasma layer was extracted using a sterile 3mL pasteur pipette. After centrifugation, the separation medium and red blood cells become trapped below the partially permeable

membrane, preventing mixing with the PBMC fraction. The PBMC fraction from each tube was then decanted into a fresh 20mL tube.

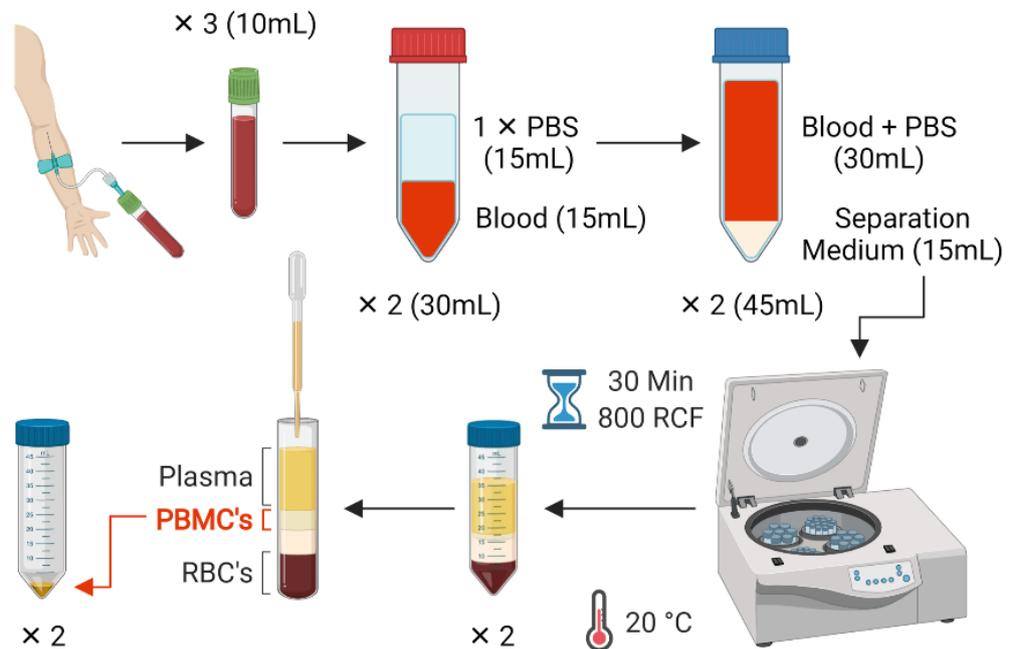


Figure 3.5 The separation of peripheral blood mononuclear cells (PBMCs) from whole blood via density gradient centrifugation. *RCF* relative centrifugal force, *RBCs* red blood cells. Created with www.BioRender.com.

3.6.3.2 PBMC Cell Counting

As outlined in Figure 3.6, each 20mL tube was made up to 20mL with sterile 1 x PBS. Samples were centrifuged at 600 rcf for 10 minutes at 20°C. The supernatant was discarded, and the pellet resuspended in 1mL of sterile 1 x PBS. One tube was made up to 20mL with sterile 1 x PBS and decanted into the other tube. The sample was centrifuged at 400 rcf for 10 minutes at 20°C. The supernatant was discarded, and the pellet resuspended in 1mL of fetal calf serum (FCS). A further 1mL of FCS was added for every 10mL of blood collected (3mL of FCS for a 30mL blood sample).

An automated cell counter was used to measure cell count and viability (NucleoCounter® NC-250™, Chemometec, Denmark). To prepare the cell counting solution, 10µL of the PBMC sample was added to a sterile 0.5mL eppendorf containing 90µL of sterile 1 x PBS. Once the PBMCs had been added to the PBS, 5µL of the fluorescent dye Solution 18 (AO-DAPI, 910-3018, Chemometec, Denmark) was added to stain all live and dead cells. Once the Solution 18 had been added, 12µL of the mixture was dispensed onto an A8 slide for analysis (NC-Slide

A8™, 942-0003, Chemometec, Denmark). The live cell count was provided as 1×10^6 cells/mL⁻¹, whereas cellular viability was expressed as a percentage of total cells.

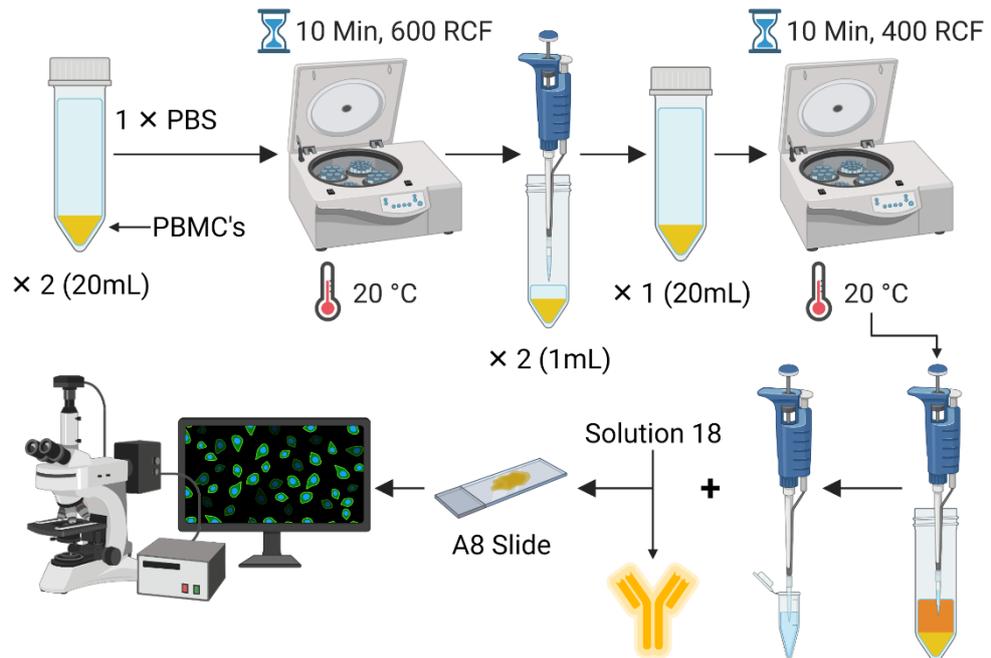


Figure 3.6 The counting of peripheral blood mononuclear cells (PBMCs) prior to storage in liquid nitrogen. *RCF* relative centrifugal force. Created with www.BioRender.com.

3.6.3.3 PBMC Storage

A typical concentration of PBMCs is $\sim 1\text{-}2 \times 10^6$ cells per mL of blood. Given that 30mL of blood was initially collected, total cell counts should be $\sim 30\text{-}60$ million cells per sample. As outlined in Figure 3.7, the cells were stored in cryogenic vials at a final concentration of 10×10^6 cells/mL⁻¹ in 90% FCS and 10% dimethyl sulfoxide (DMSO). The DMSO was added as a cryoprotectant to minimise the loss of cells due to the freeze-thaw process. Approximately 50% of PBMCs die due to the freeze-thaw process. To analyse PBMCs via flow cytometry, 4×10^6 cells are required. As a result, storage under these concentrations ensured a sufficient numbers of cells was retained for analysis.

To prevent the adverse effects of drastic temperature changes on PBMC viability, cryogenic vials were placed inside a cool cell and stored overnight in a -80°C freezer. The following morning, the cryogenic vials were transferred from the cool cell to a standard freezer box, and stored in liquid nitrogen until analysis (Mitson-Salazar et al., 2016; van de Pol et al., 2011).

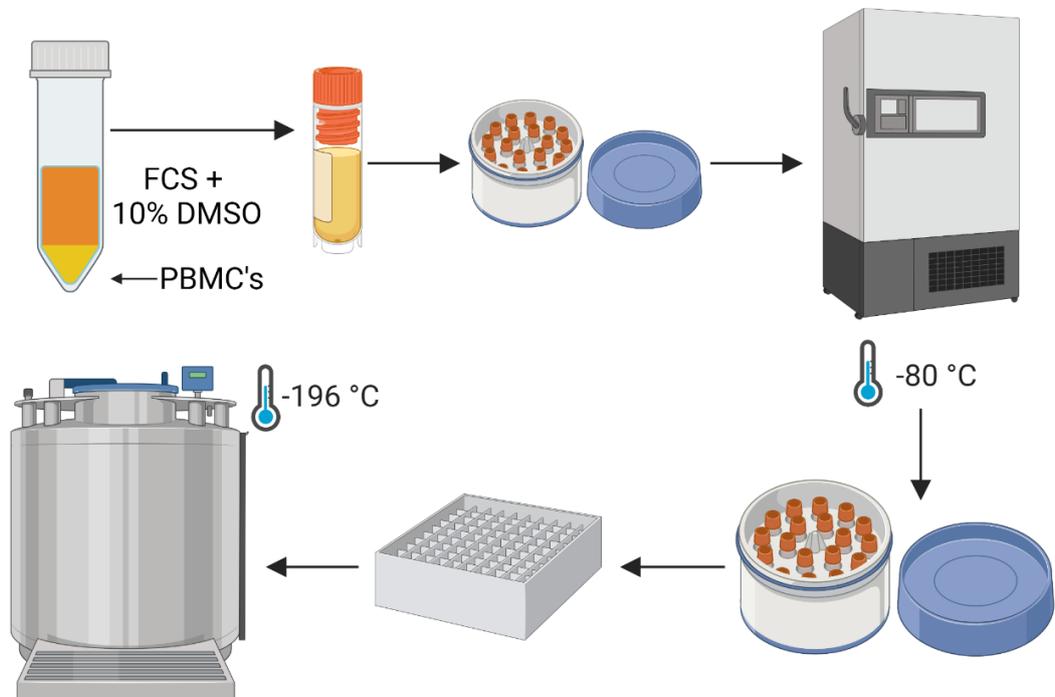


Figure 3.7 The storage of peripheral blood mononuclear cells in liquid nitrogen prior to analysis via flow cytometry. *FCS* fetal calf serum, *DMSO* dimethyl sulfoxide. Created with www.BioRender.com.

3.6.3.4 PBMC Analysis

Flow cytometry was used to assess the functional capacity of T_{REG} , $T_{\text{H}2}$, and $T_{\text{H}1}$ cells. The suppressive capacity of three T_{REG} cell subsets was assessed by analysing the expression of anti-inflammatory mediators, including $\text{IL-10}^+ \text{iT}_{\text{R}1}$ cells, $\text{CD25}^+ \text{FoxP3}^+ \text{nT}_{\text{REG}}$ cells, and $\text{CD25}^- \text{FoxP3}^+ \text{iT}_{\text{REG}}$ cells, respectively (Bohm et al., 2015; Fantini et al., 2004; Hong et al., 2018; Kearley et al., 2005; 2008; Khumalo et al., 2020; Lombardi et al., 2012; Matsuda et al., 2017; 2018; 2019; Ostroukhova et al., 2004; Polansky et al., 2010; Sakaguchi et al., 2013; Thorburn et al., 2015; Zhang et al., 2012). The pro-inflammatory capacity of one $T_{\text{H}2}$ cell subset was assessed, specifically $\text{CD161}^{\text{HIGH}} \text{hPGDS}^+ \text{peT}_{\text{H}2}$ cells (Mitson-Salazar et al., 2016). Finally, the anti-inflammatory capacity of conventional $T_{\text{H}1}$ cells was assessed by measuring the expression of $\text{IFN-}\gamma$ (Debarry et al., 2007; 2010; Ding et al., 2018). A

detailed overview of the role of these cells in the pathophysiology of exercise-induced bronchoconstriction is provided in Chapter II.

The cryogenic vials were rapidly defrosted from liquid nitrogen using a hairdryer. As outlined in Figure 3.8, up to 4 cryogenic vials from the same sample were pooled into 1 sterile 20mL tube. Each cryogenic vial was rinsed with 1mL of warm (37°C) thaw solution. The thaw solution was made fresh on the day of analysis containing 45mL of RPMI 1640 media (Scientific Laboratory Supplies, LZBE12-167F, United Kingdom), 5mL wash supplement (Cellular Technology Limited, CTLW-010, Germany), and 10µL benzonase, respectively (Merck, 71205-3, United Kingdom). Slowly, drop-by-drop, the rinse was added to the 20mL tube to prevent damage to the cells. The 20mL tube was then made up to 12mL with warm thaw solution. The samples were then centrifuged at 400 rcf for 10 minutes at 4°C. The supernatant was discarded, and the cells resuspended by adding 1mL of warm thaw solution. Up to 4mL of warm thaw solution was added to each tube, 1mL per cryogenic vial.

As outlined in Section 3.6.2 above, an automated cell counter was used to measure cell count and viability. The cell count was repeated after the defrost to calculate the number of cells required to conduct flow cytometry. A 1 in 2 dilution was prepared by adding 50µL of the PBMC sample to a sterile 0.5mL eppendorf tube containing 50µL of wash supplement (Cellular Technology Limited, Europe GmbH, CTLW-010, Germany). 50µL of the PBMC/wash supplement mixture was then transferred into a sterile 0.5mL eppendorf tube. 2.5µL of Solution 18 was added to the mixture. 12µL of the mixture was then dispensed onto an A8 slide for analysis. Following the cell count, samples were rested in an incubator at 37°C, 5% CO₂ for 1 hour.

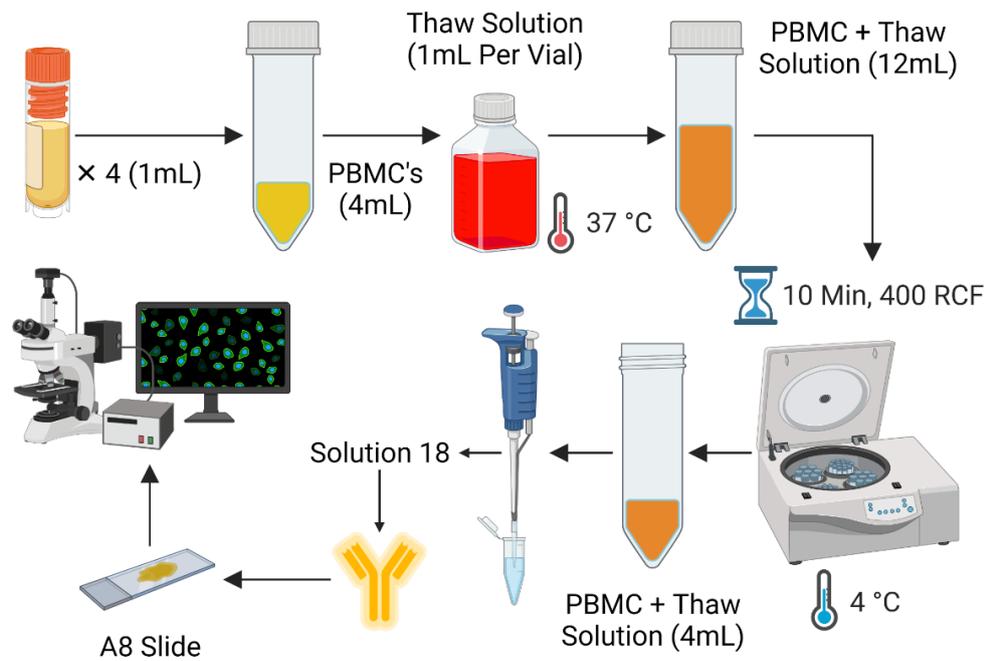


Figure 3.8 The defrosting and counting of peripheral blood mononuclear cells (PBMCs) in preparation for flow cytometry. Created with www.BioRender.com.

3.6.3.5 Cell Surface Staining

Each sample was divided between two 12 × 75mm polystyrene tubes, one for stained cells and one for unstained cells (control), respectively. Unstained cell tubes underwent the same process as stained cells, except without adding fluorescent antibodies. 2×10^6 cells were transferred into each tube. The volume in μL required to obtain 2×10^6 cells from each sample was calculated as follows:

$$\text{Volume } (\mu\text{L}) = 2 \times 10^6 \div \text{Live Cell Count} \quad \text{(Eq. 3.0)}$$

As outlined in Figure 3.9, the samples were washed by adding 2mL of 1 × PBS per tube, and centrifuged at 400 rcf for 5 minutes at 4°C. The supernatant was discarded, and the tubes dried with paper tissue. The pellet was resuspended by flicking the bottom of the tube. After resuspension, 90µL of 1 × PBS was added to each tube, followed by 10µL of FcR blocking reagent (Miltenyi Biotec, 130-059-901, Germany; Coomes et al., 2017). The FcR blocking reagent prevents the non-specific binding of fluorescent antibodies. The samples were mixed using a vortex, and incubated in the dark for 10 minutes at 4°C. Following incubation, a master mix of the cell surface antibodies was added to each stained tube, including CD3, CD25, CD161, Fixable Live-Dead violet, and CD4, respectively. The samples were mixed using a vortex, and incubated for 30 minutes at room temperature in the dark.

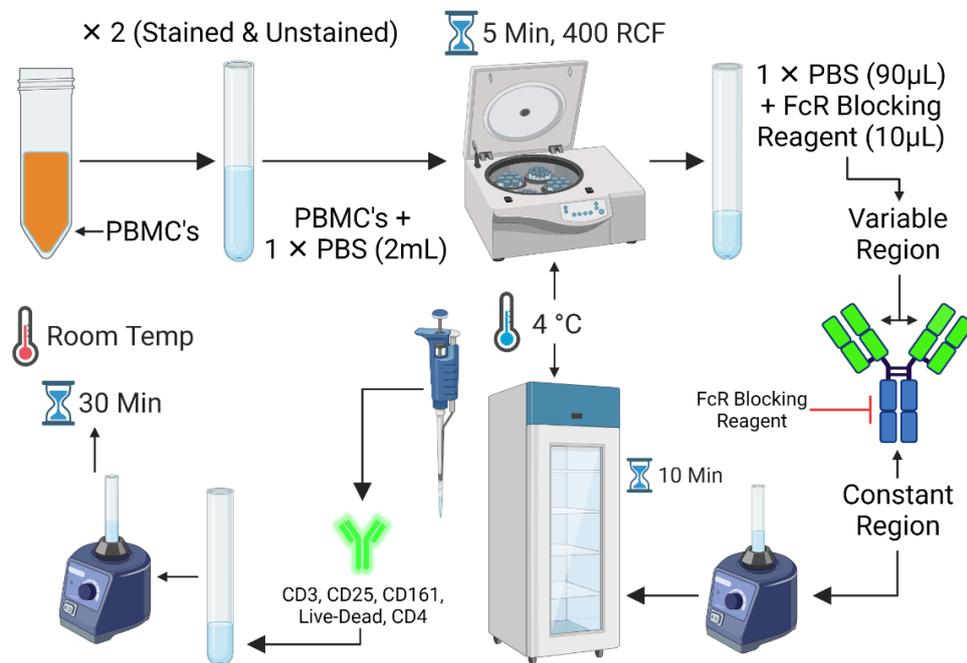


Figure 3.9 The cell surface staining of peripheral blood mononuclear cells (PBMCs) in preparation for flow cytometry. Created with www.BioRender.com. Red flat-headed arrows indicate inhibition.

3.6.3.6 Intracellular Staining

Following cell surface staining, the samples were washed by adding 2mL of 1 × PBS per tube, and centrifuged at 400 rcf for 5 minutes at 4°C. The supernatant was discarded, and the tubes dried with paper tissue. The pellet was resuspended by flicking the bottom of the tube. As outlined in Figure 3.10, to prepare for intracellular staining, 1mL of FoxP3 fixation/permeabilisation solution (Fisher Scientific, 11500597, United States; Maric et al., 2018), was added to each tube (both stained

and unstained; Lotvall et al., 2012). The samples were mixed using a vortex, and incubated for 30 minutes at room temperature in the dark. The samples were washed by adding 2mL of FoxP3 wash buffer per tube, and centrifuged at 400 rcf for 5 minutes at 4°C. The supernatant was discarded, and the tubes dried with paper tissue. The pellet was resuspended by flicking the bottom of the tube.

After resuspension, 100µL of diluted rat serum (Fisher Scientific, 15508326, United States), was added to each tube. The tubes were then incubated for 15 minutes at room temperature in the dark. Following incubation, a master mix of intracellular antibodies was added to each stained tube, including IL-10, hPGDS, TGF-β1, FoxP3, and IFN-γ, respectively. All antibodies were already conjugated except for hPGDS that was conjugated in house. Lyophilised hPGDS antibody (Bio-Techne®, MAB6487-SP, United Kingdom), was reconstituted with sterile 1 × PBS to a final concentration of 0.5mg/mL, following which 1µL of lightning-link® modifier was added for every 10µL of antibody. The mixture was pipetted directly onto the lyophilised R-PE (Abcam, AB102918, United Kingdom), resuspended, and incubated for 3 hours at room temperature in the dark. Following incubation, 1µL of quencher reagent was added for every 10µL of antibody. The hPGDS/R-PE conjugate was stores at 4°C in the dark until analysis. The samples were mixed using a vortex, and incubated for 15 minutes at room temperature in the dark.

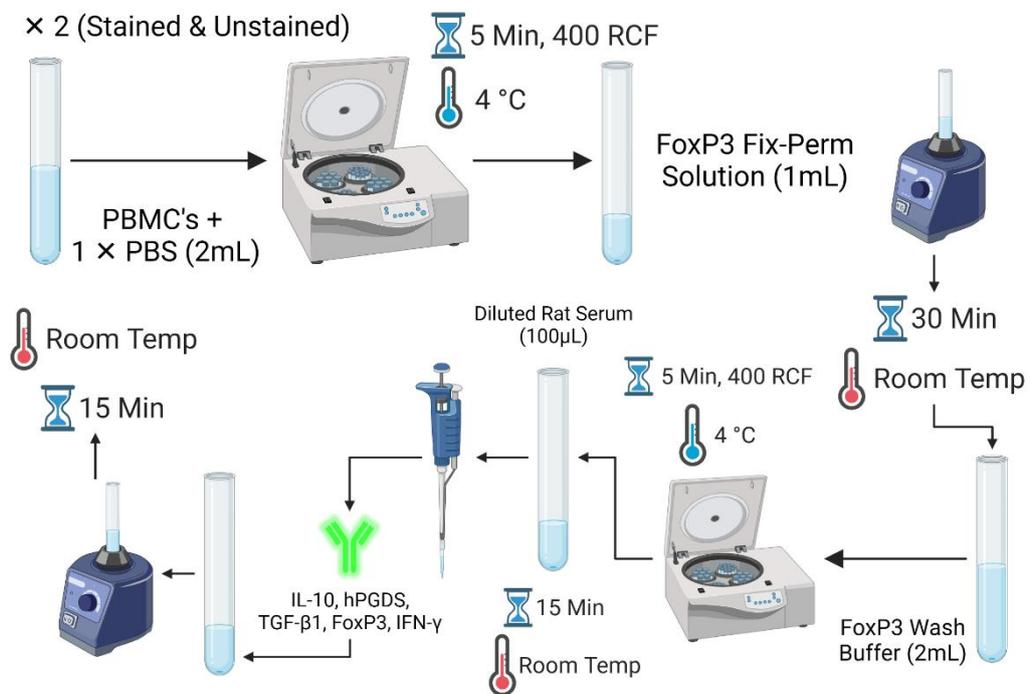


Figure 3.10 The intracellular staining of peripheral blood mononuclear cells (PBMCs) in preparation for flow cytometry. Created with www.BioRender.com.

As outlined in Figure 3.11, the samples were washed by adding 2mL of FoxP3 wash buffer per tube, and centrifuged at 400 rcf for 5 minutes at 4°C. The supernatant was discarded, and the tubes dried with paper tissue. The pellet was resuspended by flicking the bottom of the tube. 500µL of isoton (sheath fluid) was added to each tube before. The samples were mixed using a vortex before being loaded onto the flow cytometer. A summary of the antibodies used in outlined in Table 3.1 below.

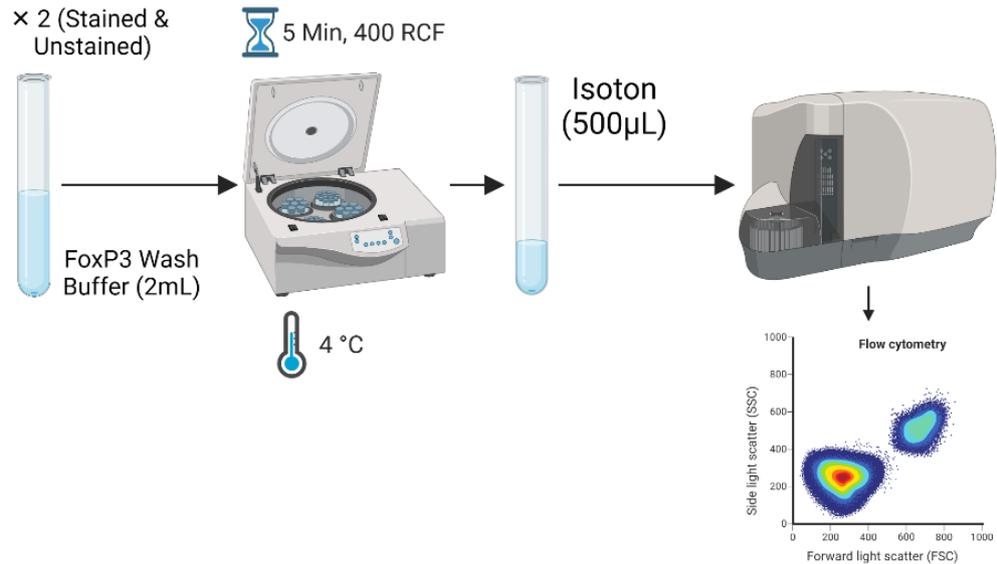


Figure 3.11 The analysis of peripheral blood mononuclear cells (PBMCs) by flow cytometry. Created with www.BioRender.com.

Table 3.1 A summary of the antibodies and dye used to characterise T_{REG}, T_{H2}, and T_{H1} cells from peripheral blood mononuclear cells in adults with and without hyperpnoea-induced bronchoconstriction.

Cell Surface Antibodies & Dye				
Antigen	Clone	Fluorochrome	Company	Catalogue Number
CD3	UCHT1	ECD	Beckman Coulter	A07748
CD25	B1.49.9	PC5.5	Beckman Coulter	A79386
CD161	HP-3G10	AF700	BioLegend	339942
CD4	13B8.2	Krome Orange	Beckman Coulter	A96417
Live-Dead	-	Violet	Fisher Scientific	15562731
Intracellular Antibodies				
Antigen	Clone	Fluorochrome	Company	Catalogue Number
IL-10	BT-10	FITC	Fisher Scientific	15581407
hPGDS	735301	PE	R & D Systems	MAB6487-SP
TGF- β 1	TW4-2F8	PE CY7	BioLegend	349610
FoxP3	PCH101	EFluor 660	Fisher Scientific	15529956
IFN- γ	4S.B3	APC EFluor 780	Fisher Scientific	15139484

T_{REG} regulatory T cells, *T_{H2}* T-helper type-2 cells, *T_{H1}* T-helper type-1 cells, *IL-10* interleukin-10, *hPGDS* haematopoietic prostaglandin D synthase, *TGF- β 1* transforming growth factor beta one, *FoxP3* fork-head box protein 3, *IFN- γ* interferon gamma. hPGDS was conjugated in house with R-PE (Bio-Techne, United States).

3.6.3.7 Flow Cytometry Analysis

A 3 laser, 10 colour Gallios flow cytometer (Beckman Coulter) was used to analyse T_{REG}, T_{H2}, and T_{H1} cell subsets isolated from PBMCs. Quality control checks were conducted daily. The same standardised protocol was used to assess all samples. For stained cells, the flow cytometer was run for the maximum acquisition time (8 minutes per sample), or the maximum cell count (160×10^6), whichever came first. For unstained cells, the acquisition was run until 2,000 cells had been sampled. The data was analysed using Kaluza (Kaluza Flow Cytometry Analysis Software, Version 2.1, Beckman Coulter, United States).

Midway through the analysis, the original IL-10 antibody was discontinued. Samples from participants N1, N4, N11, and P1 were analysed using the original IL-10 antibody, whereas samples from participants N2, N3, N10, N13, N15, N16, P3, P4, P5, P9, P11, and P14 were analysed using a new IL-10 antibody (15581407, Fisher Scientific, United States). Both IL-10 antibodies were conjugated to the same fluorochrome (FITC). No samples were collected from one participant (P12) due to haemophilia.

3.7 Pulmonary Function & The EVH Protocol

3.7.1 Prior Control Measures

During all studies (Chapters IV – VI), the EVH protocol was used to assess the severity of hyperpnoea (exercise)-induced bronchoconstriction. As outlined in Table 3.2, all participants were asked to temporarily avoid taking anti-histamines, consuming caffeinated food and drink, alcohol, and strenuous exercise prior to each experimental trial (Kennedy et al., 2019). Participants with HIB were also asked to temporarily stop taking their medication(s) prior to each experimental trial (Brummel et al., 2009; Dickinson et al., 2006A). Participants with HIB were asked to bring their reliever medication (short-acting β_2 -agonist) with them to all experimental trials. If resting FEV₁ was < 70% of percentage predicted values the EVH protocol was not undertaken (Anderson et al., 2010; Brannan & Porsbjerg, 2018; Parsons et al., 2007; Weiler et al., 2016). To control for within participant diurnal variation in pulmonary function (Rhee and Kim, 2015), experimental trials were conducted at the same time of day within 1-2 hours of previous trials (Anderson et al., 2010; Sue-Chu et al., 2010). Where possible, to control for between participant diurnal variation, experimental trials were conducted at the same time of day (Rhee & Kim,

2015). All experimental trials were conducted at least 1 hour post prandial (Anderson et al., 2010; Rhee & Kim, 2015; Sue-Chu et al., 2010). Participants became accustomed to assessments of pulmonary function and the EVH protocol during familiarisation trials.

Table 3.2 Experimental trial restriction guidelines.

Anti-Histamines	72 Hours Before
Alcohol	48 Hours Before
Caffeine	24 Hours Before
Strenuous Exercise	24 Hours Before
Inhaled Corticosteroids + Long-Acting β_2 -Agonist	24 Hours Before
Inhaled Corticosteroids	12 Hours Before
Short-Acting β_2 -Agonists	8 Hours Before

(Anderson & Kippelen, 2013; Hull et al., 2015; Simpson et al., 2016; Weiler et al., 2016).

3.7.2 Resting Pulmonary Function

Pulmonary function was assessed through spirometry via maximal flow volume loops (MFVLs) using a computerised pneumotachograph spirometer (Pneumotrac, Vitalograph, Buckingham, UK). All assessments were conducted in accordance with American Thoracic Society/European Respiratory Society guidelines (Brannan & Porsbjerg, 2018; Dickinson et al., 2006A; Enright et al., 2004; Miller et al., 2005). The spirometer, location of testing, and researcher conducting the assessments were kept consistent (Enright et al., 2004). The spirometer was checked for accuracy prior to each experimental trial using a 3-litre calibration syringe. The accuracy values were within accepted ranges across all experimental trials (Miller et al., 2005).

Spirometry was performed in a standing position with the nasal passage occluded. Participants were provided with a verbal explanation and practical demonstration of the MFVL manoeuvre before completing 2-3 practice efforts (Miller et al., 2005). Participants were instructed to passively exhale to residual volume, forcefully inhale to total lung capacity (TLC), forcefully exhale for 6 seconds, and forcefully inhale to TLC once more (Miller et al., 2005). Strong verbal encouragement was provided throughout the forced exhalation. A minimum of 3 and maximum of 8 MFVLs were completed at rest (Miller et al., 2005).

Four key parameters were assessed, forced vital capacity (FVC), forced expiratory volume in one second (FEV₁), peak expiratory flow rate (PEF), and forced expiratory flow rate between 25% and 75% of FVC (FEF_{25%-75%}; Ciprandi et al., 2012; Miller et al., 2005; Molphy et al., 2014). Forced vital capacity was defined as the total volume of air forcibly exhaled during the MFVL manoeuvre, whereas FEV₁ was defined as the total volume of air forcibly exhaled during the first second of the MFVL manoeuvre (Miller et al., 2005). Both FVC and FEV₁ are measurements of lung volume and are expressed in litres (l; Miller et al., 2005). As outlined by Miller et al. (2005), PEF was defined as the maximum flow achieved during the MFVL manoeuvre, whereas FEF_{25%-75%} was defined as the mean flow achieved between 25% and 75% of FVC. Both PEF and FEF_{25%-75%} are measurements of flow and expressed in litres per second (l·s⁻¹; Miller et al., 2005).

A reproducible assessment of FVC and FEV₁ was defined as a difference between the highest and second highest value of ≤ 0.150 litres (Miller et al., 2005). A reproducible assessment of PEF was defined as a difference between the highest and second highest value of ≤ 0.300 l·s⁻¹, whereas a reproducible assessment of FEF_{25%-75%} was defined as a difference between the highest and second highest value of ≤ 0.200 l·s⁻¹ (Miller et al., 2005). In accordance with current practice (Williams et al., 2015; 2016), FEV₁ was used as the criterion measure to assess changes in pulmonary function in response to the EVH protocol, and in response to prebiotic supplementation. The highest value obtained at rest was used for analysis (Molphy et al., 2014), whereas the lowest value obtained post-EVH was used for analysis (Anderson et al., 2001).

3.7.3 The EVH Protocol

Eucapnic voluntary hyperpnoea (EVH) is an indirect, ungraded objective test used to assess the presence and severity of hyperpnoea (exercise)-induced bronchoconstriction (Anderson et al., 2001; Anderson & Kippelen, 2013; Weiler et al., 2016; Williams et al., 2015). The EVH protocol involves 6-minutes of constant hyperventilation (hyperpnoea) at 85% of the participants predicted maximal voluntary ventilation (MVV; Anderson et al., 2001; Anderson & Kippelen, 2013; Brummel et al., 2009; Parsons et al., 2007; Stadelmann et al., 2011; Weiler et al., 2016). The ventilatory target of 85% of MVV is used to simulate the demands of high-intensity exercise (Anderson et al., 2001; Anderson & Kippelen, 2013; Parsons et al., 2007; Stadelmann et al., 2011). The highest value for FEV₁ obtained at rest

is used to calculate 85% of MVV using the following equation (Anderson et al., 2001):

$$85\% \text{ of MVV (l}\cdot\text{min}^{-1}) = 30 \times \text{highest resting FEV}_1 \text{ (l)} \quad \text{(Eq. 3.1)}$$

As outlined in Figure 3.12, participants inhaled a dry gas mixture of ~ 3-4% relative humidity from a medical grade gas cylinder (5% CO₂, 20.93% O₂, balance N₂; BOC, Guilford, UK). The gas cylinder was stored at room temperature (Brannan & Porsbjerg, 2018; Brummel et al., 2009; Dickinson et al., 2006A; Molphy et al., 2014; Parsons et al., 2007; Stadelmann et al., 2011; Weiler et al., 2016; Williams et al., 2015). 5% CO₂ was included in the gas mixture to maintain eucapnia (Anderson et al., 2001; 2011, Dickinson et al., 2011; Hallstrand et al., 2018; Hull et al., 2016; Williams et al., 2015).

With the nasal passage occluded, in a seated position, participants breathed through a standard mouthpiece (Series 9060, Hans Rudolph, Kansas City, Missouri, USA), connected to a low-resistance, two-way, non-rebreathing valve (Series 2700, Hans Rudolph, Kansas City, Missouri, USA; Brummel et al., 2009; Parsons et al., 2007; Pedersen et al., 2008). The breathing valve inlet was connected to a 150 L capacity Douglas bag (containing the dry gas mixture) via plastic corrugated tubing (internal diameter: 35 mm; Anderson et al., 2001; Anderson & Kippelen, 2013; Brannan & Porsbjerg, 2018; Molphy et al., 2014). During study one (Chapter IV), the breathing valve outlet was connected to an online breath-by-breath analyser (ZAN 600 CPET, Nspire Health, United Kingdom; Williams et al., 2015). The breath-by-breath analyser was calibrated prior to each trial using gases of known concentration (5% CO₂, 15% O₂, balance N₂, BOC, Guilford, United Kingdom), and a 3-litre calibration syringe (Williams et al., 2015). During studies two and three (Chapters V and VI), the breathing valve outlet was connected to a dry gas meter (Series 50-6164, Harvard Apparatus, Cambourne, UK) via plastic corrugated tubing (internal diameter: 35 mm). The dry gas meter was checked for accuracy before each experimental trial using a 3-litre calibration syringe. A flow meter was used to control the rate at which gas flowed from the cylinder into the Douglas bag.

During study one (Chapter IV), \dot{V}_E was monitored using the breath-by-breath analyser. During studies two and three (Chapters V and VI), a spreadsheet was created in Microsoft Excel to calculate pulmonary ventilation targets every 15 seconds. The researcher checked the participants actual ventilation from the dry gas meter against the target ventilation in the spreadsheet. Live feedback was provided throughout all studies to ensure the target \dot{V}_E was maintained (Brannan & Porsbjerg, 2018; Dickinson et al., 2006A). Strong verbal encouragement was provided throughout the EVH protocol. A test was considered valid if participants attained $\geq 21 \times$ their highest resting FEV₁ within the first 2 minutes (~ 60% of MVV; Anderson and Kippelen, 2013), and maintained their \dot{V}_E above this target for the full 6 minutes (Brummel et al., 2009; Weiler et al., 2016).

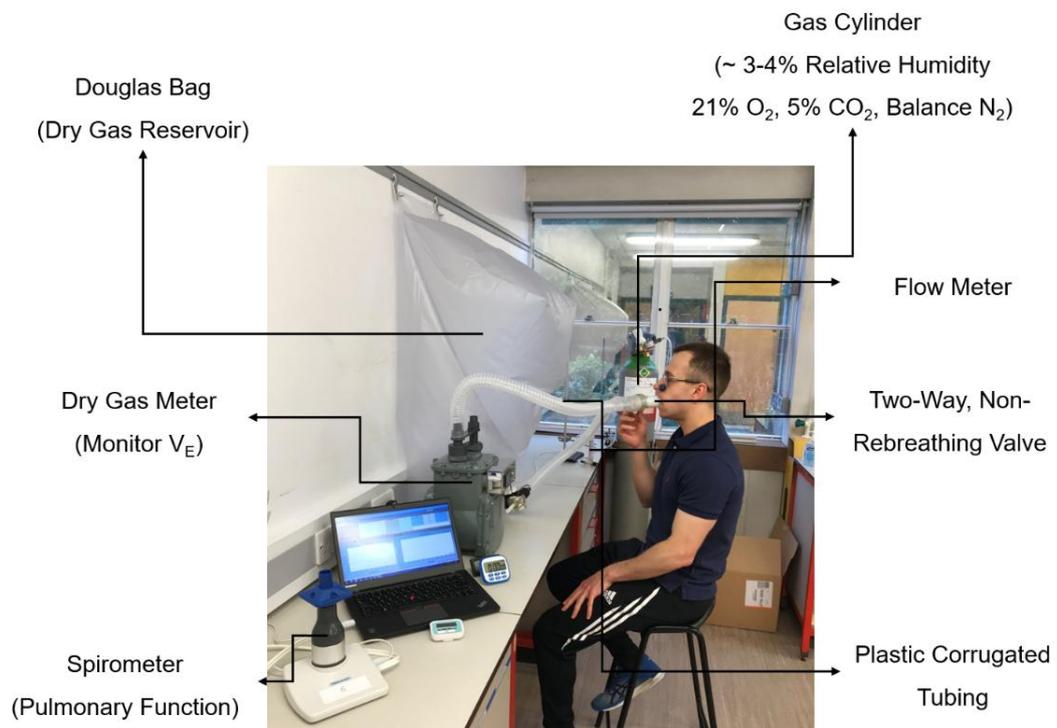


Figure 3.12 The experimental setup for the EVH protocol. *EVH* eucapnic voluntary hyperpnoea.

3.7.4 Post-EVH Pulmonary Function

For study one (Chapter IV), pulmonary function was reassessed in duplicate at 3, 5, 10, 15, 20, and 30 minutes post-EVH (Hallstrand et al., 2018; Weiler et al., 2016). For studies two and three (Chapters V and VI), pulmonary function was reassessed in duplicate at 3, 6, 10, 15, 20, and 30 minutes post-EVH (Anderson & Kippelen, 2013; Anderson et al., 2010; Dickinson et al., 2006A; Pedersen et al., 2008; Weiler et al., 2016; Williams et al., 2016). If the difference in FEV₁ between efforts at the

same time point was > 0.150 litres a third measurement was conducted (Miller et al., 2005). The presence and severity of HIB was determined using the following equation (Anderson et al., 2001; Dickinson et al., 2006A; Godfrey et al., 1999; Hallstrand et al., 2018; Pederson et al., 2008):

$$\text{Percentage Decrease in FEV}_1 \text{ Post-EVH} = \frac{100 \times (\text{Highest Resting FEV}_1 - \text{Lowest FEV}_1 \text{ Post-EVH})}{\text{Highest Resting FEV}_1} \quad \text{(Eq. 3.2)}$$

The severity of HIB was classified, according to the percentage decrease in FEV₁ post-EVH, as mild (> 10% - < 25%), moderate (> 25% - < 50%), or severe (> 30%, and > 50% reductions in FEV₁ post-EVH for participants receiving/not receiving steroid-based treatments, respectively; Anderson & Kippelen, 2013). An ≥ 13% decrease in FEV₁ post-EVH was used to diagnose HIB, as this criteria has been associated with a lower rate of false-positive diagnoses when compared to a 10% decrease in FEV₁ post-EVH (Anderson et al., 2001; Anderson & Kippelen, 2013; Godfrey et al., 1999).

This criteria was established and recommended for use by Godfrey et al. (1999), in participants up to the age of 25. Given the current thesis was conducted from a university, it was anticipated that the majority of participants may be young adults in the form of university students within this age range. In addition, the current program of work had to primarily rely on the EVH protocol for an accurate diagnosis of EIB. We did not have access to direct measures of airway inflammation, such as induced sputum or F_ENO, or medical personnel who could help with conducting tests to help differentiate different asthma phenotypes through allergen skin prick testing. These factors contributed to the decision to use a stricter cut-off value for the EVH protocol. Finally, HOST Therabiomics (who provided the prebiotic supplement for the intervention-based studies) intended to use the data collected as part of this PhD to support further applications to have their supplement registered as an “approved” product. This led to the study in Chapter VI being conducted to USFDA standards, complete with full study monitoring through site visits and established protocols. Based on these factors, the decision was made to adopt the 13% decrease in FEV₁ post-EVH as the diagnostic threshold to provide the most sensitive and specific assessment.

In accordance with standard operating procedures (Appendix J), if FEV₁ decreased by $\geq 13\%$ post-EVH, at two consecutive time points, participants were instructed to take their reliever medication using a spacer (2 puffs of Salbutamol, 100 μ g each; Brannan & Porsbjerg, 2018). Pulmonary function was reassessed ten minutes later. This process was repeated (a maximum of four times) until FEV₁ was within 10% of resting values. Participants remained supervised in the laboratory at all times (Anderson et al., 2001; Dickinson et al., 2011; Molphy et al., 2014; Williams et al., 2015). Under these circumstances, the original frequency of assessing pulmonary function post-EVH was terminated. Data used for analysis was taken from time points assessed prior to the administration of reliever medication.

3.8 Prebiotic Supplementation

During studies two and three (Chapters V and VI), prebiotic supplementation was administered following a double-blind, placebo-controlled, crossover design. During both studies, participants were administered a galactooligosaccharide-based prebiotic (Host Therabiomics, HOST-DM059, Jersey, The Channel Islands), and a taste and appearance matched placebo (maltodextrin). One prebiotic sachet weighed 3.6g and contained up to 85% galactooligosaccharides (3.1g), 6% water (0.22g), 6% glucose (0.22g), 4% galactose (0.14g), and 16% lactose (0.58g), respectively. During both studies, participants were administered one sachet of prebiotic per day. During study two (Chapter V), participants consumed one sachet in total, investigating the effects of short-term (acute) supplementation. During study three (Chapter VI), participants were administered one sachet every day for a 4-week period, investigating the effects of long-term supplementation. During study two (Chapter V), both supplements were reconstituted in an opaque bottle in 300ml of water. During study three (Chapter VI), participants were advised to reconstitute both supplements in a hot drink (such as tea or coffee) or fruit juice to help them dissolve. The supplements were blinded by HOST Therabiomics, labelled as “X” and “L”, respectively.

In the HIB group, in participants with asthma, adherence to maintenance medication and study supplements was prospectively monitored using a modified version of the MARS-A[®] completed at the end of each week throughout the nutritional intervention (Appendix I; Cohen et al., 2009). In participants without asthma (in the HIB group and control group) adherence to study supplements was prospectively monitored with the same questionnaire, but without the MARS-A[®]. Participants had to maintain

at least 80% adherence during both supplementation phases to be included in the final analysis. Unblinding was completed when the final participant completed their final experimental trial.

3.9 Statistical Analysis

Statistical analysis was conducted using the statistical package for the social sciences (IBM SPSS Inc, Version 26, Chicago IL, USA). The Shapiro-Wilk test was used to assess normality (Field, 2018). If deviations from normality were present, equivalent non-parametric tests were used where possible (Field, 2018).

During study one (Chapter IV), bootstrap corrected unpaired samples *t*-tests (or Mann-Whitney U tests) were used to assess differences between the HIB and control groups (Field, 2018). For unpaired samples *t*-tests, bias corrected accelerated (BCa) adjusted 95% confidence intervals (CI's) were calculated for the mean difference between groups (Field, 2018). Homogeneity of variance was assessed using Levene's test of equality of variance (Field, 2018). For unpaired samples *t*-tests, estimates of effect size were calculated using Cohen's *d* (Field, 2018). For Mann-Whitney U tests, estimates of effect size were calculated according to Field, (2018).

During study two (Chapters V) a series of two-way repeated measures analysis of variance (RM ANOVA) with Bonferroni post-hoc correction were used to assess changes in the HIB group across the prebiotic and placebo conditions from baseline to pre-EVH, baseline to post-EVH, and pre-EVH to post-EVH, respectively (Field, 2018). Greenhouse-Geisser corrected estimates were reported across all two-way repeated measures ANOVA's (Field, 2018). Bootstrap corrected paired samples *t*-tests (or Wilcoxon Signed-Rank tests) were used to assess changes in the HIB group between the prebiotic and placebo conditions. For paired samples *t*-tests, bias corrected accelerated (BCa) adjusted 95% confidence intervals (CI's) were calculated for the mean difference between conditions (Field, 2018). Homogeneity of variance was assessed using Levene's test of equality of variance. For two-way repeated measures ANOVA's and Wilcoxon Signed-Rank tests, estimates of effect size were calculated according to Field, (2018). For paired samples *t*-tests, estimates of effect size were calculated using Cohen's *d* (Field, 2018).

During study three (Chapter VI), a series of mixed ANOVA's with Bonferroni post-hoc correction were used to assess changes between the HIB and control groups

across the prebiotic and placebo conditions from baseline to post-intervention (Field, 2018). Estimates of effect size were calculated according to Field, (2018), based on the F statistic and error value for degrees of freedom (DF) from the tests of within-subject contracts:

$$\text{Effect Size} = \sqrt{F \div F + \text{DF (Error)}} \quad \text{(Eq. 3.3)}$$

Across all studies, statistically significant differences were accepted if $p < 0.05$ (two-tailed). Estimates of effect size were classed as small (0.2-0.5), medium (0.5-0.8), or large (> 0.8 ; Field, 2018). Values are reported as mean \pm standard deviation for parametric data, and median plus interquartile range for non-parametric data (IQR; 25th – 75th percentile). Figures demonstrating mechanisms or protocols were created using Microsoft PowerPoint or Biorender (Toronto, Ontario, Canada). Figures reporting experimental data were created using GraphPad Prism Software (Version 8.0.1, San Diego, CA, USA).

Chapter IV

Gut Bacterial Composition and Markers of Systemic Inflammation in Adults with and without Hyperpnoea-Induced Bronchoconstriction: A Pilot Study.

4.1 Introduction

Hyperpnoea (exercise)-induced bronchoconstriction is a subtype of asthma driven by type-2 inflammation (Altman et al., 2019; Anderson, 2011; Anderson & Kippelen, 2013; Hallstrand et al., 2013). It is characterised by a temporary narrowing of the lower airways that occurs during, or immediately after high-intensity aerobic exercise (Altman et al., 2019; Molphy et al., 2014; Parsons et al., 2007; Price et al., 2014; Simpson et al., 2016; Weiler et al., 2016).

In accordance with the osmotic hypothesis, the key stimulus of EIB is airway hyperosmolarity induced by high levels of ventilation (Aggarwal et al., 2018; Brummel et al., 2009; Dickinson et al., 2006A; Weiler et al., 2016). During high-intensity exercise, the switch from nasal to mouth breathing overwhelms the airways ability to condition and humidify (moisten) the inspired air (Figure 2.0, Anderson & Kippelen, 2013; Kippelen et al., 2018; Molphy et al., 2014; Weiler et al., 2016). The subsequent loss of periciliary fluid creates a hyperosmotic state in the airways (Anderson, 2011; Anderson & Kippelen, 2013; Hallstrand et al., 2012; Kippelen et al., 2018; Mannix et al., 2003; Weiler et al., 2016).

From an inflammatory perspective, systemic and pulmonary eosinophilia is a hallmark feature of exercise-induced bronchoconstriction (McBrien & Menzies-Gow, 2017). Eosinophils account for ~ 50% of pulmonary leukocyte infiltration, driving airway hyperresponsiveness and mucous hypersecretion (Matsuda et al., 2018; 2019; McBrien & Menzies-Gow, 2017). Eosinophils create the opportune environment to drive airway inflammation by making the airways more acidic (Kostikas et al., 2002; Kottyan et al., 2009), increasing the magnitude of degranulation, and resistance to apoptosis, respectively. When taking into account that eosinophils are the primary leukocyte responsible for infiltrating the airways during EIB, being able to measure inflammatory eosinophils that are highly abundant in the blood (Januskevicius et al., 2020), can be a useful surrogate marker for directly measuring airway eosinophilia and evaluating the severity of exercise-induced bronchoconstriction.

The causes of exercise-induced bronchoconstriction may originate from perturbations to the gut microbiota, the collection of microorganisms that colonise the gastrointestinal tract (Clemente et al., 2012; Fujimura & Lynch, 2015; Gibson & Roberfroid, 1999; Sbihi et al., 2019; Sender et al., 2016A; Ursell et al., 2012). Gut microbial dysbiosis is defined as an imbalance or deficiency in the number, or functional capacity, of symbiotic bacteria that modulate host immune function. Features of gut microbial dysbiosis have been identified in adults with asthma. At the genus level, Hevia et al. (2016), reported an increase in *Bifidobacterium* and *Faecalibacterium* that corresponded with an increase in *Bifidobacterium adolescentis* at the species level. Importantly, the increase in *Bifidobacterium adolescentis* was restricted to those with shorter-term diagnoses less than 11 years (Hevia et al., 2016). Those with diagnoses greater than 11 years experienced a decrease in *Bifidobacterium adolescentis* and increase in serum IgE (Hevia et al., 2016), suggesting a potential link between features of gut microbial dysbiosis and asthma. Okba et al. (2018), went on to demonstrate an increase in *Lactobacilli* and *Escherichia coli* in adults with atopic asthma, whereas Barcik et al. (2016), reported an increase in histamine secreting bacteria in non-obese adults with asthma. Histamine is a potent pro-inflammatory mediator of bronchoconstriction and type-2 inflammation (Ojiaku et al., 2018). Barcik et al. (2016), reported an increase in *Morganella morganii* in those with more severe asthma. In contrast to human derived histamine (Ojiaku et al., 2018), microbial derived histamine may have anti-inflammatory properties (Barcik et al., 2016; 2019). In an OVA-based model of asthma, gavage with a strain of *Escherichia coli* genetically modified with *Morganella morganii* to produce histamine (*E. coli* BL21_HTW), significantly attenuated airway eosinophilia (Barcik et al., 2019).

Expanding on these observations, Begley et al. (2018), were the first to demonstrate associations between specific features of gut microbial dysbiosis and both clinical and inflammatory parameters of asthma. In adults with mild-moderate asthma, features of gut microbial dysbiosis were associated with decreased pulmonary function and increased sensitisation to aeroallergens (Begley et al., 2018). For example, decreases in FEV₁ were associated with a lower *Bacteroidetes:Firmicutes* ratio, as well as decreases in *Bacteroides* and *Enterobacteriaceae*, respectively (Begley et al., 2018; Vital et al., 2015). Features of gut microbial dysbiosis were also associated with bronchial hyperresponsiveness, bronchodilator reversibility, and

decreased daily fruit intake (Begley et al., 2018). In contrast, an increase in *Bifidobacterium* was observed in obese individuals with asthma (Begley et al., 2018), highlighting the potential confounding effect of body composition on characterising asthma specific features of gut microbial dysbiosis.

Although there is growing evidence to suggest that gut microbial dysbiosis plays a critical role in asthma (Begley et al., 2018), it is unclear whether different asthma subtypes reflect different features of gut microbial dysbiosis. As a result, the primary aim of this study was to investigate whether adults with exercise-induced bronchoconstriction display features of gut microbial dysbiosis.

4.2 Methods

4.2.1 Experimental Design

This study was conducted following an observational design. Ethical approval was obtained from Nottingham Trent Universities Human Research Ethics Committee (approval number: 418). All protocols were conducted in accordance with the Declaration of Helsinki (World Medical Association, 2013), and the British Association of Sport and Exercise Sciences Code of Conduct (British Association of Sport and Exercise Sciences, 2017).

4.2.2 Participants

Recreationally active adults with ($n = 7$), and without ($n = 7$) hyperpnoea (exercise)-induced bronchoconstriction were recruited from Nottingham Trent University. Twelve ($n = 12$) were male, and two were female (both in the control group). Written informed consent was obtained prior to any procedures taking place (British Association of Sport and Exercise Sciences, 2013). All participants completed a health screen and history questionnaire prior to taking part. A detailed overview of the eligibility criteria is provided in the general methods section (Chapter III, pages 124-126). All participants attended the laboratory on two occasions, 1 week apart. Both trials were conducted at the same time of day to control for the diurnal effect on pulmonary function (Rhee & Kim, 2015). The first visit was a familiarisation trial, and the second visit the experimental trial.

4.2.3 Measurements

4.2.3.1 Pulmonary Function & The EVH Protocol

During the familiarisation trial, participants were allocated to the HIB group or the control group using the EVH protocol. The EVH protocol is an objective test used to assess the presence and severity of hyperpnoea (exercise)-induced bronchoconstriction (Anderson et al., 2001; Anderson & Kippelen, 2013; Weiler et al., 2016; Williams et al., 2015). Prior to each visit, participants were asked to temporarily avoid taking anti-histamines (72 hours before), alcohol (48 hours before), caffeine (24 hours before), and strenuous exercise (24 hours before). Participants with HIB were asked to temporarily avoid taking inhaled corticosteroids (12 hours before), and short-acting β_2 -agonists (8 hours before).

As part of the EVH protocol, height and body mass were assessed. Resting assessments of pulmonary function were conducted via spirometry using a computerised pneumotachograph spirometer (Pneumotrac, Vitalograph, Buckingham, UK). The spirometer was checked for accuracy prior to all trials using a 3-litre calibration syringe. All assessments were conducted in accordance with ATS/ERS guidelines (Brannan & Porsbjerg, 2018; Dickinson et al., 2006A; Enright et al., 2004; Miller et al., 2005). Spirometry was performed in a standing position with the nasal passage occluded. Participants completed a minimum of 3 and maximum of 8 maximal flow-volume loops (Miller et al., 2005). Four key parameters were assessed, FVC, FEV₁, PEF, and FEF_{25%-75%} (Ciprandi et al., 2012; Miller et al., 2005; Molphy et al., 2014). Reproducibility for FVC and FEV₁ was defined as a difference of ≤ 0.150 litres between the highest and second highest value (Miller et al., 2005). The highest resting values were used for analysis (Molphy et al., 2014), with the highest FEV₁ used to calculate target ventilation for the EVH protocol (85% of MVV: $30 \times$ highest resting FEV₁; Anderson et al., 2001). If resting FEV₁ was $< 70\%$ of percentage predicted values, the EVH protocol was not completed (Anderson et al., 2010; Brannan & Porsbjerg, 2018; Parsons et al., 2007; Weiler et al., 2016).

The EVH protocol involved 6-minutes of constant hyperpnoea at 85% of MVV (Anderson et al., 2001; Anderson & Kippelen, 2013; Brummel et al., 2009; Parsons et al., 2007; Stadelmann et al., 2011; Weiler et al., 2016). The EVH protocol was performed in a seated position with the nasal passage occluded. Participants

inhaled a dry gas mixture of ~ 3-4% relative humidity (5% CO₂, 20.93% O₂, balance N₂; BOC, Guilford, UK). Live feedback was provided on target \dot{V}_E (Brannan & Porsbjerg, 2018; Dickinson et al., 2006A). Minute ventilation (\dot{V}_E) was measured using an online breath-by-breath analyser (ZAN 600 CPET, Nspire Health, United Kingdom; Williams et al., 2015). The breath-by-breath analyser was calibrated prior to each trial using gases of known concentration (5% CO₂, 15% O₂, balance N₂, BOC, Guilford, United Kingdom), and a 3-litre calibration syringe (Williams et al., 2015). Strong verbal encouragement was provided throughout. A test was considered valid if participants attained $\geq 21 \times$ their highest resting FEV₁ within the first 2 minutes (Anderson and Kippelen, 2013), and maintained above this target for the full 6 minutes (Brummel et al., 2009; Weiler et al., 2016).

Pulmonary function was reassessed in duplicate at 3, 5, 10, 15, 20, and 30 minutes post-EVH (Anderson & Kippelen, 2013; Anderson et al., 2010; Dickinson et al., 2006A; Hallstrand et al., 2018; Pedersen et al., 2008; Weiler et al., 2016). The lowest values obtained post-EVH were used for analysis. Hyperpnoea-induced bronchoconstriction was diagnosed if FEV₁ decreased by $\geq 13\%$ from rest at two consecutive time points (Godfrey et al., 1999). If an $\geq 13\%$ decrease in FEV₁ occurred at two consecutive time points post-EVH, participants were instructed to take 2 puffs of their reliever inhaler using a spacer device in accordance with the asthma exacerbation protocol (Appendix J). Pulmonary function was reassessed ten minutes later. This process was repeated (a maximum of four times) until FEV₁ was within 10% of resting values. Under these circumstances, the original frequency of assessing pulmonary function post-EVH was terminated. Data used for analysis was taken from time points assessed prior to the administration of reliever medication. Participants remained supervised in the laboratory at all times (Anderson et al., 2001; Dickinson et al., 2011; Molphy et al., 2014; Williams et al., 2015).

4.2.3.2 The Asthma Control Questionnaire-7 (ACQ-7[©])

During the familiarisation trial, participants with asthma became accustomed to the ACQ-7[©] (Juniper et al., 1999; 2006). The ACQ-7[©] is a validated questionnaire used to assess self-reported perceptions of asthma control (Juniper et al., 1999; 2006). The severity of seven parameters is scored on a 7-point scale ranging from 0 indicating “least severe”, to 6 indicating “most severe” (Juniper et al., 1999; 2006). Participants rated the first 6 parameters, whereas the researcher rated the seventh

parameter (percentage predicted FEV₁; Juniper et al., 1999). The first 6 parameters included a series of statements assessing: i) night-time awakening by symptoms, ii) symptom severity upon waking, iii) activity limitation due to symptoms, iv) shortness of breath, v) wheeze, and vi) the use of short-acting β_2 -agonists, respectively (Juniper et al., 1999). The seventh parameter was measured via spirometry as described above (Miller et al., 2005). A score for asthma control was calculated by taking the average of the sum of all seven parameters (Juniper et al., 1999; 2006). The lowest score that could be obtained was 0, whereas the highest score was 6. A score of ≤ 0.75 indicated stable asthma, whereas a score of ≥ 1.50 indicated unstable asthma (Juniper et al., 2006).

4.2.3.3 Gut Bacterial Analysis

During the experimental trial, a faecal sample was collected to assess gut bacterial composition (Fecotainer[®], 460869, DaklaPack[®], London, United Kingdom). Samples were collected up to 2 hours before the trial, and processed within 4 hours of collection to minimise the adverse effects of aerobic environments on gut bacterial composition. Samples were stored at -20°C until transfer to the University of Reading for analysis. Flow cytometry was used to assess a range of key bacteria, including *Bifidobacterium*, *Lactobacillus-Enterococcus*, *Bacteroides-Prevotella*, *Eubacterium rectale-Clostridium coccoides*, *Roseburia-E rectale*, *Atopobium-Coriobacterium*, *Clostridial cluster IX*, *Faecalibacterium prausnitzii*, *Desulfovibrio*, and *Clostridium histolyticum-Clostridium perfringens*, respectively (Poveda et al., 2020; Vulevic et al., 2015). Data is presented as log₁₀ cells per gram of faeces dry weight (log₁₀ cells/g).

4.2.3.4 Markers of Systemic Inflammation

During the experimental trial, at rest, a 4mL blood sample was collected into a vacutainer tube containing EDTA (Brooks, 2014; Calfee & Farr, 2002; World Health Organisation, 2010). White blood cell subsets were quantified fresh in whole blood using an automated haematology analyser (Sysmex, XS-1000i[™], United States). Blood samples were analysed for absolute counts of total white blood cells, eosinophils and basophils ($\times 10^9/L$), as well as the relative abundance (%) of eosinophils and basophils, respectively.

4.2.4 Experimental Trial

During the experimental trial, at rest, participants provided a faecal sample and a blood sample before completing the EVH protocol. During the experimental trial, only participants with a previous diagnosis of asthma, who demonstrated EIB in response to the EVH protocol, were asked to complete the ACQ-7[®] ($n = 4$). In accordance with Nottingham Trent Universities Data Protection and Retention Policy, paper-based and electronic data was stored for up to 5 years. Participants were informed they could withdraw their participation at any point, without having to provide a reason. The familiarisation trial and experimental trial each lasted ~ 1 hour, equating to ~ 2 hours in total. An overview of the protocol for the experimental trial is outlined in Figure 4.0 below.

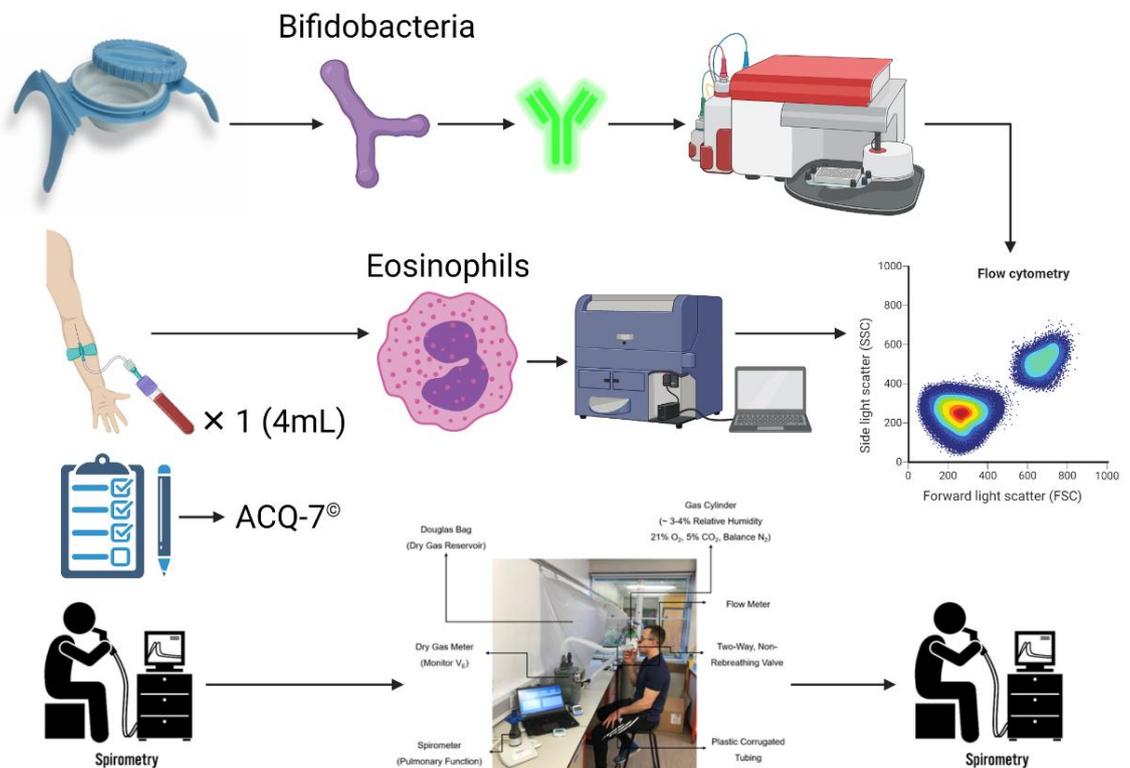


Figure 4.0 An overview of the protocol for the experimental trial, outlining the measurements in the order they were completed. Only participants who were diagnosed with hyperpnoea-induced bronchoconstriction during the familiarisation trial completed the ACQ-7[®]. Created with www.BioRender.com. ACQ-7[®] the Asthma Control Questionnaire-7.

4.2.5 Statistical Analysis

Statistical analysis was conducted using the statistical package for the social sciences (IBM SPSS Inc, Version 26, Chicago IL, USA). The Shapiro-Wilk test was

used to assess normality (Field, 2018). If deviations from normality were present, equivalent non-parametric tests were used where possible (Field, 2018). Bootstrap corrected unpaired samples *t*-tests (or Mann-Whitney U tests) were used to assess differences between the HIB and control groups in pulmonary function (FVC, FEV₁, PEF, FEF_{25%-75%}) and markers of systemic inflammation (total white blood cells, eosinophils and basophils, respectively; Field, 2018). For unpaired samples *t*-tests, bias corrected accelerated (BCa) adjusted 95% confidence intervals (CI's) were calculated for the mean difference between groups (Field, 2018). Homogeneity of variance was assessed using Levene's test of equality of variance (Field, 2018). For unpaired samples *t*-tests, estimates of effect size were calculated using Cohen's *d* (Field, 2018). For Mann-Whitney U tests, estimates of effect size were calculated according to Field, (2018).

Statistically significant differences were accepted if $p < 0.05$ (two-tailed). Estimates of effect size were classed as small (0.2-0.5), medium (0.5-0.8), or large (> 0.8 ; Field, 2018). Values are reported as mean \pm standard deviation for parametric data, and median plus interquartile range for non-parametric data (IQR; 25th – 75th percentile). Figures demonstrating mechanisms or protocols were created using Biorender (Toronto, Ontario, Canada). Figures reporting experimental data were created using GraphPad Prism Software (Version 8.0.1, San Diego, CA, USA).

4.3 Results

A total of fourteen ($n = 14$) participants completed the study, seven in the HIB group ($n = 7$), and seven in the control group ($n = 7$). In the control group, data for descriptive characteristics (age, height, body mass, and BMI) were available for six participants ($n = 6$). For the HIB and control groups, data for pulmonary function was available for six participants per group ($n = 6$ per group, $n = 12$ in total). In the HIB group, one participant did not complete the EVH protocol because they had a resting FEV₁ $< 70\%$ of percentage predicted values. In the control group, one participant did not complete the EVH protocol due to technical issues with the online breath-by-breath analyser. For the HIB and control groups, data for markers of systemic inflammation and gut bacterial composition were available from all participants ($n = 7$ per group, $n = 14$ in total). In the HIB group, data for the ACQ-7[®] was available for four participants ($n = 4$), as the remaining participants in the HIB group ($n = 3$) did not have a prior diagnosis of asthma, but were assigned to the HIB group based on the EVH protocol completed during familiarisation. No differences were observed

across all descriptive characteristics. The accuracy values for the spirometer were within accepted ranges across all experimental trials (Miller et al., 2005). The descriptive characteristics for participants in the HIB and control groups are outlined in Table 4.0 below.

Table 4.0 Descriptive characteristics of the HIB and control group.

	HIB (<i>n</i> = 7)	Control (<i>n</i> = 6)	<i>p</i>
Age (years)	23 [20, 42]	23 [21, 26]	0.628
Height (m)	1.82 ± 0.05	1.80 ± 0.15	0.780
Body Mass (kg)	81.7 ± 12.5	72.1 ± 10.8	0.173
BMI (kg/m ²)	24.6 ± 2.7	22.2 ± 2.1	0.114

Values are presented as mean ± SD for parametric data, and median plus IQR for nonparametric data. *HIB* hyperpnoea-induced bronchoconstriction, *IQR* interquartile range (25th – 75th percentile), *m* metres, *kg* kilograms.

4.3.1 Pulmonary Function

4.3.1.1 Resting Pulmonary Function

As outlined in Table 4.1, no differences were reported in resting pulmonary function between the HIB and control group when the data was expressed as either absolute or relative values.

Table 4.1 Resting pulmonary function in the HIB and control groups.

	HIB (n = 6)	Control (n = 6)	Difference	Statistical Analysis
FEV ₁ (L)	4.03 ± 0.53	4.18 ± 1.13	0.16, BCa 95% CI's [-0.81, 1.03]	<i>t</i> (7.09) = 0.30, <i>p</i> = 0.770
FEV ₁ (% Predicted)	91 ± 17	92 ± 8	0.83, BCa 95% CI's [-19.65, 15]	<i>t</i> (10) = 0.12, <i>p</i> = 0.907
FVC (L)	5.49 ± 0.59	4.87 ± 1.22	-0.58, BCa 95% CI's [-1.68, 0.58]	<i>t</i> (6.9) = -1.07, <i>p</i> = 0.321
FVC (% Predicted)	98, [94-104]	95, [81-100]		<i>U</i> = 22.5, <i>p</i> = 0.485
PEF (l·s ⁻¹)	9.73 ± 1.07	9.29 ± 1.95	-0.44, BCa 95% CI's [-2.10, 1.27]	<i>t</i> (10) = -0.48, <i>p</i> = 0.641
PEF (% Predicted)	92 ± 6	95 ± 8	-0.83, BCa 95% CI's [-9.63, 7.24]	<i>t</i> (10) = -0.17, <i>p</i> = 0.871
FEF _{25%-75%} (l·s ⁻¹)	3.35 ± 1.06	4.59 ± 1.87	1.24, BCa 95% CI's [-0.25, 2.71]	<i>t</i> (10) = 1.42, <i>p</i> = 0.187
FEF (% Predicted)	63 ± 22	90 ± 26	24.17, BCa 95% CI's [-1.20, 50]	<i>t</i> (10) = 1.81, <i>p</i> = 0.101

*FEV*₁ forced expiratory volume in one second, *FVC* forced vital capacity, *PEF* peak expiratory flow rate, *FEF*_{25%-75%} forced expiratory flow rate between 25%-75% of FVC.

Bias corrected accelerated (BCa) confidence intervals were not calculated for the difference between groups when non-parametric methods were used (Field, 2018).

4.3.1.2 Post-EVH Pulmonary Function

During the EVH protocol, participants were asked to maintain their \dot{V}_E at $30 \times$ their highest resting FEV₁ (Anderson et al., 2001). In the HIB group, the actual \dot{V}_E achieved during the experimental trial was $29 \pm 5 \times$ highest resting FEV₁ (range: 25-39), whereas in the control group, the actual \dot{V}_E achieved during the experimental trial was $28 \pm 3 \times$ highest resting FEV₁ (range: 24-31). All participants in both groups maintained their average $\dot{V}_E \geq$ the minimum $21 \times$ highest resting FEV₁ criteria used to indicate a valid test (Table 4.2; Anderson & Kippelen, 2013).

Table 4.2 Minute ventilation during the EVH protocol in the HIB and control groups.

Participant	HIB (<i>n</i> = 6)		Control (<i>n</i> = 6)	
	Absolute (l·min ⁻¹)	% MVV	Absolute (l·min ⁻¹)	% MVV
1	102.5	27	158.4	29
2	136.5	27	96.3	31
3	109.1	29	114.9	26
4	107.2	25	91.7	31
5	97.6	29	102.4	28
6	153.1	39	133.0	24

EVH eucapnic voluntary hyperpnoea.

Across all parameters, the percentage and absolute decrease in pulmonary function post-EVH was greater in the HIB group compared to the control group (Table 4.2). Figure 4.1 provides an outline of the percentage decrease in FEV₁ post-EVH for each participant, demonstrating that all participants in the HIB group were above the diagnostic threshold of 13%, and all participants in the control group were below.

In the HIB group, the severity of HIB was classed as moderate based on the peak percentage drop in FEV₁ observed post-EVH (Anderson & Kippelen, 2013). As outlined in Chapter 3, it should be noted that the “true” severity of HIB may be higher. The safety protocols followed requiring rescue medication to be prescribed if FEV₁ was $\geq 13\%$ lower than baseline after two consecutive timepoints may have meant the “true” drop in FEV₁ was underestimated, and as a result potentially the severity of HIB.

Table 4.3 Changes in pulmonary function from baseline to post-EVH in the HIB and control groups.

	HIB (n = 6)	Control (n = 6)	Difference	Statistical Analysis
FEV ₁ (L)	1.36 ± 0.47	0.23 ± 0.06	-1.12, BCa 95% CI's [-1.49, -0.72]	<i>t</i> (5.163) = -5.83, <i>p</i> = 0.002, <i>d</i> = 18.83
FEV ₁ (%)	38, [26-41]	6, [4-7%]		<i>U</i> = 36, <i>p</i> = 0.002, <i>r</i> = 0.84
FVC (L)	1.18 ± 0.52	0.12 ± 0.05	-1.06, BCa 95% CI's [-1.40, -0.66]	<i>t</i> (5.106) = -5, <i>p</i> = 0.004, <i>d</i> = 21.2
FVC (%)	25, [12-31]	3, [2-3%]		<i>U</i> = 36, <i>p</i> = 0.002, <i>r</i> = 0.84
PEF (l·s ⁻¹)	3.22 ± 1.37	1.38 ± 0.22	-1.84, BCa 95% CI's [-3.06, -0.72]	<i>t</i> (5.251) = -3.25, <i>p</i> = 0.021, <i>d</i> = 8.36)
PEF (%)	33 ± 13	15 ± 1	-17.83, BCa 95% CI's [-28.68, -5.67]	<i>t</i> (5.103) = -3.32, <i>p</i> = 0.020, <i>d</i> = 12.68
FEF _{25%-75%} (l·s ⁻¹)	1.67 ± 0.86	0.64 ± 0.23	-1.03, BCa 95% CI's [-1.86, -0.43]	<i>t</i> (10) = -2.82, <i>p</i> = 0.032, <i>d</i> = 4.48)
FEF (%)	49 ± 12	15 ± 4	-33.83, BCa 95% CI's [-44.5, -21.12]	<i>t</i> (10) = -6.27, <i>p</i> < 0.0001, <i>d</i> = 8.73

EVH eucapnic voluntary hyperpnoea, *FEV*₁ forced expiratory volume in one second, *FVC* forced vital capacity, *PEF* peak expiratory flow rate, *FEF*_{25%-75%} forced expiratory flow rate between 25%-75% of FVC. Bias corrected accelerated confidence intervals were not calculated for the difference between groups when non-parametric methods were used (Field, 2018). Effect sizes were calculated for significant differences.

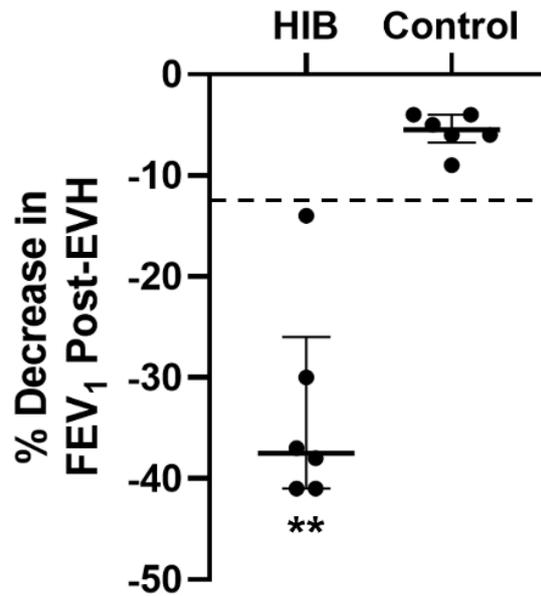


Figure 4.1 The percentage decrease in FEV₁ post-EVH was significantly greater in adults with mild-moderate hyperpnoea-induced bronchoconstriction compared to controls. FEV₁ forced expiratory volume in one second, EVH eucapnic voluntary hyperpnoea. ** $p < 0.01$ compared to control ($n = 6$ per group). Dashed line indicates diagnostic threshold. Data are presented as median and interquartile range.

4.3.2 Markers of Systemic Inflammation

When expressed as absolute values, no differences were reported between the HIB and control group for total white blood cells. In contrast, eosinophils were higher in the HIB group compared to the control group. In accordance with eosinophils, basophils were also higher in the HIB group compared to the control group. When expressed as relative values (the proportion of total white blood cells), eosinophils were higher in the HIB group compared to the control group. In contrast, no differences were reported for basophils between the HIB and control group.

Table 4.4 Resting markers of systemic inflammation in the HIB and control groups.

	HIB (<i>n</i> = 7)	Control (<i>n</i> = 7)	Difference	Statistical Analysis
WBC ($\times 10^9/L$)	5.86 \pm 1.07	5.46 \pm 1.22	-0.40, BCa 95% CI's [-1.62, 0.71]	<i>t</i> (12) = -0.65, <i>p</i> = 0.527
Eosinophils ($\times 10^9/L$)	0.46 \pm 0.16	0.19 \pm 0.11	-0.27, BCa 95% CI's [-0.4, -0.15]	<i>t</i> (12) = -3.75, <i>p</i> = 0.003, <i>d</i> = 2.45
Eosinophils (%)	8 \pm 2	4 \pm 2	-4.14, BCa 95% CI's [-6.83, -1.32]	<i>t</i> (12) = -3.20, <i>p</i> = 0.008, <i>d</i> = 1.78
Basophils ($\times 10^9/L$)	0.04, [0.01-0.04]	0.01, [0.01-0.01]		<i>U</i> = 41, <i>p</i> = 0.038, <i>r</i> = 0.63
Basophils (%)	1, [0-1]	0, [0-0%]		<i>U</i> = 38.5, <i>p</i> = 0.073

Bias corrected accelerated (BCa) confidence intervals were not calculated for the difference between groups when non-parametric methods were used (Field, 2018). Effect sizes were calculated for variables that demonstrated significant differences.

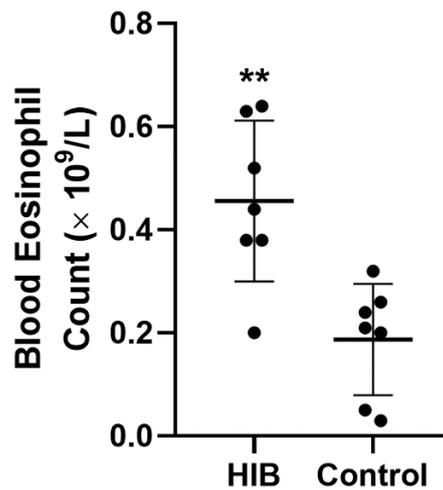


Figure 4.2 Resting blood eosinophil concentrations are significantly higher in adults with mild-moderate hyperpnoea-induced bronchoconstriction compared to controls. ** $p < 0.01$ compared to control ($n = 7$ per group). Data are presented as mean \pm SD. $n = 2$ participants in the HIB group demonstrated resting blood eosinophilia $> 0.6 \times 10^9/L$.

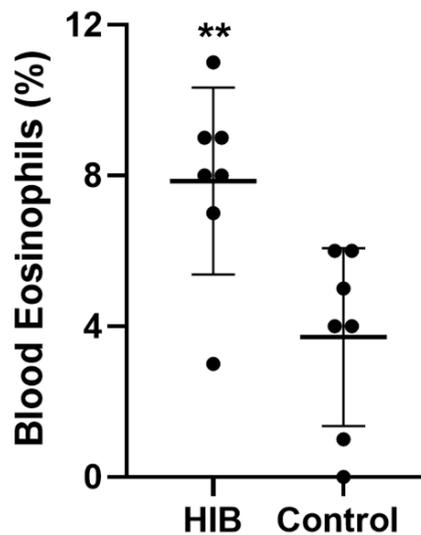


Figure 4.3 The relative abundance of eosinophils at rest is significantly higher in adults with mild-moderate hyperpnoea-induced bronchoconstriction compared to controls. ** $p < 0.01$ compared to control ($n = 7$ per group). Data are presented as mean \pm SD.

4.3.3 Gut Bacterial Composition

No differences were observed between the HIB and control groups for any of the bacterial groups analysed.

Table 4.5 Gut bacterial composition in the HIB and control groups.

	HIB (<i>n</i> = 7)	Control (<i>n</i> = 7)	Difference	Statistical Analysis
Total Bacteria	9.26 ± 0.42	9.20 ± 0.64	-0.06, BCa 95% CI's [-0.65, 0.57]	<i>t</i> (12) = -0.21, <i>p</i> = 0.835
<i>Bifidobacterium</i>	8.17 ± 0.42	8.11 ± 0.64	-0.06, BCa 95% CI's [-0.58, 0.40]	<i>t</i> (12) = -0.20, <i>p</i> = 0.842
<i>Lactobacillus-Enterococcus</i>	7.87 ± 0.42	7.81 ± 0.64	-0.06, BCa 95% CI's [-0.60, 0.51]	<i>t</i> (12) = -0.20, <i>p</i> = 0.843
<i>Bacteroides-Prevotella</i>	8.06 ± 0.42	8.00 ± 0.64	-0.06, BCa 95% CI's [-0.62, 0.58]	<i>t</i> (12) = -0.20, <i>p</i> = 0.843
<i>Eubacterium rectale-Clostridium</i> <i>coccoides</i>	8.70 ± 0.42	8.64 ± 0.64	-0.06, BCa 95% CI's [-0.56, 0.44]	<i>t</i> (12) = -0.21, <i>p</i> = 0.838
<i>Roseburia-E rectale</i>	8.06 ± 0.42	8.00 ± 0.64	-0.06, BCa 95% CI's [-0.64, 0.55]	<i>t</i> (12) = -0.21, <i>p</i> = 0.835
<i>Atopobium-Coriobacterium</i>	8.02 ± 0.42	7.96 ± 0.64	-0.06, BCa 95% CI's [-0.53, 0.43]	<i>t</i> (12) = -0.21, <i>p</i> = 0.838
<i>Clostridial cluster IX</i>	7.94 ± 0.42	7.88 ± 0.64	-0.06, BCa 95% CI's [-0.68, 0.61]	<i>t</i> (12) = -0.21, <i>p</i> = 0.838
<i>Faecalibacterium prausnitzii</i>	8.65 ± 0.42	8.59 ± 0.64	-0.06, BCa 95% CI's [-0.61, 0.53]	<i>t</i> (12) = -0.20, <i>p</i> = 0.842
<i>Desulfovibrio</i>	8.25 ± 0.42	8.19 ± 0.64	-0.06, BCa 95% CI's [-0.59, 0.42]	<i>t</i> (12) = -0.20, <i>p</i> = 0.843
<i>Clostridium histolyticum-Clostridium</i> <i>perfringens</i>	7.87 ± 0.42	7.81 ± 0.64	-0.06, BCa 95% CI's [-0.52, 0.40]	<i>t</i> (12) = -0.20, <i>p</i> = 0.843

All data is presented as log₁₀ cells/g of faeces (dry weight).

4.4 Discussion

4.4.1 Key Findings

The primary aim of the current study was to explore whether adults with hyperpnoea (exercise)-induced bronchoconstriction displayed features of gut microbial dysbiosis. Using fluorescent *in situ* hybridisation, the current study reported no features of gut microbial dysbiosis in adults with mild-moderate HIB. This observation is in contrast to previous research that has consistently demonstrated features of gut microbial dysbiosis in adults with allergic asthma (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016; Okba et al., 2018). The disparity in findings could be attributed, at least in part, to differences in study design, as well as study population, especially considering the inability of the current study to more accurately characterise the asthma phenotype(s) of the participants taking part (i.e. through further objective measures such as allergen skin prick testing).

Asthma is a heterogenous syndrome with a variety of subtypes each underpinned by different pathophysiological mechanisms (Pavord et al., 2017; Wenzel, 2016). A robust diagnosis of asthma (and its subtypes) is, therefore, essential when investigating its relationship with gut microbial dysbiosis. As such, research to date has investigated features of gut microbial dysbiosis in adults with allergic asthma only (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016; Okba et al., 2018). Given that different features of gut microbial dysbiosis have been identified in the same subtype of asthma, at different levels of severity (Begley et al., 2018), it is plausible to speculate that different subtypes of asthma may display different features of gut microbial dysbiosis, if any.

Exercise (hyperpnoea)-induced bronchoconstriction is a highly prevalent subtype of asthma ranging from 9% to 71.4% in athletic populations (Dickinson et al., 2006A; Sue-Chu et al., 2010). Traditionally, EIB has been diagnosed based on the subjective assessment of symptoms, including coughing, wheezing, chest tightness, and dyspnoea, respectively (Dickinson et al., 2011; GBD Chronic Respiratory Disease Collaborators, 2020; Juniper et al., 1999; Rundell et al., 2001; Teeter & Bleeker, 1998). Subjective assessments are less robust than objective assessments, increasing the risk of misdiagnosis or underdiagnosis, highlighting the importance of using objective measures to diagnose EIB (Aggarwal et al., 2018; Dickinson et al., 2011; Griffin et al., 2018; 2019; Johannsson et al., 2015; Juniper et

al., 2004; Kennedy et al., 2019; Parsons et al., 2007; Pavord et al., 2017; Pedersen et al., 2008; Rundell et al., 2001; Stadelmann et al., 2011; Weiler et al., 2016; Wenzel, 2016).

Exercise (hyperpnoea)-induced bronchoconstriction is assessed objectively via eucapnic voluntary hyperventilation (EVH, Anderson et al., 2001; Williams et al., 2015), a protocol advocated by the International Olympic Committee Medical Commission (IOC-MC), and Joint Task Force on Practice Parameters in EIB (JTFPP) for diagnosing EIB in athletic populations (Hull et al., 2016; Molphy et al., 2014; Weiler et al., 2016; Williams et al., 2015). A decrease in FEV₁ of $\geq 13\%$ post-EVH provides the most sensitive and specific diagnosis of EIB (Godfrey et al., 1999). In accordance with these guidelines, HIB was diagnosed in the current study by EVH. As outlined in Figure 4.1, adults with HIB demonstrated significantly greater decreases in FEV₁ post-EVH when compared to controls. The decrease in FEV₁ was of sufficient magnitude to be perceivable by participants (Santanello et al., 1999). Based on the criteria of Anderson and Kippelen, (2013), the severity of HIB was classed as mild-moderate, similar to that of previous research investigating features of gut microbial dysbiosis in adults with allergic asthma (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016; Okba et al., 2018). Given that no differences were observed between the target and actual ventilation rates obtained during the EVH protocol, the current assessment can be considered a valid diagnosis of HIB (Anderson & Kippelen, 2013; Williams et al., 2015).

The robustness of the assessment of HIB suggests that the absence of features of gut microbial dysbiosis is unlikely to be caused by misdiagnosis. This is further supported by the significant decrease in FEF_{25%-75%}, indicating the presence of lower airway disease (Ciprandi et al., 2012), and the significant increase in blood eosinophils observed in adults with mild-moderate HIB. HIB is driven by type-2 inflammation of which eosinophils are a key mediator (Altman et al., 2019; Anderson, 2011; Anderson & Kippelen, 2013; Hallstrand et al., 2013). Eosinophils account for $\sim 50\%$ of pulmonary leukocyte infiltration, playing a critical role in driving airway hyperresponsiveness and mucous hypersecretion (Matsuda et al., 2018; 2019; McBrien & Menzies-Gow, 2017). Upon translocation from the blood to the airways (Januskevicius et al., 2020; McBrien and Menzies-Gow, 2017; Yi et al., 2018; Ying et al., 1999), inflammatory eosinophils create the opportune environment to drive airway inflammation by making the airways more acidic (Kostikas et al.,

2002; Kottyan et al., 2009). In more acidic conditions, eosinophils are more capable of degranulation, and more resistant to apoptosis (Kottyan et al., 2009), increasing the severity of type-2 inflammation. Taken together, this data suggests that the current participants were a cohort of adults with mild-moderate HIB underpinned by type-2 inflammation (Altman et al., 2019; Anderson, 2011; Anderson & Kippelen, 2013; Hallstrand et al., 2013), yet further assessment could have been conducted to confirm and differentiate between atopic status and different asthma phenotypes.

Although the protocols used to assess HIB and systemic inflammation were appropriate, the method used to assess gut bacterial composition (fluorescent *in situ* hybridisation) was limited. In contrast to the technique used in this study, previous research has primarily used metagenomics-based approaches to analyse gut bacterial composition (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016). Both fluorescent *in situ* hybridisation and metagenomics employ culture-independent approaches to analyse gut bacterial composition, avoiding limitations associated with culture-dependent methods (Okba et al., 2018). In contrast to fluorescent *in situ* hybridisation however, metagenomics provides a more detailed and comprehensive assessment of gut bacterial composition. Fluorescent *in situ* hybridisation is limited, in part, by the availability of oligonucleotide probes required to analyse specific bacteria of interest, as well as the number of different bacteria that can be quantified through flow-cytometry. Metagenomics routinely assesses gut bacterial composition to the species and strain levels (Begley et al., 2018; Hevia et al., 2016), whereas fluorescent *in situ* hybridisation typically assesses key bacterial groups above the species and strain levels (Vulevic et al., 2015). Given that features of gut microbial dysbiosis in asthma relate to specific species or strains (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016), as opposed to overall community structure, it is possible the current approach lacked the required sensitivity to detect features of gut microbial dysbiosis.

4.4.2 Practical Applications

In adults with asthma, gut microbial dysbiosis is characterised by imbalances or deficiencies in certain species or strains, as opposed to overall community structure (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016; Okba et al., 2018). For example, Hevia et al. (2016), reported a significant decrease in *Bifidobacterium adolescentis* in adults with long-term allergic asthma. From a therapeutic perspective, features of gut microbial dysbiosis can be used to inform the

development of targeted therapies, such as probiotics and prebiotics (Arrieta et al., 2015; Depeint et al., 2008; Halmes et al., 2017). If analyses of gut bacterial composition demonstrate that bacteria targeted by prebiotics are not present in sufficient quantities, probiotic supplementation is initially required (Arrieta et al., 2015; Davis et al., 2011). Interestingly, different species of *Bifidobacteria* demonstrate significant variability in their ability to utilise galactooligosaccharide-based prebiotics (Davis et al., 2011), with novel species demonstrated to be more capable of utilising certain galactooligosaccharides when compared to traditional species (Davis et al., 2011). Whether prebiotics that target novel species demonstrate greater therapeutic efficacy than current prebiotics remains to be determined (Depeint et al., 2008).

What is clear, is that analyses of gut bacterial composition above the species and strain levels may not be sensitive enough to detect HIB specific features of gut microbial dysbiosis required to develop targeted probiotic and prebiotic therapies (Arrieta et al., 2015; Depeint et al., 2008). Although certain galactooligosaccharide-based prebiotics have been shown to induce clinically relevant, participant perceivable improvements in HIB, by a comparable magnitude to that observed following pharmaceutical treatments (39-60%; Kippelen et al., 2010; Rundell et al., 2005; Santanello et al., 1999; Simpson et al., 2016; Verheijden et al., 2015A; 2018; Williams et al., 2016), it is currently unclear whether this is the result of enhancing the immunomodulatory properties of traditional species of *Bifidobacteria* (Depeint et al., 2008), novel species of *Bifidobacteria* (Davis et al., 2011), or a combination of both through as yet uncharacterised mechanisms of bacterial cross-feeding. As a result, further research is required to characterise the gut bacterial composition of adults with HIB to the appropriate phylogenetic depth (Ursell et al., 2012), before the efficacy of different prebiotics can be investigated.

Metagenomics is an obvious approach to address this issue, given its broad scope and depth of sensitivity. However, traditional metagenomics-based approaches characterise gut bacterial composition based on relative abundance (Hevia et al., 2016). Such methods may be less informative than quantifying absolute cell counts, given that changes in relative abundance do not always correspond to actual quantitative changes. Fluorescence *in situ* hybridisation does provide quantitative analyses of gut bacterial composition, albeit with less sensitivity than metagenomics (Ursell et al., 2012). It is likely that a number of different approaches will be required

to address the potential complexity of gut microbial dysbiosis in HIB. Initially, studies should use recently developed quantitative metagenomics-based approaches to comprehensively assess any features of gut microbial dysbiosis that could be used to inform the development of targeted therapies. Follow up studies could then employ more targeted fluorescent *in situ* hybridisation-based techniques to develop species or strain specific oligonucleotide probes that can be used to monitor changes in features of gut microbial dysbiosis following probiotic or prebiotic supplementation (Takada et al., 2004). Such approaches would provide detailed, yet targeted, information on which bacteria may demonstrate immunomodulatory properties in HIB.

4.4.3 Strengths

The strengths of the current study relate to the robustness of the protocols used to assess HIB (Williams et al., 2015). Previously, the absence of objective assessments when diagnosing asthma subtypes increases the risk of misdiagnosis or underdiagnosis (Aggarwal et al., 2018; Dickinson et al., 2011; Griffin et al., 2018; 2019; Johannsson et al., 2015; Juniper et al., 2004; Kennedy et al., 2019; Parsons et al., 2007; Pavord et al., 2017; Pedersen et al., 2008; Rundell et al., 2001; Stadelmann et al., 2011; Weiler et al., 2016; Wenzel, 2016). The limitations of subjective assessments are highlighted by the similarity in self-reported respiratory symptoms between varsity college athletes with and without an objective diagnosis of EIB (34.9 vs. 36.5%; Parsons et al., 2007). Similar relationships have been observed in elite athletes (Pedersen et al., 2008; Stadelmann et al., 2011), winter sport athletes (Rundell et al., 2001), and elite adolescent swimmers (Kennedy et al., 2019; Rundell et al., 2001), highlighting the non-specific nature of self-reported respiratory symptoms (Heir & Oseid, 1994; Parsons et al., 2007; Rundell et al., 2001). Even when objective assessments of EIB are used, conditions such as exercise-induced laryngeal obstruction (EILO) can go undiagnosed if specialist protocols such as Continuous Laryngoscopy During Exercise (CLE Testing) is not available to help differentiate the pathology (Griffin et al., 2018; 2019; Johannsson et al., 2015). Therefore, future research should use differential diagnoses of EIB and EILO to further verify HIB specific features of gut microbial dysbiosis (Griffin et al., 2018; 2019; Johannsson et al., 2015).

4.4.4 Limitations

The current study used the drop in FEV₁ in response to EVH during an initial screening visit to assign participants to the HIB or control group. This was not supported by further assessments of atopic status or asthma phenotypes via measures such as allergen skin prick testing. As a result, the HIB group consisted of a mixture of asthma phenotypes. Future research should ensure more robust characterisation of asthma phenotypes alongside the presence of EIB to obtain more homogenous samples and inform a more targeted investigation of potential features of gut microbial dysbiosis. Furthermore, the severity of EIB may have been underestimated due to prescribing rescue medication if FEV₁ was $\geq 13\%$ lower than baseline at two consecutive time-points post EVH. Although an important safety consideration for conducting EVH, especially outside of clinical environments, future research may need to consider the time-points selected to assess pulmonary function post-EVH, or whether any additional safety measures may enable the nadir of FEV₁ to be more accurately assessed over a longer period.

Features of gut microbial dysbiosis may identify which bacteria underpin the pathophysiology of HIB, but they alone do not explain the underlying host-microbial interactions that drive immunopathology. The ability to produce short-chain fatty acids is considered an important immunomodulatory property of the gut microbiota. Short-chain fatty acids are anti-inflammatory metabolites produced by the fermentation of dietary fibre (Arpaia et al., 2013; Berthon et al., 2013; Holscher, 2017; Macia et al., 2014; Macfarlane et al., 1998; Maslowski et al., 2009; Sbihi et al., 2019; Tan et al., 2014; Thorburn et al., 2015; Trompette et al., 2014). They are particularly important in the pathophysiology of HIB, given their ability to modulate systemic and pulmonary immune function (Sbihi et al., 2019; Schroeder & Backhed, 2016). For example, acetate attenuates the severity of airway inflammation by increasing the suppressive capacity of FoxP3⁺ T_{REG} cells (Thorburn et al., 2015). Faecal concentrations of short-chain fatty acids can provide an indication of the gut microbiota's immunomodulatory capacity (Vulevic et al., 2015). Faecal short-chain fatty acids were meant to be analysed as part of the current study, however, the equipment required to analyse faecal short-chain fatty acid concentrations was broken, preventing this information from being obtained.

In accordance with current thinking (Barcik et al., 2016; Hevia et al., 2016; Okba et al., 2018), the current study is limited by the use of single faecal samples to identify

features of gut microbial dysbiosis. Without longitudinal profiling, it can be difficult to identify features of gut microbial dysbiosis that are not caused by confounding factors, such as diurnal effects (Sender et al., 2016A), temporal parameters (Flores et al., 2014), or environmental exposures, respectively (Feddema & Claassen, 2020; He et al., 2020; Khreis et al., 2017; Malik et al., 2012; Rodriguez et al., 2019; Sbihi et al., 2019; Sharpe et al., 2015). Begley et al. (2018), addressed this issue, to some extent, by assessing the short-term stability of gut bacterial composition in adults with mild-moderate asthma over a 4-week period which demonstrated no significant changes over this timeframe. Nevertheless, longitudinal investigations are required to assess the long-term stability of gut bacterial composition before HIB specific features of gut microbial dysbiosis can be identified (Hoen et al., 2015; Stokholm et al., 2016). Longitudinal, prospective designs have been used to identify biomarkers of exacerbations in adults with mild-moderate asthma (Brinkman et al., 2017; Peters et al., 2020; Silkoff et al., 2019; van der Schee et al., 2013). Combining such approaches with assessments of gut bacterial parameters may identify exacerbation specific features of gut microbial dysbiosis that could be used to inform targeted therapies.

Being a pilot study, the current project is limited by the small sample size. Similar research investigating the features of gut microbial dysbiosis in adults with allergic asthma has involved sample sizes ranging from twenty-one to eighty (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016; Okba et al., 2018). Although future research in larger populations is required, more must be done to first of all address the fundamental lack of engagement in asthma research (Delsing et al., 2016; Nwaru et al., 2016), and issues associated with using power calculations to estimate sample size (Jones et al., 2003). Alarming, ~ 26.3% of clinical trials in asthma and COPD do not achieve their recruitment targets, despite the funding and resources available for these projects when compared to academic studies (Delsing et al., 2016). Furthermore, recent attempts to develop a national recruitment database for asthma (REACH database; Nwaru et al., 2016), have since been shut down, demonstrating that new initiatives are required to resolve issues with participant recruitment at a local and national level.

4.5 Conclusion

In conclusion, the current study demonstrated that adults with mild-moderate HIB do not display features of gut microbial dysbiosis when measured using FISH-based

analyses of gut microbial composition at the genus level. This observation is in contrast to previous research in participants with a different subtype of asthma (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016; Okba et al., 2018), and could be attributed to the lack of sensitivity in the method used to quantify gut bacterial composition, compounded by the small sample size. Future research should incorporate species and strain level analyses of gut bacterial composition, in larger cohorts of adults with HIB confirmed via EVH, to investigate if adults with mild-moderate HIB demonstrate features of gut microbial dysbiosis or not based on analyses of improved sensitivity and power.

Chapter V

The Effects of Short-Term Prebiotic Supplementation on the Severity of Hyperpnoea-Induced Bronchoconstriction: A Double-Blind, Placebo-Controlled Crossover Trial.

5.1 Introduction

A variety of approaches exist to provide acute short-term relief of the severity of asthma or EIB in athletes. The most common pharmaceutical treatment used to provide protection against acute increases in asthma severity during exercise is salbutamol. Although salbutamol is routinely used by athletes with a TUE, the magnitude of bronchoprotection may be lost following repeated administration (Hancox et al., 2002; Williams et al., 2020). The loss of protection following repeated administration is referred to as tachyphylaxis, placing athletes at a greater risk of exacerbations (Hancox et al., 2002; Williams et al., 2020). Hancox et al. (2002), investigated the bronchoprotective effects of salbutamol pretreatment on the severity of EIB in non-athletic adults. Participants underwent a week of salbutamol pretreatment (total daily dose: 800µg split into 2 × 100µg (a standard puff), four times daily; Hancox et al., 2002). Alarmingly, the severity of EIB was 90% worse following salbutamol pretreatment compared to placebo (Hancox et al., 2002), as measured by the drop in FEV₁ 5-minutes post-exercise. In addition, a blunted response was reported following the administration of salbutamol post-exercise, indicating tachyphylaxis (Hancox et al., 2002; Williams et al., 2020). Although assessed in non-athletic adults, the salbutamol pretreatment protocol investigated by Hancox et al. (2002), reflects the dosing regimens used by athletes during training and competition. Taken together, these results highlight the importance of maintain the efficacy of pharmaceutical treatments by avoiding excessive use.

However, concerns around the loss of bronchoprotection in athletes following the recommended use of pharmaceutical treatments is also compounded by the potential for adverse analytical findings due to high inter-individual differences in urinary salbutamol excretion (Elers et al., 2012). When corrected for urine specific gravity, a single, moderate dose of salbutamol (800µg), produced urinary concentrations below WADA's threshold for adverse analytical findings (335 vs. 1000ng/mL; Elers et al., 2012; World Anti-Doping Agency, 2020). With a Therapeutic Use Exemption (TUE), athletes may inhale up to 1600µg of salbutamol

over a 24-hour period, with a maximum dose of 800 μ g in the first 12 hours (World Anti-Doping Agency, 2020). Typically, a 200 μ g dose of salbutamol is inhaled 15 minutes before exercise, meaning up to 8 doses could be administered over a 24-hour period. Interestingly, the 800 μ g dose administered by Elers et al. (2012), was meant to mimic those characteristic of doping, yet no adverse analytical findings were reported. These results question the validity of current thresholds employed by the WADA to detect doping, highlighting the potential margin for abuse by athletes pursuing illegal enhancements, or the risk of inadvertent doping, despite following TUE guidelines.

Short-term protection from increases in asthma severity have also been obtained through other pharmaceutical treatments. For example, when compared to placebo, taking 10mg of montelukast 6-8 hours prior to EVH attenuated the peak decrease in FEV₁ post-EVH by ~ 46% (26 \pm 17 vs. 14 \pm 11%; Rundell et al., 2005), whereas high dose beclomethasone dipropionate attenuated the peak decrease in FEV₁ post-EVH by ~ 39-41% for up to 4 hours (Kippelen et al., 2010). In addition, a standard single dose of terbutaline, administered 15 minutes prior to EVH, has been shown to attenuate the peak decrease in FEV₁ below the diagnostic threshold (14, [12-20] vs. 7, [5-9%]; Simpson et al., 2016).

To help combat tachyphylaxis, various short-term nutritional strategies have been investigated as complementary therapies to attenuate the severity of exercise-induced bronchoconstriction, including caffeine supplementation (VanHaitsma et al., 2010), and a low-salt diet (Williams et al., 2020). Caffeine was removed from WADA's prohibited list in 2004, and has since been used to attenuate the severity of EIB (Dickinson et al., 2018; VanHaitsma et al., 2010). In moderately trained recreational athletes, an inverse relationship has been observed between caffeine supplementation and EIB severity (VanHaitsma et al., 2010). When administered 1 hour prior to exercise, the peak decrease in FEV₁ post-exercise was attenuated by ~ 33% following low dose supplementation (12 \pm 4 vs. 18 \pm 2%, 3 mg·kg·bm⁻¹), and ~ 50% following moderate dose supplementation when compared to baseline (9 \pm 3 vs. 18 \pm 2%, 6 mg·kg·bm⁻¹; VanHaitsma et al., 2010). Following high dose supplementation, the peak decrease in FEV₁ was attenuated by ~ 61% (7 \pm 2 vs. 18 \pm 2%, 9 mg·kg·bm⁻¹), a comparable magnitude to that observed following pharmaceutical treatment (180 μ g of albuterol), and below the diagnostic threshold

for EIB (Anderson & Kippelen, 2013; Godfrey et al., 1999; VanHaitsma et al., 2010; Williams et al., 2015).

However, adverse side-effects have been reported following high dose caffeine supplementation, including tachycardia and muscle tremors (7-10 mg·kg·bm⁻¹; VanHaitsma et al., 2010; Williams et al., 2020), with the magnitude of bronchoprotection also demonstrated to be inconsistent (Weiler et al., 2016). Although caffeine was removed from WADA's prohibited list, it remains part of the monitoring program of supplements that may be prohibited (Williams et al., 2020), bringing into question its long-term viability as a therapeutic strategy for EIB.

More recently, a low salt diet has been proposed as a short-term strategy for decreasing the severity of asthma around major competitions (Williams et al., 2020). Adopting a low salt diet for one-week (< 1500mg of sodium·day⁻¹) can attenuate the peak decrease in FEV₁ post-exercise by up to 27% (Williams et al., 2020). However, a low salt diet may have adverse effects on rehydration and as a result, athletes are likely to exceed the proposed low salt diet guidelines (Williams et al., 2020), unless in-depth nutritional support and education is provided.

Given the limitations associated with current nutritional strategies, novel interventions may offer greater therapeutic benefits. Following the recent identification of associations between features of gut microbial dysbiosis, clinical and inflammatory parameters of asthma (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016; Okba et al., 2018), research has begun to investigate whether short-term supplementation with synbiotics decreases the severity of asthma (Halnes et al., 2017). Synbiotics are a combination of probiotics and prebiotics designed to exert synergistic effects on immune function by enhancing the gut microbiota's immunomodulatory properties. Research has demonstrated that gut microbial composition can change rapidly in response to dietary changes (i.e. within twenty-four hours (David et al. 2014)). When combined with the research of Begley et al. (2018), demonstrating associations between gut microbial dysbiosis and asthma, it is plausible to speculate that acute strategies to attenuate the severity of asthma warrant further investigation, particularly if they can yield similar benefits as other nutritional strategies but without adverse side effects. To date, only one study has investigated the short-term effects of synbiotic supplementation on the severity of asthma. Halnes et al. (2017), administered a synbiotic meal containing a multi-strain probiotic ($\geq 10^8$ colony forming units of *Lactobacillus acidophilus* LA5,

Bifidobacterium lactis Bb12, and *Lactobacillus rhamnosus* GG), and 3.5g of inulin to adults with stable asthma. Just 4 hours after consuming the synbiotic meal, a significant increase in resting FEV₁ was observed compared to placebo (Halnes et al., 2017). This was associated with a decrease in markers of airway inflammation, and an increase in the expression of GPR41 and GPR43 in sputum (Halnes et al., 2017). The mechanisms behind these improvements are unclear.

In theory, synbiotic supplementation could lead to greater therapeutic benefits than probiotics or prebiotic alone, but this is not reflected in practice, at least in humans (van de Pol et al., 2011). There is currently a lack of understanding regarding the mechanisms by which probiotics and prebiotics attenuate the severity of asthma in humans. Therefore, isolated investigations of probiotics and prebiotics are required to expand current understandings of the respective therapeutic mechanisms, supported by analyses of gut microbial composition and markers of inflammation. Given that Halnes et al. (2017), investigated the effects of an inulin-based synbiotic meal using a parallel group design, the short-term effects of galactooligosaccharides remain to be determined in robust crossover trials.

As a result, the primary aim of the current study was to investigate the short-term effects of galactooligosaccharide-based prebiotic supplementation on the severity of HIB and markers of systemic inflammation. It was hypothesised that acute supplementation would lead to reductions in both the severity of HIB, and markers of systemic inflammation (i.e. eosinophils).

5.2 Methods

5.2.1 Experimental Design

This study was conducted following a double-blind, placebo-controlled, crossover design. Ethical approval was obtained from Nottingham Trent Universities Human Research Ethics Committee (approval number: 512). All protocols were conducted in accordance with the Declaration of Helsinki (World Medical Association, 2013), and the British Association of Sport and Exercise Sciences Code of Conduct (British Association of Sport and Exercise Sciences, 2017). The study was conducted from February 2018 to April 2019.

5.2.2 Participants

Recreationally active adults with hyperpnoea (exercise)-induced bronchoconstriction ($n = 8$) were recruited from Nottingham Trent University. Six were male ($n = 6$), and two were female ($n = 2$). Written informed consent was obtained prior to any procedures taking place (British Association of Sport and Exercise Sciences, 2013). All participants completed a health screen and history questionnaire prior to taking part. A detailed overview of the eligibility criteria is provided in the general methods section (Chapter III, pages 124-126). All participants attended the laboratory on three occasions over a 3-week period. All trials were conducted at the same time of day, one-week apart, to control for the diurnal effect on pulmonary function (Rhee & Kim, 2015). The first visit was a familiarisation trial, and the next two visits the experimental trials.

All participants had a physician's diagnosis of asthma, with severity classed as Step 1 according to the British Thoracic Society and Scottish Intercollegiate Guidelines Network (British Thoracic Society & Scottish Intercollegiate Guidelines Network, 2016). Six ($n = 6$) participants were prescribed a short-acting β_2 -agonist, and two ($n = 2$) were prescribed a short-acting β_2 -agonist plus a low dose inhaled corticosteroid (beclometasone dipropionate, and beclometasone extra-fine QVAR[®], respectively).

5.2.3 Measurements

5.2.3.1 Pulmonary Function & The EVH Protocol

During the familiarisation trial, participants were screened for HIB via the EVH protocol following the same protocol outlined in Chapters 3 and 4. Participants were excluded from the study if resting FEV₁ values were < 70% of percentage predicted values. If participants demonstrated a positive response to the EVH protocol during familiarisation, they continued to the experimental trials where the EVH protocol was repeated.

5.2.3.2 Markers of Systemic Inflammation

During each experimental trial, a 4mL blood sample was collected at baseline, pre-EVH, and 60-minutes post-EVH into a vacutainer tube containing EDTA (Brooks, 2014; Calfee & Farr, 2002; World Health Organisation, 2010). White blood cell subsets were quantified fresh in whole blood using an automated haematology analyser (Sysmex, XS-1000i[™], United States). Blood samples were analysed for

absolute counts of total white blood cells, eosinophils and basophils ($\times 10^9/L$), as well as the relative abundance (%) of eosinophils and basophils, respectively.

5.2.3.3 Prebiotic Supplementation

Prebiotic supplementation was administered following a double-blind, placebo-controlled, crossover design. During one experimental trial, participants were administered a single sachet of a galactooligosaccharide-based prebiotic (Host Therabiotics, HOST-DM059, Jersey, The Channel Islands). During the other experimental trial, participants were administered a taste and appearance matched placebo (maltodextrin). In accordance with the newly refined supplement developed by Clasado Biosciences, one prebiotic sachet weighed 3.6g and contained up to 85% galactooligosaccharides (3.1g), 6% water (0.22g), 6% glucose (0.22g), 4% galactose (0.14g), and 16% lactose (0.58g), respectively. With the view of this work having practical relevance to applied settings, this dose was investigated because this is what is commercially available and is in accordance with the approach taken by previous research (Williams et al., 2016). Both supplements were reconstituted in an opaque bottle in 300ml of water. The supplements were blinded by HOST Therabiotics, labelled as “X” and “L”, respectively. Unblinding was completed when the final participant completed their final experimental trial.

5.2.4 Experimental Trials

Both experimental trials were completed one-week apart, in accordance with the complete reversal of changes in *Bifidobacteria* observed by Davis et al. (2011), one-week after galactooligosaccharide supplementation was ended. Nutritional intake in the 24-hours before the first experimental trial was monitored via a 24-hour weighed nutritional intake record (Appendix L), and repeated during the second experimental trial to standardise short-term dietary intake. Participants were asked to attend the laboratory at least 1 hour post prandial (Anderson et al., 2010; Rhee & Kim, 2015; Sue-Chu et al., 2010). Both trials consisted of baseline measurements, pre-EVH measurements, and post-EVH measurements, respectively. Baseline measurements were conducted at ~ 09:00, and involved resting assessments of pulmonary function and providing a blood sample. Following baseline measurements, participants consumed one of the nutritional supplements reconstituted in 300ml of water. Participants were given 15 minutes to consume the beverage. A stopwatch was then used to time a 4-hour ingestion period. The 4-hour

ingestion period was implemented to replicate and enable a direct comparison to the protocol of Haines et al. (2017), who reported significant improvements in the production of pro-inflammatory mediators and pulmonary function four hours after ingesting a synbiotic-based soluble fibre meal. Further data collected from David et al. (2013), demonstrate that significant changes in gut microbial composition can be achieved within twenty four hours of starting a purely animal-based diet, highlighting the rapid nature with which gut microbial composition can change in response to dietary stimuli. From the start of the ingestion period to the end of post-EVH measurements, participants were only permitted to consume plain water. The water consumed during the first experimental trial was measured and repeated during the second experimental trial. Participants remained supervised in the laboratory during the ingestion period and were not allowed to perform any strenuous exercise. Following the ingestion period, pre-EVH measurements were conducted ~ 13:00. The pre-EVH measurements were the same as baseline measurements. Once the pre-EVH measurements had been completed, the EVH protocol was carried out. One hour after the EVH protocol, a final blood sample was obtained (post-EVH measurement). The familiarisation trial lasted ~ 1 hour, and each experimental trial lasted ~ 6-7 hours, equating to ~ 15 hours in total. Environmental conditions including laboratory temperature, relative humidity, and barometric pressure were monitored across experimental trials. An overview of participant recruitment and study completion is outlined in Figure 5.0. The protocol for the experimental trials is outlined in Figure 5.1.

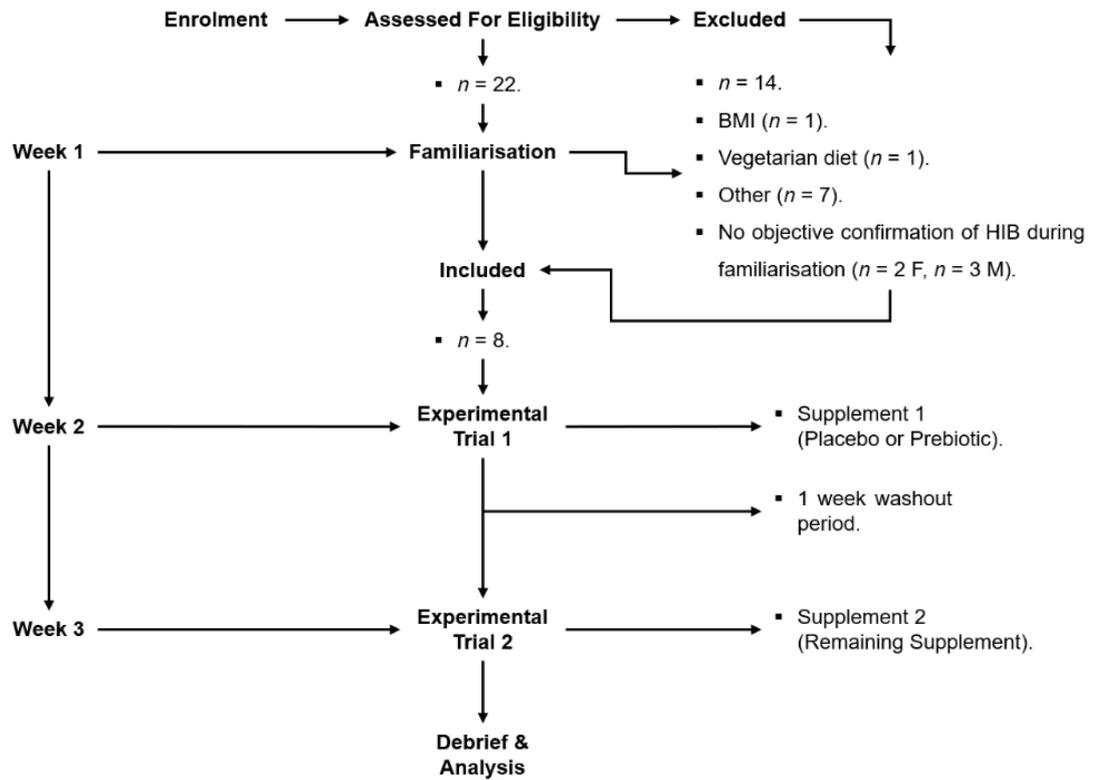


Figure 5.0 An overview of participant recruitment and study completion. *HIB* hyperpnoea-induced bronchoconstriction, *F* female, *M* male, *BMI* body mass index.

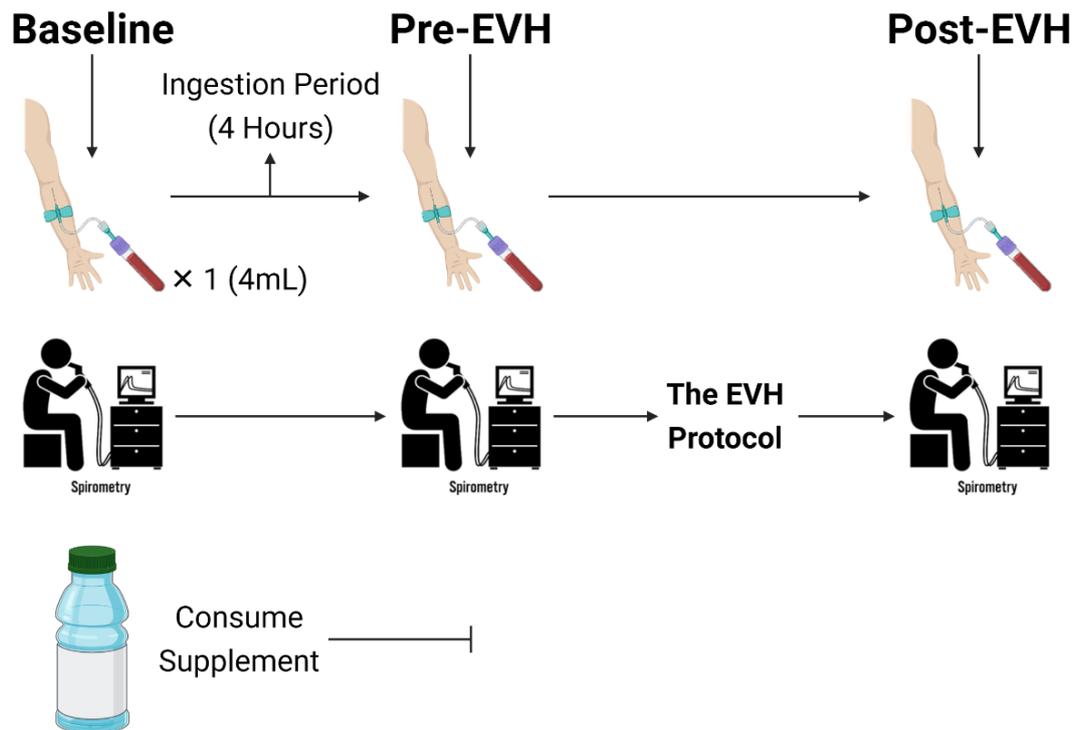


Figure 5.1 An overview of the protocol for the experimental trials. Created with www.BioRender.com. *EVH* eucapnic voluntary hyperpnoea.

5.2.5 Statistical Analysis

An estimation of sample size was conducted using an online calculator based on an α priori level of 0.05 (two-sided; MGH Biostatistics Centre, Harvard, Massachusetts). Based on a within-subject's standard deviation for the peak decrease in FEV₁ post-EVH of 100mL (Williams et al., 2015), associated power of 0.9 ($P\beta$), and a minimal detectable difference in FEV₁ that is perceivable by participants (230mL; Santanello et al., 1999), an estimated sample size of seven ($n = 7$) was calculated with a 94% probability that a change in the peak decrease in FEV₁ post-EVH of ≥ 230 mL would be detected at the specified α level (Williams et al., 2016).

Statistical analysis was conducted using the statistical package for the social sciences (IBM SPSS Inc, Version 26, Chicago IL, USA). The Shapiro-Wilk test was used to assess normality (Field, 2018). If deviations from normality were present, equivalent non-parametric tests were used where possible (Field, 2018). A series of two-way repeated measures analysis of variance (RM ANOVA) with Bonferroni post-hoc correction were used to assess changes in the HIB group across the prebiotic and placebo conditions from baseline to pre-EVH, baseline to post-EVH, and pre-EVH to post-EVH, in pulmonary function (FVC, FEV₁, PEF, FEF_{25%-75%}), and markers of systemic inflammation (total white blood cells, eosinophils and basophils, respectively; Field, 2018). Greenhouse-Geisser corrected estimates were reported across all two-way repeated measures ANOVA's (Field, 2018). Bootstrap corrected paired samples t -tests (or Wilcoxon Signed-Rank tests) were used to assess changes in the HIB group between the prebiotic and placebo conditions for the decrease in pulmonary function post-EVH (FVC, FEV₁, PEF, FEF_{25%-75%}). For paired samples t -tests, bias corrected accelerated (BCa) adjusted 95% confidence intervals (CI's) were calculated for the mean difference between conditions (Field, 2018). Homogeneity of variance was assessed using Levene's test of equality of variance. For two-way repeated measures ANOVA's and Wilcoxon Signed-Rank tests, estimates of effect size were calculated according to Field, (2018). For paired samples t -tests, estimates of effect size were calculated using Cohen's d (Field, 2018).

Statistically significant differences were accepted if $p < 0.05$ (two-tailed). Estimates of effect size were classed as small (0.2-0.5), medium (0.5-0.8), or large (> 0.8 ; Field, 2018). Values are reported as mean \pm standard deviation for parametric data,

and median plus interquartile range for non-parametric data (IQR; 25th – 75th percentile). Figures demonstrating mechanisms or protocols were created using Microsoft PowerPoint or Biorender (Toronto, Ontario, Canada). Figures reporting experimental data were created using GraphPad Prism Software (Version 8.0.1, San Diego, CA, USA).

5.3 Results

A total of eight ($n = 8$) participants completed the study. No differences were reported across any environmental conditions between the prebiotic and placebo trials, including laboratory temperature (22 ± 2.3 vs. $22.4 \pm 2.2^\circ\text{C}$; $p = 0.460$), relative humidity (51 ± 12 vs. $49 \pm 12\%$; $p = 0.430$), and barometric pressure (752 ± 5 vs. 749 ± 6 mmHg; $p = 0.305$). The baseline descriptive characteristics for participants are outlined in Table 5.0 below.

Table 5.0 Descriptive characteristics of the HIB group.

	HIB ($n = 8$)
Age (years)	22 ± 3
Height (m)	1.79 ± 0.13
Body Mass (kg)	75.7 ± 11.8
BMI (kg/m^2)	23.6 ± 0.6

Values are presented as mean \pm SD. *HIB* hyperpnoea-induced bronchoconstriction, *m* metres, *kg* kilograms.

5.3.1 Pulmonary Function

The accuracy values for the spirometer were within accepted ranges across all experimental trials (Miller et al., 2005). No differences were reported between the actual and predicted calibration values between the prebiotic and placebo trials when expressed as absolute (0.04 ± 0.02 vs. 0.04 ± 0.02 litres; $p = 0.516$), or relative values (the percentage difference between the intended calibration volume of 3-litres, and the actual measured calibration volume, 1.3 ± 0.5 vs. $1.2 \pm 0.6\%$; $p = 0.430$). No differences were reported between the actual and predicted calibration values for the dry gas meter between the prebiotic and placebo trials (median, IQR: 0.1, [0.03-0.1] vs. 0.1, [0.03-0.2 litres]; $p = 0.317$).

Table 5.1 Changes in pulmonary function during the prebiotic and placebo trials.

	Prebiotic			Placebo		
	Baseline	Pre-EVH	Post-EVH	Baseline	Pre-EVH	Post-EVH
FEV ₁ (L)	3.64 ± 0.58	3.66 ± 0.65	2.99 ± 0.53	3.72 ± 0.64	3.76 ± 0.73	3.00 ± 0.66
FVC (L)	5.06 ± 1.13	5.05 ± 1.21	4.60 ± 0.99	5.14 ± 1.23	5.12 ± 1.27	4.59 ± 1.21
PEF (l·s ⁻¹)	8.94 ± 1.88	8.92 ± 1.90	6.67 ± 1.54	8.83 ± 1.88	9.08 ± 2.17	6.42 ± 1.70
FEF _{25%-75%} (l·s ⁻¹)	2.92 ± 0.41	2.99 ± 0.49	1.82 ± 0.24	2.99 ± 0.57	3.07 ± 0.52	1.93 ± 0.34

EVH eucapnic voluntary hyperpnoea, *FEV₁* forced expiratory volume in one second, *FVC* forced vital capacity, *PEF* peak expiratory flow rate, *FEF_{25%-75%}* forced expiratory flow rate between 25%-75% of FVC.

Table 5.2 Changes in the peak decrease in FEV₁ post-EVH during the prebiotic and placebo trials (expressed as a percentage).

Participant	Prebiotic	Placebo
1	12.1	20.5
2	23.3	19.0
3	27.6	31.6
4	13.7	18.3
5	25.0	25.2
6	13.3	15.7
7	21.2	24.7
8	7.4	8.9

EVH eucapnic voluntary hyperpnoea.

A cut-off value of a 13% drop in FEV₁ post EVH was considered diagnostic of EIB (Godfrey et al., 1999). Based on this threshold, Participant 1 demonstrated a non-diagnostic response to EVH in the prebiotic (but not placebo) condition. Participant 8 demonstrated a non-diagnostic response in both the prebiotic and placebo conditions, which was in contrast to the 16.0% drop observed during their familiarisation trial. A valid assessment of EIB was conducted across all three visits, suggesting that the decrease in the drop in FEV₁ observed post-EVH in the prebiotic and placebo conditions could be an anomalous result.

5.3.1.1 Forced Expiratory Volume in One Second (FEV₁)

No differences were reported for resting FEV₁ between the prebiotic and placebo trials when expressed as absolute (0.07, BCa 95% CI's [-0.01, 0.17], $t(7) = 1.06$, $p = 0.324$, $d = -0.13$), or percentage predicted values (1.88, BCa 95% CI's [-0.38, 4.67], $t(7) = 1.23$, $p = 0.260$, $d = -0.25$). In addition, no differences were reported in the post-EVH decrease in FEV₁ between the prebiotic and placebo trials when expressed as absolute (0.67 ± 0.32 vs. 0.77 ± 0.27 litres; 0.1, BCa 95% CI's [0.01, 0.17], $t(7) = 2.4$, $p = 0.048$, $d = -0.36$), or relative values (18 ± 7 vs. $21 \pm 7\%$; 2.54, BCa 95% CI's [0.25, 4.76], $t(7) = 1.95$, $p = 0.092$, $d = -0.37$). The severity of HIB was classed as mild-moderate (Anderson & Kippelen, 2013). This was based on the peak drop in FEV₁ observed by two consecutive timepoints post-EVH of being $\geq 13\%$ lower than baseline, which may not reflect the "true" drop in FEV₁ post-EVH and subsequent severity.

In accordance with these findings, no interaction effect was observed for trial (prebiotic vs. placebo) \times time (baseline, pre-EVH, post-EVH; Greenhouse-Geisser $\epsilon = 0.661$; $F(1.32, 9.25) = 1.38$, $p = 0.284$, $r = 0.32$), and no main effect was observed for trial (Greenhouse-Geisser $\epsilon = 1.000$; $F(1, 7) = 0.97$, $p = 0.356$, $r = 0.35$). In contrast, a significant main effect was observed for time (Greenhouse-Geisser $\epsilon = 0.682$; $F(1.36, 9.55) = 46.33$, $p < 0.0001$, $r = 0.94$). As outlined in Table 5.1 and Figure 5.2, in the prebiotic trial, FEV₁ decreased from baseline to post-EVH (0.65, BCa 95% CI's [0.48, 0.83], $t(7) = 5.74$, $p < 0.001$, $d = -1.12$), and pre-EVH to post-EVH (0.67, BCa 95% CI's [0.47, 0.85], $t(7) = 5.97$, $p < 0.001$, $d = -1.04$). The same pattern was observed in the placebo trial, FEV₁ decreased from baseline to post-EVH (0.72, BCa 95% CI's [0.58, 0.86], $t(7) = 8.63$, $p < 0.0001$, $d = -1.13$), and pre-EVH to post-EVH (0.77, BCa 95% CI's [0.58, 0.95], $t(7) = 7.91$, $p < 0.0001$, $d = -1.05$).

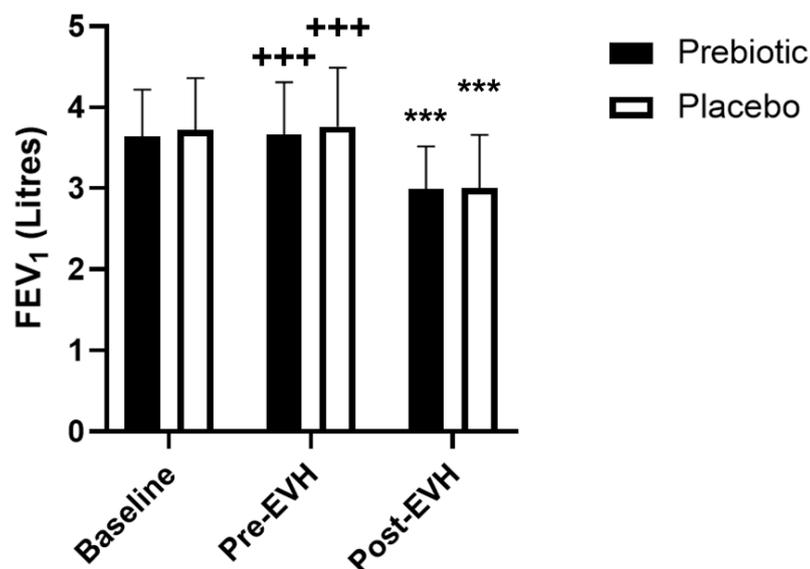


Figure 5.2 Changes in FEV₁ within the prebiotic and placebo trials. FEV₁ forced expiratory volume in one second. EVH eucapnic voluntary hyperpnoea. *** $p < 0.0001$ when compared to baseline values of the respective trial. *** $p < 0.0001$ when compared to post-EVH values of the respective trial ($n = 8$).

5.3.1.2 Forced Vital Capacity (FVC)

No differences were reported for resting FVC between the prebiotic and placebo trials when expressed as absolute ($t = -1.33$, $p = 0.183$, $r = -0.33$), or percentage predicted values (1.50, BCa 95% CI's [-0.75, 3.63], $t(7) = 1.53$, $p = 0.170$, $d = -0.17$). In addition, no differences were reported in the post-EVH decrease in FVC between the prebiotic and placebo trials when expressed as absolute (0.45 ± 0.28 vs. 0.53 ± 0.27 litres; 0.08, BCa 95% CI's [-0.10, 0.24], $t(7) = 0.70$, $p = 0.504$, $d = -0.28$), or relative values (8.3 ± 4.4 vs. $10.6 \pm 5.0\%$; of 2.3%; 2.3, BCa 95% CI's [-3.06, 7.68], $t(7) = 1.01$, $p = 0.346$, $d = -0.46$).

In accordance with these findings, no interaction effect was observed for trial (prebiotic vs. placebo) \times time (baseline, pre-EVH, post-EVH; Greenhouse-Geisser $\epsilon = 0.812$; $F(1.63, 11.38) = 0.55$, $p = 0.555$, $r = 0.41$), and no main effect was observed for trial (Greenhouse-Geisser $\epsilon = 1.000$; $F(1, 7) = 0.4$, $p = 0.549$, $r = 0.23$). In contrast, a significant main effect was observed for time (Greenhouse-Geisser $\epsilon = 0.753$; $F(1.51, 10.55) = 35.3$, $p < 0.0001$, $r = 0.93$). As outlined in Table x and Figure 5.3, in the prebiotic trial, FVC decreased from baseline to post-EVH ($t = -2.52$, $p = 0.012$, $r = -0.63$), and pre-EVH to post-EVH (0.45 , BCa 95% CI's [0.25, 0.63], $t(7) = 4.62$, $p = 0.002$, $d = -0.37$). The same pattern was observed in the placebo trial, FVC decreased from baseline to post-EVH (0.55 , BCa 95% CI's [0.41,

0.72], $t(7) = 6.4$, $p < 0.001$, $d = -0.45$), and pre-EVH to post-EVH (0.53, BCa 95% CI's [0.37, 0.68], $t(7) = 5.44$, $p < 0.001$, $d = -0.42$).

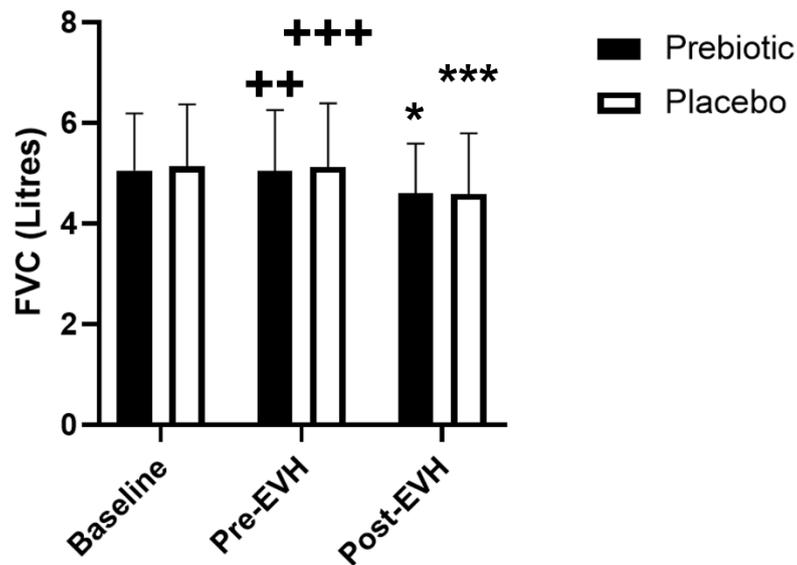


Figure 5.3 FVC within the prebiotic and placebo trials. *FVC* forced vital capacity. *EVH* eucapnic voluntary hyperpnoea. * $p < 0.05$ when compared to baseline values of the respective trial. *** $p < 0.001$ when compared to baseline values of the respective trial. ** $p < 0.01$ when compared to post-EVH values of the respective trial. *** $p < 0.001$ when compared to post-EVH values of the respective trial ($n = 8$).

5.3.1.3 Peak Expiratory Flow (PEF)

No differences were reported for resting PEF between the prebiotic and placebo trials when expressed as absolute (-0.11 , BCa 95% CI's $[-0.37, 0.13]$, $t(7) = -0.71$, $p = 0.503$, $d = 0.06$), or percentage predicted values (-0.63 , BCa 95% CI's $[-3.13, 1.63]$, $t(7) = -0.40$, $p = 0.698$, $d = 0.07$). In addition, no differences were reported in the post-EVH decrease in PEF between the prebiotic and placebo trials (25 ± 6 vs. $30 \pm 5\%$; 4.31 , BCa 95% CI's $[0.48, 7.87]$, $t(7) = 2.23$, $p = 0.061$, $d = -0.89$).

In accordance with these findings, no interaction effect was observed for trial (prebiotic vs. placebo) \times time (baseline, pre-EVH, post-EVH; Greenhouse-Geisser $\epsilon = 0.996$; $F(1.99, 13.95) = 3.38$, $p = 0.064$, $r = 0.31$), and no main effect was observed for trial (Greenhouse-Geisser $\epsilon = 1.000$; $F(1, 7) = 0.13$, $p = 0.731$, $r = 0.13$). In contrast, a significant main effect was observed for time (Greenhouse-Geisser $\epsilon = 0.678$; $F(1.36, 9.49) = 110.12$, $p < 0.0001$, $r = 0.97$). As outlined in Figure 5.4, in the prebiotic trial, PEF decreased from baseline to post-EVH (2.27, BCa 95% CI's [1.88, 2.68], $t(7) = 9.76$, $p < 0.0001$, $d = -1.21$), and pre-EVH to post-EVH (2.25, BCa 95% CI's [1.76, 2.71], $t(7) = 9.12$, $p < 0.0001$, $d = -1.18$). The same pattern was observed in the placebo trial, PEF decreased from baseline to post-EVH (2.41, BCa 95% CI's [1.96, 2.88], $t(7) = 11.41$, $p < 0.0001$, $d = -1.28$), and pre-EVH to post-EVH (2.66, BCa 95% CI's [2.17, 3.13], $t(7) = 11.55$, $p < 0.0001$, $d = -1.22$).

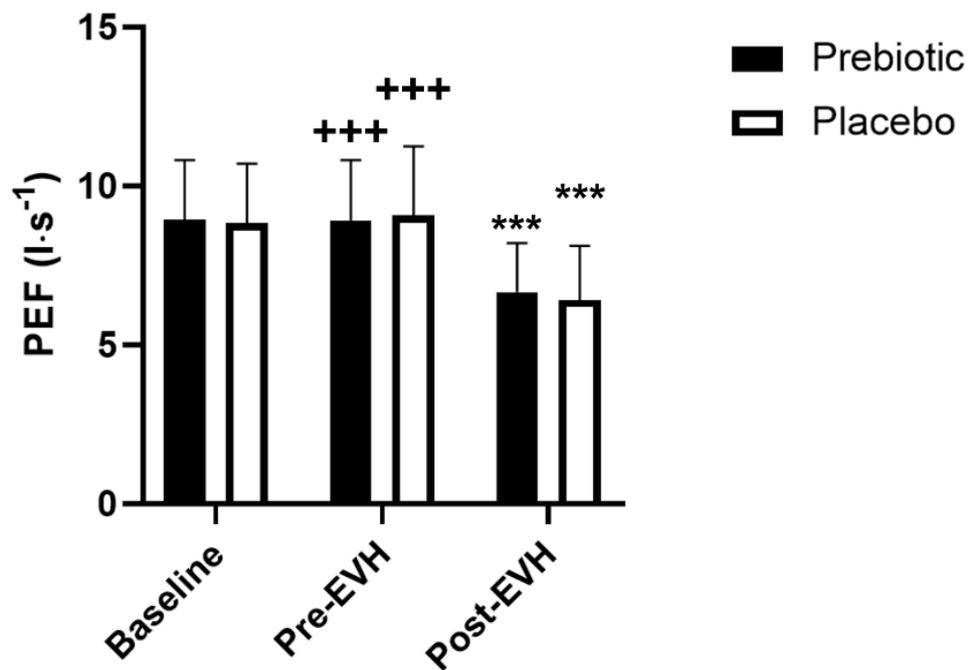


Figure 5.4 Changes in PEF within the prebiotic and placebo trials. *PEF* peak expiratory flow rate. *EVH* eucapnic voluntary hyperpnoea. *** $p < 0.0001$ when compared to baseline values of the respective trial. *** $p < 0.0001$ when compared to post-EVH values of the respective trial ($n = 8$).

5.3.1.4 Forced Expiratory Flow Rate Between 25% and 75% of FVC (FEF_{25%-75%})

No differences were reported for resting FEF_{25%-75%} between the prebiotic and placebo trials when expressed as absolute ($t = -0.7$, $p = 0.484$, $r = -0.18$), or percentage predicted values ($t = -0.56$, $p = 0.575$, $r = -0.14$). In addition, no

differences were reported in the percentage decrease in $FEF_{25\%-75\%}$ post-EVH between the prebiotic and placebo trials (38 ± 13 vs. $36 \pm 13\%$; -1.64 , BCa 95% CI's $[-10.28, 4.35]$, $t(7) = -0.52$, $p = 0.622$, $d = 0.12$).

In accordance with these findings, no interaction effect was observed for trial (prebiotic vs. placebo) \times time (baseline, pre-EVH, post-EVH; Greenhouse-Geisser $\epsilon = 0.912$; $F(1.82, 12.77) = 0.06$, $p = 0.925$, $r = 0.14$), and no main effect was observed for trial (Greenhouse-Geisser $\epsilon = 1.000$; $F(1, 7) = 1.1$, $p = 0.330$, $r = 0.37$). In contrast, a significant main effect was observed for time (Greenhouse-Geisser $\epsilon = 0.727$; $F(1.45, 10.18) = 31.57$, $p < 0.0001$, $r = 0.91$). As outlined in Figure 5.5, in the prebiotic trial, $FEF_{25\%-75\%}$ decreased from baseline to post-EVH (1.1 , BCa 95% CI's $[0.78, 1.44]$, $t(7) = 6$, $p = 0.001$, $d = -2.65$), and pre-EVH to post-EVH (1.17 , BCa 95% CI's $[0.87, 1.47]$, $t(7) = 6.12$, $p < 0.0001$, $d = -2.40$). The same pattern was observed in the placebo trial, $FEF_{25\%-75\%}$ decreased from baseline to post-EVH (1.06 , BCa 95% CI's $[0.76, 1.45]$, $t(7) = 4.97$, $p = 0.002$, $d = -1.87$), and pre-EVH to post-EVH (1.14 , BCa 95% CI's $[0.81, 1.47]$, $t(7) = 5.96$, $p = 0.001$, $d = -2.17$).

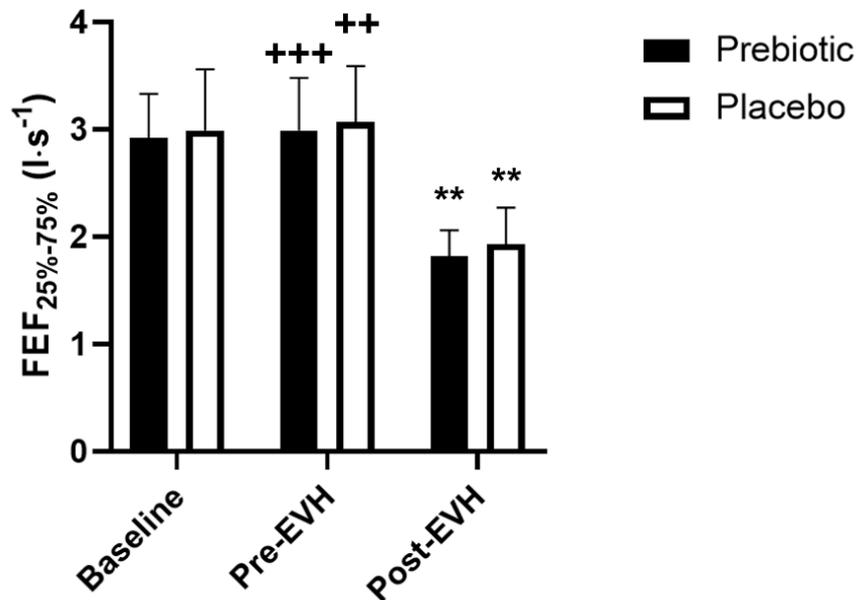


Figure 5.5 Changes in $FEF_{25\%-75\%}$ within the prebiotic and placebo trials. $FEF_{25\%-75\%}$ forced expiratory flow rate between 25% and 75% of FVC. *EVH* eucapnic voluntary hyperpnoea. ** $p < 0.01$ when compared to baseline values of the respective trial. ** $p < 0.01$ when compared to post-EVH values of the respective trial. *** $p < 0.0001$ when compared to post-EVH values of the respective trial ($n = 8$).

5.3.1.5 Minute Ventilation (\dot{V}_E)

During the EVH protocol, participants were asked to maintain their \dot{V}_E at $30 \times$ their highest resting FEV_1 (Anderson et al., 2001). During the prebiotic trial, the actual \dot{V}_E achieved was $29 \pm 2 \times$ highest resting FEV_1 (range: 26-30), whereas during the placebo trial, the actual \dot{V}_E achieved was $28 \pm 2 \times$ highest resting FEV_1 (range: 26-31). All participants in both conditions maintained their average $\dot{V}_E \geq$ the minimum $21 \times$ highest resting FEV_1 criteria used to indicate a valid test (Anderson & Kippelen, 2013).

Table 5.3 Minute ventilation during the EVH protocol in the prebiotic and placebo trials.

Participant	Prebiotic (<i>n</i> = 8)		Placebo (<i>n</i> = 8)	
	Absolute (l·min ⁻¹)	% MVV	Absolute (l·min ⁻¹)	% MVV
1	71.0	26	68.4	25
2	122.3	30	120.5	29
3	109.6	30	106.0	30
4	117.4	31	119.4	31
5	127.6	29	123.9	29
6	124.0	30	120.0	26
7	71.9	25	79.9	26
8	100.7	28	102.9	29

EVH eucapnic voluntary hyperpnoea.

5.3.2 Markers of Systemic Inflammation

5.3.2.1 Total White Blood Cells

For quality control samples, no differences were observed between the prebiotic and placebo trials (7.15 ± 0.24 vs. $7.15 \pm 0.28 \times 10^9 \cdot L$; $p = 1.00$). For experimental samples, no interaction effect was observed for trial (prebiotic vs. placebo) \times time (baseline, pre-EVH, post-EVH; Greenhouse-Geisser $\epsilon = 0.9$; $F(1.8, 9) = 0.41$, $p = 0.657$, $r = 0.18$), no main effect was observed for trial (Greenhouse-Geisser $\epsilon = 1$; $F(1, 5) = 0$, $p = 0.993$, $r = 0$), and no main effect was observed for time (Greenhouse-Geisser $\epsilon = 0.522$; $F(1.04, 5.22) = 3.44$, $p = 0.120$, $r = 0.58$).

5.3.2.2 Eosinophils

For quality control samples, no differences were observed between the prebiotic and placebo trials when expressed as absolute (0.74 ± 0.05 vs. $0.77 \pm 0.05 \times 10^9 \cdot L$; $p = 0.376$), or relative values (10 ± 1 vs. $11 \pm 1\%$; $p = 0.268$). For experimental samples expressed as absolute values, no interaction effect was observed for trial (prebiotic vs. placebo) \times time (baseline, pre-EVH, post-EVH; Greenhouse-Geisser $\epsilon = 0.561$; $F(1.12, 5.61) = 0.12$, $p = 0.766$, $r = 0.10$), no main effect was observed for trial (Greenhouse-Geisser $\epsilon = 1$; $F(1, 5) = 0.08$, $p = 0.789$, $r = 0.13$), and no main effect was observed for time (Greenhouse-Geisser $\epsilon = 0.786$; $F(1.57, 7.86) = 3.21$, $p = 0.102$, $r = 0.71$).

In accordance with absolute values, no interaction effect was observed for trial (prebiotic vs. placebo) × time (baseline, pre-EVH, post-EVH; Greenhouse-Geisser $\epsilon = 0.683$; $F(1.37, 6.83) = 0.1$, $p = 0.836$, $r = 0.10$), and no main effect was observed for trial (Greenhouse-Geisser $\epsilon = 1.000$; $F(1, 5) = 0$, $p = 0.991$, $r = 0$). In contrast, a significant main effect was observed for time (Greenhouse-Geisser $\epsilon = 0.887$; $F(1.77, 8.87) = 10.68$, $p = 0.005$, $r = 0.87$). In the placebo trial, eosinophils were highest at baseline, and significantly decreased from baseline to post-EVH (0.7, BCa 95% CI's [0.21, 1.35], $t(7) = 2.58$, $p = 0.036$, $d = -0.38$).

5.3.2.3 Basophils

For quality control samples, no differences were observed between the prebiotic and placebo trials when expressed as absolute (0.49 ± 0.06 vs. $0.49 \pm 0.05 \times 10^9 \cdot L$; $p = 1.000$), or relative values (6.7 ± 0.9 vs. $6.8 \pm 0.9\%$; $p = 0.981$). For experimental samples expressed as absolute values, no interaction effect was observed for trial (prebiotic vs. placebo) × time (baseline, pre-EVH, post-EVH; Greenhouse-Geisser $\epsilon = 0.848$; $F(1.7, 8.48) = 1.15$, $p = 0.353$, $r = 0.33$), no main effect was observed for trial (Greenhouse-Geisser $\epsilon = 1$; $F(1, 5) = 0.455$, $p = 0.530$, $r = 0.29$), and no main effect was observed for time (Greenhouse-Geisser $\epsilon = 0.856$; $F(1.71, 8.56) = 1.40$, $p = 0.292$, $r = 0$). In accordance with absolute values, no interaction effect was observed for trial (prebiotic vs. placebo) × time (baseline, pre-EVH, post-EVH; Greenhouse-Geisser $\epsilon = 0.979$; $F(1.96, 9.79) = 2.067$, $p = 0.179$, $r = 0.33$), no main effect was observed for trial (Greenhouse-Geisser $\epsilon = 1$; $F(1, 5) = 0.349$, $p = 0.580$, $r = 0.26$), and no main effect was observed for time (Greenhouse-Geisser $\epsilon = 0.994$; $F(1.99, 9.94) = 0.730$, $p = 0.505$, $r = 0.15$).

Table 5.4 Changes in markers of systemic inflammation during the prebiotic and placebo trials.

	Prebiotic			Placebo		
	Baseline	Pre-EVH	Post-EVH	Baseline	Pre-EVH	Post-EVH
WBC ($\times 10^9/L$)	5.57 ± 0.83	6.44 ± 0.64	6.28 ± 0.75	5.77 ± 0.70	6.61 ± 1.97	6.41 ± 1.44
Eosinophils ($\times 10^9/L$)	0.14 ± 0.07	0.13 ± 0.05	0.12 ± 0.05	0.17 ± 0.12	0.12 ± 0.05	0.13 ± 0.09
Eosinophils (%)	2.6 ± 1.3	2.0 ± 1.7	1.8 ± 0.7	2.8 ± 1.9	1.9 ± 1.0	2.1 ± 1.3
Basophils ($\times 10^9/L$)	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Basophils (%)	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.2	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.2

Table 5.5 Changes in systemic eosinophilia during the prebiotic and placebo trials based on individual data (expressed as a percentage of total white blood cells).

Participant	Prebiotic			Placebo		
	Baseline	Pre-EVH	Post-EVH	Baseline	Pre-EVH	Post-EVH
1	5.4		2.8	6.8		4.7
2	3.2	2.4	1.8	3.4	2.6	2.9
3	1.8	1.9	1.8	3.6	2.8	2.4
4	1.6	0.8	0.7	1.5	1.0	1.7
5	2.7	2.6	2.3	1.1	1.1	1.4
6	2.0	1.6	1.7	2.8	2.9	2.2
7	1.2		1.1	2.1		1.2
8	2.6	2.4	2.5	1.4	0.9	0.6

Data is missing for Participants 1 and 7 at the pre-EVH timepoint during both conditions as blood samples could not be collected. This was due to these participants being deemed to not having three appropriate sites for conducting venipuncture upon initial inspection at rest. In these situations, the baseline and post-EVH timepoints were prioritised.

5.4 Discussion

5.4.1 Key Findings

The primary aim of the current study was to investigate whether short-term supplementation with a single dose of galactooligosaccharide-based prebiotic (HOST-DM059) attenuated the severity of hyperpnoea (exercise)-induced bronchoconstriction (i.e. be classed as HIB⁻ based on a response to the EVH protocol of a drop in FEV₁ < 13% from baseline. Based on the current findings, short-term galactooligosaccharide-based prebiotic supplementation did not attenuate the severity of HIB, or markers of systemic inflammation. Based on an a priori power calculation, the minimum sample size required to detect participant perceivable changes in the peak decrease in FEV₁ post-EVH of $\geq 230\text{mL}$ was seven ($n = 7$; Santanello et al., 1999; Williams et al., 2015; 2016). Between the prebiotic and placebo trials, the mean difference in the peak decrease in FEV₁ post-EVH was 100mL, less than what would be perceivable or of clinical relevance (Santanello et al., 1999). Given that the current sample size exceeded the minimum sample size required to detect patient perceivable, clinically relevant improvements in FEV₁ ($n = 8$), it is likely that no adaptations occurred following prebiotic supplementation, as opposed to the current study being insufficiently powered to detect them.

Various factors associated with the current intervention may explain the disparity in findings when compared to short-term synbiotic and longer-term prebiotic interventions (Halnes et al., 2017; Williams et al., 2016). The first potential factor is the dose administered. In healthy adults, Davis et al. (2011), demonstrated that a minimum dose of 10 g/day^{-1} of galactooligosaccharides was required to cause significant increases in gastrointestinal *Bifidobacteria*. In contrast, no changes were observed following 2.5 g/day^{-1} (Davis et al., 2011), suggesting that a minimum threshold exists for galactooligosaccharides to induce bifidogenic responses, that a dose-response relationship occurs between galactooligosaccharides and increases in *Bifidobacteria*, or a combination of both. The current study administered 3.1 g/day^{-1} of galactooligosaccharides (3.6g sachet, up to 85% galactooligosaccharides). Although this was below the minimum dose suggested by Davis et al. (2011), to cause a bifidogenic response, the current supplement contained less impurities, supposedly increasing its bifidogenic properties. In theory, a lower dose of the current galactooligosaccharide may cause similar or greater adaptations to higher doses of previous galactooligosaccharides.

Previous research using a similar galactooligosaccharide-based prebiotic (Bimuno[®] galactooligosaccharide, B-GOS[®]; Williams et al., 2016), reported clinically relevant, participant perceivable improvements in the severity of HIB, and systemic markers of airway inflammation, following 3-weeks of supplementation with 5.5 g/day⁻¹ (55%) of B-GOS[®]. Although this effect could be partly attributed to the duration of supplementation, the daily dose administered was above the minimum threshold proposed by Davis et al. (2011), to induce a bifidogenic response (Williams et al., 2016). Although changes in gut bacterial composition were not assessed by Williams et al. (2016), the prebiotic administered selectively targets *Bifidobacterium bifidum* (Depeint et al., 2008), a species of *Bifidobacteria* shown to be significantly elevated in healthy controls when compared to adults with allergic asthma (Hevia et al., 2016). Given that significant increases in *Bifidobacteria* have been observed in elderly individuals following 10-weeks of supplementation with 5.5 g/day⁻¹ of B-GOS[®] (Vulevic et al., 2015), it is plausible to speculate that Williams et al. (2016), would have observed increases in *Bifidobacteria*. As a result, we can speculate that the current dose administered was insufficient to induce the bifidogenic response required to attenuate the severity of HIB and systemic markers of airway inflammation, regardless of the improved purity (Davis et al., 2011; Verheijden et al., 2015A; 2015B; 2016; 2018; Williams et al., 2016).

Another factor that may have affected the response to galactooligosaccharide supplementation is the severity of HIB in the current cohort. The severity of HIB in the cohort recruited by Williams et al. (2016), was moderate-severe (Anderson and Kippelen, 2013), similar to that of the cohort recruited for study one (Chapter IV; 28 ± 14 vs. 34 ± 10% decrease in FEV₁ post-EVH). It should be noted however that the “true” severity of HIB in the cohort recruited for study one may be underestimated by only measuring the drop in FEV₁ up to two consecutive time points post-EVH where an ≥ 13% drop was observed. In fact, the severity of HIB in the cohort recruited by Williams et al. (2016), was similar to the current cohort of mild HIB (study two, Chapter V) following 3-weeks of supplementation with B-GOS[®] (17 ± 12 vs. 21 ± 7% decrease in FEV₁ post-EVH; Anderson & Kippelen, 2013). The response to prebiotic supplementation is partly dependent on habitual dietary fibre intake (Healey et al., 2016; 2018). Significant decreases in dietary fibre intake have been reported in severe asthma (Berthon et al., 2013; Wood et al., 2015), leading to reductions in *Bifidobacteria* and impairments in immune function (De Filippo et

al., 2010; Healey et al., 2016; 2018; Holscher et al., 2017; Huang et al., 2017). Previous research has demonstrated that through the production of short-chain fatty acids, galactooligosaccharides attenuate airway inflammation and hyperresponsiveness by inhibiting the magnitude of systemic and pulmonary eosinophilia (Ni et al., 2010; Thorburn et al., 2015; Trompette et al., 2014; Verheijden et al., 2015B; 2018). The participants who took part in Study 1, Chapter V had more severe HIB (moderate) compared to those who took part in the current study (Study 2, Chapter V; mild). Participants from study one demonstrated a significant increase in resting blood eosinophils ($p < 0.001$). When taking into consideration the previous research outlined above, galactooligosaccharide-based prebiotic supplementation may be more effective in more severe cases of HIB due to higher levels of eosinophilia (Williams et al., 2016).

The synbiotic administration of probiotics and prebiotics administered by Halmes et al. (2017), may have led to greater improvements in pulmonary function and airway inflammation through a synergistic effect. Probiotic supplementation involves ingesting live bacteria (Gourbeyre et al., 2011; Huang et al., 2017), whereas prebiotic supplementation selectively stimulates the growth and development of resident gut bacteria (Depeint et al., 2008; Gibson et al., 2017; Vulevic et al., 2015). For prebiotic supplementation to be effective, the bacteria of interest must be present in sufficient quantities (De Preter et al., 2008). As outlined above, decreases in dietary fibre intake in severe asthma may lead to the loss of *Bifidobacteria*, and their ability to modulate host immune function (Berthon et al., 2013; De Filippo et al., 2010; Healey et al., 2016; 2018; Holscher et al., 2017; Huang et al., 2017; Wood et al., 2015). Galactooligosaccharides selectively target certain species of *Bifidobacteria* (Davis et al., 2011; Depeint et al., 2008), yet adults with asthma demonstrate imbalances or deficiencies in certain species of *Bifidobacteria* (Hevia et al., 2016). It is possible that the multi-strain probiotic administered by Halmes et al. (2017), may have increased the number of *Bifidobacteria* and *Lactobacilli*, whilst the inulin-based prebiotic enhanced their immunomodulatory properties. These results suggest that baseline analyses of gut bacterial composition (De Preter et al., 2008), and habitual dietary fibre intake (Healey et al., 2016; 2018), are required to adjust the dose of targeted probiotic (Arrieta et al., 2015), and prebiotic therapies (Williams et al., 2016) in adults with differing severities of HIB.

5.4.2 Strengths

As outlined above, the current study was sufficiently powered to detect clinically relevant, participant perceivable changes in the peak decrease in FEV₁ post-EVH (Williams et al., 2015; 2016). Although sufficiently powered to detect changes in pulmonary function, the same cannot be said for markers of systemic inflammation. As a result, future research should ensure studies are sufficiently powered to detect clinically relevant changes in all key parameters through appropriate sample sizes and procedures (Begley et al., 2018). In accordance with study one (Chapter IV), the current participants were a cohort of adults with objective confirmation of mild HIB (Anderson & Kippelen, 2013). In contrast, only ~ 59% of participants recruited by Halnes et al. (2017), demonstrated objective evidence of asthma in response to hypertonic saline. These results suggest that almost half of the participants administered the synbiotic intervention may not have had active asthma (Halnes et al., 2017), impairing the robustness of the study. Given the limitations associated with subjective diagnoses outlined in the previous chapter, objective protocols such as EVH should continue to be used not only to characterise, but initially screen participants for active evidence of asthma or EIB upon enrolment to intervention trials.

5.4.3 Limitations

Habitual dietary fibre intake was not monitored as part of the current study, despite its influence on prebiotic supplementation (Healey et al., 2016; 2018). Previous research has used food-frequency questionnaires to assess habitual dietary fibre intake (Berthon et al., 2013; Wood et al., 2015). However, these are limited in scope and sensitivity. Future research should prospectively monitor habitual dietary fibre intake using software capable of quantifying different sources of dietary fibre. Combining this information with the response to prebiotic supplementation may inform the development of habitual dietary fibre intake thresholds that inform the potential magnitude of response to prebiotics (Healey et al., 2016; 2018), encouraging appropriate use in targeted populations.

Habitual dietary fibre intake also plays a critical role in maintaining gastrointestinal epithelial integrity (Budden et al., 2016; Gourbeyre et al., 2011; Huang & Boushey, 2014; Josefowicz et al., 2012; Vieira & Pretorius, 2010). The fermentation of butyrate from dietary fibre provides a key source of energy for colonocytes

(McLoughlin et al., 2019). In the absence of sufficient dietary fibre intake, gastrointestinal permeability leads to the translocation of symbiotic bacteria into the bloodstream (Gourbeyre et al., 2011; Sbihi et al., 2019). Upon translocation to the bloodstream, components of symbiotic bacteria such as lipopolysaccharide are labelled as pathogenic and neutralised by the immune system (Clemente et al., 2012; Ding et al., 2018; Gourbeyre et al., 2011; Hansel et al., 2013; Holgate, 2012; Male et al., 2013; Murdoch & Lloyd, 2010; Sbihi et al., 2019; Tuohy & Scott, 2014; Warrington et al., 2011). Neutralising symbiotic bacteria has the adverse effect of driving type-2 inflammation by removing bacteria capable of attenuating the severity of asthma (Arrieta et al., 2015; Konieczna et al., 2012; Sagar et al., 2014A), highlighting the importance of maintaining gastrointestinal epithelial integrity in protecting against type-2 inflammation (Josefowicz et al., 2012).

The presence and severity of gastrointestinal permeability can be assessed through systemic markers such as lipopolysaccharide binding protein (LBP). The current study intended to investigate the effects of short-term prebiotic supplementation on gastrointestinal permeability by assessing changes in systemic LBP concentrations via enzyme linked immunosorbent assay (ELISA, Hycult Biotech, HK215-02, The Netherlands). However, the ELISA kits purchased to analyse systemic LBP concentrations expired during the laboratory closures caused by the COVID-19 pandemic from March to September 2020. As a result, data exploring the effect of short-term prebiotic supplementation on systemic markers of gastrointestinal permeability were not obtained.

Although short-term prebiotic supplementation may be beneficial for adults with HIB of various activity levels (recreational-elite), the specific contraindications of reduced responsiveness to short-acting β_2 -agonists associated with tachyphylaxis and therapeutic use exemptions in elite athletes represent the most promising application to help maintain medication responsiveness and ethical competition. Future research should initially focus on more robust and larger scale investigations in recreationally active individuals. This could then potentially be expanded to elite athletes to directly investigate the potential efficacy of short-term prebiotic supplementation in this population given the strict guidelines imposed by WADA for the use of short-acting β_2 -agonists, and potential concerns around inter-individual variability in urinary salbutamol excretion and how this may contribute to adverse analytical findings (Elers et al., 2012). Supplements such as B-GOS® are

manufactured to Informed Sport standards, enabling ethical investigations in elite populations.

5.4.4 Future Directions

A minimum threshold and dose-response relationship may exist between galactooligosaccharide supplementation, and the bifidogenic response required to modulate systemic and pulmonary immune function (Davis et al., 2011; Williams et al., 2016). This is partly dependent on baseline gut bacterial composition (De Preter et al., 2008), and habitual dietary fibre intake (Healey et al., 2016; 2018). To be effective, prebiotics must be administered in the right dose. Given the minimum threshold proposed by Davis et al. (2011), and the compelling evidence of Williams et al. (2016), future research should first investigate the effects of higher dose short-term galactooligosaccharide supplementation on the severity of HIB. It will then be interesting to see whether greater therapeutic efficacy is obtained by attenuating HIB specific features of gut microbial dysbiosis through a galactooligosaccharide-based synbiotic (Arrieta et al., 2015; Halnes et al., 2017). Given the influence of baseline gut bacterial composition (De Preter et al., 2008), and habitual dietary fibre intake on gastrointestinal *Bifidobacteria* (De Filippo et al., 2010), and response to prebiotics (Healey et al., 2016; 2018), it is likely that dosing regimens may need to be adjusted for these factors alongside the severity of HIB.

5.5 Conclusion

In conclusion, short-term supplementation with a single dose of galactooligosaccharides ($3.1 \text{ g}\cdot\text{day}^{-1}$) does not attenuate the severity of HIB, or markers of systemic inflammation in recreationally active adults. Future research is first required to investigate whether adults with HIB display features of gut microbial dysbiosis. If this is the case, then probiotics specific to the features of gut microbial dysbiosis may be explored as an initial targeted intervention before potentially combining with a prebiotic to explore if synbiotic therapy leads to greater efficacy (Arrieta et al., 2015). If designed appropriately, this research may help understand what the minimal clinically important dose of probiotic, prebiotic, or synbiotic therapy might be to attenuate the severity of HIB, using knowledge of the current bifidogenic threshold proposed by Davis et al. (2011), as a starting point.

Chapter VI

The Effects of Long-Term Prebiotic Supplementation on the Severity of Hyperpnoea-Induced Bronchoconstriction: A Double-Blind, Placebo-Controlled Crossover Trial.

6.1 Introduction

In addition to short-term supplementation strategies (Halmes et al., 2017), research has investigated whether long-term prebiotic supplementation leads to greater improvements in the severity of HIB. Williams et al. (2016), investigated the effects of 3-weeks of supplementation with B-GOS[®] on the severity of HIB, and systemic markers of airway inflammation, in adults with moderate to severe HIB. Clinically relevant, participant perceivable improvements in the severity of HIB were observed post supplementation (Santanello et al., 1999; Williams et al., 2016). Strikingly, the percentage decrease in FEV₁ post-EVH was attenuated by ~ 41%, a comparable magnitude to that observed following pharmaceutical treatments (39-60%; Kippelen et al., 2010; Rundell et al., 2005; Simpson et al., 2016; Verheijden et al., 2015A; 2018; Williams et al., 2016). The improvements in pulmonary function were attributed to the attenuation of type-2 inflammatory markers. Specifically, TNF- α was completely inhibited at rest and post-EVH (Williams et al., 2016), whereas CCL17 concentrations were significantly attenuated at rest. The proof-of-concept study conducted by Williams et al. (2016), was the first to demonstrate the therapeutic efficacy of galactooligosaccharides in attenuating the severity of HIB in humans. However, further research is required to explore the mechanisms by which galactooligosaccharides attenuate the severity of HIB.

Evidence from murine models suggests that galactooligosaccharides attenuate the severity of asthma by increasing the suppressive capacity of CD25⁺ FoxP3⁺ iTREG cells (Verheijden et al., 2015B; 2018). Galactooligosaccharides attenuated the magnitude of airway eosinophilia, and the production of mMCP-1, by a comparable magnitude to that observed following budesonide therapy (Verheijden et al., 2015A; 2018; Zhang et al., 2016).

Regulatory T cells are essential to attenuating type-2 inflammation (Bohm et al., 2015; Hong et al., 2018; Maazi et al., 2018; Van der Veecken et al., 2016). However, a reduction in the number and/or suppressive capacity of T_{REG} cells has been observed in people with asthma (Chen et al., 2018; Hansel et al., 2013; Hartl et al.,

2007; Holgate, 2012; Hong et al., 2018; Josefowicz et al., 2012; Lotvall et al., 2012; Ostroukhova et al., 2004; Thorburn & Hansbro, 2010; Verheijden et al., 2015B; 2016; Warrington et al., 2011). Alarming, T_{REG} cells can adopt a T_{H2} phenotype under inflammatory conditions by decreasing their expression of CTLA-4 and IL-10, and increasing their expression of IL-4, IL-5, IL-13, and GATA-3, respectively (Chen et al., 2017; 2018). When compared to healthy individuals, FoxP3 expression is increased in T_{REG} cells from people with asthma (Chen et al., 2018; Lotvall et al., 2012), potentially reflecting increased attempts to attenuate type-2 inflammation. In contrast, the relative expression of FoxP3 is decreased compared to GATA-3, leading to decreases in pulmonary function, and increases in airway hyperresponsiveness characteristic of a T_{H2} phenotype (Chen et al., 2018; Lotvall et al., 2012).

Various T_{REG} cell subsets play critical, yet divergent roles in attenuating type-2 inflammation. For example, IL-10⁺ iT_{R1} cells directly attenuate type-2 inflammation and airway hyperresponsiveness by decreasing the recruitment of T_{H2} cells, the magnitude of airway eosinophilia, mucous hypersecretion, and the production of IL-5, IL-13, and TGF-β1, respectively (Kearley et al., 2005; 2008; Matsuda et al., 2017; 2018; 2019). In contrast, FoxP3⁺ nT_{REG} cells indirectly attenuate type-2 inflammation by maintaining the suppressive capacity of IL-10⁺ iT_{R1} cells (Bohm et al., 2015). The direct role of FoxP3⁺ T_{REG} cells in attenuating type-2 inflammation is mediated by TGF-β1 (Bohm et al., 2015; Khumalo et al., 2020). Specifically, TGF-β1 drives the chitotriosidase-1 dependent differentiation of lung derived FoxP3⁺ iT_{REG} cells (Hong et al., 2018). Despite the critical role of FoxP3⁺ iT_{REG} cells in mediating the galactooligosaccharide-induced attenuation of type-2 inflammation (Verheijden et al., 2015B; 2018), no research has investigated the immunomodulatory properties of T_{REG} cell subsets in EIB.

It is well known that T_{H2} cells play a critical role in driving type-2 inflammation. Specifically, peT_{H2} cells are more capable of driving type-2 inflammation than cT_{H2} cells, by increasing the activation of ILC2s through PGD₂-dependent mechanisms (Maric et al., 2018; Mitson-Salazar et al., 2016). In eosinophilic gastrointestinal disease, peT_{H2} cells are more abundant in the gut than cT_{H2} cells, supported by the increased expression of gut homing receptors (Mitson-Salazar et al., 2016). Whether peT_{H2} cells also demonstrate increased expression of pulmonary homing receptors remains to be determined. What is clear, is that the dual role of peT_{H2}

cells in gut and lung inflammation could underpin the gut-lung microbial axis in asthma (Budden et al., 2017; Fujimura & Lynch, 2015; Huang & Boushey, 2014; Kozik & Huang, 2019; Mitson-Salazar et al., 2016; Singanayagam et al., 2017). Despite these observations, the role of $\text{peT}_{\text{H}2}$ cells remains unexplored in EIB, let alone the effects of prebiotic supplementation.

Current evidence suggests that longer-term prebiotic supplementation is more effective than short-term supplementation in attenuating the severity of HIB (Williams et al., 2016). This is partly attributed to the higher dose administered during longer-term interventions (Davis et al., 2011; Williams et al., 2016). Davis et al. (2011), proposed that a minimum dose of 10 g/day^{-1} of galactooligosaccharides was required to induce a bifidogenic effect. However, research in other galactooligosaccharides suggests that bifidogenic responses may be achieved from lower doses (Depeint et al., 2008), suggesting that the dose required is partly dependent on the supplement administered. Recently, the development of novel prebiotics with reduced impurities (Grimaldi et al., 2016), have led researchers to question whether similar therapeutic effects can be obtained with lower doses.

As outlined in Chapter V, although short-term supplementation with a novel prebiotic did not attenuate the severity of HIB, it remains to be determined whether longer supplementation periods are required, such as those used by previous research (Williams et al., 2016), to cause clinically relevant, participant perceivable improvements.

Taken together, these results suggest that further research is required to establish the minimal, clinically important dose and duration of galactooligosaccharide supplementation required to attenuate the severity of HIB. As a result, the primary aim of the current study was to investigate the effects of longer-term supplementation with a novel prebiotic on the severity of HIB, exploring the potential mechanisms by which T_{REG} cells inhibit type-2 inflammation.

6.2 Methods

6.2.1 Experimental Design

This study was conducted following a double-blind, placebo-controlled, crossover design. Ethical approval was obtained from Nottingham Trent Universities Human Research Ethics Committee (approval number: 484), and the NHS East Midlands

Nottingham 2 Research Ethics Committee (approval number: 17/EM/0369). All protocols were conducted in accordance with Good Clinical Practice, the Declaration of Helsinki (World Medical Association, 2013), and the British Association of Sport and Exercise Sciences Code of Conduct (British Association of Sport and Exercise Sciences, 2017). The study was conducted from November 2017 to March 2019, and registered as a clinical trial in the ClinicalTrials.Gov database (Identifier: NCT02872675).

6.2.2 Participants

Recreationally active adults with ($n = 9$), and without hyperpnoea (exercise)-induced bronchoconstriction ($n = 8$) were recruited from Nottingham Trent University. Thirteen ($n = 13$) were male ($n = 8$ in the HIB group, $n = 5$ in the control group), and 4 were female ($n = 1$ in the HIB group, $n = 3$ in the control group). Written informed consent was obtained prior to any procedures taking place (British Association of Sport and Exercise Sciences, 2013). In accordance with Good Clinical Practice, written informed consent was reassessed at each visit. All participants completed a health screen and history questionnaire prior to taking part. A detailed overview of the eligibility criteria is provided in the general methods section (Chapter III, pages 124-126). All participants attended the laboratory on six occasions over a 12-week period. All trials were conducted at the same time of day to control for the diurnal effect on pulmonary function (Rhee & Kim, 2015). The first visit was an initial consultation, the second visit a familiarisation trial, and the third to sixth visits the main experimental trials.

Participants were assigned to the HIB or control group based on the EVH protocol completed during the familiarisation trial. Participants in the HIB group who had a physician's diagnosis of asthma ($n = 6$), were classed as Step 1 ($n = 5$), or Step 2 ($n = 1$) according to the British Thoracic Society and Scottish Intercollegiate Guidelines Network (British Thoracic Society & Scottish Intercollegiate Guidelines Network, 2016). Participants continued to take their usual asthma medication throughout the intervention. Two ($n = 2$) were prescribed a short-acting β_2 -agonist, two ($n = 2$) were prescribed a short-acting β_2 -agonist plus a low dose inhaled corticosteroid, and two ($n = 2$) were prescribed a short-acting β_2 -agonist plus a combined inhaler containing a low dose inhaled corticosteroid and a long-acting β_2 -agonist. The remaining participants in the HIB group ($n = 3$) did not have a physician's diagnosis of asthma.

6.2.3 Measurements

6.2.3.1 Pulmonary Function & The EVH Protocol

During the familiarisation trial, participants were screened for HIB via the EVH protocol following the same protocol outlined in Chapters 3-5. Participants were excluded from the study if resting FEV₁ values were < 70% of percentage predicted values. Assessments of pulmonary function and the EVH protocol were repeated during all experimental trials.

6.2.3.2 Markers of Systemic Inflammation

During experimental trials, at rest, a 4mL blood sample was collected into a vacutainer tube containing EDTA (Brooks, 2014; Calfee & Farr, 2002; World Health Organisation, 2010). White blood cell subsets were quantified fresh in whole blood using an automated haematology analyser (Sysmex, XS-1000i™, United States). Blood samples were analysed for absolute counts of total white blood cells, eosinophils and basophils ($\times 10^9/L$), as well as the relative abundance (%) of eosinophils and basophils, respectively.

At rest, a 30mL blood sample was also collected into 3 \times 10mL vacutainer tubes containing lithium heparin to analyse T_{REG}, T_{H2}, and T_{H1} cells. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood via density gradient centrifugation, and stored in liquid nitrogen until analysis by flow cytometry. A detailed overview of the protocol used to isolate and analyse PBMCs is provided in the general methods section (Chapter III, pages 135-145). An overview of the markers used to characterise regulatory T cells and effector T cells is outlined in Figure 6.0 below.

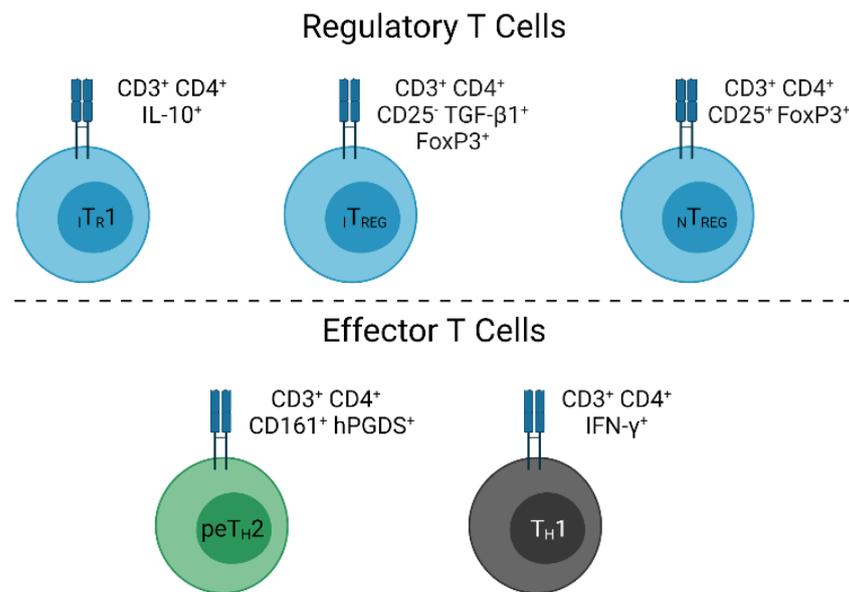


Figure 6.0 An overview of the markers used to characterise regulatory T cells and effector T cells via flow cytometry. *i*T_{R1} type-1 IL-10 producing regulatory T cell, *i*T_{REG} induced regulatory T cell, *N*T_{REG} natural regulatory T cell, IL-10 interleukin-10, TGF-β1 transforming growth factor beta-one, FoxP3 fork-head box protein 3, peT_{H2} pathogenic effector T-helper type-2 cell, T_{H1} T-helper type-1 cell, hPGDS haematopoietic prostaglandin D synthase, IFN-γ interferon gamma. Created with www.BioRender.com.

The samples were initially analysed by the PhD student for the total number of lymphocytes by visualising the data on a density plot. Forward scatter was plotted on the x axis and side scatter on the y axis, both presented using a linear scale. As outlined in Figure 6.1, this enabled the lymphocytes (gated population) to be distinguished from the debris in the sample (lower left corner) and monocytes (above the gated population).

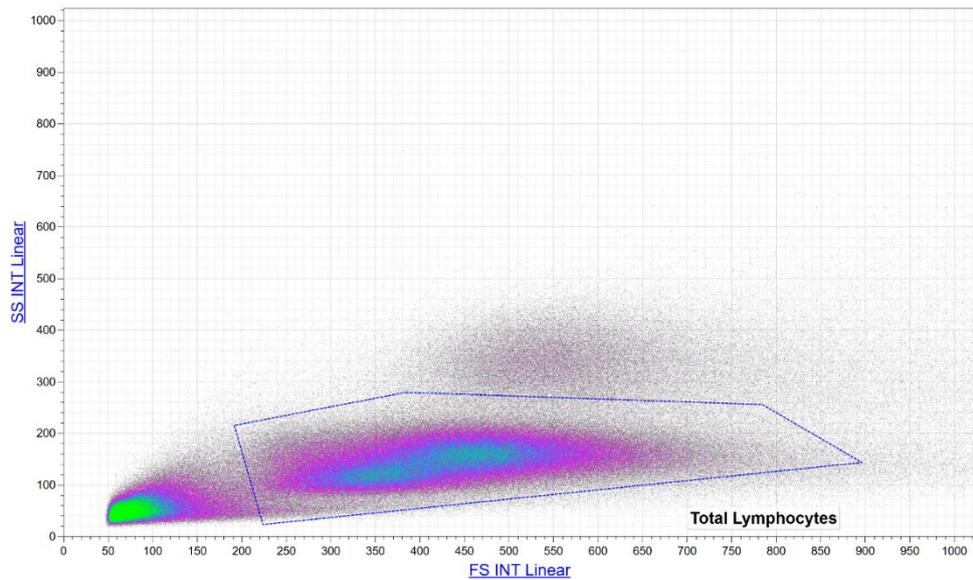


Figure 6.1 Representative example of the protocol used to identify the total number of lymphocytes from the isolated PBMC's. *PBMC's* peripheral blood mononuclear cells.

Viable lymphocytes were then identified within the total lymphocyte population by presenting the live-dead antibody on the x axis using a logarithmic scale, and side scatter on the y axis using a linear scale (Figure 6.2).

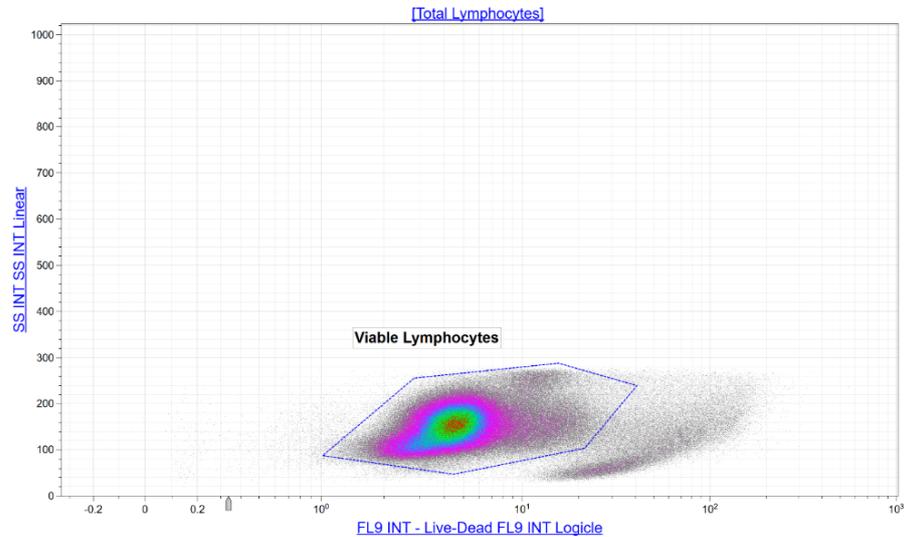


Figure 6.2 Representative example of the protocol used to identify viable lymphocytes within the total lymphocyte population.

T cells were then identified within the viable lymphocyte population by presenting the CD4 antibody on the x axis, and CD3 antibody on the y axis, both using a logarithmic scale. As outlined in Figure 6.3 below, the populations of cells to the left

of the gated region demonstrate high fluorescence for CD3, but not CD4, hence the gated region only includes cells with high fluorescence for both markers.

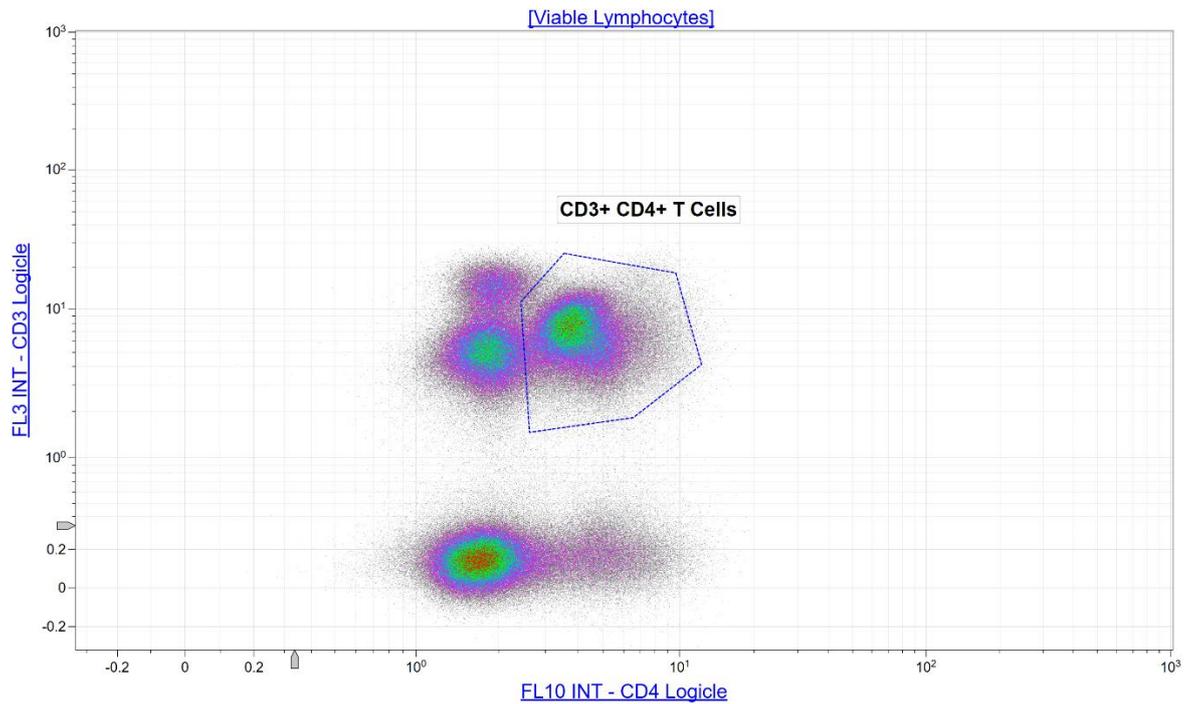


Figure 6.3 Representative example of the protocol used to identify CD3⁺ CD4⁺ T cells from the viable lymphocyte population.

Once the CD3⁺ CD4⁺ T cells had been identified, it was possible to characterise regulatory and effector T cell subsets within this population. However, peT_{H2} cells could not be identified based on the dual expression of hPGDS and CD161. Furthermore, IFN- γ alone was insufficient to identify T_{H1} cells within the CD3⁺ CD4⁺ T cell population. nT_{REG} cells could be identified from the CD3⁺ CD4⁺ T cell population based on the dual expression of CD25 and FoxP3 (as outlined in Figure 6.4). However, iT_{REG} cells that express FoxP3, but not CD25, could not be identified.

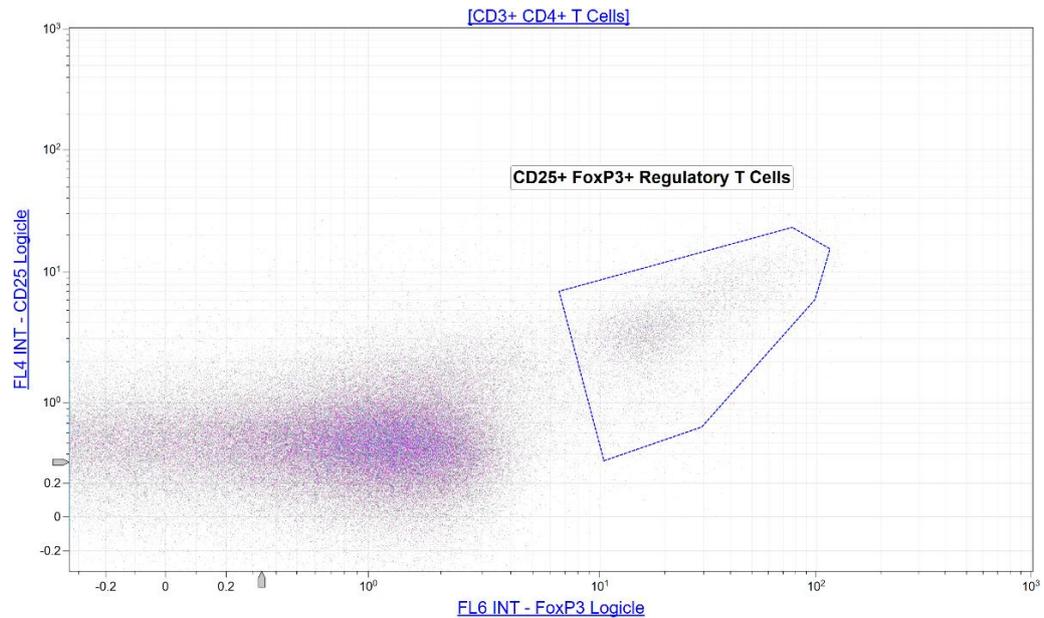


Figure 6.4 Representative example of the protocol used to identify CD25⁺ FoxP3⁺ nT_{REG} cells from the CD3⁺ CD4⁺ T cell population. nT_{REG} natural (thymically derived) regulatory T cells.

Using the CD3⁺ CD4⁺ T cell population as a baseline, the number of iT_{R1} cells was identified by assessing the expression of IL-10 (Figure 6.5). Those cells that demonstrated fluorescence for IL-10, but not FoxP3 (top left rectangle) were classified as CD3⁺ CD4⁺ IL-10⁺ iT_{R1} cells.

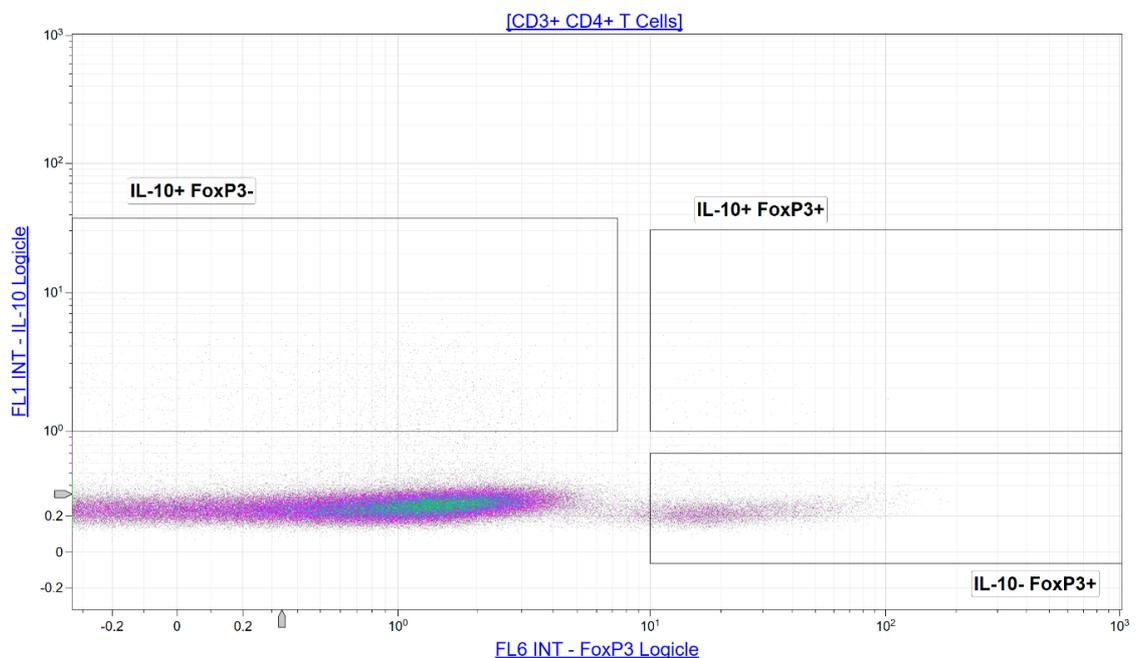


Figure 6.5 Representative example of the protocol used to identify IL-10⁺ iT_{R1} cells from the CD3⁺ CD4⁺ T cell population. iT_{R1} Type-1 IL-10 producing regulatory T cells.

6.2.3.3 Nutritional Intake

To standardise nutritional intake prior to each experimental trial, participants were asked to complete a 24-hour weighed nutritional intake record before the first experimental trial which was replicated before experimental trials 2-4 (Appendix L). Data for total calories, fat, carbohydrate, protein, and fibre were analysed using specialist nutritional software (Nutrium, Version 2.149.0, Portugal).

6.2.3.4 The Nutritional Intervention

The nutritional intervention was conducted following a double-blind, placebo-controlled, crossover design. The intervention consisted of two, 4-week supplementation phases, each separated by a 2-week washout period (Davis et al., 2011; Depeint et al., 2008; Vulevic et al., 2015; Williams et al., 2016). Participants completed four experimental trials, one at the start, and one at the end of each supplementation phase, respectively. During each experimental trial, at rest, participants provided a blood sample (34mL), and completed the EVH protocol. During the first supplementation phase, participants were administered a galactooligosaccharide-based prebiotic ($3.1 \text{ g}\cdot\text{day}^{-1}$, Host Therabiomics, HOST-DM059, Jersey, The Channel Islands), or a taste and appearance matched placebo. During the second supplementation phase, participants were administered the remaining supplement. One prebiotic sachet weighed 3.6g and contained up to 85% galactooligosaccharides (3.1g), 6% water (0.22g), 6% glucose (0.22g), 4% galactose (0.14g), and 16% lactose (0.58g), respectively.

Participants were allocated to receive the prebiotic or placebo during the first supplementation phase using a 1:1 allocation ratio to control for any order effects (Vulevic et al., 2015). For the prebiotic and placebo, participants were instructed to take one sachet per day in the morning, and to reconstitute the sachet in a hot drink or fruit smoothie to help it dissolve. The supplements were blinded by HOST Therabiomics, and provided in opaque sachets labelled as “X” and “L”, respectively. Adverse events were prospectively monitored in accordance with USFDA guidelines. In the HIB group, in participants with asthma, adherence to maintenance medication and study supplements was prospectively monitored using a modified version of the MARS-A[®] completed at the end of each week throughout the nutritional intervention (Appendix I; Cohen et al., 2009). In participants without asthma (in the HIB group and control group) adherence to study supplements was

prospectively monitored with the same questionnaire, but without the MARS-A[®]. Participants had to maintain at least 80% adherence during both supplementation phases to be included in the final analysis. Unblinding was completed when the final participant completed their final experimental trial. A schematic overview of the nutritional intervention is provided in Figure 6.6 below.

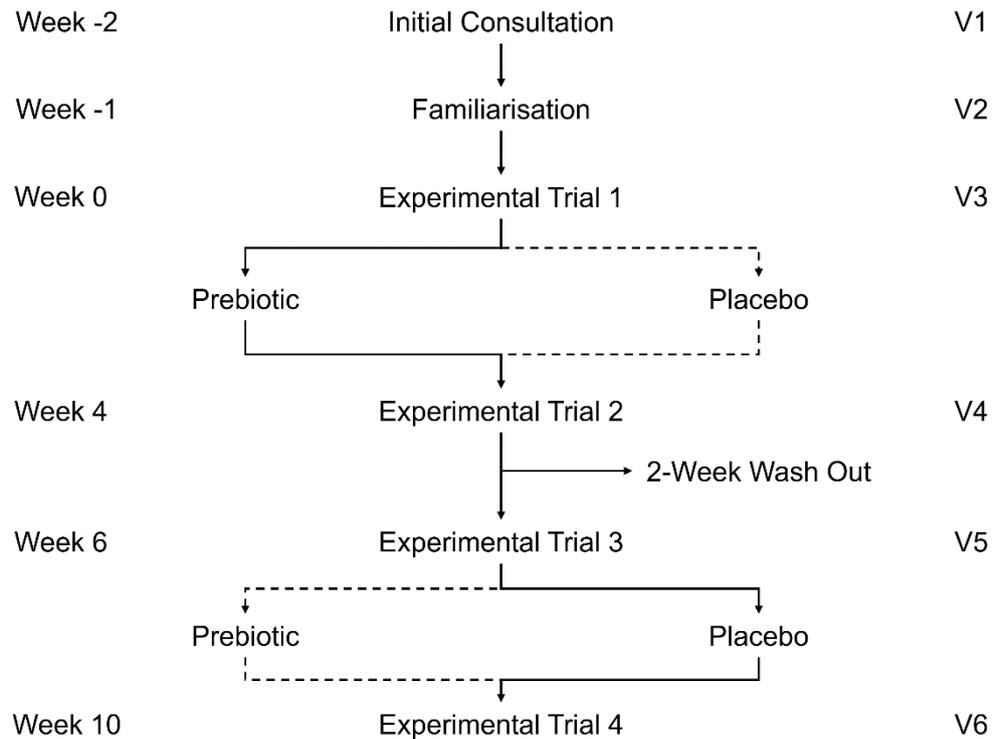


Figure 6.6 A schematic overview of the nutritional intervention. Solid and dashed arrows indicate participant flow through the intervention depending on the supplement administered during the first supplementation phase. “V” refers to visit number.

6.2.4 Statistical Analysis

An estimation of sample size was conducted using an online calculator based on an α priori level of 0.05 (two-sided; MGH Biostatistics Centre, Harvard, Massachusetts). Based on a within-subject’s standard deviation of 1.0, associated power of 0.9 ($P\beta$), and a minimal detectable difference in the expression of FoxP3 by CD3⁺ CD4⁺ CD25^{+/-} TGF- β 1^{+/-} T_{REG} cells of 1.05%, from baseline to post intervention, an estimated sample size of twenty two ($n = 22$) was calculated in the HIB group, with a 91% probability that a significant difference would be detected at the specified α level. When adjusted for the dropout rate observed by Williams et al. (2016; 18.2%), an adjusted sample size of twenty-six ($n = 26$) was calculated in the HIB group. The target sample size for the control group was twelve ($n = 12$), in

accordance with the control group recruited by Konieczna et al. (2012), leading to a total of thirty-eight ($n = 38$) participants.

Konieczna et al. (2012), assessed changes in the expression of FoxP3 in CD4⁺ CD25⁺ T_{REG} cells in healthy adults ($n = 10$) administered 8-weeks of probiotic supplementation (*Bifidobacterium infantis* 35624, 1×10^9 live bacteria per day). No prebiotic supplementation trials have been conducted assessing changes in the expression of FoxP3 in T_{REG} cells from adults with HIB. Although Konieczna et al. (2012), administered a probiotic intervention to healthy adults, the target of their probiotic intervention (*Bifidobacterium infantis*) is similar to the target of the current prebiotic intervention (*Bifidobacterium bifidum*; Depeint et al., 2008). As a result, the research of Konieczna et al. (2012), provided the most relevant information to estimate the sample size required to detect meaningful changes in systemic immune function in adults with HIB following prebiotic supplementation.

As outlined in study two (Chapter V), a sample size of seven ($n = 7$) was required to detect participant perceivable changes in the peak decrease in FEV₁ post-EVH (Williams et al., 2015; 2016). Basing the estimation of sample size on the number of participants required to detect meaningful changes in systemic immune function (Konieczna et al., 2012), ensured the current study would be sufficiently powered to also detect meaningful changes in pulmonary function.

Statistical analysis was conducted using the statistical package for the social sciences (IBM SPSS Inc, Version 26, Chicago IL, USA). The Shapiro-Wilk test was used to assess normality (Field, 2018). If deviations from normality were present, equivalent non-parametric tests were used where possible (Field, 2018). A series of mixed ANOVA's with Bonferroni post-hoc correction were used to assess changes between the HIB and control groups across the prebiotic and placebo conditions from baseline to post-intervention (condition \times time \times group; Field, 2018). Estimates of effect size were calculated according to Field, (2018), based on the F statistic and error value for degrees of freedom from the tests of within-subject contracts:

$$\text{Effect Size} = \sqrt{F \div F + DF (\text{Error})} \quad (\text{Eq. 3.3})$$

Across all studies, statistically significant differences were accepted if $p < 0.05$ (two-tailed). Estimates of effect size were classed as small (0.2-0.5), medium (0.5-0.8), or large (> 0.8 ; Field, 2018). Values are reported as mean \pm standard deviation for parametric data, and median plus interquartile range for non-parametric data (IQR; 25th – 75th percentile). Figures demonstrating mechanisms or protocols were created using Microsoft PowerPoint or Biorender (Toronto, Ontario, Canada). Figures reporting experimental data were created using GraphPad Prism Software (Version 8.0.1, San Diego, CA, USA). The protocol for the experimental trials is outlined in Figure 6.7 below.

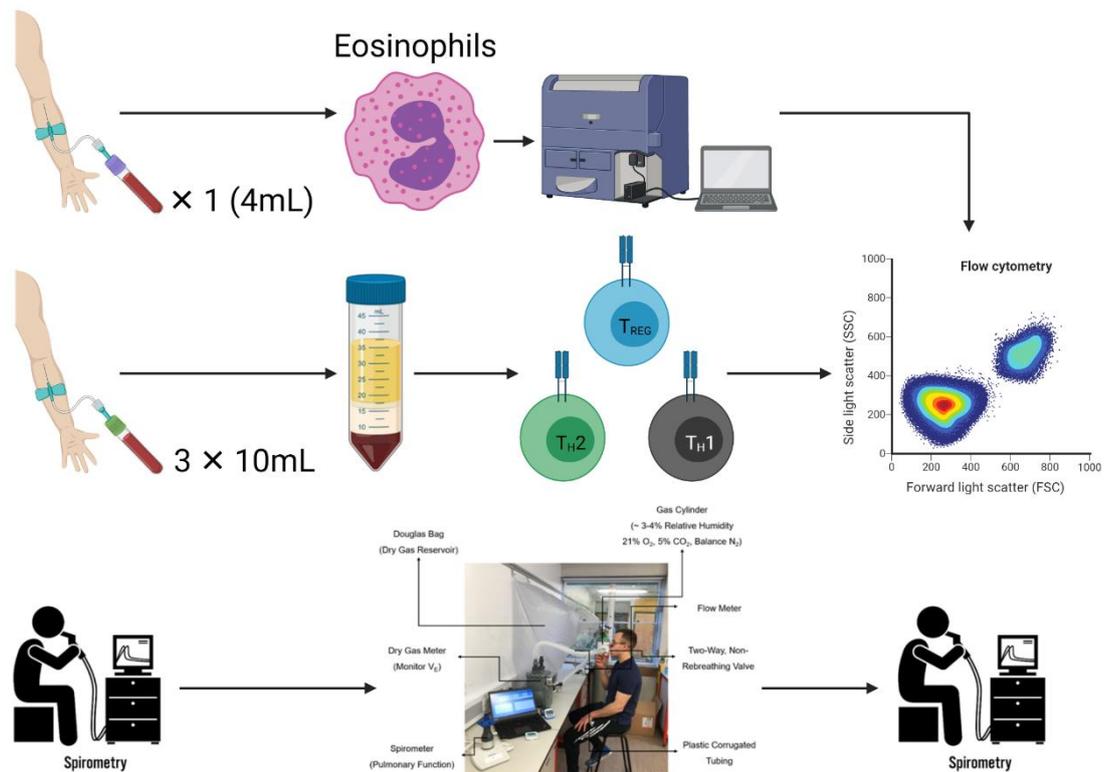


Figure 6.7 An overview of the protocol for the experimental trials. Created with www.BioRender.com. T_{REG} regulatory T cell, T_{H2} T-helper type-2 cell, T_{H1} T-helper type-1 cell.

6.3 Results

A total of seventeen ($n = 17$) participants completed the study, nine in the HIB group ($n = 9$), and eight in the control group ($n = 8$). Adherence to the prebiotic phase of the nutritional intervention was $95 \pm 6\%$ in the HIB group, and $99 \pm 1\%$ in the control group, whereas adherence to the placebo phase was the same for both groups ($98 \pm 4\%$). Adherence across both phases for all participants was above the minimum adherence criteria of 80% to be included in the analysis. An overview of participant recruitment and study completion is outlined in Figure 6.8 below, with baseline descriptive characteristics outlined in Table 6.0.

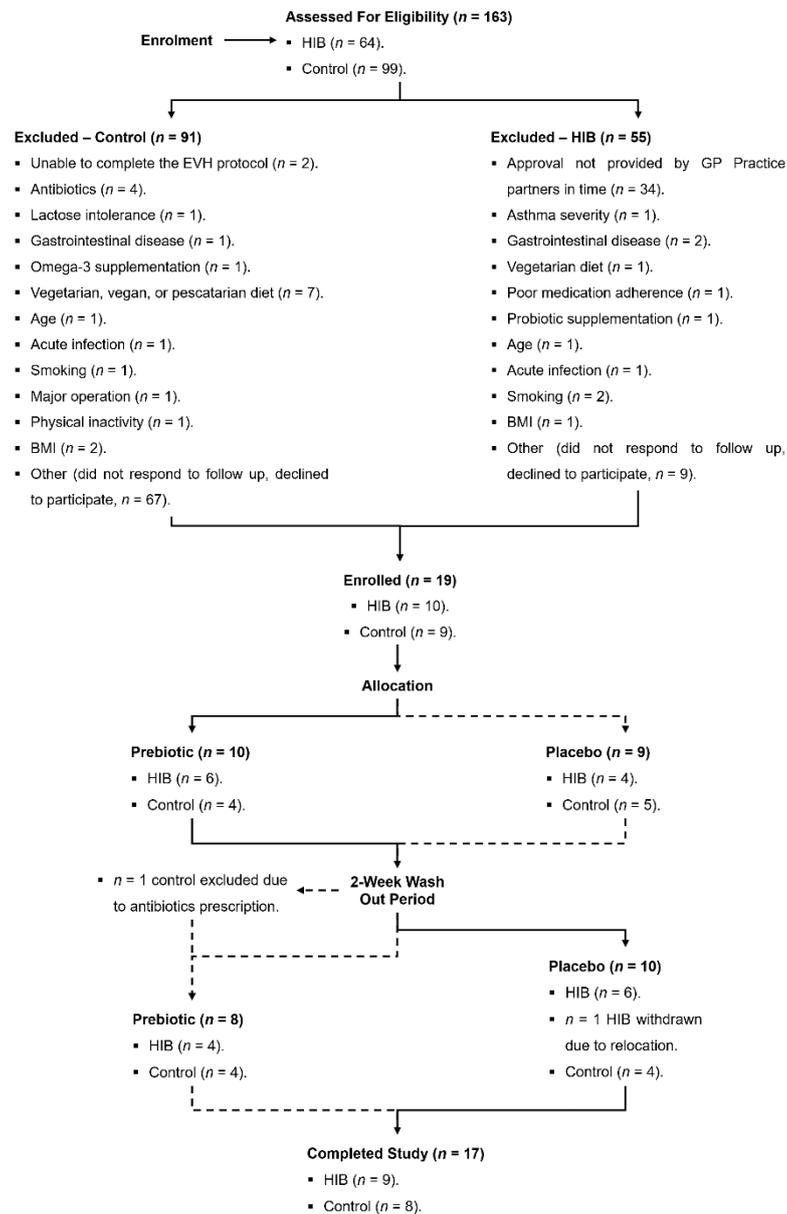


Figure 6.8 An overview of participant recruitment and study completion. Solid and dashed arrows indicate participant flow through the intervention depending on the supplement administered during the first supplementation phase.

Table 6.0 Descriptive characteristics of the HIB and control groups.

	HIB (n = 9)	Control (n = 8)	p
Age (years)	24 ± 5	22 ± 3	0.349
Height (m)	1.78 ± 0.09	1.75 ± 0.12	0.614
Body Mass (kg)	76.4 ± 13.2	67.9 ± 7.3	0.066
BMI (kg/m ²)	24 ± 2	22.2 ± 2	0.104

Values are presented as mean ± SD. Data for height, body mass and BMI are presented for eight participants in the HIB group ($n = 8$). *HIB* hyperpnoea-induced bronchoconstriction, *m* metres, *kg* kilograms.

For descriptive characteristics, no differences were reported between groups across conditions for height (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 0.3$, $p = 0.594$, $r = 0.14$), body mass (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 1.66$, $p = 0.218$, $r = 0.33$), or BMI (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 1.33$, $p = 0.268$, $r = 0.29$). In addition, no differences were reported for laboratory temperature (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 0.04$, $p = 0.843$, $r = 0.05$), or humidity (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 1.95$, $p = 0.183$, $r = 0.34$). In contrast, a time × group interaction was reported for barometric pressure (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 5.66$, $p = 0.031$, $r = 0.52$). For barometric pressure, a main effect of time was observed in the control group (Greenhouse-Geisser $\epsilon = 0.664$; $F(1.99, 13.94) = 5.45$, $p = 0.018$, $r = 0.53$) due to a significant difference between baseline in the placebo condition and post-intervention in the prebiotic condition ($p = 0.012$).

6.3.1 Pulmonary Function

When expressed as relative values, a main effect of time was reported for accuracy of the spirometer (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 9.11$, $p = 0.009$, $r = 0.61$), but post hoc analyses did not reveal any differences between or within groups. When expressed as absolute values, no differences were reported for accuracy of the spirometer (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 4.15$, $p = 0.06$, $r = 0.47$), or the dry gas meter (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 0.05$, $p = 0.825$, $r = 0.06$). The accuracy values for the spirometer were within accepted ranges across all experimental trials (Miller et al., 2005).

Table 6.1 Changes in resting and post-EVH pulmonary function throughout the nutritional intervention in the HIB and control groups.

	Resting							
	HIB (<i>n</i> = 9)				Control (<i>n</i> = 8)			
	B-Placebo	PI-Placebo	B-Prebiotic	PI-Prebiotic	B-Placebo	PI-Placebo	B-Prebiotic	PI-Prebiotic
FEV ₁ (L)	4.16 ± 0.76	4.16 ± 0.78	4.09 ± 0.70	4.19 ± 0.73	4.03 ± 0.97	4.09 ± 1.00	4.03 ± 1.06	4.04 ± 1.03
FVC (L)	5.29 ± 0.87	5.28 ± 0.89	5.26 ± 0.80	5.30 ± 0.82	4.86 ± 1.08	4.92 ± 1.12	4.90 ± 1.15	4.85 ± 1.09
PEF (l·s ⁻¹)	9.89 ± 1.60	9.79 ± 1.74	9.69 ± 1.61	9.69 ± 1.52	9.18 ± 2.06	9.56 ± 1.89	9.12 ± 1.69	9.61 ± 1.86
FEF _{25%-75%} (l·s ⁻¹)	3.69 ± 1.20	3.79 ± 1.25	3.76 ± 1.14	3.75 ± 1.26	4.17 ± 1.73	4.37 ± 1.44	4.14 ± 1.61	4.24 ± 1.72
	Post-EVH							
FEV ₁ (L)	3.38 ± 0.74	3.32 ± 0.72	3.21 ± 0.76	3.26 ± 0.74	3.74 ± 0.88	3.81 ± 0.99	3.64 ± 0.98	3.66 ± 1.00
FVC (L)	4.77 ± 0.82	4.74 ± 0.77	4.69 ± 0.76	4.72 ± 0.72	4.58 ± 1.01	4.60 ± 1.08	4.47 ± 1.06	4.46 ± 1.06
PEF (l·s ⁻¹)	7.57 ± 1.47	7.42 ± 1.38	7.30 ± 1.43	7.26 ± 1.31	7.80 ± 1.76	8.30 ± 1.66	7.50 ± 1.39	7.94 ± 1.86
FEF _{25%-75%} (l·s ⁻¹)	2.42 ± 0.82	2.34 ± 0.88	2.13 ± 0.93	2.27 ± 0.95	3.49 ± 1.29	3.52 ± 1.35	3.23 ± 1.25	3.41 ± 1.46

EVH eucapnic voluntary hyperpnoea, *FEV₁* forced expiratory volume in one second, *FVC* forced vital capacity, *PEF* peak expiratory flow rate, *FEF_{25%-75%}* forced expiratory flow rate between 25%-75% of FVC.

Table 6.2 Changes in the peak decrease in FEV₁ post-EVH throughout the nutritional intervention in the HIB group (expressed as a percentage decrease from baseline).

Participant	B-Placebo	PI-Placebo	B-Prebiotic	PI-Prebiotic
1	-17.0	-15.5	-15.6	-13.2
2	-5.6	-10.1	-13.6	-11.0
3	-7.4	-13.3	-9.3	-13.1
4^A	-24.3	-45.1	-45.0	-32.4
5^A	-20.8	-22.2	-25.9	-35.1
6^A	-14.8	-13.3	-10.9	-18.5
7^A	-38.7	-29.1	-37.2	-39.1
8^A	-24.5	-23.1	-19.1	-18.8
9^A	-16.3	-7.1	-17.6	-19.3

^ARefers to participants who had a prior diagnosis of asthma before taking part in the study.

EVH eucapnic voluntary hyperpnoea. A cut-off value of a 13% drop in FEV₁ post EVH was considered diagnostic of EIB (Godfrey et al., 1999).

Participant 2 showed a non-diagnostic response to the EVH protocol during 3 out of 4 experimental visits across the placebo and prebiotic conditions.

Participant 3 showed a non-diagnostic response to the EVH protocol during 2 out of 4 experimental visits across the placebo and prebiotic conditions. In both cases this was observed post-intervention.

Participant 6 showed a non-diagnostic response to the EVH protocol during 1 out of 4 experimental visits at baseline during the prebiotic condition (but not post-intervention).

Participant 9 showed a non-diagnostic response to the EVH protocol during 1 out of 4 experimental visits post-intervention during the placebo condition.

Table 6.3 Changes in the peak decrease in FEV₁ post-EVH throughout the nutritional intervention in the control group (expressed as a percentage decrease from baseline).

Participant	B-Placebo	PI-Placebo	B-Prebiotic	PI-Prebiotic
1	-5.3	-9.0	-10.6	-4.2
2	-8.7	-11.7	-12.5	-23.4
3	-9.8	-12.0	-11.9	-7.2
4	-7.7	-3.5	-7.3	-12.5
5	-10.5	-8.7	-10.4	-8.7
6	-3.1	-2.0	-9.0	-10.2
7	-4.7	-5.0	-8.8	-9.3
8	-7.8	-6.9	-8.7	-3.0

EVH eucapnic voluntary hyperpnoea. A cut-off value of a 13% drop in FEV₁ post EVH was considered diagnostic of EIB (Godfrey et al., 1999).

6.3.1.1 Forced Expiratory Volume in One Second (FEV₁)

No differences were reported for resting FEV₁ between groups across conditions when expressed as absolute values (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 1.9$, $p = 0.189$, $r = 0.33$), or percentage predicted values (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 1.26$, $p = 0.280$, $r = 0.29$). In contrast, when expressed as relative values, a main effect of condition was reported for the percentage decrease in FEV₁ post-EVH (Figure 6.9, Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 6.85$, $p = 0.019$, $r = 0.56$). The decrease in FEV₁ post-EVH was higher in the HIB group compared to the control group at all time points throughout the nutritional intervention, including baseline in the placebo condition (19 ± 10 vs. $7 \pm 3\%$; 11.62 , BCa 95% CI's [5.82, 17.58], $t(9.2) = 3.38$, $p = 0.008$, $d = 4$), post-intervention in the placebo condition (20 ± 12 vs. $7 \pm 4\%$; 12.52 , BCa 95% CI's [5.36, 21.37], $t(9.7) = 3.04$, $p = 0.013$, $d = 3.25$), baseline in the prebiotic condition (22 ± 12 vs. $10 \pm 2\%$; 11.67 , BCa 95% CI's [4.62, 20.36], $t(8.4) = 2.83$, $p = 0.021$, $d = 6$), and post-intervention in the prebiotic condition (22 ± 10 vs. $10 \pm 6\%$, 12.46 , BCa 95% CI's [4, 20.29], $t(15) = 2.92$, $p = 0.011$, $d = 2$). There was no effect of condition on FEV₁ in either group throughout the nutritional intervention.

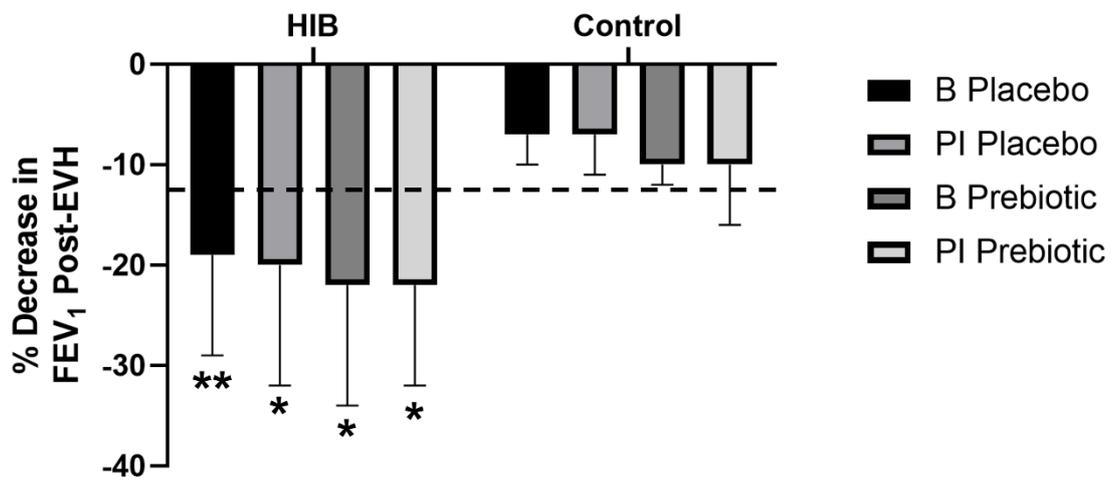


Figure 6.9 The percentage decrease in FEV₁ post-EVH was significantly greater in the HIB group compared to the control group throughout the nutritional intervention. FEV₁ forced expiratory volume in one second, EVH eucapnic voluntary hyperpnoea. B Placebo baseline placebo, PI Placebo post-intervention placebo. * $p < 0.05$ compared to control, ** $p < 0.01$ compared to control ($n = 9$ in the HIB group, $n = 8$ in the control group). Dashed line indicates diagnostic threshold. Data are presented as mean \pm SD.

In accordance with relative values, a main effect of condition was reported for the decrease in FEV₁ post-EVH when expressed as absolute values (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 5.44$, $p = 0.034$, $r = 0.52$). The decrease in FEV₁ post-EVH was higher in the HIB group compared to the placebo group at all time points throughout the nutritional intervention, including baseline in the placebo condition (0.78 ± 0.41 vs. 0.29 ± 0.15 litres; -0.48 , BCa 95% CI's $[-0.77, -0.19]$, $t(10.3) = 3.29$, $p = 0.008$, $d = 3.27$), post-intervention in the placebo condition (0.84 ± 0.53 vs. 0.29 ± 0.13 litres; -0.56 , BCa 95% CI's $[-0.96, -0.2]$, $t(9.1) = -3.06$, $p = 0.013$, $d = 4.23$), baseline in the prebiotic condition (median, IQR: 0.66 , $[0.52, 1.26]$ vs. 0.38 , $[0.32, 0.41]$ litres), $U = 62.5$, $p = 0.008$, $r = 0.62$), and post-intervention in the prebiotic condition (median, IQR: 0.74 , $[0.53, 1.42]$ vs. 0.44 , $[0.15, 0.53]$ litres), $U = 64.5$, $p = 0.004$, $r = 0.67$). No differences were reported within either group throughout the nutritional intervention.

6.3.1.2 Forced Vital Capacity (FVC)

No differences were reported for resting FVC between groups across conditions when expressed as absolute values (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 1.62$, $p = 0.223$, $r = 0.31$), or percentage predicted values (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 2.53$, $p = 0.132$, $r = 0.38$). In addition, no differences were reported in the post-EVH decrease in FVC when expressed as relative values (percentage decrease; Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 0.13$, $p = 0.721$, $r = 0.09$), or absolute values (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 0.07$, $p = 0.8$, $r = 0.07$).

6.3.1.3 Peak Expiratory Flow

No differences were reported for resting PEF between groups across conditions when expressed as absolute values (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 0$, $p = 0.968$, $r = 0.01$), or percentage predicted values (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 1.02$, $p = 0.329$, $r = 0.26$). In addition, when expressed as relative values, no differences were reported in the post-EVH decrease in PEF between groups across conditions (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 0.1$, $p = 0.760$, $r = 0.08$). No differences were reported within either group throughout the nutritional intervention.

6.3.1.4 Forced Expiratory Flow Rate Between 25% and 75% of FVC (FEF_{25%-75%})

No differences were reported for resting FEF_{25%-75%} between groups across conditions when expressed as absolute values (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 0$, $p = 0.978$, $r = 0.01$), or percentage predicted values (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 0.1$, $p = 0.759$, $r = 0.08$). In contrast, when expressed as relative values, a main effect of condition was reported for the percentage decrease in FEF_{25%-75%} post-EVH (Greenhouse-Geisser $\epsilon = 1$; $F(1, 15) = 6.79$, $p = 0.02$, $r = 0.56$). The decrease in FEF_{25%-75%} post-EVH was higher in the HIB group compared to the placebo group at all time points throughout the nutritional intervention, including baseline in the placebo condition (median, IQR: -38.6, [-44.1, -23.7] vs. -14.1, [-15.3, -11.1%], $U = 8$, $p = 0.006$, $r = -0.65$), post-intervention in the placebo condition (median, IQR: -38.3, [-53.9, -24.2] vs. -17.8, [-22.3, -14.6%], $U = 9$, $p = 0.008$, $r = -0.63$), baseline in the prebiotic condition (-43 ± 17 vs. $-22 \pm 5\%$; 21.37, BCa 95% CI's [11.5, 32.48], $t(9.6) = 3.54$, $p = 0.006$, $d = 4.2$), and post-intervention in the prebiotic condition (-40 ± 12 vs. $-20 \pm 8\%$; 20, BCa 95% CI's [9.61, 31.46], $t(15) = 4.07$, $p = 0.001$, $d = 2.5$). No differences were reported within either group throughout the nutritional intervention.

6.3.1.5 Minute Ventilation (\dot{V}_E)

During the EVH protocol, participants were asked to maintain their \dot{V}_E at $30 \times$ their highest resting FEV₁ (Anderson et al., 2001). An overview of the actual \dot{V}_E achieved in the HIB and control groups throughout the nutrition intervention is outlined in Table 6.1 below. All participants in both groups maintained their average $\dot{V}_E \geq$ the minimum $21 \times$ highest resting FEV₁ criteria used to indicate a valid test during each experimental trial (Anderson & Kippelen, 2013).

Table 6.1 The actual \dot{V}_E achieved during the EVH protocol in the HIB and control groups throughout the nutrition intervention, expressed as \times highest resting FEV₁, and l·min⁻¹.

	Baseline Placebo	Post-Intervention Placebo	Baseline Prebiotic	Post-Intervention Prebiotic
\times highest resting FEV₁				
HIB	27 ± 4 (21-32)	28 ± 3 (24-33)	28 ± 4 (24-35)	28 ± 3 (24-33)
Control	27 ± 3 (22-31)	27 ± 3 (21-31)	28 ± 4 (21-33)	27 ± 2 (22-30)
l·min⁻¹				
HIB	112.4 ± 15.9	114.3 ± 15.0	112.7 ± 14.1	117.1 ± 15.6
Control	109.8 ± 30.4	112.2 ± 31.6	111.1 ± 28.5	109.4 ± 30.8

Data are presented as mean ± SD (range) for \times highest resting FEV₁, and mean ± SD for l·min⁻¹. FEV₁ forced expiratory volume in one second.

6.3.2 Markers of Systemic Inflammation

6.3.2.1 White Blood Cells

Quality Control Samples

For quality control samples, when expressed as absolute values, no differences were reported between groups across conditions for white blood cells (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 2.27$, $p = 0.154$, $r = 0.37$), or eosinophils (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 0.81$, $p = 0.385$, $r = 0.23$). In contrast, a main effect of time \times group was reported for basophils (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 8.91$, $p = 0.01$, $r = 0.62$). However, post-hoc analyses revealed no significant differences in basophils based on time or group ($p > 0.05$). When expressed as relative values, no differences were reported for eosinophils (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 1.07$, $p = 0.318$, $r = 0.27$), whereas a main effect of time \times group was reported for basophils (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 6.97$, $p = 0.019$, $r = 0.58$). However, post-hoc analyses revealed no significant differences in basophils based on time or group ($p > 0.05$).

Experimental Samples

For experimental samples, when expressed as absolute values, no interaction effects were reported for white blood cells (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 3.05$, $p = 0.102$, $r = 0.42$), eosinophils (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 0.55$, $p = 0.471$, $r = 0.19$), or basophils (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 0.06$, $p =$

0.816, $r = 0.06$). In accordance with these findings, no differences were reported for eosinophils (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 0.1$, $p = 0.758$, $r = 0.08$), or basophils when expressed as relative values (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 0.27$, $p = 0.610$, $r = 0.14$). Furthermore, no main effects were reported for time, group, or condition for white blood cells, eosinophils, or basophils, respectively ($p > 0.05$).

Table 6.5 Changes in resting markers of systemic inflammation throughout the nutritional intervention in the HIB and control groups.

	HIB				Control			
	B-Placebo	PI-Placebo	B-Prebiotic	PI-Prebiotic	B-Placebo	PI-Placebo	B-Prebiotic	PI-Prebiotic
WBC ($\times 10^9/L$)	4.96 \pm 1.05	5.54 \pm 1.23	5.46 \pm 1.35	5.53 \pm 1.35	5.12 \pm 1.42	4.57 \pm 1.45	4.92 \pm 1.63	4.86 \pm 1.72
Eosinophils ($\times 10^9/L$)	0.14 \pm 0.06	0.14 \pm 0.07	0.14 \pm 0.12	0.14 \pm 0.07	0.09 \pm 0.04	0.08 \pm 0.08	0.07 \pm 0.03	0.09 \pm 0.06
Eosinophils (%)	2.9 \pm 1.2	2.6 \pm 1.3	2.7 \pm 2.1	2.5 \pm 1.0	2.0 \pm 1.3	2.2 \pm 2.5	1.6 \pm 0.9	2.1 \pm 1.8
Basophils ($\times 10^9/L$)	0.02 \pm 0.02	0.02 \pm 0.01	0.02 \pm 0.01	0.03 \pm 0.02	0.02 \pm 0.01	0.01 \pm 0.01	0.01 \pm 0.00	0.01 \pm 0.01
Basophils (%)	0.4 \pm 0.4	0.3 \pm 0.2	0.4 \pm 0.2	0.4 \pm 0.4	0.3 \pm 0.2	0.3 \pm 0.2	0.3 \pm 0.1	0.3 \pm 0.2

EVH eucapnic voluntary hyperpnoea, *FEV₁* forced expiratory volume in one second, *FVC* forced vital capacity, *PEF* peak expiratory flow rate, *FEF_{25%-75%}* forced expiratory flow rate between 25%-75% of FVC.

6.3.2.2 Peripheral Blood Mononuclear Cells (PBMCs)

As outlined in Table 6.2, When expressed as relative values, no interaction effects were reported for total lymphocytes (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 0.41$, $p = 0.53$, $r = 0.17$), viable lymphocytes (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 0.01$, $p = 0.94$, $r = 0.02$), CD25⁺ FoxP3⁺ nT_{REG} cells (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 3.88$, $p = 0.07$, $r = 0.47$), or IL-10⁺ iT_R1 cells (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 0.18$, $p = 0.68$, $r = 0.11$). No main effects were reported for condition, time, or group for total lymphocytes, viable lymphocytes, CD25⁺ FoxP3⁺ nT_{REG} cells, or IL-10⁺ iT_R1 cells, respectively. In contrast, an interaction effect was reported for CD3⁺ CD4⁺ T cells (Greenhouse-Geisser $\epsilon = 1$; $F(1, 14) = 7.9$, $p = 0.01$, $r = 0.60$). However, no main effects were reported for condition, time, or group ($p > 0.05$).

Table 6.6 Changes in lymphocyte and T cell subsets in the HIB and control groups throughout the nutritional intervention.

	Placebo		Prebiotic	
	Baseline	Post-Intervention	Baseline	Post-Intervention
	Total Lymphocytes			
Control	59.7 ± -1.9%	58.1 ± -4.6%	58.7 ± -10.3%	61.7 ± -8.5%
HIB	64.7 ± -7.7%	63.2 ± -13.3%	67.3 ± -11.7%	64.6 ± -10.2%
	Viable Lymphocytes			
Control	82.7 ± -7.0%	86.9 ± -3.5%	81.5 ± -9.4%	83.4 ± -12.9%
HIB	90.6 ± -5.0%	86.5 ± -7.5%	90.4 ± -5.2%	92.1 ± -3.9%
	CD3⁺ CD4⁺ T Cells			
Control	51.6 ± -10.9%	46.4 ± -7.3%	45.6 ± -8.2%	49.8 ± -10.6%
HIB	49.3 ± -12.3%	49.4 ± -14.9%	51.0 ± -13.1%	48.3 ± -12.5%
	CD25⁺ FoxP3⁺ nT_{REG} Cells			
Control	5.7 ± -0.7%	6.0 ± -0.7%	5.7 ± -1.0%	5.6 ± -1.2%
HIB	6.2 ± -3.3%	6.0 ± -3.0%	6.5 ± -3.2%	6.4 ± -3.4%
	IL-10⁺ iT_{R1} Cells			
Control	1.1 ± -0.9%	1.2 ± -1.1%	0.7 ± -0.4%	0.7 ± -0.4%
HIB	0.9 ± -1.1%	0.7 ± -1.0%	1.5 ± -2.5%	1.4 ± -2.0%

nT_{REG} natural (thymically derived) regulatory T cell, *iT_{R1}* type-1 IL-10 producing regulatory T cell.

The control group consisted of eight ($n = 8$) participants. The HIB group consisted of nine ($n = 9$) participants. *HIB* hyperpnoea-induced bronchoconstriction. One participant in the control group ($n = 1$) did not provide blood samples due to haemophilia. As a result, data is presented for seven ($n = 7$) participants in the control group.

For the percentage of viable lymphocytes, anomalous data was reported for one participant ($n = 1$) in the control group following the placebo arm (post-intervention placebo). As a result, data is presented for six ($n = 6$) participants in the control group following the placebo arm for the following parameters: Viable Lymphocytes, CD3⁺ CD4⁺ T Cells, CD25⁺ FoxP3⁺ N_TREG Cells, and IL-10⁺ i_TR1 Cells, respectively.

6.3.3 Nutritional Intake

In the 24-hours prior to each experimental trial, no differences were reported between groups across conditions for total calorie intake (Greenhouse-Geisser $\epsilon = 1$; $F(1, 13) = 1.9$, $p = 0.19$, $r = 0.36$), fat (Greenhouse-Geisser $\epsilon = 1$; $F(1, 13) = 0.30$, $p = 0.591$, $r = 0.15$), carbohydrate (Greenhouse-Geisser $\epsilon = 1$; $F(1, 13) = 2.72$, $p = 0.123$, $r = 0.42$), protein (Greenhouse-Geisser $\epsilon = 1$; $F(1, 13) = 2.90$, $p = 0.112$, $r = 0.43$), or fibre, respectively (Greenhouse-Geisser $\epsilon = 1$; $F(1, 13) = 2.19$, $p = 0.163$, $r = 0.38$).

6.4 Discussion

6.4.1 Key Findings

The primary aim of the current study was to investigate whether long-term supplementation with a galactooligosaccharide based prebiotic (HOST-DM059) attenuated the severity of hyperpnoea (exercise)-induced bronchoconstriction. Based on the current findings, long-term galactooligosaccharide-based prebiotic supplementation did not attenuate the severity of HIB, or markers of systemic inflammation and immune function.

The current observations may be explained by a variety of factors, including the power of the study, the prebiotic dose administered, and the severity of HIB. Based on an *a priori* power calculation, the minimum sample size required to detect participant perceivable changes in the peak decrease in FEV₁ post-EVH of ≥ 230 mL was seven ($n = 7$; Williams et al., 2015; 2016). In the HIB group, from baseline to post-supplementation in the prebiotic condition, the peak decrease in FEV₁ post-EVH did not change, and therefore would not have been perceivable by participants, or of clinical relevance (880 ± 500 vs. 930 ± 460 mL; Santanello et al., 1999). Given that the current sample size exceeded the minimum sample size required to detect participant perceivable improvements in FEV₁ ($n = 9$), it is more likely that no adaptations in pulmonary function occurred following prebiotic supplementation, as opposed to the current study being insufficiently powered to detect them.

In contrast, the current study was not sufficiently powered to detect significant changes in the expression of FoxP3 by CD3⁺ CD4⁺ CD25^{+/-} TGF- β 1^{+/-} T_{REG} cells. Based on an *a priori* power calculation, the minimum sample size required to detect a significant difference in the expression of FoxP3 by CD3⁺ CD4⁺ CD25^{+/-} TGF- β 1^{+/-}

T_{REG} cells was twenty-two ($n = 22$; Konieczna et al., 2012). The sample sizes in the HIB group ($n = 9$), and control group ($n = 8$), were below the minimum sample size required to detect significant changes in the expression of FoxP3 by CD3⁺ CD4⁺ CD25⁺ TGF- β 1⁺ T_{REG} cells. Given the absence of improvement in pulmonary function, despite the current study being sufficiently powered to detect such changes, it is unlikely that prebiotic supplementation improved the suppressive capacity of T_{REG} cells.

In addition to being insufficiently powered to detect changes in the suppressive capacity of T_{REG} cells, the prebiotic dose administered may have been insufficient to elicit any adaptations in pulmonary or immune function. As outlined in study two (Chapter V), short-term, low dose supplementation with the same galactooligosaccharide-based prebiotic (3.1g) did not attenuate the severity of HIB or markers of systemic inflammation. The current study extends these findings by demonstrating that long-term supplementation with the same prebiotic did not attenuate the severity of HIB or markers of systemic inflammation. Using a different galactooligosaccharide-based prebiotic, Davis et al. (2011), demonstrated that 3-weeks of 10 g/day⁻¹ was required to induce a bifidogenic response. No increases in gastrointestinal *Bifidobacteria* were observed following 3-weeks of 2.5 g/day⁻¹ of galactooligosaccharides (Davis et al., 2011). In accordance with these findings, Williams et al. (2016), observed participant perceivable, clinically relevant improvements in pulmonary function following 3-weeks of supplementation with 5.5 g/day⁻¹ of a different galactooligosaccharide (B-GOS[®]). Although Williams et al. (2016), did not assess changes in gut bacterial composition, the dose administered was above the minimum threshold proposed by Davis et al. (2011), required to induce a bifidogenic response. Furthermore, research in elderly individuals has demonstrated a significant increase in gastrointestinal *Bifidobacteria*, and natural killer cell activity, following 10-weeks of supplementation with 5.5 g/day⁻¹ of B-GOS[®] (Vulevic et al., 2015). As a result, it is plausible to speculate that the improvements in pulmonary and immune function observed by Williams et al. (2016), may be partly attributed to gut microbiota-mediated adaptations. In contrast, the prebiotic dose administered during the current study (3.1 g/day⁻¹) was similar to the low dose demonstrated by Davis et al. (2011), to be insufficient in eliciting a bifidogenic response (2.5 g/day⁻¹). Taken together, these results suggest that the prebiotic dose administered during the current study was insufficient to elicit any changes in

gastrointestinal *Bifidobacteria*, potentially explaining why no improvements in pulmonary or immune function were observed.

As part of the current PhD, the primary aim of investigating low dose supplementation was to explore whether increasing the purity of a supplement leads to increases in potency (Grimaldi et al., 2016). The prebiotic investigated during the current PhD (HOST-DM059) contained up to 85% galactooligosaccharides, whereas the prebiotic investigated by Williams et al. (2016; B-GOS®), contained ~ 55% galactooligosaccharides. It was hypothesised that the removal of impurities would increase the potency of the current prebiotic, potentially leading to similar improvements in pulmonary and immune function observed following higher doses of less pure supplements (Williams et al., 2016). Despite the increase in purity (Grimaldi et al., 2016), the current supplement did not lead to any improvements in pulmonary or immune function. These results provide further support for the concept of a minimum threshold required for galactooligosaccharide-based prebiotics to induce improvements in pulmonary and immune function through increases in gastrointestinal *Bifidobacteria* (Davis et al., 2011; Williams et al., 2016).

However, it should be noted that the minimum threshold required to induce improvements in pulmonary and immune function through increases in gastrointestinal *Bifidobacteria* is likely to vary depending on the galactooligosaccharide-based prebiotic administered. For example, Depeint et al. (2008), reported a significant increase in gastrointestinal *Bifidobacteria* following 1-week of supplementing with 3.6 g/day⁻¹ of B-GOS®. Bimuno® galactooligosaccharide (B-GOS®) was developed from the β-galactosidase enzyme isolated from *Bifidobacterium bifidum* NCIMB 41171 (Depeint et al., 2008). As a result, it is possible that Depeint et al. (2008), observed a bifidogenic response to 3.6 g/day⁻¹ of B-GOS® due to increases in potency and selectivity compared to the galactooligosaccharide investigated by Davis et al. (2011). In addition, variation in the dose of different prebiotics required to elicit bifidogenic responses may be attributed to the method used to quantify changes in gut bacterial composition. In accordance with established washout periods (Davis et al., 2011; Depeint et al., 2008), and double-blind, placebo-controlled, crossover designs (Vulevic et al., 2015; Williams et al., 2016), future research should directly investigate changes in gut bacterial composition, in response to equal doses of different prebiotics, to establish

to what extent differences in the minimum dose required are due to supplement potency, selectivity, and the methods used to quantify gut bacterial composition.

As outlined in murine models (Verheijden et al., 2015B; 2018), galactooligosaccharide supplementation may be more effective in moderate-severe cases of HIB. Eosinophils are a key target of galactooligosaccharides. Following the fermentation of galactooligosaccharides, short-chain fatty acids attenuated airway inflammation and hyperresponsiveness by inhibiting the magnitude of systemic and pulmonary eosinophilia (Ni et al., 2010; Thorburn et al., 2015; Trompette et al., 2014; Verheijden et al., 2015B; 2018). In accordance with murine models, Williams et al. (2016), demonstrated participant perceivable, clinically relevant improvements in pulmonary function in adults with moderate-severe HIB (Anderson & Kippelen, 2013; Santanello et al., 1999). This could be attributed to significant increases in systemic eosinophils in adults with moderate as opposed to mild HIB (study one vs. study two; $p < 0.001$), or the fact that some participants may have had atopic asthma, and some did not. However, due to limitations in the current methods we were unable to define atopic status or asthma phenotype more accurately. Both the supplementation studies conducted as part of the current PhD investigated the effects of prebiotic supplementation in mild HIB. When combined with the low dose administered, it is possible the underlying pathophysiology of mild HIB is not severe enough to warrant positive modulation by galactooligosaccharides.

6.4.2 Practical Applications

The results of the current study raise important questions regarding the factors that influence the responsiveness to prebiotic supplementation. Based on the results of the current PhD, and those of previous research (Davis et al., 2011; Depeint et al., 2008; Verheijden et al., 2015B; 2018; Vulevic et al., 2015; Williams et al., 2016), it appears that a minimum dose of $\sim 3.6\text{-}5\text{g/day}^{-1}$ of galactooligosaccharides is required to elicit bifidogenic responses in healthy individuals, depending on the prebiotic administered. However, future research is required to clarify the minimum, clinically important dose of different prebiotics required to elicit bifidogenic responses in adults with HIB, before exploring the efficacy of increased doses. The approaches of Davis et al. (2011), and VanHaitsma et al. (2012), in prebiotics and caffeine respectively could be replicated by investigating incremental absolute doses of the type of galactooligosaccharide-based prebiotic investigated as part of the current work. This could include aspects of the current methodology, such as

measuring the severity of HIB via the EVH protocol, but should also include assessments of both gut microbial composition and airway inflammation.

A dose-response relationship has been observed between galactooligosaccharide supplementation and gastrointestinal *Bifidobacteria* with doses up to 10g/day⁻¹ (Davis et al., 2011; Depeint et al., 2008). Based on material safety data sheets, up to 15g/day⁻¹ of galactooligosaccharides can be consumed without adverse side effects. However, studies investigating the dose-response relationship between galactooligosaccharide supplementation and gastrointestinal *Bifidobacteria* remain confined to healthy individuals (Davis et al., 2011; Depeint et al., 2008). As a result, it remains to be determined whether doses of galactooligosaccharides > 5.5g/day⁻¹ (Williams et al., 2016), lead to further improvements in the pulmonary and immune function of adults with HIB. Future research should investigate the effects of short-term and long-term supplementation with increased doses of galactooligosaccharides, with a particular focus on the ability of T_{REG} cells to attenuate type-2 inflammation. As part of these investigations, the increased costs associated with increased doses, and potential issues with compliance, should also be assessed to inform the efficacy of high dose galactooligosaccharide supplementation.

6.4.3 Strengths

A key strength of the current study was the adherence to the nutritional supplements. Across both experimental groups, adherence to both supplements (prebiotic or placebo) was ≥ 95%, well above the 80% criteria outlined by the USFDA for data to be included in statistical analyses. Furthermore, throughout the nutritional intervention, all assessments of EIB conducted through the EVH protocol were considered valid (Anderson & Kippelen, 2013). Across both experimental groups, participants maintained an average \dot{V}_E during the EVH protocol of ≥ 27 × their highest resting FEV₁, similar to the target ventilation rate of 30 × highest resting FEV₁ (Anderson & Kippelen, 2013). Finally, the double-blind, placebo-controlled, crossover design was of stronger methodological quality than parallel group designs adopted by previous research (Halnes et al., 2017; van de Pol et al., 2011), reducing the potential influence of placebo effects and expectancies. When combined with the robust assessments of EIB, the high adherence rates suggest that the absence of improvement in pulmonary and immune function following prebiotic

supplementation may be attributed to the dose administered as opposed to methodological limitations.

6.4.4 Limitations

A key limitation faced by the current study was participant recruitment. Of the 163 potential participants initially screened ($n = 64$ HIB, $n = 99$ control), only 13.8% completed the study. By obtaining NHS ethical approval, the current study was able to recruit participants with EIB from a wider population than studies one and two. For example, the Register of Asthma Research Volunteers (REACH) database was screened to identify potentially eligible participants in the local area (Nwaru et al., 2016), advertisements were placed in Asthma UK's research and policy bulletin, and applications were made to recruit participants from GP practices in Nottingham City's Clinical Commissioning Group.

Screening the REACH database did not identify any potentially eligible participants. Although the database was only developed in 2018, it has already been shut down, potentially highlighting the wider problems associated with engagement in asthma research (Delsing et al., 2016). Advertisement in Asthma UK's research and policy bulletin proved ineffective, and of the 70-80 GP practices in Nottingham City's Clinical Commissioning Group, only one agreed to assist with participant recruitment. From this GP practice, thirty-four ($n = 34$) potentially eligible participants were identified. However, long delays were experienced when trying to obtain approval from GP practice partners, ultimately surpassing the timeline for participant recruitment. As a result, none of the additional recruitment options available to the current study recruited any participants.

It should be noted, however, that issues with participant recruitment are not confined to studies conducted in academic settings. Alarming, ~ 26.3% of clinical trials in asthma and COPD do not achieve their recruitment targets despite the funding and resources available (Delsing et al., 2016). It is evident that qualitative explorations of potential barriers to participation are required to understand how to encourage more effective engagement in asthma research before more intervention-based trials are carried out. Given current issues associated with the replicability crisis (Munafò et al., 2017), conducting future intervention-based trials across multiple sites could expand recruitment populations, and increase the quality of research

outputs by ensuring a small number of sufficiently powered projects are carried out that can inform impactful outcomes.

Another limitation of the current study was the absence of assessing changes in gut bacterial composition in response to prebiotic supplementation. As outlined in study one (Chapter IV), fluorescent *in situ* hybridisation is a time-consuming process. Although multiple samples can be analysed at once using flow cytometry, the initial process of preparing samples takes ~ 8 hours. Given the time critical nature of sample processing, and the logistics of arranging participant trials, sample processing cannot be made more efficient by processing multiple samples at once. Although metagenomics can be more efficient, this approach is far more expensive. Without multi-disciplinary teams, or access to external funding, it is impractical to include assessments of gut bacterial composition, markers of systemic inflammation, and objective assessments of EIB in intervention-based trials. An alternative approach to assess whether prebiotic supplementation induces gut microbiota-mediated improvements in pulmonary and immune function could be to analyse anti-inflammatory microbial metabolites in the urine.

Urinary metabolomics can provide extensive information on prebiotic induced functional interactions between the gut microbiota and host immune system (Claus & Swann, 2013; Marcobal et al., 2013). As part of the current study, ^1H NMR-based urinary metabolomics was used to investigate whether prebiotic supplementation induced gut microbiota-mediated improvements in pulmonary and immune function (Beckonert et al., 2007; Claus & Swann, 2013; Dona et al., 2014; Nicholson et al., 2007; Paris et al., 2018; Sheedy et al., 2010; Swann & Claus, 2014). ^1H NMR-based urinary metabolomics is non-invasive (Nicholson et al., 2007), highly reproducible (Dona et al., 2014; Sheedy et al., 2010), and permits a greater frequency of measurement than faecal samples (Beckonert et al., 2007). During each experimental trial, urine samples were collected at rest and 60-minutes post-EVH. Processing urine samples for analysis via NMR only takes a few minutes, unlike the 8 hours required to process faecal samples for fluorescent *in situ* hybridisation-based flow cytometry. Once processed, samples were transferred to Imperial College London for analysis. Unfortunately, the data from this analysis was never provided, preventing inclusion in the current PhD. Although the data was never provided, it was made apparent during a meeting with colleagues from Imperial College London who had analysed the samples that the samples collected 60-

minutes post-EVH could not be used for analysis. The dilution of samples caused by participants drinking water following the EVH protocol prevented metabolites from being detected. If post-EVH analyses of urinary metabolites are required, future analyses must restrict fluid intake following the EVH protocol, or stick to analysing urine samples collected at rest.

In addition to the urine samples, plasma samples were also collected at rest throughout the nutritional intervention and sent to Imperial College London to analyse short-chain fatty acids. The presence of short-chain fatty acids in systemic circulation can be a key indicator of whether the gut microbiota is capable of inducing peripheral changes in immune function. For example, acetate, butyrate and propionate play critical roles in attenuating the severity of asthma by increasing the suppressive capacity of FoxP3⁺ iT_{REG} cells, decreasing the magnitude of airway eosinophilia (Arpaia et al., 2013; Thorburn et al., 2015; Trompette et al., 2014). Assessing changes in plasma short-chain fatty acids can also indicate whether the dose of prebiotic administered was sufficient to potentially support peripheral adaptations in immune function. Unfortunately, this data was also not provided, preventing information from being obtained regarding the effect of systemic short-chain fatty acids on pulmonary and immune function.

Finally, the flow cytometry-based analysis of T_{REG}, T_{H2}, and T_{H1} cells could have been more informative if assessed in response to bronchoprovocation. Originally, the current study planned to replicate the protocol of Williams et al. (2016), by assessing changes in blood-based markers of systemic inflammation at rest and post-EVH. However, concerns raised by the NHS Research Ethics Committee regarding the burden placed on participants meant post-EVH blood samples were not collected. Importantly, when measured at rest, the functional capacity of T cells does not reflect the key changes that occur in response to bronchoprovocation.

Future studies aiming at investigating changes in the functional capacity of T cells at rest, and post-EVH, could use cannulation to collect blood samples in a less invasive manner compared to venepuncture. If conducting objective assessments is impractical due to the age or severity of the cohort, *in vitro* suppression assays (Chen et al., 2017; 2018), could be applied to resting blood samples to assess changes in the functional capacity of T cells. Furthermore, given the limited number of markers that can be assessed via flow cytometry, it might be more appropriate for future research to focus on a single T cell subset. Adopting such an approach as

part of the current study would have enabled a more comprehensive investigation of the effect of prebiotic supplementation on the functional capacity of T_{REG} cells by allowing the expression of key markers such as ST2 (IL-33) and IL-4R α to be investigated (Chen et al., 2017; Khumalo et al., 2020). Recently, antigens have been developed that permit the expression of short-chain fatty acid receptors on T_{REG} cells to be assessed. The antigens commonly used to analyse T cells in humans are developed in mice. Unfortunately, the antigens currently available to assess short-chain fatty acid receptors are developed in goats, and therefore have the potential to cross-react with markers developed in mice. The development of such antigens in mice could be integral to understanding whether prebiotics improve the suppressive capacity of T_{REG} cells through direct or indirect mechanisms.

6.4.5 Conclusion

In conclusion, long-term supplementation with a low dose galactooligosaccharide (3.1 g·day⁻¹; HOST-DM059) does not attenuate the severity of HIB, or markers of systemic inflammation. Future research is required to establish the minimum, clinically important dose required to induce bifidogenic responses, and to explore the mechanisms by which moderate-high dose short-term and long-term supplementation with prebiotic galactooligosaccharides attenuate the severity of HIB.

Chapter VII

General Discussion

7.1 Key Findings

The studies conducted as part of the current PhD explored if adults with EIB display features of gut microbial dysbiosis, and whether short-term or long-term supplementation with a low dose galactooligosaccharide-based prebiotic attenuated the severity of HIB. The key findings are outlined below:

Chapter IV

- Adults with moderate EIB do not demonstrate features of gut microbial dysbiosis based on fluorescent *in situ* hybridisation-based flow cytometry.

Chapters V & VI

- Both short-term and long-term supplementation with a low dose galactooligosaccharide-based prebiotic (HOST-DM059) does not attenuate the severity of HIB or markers of systemic inflammation.

7.2 Practical Applications

When investigating whether specific asthma subtypes, such as EIB, display features of gut microbial dysbiosis, the methods used to quantify gut bacterial composition must be applied at the appropriate phylogenetic depth (Han et al., 2012; Ursell et al., 2012). During study one (Chapter IV), fluorescent *in situ* hybridisation was used to analyse key bacterial groups above the species and strain levels (Poveda et al., 2020; Vulevic et al., 2015). In contrast, previous research has used metagenomics-based approaches to identify features of gut microbial dysbiosis in adults with allergic asthma at the species and strain levels (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2018). Metagenomics provides a more detailed, comprehensive, and sensitive assessment of gut bacterial composition than fluorescent *in situ* hybridisation. Given that features of gut microbial dysbiosis in adults with allergic asthma relate to specific species or strains, as opposed to overall community structure (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016), it is possible fluorescent *in situ* hybridisation lacks the required sensitivity to detect features of gut microbial dysbiosis in adults with EIB.

As a result, further research is required using more sensitive metagenomics-based approaches to investigate whether adults with EIB display features of gut microbial dysbiosis at the species or strain levels. If features of gut microbial dysbiosis are identified at the species or strain levels then probiotic supplementation may be required before prebiotic supplementation (Arrieta et al., 2015; Davis et al., 2011; Williams et al., 2016).

The prebiotic dose administered during the current PhD (3.1 g/day⁻¹ of pure galactooligosaccharide) may have been too low to induce a bifidogenic response, regardless of the increased purity (Davis et al., 2011; Grimaldi et al., 2016), potentially explaining why no improvements were observed in pulmonary or immune function. Using a different galactooligosaccharide-based prebiotic, Davis et al. (2011), demonstrated that 3-weeks of 10 g/day⁻¹ was required to induce a consistent bifidogenic response, although some improvements were observed in response to 5 g/day⁻¹. No increases in gastrointestinal *Bifidobacteria* were observed following 3-weeks of 2.5 g/day⁻¹ of galactooligosaccharides (Davis et al., 2011). In accordance with these findings, Williams et al. (2016), observed participant perceivable, clinically relevant improvements in pulmonary function following 3-weeks of supplementation with 5.5 g/day⁻¹ of a different galactooligosaccharide (B-GOS®). Although Williams et al. (2016), did not assess changes in gut bacterial composition, the dose administered was in accordance with the dose proposed by Davis et al. (2011), required to induce a bifidogenic response. Furthermore, research in elderly individuals has demonstrated a significant increase in gastrointestinal *Bifidobacteria*, and natural killer cell activity, following 10-weeks of supplementation with 5.5 g/day⁻¹ of B-GOS® (Vulevic et al., 2015). As a result, it is plausible to speculate that the improvements in pulmonary and immune function observed by Williams et al. (2016), may be partly attributed to gut microbiota-mediated adaptations.

The prebiotic investigated during the current PhD (HOST-DM059) contained up to 85% galactooligosaccharides, whereas the prebiotic investigated by Williams et al. (2016; B-GOS®), contained ~ 55% galactooligosaccharides. It was hypothesised that the removal of impurities would increase the potency of the current prebiotic, potentially leading to similar improvements in pulmonary and immune function observed following higher doses of less pure supplements (Williams et al., 2016). Despite the increase in purity (Grimaldi et al., 2016), the current supplement did not

lead to any improvements in pulmonary or immune function. These results provide further support for the concept of a minimum threshold required for galactooligosaccharide-based prebiotics to induce improvements in pulmonary and immune function through increases in gastrointestinal *Bifidobacteria* (Davis et al., 2011; Williams et al., 2016).

It should be noted, however, that the minimum threshold required to induce improvements in pulmonary and immune function through increases in gastrointestinal *Bifidobacteria* is likely to vary depending on the galactooligosaccharide-based prebiotic administered. Depeint et al. (2008), reported a significant increase in gastrointestinal *Bifidobacteria* following 1-week of supplementing with 3.6 g/day⁻¹ of B-GOS[®]. Bimuno[®] galactooligosaccharide (B-GOS[®]) was developed from the β -galactosidase enzyme isolated from *Bifidobacterium bifidum* NCIMB 41171 (Depeint et al., 2008). As a result, it is possible that Depeint et al. (2008), observed a bifidogenic response to 3.6 g/day⁻¹ of B-GOS[®] due to increases in potency and selectivity compared to the galactooligosaccharide investigated by Davis et al. (2011).

A key caveat to interpreting the potential role of the dose of prebiotic administered on immune and pulmonary function is being able to accurately compare the actual proportion of “active ingredient” across the range of galactooligosaccharide-based prebiotics used in previous research (Davis et al., 2011; Depeint et al., 2008; Vulevic et al., 2015; Williams et al., 2016). Typically, doses are reported as the total weight of a given commercially available supplement administered as a powdered sachet or confectionary (Davis et al., 2011; Depeint et al., 2008; Vulevic et al., 2015). Material safety data sheets are required to calculate the proportion of active ingredient, however these are not routinely published. The proportion of active ingredient was able to be calculated for the research of Williams et al. (2016), given that this research was conducted by our research group. If we take the difference in active ingredient between the current programme of work and the research of Williams et al. (2016), as an example (85% vs. 55% galactooligosaccharide), it is clear how conclusions regarding the importance of dose may be misinterpreted by the unexplained variation in the proportion of active ingredient administered by previous research, especially considering the breadth of different galactooligosaccharide-based prebiotics administered (Davis et al., 2011; Depeint et al., 2008; Vulevic et al., 2015). Future research should attempt to be more

transparent when reporting details of prebiotic interventions being investigated, specifically stating the proportion of the active ingredient being explored, especially if the supplement being studied is commercially available.

As outlined in murine models (Verheijden et al., 2015B; 2018), galactooligosaccharide supplementation may be more effective in moderate-severe cases of HIB or different phenotypes of asthma. Eosinophils are a key target of galactooligosaccharides. Following the fermentation of galactooligosaccharides, short-chain fatty acids attenuated airway inflammation and hyperresponsiveness by inhibiting the magnitude of systemic and pulmonary eosinophilia (Ni et al., 2010; Thorburn et al., 2015; Trompette et al., 2014; Verheijden et al., 2015B; 2018). In accordance with murine models, Williams et al. (2016), demonstrated participant perceivable, clinically relevant improvements in pulmonary function in adults with moderate-severe HIB (Anderson & Kippelen, 2013; Santanello et al., 1999). This could be attributed to significant increases in resting systemic eosinophils in adults with moderate as opposed to mild HIB, as outlined by the difference observed between participants who took part in study one vs. study two. Both the supplementation studies conducted as part of the current PhD investigated the effects of prebiotic supplementation in mild HIB. When combined with the low dose administered, it is possible the underlying pathophysiology of mild HIB is not severe enough to warrant positive modulation by galactooligosaccharides.

7.4 Strengths

Throughout all of the studies conducted during the current PhD, assessments of EIB using the EVH protocol were considered valid in adults with and without HIB, preventing the validity of the current conclusions from being reduced due to limitations associated with subjective diagnoses (Aggarwal et al., 2018; Anderson & Kippelen, 2013; Dickinson et al., 2011; Juniper et al., 2004; Pavord et al., 2017; Wenzel, 2016). During study two (Chapter V), adherence to the nutritional supplements was $\geq 95\%$ across both experimental groups, well above the minimum 80% criteria proposed by the USFDA for data to be included in statistical analyses. Finally, the double-blind, placebo-controlled, crossover design was of stronger methodological quality than parallel group designs adopted by previous research (Halnes et al., 2017; van de Pol et al., 2011), reducing the potential influence of placebo effects and expectancies. Taken together, these observations demonstrate

the robust nature of the cohorts recruited, the methods used, and the quality of data collected.

7.5 Limitations

The current PhD attempted to identify features of gut microbial dysbiosis based on single faecal samples. Without longitudinal profiling, it can be difficult to identify features of gut microbial dysbiosis that are not caused by confounding factors, such as diurnal effects (Sender et al., 2016A), temporal parameters (Flores et al., 2014), or environmental exposures, respectively (Feddema & Claassen, 2020; He et al., 2020; Khreis et al., 2017; Malik et al., 2012; Rodriguez et al., 2019; Sbihi et al., 2019; Sharpe et al., 2015). Begley et al. (2018), addressed this issue, to some extent, by assessing the short-term stability of gut bacterial composition in adults with mild-moderate asthma over a 4-week period. Nevertheless, longitudinal investigations are required to assess the long-term stability of gut bacterial composition before HIB specific features of gut microbial dysbiosis can be identified (Hoen et al., 2015; Stockholm et al., 2016). Longitudinal, prospective designs have been used to identify biomarkers of exacerbations in adults with mild-moderate asthma (Brinkman et al., 2017; Peters et al., 2020; Silkoff et al., 2019; van der Schee et al., 2013). Combining such approaches with assessments of gut bacterial parameters may identify exacerbation specific features of gut microbial dysbiosis that could be used to inform targeted therapies.

For prebiotic supplementation to be effective, the bacteria of interest must be present in sufficient quantities (De Preter et al., 2008). Decreases in dietary fibre intake in severe asthma may lead to the loss of *Bifidobacteria*, and their ability to modulate host immune function (Berthon et al., 2013; De Filippo et al., 2010; Healey et al., 2016; 2018; Holscher et al., 2017; Huang et al., 2017; Wood et al., 2015). Galactooligosaccharides selectively target certain species of *Bifidobacteria* (Davis et al., 2011; Depeint et al., 2008), yet adults with asthma demonstrate imbalances or deficiencies in certain species of *Bifidobacteria* (Hevia et al., 2016). Baseline analyses of gut bacterial composition (De Preter et al., 2008), and habitual dietary fibre intake (Healey et al., 2016; 2018), may be required to adjust the dose of targeted probiotic (Arrieta et al., 2015), and prebiotic therapies (Williams et al., 2016) in adults with differing severities of HIB.

The absence of assessing changes in gut bacterial composition in response to prebiotic supplementation leads to uncertainty regarding the mechanisms by which prebiotics may improve pulmonary and immune function. Urinary metabolomics can be used to assess whether prebiotic supplementation induces gut microbiota-mediated improvements in pulmonary and immune function by analysing changes in anti-inflammatory microbial metabolites. Furthermore, the presence of short-chain fatty acids in systemic circulation can be a key indicator of whether prebiotic induced changes in the gut microbiota are capable of mediating peripheral changes in immune function. Acetate, butyrate and propionate play critical roles in attenuating the severity of asthma by increasing the suppressive capacity of FoxP3⁺ iT_{REG} cells, decreasing the magnitude of airway eosinophilia (Arpaia et al., 2013; Thorburn et al., 2015; Trompette et al., 2014). Both urine samples and plasma samples were sent to Imperial College London for analysis. Unfortunately, data from these analyses was not provided. As a result, it remains to be determined whether galactooligosaccharide-based prebiotic supplementation induces changes in the gut microbiota that could mediate pulmonary or immune function. Given that no formal assessments were carried out to assess how successful the supplement blinding was, influences of the placebo effect and expectancy cannot be ruled out.

The methods used to estimate the sample size required for the two supplementation studies conducted (Chapters V and VI) were based on the research of Santanello et al. (1999), regarding what is classed as the minimum detectable difference in resting FEV₁ that is perceivable by patients. However, the focus of the current research was on exploring to what extent short-term or long-term prebiotic supplementation may attenuate the severity of the peak drop in FEV₁ post-EVH, not the change in resting pulmonary function. Dahlen et al. (2001), demonstrated that sample size estimations based on the peak drop in FEV₁ post-EVH were more reproducible than area under the curve measurements, as well as being more valid than estimates based on resting pulmonary function (Santanello et al., 1999). Estimates of the sample sizes required to demonstrate the attenuation of EIB by different magnitudes are also provided in the form of a sample size estimation curve. Future research looking to explore the potential bronchoprotective effects of prebiotic supplementation should therefore use these approaches in future to obtain more accurate estimates of the sample sizes required to detect patient perceivable, clinically relevant improvements.

As well as changes to the approach to estimating sample size, future research should also consider adopting a different cut-off value for diagnosing HIB. An $\geq 13\%$ decrease in FEV₁ post-EVH was used to diagnose HIB in the current thesis as this criteria has been associated with a lower rate of false-positive diagnoses when compared to a 10% decrease in FEV₁ post-EVH (Anderson et al., 2001; Anderson & Kippelen, 2013; Godfrey et al., 1999), providing the most sensitive and specific assessment of EIB. This criteria was established and recommended for use by Godfrey et al. (1999), in participants up to the age of 25. That being said, the vast majority of participants who took part in the study conducted by Godfrey et al. (1999), were children under the age of 18. As a result, this diagnostic threshold may be less appropriate to use in adult populations, such as the 10% cut-off value adopted by other research conducted by different members of our research group at Nottingham Trent University who largely recruited the same demographic as the current program of work (Needham et al., 2022; Williams et al., 2015; 2016; 2017). Although the 13% criteria was applied in the current thesis with the aim of providing the most sensitive and specific assessment of EIB (Godfrey et al., 1999), it leads to three key limitations, namely the potential misdiagnosis of participants as EIB⁻ based on a peak decrease in FEV₁ post-EVH between 10.0-12.9%, potentially misclassifying the magnitude of bronchoprotection observed in response to prebiotic supplementation, and the inability to accurately compare and contrast to the larger body of existing research in adults that has primarily focused on using 10% as a cut-off value.

7.6 Conclusions

In conclusion, the current study demonstrated that adults with mild-moderate HIB do not display features of gut microbial dysbiosis. This observation must be viewed with caution regarding the limitations of the current work, such as the sample size investigated, and the methods used to analyse gut microbial composition down to the genus level. This is in contrast to previous research in participants with a different subtype of asthma (Barcik et al., 2016; Begley et al., 2018; Hevia et al., 2016; Okba et al., 2018), and could be attributed to the lack of sensitivity in the method used to quantify gut bacterial composition, compounded by the small sample size.

Both short-term and long-term supplementation with a low dose galactooligosaccharide (3.1 g·day⁻¹; HOST-DM059) does not attenuate the severity

of HIB, or markers of systemic inflammation. A minimum threshold and dose-response relationship may exist between galactooligosaccharide supplementation, and the bifidogenic response required to modulate systemic and pulmonary immune function (Davis et al., 2011; Williams et al., 2016). This is partly dependent on baseline gut bacterial composition (De Preter et al., 2008), and habitual dietary fibre intake (Healey et al., 2016; 2018). Given the influence of baseline gut bacterial composition (De Preter et al., 2008), and habitual dietary fibre intake on gastrointestinal *Bifidobacteria* (De Filippo et al., 2010), and the responsiveness to prebiotics (Healey et al., 2016; 2018), it is likely that dosing regimens may need to be adjusted for these factors.

Future research is required to establish the minimum, clinically important dose required to induce bifidogenic responses, and to explore the mechanisms by which moderate-high dose short-term and long-term supplementation with prebiotic galactooligosaccharides attenuate the severity of HIB.

7.7 Directions for Future Research

Certain galactooligosaccharide-based prebiotics have been shown to induce clinically relevant, participant perceivable improvements in HIB, by a comparable magnitude to that observed following pharmaceutical treatments (39-60%; Kippelen et al., 2010; Rundell et al., 2005; Santanello et al., 1999; Simpson et al., 2016; Verheijden et al., 2015A; 2018; Williams et al., 2016). However, it is currently unclear whether these improvements in pulmonary and immune function are attributed to the gut microbiota.

Future research is required to longitudinally investigate whether adults with EIB display features of gut microbial dysbiosis at the species and strain levels (Almonacid et al., 2017; Begley et al., 2018; Flores et al., 2014; Hoen et al., 2015; Stockholm et al., 2016). If present, follow on studies could investigate whether prebiotic supplementation attenuates the severity of EIB by resolving features of gut microbial dysbiosis. Whether observational or intervention-based, this research should look to adopt a 10% cut-off in the peak decrease in FEV₁ post-EVH as the diagnostic threshold for assessing EIB to enable more effective comparisons with previous research conducted in similar demographics, and help minimise the risk of misdiagnosis. Assessing the expression of short-chain fatty acid receptors on T_{REG}

cells will provide further clarity regarding the direct or indirect mechanisms by which prebiotic supplementation modulates systemic immune function.

Variation in the dose of different prebiotics required to elicit bifidogenic responses may be attributed to the method used to quantify changes in gut bacterial composition. In accordance with established washout periods (Davis et al., 2011; Depeint et al., 2008), and double-blind, placebo-controlled, crossover designs (Vulevic et al., 2015; Williams et al., 2016), future research should directly investigate changes in gut bacterial composition, in response to equal doses of different prebiotics, to establish to what extent differences in the minimum dose required are due to supplement potency, selectivity, and the methods used to quantify gut bacterial composition.

Habitual dietary fibre intake was not monitored as part of the current PhD, despite its influence on prebiotic supplementation (Healey et al., 2016; 2018). Future research should prospectively monitor habitual dietary fibre intake using software capable of quantifying different sources of dietary fibre. Combining this information with the response to prebiotic supplementation may inform the development of habitual dietary fibre intake thresholds that inform the potential magnitude of response to prebiotics (Healey et al., 2016; 2018), encouraging appropriate use in targeted populations.

As outlined in Chapter VI, future studies aimed at investigating changes in the functional capacity of T cells at rest, and post-EVH, could use cannulation to collect blood samples in a less invasive manner compared to multiple venepuncture procedures, supported by *in vitro* suppression assays and targeted investigations of T cell subsets and the expression of key mouse anti-human markers such as ST2 (IL-33) and IL-4R α to be investigated (Chen et al., 2017; Khumalo et al., 2020).

Although more targeted investigations of the role of T_{REG} cells in galactooligosaccharide mediated improvements in EIB will prove vital to understanding the mechanisms by which prebiotics attenuate type-2 inflammation, blood-based assessments of T cells are generally limited by the number of markers that can be analysed. An alternative approach could be to use breathomics to measure systemic and pulmonary inflammatory metabolites of host and microbial origin when attempting to characterise features of gut microbial dysbiosis and responses to prebiotic supplementation.

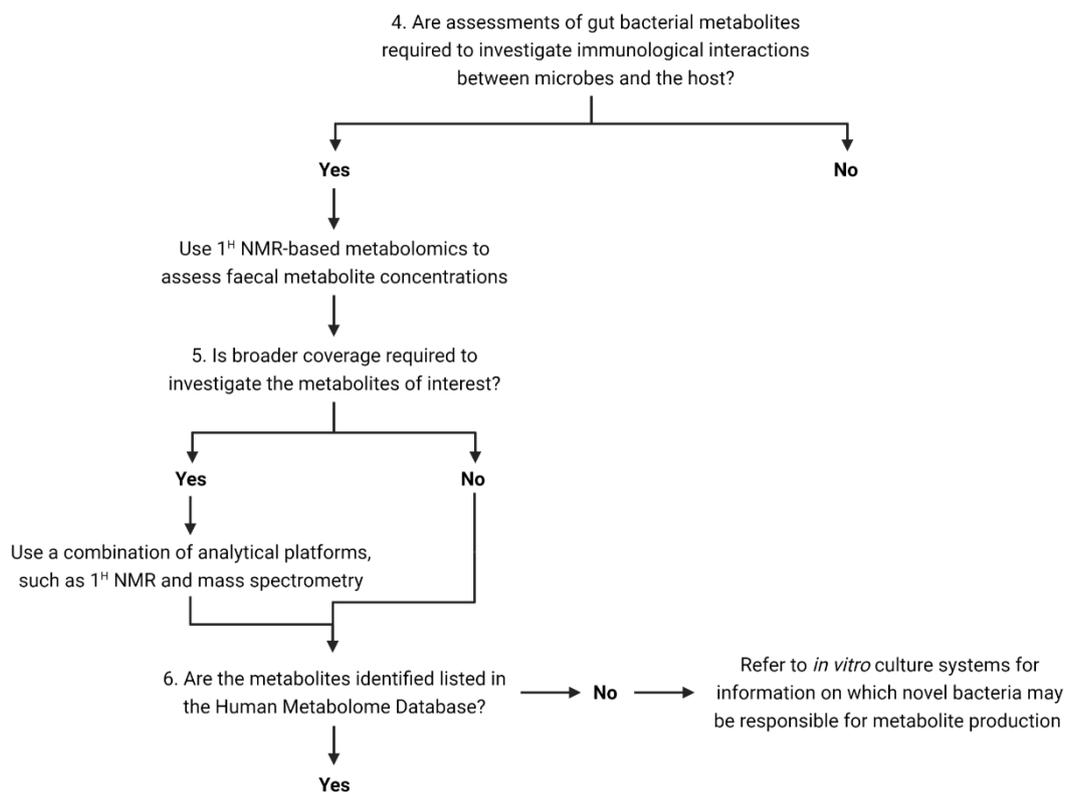
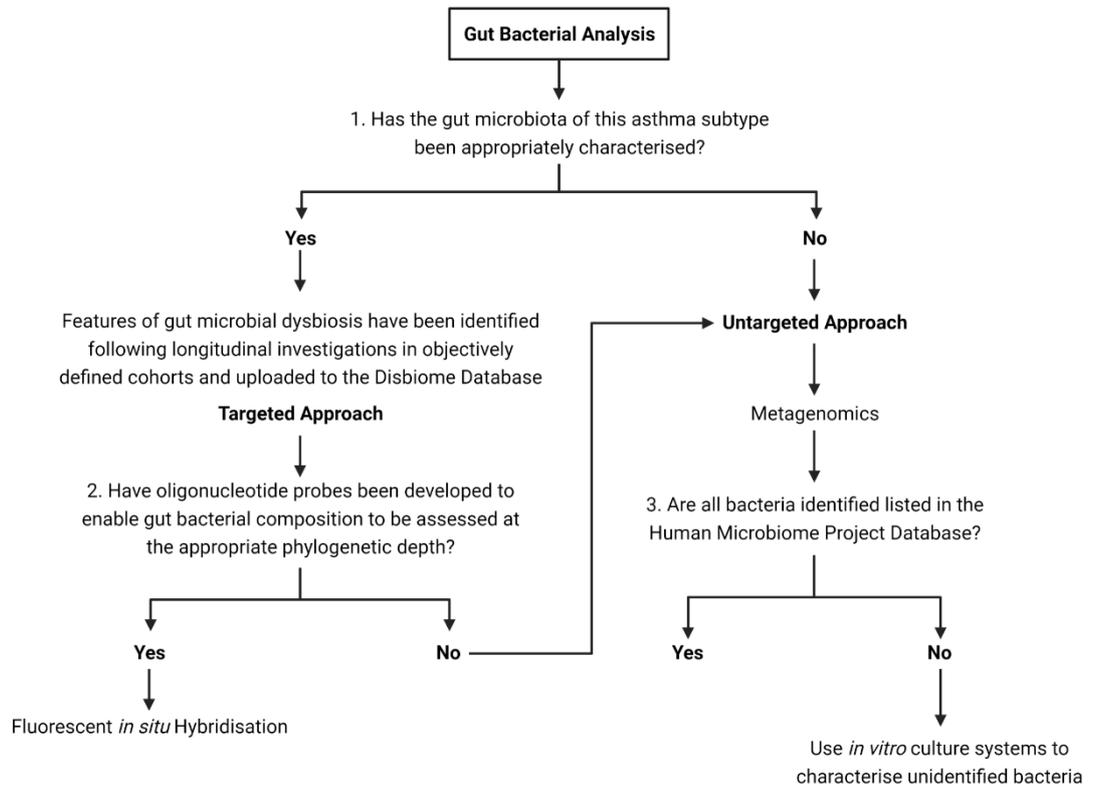
The current programme of work is limited by its inability to provide direct measures of airway inflammation, having to settle for speculation based on systemic markers. This was partly due to the level of expertise within the research team, and the inability to conduct direct measurements of airway inflammation via techniques such as induced sputum. Breathomics detects volatile organic compounds (VOCs) in exhaled breath that are associated with local and systemic inflammatory processes in a simple and non-invasive manner (Brinkman et al., 2019A; 2020; de Vries & Sterk, 2020; van der Schee et al., 2013). When compared to exhaled breath, blood and sputum are less suitable biofluids for regular, prospective monitoring (de Vries and Sterk, 2020), given the technical expertise, time required for sample collection, and classification as “Relevant Material” by the Human Tissue Authority (Brinkman et al., 2020). Aided by its simple and non-invasive nature (Brinkman et al., 2020; van der Schee et al., 2013; de Vries and Sterk, 2020), breathomics can discriminate between eosinophilic and neutrophilic phenotypes of asthma (Brinkman et al., 2019A), monitor the occurrence of exacerbations (Brinkman et al., 2017; van der Schee et al., 2013), assess medication adherence (Brinkman et al., 2020), and predict responsiveness to steroid-based treatments (Brinkman et al., 2019B; van der Schee et al., 2013), preventing the prescription of unnecessary and expensive therapies with potential adverse side effects. When combined with the protocol of Begley et al. (2018), the inclusion of breathomics could be invaluable in understanding host-microbial interactions in the pathophysiology of EIB, helping bridge the gap between current phenotyping approaches and optimal asthma management (Silkoff et al., 2019).

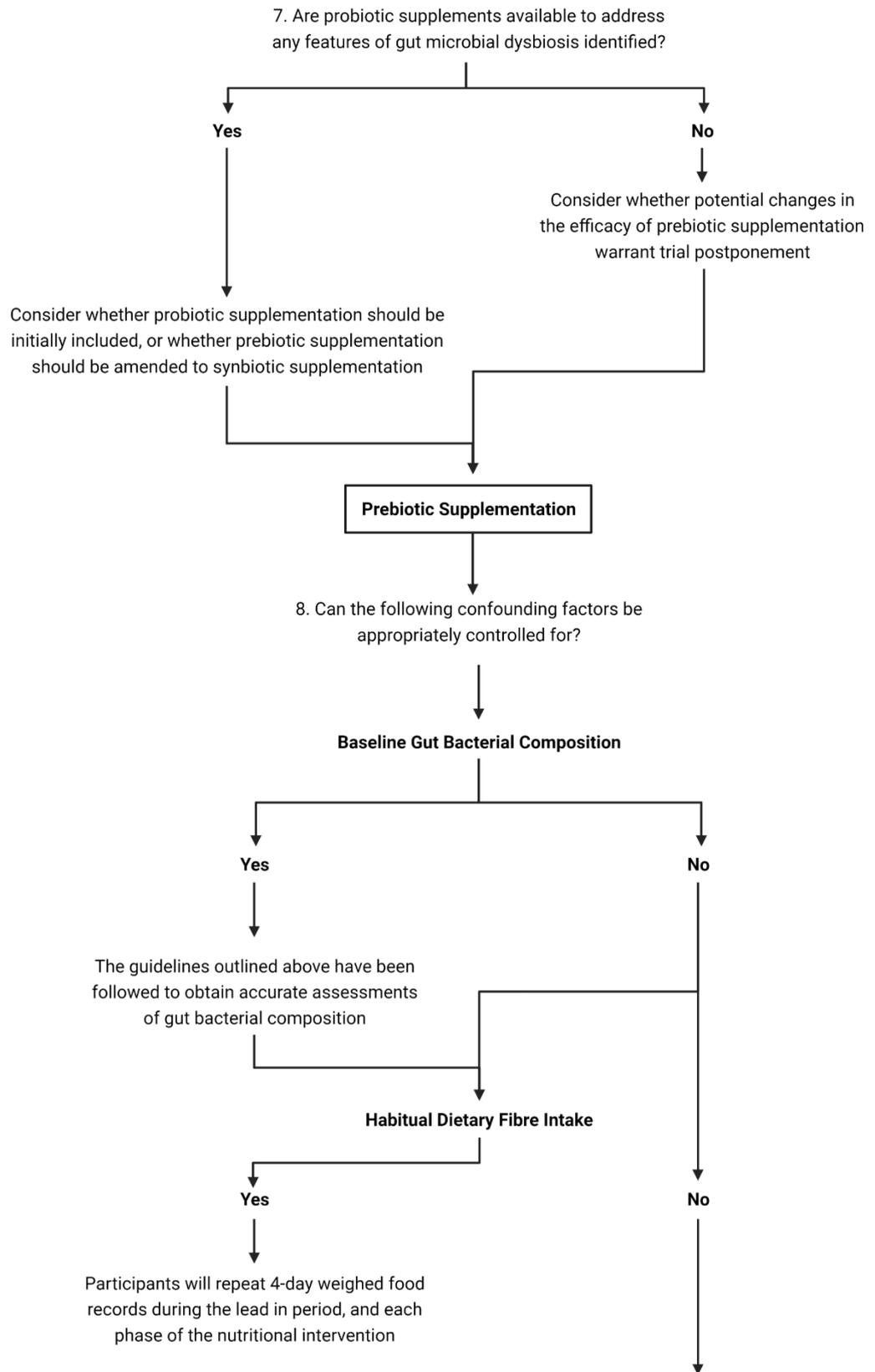
Breathomics could extend the scope of current phenotyping approaches by profiling the local and systemic pathophysiology of EIB in a single, easy to collect sample (de Vries & Sterk, 2020). Breathomics demonstrates excellent accuracy in discriminating between phenotypes of severe asthma based on induced sputum (0.93; Brinkman et al., 2019A), yet provides a more clinically accessible means of analysis, especially during exacerbations. Novel, composite biomarkers can be developed with greater diagnostic accuracy than current singular biomarkers (Peters et al., 2020; van der Schee et al., 2013). For example, breathomics demonstrates greater diagnostic accuracy than $F_{E}NO$ and resting assessments of pulmonary function (88% vs. 83%; Montuschi et al., 2010). When combined with $F_{E}NO$, breathomics improves the diagnostic accuracy even further (96%; Montuschi

et al., 2010). Due to the ease of sample collection (Brinkman et al., 2020; de Vries and Sterk, 2020; van der Schee et al., 2013), and online, cloud-based data delivery platforms, breathomics has the potential to be widely integrated into clinical practice, and provide real-time data to inform personalised diagnosis, monitoring and treatment (Brinkman et al., 2019A; de Vries & Sterk, 2020; Silkoff et al., 2019).

The clinical utility of breathomics in monitoring the occurrence of exacerbations has been demonstrated in adults with mild-moderate asthma (Brinkman et al., 2017). Biomarkers of exacerbations were detected in exhaled breath that were associated with changes in clinical and inflammatory characteristics (Brinkman et al., 2017), including post-bronchodilator percentage predicted FEV₁, sputum eosinophils, and F_ENO, respectively. By assessing metabolites of microbial origin (e.g. acetate, propionate and butyrate; Arpaia et al., 2013; Cait et al., 2018; Thorburn et al., 2015), breathomics could be used to assess the relative contribution of changes in gut bacterial composition and metabolic function to exacerbations of EIB, helping identify potential targets for exacerbation specific probiotic and prebiotic interventions.

In conclusion, future research attempting to explore the potential role of gut microbial dysbiosis in EIB, and the clinical utility of prebiotic supplementation may benefit greatly from the inclusion of breathomics based approaches to further understanding the influence of baseline gut bacterial composition (De Preter et al., 2008), habitual dietary fibre intake (Healey et al., 2016; 2018), the dose administered (Davis et al., 2011; Depeint et al., 2008), and the severity of EIB on the efficacy of prebiotic supplementation (Williams et al., 2016), as more severe phenotypes could be more likely to display features of gut microbial dysbiosis and therefore potentially respond better to prebiotic supplementation. A flow diagram illustrating the critical questions that must be asked when planning future research is outlined in Figure 7.1 below.





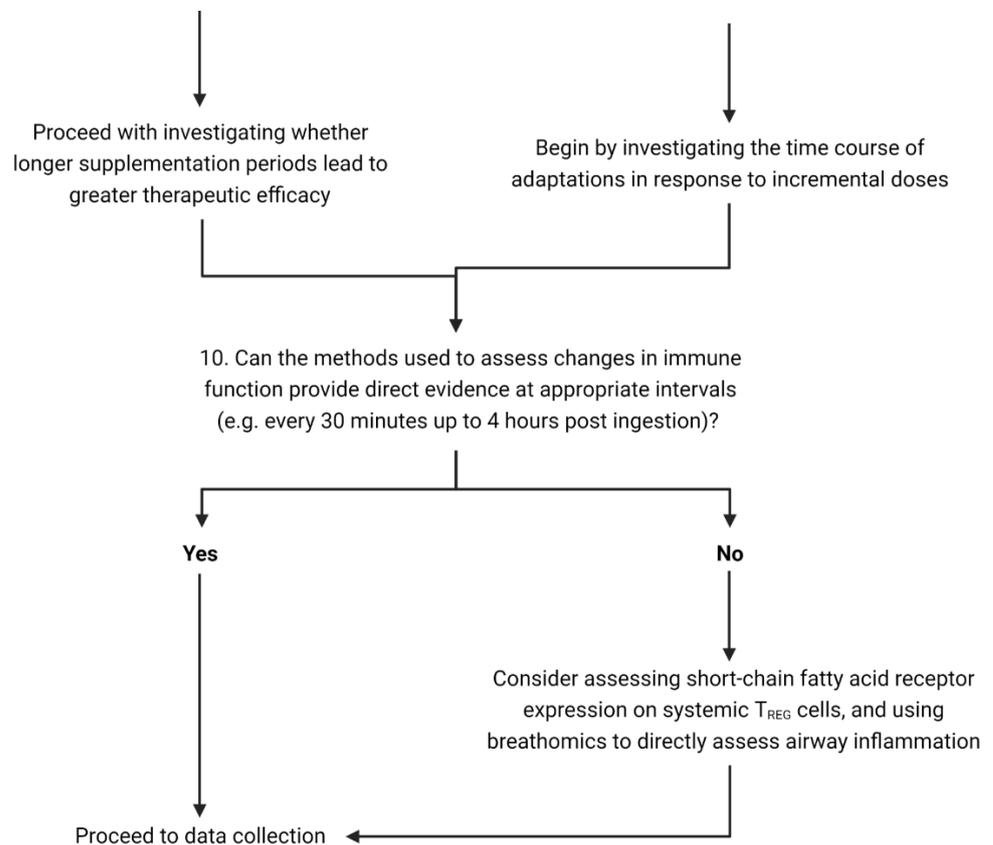
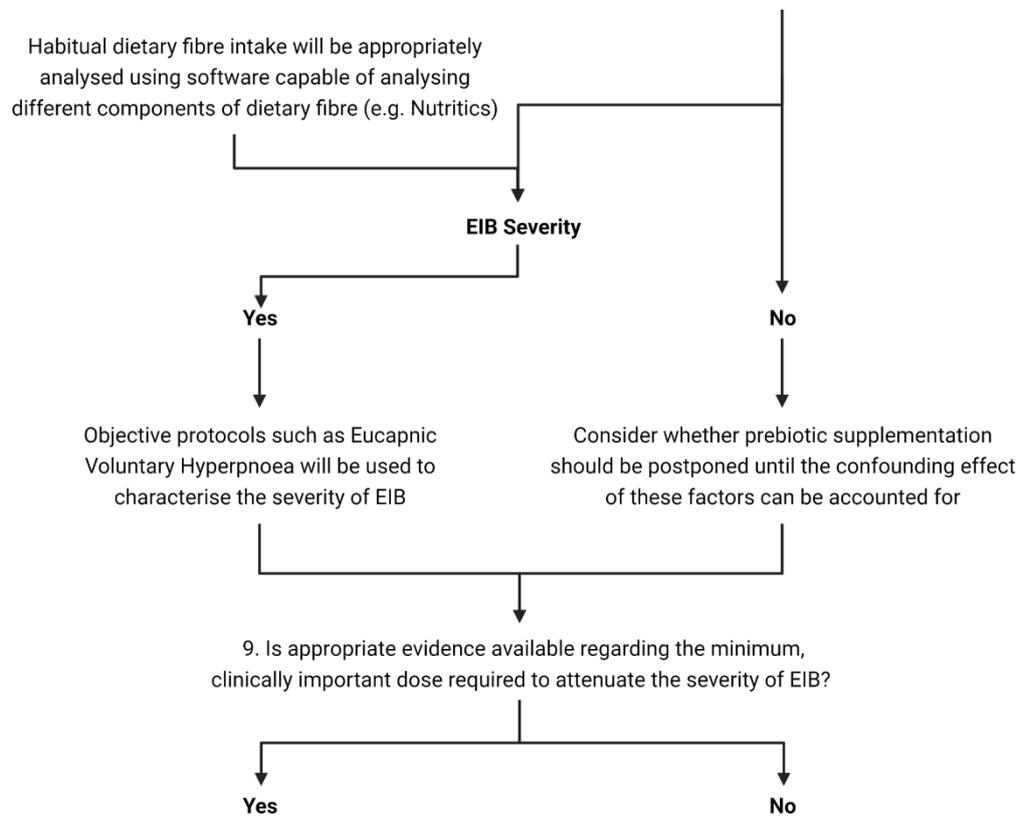


Figure 7.1 A flow diagram illustrating critical considerations for future research when attempting to explore the role of gut microbial dysbiosis and prebiotic supplementation in different asthma subtypes. Created with www.BioRender.com.
NMR nuclear magnetic resonance.

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Appendices

Abstract (Loose Copy 1)

Paul Lester

Doctor of Philosophy (PhD)

The Gut Microbiota in the Pathophysiology of Hyperpnoea-Induced Bronchoconstriction: Investigating the Effects of Short and Long-Term Prebiotic Supplementation.

In adults with allergic asthma, clinical and inflammatory parameters have been associated with features of gut microbial dysbiosis. This has led researchers to investigate whether the severity of asthma can be attenuated through gut-microbiota mediated nutritional interventions, such as prebiotics. Strikingly, following 3-weeks of supplementation with a galactooligosaccharide-based prebiotic, clinically relevant, participant perceivable improvements have been observed in adults with moderate-severe exercise-induced bronchoconstriction. However, given that gut bacterial composition has not been assessed in adults with EIB, it remains to be determined whether prebiotics attenuate the severity of EIB via the gut microbiota. Furthermore, the minimum, clinically important dose required to attenuate the severity of EIB remains to be determined. To address these questions, the current thesis investigated whether adults with EIB displayed features of gut microbial dysbiosis, and whether short-term and long-term supplementation with a low dose galactooligosaccharide-based prebiotic attenuated the severity of EIB.

The first experimental chapter (Chapter IV) investigated whether adults with moderate EIB displayed features of gut microbial dysbiosis through fluorescent *in situ* hybridisation (FISH). A total of seven ($n = 7$) adults with EIB, and seven ($n = 7$) controls took part. Participants provided a faecal sample to assess gut bacterial composition, a blood sample to assess markers of systemic inflammation, and completed the EVH protocol to determine the presence and severity of EIB. Despite significant increases in the percentage decrease in FEV₁ post-EVH (median, IQR: 38, [26-41] vs. 6, [4-7%]; $p = 0.002$), and resting blood eosinophils (8 ± 2 vs. $4 \pm 2\%$; $p = 0.008$), no features of gut microbial dysbiosis were reported in adults with moderate EIB across any bacterial groups when compared to controls ($p > 0.05$). It is possible the current method used to profile the gut microbiota lacked the required

sensitivity to detect features of gut microbial dysbiosis. In contrast to the current method, previous research has used metagenomics-based approaches to profile the gut microbiota of adults with allergic asthma to the species and strain levels, identifying features of gut microbial dysbiosis. This study highlights the importance of using techniques capable of profiling the gut microbiota to the appropriate phylogenetic depth.

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Key Words: Gut Microbiota, Asthma, Exercise/Hyperpnoea-Induced Bronchoconstriction, Prebiotic Supplementation, Galactooligosaccharides, Pulmonary Function, Eosinophils, Regulatory T Cells, T-Helper Type-2 (T_{H2}) Cells

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Paul Lester

Doctor of Philosophy (PhD)

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In adults with allergic asthma, clinical and inflammatory parameters have been associated with features of gut microbial dysbiosis. This has led researchers to investigate whether the severity of asthma can be attenuated through gut-microbiota mediated nutritional interventions, such as prebiotics. Strikingly, following 3-weeks of supplementation with a galactooligosaccharide-based prebiotic, clinically relevant, participant perceivable improvements have been observed in adults with moderate-severe exercise-induced bronchoconstriction. However, given that gut bacterial composition has not been assessed in adults with EIB, it remains to be determined whether prebiotics attenuate the severity of EIB via the gut microbiota. Furthermore, the minimum, clinically important dose required to attenuate the severity of EIB remains to be determined. To address these questions, the current thesis investigated whether adults with EIB displayed features of gut microbial dysbiosis, and whether short-term and long-term supplementation with a low dose galactooligosaccharide-based prebiotic attenuated the severity of EIB.

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Appendix A: GP Practice Research Invitation Letter

Research Study – Improving the Clinical Management of Exercise-Induced Asthma through Prebiotics

Nottingham Trent University is looking for GP practices to be involved in a trial that is investigating novel methods of improving the clinical management of exercise-induced asthma through the use of prebiotics.

Prebiotics feed certain good bacteria in the gut, increasing their ability to regulate inflammatory responses in the airways during asthma. As a result, dietary strategies aimed at increasing the number of good bacteria in the gut have emerged as an exciting new strategy to increase the exercise capacity and overall quality of life of people with asthma. This research will develop our current understanding of which types of asthma, prebiotic supplementation would be most suitable for.

Patients will receive an in-depth assessment of their asthma using objective techniques not typically available as part of routine care. Patients will be provided with a personalised report. They will be reimbursed for the costs of travel to Nottingham Trent University's Clifton Campus.

Practice staff will be asked to review their electronic patient records against the trial's inclusion and exclusion criteria, and to distribute a Research Invitation Letter and Participant Information Sheet to potentially eligible participants. The practice will receive £10.00 for every participant successfully recruited into the trial. The costs of any documents to be displayed at/distributed by the practice will be covered by the researchers.

The study has received full approval from the NHS Health Research Authority.

If you are interested in finding out more, please contact:

Mr Paul Lester (PhD Student & Principal Investigator)

Paul.Lester2015@my.ntu.ac.uk (Telephone: 07564329039)

Appendix B: Participant Research Invitation Letter



Dear Sir/Madam,

You are invited to take part in research being conducted by the Sport Science department at Nottingham Trent University into Asthma.

The research, titled:

The Effects of HOST-DM059 Prebiotic Supplementation on Gut Bacterial Metabolites, and Markers of Systemic Inflammation in Adults with and without Hyperpnoea-Induced Bronchoconstriction: A Double-Blind, Placebo-Controlled, Crossover Trial.

Is looking to investigate the role of the gut bacteria in the development of asthma. The trial will explore if adults with asthma display abnormalities in the activity of their gut bacteria. We will investigate if changing a person's diet through prebiotic supplementation can improve gut bacterial activity, and whether this leads to improvements in the management of asthma. The aim of the current project is to increase current understandings of the development of asthma, and to explore the therapeutic benefits of complimentary nutritional supplements in reducing the severity of asthma.

Please ask a member of your clinical care team for a copy of the Participant Information Sheet (if not already provided) for further information on the study and how you could get involved.

If you are interested in taking part, or have any questions please contact the Chief Investigator:

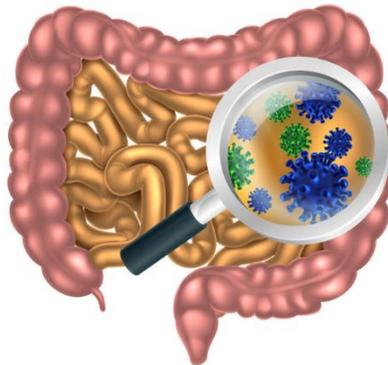
Mr. Paul Lester (Chief Investigator & PhD Student)

Nottingham Trent University, Sport Science Department

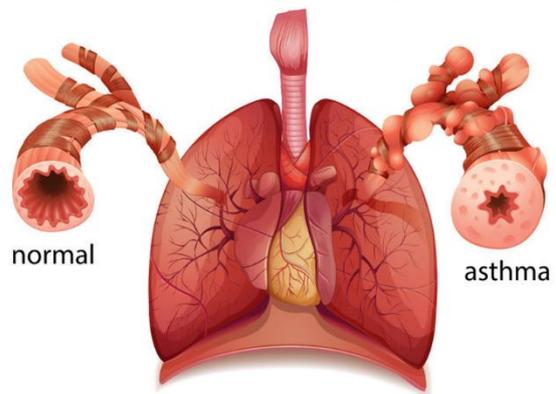
Telephone: 07564329039, E-Mail: Paul.Lester2015@my.ntu.ac.uk.



Can This



Influence This?



Can changing your diet increase the activity of beneficial gut bacteria and improve asthma management?

YOU COULD HELP US FIND OUT!

Aged 18-50? Whether you have mild asthma or not,
please get in touch!

Paul Lester (PhD Student & Chief Investigator)

Nottingham Trent University

Paul.Lester2015@my.ntu.ac.uk, 07564329039.

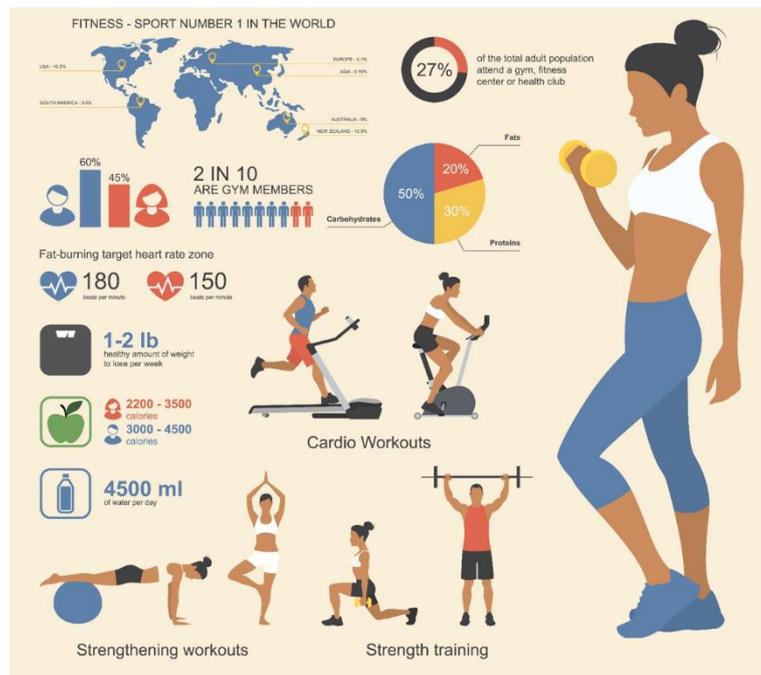


Appendix D: Participant Recruitment Leaflet



Exercise & Health Awareness!

On average people gain 6.8 kg (15 pounds) of weight
in the first year of university



Do you want to stay/get fit and healthy at university?

Come and chat to the Sport Science department!



Appendix E: Participant Information Sheet

Mr. Paul Lester

Erasmus Darwin Building Room 259

Nottingham Trent University

Clifton Campus

Clifton Lane

Nottingham

NG11 8NS

Telephone: 07564329039

E-Mail: Paul.Lester2015@my.ntu.ac.uk

Participant Information Sheet

Study Title:

The Effects of HOST-DM059 Prebiotic Supplementation on Gut Bacterial Metabolites, and Markers of Systemic Inflammation in Adults with and without Hyperpnoea-Induced Bronchoconstriction: A Double-Blind, Placebo-Controlled, Crossover Trial.

Please read the following information before discussing question/concerns with the chief investigator.

Participation is voluntary. You may choose not to participate, or withdraw your participation at any point, without having to specify a reason.

Introduction & Purpose

This study will investigate how prebiotic supplementation affects the severity of exercise-induced airway restriction in adults with asthma, and healthy controls. Prebiotics are a carbohydrate that feed the 'good bacteria' in the gut. The gut bacteria play a vital role in supporting our immune system. A reduced number/activity of 'good bacteria' may lead to the development of asthma. This study aims to increase our understanding of how the gut bacteria contribute to the development of asthma. The results of this study may lead to a better understanding of the mechanisms of asthma, helping to develop new strategies based on dietary changes to assist with asthma management.

Participant Requirements

If you object to any of the procedures in the current trial, please inform the chief investigator as soon as possible. Your GP will be contacted to inform them of your participation in the current study.

Inclusion Criteria

To be eligible to take part, you must:

- Be 18-50 years of age at the date of your first visit.
- Have a body mass index (BMI) of 18.5-25 kg/m² (we can work this out for you using your height and body mass).

- Be physically active (completing 3 or more exercise sessions a week lasting at least 45 minutes each).
- Be a non-smoker.
- Your asthma is defined as Steps 1, 2, or 3 based on British Thoracic Society guidelines.
- You must have a current medication prescription from your GP if diagnosed with asthma (e.g., maintenance or reliever inhalers including short-acting β_2 -agonist).
- You must in the researcher's opinion, be able and willing to follow all trial requirements.

Exclusion Criteria

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

- Your asthma is defined as Steps 4 or 5 based on British Thoracic Society guidelines.
- You suffer from asthma but do not have a **current** medication prescription from your GP (e.g., maintenance and reliever inhalers).
- You regularly consume Omega-3 supplements, and/or eat high levels of Omega-3 (e.g., more than 1-2 portions of oily fish such as salmon or mackerel a week).
- You take a daily dose of aspirin or other non-steroidal anti-inflammatory drugs such as ibuprofen.
- Have consumed prebiotics and/or probiotics (supplements), drugs that affect gastrointestinal mobility, or laxatives in the 4 weeks before signing the consent form.
- You are currently taking a daily dose of anti-histamine, which you could not temporarily avoid for 72 hours before each testing session without exacerbation of symptoms.
- Unable to temporarily avoid taking asthma medication(s) before study visits without an exacerbation of symptoms, e.g., short-acting β_2 -agonists (8 hours before), and standard inhaled corticosteroids (12 hours before).
- You have a vegetarian or vegan diet.
- You have been previously diagnosed with chronic obstructive pulmonary disease (COPD), emphysema, chronic bronchitis, or similar respiratory (breathing-related) illness.
- You have ever been hospitalised due to asthma (e.g., intensive care unit).
- You have received treatment with oral corticosteroids/been admitted to hospital during the past 12 months for your asthma.
- Your asthma medication is increased/stepped-up during participation in the current study (e.g., from Step 1 to Step 2, Step 2 to Step 3 etc.).
- You have a history of heart failure, pulmonary hypertension, embolism, or other pulmonary heart disease.
- You have a history of recurrent chest infections.
- You currently smoke.
- You are pregnant, planning to become pregnant during the study, or lactating.
- You have had an acute infection in the last four weeks, and/or major operation in the past four months.
- You have a history of gastrointestinal drug reaction.
- You have taken antibiotics in the past 3 months.

- You have a history or current evidence of gastrointestinal disease (e.g., chronic constipation, diarrhoea, irritable bowel syndrome, Crohn's disease).
- You have recently taken part in other research projects. Please notify the chief investigator.
- You are or you believe you may be lactose intolerant.
- You regularly take antioxidant supplements.
- Standard multivitamin and mineral supplements are acceptable, as long as the product label states the recommended Dietary Reference Values (DRV's).
- If a single antioxidant supplement (e.g., Vitamin C), is more than the recommended daily DRV's this must be checked with the chief investigator.

Study Location

You will be asked to attend Nottingham Trent University's Clifton Campus on 6 occasions over a 10-week period. Visit one will last 30 minutes, visit two 60 minutes, and visits 3-6 approximately 2 hours (around 8-10 hours in total).

Testing Restrictions

Participants with asthma will be allowed to continue to use their medication as needed during the course of the study. However, when we conduct tests (visits 2-6) to assess the severity of exercise-induced airway restriction, you will be asked to temporarily refrain from using your medication(s), consuming alcohol/caffeine, and strenuous exercise beforehand as outlined below (See Table 1). Restrictions for anti-histamine, alcohol, caffeine, and strenuous exercise apply to all participants.

Restriction durations for asthma medication will vary from 8-96 hours before each visit, depending on the type of inhaler(s) you have been prescribed. During visit one, you will be asked to provide the chief investigator with the generic name (e.g., Salbutamol), and brand name (e.g., Ventolin®) of all inhalers, as well as other treatments (e.g., oral steroid tablets). You will be provided with an asthma inhaler information leaflet. This will explain how long to avoid taking each inhaler before your visits.

	Restriction Duration (Before Visits)
Anti-Histamines	72 Hours
Caffeine	24 Hours
Alcohol	48 Hours
Strenuous Exercise	24 Hours

These are standard requirements for assessing exercise-induced airway restriction. Participants with asthma may experience a mild-moderate worsening of their symptoms (e.g., chest tightness, wheezing, coughing, shortness of breath) during temporary medication restriction, and experimental trials. **If you struggle to control your symptoms during this period, you must take up your medication immediately, and subsequently will be withdrawn from the study.** The reduction in medication use may have a very short-term impact on your exercise capacity. This will be reversed following the testing session and when you take your medication again. Please notify the chief

investigator of any changes to your medication. If you have an asthma diagnosis, but fail to present your reliever medication to the research team during your visits, you will not complete the Eucapnic Voluntary Hyperpnoea (EVH Protocol), on that occasion (explained in more detail below under 'Research Protocol'). You will be invited back at a later date, and asked to bring your medication with you. If you have a previous diagnosis of asthma, but no current prescription for a reliever inhaler, you will be excluded from the study until you can present a current prescription.

Study Protocol

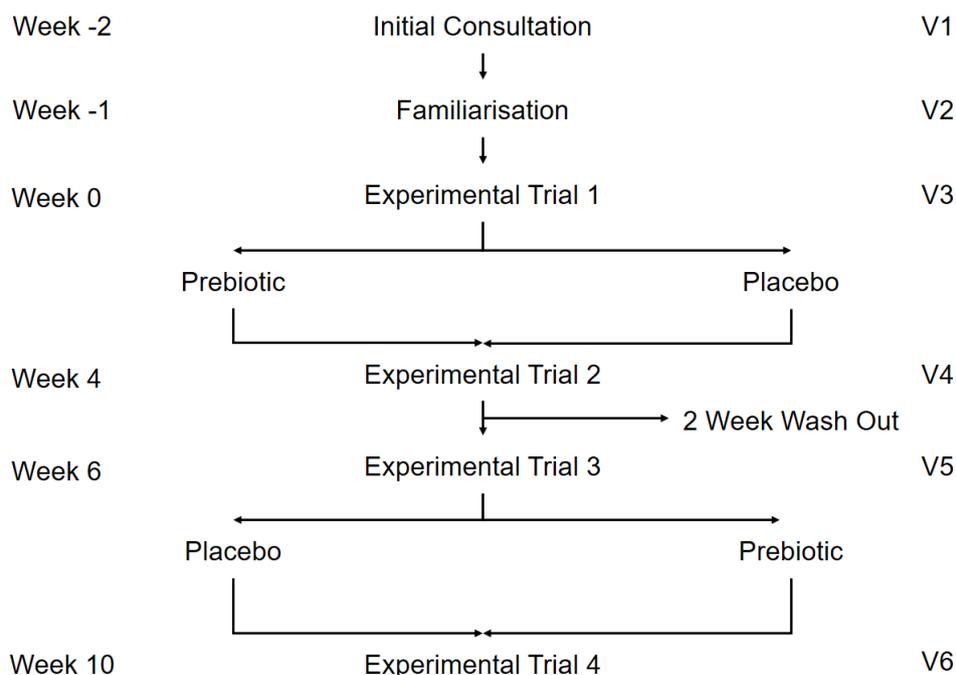


Figure 1.0 Outline of the study design and duration. V = Visit Number.

You be asked to continue with your regular diet throughout the course of the study. You will be asked to record your diet 24 hours before visit 2, and to repeat this diet 24 hours before visits 3-6, using a weighed food log.

Participants will be asked to consume their final meal 3 hours before visits 3-6, and to consume 500 millilitres of water one hour beforehand.

Visit 1: Initial Consultation

The chief investigator will explain what participation would involve, and how data obtained from participants will be used/stored. The procedures for documenting adverse/serious adverse events throughout the study will be explained. You will have the opportunity to ask any questions/raise any concerns regarding taking part. If satisfied, you will be asked to provide written informed consent to participate, and to complete a health screen and history questionnaire, both of which will be reassessed at each visit. You will be provided with instructions for your second visit. The chief investigator will explain how and when to complete the 24-hour nutritional intake record.

Visit 2: Familiarisation

Your height and body mass will be measured, and BMI calculated. You will be familiarised with all the measures to be completed during visits 3-6. The chief investigator will explain how the nutritional supplements will be administered, and how/when to complete certain questionnaires/scales throughout the study. These will include a questionnaire to assess your perceptions of asthma control, and adherence to your asthma medication/the nutritional supplements. The procedures for collecting urine and blood samples during visits 3-6 will be explained. To develop correct technique, you will have the opportunity to practice assessments of lung function at rest. This will involve breathing out through a mouthpiece connected to the computer as quickly as possible for 6 seconds, up to a maximum of 8 times. The technique is similar to peak flow assessments which you may have completed with your GP.

Exercise-Induced Airway Restriction Procedure:

The Breathing Challenge Test/Eucapnic Voluntary Hyperpnoea (EVH Protocol)

All participants will complete a practice of the EVH protocol, providing resting assessments of lung function meet the testing criteria. Participants with asthma must have their reliever medication available. The EVH protocol is a seated, 6-minute hyper-ventilatory breathing challenge used to assess the severity of exercise-induced airway restriction. The protocol is designed to cause airway restriction/inflammation in susceptible individuals, and involves maintaining a breathing rate of 85% of your maximum breathing capacity (designed to simulate the breathing demands of high-intensity exercise), whilst breathing in a dry (~ 3% relative humidity) gas mixture with a fixed CO₂ concentration. The purpose of the dry gas mixture is to maintain eucapnia (normal blood CO₂ concentrations to prevent fainting/loss of consciousness), and to simulate the extremes of what individuals susceptible to exercise-induced airway restriction might experience during exercise. Targets for breathing rate will be provided throughout the test to help maintain intensity. Verbal encouragement and coaching will be provided throughout. Following completion of the EVH protocol assessments of lung function will be repeated in duplicate at 3, 6, 10, 15, 20, and 30 minutes post EVH, to classify the severity of exercise-induced airway restriction. You may request a report of the EVH assessment, which can be passed on to your GP to inform future treatment.

Visits 3-6: The Main Experimental Trials

For an overview of the procedures during each experimental trial, please see Figure 2.0 below.

START —————> END

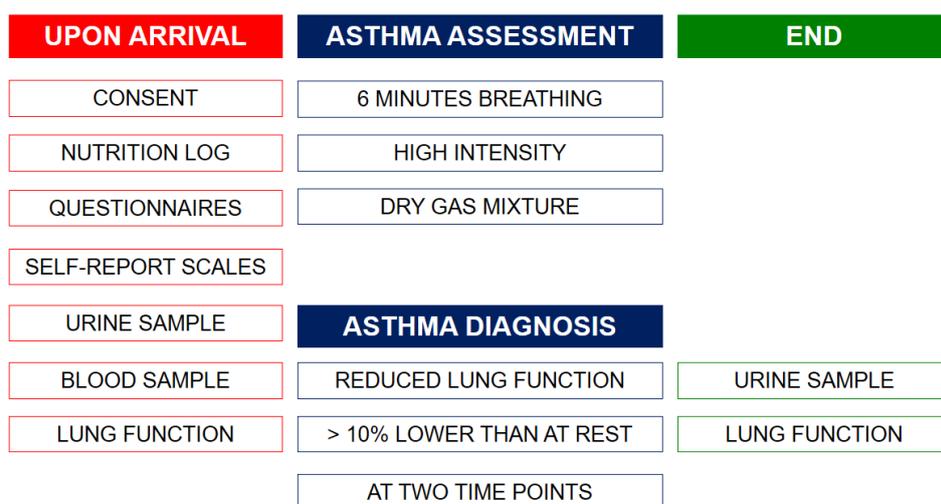


Figure 2.0 Experimental Trial Outline.

- Measurements of height, body mass, BMI, lung function, and the EVH protocol will be conducted during each experimental trial as per visit 2.
- Participants will be asked to present their 24-hour diet log and reliever medication to the chief investigator upon arrival.
- You will be asked to complete a questionnaire to assess asthma control, and a scale to monitor asthma medication/nutritional supplement adherence.
- Participants will be asked to provide a urine sample, and blood sample at rest, and a second urine sample 60 minutes after the EVH protocol. The same procedure will be followed during visits 3-6.

Blood Sample Collection

Blood samples will be collected using a needle inserted into a vein in the mid-arm by trained personnel following sterile protocols (similar procedure to blood donation). The procedure will be explained in full before sample collection. Participants will be asked to provide written informed consent for the procedure to be conducted. You will be asked to state any fears/phobias (e.g., blood/needles), or allergies (e.g., elastoplast, alcohol, latex) before blood samples are collected. A 40ml blood sample will be taken during each visit (approximately 6-8 teaspoons of blood). This will be used to assess markers of immune function, inflammation, and intestinal permeability.

Urine Sample Collection

Urine samples will be obtained using a bathroom adjacent to the laboratory in private, unaccompanied by the researcher, to analyse markers of gut bacterial activity/human immune function.

Nutritional Intervention

You will be asked to consume two nutritional supplements in total, for 4 weeks each, separated by a 2-week washout period (no consumption of nutritional supplements). The total duration of the nutritional intervention is 10 weeks. The supplements will include a prebiotic, and a taste/appearance-matched placebo. The prebiotic may contain up to 16% lactose. The supplements will be provided in sequential order using an allocation ratio. Neither you nor the chief investigator will be aware of which supplement will be consumed during each supplementation phase. You will be asked to consume one powdered sachet in the morning at the same time of day. Sachets can be consumed in tea, coffee, fruit juice, or sprinkled over cereal etc. The method of consumption must be kept consistent throughout.

You will be asked to complete a questionnaire to assess asthma control, and a scale to assess medication/nutritional supplement adherence each week throughout the intervention. Continued supply of the nutritional supplement will not be provided by the research team after trial completion. The chief investigator will advise participants where to purchase similar products if requested.

After Participation: Formal Debrief

During your final visit, the chief investigator will explain how data obtained from you will be used (e.g., research publications, conference presentations), and how you will be able to access any publications/reports of the research. Procedures for withdrawing yourself and/or your data following trial completion will be explained.

Participant Responsibility

You are kindly asked to complete all documents accurately, and to follow all control measures/testing restrictions/intervention guidelines throughout the study. If completed accurately, the information from this study may help develop new methods of managing asthma for people in the future. Consume the nutritional supplements as instructed. If sachets are missed, do not compensate by consuming multiple sachets. Proceed with the instructed dosing schedule, and document any discrepancies. Please notify the chief investigator of any deviations from these instructions, and/or changes relevant to the inclusion/exclusion criteria (e.g., antibiotics prescription).

Potential Benefits

You will undergo an in-depth assessment of exercise-induced airway restriction. In terms of the nutritional supplements, no specific benefits are anticipated.

Risks

Blood Sampling

Some discomfort may be caused during blood sample collection. The procedure has been risk assessed and in relation to current experience at NTU is considered safe. Staff who perform the procedure are appropriately trained, and sterile procedures are used at all times. The possibility of adverse events such as bruising, swelling are low given the research teams experience.

The EVH Protocol

The EVH protocol will cause airway restriction/inflammation in susceptible individuals. The EVH protocol will be stopped early if you experience excessive breathlessness/discomfort. During the test you may experience symptoms of wheezing, coughing, chest tightness, and shortness of breath. These symptoms will usually be reversed over the 30 minutes after the EVH protocol. At any point during/after the EVH protocol, you will be free to use your reliever medication which should relieve any remaining symptoms. These symptoms, although very unlikely, may result in a slight detriment to exercise performance immediately following the test. You will not be allowed to complete the EVH protocol without your reliever medication. You will remain in the laboratory until your lung function recovers to within 10% of your resting value. 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.

Nutritional Supplements Side-Effects

Although mainly associated with consuming a higher daily dose, or in participants with gastrointestinal disorders, temporary gastrointestinal discomfort may be experienced (e.g. abdominal bloating, flatulence, abdominal cramps/pains, nausea).

Injury & Trial Complaints Procedure

In the event of injury/illness caused by negligence by the research team, you may contact the Research Sponsor for independent advice (Professor Barbara Pierscionek, Associate Dean for Research, Barbara.Pierscionek@ntu.ac.uk).

Travel Reimbursement

Travel reimbursements will be provided for all participants in accordance with NTU's policy (e.g., £0.25 per mile for travel by car, regardless of distance covered).

Pregnancy During Participation

If subjects become pregnant during the trial, they will be withdrawn immediately.

Data Protection

Electronic data will be collected using a unique code, preventing participant identification, and stored on password-protected computers/user accounts at Nottingham Trent University, using a secure online server only accessible by the research team. Data may be retained for up to 5-10 years, but will be destroyed when no longer required, in line with Data Protection Legislation. Destruction of data excludes Written Informed Consent Forms. If publications containing your data have already been submitted/approved following your withdrawal from the trial, the chief investigator will ensure your data is not included in any future publications. If you would like a copy of the data obtained from you, please contact the Chief Investigator. Confidential, electronic trial data (not personal information) will be transferred in pseudonymised format to HOST Therabiomics, based in Jersey, The Channel Islands. HOST Therabiomics has provided the nutritional supplements for the trial. Data

would be transferred as part of auditing procedures. Identification of individual participants will not be possible. Data will be retained for the duration of the trial/until publication of the research.

Supplement Disclaimer

As part of this study, you will be consuming multiple nutritional supplements. If you are an elite sportsperson (e.g., international or national standard who may undergo 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 accidentally with the prohormones of testosterone and nandrolone. Even well-known brands from the 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.

Participant Signature:	Date:
Chief Investigator Signature:	Date:

If you have any questions/concerns, please contact the research team using the contact details below.

Mr. Paul Lester (Chief Investigator)	Dr. Graham Sharpe (Senior Researcher)
Nottingham Trent University	Nottingham Trent University
School of Science and Technology	School of Science and Technology
Erasmus Darwin, Room 259	Erasmus Darwin, Room 245
Clifton, Nottingham	Clifton, Nottingham
NG11 8NS	NG11 8NS
Telephone: 07564329039	Telephone: 01158483340
E-Mail: Paul.Lester2015@my.ntu.ac.uk	E-Mail: Graham.Sharpe@ntu.ac.uk

Appendix F: Written Informed Consent Form

IRAS Project ID: 233556

Mr. Paul Lester

Participant Identification

Erasmus Darwin Building Room 259

Number:

Nottingham Trent University

Clifton Campus

Clifton Lane, Nottingham

NG11 8NS

Telephone: 07564329039

E-Mail: Paul.Lester2015@my.ntu.ac.uk

CONSENT FORM

Project Title:

The Effects of HOST-DM059 Prebiotic Supplementation on Gut Bacterial Metabolites, and Markers of Systemic Inflammation in Adults with and without Hyperpnoea-Induced Bronchoconstriction: A Double-Blind, Placebo-Controlled, Crossover Trial.

Researcher Name:

Mr. Paul Lester (PhD Student)

Initials

1. I confirm that I have read the information sheet dated: (Version 2.9, Date: 25-11-17) for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered satisfactorily.
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.
3. I understand that pseudonymised data collected from me during the study, may be looked at by individuals from the Imperial College of Science, Technology, and Medicine, Clasado Biosciences, and HOST Therabiomics, or from regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my pseudonymised data.

Appendix G: Written Informed Consent From (Venepuncture)

Mr. Paul Lester

Erasmus Darwin Building Room 259

Nottingham Trent University

Clifton Campus

Clifton Lane

Nottingham

NG11 8NS

Telephone: 07564329039

E-Mail: Paul.Lester2015@my.ntu.ac.uk

BLOOD SAMPLE FORM FOR VENOUS BLOOD SAMPLES ONLY

PARTICIPANTS NAME:

PARTICIPANTS D.O.B:

DATE OF DONATION	TAKEN BY	COMMENTS

- Is there any reason why you cannot give blood for the purposes of this study?
- Have you ever experienced any problems giving blood, e.g., fainting or dizziness?
- I am willing to provide blood samples for the purposes of this study:

PARTICIPANTS SIGNATURE:

DATE:

ANALYSIS:

CODE:

Appendix H: The Asthma Control Questionnaire-7 (ACQ-7[©])

The Asthma Control Questionnaire [©]

The Asthma Control Questionnaire is Copyrighted (Juniper et al., 1999).

PARTICIPANT CODE	SUPPLEMENT LETTER	EXPERIMENTAL TRIAL/WEEK NUMBER	DATE

Circle the number of the response that best describes how you have been during the past week:

1. On average, during the past week, how often were you woken by your asthma during the night?

0	Never
1	Hardly Ever
2	A Few Minutes
3	Several Times
4	Many Times
5	A Great Many Times
6	Unable to Sleep Because of Asthma

2. On average, during the past week, how bad were your asthma symptoms when you woke up in the morning?

0	No Symptoms
1	Very Mild Symptoms
2	Mild Symptoms
3	Moderate Symptoms
4	Quite Severe Symptoms
5	Severe Symptoms
6	Very Severe Symptoms

3. In general, during the past week, how limited were you in your activities because of your asthma?

0	Not Limited at All
1	Very Slightly Limited
2	Slightly Limited
3	Moderately Limited
4	Very Limited
5	Extremely Limited
6	Totally Limited

4. In general, during the past week, how much shortness of breath did you experience because of your asthma?

0	None
1	A Very Little
2	A Little
3	A Moderate Amount
4	Quite a Lot
5	A Great Deal
6	A Very Great Deal

5. In general, during the past week, how much of the time did you wheeze?

0	Not at All
1	Hardly Any of the Time
2	A Little of the Time
3	A Moderate Amount of Time
4	A Lot of the Time
5	Most of the Time
6	All of the Time

6. On average, during the past week, how many puffs of Short-Acting Bronchodilator (e.g., Ventolin) have you used on each day?

0	None
1	1-2 Puffs Most Days
2	3-4 Puffs Most Days
3	5-8 Puffs Most Days
4	9-12 Puffs Most Days
5	13-16 Puffs Most Days
6	> 16 Puffs Most Days

7. FEV ₁ Pre-Bronchodilator:	0	> 95% Predicted
FEV ₁ Predicted:	1	95%-90%
FEV ₁ % Predicted:	2	89%-80%
	3	79%-70%
	4	69%-60%
	5	59%-50%
	6	< 50% Predicted

Appendix I: The Medication Adherence Report Scale for Asthma (MARS-A)®

The Medication Adherence Report Scale for Asthma (MARS-A)®

Participant Code	Supplement Letter	Experimental Trial & Week Number	Date

© The Medication Adherence Report Scale for Asthma (MARS-A) is Copyrighted by the originator: Dr. Robert Horne (Cohen et al., 2009).

Please complete the sections below at the end of each week of the nutritional intervention. Please complete Questions 1-10 in relation to your Preventer, not Rescue/Reliever Medication.

How often do you do the following?

Answers: 1 = Always, 2 = Often, 3 = Sometimes, 4 = Rarely, 5 = Never

QUESTIONS	RATING (1-5)
1. I only use my medication [insert name of medication] when I need it.	
2. I only use it when I feel breathless.	
3. I decide to miss out a dose.	
4. I try to avoid using it.	
5. I forget to take it.	
6. I alter the dose.	
7. I stop taking it for a while.	
8. I use it as a reserve, if my other treatment doesn't work.	
9. I use it before doing something which might make me breathless.	
10. I take it less than instructed.	
	TOTAL

Additional Questions

11. Have you missed any doses of your prescribed medication(s) over the last week?

Yes No

If yes, please state during which week/phase, and day of the nutritional intervention this occurred, which dose(s)/medication(s) this relates to, specifying any particular reasons for this (please see example below):

Intervention Week Number/Phase: 2/1

Day(s): Tuesday, Friday

Dose(s) Missed: Morning & Afternoon, Morning

Medication Name(s): Budesonide

Reasons:

I was away from home at a friend's house and forgot to take my medication with me.

I felt as though I did not need my medication on that particular day.

Intervention Week Number/Phase:

Day(s):

Dose(s) Missed:

Medication Name(s):

Reasons:

12. Have you missed any sachets of the nutritional supplements over the last week?

Yes No

If yes, please state during which week/phase, and day of the nutritional intervention this occurred, and how many sachets were missed, specifying any particular reasons for this:

Intervention Week Number/Phase: 2/1

Day(s): Friday

Supplement Label: L

Sachet(s) Missed: 1

Reasons:

I was called away to work last minute and forgot to take my supplements with me.

Intervention Week Number/Phase:

Day(s):

Supplement Label:

Sachet(s) Missed:

Reasons:

Appendix J: The Asthma Exacerbation Protocol

Asthma Exacerbation Response Protocol

- The following guidelines are not to be implemented for patients on a 'SMART' or 'MART' Regime.

1.0 Response Protocol Preparations

- Ensure researchers, participants, and supporting technicians/first aiders have a copy of the 'Asthma Attack Guidelines' with them at all times.
- Ensure a suitable chair is available in the laboratory.
- Make sure a stopwatch is on hand for timing reliever inhaler usage, and monitoring the time frame from contacting the emergency services to paramedic/ambulance arrival.

2.0 Asthma Attack Indicators

- The participant's reliever/rescue medication is not helping, or lasting/providing relief for over four hours.
- The participants symptoms are getting worse (e.g., cough, breathlessness, wheeze, and/or tight chest).
- The participant is too breathless, and finds it difficult to speak, eat, or sleep.
- The participant begins to breathe faster, and feels as though they cannot get their breath properly.

3.0 The EVH Protocol Termination Criteria

- Follow standard guidelines during the EVH protocol (e.g., if the participant experiences significant symptom exacerbation, or for any reason wishes to stop the test, terminate the test immediately).

3.1 Post EVH Pulmonary Function Termination Criteria

- Pulmonary function will be reassessed in duplicate at 3, 6, 10, 15, 20, and 30 minutes post EVH.
- If FEV₁ is < 10% of baseline at two consecutive time points (e.g., 3- and 5-minutes post EVH), terminate the test immediately.
- Ask the participant to take (at least) 200 mcg of Inhaled Salbutamol.
- Measure Flow-Volume Loops/Pulmonary Function 10 minutes after Salbutamol administration.
- If FEV₁ is ≥ 10% of baseline 10 minutes post Salbutamol administration, ask the participant to take a further 200 mcg dose of Inhaled Salbutamol.
- **Measure Flow-Volume Loops/Pulmonary Function 10 minutes after the second Salbutamol administration.**

- The participant must not leave the laboratory until FEV₁ has recovered to within 10% of baseline.
- **If FEV₁ continues to fall despite the administration of Salbutamol as described above, proceed with the guidelines in 4.0 below.**

4.0 Asthma Attack Response Protocol

1. Sit the participant up straight, do not lie down. Provide reassurance and try to keep them calm.
2. Instruct the participant to take one puff of their reliever/rescue inhaler (usually a blue color), every 30-60 seconds, up to a maximum of 10 puffs. A spacer will be available for metered dose inhalers.
3. If at any point the participant feels worse whilst using their inhaler, or they do not feel any better after 10 puffs, or you are worried/concerned for their welfare, **Call 999 for an Ambulance. If the participants rescue/reliever inhaler is unavailable/unable to be administered correctly, or you are concerned, Call 999 for an Ambulance.**
4. If the ambulance is taking longer than 15 minutes, repeat step 2.

Reference List

Asthma UK. (2016). *Asthma Attacks*. Retrieved July 25, 2017. From <https://www.asthma.org.uk/advice/asthma-attacks/>.

Appendix K: Health Screen & History Questionnaire

Health Screen & History Questionnaire

Participant Name	Participant Code	Visit/Trial Number	Date

Please complete this brief questionnaire to confirm fitness to participate:

For certain questions, further information/clarification beyond 'Yes/No' answers may be required. For any additional information you feel the research team should be aware of, please use Table 1 at the end of the questionnaire following the example provided.

1. **At present**, do you have any health problem(s) 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

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

- (a) consult your GP Yes No
- (b) attend a hospital outpatient department Yes No
- (c) be admitted to hospital Yes No

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

- (a) Convulsions/Epilepsy Yes No
- (b) Asthma/Exercise-Induced Bronchoconstriction (EIB) Yes No
- (c) Eczema Yes No
- (d) Diabetes Yes No
- (e) A Blood Disorder Yes No
- (f) Head Injury Yes No

- (g) Digestive Problems
(e.g., Chronic Constipation, Diarrhoea, Irritable Bowel Syndrome, Crohn's Disease) 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

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

Females Only:

- 8.1 Are you currently on any form of contraceptive Yes No
If yes, please state _____

- 8.2 Have you been taking the form of contraception stated above for at least the last 6 months?

Yes No

If known, please state consumption duration _____

8.3 Have you had a consistent menstrual cycle (e.g., between 26-30 days) for at least the last 3 months?

Yes No

Asthma Specific Questions:

9. When was your last Asthma Exacerbation (Attack) _____

10. Have you ever been admitted to hospital for your Asthma Yes No

11. Have you ever been prescribed a course of steroids for your Asthma
Yes No

12. Do you suffer from Asthma symptoms post exercise Yes No

13. Do you suffer from Asthma symptoms when exposed to dry/cold air
Yes No

14. Do you suffer from Asthma symptoms when exposed to allergens (pollen, animal fur, house dust mite etc.), pollution, or fungi?
Yes No

If so, please state which particular triggers exacerbate your symptoms (include any not stated above):

15. Have you had any pets throughout your life (e.g., dogs, cats) Yes No

If so, please specify how old you were (e.g., from 2-14 years old):

16. Did you/your mother ever visit farm yards or associated environments that allow exposure to animals (e.g., cows) during infancy/pregnancy? Yes No

17. Do you have any siblings?

Yes No

If so please specify if these are brother(s), sister(s), and if they are younger/older:

18. At what age were you diagnosed with Asthma?

19. Have you, or are you currently participating in Swimming, Ice-Skating, Cross-Country Skiing, or any General Endurance Based Events (e.g., Running, Road Cycling, Triathlon)?

If so, please specify when, and how long each of these activities were participated in for:

Infant History Questions:

20. Were you born early, on-time, or late in relation to your due date?

If known, please specify:

21. Were you classified as a low weight at birth?

If known, please specify and state your birth weight:

22. What was your mode of birth? (if known)

Natural Birth Caesarean Birth

If known, please specify (Caesarean Only): Elective Emergency

23. What was your infant feeding method? (if known)

Breast Milk Formula Milk Both

Antibiotic Questions:

24. Have you taken antibiotics in the past 3 months?

Yes No

25. Have you taken antibiotics in the past 12 months?

Yes No

26. Did you/your mother experience exposure to antibiotics during infancy/pregnancy?

Yes No

If known, please specify:

Table 1 Additional Question Information.		
Question Number	Section	Additional Information
3	B	I was diagnosed with Asthma as a child (around 6 years old), but have not needed by medication since I was 18. As a result, I have not attended regular check-ups since, and do not have a current rescue/reliever medication prescription.

Appendix L: 24-Hour Weighed Nutritional Intake Record

24 Hour Weighed Nutritional Intake Record

PARTICIPANT CODE	EXPERIMENTAL TRIAL	SUPPLEMENT LETTER

Diet diaries are a very important part of the study as they help us to interpret your nutritional intake. It is **essential that you eat as normal**; for the information to be of use to us, please do not make a special effort to change your diet during the study. The more detail you add to your food and drink descriptions, the more accurate our analysis will be so please add as many details as possible.

Below are some instructions that we ask you follow carefully. An example of a correctly completed diary has been included as a guide. Please contact the chief investigator if you have any questions. If needed, we will ask you to clarify any missing details during your next visit.

Key Information for Accurately Recording Food & Drink Consumption

1. **AT HOME ALL FOODS AND DRINKS MUST BE WEIGHED**. Include **everything** you **eat and drink** no matter how small the amount. Snacks, nibbles, table sauces, butters, spreads, cooking oils, and salad dressings are easily forgotten.
2. **Record as you eat and drink**. Please do not rely on memory at the end of the day.
3. If you are **eating out** it is important that you **take the diary with you** (do not rely on memory). If you will be eating out during the monitoring of nutritional intake, please notify the chief investigator. Further guidance for the accurate monitoring of portion sizes will be provided. If you cannot use the food scales, provide **details** of the **portion size and ingredients**. Use **household measures** to breakdown the dish into ingredients (e.g. one heaped tablespoon or steamed carrots). A **photo guide** will be provided to help you estimate the portion size if eating out (write down which photo you are referring to). Alternatively, look at the **packaging**, which shows the weight of the item and ingredients, including their % weight, all of which are helpful to us, you may even want to give the packaging to us.
4. Tell us if the food was **regular or low fat/reduced calorie/reduced sugar/economy**.
5. **Break down** foods with multiple ingredients, such as salads, stews, pasta dishes, and sandwiches, into **individual ingredients** and add each ingredient on a separate line, or use the recipe pages (**you must weigh each ingredient, or estimate if eating out**).
6. For homemade meals or desserts where you are not eating 100% of the dish, you can **write recipes** in the recipe section. At the top of the recipe page, **state how much you ate as a proportion (e.g. ¼ or 25%)**, rather than weighing your portion. Add the name of this recipe to the main food diary every time it was eaten (e.g. Recipe 1: Lasagne).
7. For **tea/coffee**, add the total weight with water, not the dried weight. Remember to **weigh milk and sugar/sweeteners separately**. **Record all alcoholic and soft drinks**.
8. For items that are rehydrated (e.g. pasta, rice, noodles, pulses, and couscous), it is important to note whether the weight is for the **dried or cooked** item.

9. The same applies to **meat, fish, fruit, and vegetables** (e.g. **raw or cooked** weight). Also, for meat/poultry, was a bone included, or the fat/skin eaten/removed?

THERE ARE 7 SECTIONS TO THE NUTRITIONAL INTAKE DIARY:

1. Time of Eating

2. Brand Name or Place Where Eaten

- Please write brand names (e.g. Kellogg's, Walkers, Sainsbury's).
- When eating out, please state the restaurant, coffee shop, or fast food outlet.
- If a food is homemade, please write this here. The recipes are required on a separate sheet.
- This information is not required for basic items such as fresh fruit and vegetables, sugar or flour.

3. Description of Food or Drink

- Please be as **descriptive as possible** (use the labels on food to help you).
- Use terms such as 'luxury/finest', 'reduced fat', 'no added sugar', or 'healthy eating'.
- State whether the food was fresh, frozen, dried, canned, etc.
- Remember to include **any fats/oils added to food** (e.g. butter, spreads, oils, mayonnaise, salad dressing, salad cream, marinades).
- Remember to include any **condiments/sauces** added to food (e.g. ketchup, mayonnaise, olive oil, vinegar; balsamic or malt, mustard, mint sauce, gravy, horseradish, salad dressing).
- If you eat **ready meals**, please save packages and bring along with your diary.
- For **tea and coffee**, don't forget to add any milk and sugar used.

4. State when Weighed

- Please add for foods that can be dried/cooked (e.g. rice, pulses, pasta), or **concentrated/diluted with water** (e.g. fruit squash, cup-a-soup, tea, coffee) or raw/cooked (e.g. meat, poultry, fish, fruit, and vegetables).
- Also state if weighed **peeled/unpeeled** (e.g. potatoes, bananas) or **with a bone/without a bone** (e.g. pork chops), or **fat trimmed/skin removed** (e.g. chicken).

5. Cooking Method

- If you have cooked a food then add the method used (e.g. grilled, baked, boiled, fried, toasted, steamed, microwaved).
- If **fried/roasted**, remember to give details of the fat/oil used (type and quantity) on a separate line.
- Specify whether the weight given was for the **cooked or uncooked** item.

6. Weight Consumed

- Please **weigh all food and drink**, and record in **grams**.
- Alternatively, use the **manufacturer's weights** that are printed on the food labels (e.g. confectionary, crisps, sandwiches, ready meals). Drink labels show volumes rather than weights, you may write the volumes for drinks.
- When **eating out** and you cannot weight your food/drink, please **describe** the portion size in detail (e.g. large skinny latte, regular fries, matchbox-sized cheese).

- Use the **portion and glass size guides** only when eating out.
- **Household measures** (e.g. two heaped tablespoons, ½ cup), should also be used to breakdown the ingredients of a meal eaten outside of the home.

7. Leftovers

- Please weigh your leftovers and record in the diary.

The table below lists common foods/drinks, and gives examples of the words that can be used to describe these items if you are unsure of details to include.

(Note: You may wish to use other relevant words that are not on this list).

Food/Drink	Description Examples
Alcoholic Drinks	Red, White, Rose, Sparkling, Alcopop, Low Alcohol. If measured in a pub: Pint, Half-Pint, Single/Double, Large/Small.
Soft Drinks	No Added Sugar, Diet, Diluted/Undiluted Volume. If measured in a pub: Pint/Half-Pint.
Biscuits	Variety Examples: Shortbread, Digestive, Rich Tea, Custard Cream, With/Without Chocolate, Cream-Filled.
Bread	Wholemeal/Brown, White, 50:50, Seeded, Granary, Fruit. Not Sliced, Medium Slice/Thick Slice, Small Loaf, Toasted. Crusty/Soft, Baguette, French Stick, Bagel, Pitta.
Breakfast Cereals	Include Details About Milk & Sugar (Type & Quantity). Porridge: Instant/Homemade, Raw Oats Quantity, Toppings.
Butter	Hard, Spreadable, Salted/Unsalted. Include Brand Name.
Cakes & Buns	Type Examples: Victoria, Chocolate, Fruit, Carrot, Muffin, Sponge. Filling Examples: Frosting, Royal Icing, Marzipan, Buttercream, Jam.
Cheese	Variety Examples: Cheddar (Mature, Mild), Parmesan, Brie. Hard, Soft, Cream, Cottage, Processed, Triangle. Half-Fat, Light.
Cream	Double, Clotted, Single, Sour, Long-Life, Spray, Crème Fraiche. Half-Fat, Light.
Coffee & Tea	Include Milk & Sugar/Sweetener Details (Type & Quantity). Cup, Mug. Fruit, Herbal, Green. Filter, Instant, Decaffeinated, Cappuccino, Espresso.
Eggs	Scrambled (Include Milk & Butter Details), Boiled, Fried (Include Oil Details).
Fish & Shellfish	Type Examples: Salmon, Cod, Sardines, King Prawns. Fresh, Canned (Oil, Brine, Water), Frozen. Breaded, Batter, Cakes, Fingers.

Food/Drink	Description Examples
Fruit, Vegetables & Fruit Juice	Variety Examples: Apples: Braeburn, Cox, Gala. State If Peeled, Peeled Item Weight (e.g. Apples, Potatoes, Kiwi, Banana). Canned (Juice/Syrup), Fresh, Stewed, Dried, Frozen. Fruit Juice: Freshly Squeezed, Fresh (e.g. From Chilled Section)/From Concentrate (e.g. Long-Life).
Gravy & Sauces	Instant, Sachet, Jar, Fresh, Homemade. With Meat Juices, Milk (Type & Amount), Thick, Thin.
Meat & Poultry	Type Examples: Pork, Chicken, Beef, Lamb. With/Without Fat/Skin, Well-Done/Rare. Cut: Chop, Steak, Leg, Drumstick, Mince (Lean, 20% Fat), Sausages (Pork/Beef, Thick/Thin, Low-Fat), Bacon (Streaky/Back).
Milk	Skimmed, 1% Fat, Semi-Skimmed, Whole/Full-Fat. UHT, Soya, Rice, Flavoured, Condensed, Sweetened/Unsweetened.
Nuts & Seeds	Salted, Unsalted, Honey Roasted. Nut/Seeds Type: Walnuts, Peanuts, Sesame/Pumpkin Seeds.
Oils	Variety Examples: Olive Oil, Extra-Virgin Olive Oil, Vegetable Oil, Sunflower Oil, Rapeseed Oil, Sesame Oil.
Pasta, Rice, Noodles & Couscous	Wholemeal/Brown, White, Pilau, Egg-Fried. State Weight: Dried/Cooked .
Pies & Quiches	Shortcrust, Puff Pastry, Suet, Filo. Individual/Family Sized. Filling Details: Steak, Chicken & Gravy, Minced-Steak & Onion.
Pizza	Size (Inches): 8, 10, 12. Takeaway, Chilled, Frozen, Homemade. Thick/Thin/Stuffed Crust. List Toppings.
Potatoes	Chips: Frozen, Fresh, Oven-Baked, Fried, Thick-Cut, Crinkle Cut, French Fries. Mash: Include Butter, Milk, Cheese. Roast/Deep Fried: Include Type & Fat Amount Used. New & Baked: With/Without Skin, Butter.
Sandwiches	Butter, Spread, No Spread, Mayonnaise. List All Fillings.
Spreads/Margarines	Varieties: Brand Names, Low-Fat/Full Fat.
Yoghurts	Creamy, Full-Fat, Low-Fat, Fat-Free. Natural, Greek, Fruit, Toffee, Fromage Frais.

Recipes

If you make a dish containing more than one portion (e.g. Lasagne, Casserole, Meat Pie, Stir Fry, Apple Crumble), then you may wish to add this as a recipe (see example below).

On the **Recipe Sheet**, Include:

- The **Name** of the dish.
- **Date(s)** of when the meal was eaten.
- **Portion** of the recipe consumed each time it was eaten (e.g. $\frac{1}{4}$, or 25%), please add this as a fraction or percentage, not weight.
- **Leftovers** from the portion eaten.
- **Ingredients** (it is important to include cooking oils/fats added to your ingredients).
- **Ingredients Status When Weighed** (e.g. Raw, Dried, Boiled).
- **Ingredients Weight Used (In The Recipe)**.

In the **Main Diary**, Include:

- A record of the **Recipe Name** each time it was eaten (e.g. Lasagne – Recipe 1).

If you do not have access to **electronic food weighing scales at home**, please notify the chief investigator.

Example Nutritional Intake Diary			Participant Code: A10				Date: 21-11-16		
WEIGH ALL FOOD & DRINK		Day (Circle):	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Time Eaten	Brand Name	Food/Drink Description			State When Weighed	Cooking Method	Weight (G)	Leftovers (G)	
07:45	Kellogg's	Crunchy Nut Cornflakes					45	0	
	Tesco	Milk (Semi-Skimmed) (Fresh)					113	40	
	Princes	Orange Juice (Long-Life Carton)					195	0	
10:00	Tesco	Apple (Golden Delicious)			Peeled		110	25 (Core)	
	Kenco	Coffee (Instant)			With Water		258	0	
	Sainsbury's	Milk (Semi-Skimmed) (Fresh)					32	0	
12:30	Hovis	7 Seed Wholemeal Bread (Thick Sliced)			Untoasted	Toasted	98	12	
	Flora	Spread (Light)					15	0	
	Cathedral City	Cheddar Cheese (Mature)			Untoasted	Toasted	43	0	
	Muller	Yoghurt Fruit Corner (Strawberry, Full-Fat)					175	0	
	Waitrose	Chicken Drumstick			Cooked (With Bone, No Skin)	Roasted	105	15	
	PG Tips	Tea			With Water		190	0	
	Tesco	Milk (Skimmed) (Fresh)					17	0	
	Sainsbury's	Sugar (Brown)					6	0	
14:15	McVitie's	Digestives x 3 (With Milk Chocolate, Reduced Calorie)					54	0	

Example Nutritional Intake Diary			Participant Code: A10				Date: 21-11-16		
WEIGH ALL FOOD & DRINK		Day (Circle):	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Time Eaten	Brand Name	Food/Drink Description			State When Weighed	Cooking Method	Weight (G)	Leftovers (G)	
	Kenco	Coffee (Instant, No Milk)			With Water		258	0	
16:00	Starbucks	Hot Chocolate (Semi-Skimmed Milk, Aerosol Cream)					Small (~ 1 Heaped Table Spoon Sugar)	~ 2 Table Spoons	
		Bakewell Tart					Specific Picture Guidance Number		
18:40	Asda Economy	Chicken & Mushroom Pie (Individual, Puff Pastry Top & Bottom)			Uncooked	Baked	235		
		New Potatoes (With Skins)			Raw	Roasted	181	27	
	Sainsbury's	Extra Virgin Olive Oil (For Roasting Potatoes)				Roasted	11		
		Broccoli			Raw	Steamed	74	15	
		Carrots			Cooked	Boiled	66		
		Salt					< 1 G (1 Small Pinch)		
	Robinsons	Orange Squash (No Added Sugar)			No Water Added		56		
19:30	Carte D'Or	Ice Cream (Vanilla)					160		
	Recipe	Apple Pie (Recipe 1)					¼		
	Blossom Hill	Wine (Red)					264		
21:10	Lindt	Plain Chocolate (70% Cocoa With Almonds)					25		

Weighing Food & Drink Leftovers: Helpful Hints

- When **weighing your meal**, place your plate on the scales, then press zero (tare) on the scales.
- Add each food item one by one, remembering to tare the scales after each food item to show the weight of each item.
- Do the same with your mug/glass for **drinks** (e.g. add the empty mug/glass to the scales, press tare, and then add the liquid).
- Please note that if you leave the scales untouched for a while, they may automatically switch off.
- When **weighing your leftovers**, either weigh your plate with the leftovers, as well as an identical empty plate and the difference of the leftovers, or add an empty plate to the scales, tare the scales and then weigh the leftovers.

Example Nutritional Intake Diary Recipe Record		Participant Code: A10	Dates Consumed:	25-11-16	28-11-16
Brand Name	Food Description	State When Weighed		Total Weight (G)	
Sainsbury's	Beef Mince (Extra Lean, 5% Fat) (Fresh)	Raw		500	
Sainsbury's	Olive Oil			15	
	Onion (White)	Raw		105	
	Pepper (Red)	Raw		145	
	Mushrooms (Closed Cup)	Raw		123	
Dolmio	Bolognese Sauce			500	
Schwartz	Basil (Dried)			2	
Asda	Lasagne Sheets	Dried		198	
Dolmio	White Lasagne Sauce			500	
Tesco	Cheese (Mozzarella, Grated)			70	

Important Reminders

- **Breakdown all dishes into individual ingredients (including those eaten outside of the home) and weigh each item separately** (or estimate if eating out) since everybody makes their dishes slightly different to others (recipes pages can be used).
- The accuracy of the nutritional intake diary is dependent on the weight column being completed for **all** foods and drinks, please **weigh everything** (avoid writing the number of items consumed, e.g. one banana), and please do not leave this column blank to prevent incorrect estimates being made by the researchers who analyse the diet diaries.

Thank You!

Appendix M: HOST-DM059 Galactooligosaccharide-Based Prebiotic – Material Safety Data Sheet

SECTION 1: PRODUCT AND COMPANY IDENTIFICATION		
PRODUCT NAME	HOST-DM059	
CONTACT ADDRESS	2-6 Church Street, St Helier, Jersey, JE2 3NN, U.K	
EMERGENCY PHONE	+44 787 2003 958	
OTHER CALLS	+44 771 7847 551	
FAX PHONE	+44 1534 715100	
CHEMICAL NAME	Trans Galactooligosaccharide	
CHEMICALY FAMILY	Complex Sugar	
CHEMICAL FORMULA	C ₆ H ₁₂ O ₆	
PRODUCT USE	Oral	
PREPARED BY	The enzymic treatment of lactose followed by enzyme removal and concentration by evaporation and drying. Prepared using Good Manufacturing Practice. Heat treated and filtered to render food safe.	
SECTION 1 NOTES:		
SECTION 2: COMPOSITION/INFORMATION ON INGREDIENTS		
Component	Max (%)	CAS Number
Galactooligosaccharide (GOS)	85	6587-31-1
Water	6	7732-18-5
Glucose (D & L)	6	921-60-8 AICS: 50-99-7
Galactose	4	15572-79-9 AICS: 59-23-4
Lactose	16	5989-81-1/5865-66-2 AICS: 63-42-3
SECTION 2 NOTES:		
General CAS Number given for GOS. Levels are set at maximum.		
SECTION 3: HAZARDS IDENTIFICATION		
EMERGENCY OVERVIEW:	Low Hazard	
ROUTES OF ENTRY:		
POTENTIAL HEALTH EFFECTS		
EYES:	Mild irritant.	
SKIN:	May be a mild irritant in some cases.	
INGESTION:	May cause a temporary stomach upset if > 15 grams ingested in one sitting.	

INHALATION:	Can cause mild irritation of the respiratory tract, eyes, or skin when these areas are exposed to quantities of the neat powder.
ACUTE HEALTH HAZARDS:	None Known
CHRONIC HEALTH HAZARDS:	None Known
MEDICAL CONDITIONS GENERALLY AGGRAVATED BY EXPOSURE:	None Known
CARCINOGENECITY:	None Known
SECTION 4: FIRST AID MEASURES	
EYES:	Flush thoroughly with clean running water. Seek medical attention if symptoms persist.
SKIN:	Wash with water.
NOTES TO PHYSICIANS OR FIRST AID PROVIDERS:	Ingestion – Treat symptomatically, people with Diabetes may need stabilising.
SECTION 5: FIRE FIGHTING MEASURES	
SPECIFIC HAZARDS:	Incompatible with strong oxidising agents and acids (particularly Nitric Acid).
FLAMMABILITY:	As with many powders, mixing of the dust from the powder with air can cause an explosive mixture under certain circumstances. The ignition properties have yet to be characterized however these are likely to be similar to lactose and whey powder.
EXTINGUISHING MEDIA:	Fire extinguishers which use water or carbon dioxide.
HAZARDS FROM COMBUSTION PRODUCTS:	Carbonizes at high temperatures and generates carbon dioxide or carbon monoxide depending on oxygen availability.
SECTION 6: ACCIDENTAL RELEASE MEASURES	
Spillages may be cleared up using shovels, vacuum cleaners or washed to effluent drains.	
Wash effected area with water. Notify relevant waste and/or environmental authority if there is a risk to surface water quality.	

SECTION 7: HANDLING AND STORAGE	
Tanks containing contaminated residues of the product may also have a reduced oxygen atmosphere.	
Entrance into confined space procedures should be adopted if tank maintenance or inspections are required.	
Spillages may create a sticky surface if spilt thus creating a slip hazard. Spillages should be dealt with promptly and in accordance with local procedures.	
The product may be stored in factory packaging (IBC's or Pallecons) or in hygienic and vented tanks at ambient temperatures. Contamination from mould and yeast sources and dilution from water/condensation should be avoided during storage.	
In solution however the material may ferment over a number of days if contaminated with yeast. Fermentation can yield carbon dioxide, traces of ethanol and low molecular weight organic acids. These in turn may have the potential to form explosive vapors if the area concerned is not well ventilated and the vapors are exposed to an ignition source. This combination of conditions must be avoided.	
Tanks containing contaminated pre-dissolved residues of the product may also have a reduced oxygen atmosphere. Controlled entrance into confined space procedures should be adopted if tank maintenance or inspections are required.	
The product has a density of ca 0.7 g·cc. Standard supporting floors and structures, forklift trucks and pallet trucks used in the food industry are generally fit for purpose.	
May create a slippery or sticky surface if spilt and mixed with water thus creating a slip hazard. Spillages should be dealt with promptly and in accordance with local procedures.	
SECTION 8: EXPOSURE CONTROLS/PERSONAL PROTECTION	
EXPOSURE STANDARDS:	None allocated
ENGINEERING CONTROLS:	None allocated
VENTILATION:	Use a well-ventilated area (LEV preferable) free from ignition sources.
RESPIRATORY PROTECTION:	Approved respirator conforming to U.K or equivalent standards will be required when handling this product or when entering into a contaminated confined space.

EYE PROTECTION:	Goggles or visors should be worn.
SKIN PROTECTION:	General food factory hygienic clothing is satisfactory.
WORK HYGIENE PRACTICES:	General Food Factory Goods Manufacturing Practice is Satisfactory.
SECTION 8 NOTES:	
SECTION 9: PHYSICAL AND CHEMICAL PROPERTIES	
APPEARANCE:	Creamy White Powder
ODOUR:	None
TASTE:	Bland/Flavorless
PHYSICAL STATE:	Powder
pH AS SUPPLIED:	6.0 – 7.0
BOILING POINT:	N/A
FREEZING POINT:	N/A
DENSITY (H₂O = 1):	0.6 g·cc
EVAPORATION RATE:	N/A
SOLUBILITY IN WATER:	Completely Miscible.
PERCENT SOLIDS BY WEIGHT:	94%
PERCENT VOLATILE:	None in fresh and adequately stored product.
VOLATILE ORGANIC COMPOUNDS (VOC):	None in fresh and adequately stored product.
VISCOACITY:	N/A.
SECTION 9 NOTES:	
SECTION 10: STABILITY AND REACTIVITY	
CHEMICAL STABILITY:	Stable
MICROBIOLOGICAL STABILITY:	Aw = ca 0.23: Generally resistant to microbial growth.
CONDITIONS TO AVOID (STABILITY):	Condensation, water ingress and poor environmental hygiene.
INCOMPATIBILITY (MATERIALS TO AVOID):	Strong oxidizing agents and neat mineral acids.
HAZARDOUS DECOMSPOTION OR BY-PRODUCTS:	None in powdered format.

HAZARDOUS POLYMERISATION:	N/A
CONDITIONS TO AVOID (POLYMERISATION):	N/A
SECTION 11: TOXICOLOGICAL INFORMATION	
TOXICOLOGICAL INFORMATION:	Non-Toxic Food Stuff Typical LD50 (ingestions) of sugar compounds ca 25 – 30 g·kg (rat).
SECTION 12: ECOLOGICAL INFORMATION	
The COD is approximately 0.95 kg·kg of product. Will decompose and temporarily deplete oxygen levels in water ways and lakes.	
SECTION 12 NOTES:	
SECTION 13: DISPOSAL CONSIDERATIONS	
Can be treated as 'common waste' for disposal to civil or industrial effluent plants employing high rate or activated sludge technology. Dissolved Air Flocculation units will be ineffective at reducing the COD load.	
The material has a very low nitrogen content, may ferment and oxidize to product organic acids and therefore may not be suitable for land spread. Will have some mobility within land fill sites if disposed of without suitable packaging/containers.	
SECTION 14: TRANSPORT INFORMATION	
TRANSPORT REQUIREMENTS:	General clean/hygienic vehicles/vessels used for food transport are suitable.
LAND WATER & AIR TRANSPORT	
PROPER SHIPPING NAME:	Non-Allocated
UN NUMBER:	Non-Allocated
HAZARD CLASS:	Low
ID NUMBER:	Non-Allocated
PACKING GROUP:	Non-Allocated
SECTION 14 NOTES:	
SECTION 15: REGULATORY INFORMATION	
Low Hazard – Not Registered.	

SECTION 16: OTHER INFORMATION
DISCLAIMER:
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