Dietary Fibre as a Modulator of Inflammation in Asthma

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STATEMENT OF ORIGINALITY

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Rebecca F McLoughlin

By signing below I confirm that Rebecca McLoughlin contributed to the study design, data acquisition, analysis and interpretation, drafting of the manuscript and final approval of the version of the manuscript; *Short-chain fatty acids, prebiotics, synbiotics, and systemic inflammation: a systematic review and meta-analysis. Am J Clin Nutr 2017.*

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Publications relating to this thesis to date

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Abbreviations

AAA	Adult acute asthma
ACQ	Asthma Control Questionnaire
ADD	Allergic airways disease
ADGs	Australian Dietary Guidelines
AHR	Airway hyper responsiveness
ASA	Adult stable asthma
Ax	Area under the reactance curve
BMI	Body mass index
CAA	Childhood acute asthma
CBP	CREB-binding protein
CDC	Centre for Disease Control and Prevention
cDNA	complementary Deoxyribonucleic acid
CFU	Colony-forming unit
CI	Confidence Interval
CINAHL	Cumulative Index to Nursing and Allied Health Literature
CS	Corticosteroid
CSA	Childhood stable asthma
DNA	Deoxyribonucleic acid
DP	Degrees polymerization
DRS	Dose response slope
EAACI	European Academy of Allergy and Clinical Immunology
ED	Emergency Department

EDTA	Ethylenediaminetetraacetic acid
ELP3	Elongator Complex protein 3
EMBASE	Excerpta Medica database
F&V	Fruit and vegetables
FeNO	Exhaled nitric oxide
FEV_1	Forced expiratory volume in one second
FFAR	Free fatty acid receptor
FOS	Fructooligosaccharide
Fres	Resonance frequency
FVC	Forced vital capacity
Gcn5	General Control Nonderepressible
GINA	Global Initiative for Asthma
GNAT	GCN5-related N-acetyltransferase
GOS	Galactooligosaccharide
GP	General practitioner
GPR	G-protein-coupled receptor
GSRS	Gastrointestinal Symptom Rating Scale
HAT	Histone acetyltransferase
HBSS	Hanks' Balanced Salt Solution
HDAC	Histone deacetylase
HMRI	Hunter Medical Research Institute
HNEH	Hunter New England Health
HPLC	High performance liquid chromatography
hsCRP	High sensitivity C-reactive Protein
hsIL-6	High sensitivity Interleukin-6

hsTNF-a	High sensitivity tumour necrosis factor alpha
Hz	Herts
ICS	Inhaled corticosteroid
IgE	Immunoglobulin E
IL	Interleukin
IOS	Impulse Oscillometry
Iκβ	Inhibitor of kappa beta
JHH	John Hunter Hospital
LABA	Long-acting β2-agonist
LMMs	Linear mixed models
LTB4	Leukotriene B4
LTB5	Leukotriene B5
MCID	Minimum clinically important difference
MeSH	Medical Subject Headings
MORF	Monocytic leukemia zinc finger protein-related factor
MOZ	Monocytic leukemia zinc-finger protein
n-3 PUFA	n-3 polyunsaturated fatty acids
n-6 PUFA	n-6 polyunsaturated fatty acids
NAD	Nicotinamide adenine dinucleotide
NF-κβ	Nuclear Factor Kappa Beta
NHANES	National Health and Nutrition Examination Survey
NHMRC	National Health and Medical Research Council
NK	Natural killer cells
NRC	Nuclear Receptor Cofactors
OCS	Oral corticosteroids

OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCAF	p300/CREB-binding protein associated factor
PD15	Provocation dose resulting in 15% fall in baseline FEV1.
PGD2	Prostaglandin D2
PGE3	Prostaglandin E3
PMN	Polymononuclear cells
ppb	Parts per billion
PUFA	Polyunsatured fatty acids
qPCR	quantitative Polymerase Chain Reaction
RCT	Randomised controlled trial
RevMan	Review Manager
RNA	Ribonucleic acid
RR	Relative risk
RT-PCR	Reverse Transcriptase -Polymerase Chain Reaction
SABA	Short acting β2-agonist
SAS3	Something About Silencing protein 3
SCFA	Short chain fatty acid
SD	Standard deviation
SE	Standard error
SFA	Saturated fatty acids
SMD	Standard mean difference
T2DM	Type 2 Diabetes Mellitus
TAF1	TATA-binding protein Associated Factor 1

TCC	Total cell count
TF	Transcription factor
Th0	Naïve T-helper lymphocytes
Th1	T-helper type 1
Th2	T-helper type 2
TIP60	Tat interative protein-60
TLR	Toll-like receptors
TNF-α	Tumour Necrosis Factor alpha
Treg	T regulatory cell
TSA	Trichostatin A
UK	United Kingdom
USA	United States of America
XOS	Xylooligosaccharide

Synopsis

Asthma is a common chronic respiratory disease, affecting approximately 334 million people worldwide (1). Asthma management places a substantial economic burden on both the health system and the individual. In Australia, it has been estimated that asthma management costs >$600million per year, 50% of which is attributed to inhaled corticosteroids which are the mainstay of asthma therapy (2). However, long-term, high dose treatment can predispose individuals to side effects such as osteoporosis, hypertension, insulin resistance and neuropsychiatric effects (3), and some patients respond poorly (4). Evidently new ways to improve asthma management are needed.

While genetics play an important role in asthma development and progression, it is apparent that there is an association between asthma prevalence and the westernised lifestyle, including the western diet (5, 6). Westernisation of diets has led to reduced dietary fibre intake (7). This is undesirable as dietary fibre has been shown to be inversely associated with the severity of airway inflammation and positively associated with lung function in asthma (8). The anti-inflammatory benefits of dietary fibre intake have been ascribed to the effects of soluble fibres (9), which are fermented by beneficial gut bacteria in the colon generating biologically active by-products, including the short chain fatty acids (SCFA) (9). SCFAs are suggested to have anti-inflammatory effects via mechanisms including the activation of G protein-coupled receptor (GPR) 41 and GPR43 in immune cells (10, 11), and the inhibition of histone deacetylase (HDAC) enzyme activity (12, 13).

This thesis aims to: examine the available evidence for the effect of SCFAs, prebiotics (soluble fibre) and synbiotics (prebiotic and probiotic combinations) on systemic inflammation in humans; examine the effects of soluble fibre supplementation, with

and without a probiotic, in adults with asthma; investigate the effects of soluble fibre intake (via fruit and vegetable intake) in children with asthma; and to examine molecular and epigenetic mechanisms that are modulated by soluble fibre, including HDAC enzyme activity and GPR41/43 and HDAC1-11 gene expression.

Chapter 2 presents the current evidence for the effect of SCFAs, prebiotics and synbiotics on systemic inflammation in humans. This systematic literature review and meta-analysis revealed that the evidence from individual studies is conflicting, with approximately 50% of included studies reporting a significant decrease in more than one inflammatory biomarker. Nonetheless, results of the meta-analyses of combined studies provide evidence to support the systematic anti-inflammatory benefits of prebiotic and synbiotic supplementation.

In chapter 4, the effects of soluble fibre supplementation, with and without a probiotic, in adults with asthma (n=17) were investigated. Following 7 days of soluble fibre (inulin) supplementation, we observed a reduction in airway eosinophils (Δ -1.0 [-2.5, 0.0], p=0.006), an improvement in asthma control (Δ -0.35 [-0.50, -0.13], p=0.006) and a trend towards improvement in lung function (Δ 1 [-0.5, 4], p=0.131), in adults with stable asthma. No significant changes in clinical outcomes or airway inflammation were observed with inulin+probiotic supplementation. Down regulation of sputum HDAC9 gene expression (p=0.008) was identified as a potential anti-inflammatory mechanism of soluble fibre in asthma, which was strongest in individuals with eosinophilic airway inflammation.

Chapter 5 investigated the effect of soluble fibre intake (via fruit and vegetable intake) in children with asthma (n=47). In this randomised controlled trial (RCT), participants were randomised to either a high or low fruit and vegetable diet for 6 months.

Increasing fruit and vegetable intake in children with asthma was found to improve lung function. Furthermore, in the absence of the protective effect of a high fruit and vegetable diet, over a 6 month period, systemic inflammation (CRP) increased in asthmatic children (Δ 0.37 [-0.04, 1.58], p=0.04). Down regulation of GPR41 gene expression (Δ -0.17 [-3.64, 0.47], p=0.01) and increased HDAC activity (Δ 0.80 [-0.26, 1.71], p<0.01) are suggested to contribute to this increase in systemic inflammation.

Overall, the research conducted as part of this thesis has contributed to the understanding of the benefits of soluble fibre intake in both adults and children with asthma, and the potential anti-inflammatory mechanisms involved. Delivery of soluble fibre, in both supplemental form and by increasing fruit and vegetable intake, has led to improvements in clinical outcomes and inflammatory pathways, in adults and children respectively. Hence we conclude that soluble fibre supplementation is a promising new therapeutic approach for asthma.

Chapter 1: Introduction

1.1 Asthma

1.1.1 Asthma defined

Asthma is a common chronic respiratory disease characterised by airway inflammation, airway hyper responsiveness (AHR), increased mucous production and variable airflow obstruction/airway narrowing (14). The complex interaction of these features give rise to the symptoms of asthma which include shortness of breath, wheezing, coughing, chest tightness, increased sputum production and variable expiratory airflow limitation (14). Asthma symptoms vary in intensity over time, and occur following activation of the immune system by specific stimuli, in genetically susceptible individuals (14).

1.1.2 Epidemiology of asthma

Asthma affects approximately 300 million people worldwide, and is estimated to contribute to 346 000 deaths per year (15). Interestingly, prevalence rates vary across countries ranging from 1 to 16% of the population, with asthma prevalence reported to be higher in Westernised countries such as the United Kingdom (UK), United States of America (USA), Canada, and Australia, compared to non-westernised countries (15). Over the years, there has been an increase in asthma prevalence worldwide. From 1950 to 2015 there was a 12.6% increase in asthma prevalence globally (16), and although the prevalence of asthma appears to have now plateaued in some countries such as Australia and North-West Europe, it is still on the rise in other parts of the world (17).

Nonetheless, in Australia asthma remains a significant health problem with 1 in 10 individuals reported to suffer from asthma (~10.2% of the population) (18). Furthermore, mortality rates due to asthma in Australia (~0.3% of all death) are

relatively high in comparison with many countries including Italy (~0.06%), Poland (~0.05%), Germany (~0.1%), France (~0.12%) and Japan (~0.09) (19). Asthma is also the most common chronic disease in children, with a prevalence of ~9.3% in 0-14 year olds in Australia (18). In fact, asthma prevalence rates are higher in children compared to adults, with children also reported to experience higher rates of asthma exacerbations (20). Differences in asthma prevalence are also seen between genders. In children 0-14 years of age, asthma is almost twice as prevalent in males than females (20), whereas in adults and adolescents asthma is more prevalent among females (21). It has been proposed that hormonal changes and gender-specific differences in environmental exposures may in part explain the change in asthma prevalence around puberty between males and females (21). Differences in lung and airway size may also play a role (20). In infancy, females have larger lungs and airways than males, however the opposite is true in adulthood (20).

From these statistics, it is not surprising that asthma management places a substantial economic burden on both the government and the individual. In Australia, it has been estimated that asthma management costs >$600million per year, 50% of which is attributed to prescription pharmaceuticals (2). In addition to the direct cost of health care (i.e. medications and hospital admissions), indirect costs associated with asthma arise due to its impact on an individual's social and economic participation, resulting in loss of productivity and loss of income due to time spent away from work (15). Furthermore, poorly controlled asthma is associated with both higher direct and indirect costs compared to asthma that is well controlled (15). Therefore it is evident that there is a need to better understand the molecular mechanisms that are causing and driving asthma. This will allow for the development of more effective, affordable and targeted strategies for managing the disease.

1.1.3 Clinical presentation and disease pathophysiology

The symptoms characteristic of asthma, which include shortness of breath, wheezing, coughing, chest tightness, increased sputum production and variable expiratory airflow limitation, can be triggered or aggravated by a wide array of stimuli. These include; anxiety, fatigue, physiological and psychological stress, viral infections (e.g. rhinovirus and influenza virus), bacterial infections (e.g. Chlamydia pneumonia), allergens (e.g. pollen, house dust mites and animal fur), irritants (e.g. smoke and strong smells), occupational exposures (e.g. chemicals), environmental air pollutants (e.g. particular matter), drugs (e.g. aspirin), exercise, dietary factors (e.g. food additives containing sulphites) and changes in weather conditions (14, 22). It is estimated that more than 80% of intermittent asthma exacerbations in children are triggered by respiratory infections (23). Although the origins of asthma are yet to be completely elucidated, it is proposed that it involves a complex interplay between genetic and environmental factors (22).

As mentioned earlier, asthma symptoms occur as a result of the complex interaction between four biological responses; AHR, mucus hypersecretion, variable airflow obstruction/airway narrowing and airway inflammation (**Figure 1-1)** (22).

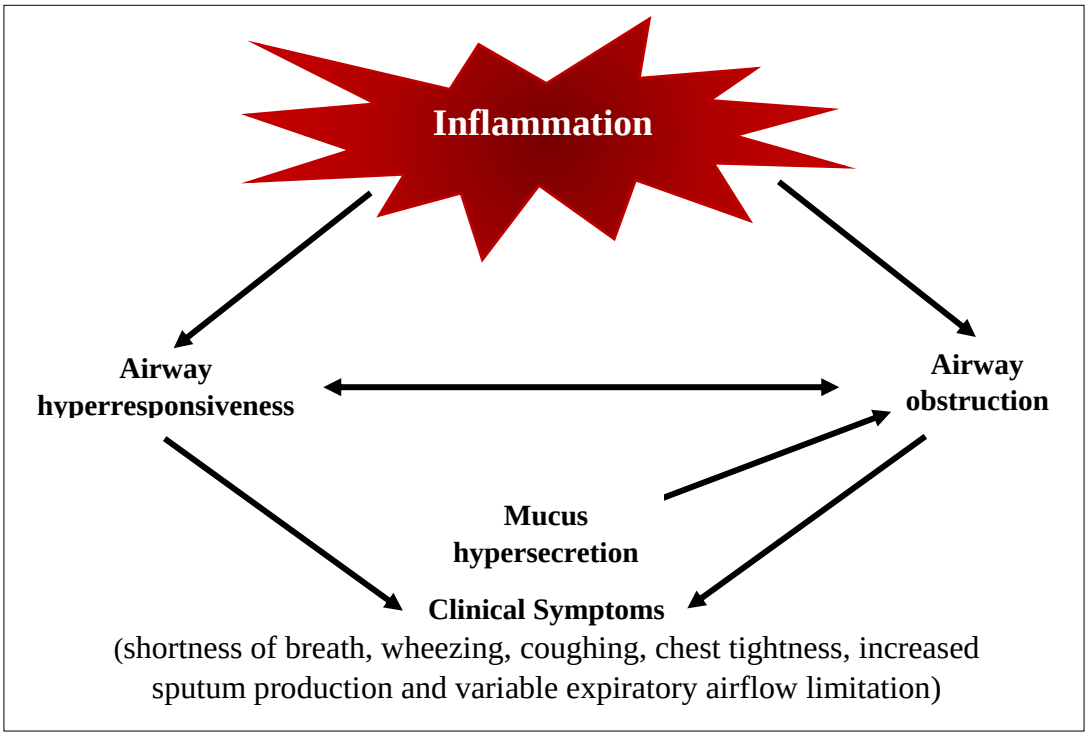

Figure 1-1: Interplay and interaction between asthma characteristics and clinical symptoms (*adapted from the National Heart, Lung and Blood Institute. Expert Panel Report 3 (2007) (22)*)

Airway hyper-responsiveness (AHR)

Airway hyper-responsiveness is an exaggerated response to stimuli such as cold air, smoke or allergens (i.e. dust), that would produce little to no effect in individuals without asthma (15). This results in the constriction and narrowing of the airway smooth muscle, and subsequently airflow obstruction and symptoms such as difficulty breathing (15). There is increasing evidence that airway inflammation and airway remodelling, which are characteristic of asthma, may contribute to the manifestation of AHR (15, 24).

The measurement of AHR using either direct airway challenges (e.g. histamine or inhaled methachlorine) or indirect airway challenges (e.g. an exercise challenge or inhaled mannitol), is used in the diagnosis of asthma of patients who have normal lung function, but present with symptoms consistent with asthma (15).

Mucus hypersecretion

The airway epithelium of a healthy, non-asthmatic individual is typically covered by a 7μM liquid layer which is overlaid by a 5-10μm thick mucus gel layer (25). Mucus secretion is the respiratory tract's first line of defence, designed to protect the lungs from inhaled foreign particles, bacteria and viruses (26). However, excess production, referred to as mucous hypersecretion which occurs in asthma, can have detrimental effects such as the development of mucus plugs, impairment of mucociliary clearance, mucus accumulation in the lungs, and subsequently airflow limitation and difficulty breathing (26).

Variable airflow obstruction/airway narrowing

There are several factors which contribute to the development of airway narrowing and subsequently airflow obstruction in asthma (15). Contraction of airway smooth muscle is the predominant mediator of airway narrowing (15, 27). This occurs in response to numerous neurotransmitter and bronchoconstriction mediators, and therefore can be reversed by bronchodilators such as salbutamol (27). As discussed above, mucus hypersecretion can also cause narrowing of the airways, largely due to the formation of mucus plugs (15, 26). Furthermore, inflammatory mediators in the airways can cause increased microvascular leakage resulting in airway oedema and subsequently airway narrowing. Airway thickening which results from airway remodelling is also suggested to contribute (15).

Airway inflammation

Inflammation is a protective response involving numerous immune cells and mediators designed to protect the body by removing harmful pathogens and initiating the healing process. However, inflammation of the airways in response to harmless stimuli is central to the pathophysiology of asthma (14). This inflammation predominantly

occurs in the conducting airways (bronchi and bronchioles), however, as the disease progresses, the inflammation spreads both distally to the smaller airways and proximally to the trachea (**Figure 1-2**) (28, 29).

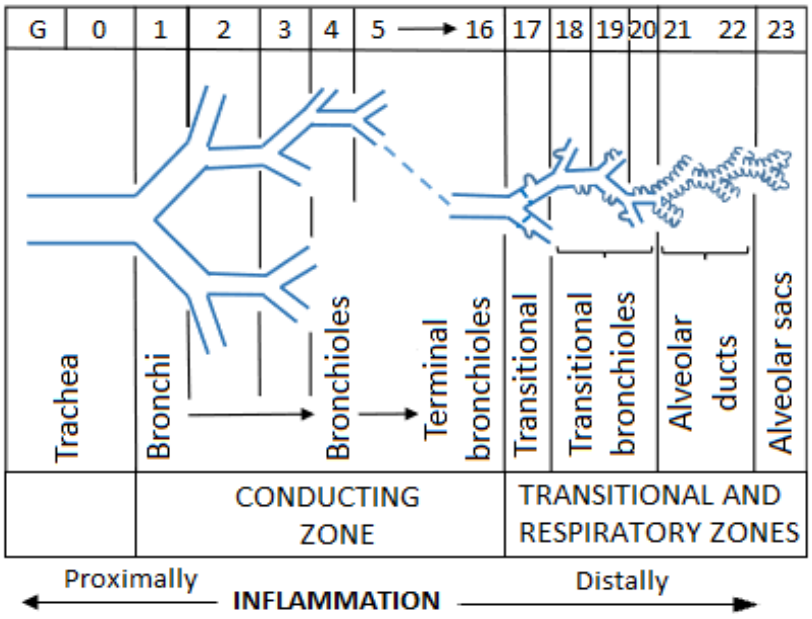

Figure 1-2: Airway inflammation in asthma *(adapted from Han et al 2016 (29))*

1.1.4 Airway inflammation in asthma and inflammatory phenotypes

The inflammatory response in asthma involves a complex interplay between various inflammatory cells (i.e. mast cells, granulocytes [eosinophils, neutrophils, and basophils], lymphocytes [T-lymphocytes, B-lymphocytes and natural killer cells (NK)], dendritic cells and macrophages), mediators (including histamine, leukotrienes and cytokines, such as interleukins) and the respiratory epithelium (28).

Asthma is heterogeneous and can be classified as one of four inflammatory phenotypes based on the predominant cell type present in the airways: neutrophilic, eosinophilic, mixed granulocytic (both increased neutrophils and eosinophils) and paucigranulocytic (normal levels of eosinophils and neutrophils) (30) . The two main

inflammatory pathways which shape these different phenotypes are the T-helper type 2 (Th2) and T-helper type 1 (Th1) mediated pathways.

T-helper type 2 mediated inflammation

Extrinsic asthma (also termed atopic, allergic or eosinophilic asthma) is driven by the acquired immune system, mediated via the Th2 inflammatory pathway(31). This inflammatory process involves an interplay between the innate and adaptive immune systems, as well as an abnormal immune response to specific environmental allergens (e.g. pollen, animal fur and house dust mites) (28). Upon entry into the airways, allergens are processed into small peptides by dendritic cells and presented to naïve T-helper (Th0) lymphocytes(32). Once activated, Th0 lymphocytes differentiate and mature into antigen-specific Th2 lymphocytes (CD4+ Th2) which release interleukins (IL), IL-4, IL-5, IL-9 and IL-13, that mediate the activation and recruitment of inflammatory cells in the airways (33). IL-4 and IL-13 stimulate the activation of B lymphocytes and subsequently the production of immunoglobulin E (IgE) (33).

Airway inflammation is predominantly mediated by IL-5, which plays an integral role in stimulating the synthesis and maturation of eosinophils in bone marrow, and their subsequent activation and survival (31). Once activated, eosinophils release a number of mediators which induce airway inflammation, AHR, and subsequently asthma symptoms (31).

T-helper type 1 mediated inflammation

Asthma can also present via activation of the innate immune system, mediated via the Th1 inflammatory pathway. This sub-type of asthma is typically more severe and is characterized by increased levels of neutrophils in the airways, in the absence of atopy (31). The inflammatory response is activated by various stimuli such as air pollutants,

viruses, bacteria and tobacco smoke (34). These stimuli bind to pattern-recognition receptors such as toll-like receptors (TLR) in the airways, which trigger the nuclear factor kappa-B (NF-κB) inflammatory pathway resulting in the production of Th1 pro-inflammatory cytokines (e.g. tumour necrosis factor alpha (TNF-α), IL-8 and IL-1B) (35). Inflammation in this sub-type of asthma is primarily driven by IL-8, which mediates the activation and recruitment of neutrophils (36).

1.1.4.1 Inflammatory phenotypes in asthma

Recent studies have focussed on exploring pathways which could link asthma inflammatory phenotypes to underlying cellular and molecular mechanisms. Greater understanding of mechanistic links between clinical characteristics and biological mechanisms of various inflammatory phenotypes may help us to target patient management more effectively.

As mentioned earlier, individuals can be categorised into the four asthma inflammatory phenotypes; eosinophilic, neutrophilic, mixed granulocytic and paucigranulocytic asthma (37) (**Table 1-1**). This is achieved using non-invasive techniques to determine the presence and/or absence of eosinophils and neutrophils in an individuals induced sputum sample.

Table 1-1: Inflammatory phenotypes in asthma

Inflammatory phenotype	**Induced sputum cut-points**
Eosinophilic asthma	Eosinophils >3%
Neutrophilic asthma	Neutrophils >61%
Paucigranulocytic asthma	Eosinophils <3% and Neutrophils <61%
Mixed granulocytic asthma	Eosinophils >3% and Neutrophils >61%

The prevalence of these asthma inflammatory phenotypes however, appear to vary between adults and children, with differences also observed between stable and acute asthma (30). In adult stable asthma (ASA) it has been reported that the most common inflammatory phenotype is paucigranulocytic asthma (51.7%), followed by neutrophilic (27.2%) (**Figure 1-3a)** (30)**.** Similarly, in childhood stable asthma (CSA) paucigranulocytic asthma (49%) has been reported to be the most common inflammatory phenotype, followed by eosinophilic asthma (28.6%) (**Figure 1-3b)** (30). Differences are also observed between adults and children with acute asthma. In adults with acute asthma (AAA), it has been demonstrated that neutrophilic asthma is the predominant inflammatory phenotype (81.8%) **(Figure 1-3c)**, whereas in childhood acute asthma (CAA), eosinophilic asthma is the most common (50%) (**Figure 1-3d)** (30).

However, although asthma inflammatory phenotype is considered stable in adults, in children is has been suggested that this is not the case (38). In a study conducted by Flemming *et al*, significant phenotypic variability was observed in 63% of children, demonstrating a change in inflammatory phenotype on repeated assessment (39). This demonstrates the complexity of defining inflammatory phenotypes in children.

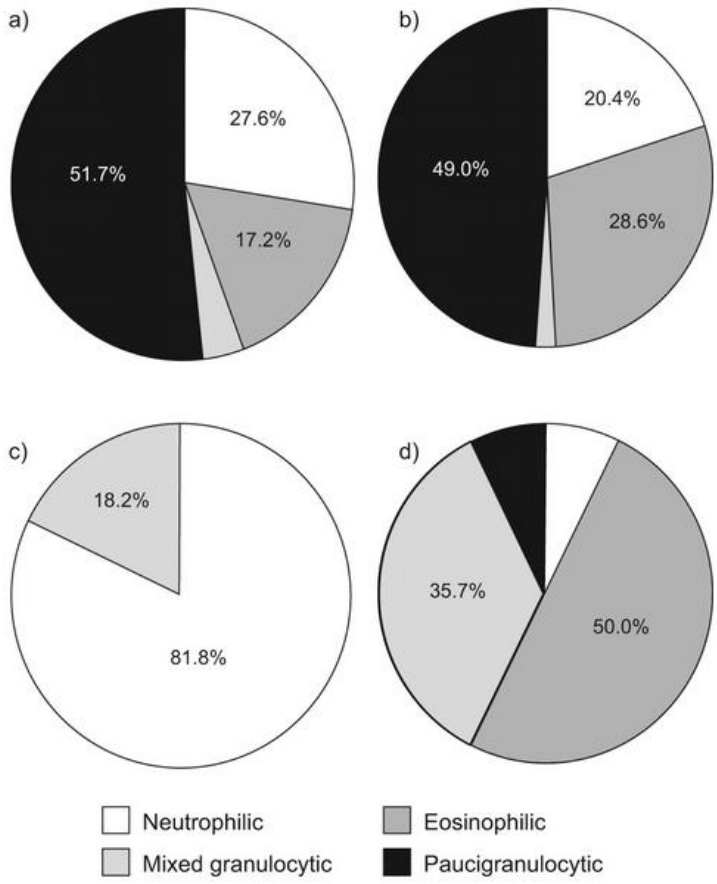

Figure 1-3: Inflammatory phenotypes a) adults with stable asthma (n=29), b) children with stable asthma (n=49), c) children with acute asthma (n=28) and d) adults with acute asthma (n=22). *Figure from Wang et al 2011* (30).

Eosinophilic asthma

As described in **Table 1**-1, patients are classified as having eosinophilic airway inflammation if they have %sputum eosinophils >3.0 (37). Eosinophilic asthma is often associated with greater asthma severity than the other inflammatory phenotypes, atopy, and late-onset disease (40). In addition to this, patients with eosinophilic asthma typically respond better to inhaled and oral corticosteroids, which are commonly used in the pharmacological management of asthma, compared to those with non-eosinophilic asthma (41).

Neutrophilic asthma

Neutrophils are the most abundant cell type present in sputum induced from both asthmatic and non-asthmatic individuals (41). Furthermore neutrophils are considered regulatory cells in asthma, which release numerous inflammatory mediators following infiltration of the airways in response to either airway injury of an allergen challenge (42, 43). Neutrophilic asthma is defined as %sputum neutrophils >61% (37). Individuals with this phenotype are typically unresponsive to inhaled and oral corticosteroids (41). Triggers of exaggerated neutrophilic inflammation include occupational exposure (44), viral respiratory infections (42), environmental factors (e.g. air pollution and ozone) (42), and exposure to tobacco smoke (44).

Paucigranulocytic asthma

Patients are classified as having paucigranulocytic asthma if they have both normal %sputum eosinophils (<3%) and %sputum neutrophils (<61%) (37). It has been suggested that variable airflow obstruction and airway hyper responsiveness in individuals with this asthma inflammatory phenotype occur independently of airway inflammation (45). Infiltration of the airway smooth muscle by mast cells has been proposed to be implicated in the onset of airway hyper-responsiveness in this asthmatic population (46).

Mixed granulocytic asthma

Patients with both elevated %sputum eosinophils (>3%) and %sputum neutrophils (>61%) are classified as having mixed granulocytic asthma (37). Of the four asthma inflammatory phenotypes, this phenotype is the least common and is typically seen in refractory or complicated asthma (37).

1.1.5 Treatment options for asthma

Following exposure to an asthma trigger and the subsequent activation of an inflammatory response, symptoms and airflow limitation may be mild and resolve either spontaneously or through the administration of asthma medication(s) (1). Alternatively an asthma exacerbation, indicating loss of asthma control, may occur. An asthma exacerbation refers to an acute episode characterized by a progressive increase in asthma symptoms and a progressive decrease in lung function (1). The severity of an exacerbation can range from mild which can be treated with asthma medication (i.e. a short acting bronchodilator), to life-threatening, which requires urgent medical attention (1).

A number of treatment options are available to control asthma and decrease the frequency and severity of symptoms (1). These include strategies such as the identification and treatment of modifiable risk factors (i.e. exposure to tobacco smoke, confirmed food allergies and exposure to known allergens), and the use of pharmacological and non-pharmacological interventions (1).

Pharmacological interventions

Pharmacological treatment is the most commonly used asthma treatment method. Inhaled corticosteroids are the mainstay of asthma therapy, costing >$300m per year alone in Australia (2). However, treatment can predispose individuals to long-term side effects such as osteoporosis, hypertension, insulin resistance and neuropsychiatric effects (3), and some patients respond poorly (4). Furthermore, non-adherence to prescribed therapy is a significant problem in asthma management, and is associated with loss of asthma control (47).

Non-pharmacological interventions

Non-pharmacological interventions include, but are not limited to; allergen avoidance (1), engagement in regular physical activity (1), consumption of a healthy diet high in fruit and vegetables (1, 48), and weight reduction (1). Whilst our understanding of the potential benefits of such strategies has increased in recent years, there is great potential for new approaches to treat inflammation and improve asthma management.

1.2 Westernisation of diets and asthma

While genetics play an important role in asthma development and progression, it is apparent that there is an association between asthma prevalence and westernisation. Indeed, westernised countries (i.e. the UK, New Zealand, Australia, Canada and the USA) and those making the transition towards a westernized lifestyle (i.e. Africa, Central and South America, and the Asia and Pacific regions) have a higher asthma prevalence compared to less developed countries (5, 6) **(Figure 1-4**). This suggests that environmental exposures may play a role in the development of asthma.

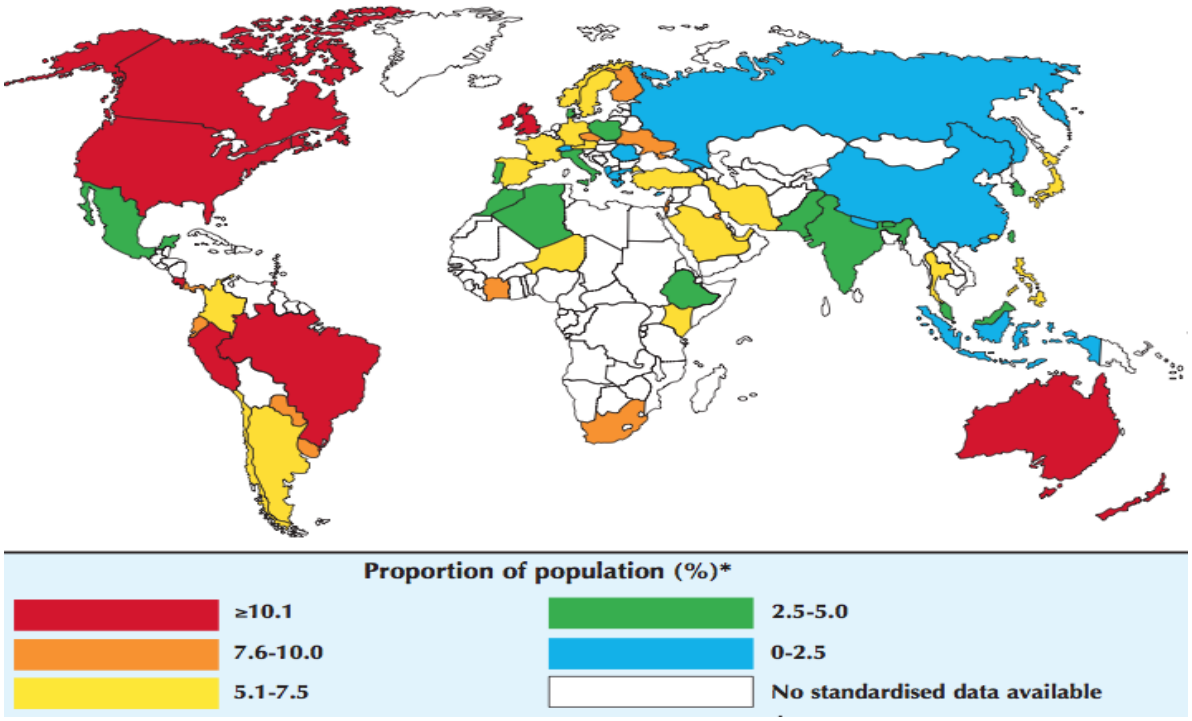

Figure 1-4: Global Burden of Asthma. *Figure from Masoli et al, 2004 (6)*

Epidemiological studies report that asthma risk is increased with consumption of a Western diet (49, 50). This diet is typically dominated by highly processed and convenience foods, which includes a high intake of processed and red meats,

desserts/sweets, refined grains, high-fat dairy products, fried foods, with a low intake of fruit, vegetables, seafood and poultry (51) (**Figure 1-5**).

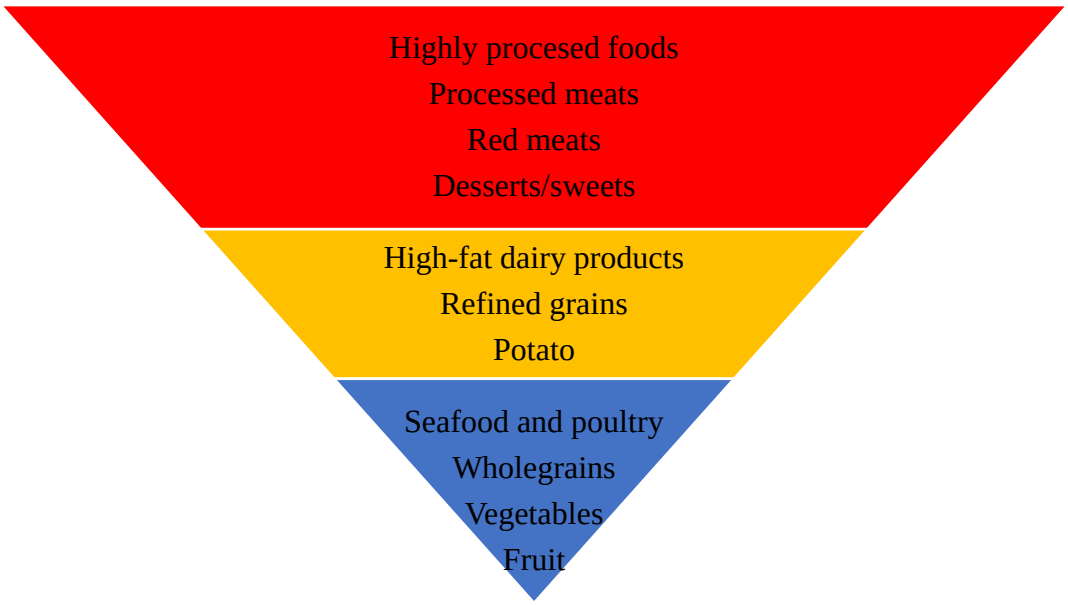

Figure 1-5: Characteristics of the Western diet. The Western diet is typically characterised by a high intake of processed and convenience foods (including processed and red meats, desserts/sweets, high-fat dairy products and refined grains), and a low intake of fruit, vegetables, seafood and poultry. *Adapted from Hu et al 2002* (51).

There are various components of this diet, notably the high saturated fat content, increased omega-6:omega-3 polyunsaturated fat ratio and low antioxidant content, which promote inflammation and oxidative stress, and may therefore contribute to the association between the Western diet and asthma prevalence (52). Of particular interest in recent research however, is the relationship between the low fibre content of the Western diet and inflammation in asthma.

1.2.1 Dietary fat intake and asthma

It has been hypothesised that the association between asthma prevalence and westernisation may be related to a high intake of saturated fat and n-6 polyunsaturated fatty acids (n-6 PUFA), and a low intake of n-3 polyunsaturated fatty acids (n-3 PUFA), which is typical of a Western diet. In general saturated fatty acids (SFA) and

n-6 PUFA are considered pro-inflammatory, whereas n-3 PUFA have been shown to have anti-inflammatory effects (34).

There is evidence that dietary fat intake can activate the innate immune response which is a significant contributor to airway inflammation in asthma (34). Saturated fatty acids in particular have been shown to directly activate toll-like receptor 4 (TLR-4), which is present on immune cells such as monocytes, neutrophils, dendritic cells and macrophages, as well as recruit protein myeloid differentiation factor 88 (34). This results in a signalling cascade that activates the NF-κB pathway and subsequently the production of various pro-inflammatory mediators such as TNF-α and IL-6 (34). Indeed, asthmatics are reported to have higher total fat intake than their non-asthmatic counterparts (8), with a higher total fat intake associated with increased eosinophilic airway inflammation (8), asthma diagnosis and AHR (53). Furthermore, our research team has previously demonstrated that a single meal high in total fat augments airway inflammation, with increased TLR-4 gene expression and increased sputum %neutrophils observed (54).

There is also evidence from epidemiological studies that populations with higher n-6 PUFA intake have greater asthma prevalence than populations with higher n-3 PUFA intake (55). Furthermore, individuals with asthma have been reported to have lower ratios of n-3:n-6 PUFA consumption than healthy controls (56). Following consumption, dietary fatty acids are incorporated into cellular membranes leading to alterations in eicosanoid synthesis (34); n-3 PUFAs produce eicosanoids such as prostaglandin E3 (PGE_3) and leukotriene B5 (LTB_5) which are less pro-inflammatory than those derived from n-6 PUFAs such as prostaglandin D2 (PGD_2) and leukotriene B4 (LTB_4) (57). Furthermore, n-3 PUFA have been shown to exert anti-inflammatory effects by reducing the activity of transcription factors such as NF-κB (34)

1.2.2 Antioxidant intake and asthma

Oxidative stress is believed to play a role in the pathogenesis of asthma (58), with evidence that it contributes to mucus hypersecretion (59), airway smooth muscle contraction (60), induction of AHR (61) and epithelial shedding (62). Furthermore, it is reported that there is an oxidant-antioxidant imbalance in asthma (63) with further increases in oxidative stress occurring during acute asthma exacerbations (58, 63, 64). Fruit and vegetable intake in many westernised countries fall well below current recommendations (65). Therefore, as fruit and vegetables are a rich source of antioxidants including vitamin C, vitamin E, carotenoids (e.g. lutein, lycopene, β-cryptoxanthin, α-carotene and β-carotene), flavonoids (e.g. anthocyanins), organosulfur compounds, indoles and isothiocyanates (66), this may contribute to the association between the Western diet and asthma prevalence.

According to the most recent National Healthy Survey results (2014-2015) (67), it was estimated that only 49.8% of Australian adults (≥ 18 years old) were meeting the guidelines for daily fruit consumption (**Table 1-2**) (68), 7% were consuming the recommended daily serves of vegetables (**Table 1-2**) (68), and only 5.1% (1 in 20) were meeting both guidelines. While women were more likely to meet the guidelines than men, average daily fruit consumption was estimated at 1.8 serves a day (compared to 1.6 serves in men) and 2.5 serves of vegetables a day (compared to 2.3 serves of vegetables in men), which is well below the guidelines (**Table 1-2)** (68) **(**Recommended serving sizes are outlined in **Table 1-3)** (68). Furthermore, although 68.1% of children (aged 2-18 years) in Australia were estimated to be meeting the guidelines for recommended daily serves of fruit (**Table 1-2)** (68), only 5.4% were meeting the guidelines for recommended daily serves of vegetables (**Table 1-2)**, with 5.1% meeting both guidelines**.**

Table 1-2: Australian Guide to Healthy Eating recommended daily fruit and vegetable serves (68)

Age	Fruit	Vegetables
2-3 years	1	2.5
4-8 years	1.5	4.5
9-11 years	2	5
12-13 years	2	5.5 males, 5 females
14-18 years	2	5.5 males, 5 females
19-50 years	2	6 males, 5 females
51-70 years	2	5.5 males, 5 females
70+ years	2	5

Table 1-3: The Australian Guide to Healthy Eating recommended fruit and vegetable serving sizes (68)

Standard serve of fruit (150g or 350kJ):	**Standard serve of vegetables (75g or 100-350kJ):**
- 1 medium piece. E.g. apple, banana, orange or pear - 2 small pieces. E.g. apricots, kiwi fruits or plums - 1 cup diced or canned fruit (no added sugar) - 125ml (1/2 cup) fruit juice (no added sugar) - 30g dried fruit	- 1/2 cup green or orange vegetables (E.g. broccoli, spinach, carrots or pumpkin) - 1/2 cup cooked dried or canned beans, peas or lentils - 1 cup green leafy salad vegetables - 1/2 cup sweet corn (1 ear of corn) - 1/2 medium starchy vegetable (E.g. sweet potato) - 1 medium tomato

Epidemiological studies show that fruit and vegetable intake plays an integral role in the development and progression of asthma. In one meta-analysis, both fruit and vegetable intake were found to be inversely associated with wheeze (Fruit intake: RR=0.81; 95% CI: 0.74-0.88. Vegetable intake: RR=0.88; 95% CI: 0.79-0.97) and asthma (Fruit intake: RR=0.90; 95% CI: 0.86-0.94. Vegetable intake: RR=0.91; 95% CI: 0.82-1.00) (69). These findings are consistent with a recent systematic review and meta-analysis conducted by our research group which found inverse associations between fruit intake and risk of prevalent wheeze (OR=0.94; 95% CI: 0.91–0.97, $p < 0.0001$) and asthma severity (OR=0.61; 95% CI: 0.44–0.87, $p = 0.005$) (70). Vegetable intake was also negatively associated with asthma prevalence (OR = 0.95; 95% CI: 0.92–0.98, $p = 0.003$) (70). Furthermore, fruit consumption at an early age (2-3 years) and long-term fruit intake has been associated with reduced asthma symptoms (OR = 0.93, 95% CI 0.85–1.00 and 0.90, 95% CI: 0.82–0.99 respectively) (71), while a RCT conducted by our research team demonstrated that a high fruit and vegetable diet for 14 days can improve measures of lung function (FEV_1 and FVC) in adults with asthma (72).

Dietary antioxidants found in fruit and vegetables are purported to decrease airway inflammation in asthma by protecting the airways from oxidants generated both exogenously (e.g. air pollution and cigarette smoke) and endogenously (e.g. produced by activated immune cells) (73). It is suggested that these antioxidants scavenge reactive oxygen species, thus inhibiting NF-κB-mediated inflammation (34).

In addition to being a rich source of antioxidants, fruit and vegetables also contain soluble fibre which may contribute to the protect effects of fruit and vegetable consumption in asthma. The association between dietary fibre intake and asthma will be explored further in this thesis.

1.2.3 Dietary fibre intake and asthma

According to the Australian Health Survey 2011-2012, the overall average daily fibre intake in Australia is well below the recommendations for both males and females (**Table 1-4)** (74, 75). These findings are similar to other westernised countries including the USA (76) and the UK (77).

Table 1-4: Comparison of mean dietary fibre intakes in Australia with the NHMRC recommendations

Age	Recommended intake (AI) (75)	Mean intake in males (74)	Mean intake in females(74)
4-8yrs	18g/day	20.1g/day	17.3g/day
9-13yrs	Males: 24g/day Females: 20g/day	22.8g/day	19.2g/day
14-18yrs	Males: 28g/day Females: 22g/day	22.4g/day	19.3g/day
>19yrs	Males: 30g/day Females: 25g/day	24.8g/day	21.1g/day

Inadequate fibre consumption is typical of the Western diet due to a low intake of fruit, vegetables and whole grains, and high intake of processed convenience foods (7). Low fruit and vegetable intake is of particular concern, as compared to whole grains on an energy basis, fruit is estimated to contain twice the amount of fibre and non-starchy vegetables are estimated to contain almost 8 times the amount of fibre (7).

Dietary fibre is defined as "*the fraction of the edible parts of plants or their extracts, or synthetic analogues, that are resistant to the digestion and absorption in the small intestine, usually with complete or partial fermentation in the large intestine"(75).* The most widely accepted method of categorising dietary fibre is based on its solubility and/or its fermentability. As such, dietary fibre is typically categorised as either insoluble or soluble, with most fibre-containing foods comprised of approximately two-thirds insoluble fibre and one-third soluble fibre (78).

Insoluble fibre

Fibre that does not dissolve in water and is predominantly resistant to bacterial fermentation in the colon is referred to as insoluble fibre (78). It is considered biologically inert, and provides bulk for the production of faeces and thus facilitates defecation (78). Examples of insoluble fibre include cellulose, hemicellulose, lignin and chitin. Food sources of insoluble fibre include the outer skin of fruit and vegetables, the bran portion of whole grains, nut and seeds, and legumes (peas and beans) (78).

Soluble fibre

Soluble fibre dissolves in water forming a viscous gel, and is fermented to physiologically active by-products such as short chain fatty acids (e.g. acetate, butyrate and propionate) by beneficial bacteria in the colon (78). Soluble fibre has also been shown to assist in decreasing circulating cholesterol levels in the body via mechanisms such as preventing the reabsorption of bile salts from the small intestines, and reducing the glycaemic response which decreases insulin stimulated hepatic cholesterol synthesis (79). Examples of soluble fibres include pectin, gums (e.g. guar gum), psyllium, raffinose and fructans (e.g. inulin). Food sources of soluble fibre include the flesh of fruits, vegetables and legumes (peas and beans), oats, oatmeal, nuts and seeds (78).

Dietary fibre, inflammation and asthma

An inverse relationship between both soluble and insoluble fibre intake and biomarkers of systemic inflammation (i.e. IL-6, TNF-α, and C-reactive protein (CRP)) has been demonstrated in numerous studies (80-85). This inverse relationship has been shown in healthy populations, as well as populations with chronic diseases such as

diabetes, cardiovascular disease and kidney disease (80-85). However, studies examining the relationship between dietary fibre and inflammation in asthma in humans are limited.

A cross sectional study conducted by our research team at the Hunter Medical Research Institute (HMRI, Newcastle NSW, Australia) demonstrated that dietary fibre was inversely associated with airway inflammation in asthma (8). This study found that dietary fibre intake was ~5g/day lower in adults with severe asthma (n=137) compared to healthy controls (n=65). Furthermore, multiple regression analysis demonstrated that fibre intake was inversely associated with airway eosinophils (**Figure 1-6a**) and positively associated with all lung function measures, including forced expiratory volume in one second (FEV_1) %predicted (**Figure 1-6b**), forced vital capacity (FVC) %predicted and FEV_1/FVC, when adjusting for energy intake, age, gender and body mass index (BMI) (8).

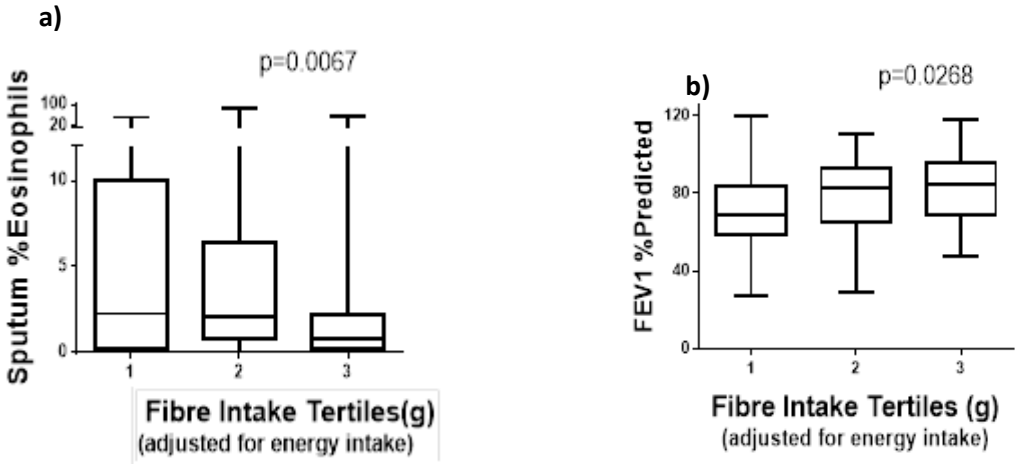

Figure 1-6: Association between dietary fibre intake and a) airway eosinophils, and b) FEV1 % predicted. *Figures from Berthon et al, 2013* (8).

Clinical interventions are required to establish a causative relationship between fibre intake, lung function and airway inflammation, to provide further insight into the type of dietary fibre responsible for these associations, and to examine potential anti-inflammatory mechanisms/pathways.

1.3 Proposed anti-inflammatory mechanisms of dietary fibre

The exact mechanisms behind the anti-inflammatory properties of dietary fibre are yet to be elucidated, however a number of explanations have been proposed.

Weight loss

Dietary fibre assists with weight loss by slowing gastric emptying, increasing satiety, and decreasing the insulin response (86). Studies have shown that weight loss is associated with a decrease in inflammatory markers (e.g. IL-6 and CRP) (84, 87, 88), with one study reporting that a 1-kg weight loss was associated with a 0.13mg/L decrease in CRP (88).

Lowering glycaemia

Consumption of refined carbohydrates low in dietary fibre contributes to hyperglycaemia, which is associated with increased levels of pro-inflammatory cytokines such as IL-6, IL-1β and CRP (89, 90). Dietary fibre may reduce inflammation through its ability to lower glycaemia by slowing gastric emptying and macronutrient absorption (90, 91).

Increasing adiponectin

Adiponectin is an adipocyte-derived protein secreted from adipose tissue. It is considered to be anti-inflammatory due to its ability to inhibit NF-kB activation in endothelial cells and macrophages, resulting in the downregulation of pro-inflammatory cytokines (e.g. CRP and IL-8) and the attenuation of TNF-α production, respectively(92). It has been suggested that fibre exerts its anti-inflammatory effects via increasing the secretion of adiponectin (93).

Short chain fatty acid production

The fermentation of indigestible carbohydrates (primarily soluble fibre and resistant starches) results in the production of physiologically active by-products, such as short chain fatty acids (SCFA), that are proposed to have systemic anti-inflammatory effects (9). This anti-inflammatory mechanism has been gaining recent research interest and will be discussed in detail below.

1.3.1 Short chain fatty acids and the gut microbiome

1.3.1.1 Short chain fatty acids

Short chain fatty acids (SCFA), also referred to as volatile fatty acids, are saturated aliphatic organic acids 1-6 carbons in size (94). They are produced primarily from the fermentation of indigestible carbohydrates by commensal bacteria in the colon (94). Amino acids such as leucine, isoleucine and valine can also be converted to branched-chain SCFAs (i.e isobutyrate, 2-methyl butyrate and isovalerate), however this only contributes to approximately 5% of total SCFA production (95). The most abundant short chain fatty acids are acetate, propionate and butyrate (typically in a 3:1:1 ratio), representing 90-95% of SCFAs produced (95).

Acetate

Acetate (C2) accounts for approximately 50% of SCFAs (96), and is the primary SCFA that enters circulation (97). Indeed, it has been demonstrated that individuals consuming a high fibre diet have high blood acetate levels (97). While the fermentation of indigestible carbohydrates is the main source of acetate, it is estimated that approximately one-third of acetate is produced via the Wood-Ljunghadl pathway by acetogenic bacteria in the colon such as *Blautia hydrogenotrophica,* which can

synthesise acetate from hydrogen and either formic acid or carbon dioxide (98) (**Figure 1-7)**.

Propionate

There are three pathways by which colonic bacteria produce the SCFA propionate (C3); the succinate, acrylate and propanediol pathways (98). The majority of propionate is produced via the succinate pathway following the fermentation of indigestible carbohydrates (**Figure 1-7)** (98). This pathway is used by bacteria of the *Firmicutes* and *Bacteriodetes* phylum (98). In the acrylate pathway, colonic bacteria from the *Veillonellaceae* and *Lachnospiraceae* families convert lactate to propionate, whereas bacteria including *Proteobacteria* and members of the *Lachnospiraceae* family produce propionate from deoxyhexose sugars such as rhamnose and fucose via the propanediol pathway (98). Once it enters circulation, propionate is primarily absorbed by the liver (97).

Butyrate

Butyrate (C4), a major energy source for colonocytes (97), can be produced via two different pathways following the fermentation of indigestible carbohydrates. The most common pathway, which is used by the majority of butyrate-producing bacteria (i.e. *Roseburia Eubacterium* and *Faecalibacterium),* is the butyryl-CoA:acetate CoA-transferase pathway (98) **(Figure 1-7)**. This pathway uses a single step enzymatic reaction to convert butyryl-CoA to butyrate, whereas the butyrate kinase pathway used by some bacterial species from the *Coprococcus* genus, requires butyrate kinase and phosphotransbutyrylase enzymes to perform the conversion.

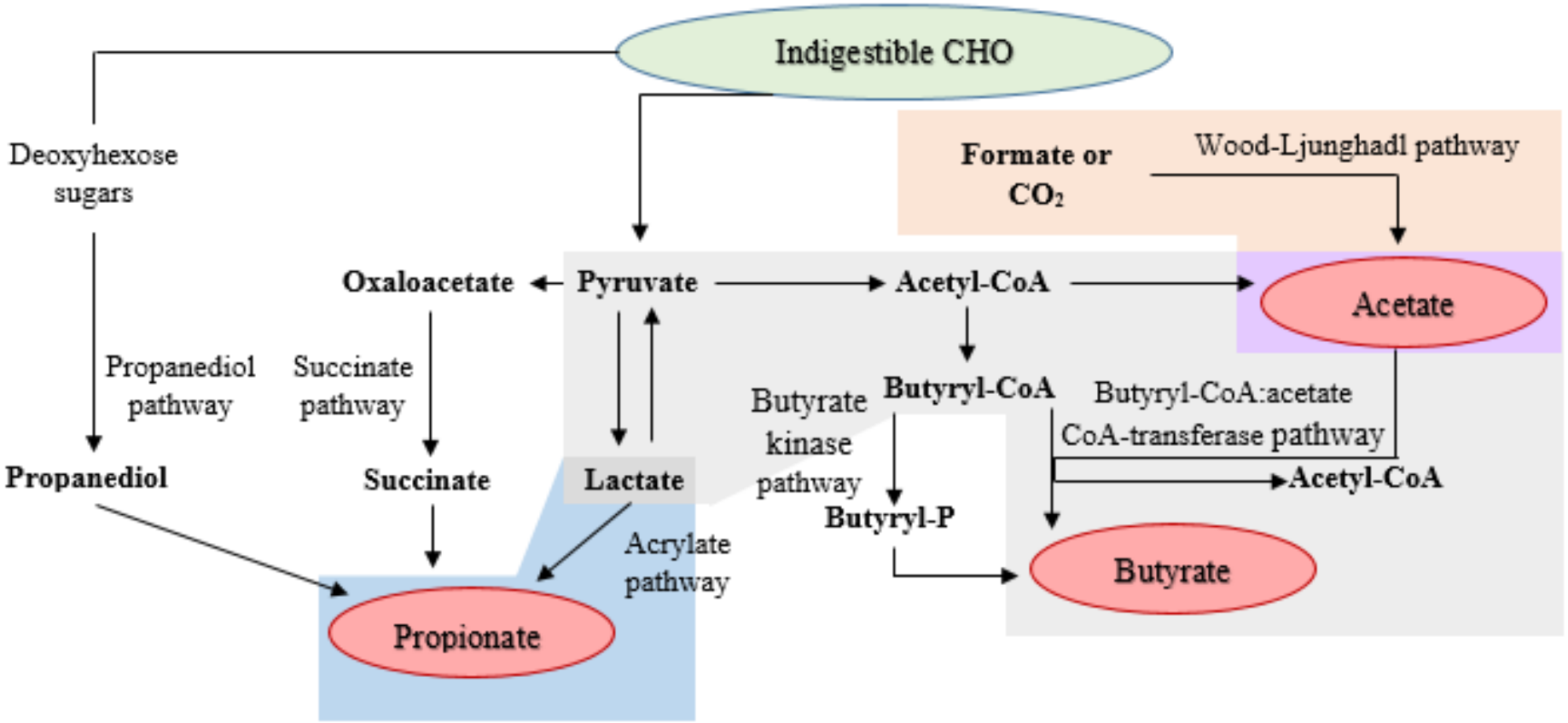

Figure 1-7: Pathways involved in the synthesis of short chain fatty acids following the fermentation of indigestible carbohydrates (primarily soluble fibre and resistant starch *(adapted from Rios-Covian et al, 2016)* (95).

The quantity, type and rate of SCFA production is determined by a number of factors including: the quantity and species of bacteria present in the colon, the types and amount of substrate available for fermentation, and intestinal transit time (97).

1.3.1.2 The gut microbiome

The human colon contains a large and diverse microbiota, with an estimated 100 trillion bacterial cells (94). Although 50 bacterial phyla have been identified to date, the human gut microbiome is dominated by two phyla, the *Bacteriodetes* (17-60%) and the *Firmicutes* (35-80%), with the *Actinobacteria* and *Proteobacteria* phyla less dominant (99). Bacterial density, and subsequently substrate fermentation, varies along the colon. As depicted in **Figure 1-8**, bacterial density is highest in the proximal colon, where substrate availability is highest, and decreases distally along the length of the colon (100).

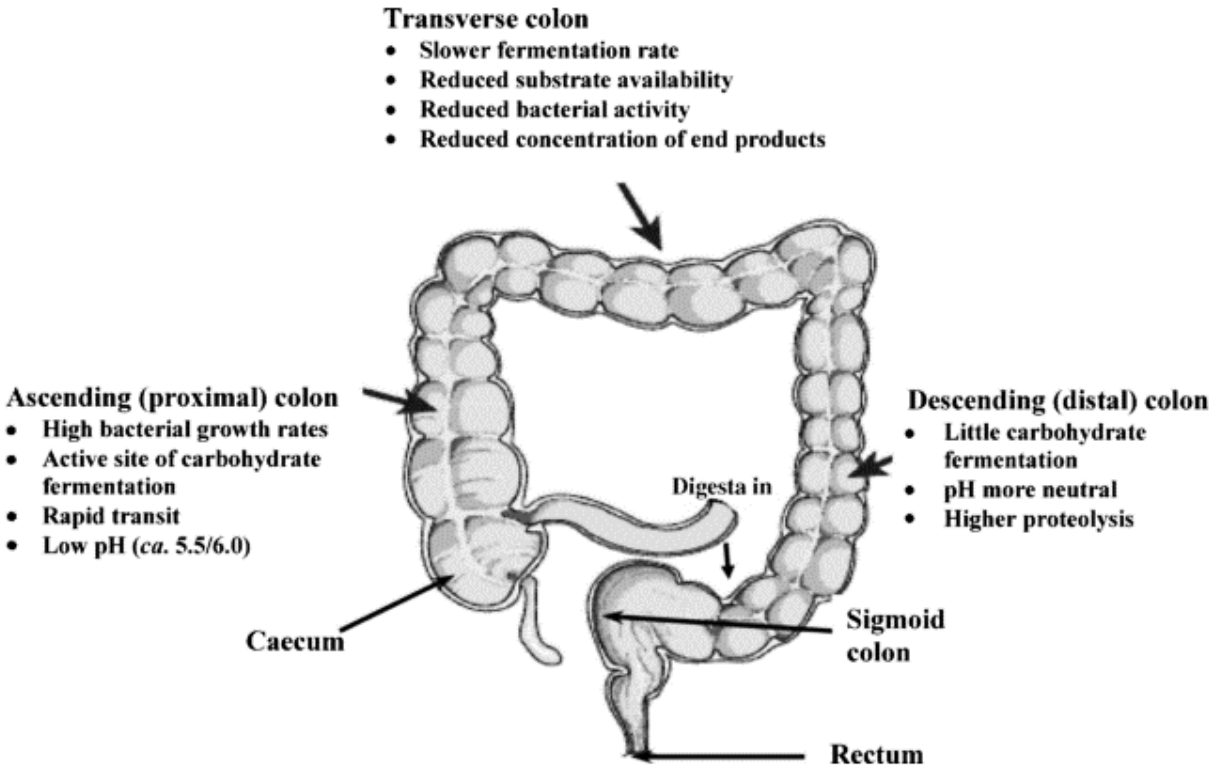

Figure 1-8: Regions of the human large intestine with corresponding bacterial activities.

The composition of the gut microbiota is important as specific species of colon bacteria such as *Bifidobacterium* and *Lactobacillus*, are potent SCFA producers (100). The predominant type of SCFAs produced also depends on the bacteria species present. For example bacteria from the *Bacteriodetes* phyla are suggested to predominantly produce acetate and propionate, whereas those from the *Firmicutes* phyla (e.g. *Lactobacilli*) predominantly produce butyrate (94).

Colonization of the gut microbiome begins at birth and is thought to be influenced by many factors including: the hosts genotype (101), gender (102), mode of delivery (natural birth versus caesarean), feeding practices (breast feeding versus formula), hygiene conditions, exposure to antibiotics, and the nature and frequency of illnesses such as gastrointestinal infections (103). This initial colonization is suggested to shape the composition of the gut microbiota, which can then be altered throughout one's lifetime by factors such as health status, stress, antibiotic therapy, smoking status, and weight (86).

The composition of the gut microbiome can also be temporarily altered with the use of probiotics, defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (104). This involves the direct delivery of live bacteria to the host, either in supplemental form (tablet/powder) or via functional foods (e.g. dairy products such as yogurts and cultured drinks). The most commonly available probiotics are strains of *Bifidobacterium* and *Lactobacillus* (105).

1.3.2 Dietary fibre/prebiotics and short chain fatty acid production

Of all the environmental factors proposed to alter the composition of the gut microbiota, dietary intake, in particular the consumption of indigestible carbohydrates, is suggested to have the largest impact (102). The interaction between indigestible

carbohydrates, the gut microbiota composition, and the production of SCFAs is complex. Fermentation of indigestible carbohydrates by gut bacteria results in the production of SCFAs which modulate colonic and intracellular pH (86). That is, an increase in SCFAs results in a decrease in pH (86). Colonic pH subsequently influences the types of SCFA-producing bacteria present (106). The lower pH in the proximal colon favours butyrate producing bacteria, and as pH increases distally, acetate- and propionate-producing bacteria become dominant (106). Overall, most SCFA production occurs in the proximal colon, where substrate availability and bacterial density is the highest and decreases distally (94).

In addition to changing the composition of the microbiome in the colon by promoting the growth of beneficial SCFA-producing bacteria such as *Lactobacillus* and *Bifidobacterium*, lowering colonic pH also inhibits the growth of potential pathogens such as clostridia (86, 97). Indigestible carbohydrates (namely soluble fibres) that selectively stimulate the growth and/or activity of beneficial gut bacteria, are referred to as prebiotics (86). Supplementation with both prebiotic soluble fibre and probiotics is hypothesised to be more beneficial than prebiotic supplementation alone. This is due to their superior ability to increase SCFA producing bacterial numbers by improving their survival and implantation in the gut (107), as well as providing substrates for fermentation (108).

SCFA production, as measured by faecal excretion, is typically in the ratio of 60:20:20 (acetate > propionate ≥ butyrate, respectively) (97). However, different indigestible carbohydrates have been shown to alter this ratio, which may be related to a number of factors (109). The monomeric composition of the indigestible carbohydrate (i.e. the type(s) of monosaccharides [glucose, galactose, fructose] the carbohydrate is composed of) is one factor (109). For example, studies have reported that resistant

starch and B-glucan (polysaccharides of glucose) primarily produce butyrate, whereas pectin (primarily composed of galactose) and inulin (polysaccharide of fructose) are mainly fermented to acetate (109). Other studies have suggested that the structure of the carbohydrate and glycosidic bond configuration may have an effect, with different SCFA profiles produced depending on the presence of α or β linkages (110). Another contributing factor is the carbohydrate chain length/degree of polymerization (DP). Short chain molecules with lower DP, such as oligosaccharides (i.e. FOS, GOS, XOS and inulin), are generally fermented more rapidly than longer chain molecules with higher DP such as acacia gum and partially hydrolysed guar gum (86).

It is estimated that up to 90-95% of SCFAs produced by the gut microbiome are absorbed by the mucosa of the colon (97). SCFAs that enter circulation (predominantly acetate) have the capacity to influence cells within peripheral tissues and have attracted much attention in recent years due to their anti-inflammatory properties. Recently published data in animal models show that orally delivered SCFAs improve the composition of the gut microbiota and reduce both systemic and airway inflammation (9).

1.4 Potential anti-inflammatory mechanisms of short chain fatty acids

Evidence suggests that SCFAs have anti-inflammatory effects due to their influence on the regulation of various molecular signalling pathways (10). To date, the anti-inflammatory properties of SCFA have mainly been studied *in vitro* (10) and in animal models (9, 10, 111-113); studies in humans are limited. Of the three SCFAs, butyrate is by far the most extensively studied in regards to anti-inflammatory properties within the colon (114). Two potential anti-inflammatory mechanisms of SCFAs are: the activation of G protein-coupled receptors (GPRs) and epigenetic regulation.

1.4.1 G protein-coupled receptor/Free fatty acid receptor activation

G protein-coupled receptors (GPRs) also known as free fatty acid receptors (FFARs) are endogenous seven-trans-membrane receptors that are involved in signalling pathways in numerous physiological and pathological processes. It is proposed that SCFAs induce anti-inflammatory effects through the activation of GPR41 and GPR43 (10, 11).

SCFAs can bind to GPR43 (also known as FFAR2), and GPR41 (also known as FFAR3) (9). GPR43 expression is highly enriched in immune cells such as peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMN), particularly neutrophils, as well as being expressed in cells of the gastrointestinal tract and adipocytes. GPR43 is preferentially activated by acetate and propionate (11), whereas GPR41 is primarily expressed in the gastrointestinal tract, as well as by immune cells, and is preferentially activated by propionate and butyrate (11).

SCFAs have been shown to have anti-inflammatory effects on immune cells via GPR43 activation. A recent study in mice showed that SCFA supplementation led to

an increase in the size and function of the colonic T regulatory cell (Treg) pool, which was dependent on GPR43 activity (111). Treg cells are involved in modulating the immune system, and as Treg function has been shown to be impaired in allergic airways disease (AAD) in mice (113), which is of particular relevance to asthma. Studies have also shown that GPR43/41 stimulation by SCFAs is necessary for the resolution of airway inflammation in animal models (112). One such study using an allergic airway inflammation model in mice, demonstrated that those deficient in GRP43 had more severe inflammation, with increased numbers of inflammatory cells present in the fluid lining the lungs and higher levels of eosinophil peroxidase activity and inflammatory cells in lung tissue (112).

In another study in mice with AAD (9), a diet rich in fibre altered the composition of the gut microbiota, by increasing proportions of the Bacteroidaceae and Bifidobacteriaceae families. Bacteria from these families are potent fermenters of fibre into SCFAs (9). Indeed, increased circulating SCFA levels were observed in this study, airway inflammation was attenuated, and dendritic cells were shown to have impaired ability to activate Th2 effector cells in the lung (9). Consequently, in mice either directly administered with acetate or propionate in their drinking water or fed a diet high in fibre, airway inflammation was attenuated (9, 111). After allergen challenge, cellular infiltration (eosinophils), IL-4, IL-5, IL-13 and IL-17A levels were reduced in the lungs and AHR improved (9). These effects were dependent on GPR41, but not GPR43 (9).

Together, these animal models suggest that soluble fibre derived SCFAs may modulate airway inflammation, via mechanisms involving the free fatty acid receptors GPR41/43. There is little data describing the role of SCFA in stimulating GPR41/43 and modulating immune responses in human airways disease.

1.4.2 Epigenetic regulation

Epigenetics are heritable changes in gene expression or changes in gene expression induced by environmental factors, that do not involve direct changes to the deoxyribonucleic acid (DNA) sequence (115). Gene expression refers to the translation of information encoded in the DNA into gene products (proteins or ribonucleic acid (RNA) structures). It is proposed that SCFAs are able to reduce inflammation through their ability to modulate this process.

DNA is stored as chromatin and organized in ~147 base pair segments around structural units called nucleosomes, which are composed of an octamer of 4 core histones. Gene expression occurs when the chromatin structure is opened up, allowing a class of proteins known as the basal general transcription machinery, to interact with the DNA and initiate transcription. While there are many epigenetic modifications that regulate gene expression, the two major reversible modifications are DNA methylation and histone modification (such as acetylation, phosphorylation, methylation, sumoylation and ubiquitination (115).

Histone acetylation is a fundamental process in epigenetics. It refers to the addition of an acetyl group from acetyl CoA to specific residues (predominantly lysine) located within the N-terminal tails of core histones (116). This changes the conformation of the chromatin, converting it from heterochromatin (closed conformation) to euchromatin (open conformation); allowing gene expression to occur (116). Acetylation of core histones is controlled by enzymes known as histone acetyltransferases (HATs) and histone deacetylases (HDACs) (116) (**Figure 1-9).**

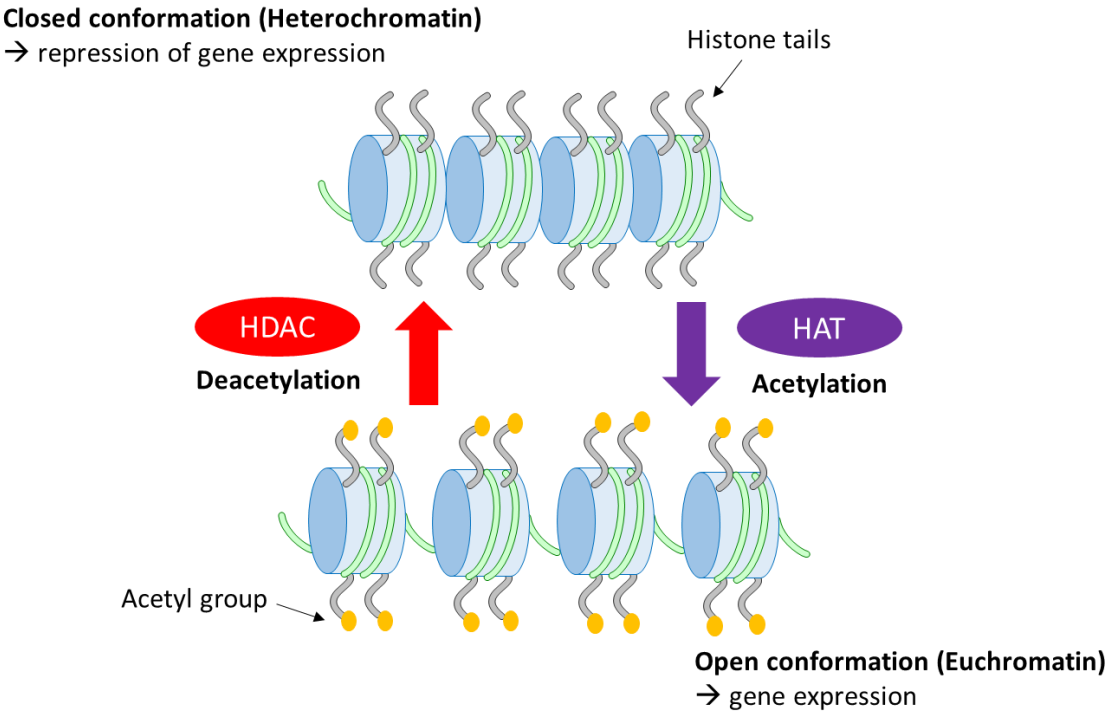

Figure 1-9: Role of HAT and HDAC enzymes in the regulation of gene expression. HAT enzymes acetylate lysine resides located within the N-terminal tails of the core histones. This allows the chromatin to open up and promotes the recruitment of transcription machinery, thus allowing gene expression to occur. HDAC enzymes remove the acetyl groups from the histones, which allows the chromatin to convert back to its closed conformation, thus repressing gene expression. *Figure created using MOTIFOLIO (Biomedical PowerPoint Tool Kits) – http:www.motifolio.com*

HAT and HDAC enzymes are also involved in the acetylation/deacetylation of non-histone proteins including transcriptional factors such as NF-κβ. These enzymes are therefore able to both directly (via chromatin remodelling) and indirectly (via regulation of transcriptional factors) influence gene expression (116).

Histone acetyltransferase enzymes (HATS)

Histone acetylation is catalysed by histone acetyltransferase (HAT) enzymes. Over 30 different enzymes have been identified to date, which can be broadly classified based on their cellular location as Type A HATs (located in the nucleus) or Type B HATs (located in the cytoplasm) (117) (**Figure 1-10)**.

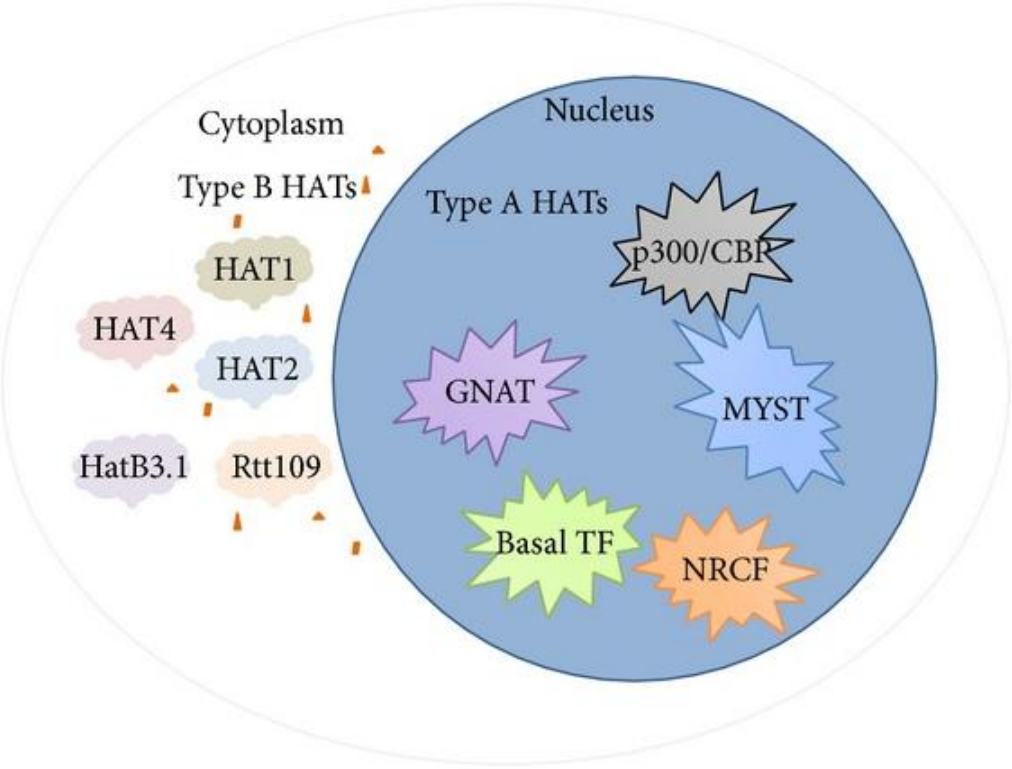

Figure 1-10: HAT enzyme classification. There are two main classes of HATs: Type A (located in the nucleus) and Type B (located in the cytoplasm. Type A HATs are further divided into 5 families: General control non-depressible related acetyltransferase (GNAT) HATs, p300/CBP HATs, MYST HATs, basal TF HATs and Nuclear Receptor Cofactor (NRCF) HATs. Type B HATs include: HAT1, HAT2, Rtt109, HATB3.1 and HAT4. *Figure from Wang et al, 2014* (117).

Type A HATs acetylate nucleosomal histones and chomatin-associated proteins and are therefore involved in regulating transcription (117). Type A HATs can be further divided into five families (117, 118) (**Figure 1-10)**: **(1)** GCN5-related *N*-acetyltransferase (GNAT) family (which includes: Elongator Complex protein 3 (ELP3), General Control Nonderepressible (Gcn5) and p300/CREB-binding protein (CBP) associated factor (PCAF); **(2)** p300/CBP family (which includes: CBP and E1A binding protein p300); **(3)** MYST family (which includes: monocytic leukemia zinc-finger protein (MOZ), Something About Silencing protein 3 (SAS3), yeast binding factor 2/SAS3 (YBF2/SAS2), Tat interative protein-60 (TIP60) and monocytic

leukemia zinc finger protein-related factor (MORF); **(4)** basal transcription factor (TF) family (which includes: TFIIIC and TATA-binding protein Associated Factor 1 (TAF1); and **(5)** Nuclear Receptor Cofactors (NRC) family (which includes steroid receptor coactivator ACTR/NCOA3 (nuclear receptor coactivator 3) (117).

Type B HATs, located in the cytoplasm, acetylate newly synthesised free histones before they are assembled into nucleosomes (117). To date, only several Type B HATs have been characterised: HAT1, HAT2, Rtt109, HatB3.1 and HAT4 (117).

Histone deacetylase enzymes (HDACS)

Histone deacetylases (HDACs) are enzymes that work in opposition to HATs by removing acetyl groups from histones (119). This allows the chromatin to transform back to its heterochromatin conformation, preventing transcriptional factors and RNA polymerase from accessing the DNA and thereby repressing gene expression. There are two major classes of HDACs, zinc-dependent and Nicotinamide adenine dinucleotide (NAD)(+)-dependent (117) (**Figure 1-11**). To date, 11 classical zinc-dependent HDAC enzymes have been identified (divided into classes I, II and IV) (119). Class I HDACs (HDAC 1-3, and 8) are found predominantly in the nucleus and are present in most cell types, whereas class II HDACs (IIA; HDAC 4, 5, 7 and 9. IIB; HDAC 6 and 10) have the ability to shuttle between the nucleus and cytoplasm (117, 120). HDAC11 is currently the only member of the Class IV HDAC subgroup, and shares similarities with both Class I and II HDACs (117, 119).

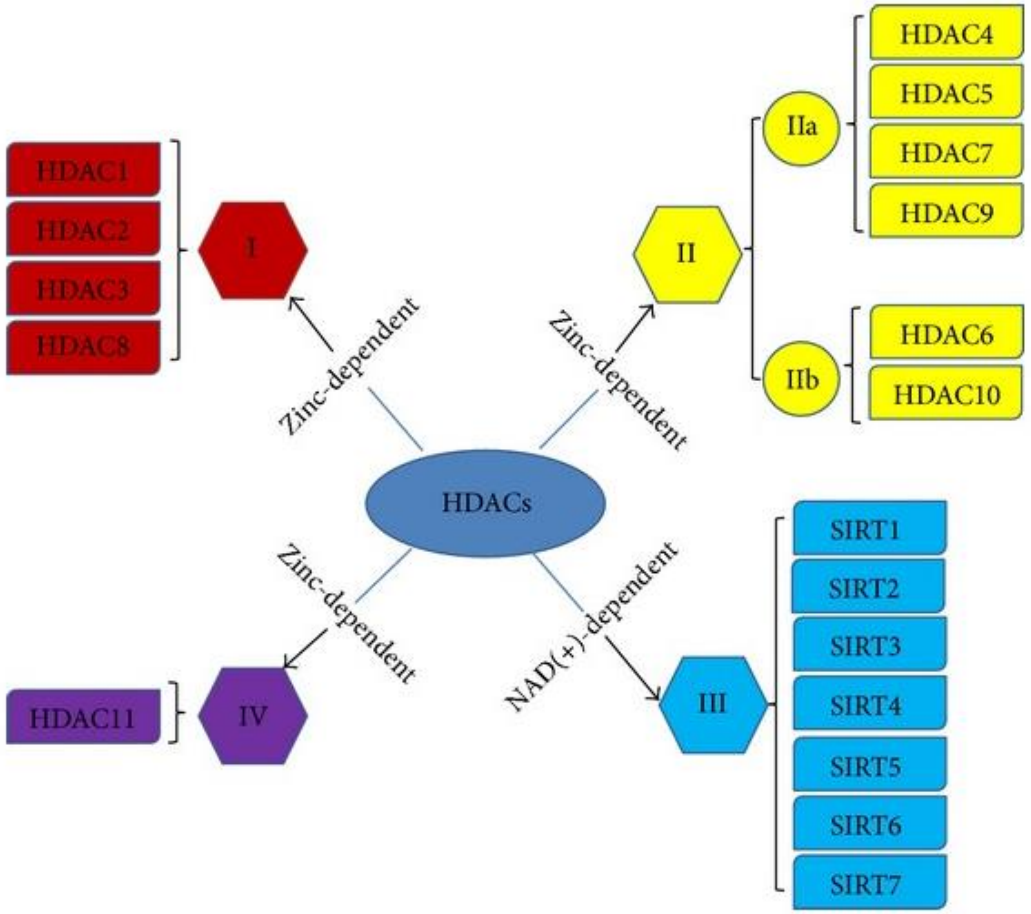

Figure 1-11: HDAC enzyme classification. There are two main categories of HDACs: NAD (+)-dependent and zinc-dependent, which are further divided into 4 classes: Class I HDACs (HDAC1, 2, 3 and 8); Class II HDACs (IIa: HDAC4, 5, 7 and 9. IIb: HDAC6 and 10); Class III HDACs (SIRT1-7); and Class IV HDAC (HDAC11). *Figure from Wang et al, 2014* (117).

1.4.2.1 Altered HAT/HDAC enzyme activity in asthma

Altered HAT/ HDAC activity is observed in humans with asthma (115, 121). It has been reported that there is an increase in total HAT activity and decrease in total HDAC activity in asthma patients compared to those without asthma, with one study suggesting that this altered ratio contributes in part to the increased inflammation observed (115). Decreased expression and activity of HDAC2 in particular has been

reported in lung macrophages, and peripheral blood cells from individuals with asthma, associated with glucocorticoid insensitivity (122). HDAC9 gene expression however has been reported to be elevated in individuals' asthma, and negatively correlated with FEV_1 %predicted in moderate-severe asthma (123). Furthermore, our group has recently collaborated on a comprehensive study of dietary fibre and SCFA using an allergic mouse model of airways disease. In this study, it was discovered that HDAC9 knockout mice were resistant to the development of AAD. This suggests that HDAC9 in particular plays an important role in enhancing airway inflammation (124), and highlights the importance of examining the effect of individual HDAC subtypes on inflammation in addition to total HDAC activity.

Due to the paucity of research in this area, the use of HDAC inhibitors in inflammatory diseases such as asthma is controversial (118). However, as discussed, HDAC inhibitors have been found to reduce the production of pro-inflammatory cytokines in animal models of AAD (125).

1.4.2.2 Short chain fatty acids as HDAC inhibitors

SCFAs are proposed to modulate inflammation due to their role as HDAC inhibitors, with butyrate the most potent HDAC inhibitor of the SCFAs (13). Many studies show that SCFAs reduce inflammation by inhibiting HDAC activity, which in turn suppresses NF-κB activity (126, 127) (**Figure 1-12**). NF-κB is a family of transcription factors that play a central role in immune and inflammatory responses (126). This is in part through their involvement in the transcriptional regulation of many pro-inflammatory cytokine genes (e.g. TNF-α, IL-1 and IL-6)(128). Indeed, butyrate has been shown to inhibit cytokine-stimulated (TNF-α, and IL-1) NF-κB activation in

epithelial and colonic cells, and lipopolysaccharide (LPS) stimulated NF-κB activation in peripheral mononuclear cells (129).

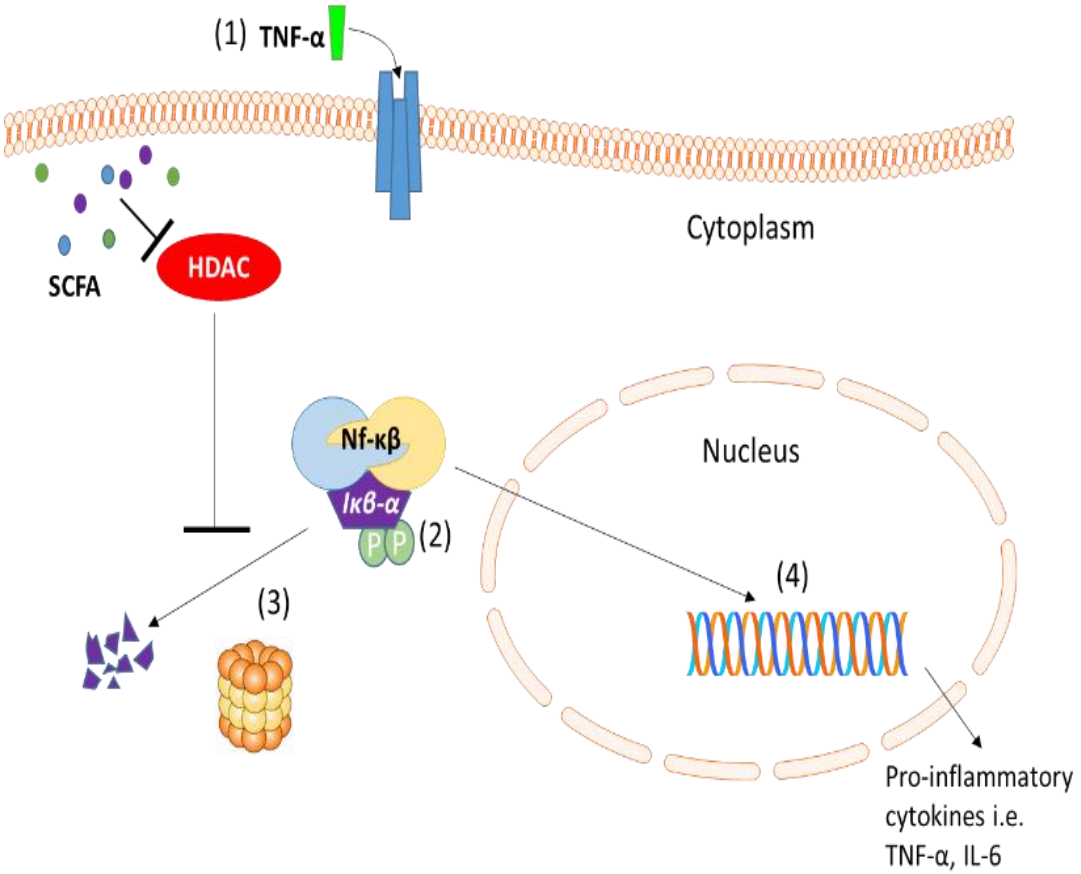

Figure 1-12: HDAC inhibitors regulate NF-κB activity. 1) Pro-inflammatory mediators such as TNF-α activate the NF-κβ signalling cascade. 2) NF-κB resides in the cytoplasm in an inactive state through association with an inhibitor of kappa beta (Iκβ) protein such as Iκβα. Upon stimulation by a pro-inflammatory mediator, Iκβα is phosphorylated and undergoes polyubiquination. 3) Iκβα is then degraded by the 26S proteasome, 4) allowing NF-κB to translocate to the nucleus where is activates the transcription of pro-inflammatory cytokines. HDAC inhibitors such as SCFAs have been shown to reduce inflammation by inhibiting NF-κB activity. One possible mechanism is by down-regulating the expression of three catalytic proteasome subunits (β1, β2 and β5), preventing the protease-depended degradation of Iκβα and subsequently preventing NF-κB from translocating to the nucleus. *Figure created using MOTIFOLIO (Biomedical PowerPoint Tool Kits) – http:www.motifolio.com*

Different HDAC enzymes have been found to be differentially inhibited by SCFA. One study demonstrated that in colonic Treg cells, propionate reduced HDAC6 and 9, but not HDAC1, 2 or 7 expression (111), whereas another study demonstrated that butyrate inhibits all class I and II HDACs with the exception of HDAC6 and HDAC10

(118). This highlights the need to focus on specific HDAC subtypes in order to understand the impact SCFAs have on their role in gene expression and inflammation. Furthermore, data from *in vitro* or *ex vivo* studies of airway cells is very limited. A study using a mouse model of AAD shows the potential for HDAC inhibition to alleviate airway inflammation (125). Treatment with the HDAC inhibitor Trichostatin A (TSA) suppressed airway levels of eosinophils, lymphocytes, IL-4 and IL-5 and reduced AHR (125). Human studies of SCFAs as HDAC inhibitors in airways disease are urgently needed to gain a better understanding of their potential role in asthma therapy.

1.5 Clinical interventions to increase short chain fatty acid production and reduce inflammation

In humans, most intervention studies using SCFAs have been designed to improve mucosal inflammation and symptoms in patients with inflammatory bowel conditions such as ulcerative and diversion colitis (97, 130). SCFA delivery methods commonly used in these studies include enemas and slow release tablets (primarily containing butyrate) designed to release the SCFA into the colon (114). Delivery of SCFAs via these methods has been reported to reduce mucosal inflammation and clinical symptoms (97, 130).

To impact diseases of the peripheral organs such as the lungs however, the goal is to improve circulating SCFA levels. In animal studies this has been done by directly delivering large doses of oral SCFA (131). However in humans, oral delivery of SCFA is not optimal, as >80% of acetate delivered orally is oxidized, with plasma acetate levels only remaining elevated for ~60 min (132). An alternative approach is supplementation with prebiotic soluble fibres, which successfully increase circulating SCFA levels (133), modulate the microbiome and modify aspects of the immune system (134).

One of the most rigorously designed clinical studies utilizing prebiotics to date is a double-blind, placebo controlled trial in 44 elderly subjects, who consumed oligosaccharides (5.5g/day) for 10 weeks (135). The supplement significantly increased *Bifidobacterium* numbers, increased phagocytosis, NK cell activity and IL-10 production and decreased IL-6, IL-1β and TNF-α production, with the majority of anti-inflammatory effects evident at 5 weeks. In another 3 week study in the elderly, oligosaccharides (8g/day) enhanced *Bifidobacterium* numbers and decreased IL-6 gene expression in peripheral blood monocytes (136). Although neither serum SCFA

concentrations nor faecal SCFA excretion was measured in these studies, these results provide evidence supporting the anti-inflammatory effects of prebiotics.

In the airways, human data describing the effects of prebiotics is lacking, though animal studies have shown that oligosaccharides attenuate eosinophilic airway inflammation and reduce IL-4 and IL-5 in lung tissue (137, 138). One study in humans demonstrated that a specific prebiotic oligosaccharide mixture, that increases the amount of *Bifidobacterium* in the colon, significantly reduced the incidence of recurrent wheezing in n=134 2-year-old children with atopic parents (139). This is important as recurrent early wheeze has been associated with the later development of asthma (139).

Furthermore, pilot data from our group at HMRI has demonstrated that a single 3.5g dose of the soluble fibre inulin (in a pre-/ probiotic combination) modified systemic and airway inflammation in adults (≥18 years) with stable asthma (n=17) (140). Four hours after the administration of the pre-/probiotic combination, GPR43 and GPR41 gene expression were upregulated sputum cells (**Figure 1-13**), which corresponded with decreases in plasma TNF-α, total sputum cell count, sputum neutrophils, macrophages and lymphocytes, sputum IL-8 and exhaled nitric oxide (140).

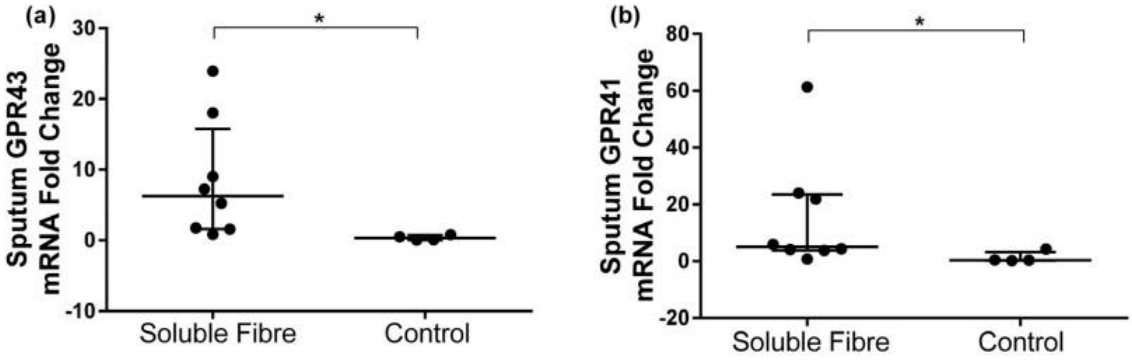

Figure 1-13: Change in sputum (a) GPR43 and (b) GPR41 gene expression 4 h following soluble fibre challenge (soluble fibre group n = 8) or control meal challenge (control group n = 4). Data expressed as median (IQR) and analyzed by Wilcoxon rank-sum test.* $p < 0.05$. *Data from Halnes et al, 2017* (140).

While SCFA levels were not measured in this study, these findings do support observations from an animal model which demonstrated that SCFA dependent GPR41 stimulation is required for airway inflammation resolution (9).

1.6 Summary

Asthma is a common chronic respiratory disease that affects >300million people worldwide. Although inhaled glucocorticoids are currently the mainstay of asthma management, long-term use has been associated with increased risk of adverse side effects such as osteoporosis. Additionally some patients are unresponsive to such treatment. Evidently, new ways to manage asthma are urgently required.

It is proposed that the development and progression of asthma is driven by a complex interplay between genetic and environmental factors. Indeed, there appears to be an association between increased asthma prevalence and the westernised lifestyle, including the Western diet that is characteristically low in dietary fibre. This is undesirable as dietary fibre intake is not only inversely associated with systematic inflammation, but, as demonstrated by our research team at HMRI, it is also inversely associated with the severity of airway inflammation and positively associated with lung function.

Soluble fibre in particular has been shown to promote the growth of beneficial gut bacteria which are strongly involved in maintaining healthy immune responses. Furthermore, soluble fibre is fermented by these beneficial bacteria, producing anti-inflammatory metabolites, namely SCFAs. Recently, it has been demonstrated in animal studies that SCFAs have the ability to reduce both systemic and airway inflammation, by activating GPRs (e.g. GPR41/43), and by modulating epigenetic mechanisms through the inhibition of HDAC enzyme activity. Whether SCFAs can modulate inflammation in humans with asthma is currently unknown.

This thesis will investigate the efficacy of soluble fibre-derived SCFAs in reducing inflammation in both adults and children with asthma. This will involve examining the

potential anti-inflammatory mechanisms of SCFAs (i.e. the activation of GPRs, and epigenetic regulation through the inhibition of HDAC activity). Overall, it is anticipated that this thesis will provide evidence for the therapeutic potential of SCFAs in asthma management, in addition to generating new mechanistic insights into the regulation of inflammation in humans.

1.7 Aims and Hypothesis

1.7.1 Key points of background to thesis

1. The burden of asthma is high; >3 million people are affected worldwide.
2. There appears to be an association between increased asthma prevalence and the westernised lifestyle, including the westernised diet.
3. Westernisation of diets has led to reduced dietary fibre intake and alterations in the gut microbiota.
4. Dietary fibre intake has been shown to be inversely associated with severity of airway inflammation and positively associated with alterations in lung function in asthma.
5. SCFAs produced from the fermentation of soluble dietary fibre by gut microbiota reduce airway inflammation in animal models, however whether SCFAs can modulate inflammation in humans with asthma is currently unknown and the mechanisms responsible are unclear.

1.7.2 Hypothesis of thesis

Study 1: A study of the literature will show that SCFAs, prebiotics and synbiotics have systemic anti-inflammatory effects in humans.

Study 2: In adults with asthma, increasing soluble fibre intake (via a dietary supplement) will;

- **i.** improve lung function
- **ii.** increase faecal and plasma SCFA levels
- **iii.** reduce airway inflammation via mechanisms involving SCFAs, such as the inhibition of HDACs and activation of G-protein coupled receptors (GPR41/43)

Study 3: In children with asthma, increasing soluble fibre intake (via increasing fruit and vegetable consumption) will:

- **i.** reduce time to first asthma exacerbation and frequency of asthma exacerbations and improve clinical outcomes
- **ii.** reduce systemic and airway inflammation via mechanisms involving SCFAs, such as the inhibition of HDACs and activation of G-protein coupled receptors

1.7.3 Aims of thesis

Study 1: To examine the available evidence for the effect of SCFAs, prebiotics and synbiotics on systemic inflammation in humans

Study 2:

- **i.** To examine the effect of soluble fibre supplementation, with and without a probiotic, on lung function in asthma.
- **ii.** To examine the effect of soluble fibre supplementation, with and without a probiotic, on plasma and faecal SCFA levels, and systemic and airway inflammation in asthma.
- **iii.** To examine molecular mechanisms that are modulated by soluble fibre supplementation in asthma, including HAT/HDAC enzyme activity in PBMCs, and GPR41/43 and HDAC1-11 gene expression in PBMCs and sputum cells.

Study 3:

- **i.** To examine the effects of a high fruit and vegetable diet, versus a standard fruit and vegetable diet on clinical outcomes in children with asthma
- **ii.** To examine the effect of a high F&V, versus a standard F&V diet on systemic and airway inflammation.
- **iii.** To examine molecular and epigenetic mechanisms that are modulated by a high F&V diet in children with asthma, including HAT/HDAC enzyme activity and GPR41/43 and HDAC1-11 gene expression in PBMCs.

Chapter 2: Effect of short chain fatty acids, prebiotics and synbiotics on systemic inflammation: a systematic review and meta-analysis

This chapter has been published:

McLoughlin, R. F.; Berthon, B. S.; Jensen, M. E.; Baines, K. J.; Wood, L. G. Short-chain fatty acids, prebiotics, synbiotics, and systemic inflammation: a systematic review and meta-analysis. Am J Clin Nutr 2017.

2.1 Abstract

Background: Prebiotic soluble fibers are fermented by beneficial bacteria in the colon to produce short-chain fatty acids (SCFAs), which are proposed to have systemic anti-inflammatory effects. **Objective:** This review examines the effect of SCFAs, prebiotics, and pre- and probiotic combinations (synbiotics) on systemic inflammation. **Design:** Relevant English language studies from 1947 to May 2017 were identified with the use of online databases. Studies were considered eligible if they examined the effects of SCFAs, prebiotics, or synbiotics; were delivered orally, intravenously, or per rectum; were on biomarkers of systemic inflammation in humans; and performed meta-analysis where possible. **Results:** Sixty-eight studies were included. Fourteen of 29 prebiotic studies and 13 of 26 synbiotic studies reported a significant decrease in ≥1 marker of systemic inflammation. Eight studies compared prebiotic and synbiotic supplementation, 2 of which reported a decrease in inflammation with synbiotics only, with 1 reporting a greater anti-inflammatory effect with synbiotics than with prebiotics alone. Meta-analyses indicated that prebiotics reduce C-reactive protein (CRP) [standardized mean difference (SMD): 20.60; 95% CI: 20.98, 20.23], and synbiotics reduce CRP (SMD: 20.40; 95% CI: 20.73, 20.06) and tumor necrosis factor-a (SMD 20.90; 95% CI: 21.50, 20.30). **Conclusions:** There is significant heterogeneity of outcomes in studies examining the effect of prebiotics and synbiotics on systemic inflammation. Approximately 50% of included studies reported a decrease in ≥1 inflammatory biomarker. The inconsistency in reported outcomes may be due to heterogeneity in study design, supplement formulation, dosage, duration, and subject population. Nonetheless, meta-analyses provide evidence to support the systemic anti-inflammatory effects of prebiotic and synbiotic supplementation.

2.2 Introduction

Systemic inflammation is associated with the development and progression of a number of chronic health conditions. These include the leading causes of mortality worldwide: ischaemic heart disease, dementia, cerebrovascular diseases, cancer and chronic lower respiratory diseases (141). Systemic inflammation results from the chronic activation of the immune system and release of pro-inflammatory mediators such as C-reactive protein (CRP), tumour necrosis factor-alpha (TNF-α) and interleukins (ILs) (i.e. IL-6 and IL-1β). An inverse relationship between dietary fibre intake and biomarkers of systemic inflammation (i.e. IL-6, TNF-α, and CRP) has been demonstrated in numerous studies (8, 81-84, 87). Although the anti-inflammatory mechanisms of dietary fibre are yet to be elucidated, short chain fatty acids (SCFAs) may be a contributing factor (10).

SCFAs (i.e. acetate, butyrate and propionate) are physiologically active by-products primarily produced from the fermentation of soluble dietary fibre and resistant starch by commensal bacteria in the colon (94). A number of factors can influence SCFA production including the fermentation substrate available. For example, oligosaccharide soluble fibres (i.e. fructo-oligosaccharides, FOS) produce a higher SCFA yield than longer chain polysaccharide soluble fibres (i.e. pectin) (86). SCFAs lower the colonic pH, which can promote the growth of beneficial bacteria, such as *Lactobacilli* and *Bifidobacterium*. These bacteria are potent SCFA producers and play a role in maintaining healthy immune responses (87, 142). Soluble fibres such as FOS that selectively stimulate the growth and/or activity of commensal bacteria associated with health benefits to the host, are referred to as prebiotics (86). The composition of the colonic microbiome can also be temporarily altered using probiotics. This involves the direct delivery of live bacteria (commonly strains of *Bifidobacterium* and

Lactobacillus (105)) to the host, either in supplemental form or via functional foods (e.g. yoghurts). Synbiotics are a mixture of prebiotics and probiotics, and therefore are considered to have synergistic effects.

It is estimated that up to 90% of SCFAs are absorbed from the intestinal lumen, with the majority either metabolised by colonocytes or delivered to the liver via the hepatic portal vein. A small portion of SCFAs (primarily acetate) also enter systemic circulation where they have the capacity to influence cells within peripheral tissues. One proposed effect of SCFAs is the reduction of systemic inflammation (9), through the modulation of molecular signalling pathways including the activation of G-protein coupled receptors (GPR) 41 and GPR43 (10) and the inhibition of histone deacetylase (HDAC) enzymes (13). Previous reviews have examined the metabolic and immunomodulatory properties of synbiotics and selected prebiotics (primarily inulin and FOS) (134, 143). To our knowledge this is the first systematic review to examine the effect of SCFAs and a wide range of prebiotics and synbiotics on systemic inflammation in humans.

2.3 Methods

2.3.1 Search Strategy

The electronic databases Medline, Excerpta Medica database (EMBASE), PubMed, Cumulative Index to Nursing and Allied Health Literature (CINAHL) and Cochrane were searched for English language articles from 1947 to May 2015, using keywords and Medical Subject Headings of the National Library of Medicine (MeSH). Hand searching of reference lists of retrieved articles and relevant systematic reviews was conducted, as well as cited reference searches of retrieved articles through the Web of Science database. The MeSH search terms included: dietary fibre/fiber, soluble fibre/fiber, indigestible carbohydrates, fermentable carbohydrates, short chain fatty acids, volatile fatty acids, butyrate, propionate, acetate, prebiotics, synbiotics, inflammatory markers, interleukin, CRP, TNF-α, IL-6. See **Figure 1** for an example of the search strategy used. The search was conducted again in 2017 to ensure any relevant articles published since the initial search were identified.

2.3.2 Article Inclusion and Exclusion Criteria

Studies were included if they examined the effects of SCFAs, prebiotics and/or synbiotics delivered orally, intravenously or per rectum (enema) on any biomarker of systemic inflammation in human participants of any age and gender. Randomised controlled trials (RCTs), quasi-experimental studies, cohort studies, case-control studies, before and after studies and observational cross-sectional studies were included. Exclusion criteria were; animal studies, *in vitro* studies, studies examining the effects of probiotics alone on inflammation, and studies which did not report on systemic inflammation i.e. inflammation outside the gastrointestinal tract. Systematic reviews, narrative reviews, opinion papers and case-studies were also excluded.

1. inflammation OR inflammatory marker OR inflammation mediator OR interleukin OR interleukin 6 OR tumor necrosis factor alpha OR tumour necrosis factor alpha OR TNF-alpha OR c reactive protein OR CRP OR IL-6
2. soluble fibre OR soluble fiber OR soluble fiber psyllium OR soluble fiber supplementation OR soluble fiber supplements OR beta glucan OR psyllium OR pectin OR dietary fiber OR dietary fibre OR indigestible carbohydrate OR indigestible fiber OR indigestible fiber OR fermentable carbohydrate OR fermentable carbohydrate supplementation OR fermentable dietary carbohydrate OR fermentable dietary fibers OR fermentable dietary fibres OR fermentable fructo oligosaccharides OR fermentable galactooligosaccharides OR undigested carbohydrate OR undigested dietary fiber OR undigested dietary fibre OR fructan OR inulin OR oligofructose OR fructooligosaccharide OR FOS OR GOS OR galactooligosaccharide OR prebiotic OR prebiotic fibre supplement OR prebiotic fiber supplement OR prebiotic food OR resistant starch OR non starch polysaccharide OR probiotic/prebiotic OR probiotic/prebiotic supplement OR synbiotic OR synbiotic supplement OR short chain fatty acid OR scfa OR volatile fatty acid OR vfa OR acetate OR acetic acid OR butyrate OR butyric acid OR propionate OR propionic acid
3. 1 AND 2
4. Filters: humans

Figure 2-1: Example of search strategy using PubMed for studies investigating the effect of SCFA, prebiotics and synbiotics on systemic inflammation in humans.

2.3.3 Article Appraisal and Data Extraction

Studies retrieved by the search strategy were independently assessed for relevance to the review by two reviewers (RM and BB), based on title, abstract and full-text. Where there was a disagreement on the inclusion of a study, a third independent reviewer (LW) was involved. At each stage, reasons for exclusion were documented. Following the full-text appraisal, all included studies were independently assessed by the two reviewers (RM and BB) for methodological quality using a standardized critical appraisal checklist designed by the American Dietetic Association (144). This tool incorporates four relevance questions which address applicability of the study findings to practice, and ten validity questions that address scientific rigour. Based on the responses to these questions as determined by the reviewers (RM and BB), each study was rated as having negative, positive or neutral quality. Studies of negative quality (response to ≥6 validity questions was "no"), were excluded. The level of evidence for each article was determined according to the study design based on the National Health and Medical Research Council (NHMRC) of Australia levels of evidence hierarchy (145).

The following data were extracted from included studies using a standardized data extraction tool: country, participant characteristics, study design, sample size, intervention details (SCFA, prebiotic or synbiotic composition and dose), treatment duration, assessment of compliance to intervention, assessment of background dietary intake and outcomes of interest (mean and standard deviation (SD) before and after supplementation period of each group). Outcomes of interest were plasma/serum inflammatory markers (e.g. interleukins, TNF-α, CRP). When SD values for any outcomes of interest were not reported, these values were calculated from the reported standard error (SE) or 95% confidence interval (CI). Included studies were categorized

according to the intervention described, to assess the evidence relating to SCFAs, prebiotics and synbiotics.

2.3.4 Meta-analysis

Meta-analysis was performed using Review Manager (RevMan) Version 5.3 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). Heterogeneity was examined using the chi-squared test ($P<0.1$ considered to indicate significant heterogeneity), and the I^2 parameter (with 30-60% indicating moderate, 50-90% indicating substantial and 75-100% indicating considerable heterogeneity (146)). Where considerable heterogeneity was identified, sub-group analyses were conducted to investigate possible contributing factors. As the studies were considered heterogeneous in relation to the type and dosage of prebiotic/synbiotic supplementation, treatment duration and study population (i.e. disease status and country of origin), the random effects meta-analysis model was applied to all meta-analyses. The inverse-variance statistical method was used, and the standard mean difference (SMD – effect size) and corresponding 95% CI calculated. The Cochrane Handbook for Systematic Reviews of Interventions was used to determine whether it was appropriate to include data from cross-over studies in the meta-analyses (146). Cross-over studies were excluded from the meta-analysis if insufficient data were available to eliminate the possibility of carry-over effects, results from paired analyses were not reported, or data were not reported in a suitable form (i.e. individual participant data or within-patient differences) to allow paired analysis to be approximated. *Ex vivo* trials were also excluded due to inherent differences to *in vivo* studies.

2.4 Results

A total of 8609 articles were identified, with 2744 excluded as they were duplicates **(Figure 2)**. The titles of the remaining 5865 articles were reviewed, with 843 articles (~14%) retrieved for abstract appraisal. Abstracts from 150 articles met the inclusion criteria and the full texts were retrieved for further review. Data extraction and assessment of methodological quality were performed on the 75 articles that met the review criteria, of which seven were excluded due to negative methodological quality (91, 136, 147-151).

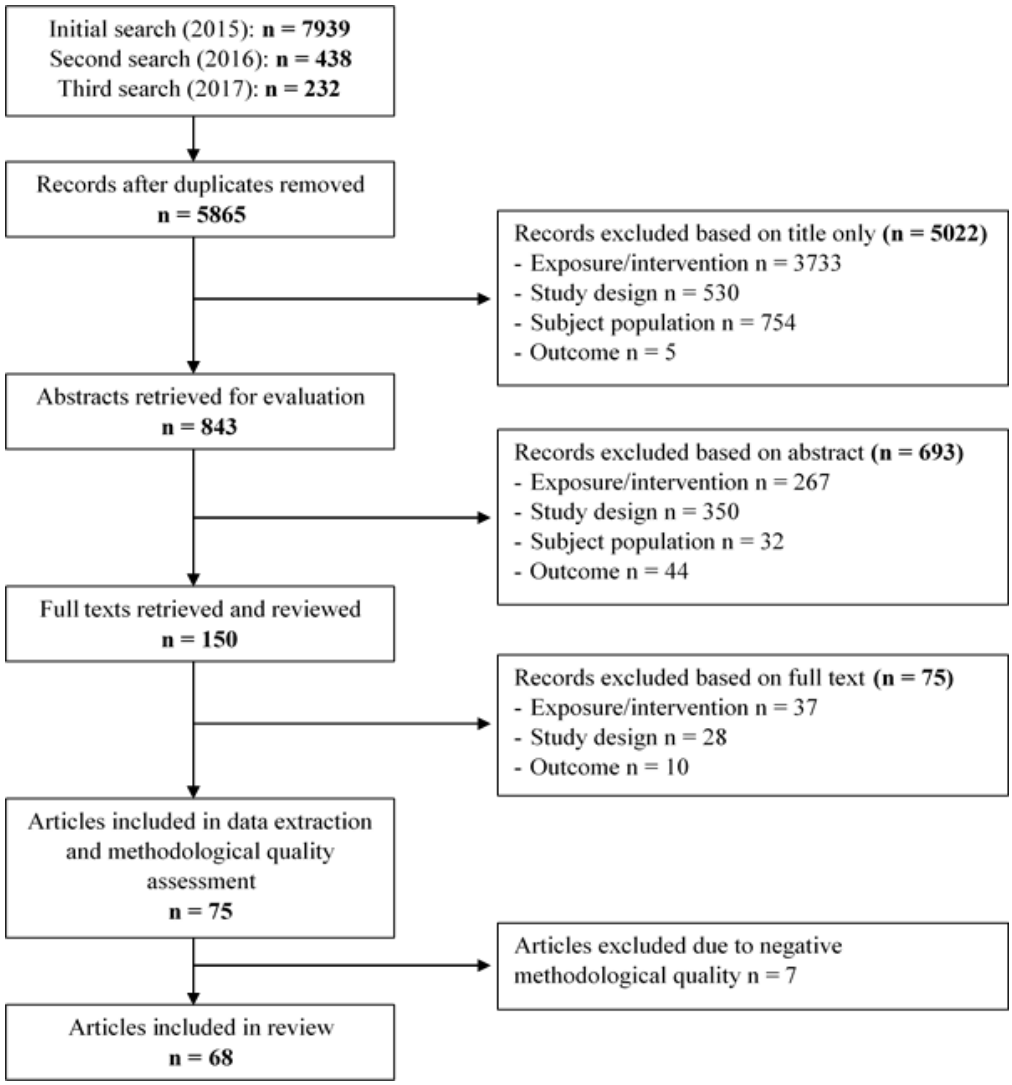

Figure 2-2 PRISMA flowchart of articles for inclusion in a systematic review of the effect of SCFA, prebiotics and synbiotics on systemic inflammation.

2.4.1 Description of included studies

Of the 68 included studies, 65 (95%) were RCTs (n= 18 cross-over trials), two were observational cross-sectional studies, and one a pre-test/post-test intervention study. The publication year ranged from 2004 to 2017. Twenty-nine studies (43%) were from Europe (152-180); 20 (30%) from Asia (181-200); 16 (25%) from America (81, 201-215) and three (5%) from Australia (216-218). The majority of studies (97%) were conducted in adults (≥18 years). In 55 (81%) studies, participants had existing inflammatory conditions; these were most commonly Type 2 Diabetes Mellitus (T2DM), hypercholesterolemia, cancer, inflammatory bowel diseases, liver diseases and overweight/obesity.

Of the 68 included studies, five (7%) used SCFAs (152, 153, 179, 180, 201) (**Table 1**), 29 (44%) used prebiotics (81, 154-164, 177, 183, 187-189, 199, 200, 202-211) (**Table 2)**, 26 used synbiotics (39%) (165-174, 181, 182, 184-186, 190, 192-198, 214, 215, 218) (**Table 3)** and eight (12%) compared the effect of prebiotic and synbiotic supplementation (175, 176, 178, 191, 212, 213, 216, 217) (**Table 4).** Intervention duration ranged from 1hr to 24wks (median: 6wks). In 42 intervention studies (65%), participants were instructed to maintain usual diet habits; however, only 32 studies (50%) reported measuring background diet intake before and after the intervention. Of these studies, 23 reported no significant difference in dietary intake between study groups at baseline and at the end of the study; the other nine did not report the findings of the background diet analysis.

Approximately 50% of the studies had positive methodological quality. These studies were methodologically strengthened by the use of random allocation to the intervention/control group or treatment sequence (cross-over trials), double-blinding

and comparability of study groups. Factors which limited the methodological quality of the 29 studies rated as neutral included insufficient detail provided regarding the intervention protocol, the measurement of potential intervening factors (e.g. background diet intake), and the comparability of study groups (i.e. limited/lack of subject characteristics reported). A biomarker of inflammation was a primary outcome in 45 (66%) of the included studies.

2.4.2 Effects of Short Chain Fatty Acids on Systemic Inflammation

Characteristics of the five included SCFA studies are presented in **Table 2-1** (152, 153, 179, 180, 201). Canfora et al (179) examined the effect of three SCFA mixtures (high acetate, high propionate, and high butyrate) delivered via enema, on systemic inflammation during fasting and postprandial conditions. While fasting IL-1β was significantly decreased by the high acetate mixture compared to the high propionate mixture, there was no significant difference compared to placebo or in IL-1β postprandially between treatments (179). Furthermore, changes in other pro-inflammatory cytokines (TNF-α, IL-6 and IL-8) did not significantly differ between treatments, in either the fasting or postprandial conditions (179). Van der Beek et al (180) reported lower fasting plasma TNF-α levels after distal administration of acetate compared to placebo, however this did not reach significance. Two studies by Hamer et al reported no significant change in plasma CRP following the delivery of the SCFA butyrate via enema (152, 153). However, Freeland et al (201) reported a significant decrease in plasma TNF-α following administration of the SCFA acetate both via enema and intravenously, with no significant difference between administration routes (201).

Dietary intake was similar between study groups at baseline and follow-up, in all of the studies. Overall, a significant decrease in inflammation was observed in 40% of the included SCFA studies. Meta-analysis was not possible due to small study numbers and heterogeneity in study design.

Table 2-1: Summary of included studies examining the effect of short chain fatty acids on systemic inflammation

Reference (country)	Design/ level of evidence	Quality	SCFA - daily dose	Control – daily dose	Participants	Duration	Effect of intervention on inflammation
Freeland 2010 (Canada)(201)	RCT(x-over)/II	∅	Acetate - 60mmol enema or 20mmol IV	Normal saline - 300mL enema or 100ml IV	N = 6 Hyperinsulinemia	1h	↓ TNF-α
Hamer 2009 (Netherlands)(152)	RCT(x-over)/II	+	Butyrate - 60mL enema (100mM)	NaCl solution – 60mlL (140mM) enema	N = 16 Healthy	2wk	↔ CRP
Hamer 2010 (Netherlands)(153)	RCT/II	∅	Butyrate - 60mL enema (100mM)	NaCl solution – 60mL (140mM) enema	N = 35 UC	20d	↔ CRP
Canfora 2017 (Netherlands)(179)	RCT(x-over)II	+	SCFA mixtures high in acetate/butyrate/ propionate - 200mL enema (200mM)	NaCl - 200mL enema (40mmol NaCl)	N = 12 overweight/obese	1d/arm 5d w/o	↓ fasting IL-1β (acetate v propionate)
Van der Beek 2016 (Netherlands)(180)	RCT(x-over)/II	+	Acetate - via distal or proximal feeding catheter (100 or 180mM in 120ml 0.9% NaCl)	120ml 0.9% NaCl via distal or proximal feeding catheter	N = 6 overweight/obese	3d/arm 7d w/o	↔ TNF-α, IL-6, IL-8

Methodological study quality was determined using the American Dietetic Association critical appraisal checklist; ∅ represents neutral study quality; + represents positive study quality. N= total number of participants in study; ↓ indicates decrease, ↑ indicates increase, ↔ indicates no change. Abbreviations: CRP, C-reactive protein; IV, intravenous; NaCl, sodium chloride; RCT, randomised controlled trial; SCFA, short chain fatty acid; UC, ulcerative colitis; w/o, wash out; x-over, cross-over study design.

2.4.3 Effect of Prebiotics on Systemic Inflammation

Characteristics of the 29 included prebiotic studies are presented in **Table 2**, of which 14 (48%) showed a decrease in inflammation. Of the 13 studies investigating the effect of an oligosaccharide prebiotic (154, 157, 158, 161-164, 177, 187-189, 199, 202), nine (69%) reported a significant decrease in at least one biomarker of systemic inflammation (primarily TNF-α, IL-6, CRP and/or interferon gamma; IFN-γ) compared with control. An increase in inflammation (CRP, TNF-α and IL-6) following oligosaccharide supplementation was reported in two studies, both of which were cross-over studies conducted in healthy populations (164, 202). No change in inflammation was reported in the remaining two studies using oligosaccharide prebiotics, which were conducted in populations with a gastrointestinal tract condition (Crohn's Disease and acute diarrhoea) (154, 199).

Two (20%) of the 10 polysaccharide prebiotic studies (155, 159, 160, 200, 203-205, 207, 208, 211) reported a significant decrease in inflammation compared to control and/or baseline (174, 190), with the remaining eight studies reporting no anti-inflammatory effect. Of the two studies investigating the effect of resistant starch supplementation (183, 206), one reported a significant decrease in inflammation (TNF-α and IL-6) (183). A significant decrease in CRP was reported following a high (10.7g) versus low (2.5g) soluble fiber diet (156), with an inverse association between soluble fiber intake and systemic inflammation (IL-6; $p = 0.004$ and TNF-α-R2; $p = 0.02$) observed in the cross-sectional study by Ma et al (81).

Table 2-2: Summary of included studies examining the effect of prebiotics on systemic inflammation

Reference (country)	Design/ level of evidence	Quality	Participants	Prebiotic – daily dose	Control – daily dose	Duration	Effect of intervention on inflammation
Studies examining oligosaccharide prebiotics							
Dehghan 2014a (Iran)(188)	RCT/II	∅	N = 49 T2DM	INU - 10g	MDX – 10g	8wk	↓ CRP
Dehghan 2014b (Iran)(189)	RCT/II	∅	N = 52 T2DM	OF-enriched INU – 10g	MDX – 10g	8wk	↓ TNF-α & IL-6
Dehghan 2016 (Iran)(187)	RCT/II	+	N = 49 T2DM	OF-enriched INU – 10g	MDX – 10g	8wk	↓ IL-12 & IFN-y
Lecerf 2012 (France)(157)	RCT/II	∅	N = 40 Healthy	INU-XOS – 6.64g	MDX – 6.64g	4wk	↓ IL-1β
Morel 2015 (France)(158)	RCT/II	+	N = 88 Overweight	α-GOS – 12g	12g glucose	2wk	↓ CRP
Van den Berg 2013 (Netherlands)(161)	RCT/II	+	N = 113 Pre-term infants	GOS/FOS – 2.25g	MDX – 2.25g	27d	↓ TNF-α, IFN-γ & IL-1β
Vulevic 2008 (UK)(162)	RCT(x-over)/II	∅	N = 82 Healthy	β-GOS -5.5g	MDX – 5.5g	10wk/arm 4wks w/o	↓ TNF-α, IL-6 & IL-1β
Vulevic 2013 (UK)(163)	RCT(x-over)/II	∅	N = 90 Overweight	β-GOS – 5.5g	MDX – 5.5g	12wk/arm 4wk w/o	↓ CRP
Benjamin 2011 (UK)(154)	RCT/II	+	N = 41 CD	FOS – 15g	MDX – 15g	4wk	↔ any biomarkers
Vaisman 2010 (Israel)(199)	RCT/II	∅	N = 42 Acute diarrhoea (9mo -2yrs)	Oligosaccharides & pectin – 6g	MDX – 6g	12d	↔ any biomarkers
Clarke 2016 (Canada)(202)	RCT(x-over)/II	+	N = 60 Healthy	β1-2 fructan (INU & OS) – 15g	MDX – 15g	28d/arm 2wk w/o	↑ TNF-α & IL-6
Vulevic 2015 (UK)(164)	RCT(x-over)/II	+	N = 80 Healthy	β-GOS – 5.5g	MDX – 5.5g	10wk/arm 4wk w/o	↑CRP
Williams 2016 (UK)(177)	RCT(x-over)/II	+	N = 10 Asthma, N = 8 Healthy	B-GOS - 5.5g	MDX – 5.5g	3wk/arm 2wk w/o	↓ TNF-α & CRP
Studies examining polysaccharide prebiotics							
Smith 2008 (USA)(208)	RCT/II	+	N = 90 Hyper cholesterol	β-glucan – 6g		6wk	↓ CRP
Xie 2015 (China)(200)	RCT/II	∅	N = 83 Kidney disease	SF (NS) – 20g	Starch – 20g	6wk	↓ CRP, IL-6, IL-8
Brouns 2012 (Netherlands)(155)	RCT(x-over)/II	∅	N = 108 Hyper cholesterol	Pectin – 6g	Cellulose – 6g	3wk/arm ≥1wk w/o	↔ any biomarkers
Dall'Alba 2013 (Brazil)(203)	RCT/II	+	N = 44 T2DM	PHGG – 10g	No supplement	6wk	↔ any biomarkers

King 2008 (USA)(204)	RCT/II	+	N = 87 Overweight	Psyllium – 14g	No supplement	12wk	↔ any biomarkers
Nieman 2008 (USA)(205)	RCT/II	+	N = 36 Healthy	β-glucan – 5.6g	5.6g cornstarch	2wk	↔ any biomarkers
Queenan 2007 (USA)(207)	RCT/II	∅	N = 75 Hyper cholesterol	Oat β-glucan – 6g	Dextrose – 6g	6wk	↔ any biomarkers
Salas-Salvado 2008 (Spain)(159)	RCT/II	+	N = 113 Overweight	Plantago ovata seed husks – 9g, glucomannan – 3g	Microcrystalline cellulose – 3g	16wk	↔ any biomarkers
Theuwissen 2009 (Netherlands)(160)	RCT(x-over)/II	∅	N = 42 Hyper cholesterol	Oat β-glucan – 4.8g	Control fibre (NS) – 4.8g	4wk/arm 2wk w/o	↔ any biomarkers
Wood 2006 (USA)(211)	RCT/II	∅	N = 29 Overweight	Glucomannan – 3g	MDX – 3g	12wk	↔ any biomarkers
Studies examining resistant starch							
Aliasgharzadeh 2015 (Iran)(183)	RCT/II	+	N = 55 T2DM	Resistant dextrin – 10g	MDX – 10g	8wk	↓ TNF-α & IL-6
Penn-Marshall 2010 (USA)(206)	RCT(x-over)/II	∅	N = 34 Pre-diabetes	RS bread - 12.39g	Control bread	6wks/arm 2wk w/o	↔ any biomarkers
Other studies							
Stewart 2010 (USA)(209)	RCT(x-over)/II	∅	N = 36 Healthy	Pullulan, RS, SF dextrin, soluble corn fibre – 12g	MDX – 12g	2wk/arm 3wk w/o	↔ any biomarkers
Johansson-Pearson 2014 (Finland)(156)	RCT(x-over)/II	+	N = 25 Hyper-cholesterol	HF diet - 10.7g SF	LF diet - 2.5g SF	5wk/arm 3wk w/o	↓ CRP
Villasenor 2011 (USA)(210)	Cross-sectional/IV	+	N = 40 Breast cancer	N/A	N/A	N/A	No association
Ma 2008 (USA)(81)	Cross-sectional/IV	∅	N = 1958 Post-menopausal	N/A	N/A	N/A	Inverse association

Methodological study quality was determined using the American Dietetic Association critical appraisal checklist; ∅ represents neutral study quality; + represents positive study quality. N= total number of participants in study; ↓ indicates decrease, ↑ indicates increase, ↔ indicates no change. Abbreviations: B-GOS, Bimuno-galatooligosaccharide; CD, Crohn's Disease; CRP, c-reactive protein; FOS, fructooligosaccharides; GOS, galactooligosaccahride; HF, high fibre; IFN-γ, interferon gamma; IL, interleukin; INU; inulin; LF, low fibre; MDX, maltodextrin; N/A, not applicable; NS, not specified; OF, oligofructose; RCT, randomised controlled trial; RS, resistant starch; SF, soluble fibre; T2DM, Type 2 Diabetes Mellitus; w/o, wash out; x-over, cross-over study design; XOS, xylooligosaccharide.

Meta-analyses were performed to examine the effect of prebiotic supplementation on CRP (n=7), IL-6 (n = 6), and TNF-α (n = 4). Results of the meta-analysis indicate that prebiotics significantly decrease CRP compared to placebo/control (SMD -0.60, 95% CI -0.98, -0.23, I^2= 64%, P = 0.002) (**Figure 2-3)**.

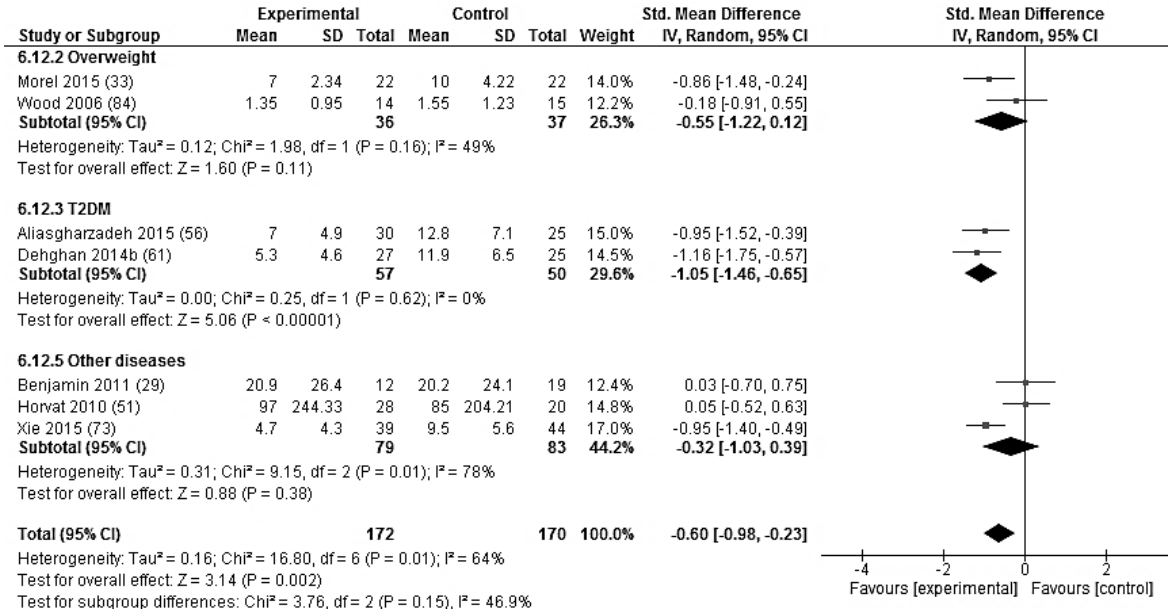

Figure 2-3: Forest plot of randomized controlled trials investigating the effect of prebiotic supplementation on circulating c-reactive protein (CRP), sub-grouped by disease. Pooled effect estimates (diamonds) for CRP are shown. Values are standard mean differences with 95% confidence intervals determined using generic inverse-variance random-effects models. Heterogeneity was quantified by I^2 at a significance of P < 0.10.

Subgroup analyses by prebiotic fiber type provided evidence for a decrease in CRP levels with oligosaccharides (n = 3, SMD -0.49, 95% CI -0.83, -0.15, I^2= 69%, P=0.04), but no effect was observed with polysaccharide supplementation (SMD -0.38, 95% CI -1.05, 0.28, I^2 = 75%, P = 0.26) (**Table 2-3).** There was no effect of prebiotics on IL-6 (SMD -0.35, 95% CI -0.84, 0.13, I^2 = 75%, P = 0.15) (**Figure 2-4**), or TNF-α (SMD -0.49, 95% CI -1.20, 0.22, I^2 = 84%, P = 0.18) (**Figure 2-5**).

Investigation into the effects of prebiotics on other inflammatory biomarkers by meta-analysis was not possible due to small study numbers.

Table 2-3: Additional sub-group meta-analysis of prebiotic studies

Outcome	N	n (experiment/ control)	SMD (95% CI)	I^2	P value
CRP (end)					
Oligosaccharide	3	61/66	-0.49[-0.83, -0.15]	69% (p = 0.04)	0.04
Polysaccharide	3	81/79	-0.38[-1.05, 0.28]	75% (p = 0.02)	0.26
IL-6 (end)					
Oligosaccharide	3	60/60	-0.13[-0.61, 0.34]	33% (p = 0.23)	0.58
Polysaccharide	4	100/96	-0.41[-1.23, 0.40]	78% (p = 0.0001)	0.32
TNF-α (end)					
Oligosaccharide	2	57/55	-0.30[-0.92, 0.31]	63% (p = 0.10)	0.34
Polysaccharide	2	53/59	-0.25[-2.16, 1.66]	95% (p < 0.0001)	0.80

Values are standard mean differences with 95% confidence intervals determined using generic inverse-variance random-effects models. Heterogeneity was quantified by I^2 at a significance of P < 0.10. N= number of studies included in meta-analysis; n= number of subjects. Abbreviations: CRP, C reactive protein; CI, confidence interval; IL, interleukin; SMD, standard mean difference.

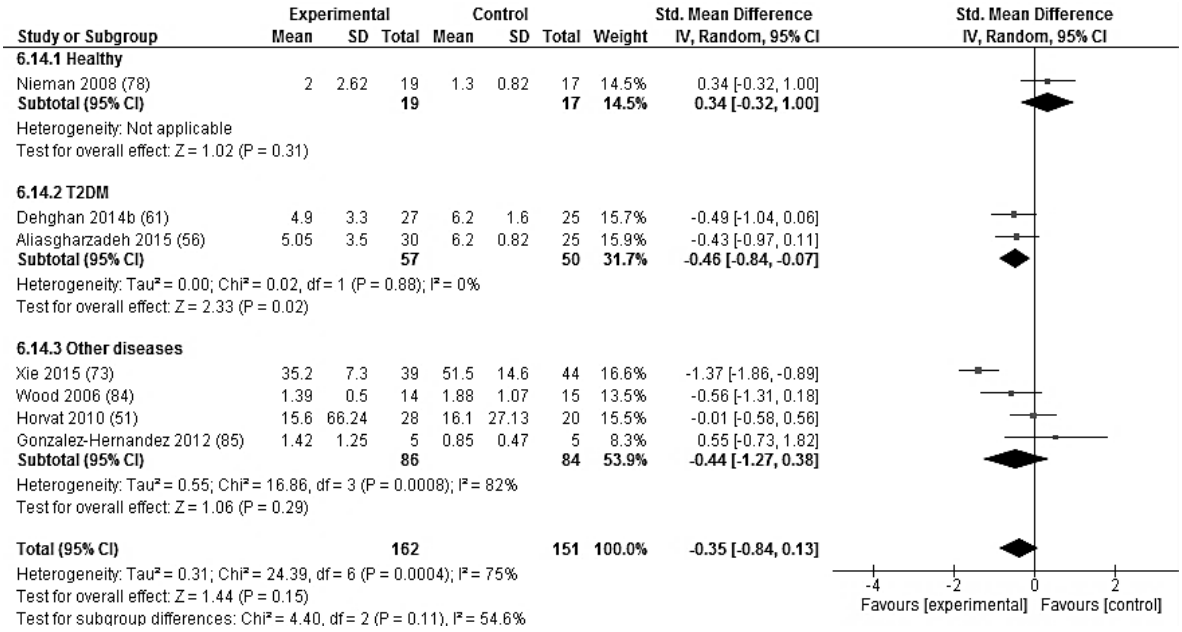

Figure 2-4: Forest plot of randomized controlled trials investigating the effect of prebiotic supplementation on circulating interleukin-6 (IL-6), sub-grouped by disease status. Pooled effect estimates (diamonds) for IL-6 are shown. Values are standard mean differences with 95% confidence intervals determined using generic inverse-variance random-effects models. Heterogeneity was quantified by I^2 at a significance of P < 0.10.

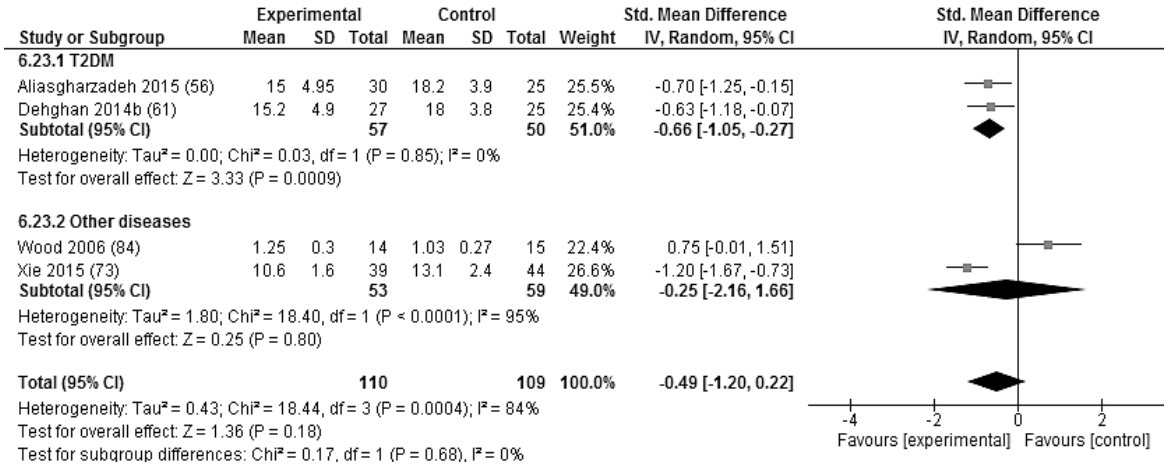

Figure 2-5: Forest plot of randomized controlled trials investigating the effect of prebiotic supplementation on circulating Tumour necrosis factor-alpha (TNF-α), sub-grouped by disease status. Pooled effect estimates (diamonds) for TNF-α are shown. Values are standard mean differences with 95% confidence intervals determined using generic inverse-variance random-effects models. Heterogeneity was quantified by I^2 at a significance of $P < 0.10$.

2.4.4 Effect of Synbiotics on Systemic Inflammation

Characteristics of the 26 included synbiotic studies are presented in **Table 2-4.** The average daily prebiotic dose was 8.7g (range: 0.1-32g), and the median daily probiotic dose was $2.2x10^9$ colony forming units (CFU) (range: $2x10^7$ - $4x10^{11}$). The majority of studies used a probiotic supplement comprised primarily of Lactobacillus and/or Bifidobacterium, combined with a single oligosaccharide prebiotic. Fourteen studies (53%) reported a significant decrease in at least one biomarker of inflammation (primarily CRP, TNF-α, and/or IL-6) following synbiotic supplementation (167-170, 173, 181, 182, 184, 185, 190, 193-195, 198). However, 10 studies reported no significant change in inflammation. A significant increase in inflammation was reported by Amati et al (165) (IL-6, IL-8 and IL-1β) and Riordan et al (218) (IL-6 and TNF-α).

Table 2-4: Summary of included studies examining the effect of synbiotics on systemic inflammation

Reference (country)	Study design/ level of evidence	Quality	Population	Intervention - daily dose	Control – daily dose	Duration	Effect of intervention on inflammation
Abbas 2012 (Pakistan)(181)	RCT/II	+	N = 72 IBS	S.bourardii capsule (750mg) + psyllium – 5g	Placebo capsule + psyllium – 5g	12wk	↓ IL-8, TNF-α
Akram 2015 (Iran)(182)	RCT/II	∅	N = 44 T2DM	1x Synbiotic capsule (dose NS)	Placebo capsule	8wk	↓ CRP, IL-6, TNF-α
Amati 2010 (Italy)(165)	Pre-post/IV	∅	N = 10 Elderly (>66yrs)	$2x10^7$ CFU LGG + OF (dose NS)	-	4wk	↑IL-6, IL-8, IL-1β
Anderson 2004 (UK)(166)	RCT/II	+	N = 137 Elective surgery	$12x10^9$ CFU L. acidophilus, L. bulgaricus, B.lactis Bb-12, S. thermophiles + 32g OF	Placebo capsule + 32g sucrose	1-2wk	↔ any biomarkers
Asemi 2014 (Iran)(184)	RCT(x-over)/II	+	N = 124 T2DM	$27x10^7$ CFU L. sporogenes + 1.08g INU	Placebo supplement	6wk/arm 3wk w/o	↓ CRP
Asemi 2013 (Iran)(185)	RCT/II	+	N = 54 T2DM	$2x10^9$ CFU L.acidophils, $7x10^9$ CFU L.casei, $1.5x10^9$ CFU L.rhamnosus, $2x10^8$ CFU L.bulgaricus, $2x10^{10}$ CFU B.breve, $7x10^9$ CFU B.longum, $1.5x10^9$ CFU S.thermophilus + 100mg FOS	Placebo supplement	8wk	↓ CRP
Asgharian 2016 (Iran)(186)	RCT/II	∅	N = 74 NAFLD	1x 500mg capsule (L casei, L.acidophilus, L.rhamnosus, L.bulgaricus, B.breve, B.longum, S.thermophilus + FOS (dose NS))	Placebo capsule (120mg starch)	8wk	↔ any biomarkers
Eslamparast 2014 (Iran)(190)	RCT/II	+	N = 52 NAFLD	$4x10^8$ CFU of 7 bacterial strains (L.casei, L.rhamnosus, S. thermophilus, B.breve, L.acidophilus, B.longum, L. bulgaricus) + FOS (dose NS)	MDX capsule	28d	↓ CRP, TNF-α
Mofidi 2017 (Iran)(193)	RCT/II	+	N = 50	$4X10^8$ CFU L.casei, L. rhamnosus, S.thermophilus, B.breve, L.acidophilus, B.longum, L.bulgaricus + 125mg FOS	MDX capsule	28wk	↓ CRP
Federico 2009 (Italy)(167)	RCT/II	∅	N = 18 UC	$10x10^9$ CFU L.paracasei + 1g XOS and 6g INU	Placebo supplement (Starch)	8wk	↓ IL-6, IL-8

Fernandes 2016 (Brazil)(214)	RCT/II	∅	N = 6 Obesity	10^9 CFU of each: L.paracasei, L.rhamnosus, L.acidophilus, B.um lactis + 6g FOS	MDX – 6g	15d	↔ any biomarkers
Giamarellos-Bourbouli 2009 (Greece)(168)	RCT/II	∅	N = 72 Multiple organ injury	12g sachet: 10^{11} CFU of each: P.pentoseceus, L.mesenteroides, L.paracasei, L.plantarum + INU, β-glucan, pectin & RS	Placebo sachet – 12g	15d	↓ CRP
Kelishadi 2014 (Iran)(192)	RCT/II	+	N = 55 Overweight/ obese	$2x10^8$ CFU of: L.casei, B.breve, L.rhamnosus, B. longum, S.thermophilus, L.acidophilus, L.Bulgaricus) + FOS (dose NS)	MDX capsule	8wk	↔ any biomarkers
Macfarlane 2013 (UK)(169)	RCT(x-over)/II	+	N = 43 Healthy	$4x10^{11}$ CFU B.longum + 12g prebiotic (inulin + OF)	Starch capsule + 12g MDX	4wk/arm 4wk w/o	↓ TNF-α, IL-6, IL-8
Malaguarnera 2012 (Italy)(170)	RCT/II	+	N = 66 NASH	B.longum + 2.5g FOS	Placebo supplement	24wk	↓ CRP, TNF-α
Neto 2013 (Brazil)(215)	RCT/II	∅	N = 17 Healthy	10^8-10^9 CFU of each: L.paracasei, L.rhamnosus, L.acidophilus, B.lactis + 6g FOS	Placebo supplement - 6g MDX	12wk	↔ any biomarkers
Nova 2011 (Spain)(171)	RCT/II	+	N = 36 Healthy	$2.4x10^9$ CFU of 5 bacterial strains (L. acidophilus, B. animalis, L. delbrueckii, S.thermophilus, L. paracasei) + 1.4g FOS	3x placebo capsules (sucrose, talcum powder, and stearic acid magnesium salt)	6wk	↔ any biomarkers
Rajkumar 2014 (India)(194)	RCT/II	∅	N = 30 Healthy	$2x10^9$ CFU L.salivarius +10g FOS	Gelatine capsule	6wk	↓ CRP, TNF-α, IL-1β
Riordan 2007 (Australia)(218)	RCT/II	∅	N = 30 Cirrhosis	10^{10} CFU of each: P.pentoseceus, L.plantarum, L.mesenteroides, L.paracasei, + 2.5g of each: β-glucan, INU, pectin, RS.	Placebo sachet – crystalline	1wk	↑IL-6, TNF-α
Roller 2007 (Ireland)(172)	RCT/II	+	N = 34 Colon cancer, N = 40 Polypectomised	10^{10} CFU L.rhamnosus, 10^{10} CFU of Bb12 + 10g prebiotic (INU & OF)	MDX capsules, MDX sachet - 10g	12wk	↔ any biomarkers
Sugawara 2006 (Japan)(195)	RCT/II	∅	N = 81 Biliary cancer	$3x10^8$ CFU L.casei, $3x10^8$ CFU B.breve + 15g GOS	-	4wk	↓ IL-6

Taghizadeh 2014 (Iran)(196)	RCT/II	+	N = 52 Pregnant	Synbiotic food containing: $18x10^7$ CFU L.sporogenes + 0.72g INU	Same food, no synbiotics	9wk	↔ any biomarkers
Tajadadi-Ebrahimi 2011 (Iran)(197)	RCT/II	+	N = 54 T2DM	Bread containing: $12x10^9$ CFU L.sporogenes + 8.4g INU	Same bread, no synbiotics	8wk	↔ any biomarkers
Usami 2011 (Japan)(198)	RCT/II	∅	N = 61 Hepatic cancer	$3x10^8$ CFU B.breve, $3x10^8$ CFU L.casei Shirota + 15g GOS	Nil supplement	2wk	↓ CRP, IL-6
Van De Pol 2011 (Netherlands)(173)	RCT/II	+	N = 26 Asthma & HDM allergy	Food supplement with: $2x10^{10}$ CFU B.breve M-16V + 14.4g scGOS, 1.6glcFOS	Food supplement with: MDX	4wk	↓ IL-5
Va der Aa 2012 (Netherlands)(174)	RCT/II	+	N = 90 Atopic dermatitis (infants <7mo)	$1.3x10^9$ CFU B.breve M-16V + 0.8g (90% scGOS, 10% lcFOS)/100ml formula	No synbiotic	12wk	↔ any biomarkers

Methodological study quality was determined using the American Dietetic Association critical appraisal checklist; ∅ represents neutral study quality; + represents positive study quality. N= total number of participants in study; ↓ indicates decrease, ↑ indicates increase, ↔ indicates no change. Abbreviations: CFU, colony forming units; CRP, c-reactive protein; FOS, fructooligosaccharides; GOS, galactooligosaccharide; HDM, house dust mite; HIV, human immunodeficiency virus; hr, hour; IBS, irritable bowel syndrome; IL, interleukin; INU, inulin; lc, long chain; MDX, maltodextrin; NAFLD, non-alcoholic fatty liver disease; NS, not specified; NASH, non-alcoholic steatohepatitis; OF, oligofructose; RCT, randomised controlled trial; sc, short chain; UC, ulcerative colitis; XOS, xylooligosaccharides; x-over, cross-over study design.

Meta-analyses were conducted to examine the effect of synbiotics on CRP (n = 11), IL-6 (n = 5) and TNF-α (n = 6). The results indicate that CRP and TNF-α levels are lower following synbiotic supplementation when compared with placebo (**Figure 2-6;** SMD -0.40, 95% CI -0.73, -0.06, I^2 = 78%, P = 0.02 and **Figure 2-7;** SMD -0.90, 95% CI -1.50, -0.30, I^2 = 78%, P = 0.003 respectively). While there was significant heterogeneity between studies in both analyses (I^2 = 76%, P = 0.002 and I^2 = 78%, P = 0.0003 respectively), subgroup analysis of studies reporting on CRP suggests that disease is a possible source of heterogeneity. There was no significant difference in IL-6 between synbiotic and placebo supplementation (**Figure 2-8;** SMD -0.21, 95% CI -0.71, 0.33, I^2 = 71, P = 0.45). Investigation into the effects of synbiotics on other inflammatory biomarkers by meta-analysis was not possible due to small study numbers.

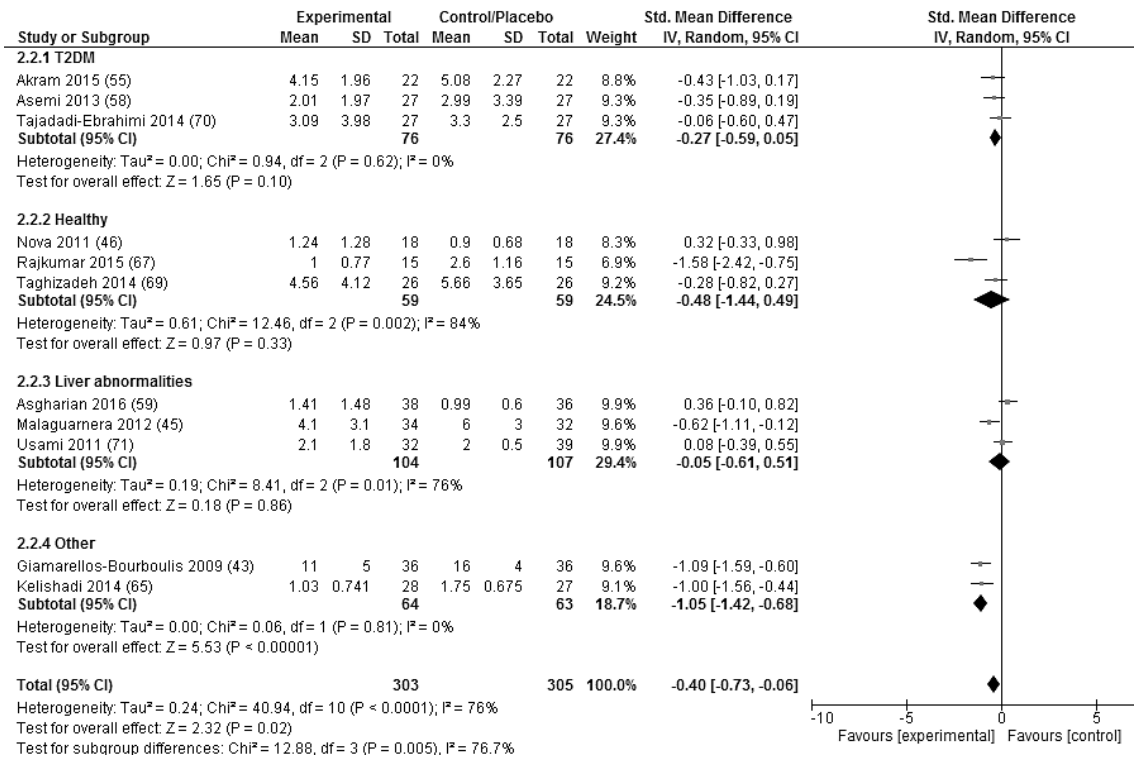

Figure 2-6: Forest plot of randomized controlled trials investigating the effect of synbiotic supplementation on circulating c-reactive protein (CRP), sub-grouped by disease. Pooled effect estimates (diamonds) for CRP are shown. Values are standard mean differences with 95% confidence intervals determined using generic inverse-variance random-effects models. Heterogeneity was quantified by I^2 at a significance of $P < 0.10$.

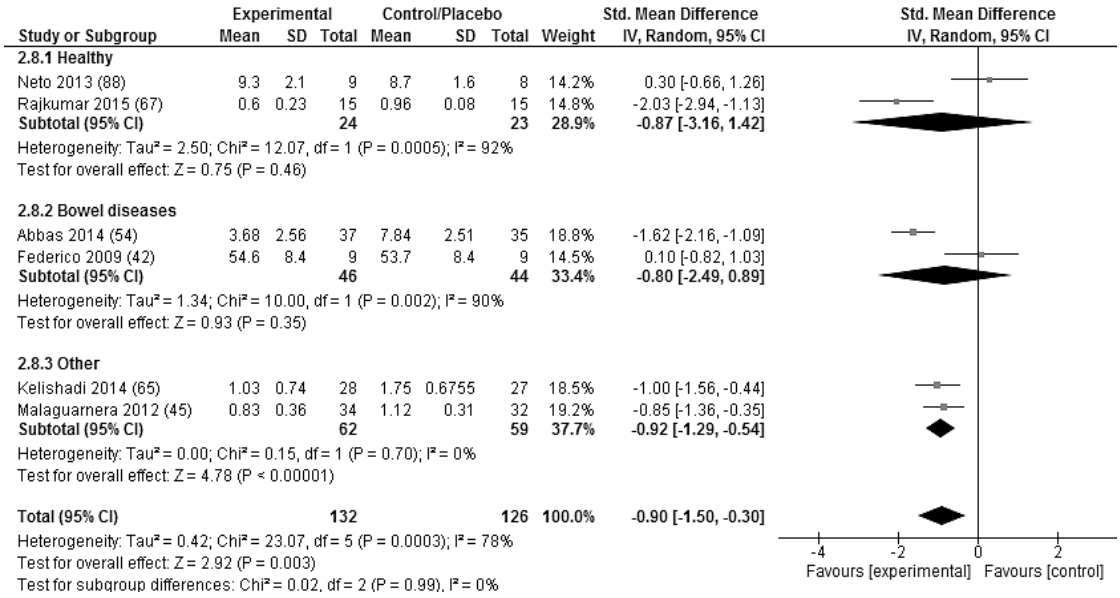

Figure 2-7: Forest plot of randomized controlled trials investigating the effect of synbiotic supplementation on circulating Tumour necrosis factor-alpha (TNF-α), sub-grouped by disease status, sub-grouped by disease status. Pooled effect estimates (diamonds) for TNF-α are shown. Values are standard mean differences with 95% confidence intervals determined using generic inverse-variance random-effects models. Heterogeneity was quantified by I^2 at a significance of $P < 0.10$.

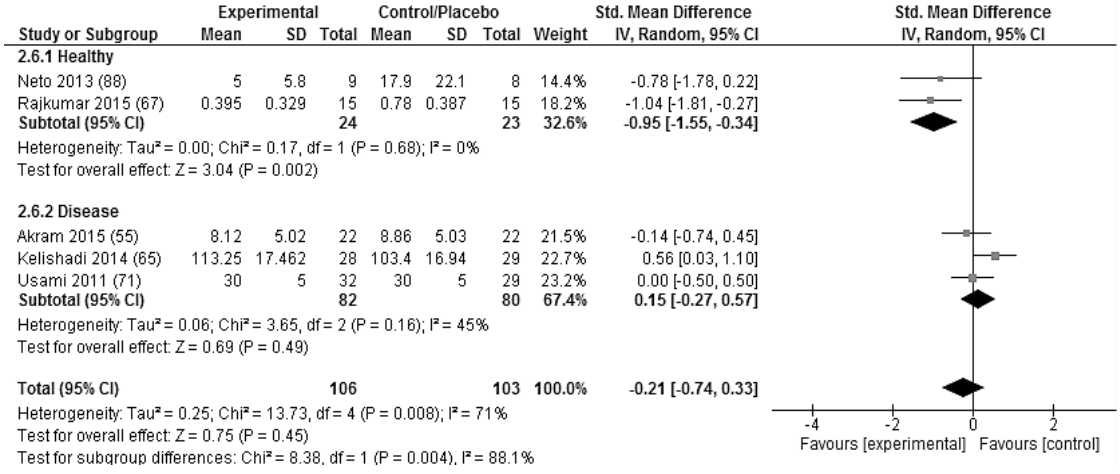

Figure 2-8: Forest plot of randomized controlled trials investigating the effect of synbiotic supplementation on circulating interleukin-6 (IL-6), sub-grouped by disease status. Pooled effect estimates (diamonds) for IL-6 are shown. Values are standard mean differences with 95% confidence intervals determined using generic inverse-variance random-effects models. Heterogeneity was quantified by I^2 at a significance of $P < 0.10$.

2.4.5 Effects of Prebiotics versus Synbiotics on Systemic Inflammation

Characteristics of the eight RCTs of prebiotic and synbiotic supplementation are presented in **Table 2-5** (175, 176, 178, 191, 212, 213, 216, 217). The average daily prebiotic dose was 10g (range, 0.57g - 20g) and the median daily probiotic dose was $5x10^9$ CFU (range, $1x10^9$ - $8x10^{10}$ CFU). Fujimori et al (191) and Gonzalez-Hernandez et al (212) reported a significant decrease in systemic inflammation (CRP and IL-6 respectively), in the synbiotic group only. West et al (216) observed an increase in pro-inflammatory IL-16 following both prebiotic and synbiotic supplementation; however, synbiotic supplementation was found to be less inflammatory, leading to an increase that was only 50% of the increase following prebiotic supplementation. Four studies, two of which were conducted in healthy participants, reported no significant change in inflammation in either the prebiotic or synbiotic treatment arm (175, 178, 213, 217).

Table 2-5: Summary of included studies comparing the effect of prebiotics and synbiotics on systemic inflammation

Reference (country)	Design/ level of evidence	Quality	Population	Synbiotic – daily dose	Prebiotic – daily dose	Duration	Effect on inflammation
Childs 2014 (UK)(175)	RCT (x-over)/II	+	N = 120 Healthy	10^9 CFU B.lactis + 8g XOS Prebiotic: 8g XOS	XOS - 8g	3wk/arm 4wk w/o	↔ any biomarkers
Fujimori 2009 (Japan)(191)	RCT/II	∅	N = 22 UC	$2x10^9$ CFU B/longum + 8g psyllium	Psyllium - 8g	4wk	↓ CRP (synbiotic group only)
Gonzalez-Hernandez 2012 (Mexico)(212)	RCT/II	+	N = 15 HIV	L.rhamnosus & B.lactis at 10^9 CFU/ml + 10g FOS	FOS - 10g	16wk	↓ IL-6
Horvat 2010 (Slovenia)(176)	RCT/II	+	N = 68 Colorectal cancer	$2x10^{10}$ CFU each of P.pentosaceus, L.mesenteroides, L.paracasei, L.plantarum + 20g prebiotic (5g each:β-glucan, RS, INU, pectin)	5g each of β-glucan. RS, INU, pectin	3d	↑ IL-6 (synbiotic group only)
Shunter 2012 (USA)(213)	RCT/II	+	N = 27 HIV	10^{10} CFU each of P. pentosaceus L.mesenteroides, L.paracasei, L.plantarum + 10g prebiotic (2.5g each: β-glucan, INU, pectin & RS)	2.5g each of β-glucan, INU, pectin & RS	4wk	↔ any biomarkers
West 2012 (Australia)(216)	RCT/II	+	N = 25 Healthy	$13.8x10^8$ CFU each of L.paracasei, L.acidophilus, & L.rhamnosus GG, $18x10^8$ CFU B.animalis + 270mg Raftiline & 30mg Raftilose	Acacia powder - 348g	3wk	Synbiotics limited IL-6 ↑ by 50% relative to prebiotics
Worthley 2009 (Australia)(217)	RCT (x-over)/II	∅	N = 36 Colorectal cancer	$5x10^9$ CFU B.lactis + 12.5g RS	RS - 12.5g	4wk/arm No w/o	↔ any biomarkers
Krebs 2016 (Slovenia)(178)	RCT/II	+	N = 73 Colorectal cancer	$2x10^{11}$ CFU each of P.pentosaceus, L.mesenteroides, L.paracasei, L.plantarum + 20g prebiotic (5g each of β-glucan, INU, pectin, RS).	5g each of β-glucan, INU, pectin, RS	3d	↔ any biomarkers

Methodological study quality was determined using the American Dietetic Association critical appraisal checklist; ∅ represents neutral study quality; + represents positive study quality. N= total number of participants in study; ↓ indicates decrease, ↑ indicates increase, ↔ indicates no change. Abbreviations: CFU, colony forming units; CRP, c-reactive protein; FOS, fructooligosaccharides; HIV, human immunodeficiency virus; IL, interleukin; INU, inulin; RCT, randomised controlled trial; RS, resistant starch; UC, ulcerative colitis; w/o, wash out; XOS, xylooligosaccharides; x-over, cross-over study design.

2.5 Discussion

This review examined evidence for the effect of SCFAs, prebiotics and synbiotics on systemic inflammation in healthy populations, diabetes, overweight/obesity, kidney disease, cancer, liver disease and bowel diseases. Approximately half of the included studies reported a significant decrease in at least one systemic inflammatory biomarker. Meta-analyses show prebiotic and synbiotic supplementation are associated with decreased systemic inflammation, including CRP, IL-6 and TNF-α; although the association was stronger with certain supplement types (particularly oligosaccharides).

Various methods can be used to deliver SCFAs. In animal studies, large oral doses of SCFA can reduce systemic inflammation (131). In humans SCFAs can be administered by enemas and tablets designed to release SCFAs into the colon (114). In this review, five studies directly delivered SCFAs. One study which used an acetate enema reported a significant decrease in systemic inflammation (201), with another study reporting a trend towards a decrease in systemic inflammation compared to control, following the delivery of acetate to the distal colon (180). Acetate is the primary SCFA to enter circulation, thus having the most potential to exert systemic anti-inflammatory effects (100). On the other hand, butyrate is primarily absorbed by colonocytes (100), which may explain why there were no significant changes in inflammation in studies using butyrate (152, 153, 179).

Circulating SCFA levels in humans can also be increased via gut fermentation of prebiotic soluble fibers (94). In a previous review of prebiotic supplementation by Kellow et al (2014), three of four studies reported significant reductions in CRP in overweight/obese adults, and women with T2DM, compared with controls (143).

However, a pooled analysis of these studies (n=181 participants) indicated a non-significant decrease in CRP (143). This review also reported conflicting results for the effect of prebiotics on TNF-α and interleukins (143). Similarly, 50% of prebiotic studies included in this current review reported a significant decrease in at least one inflammatory marker, with pooled analysis showing no significant effect on TNF-α or IL-6. In contrast to the previous review, our pooled analysis of seven prebiotic supplementation studies (n = 172) indicated a significant decrease in CRP. Heterogeneity between studies included in the previous meta-analysis and our review, in regards to supplement formulation and dosage, intervention duration and study population may explain these different findings.

Subgroup meta-analyses by prebiotic fiber type demonstrated strong evidence that oligosaccharide supplementation reduces CRP levels, whereas there was no effect with polysaccharide supplementation. Studies suggest that short chain substrates with lower degrees of polymerisation (DP) such as oligosaccharides (e.g. FOS, GOS and inulin), are more rapidly fermented and produce a greater SCFA yield than molecules with higher DP (86). While SCFA production was not measured in the majority of the included studies, the type of prebiotic fiber used and resulting SCFA levels may explain the divergent effects on inflammation. In addition to substrate type, other factors can influence SCFA production including; gut transit time (219), the composition of the colonic microbiota and the site of substrate fermentation (100). Most SCFA production occurs in the proximal colon where substrate availability and bacterial density is the highest, and decreases distally (94). Luminal pH is modulated by SCFA concentrations, which subsequently influences the types of SCFA-producing bacteria present (106). The lower pH in the proximal colon favours butyrate-producing bacteria, and as pH increases distally acetate and propionate producing bacteria

become dominant (106). Future studies investigating prebiotic supplementation that include the measurement of circulating SCFAs are warranted.

Supplementation with synbiotics is hypothesised to have a greater effect on systemic inflammation than prebiotics alone, due to their superior ability to increase SCFA producing bacteria numbers, as well as providing substrates for fermentation (108). Yet, there is conflicting evidence in the literature regarding their systemic anti-inflammatory effects. Heterogeneity was also observed in this review where 50% of synbiotic studies reported a significant decrease in systemic inflammation. Furthermore, only 43% of included studies comparing prebiotic and synbiotics reported greater anti-inflammatory effects with synbitoic versus prebiotic supplementation. Nonetheless, meta-analyses indicate that synbiotic supplementation significantly reduces CRP and TNF-α levels. Supplement formulation and dosage is likely to have an effect on whether anti-inflammatory effects are observed, which may explain the conflicting evidence.

While the anti-inflammatory effects observed following prebiotic and synbiotic supplementation may be attributed to the production of SCFAs, other mechanisms may be involved. Prebiotic and synbiotic supplementation have both been shown to stimulate the growth of beneficial bacteria in the colon (86). Certain bacteria (e.g. *Lactobacillus* species) indirectly regulate inflammation through maintaining and repairing epithelial barriers, which subsequently reduces the impact of pro-inflammatory stimuli such as lipopolysaccharide (220). Commensal bacteria can also increase synthesis of antimicrobial peptides involved in inflammation resolution pathways (220, 221). Furthermore, specific bacterial species and their metabolic products have a direct influence on pro-inflammatory signalling pathways (e.g.

Nuclear Factor Kappa B) by acting as ligands for innate immune system receptors (e.g. Toll-like receptors) (222). Another potential anti-inflammatory mechanism of gut bacteria is via modulating the differentiation and activity of immune cells such as dendritic cells, promoting the production of cytokines such as IL-10 (222).

This systematic review has a number of limitations. Primarily, heterogeneity between studies in regards to supplement formulation, dosage, study duration, and systemic inflammatory outcome variables, limited the number of studies included in the meta-analyses. This also inhibited the ability to perform meta-regression to investigate reasons for heterogeneity. Furthermore, background dietary nutrient intake can influence changes in systemic inflammation biomarkers making it difficult to assess the effect of supplementation alone, and should be considered in the design of future studies. Although most studies advised participants to maintain usual dietary habits, only 36 (~57%) studies measured background dietary intake. It is possible that changes or group differences in other nutrients (e.g. soluble fibre, pro-inflammatory nutrients such as saturated fat, or anti-inflammatory nutrients such as vitamin A, C, E, lycopene, lutein and omega-3) may have also influenced the changes in inflammation reported in these studies. The measurement of background dietary intake in future studies is warranted. Despite these limitations, to our knowledge this is the first systematic review to comprehensively examine available evidence for the effect of SCFA, prebiotics and synbiotics on systemic inflammation in humans. Furthermore, the majority of studies included in this review (95%) were classified as level II evidence as per the NHMRC evidence hierarchy, thus strengthening the findings of the review.

In summary, this review has demonstrated that there is promising evidence supporting the anti-inflammatory benefits of synbiotics and prebiotics, in particular

oligosaccharides, in humans. However, due to the heterogeneity between studies, it is difficult to determine the most beneficial supplement dosage/formulation and intervention duration. Further research is needed to confirm the association between SCFA, prebiotics and synbiotics, and systemic inflammation and to elucidate the responsible mechanisms.

Chapter 3: General methods

This chapter describes in detail the common methods used in this thesis.

3.1 Blood processing

Within 30min of blood collection, the samples were centrifuged at 20^{o}C, 3000g, for 10min and the plasma fraction aliquoted and stored at -80^{o}C until analysis. The remaining cellular fraction was used for the isolation of peripheral blood cells.

3.2 Peripheral Blood Cell Isolation

Peripheral blood monocytes (PBMCs) were isolated from venous blood samples using Ficoll-Paque PLUS (GE healthcare, Sydney Australia) as per the manufacturer recommendations with some minor modifications. Briefly, following the removal of the plasma fraction during blood processing, the cellular fraction of the sample was diluted in 1:1 Hanks' Balanced Salt Solution (HBSS), carefully layered on 3ml of Ficoll-Paque PLUS and centrifuged at 1700rpm for 20min at 20^{o}C without the brake. The buffy coat containing the PBMCs was removed, and incubated in buffer EL (30ml) at 4^{o}C for 15min. The sample was then centrifuged at 1900rpm for 10min at 20^{o}C with the brake and the supernatant was discarded.

The PBMC cell pellet was then washed in 30ml HBSS, and counted using the Countess^{TM} Automated Cell Counter (Invitrogen, Carlsbad, California, USA). The cell suspension was aliquoted, with 2 x 10^{6} cells in buffer RLT and stored at -80^{o}C for later gene expression analysis, and the remaining cells (8 x 10^{6} cells per aliquot) were used immediately for nuclear protein extraction.

3.3 Isolation of nuclear proteins from PBMCs

Nuclear proteins were extracted from the isolated PBMCs using the Active Motif Nuclear Extract kit (Active Motif, Carlsbad, California, USA) according to the

manufacturer's recommendations. All reagents and buffers required to perform the assay were provided within the kit, and were prepared as recommended.

Briefly, the PBMC cell pellet (prepared as described above in 3.2 Peripheral blood cell isolation) was re-suspended in 3ml ice-cold Phosphate buffered saline (PBS) in the presence of Phosphate Inhibitors, to minimise further protein modifications such as proteolysis and de-phosphorylation. The cells were then centrifuged at 1100rmp for 5mins at 4^{o}C and the supernatant discarded. The pellet was re-suspended in 500µL of hypotonic buffer and incubated for 15min on ice to allow the cell membranes to swell and become fragile. 25µL of detergent was added to cause leakage of the cytoplasmic proteins into the supernatant which was collected and stored at -80^{o}C, after centrifuging the cells at 14 000xg for 1min at 4^{o}C. The cell pellet containing the nuclear proteins were solubilized in 50µL of Lysis Buffer in the presence of Protease Inhibitor Cocktail, and incubated on a rocking platform for 30mins at 4^{o}C. The cells were then centrifuged at 14 000xg for 10min at 4^{o}C, and the supernatant fraction containing the nuclear proteins removed and stored at -80^{o}C.

3.4 Quantification of nuclear protein extracts

Nuclear proteins extracted from PBMCs were quantified using the Bio-Rad Protein Assay (Bio-rad, Hercules, CA, USA) as per the manufacturer's recommendations. A bovine serum albumin (BSA) protein standard curve ranging from 0.03mg/ml to 0.5mg/ml was prepared. Nuclear protein samples were diluted 1:5 using Milli-Q ultrapure water, and duplicate reactions of 10µL for each standard and sample were added to individual wells of a 96-well plate. Using a multi-channel pipette, 200µL of diluted dye reagent (1:5 dilution) was added to each well, and the solutions were mixed by pipetting up and down repeatedly. Following a 5min incubation at room

temperature, absorbance was measured at 595nm (FLUOstar Optima, BMG Labtech, Durham, NC), and nuclear protein concentrations were calculated using the standard curve.

3.5 Histone Deacetylase (HDAC) Enzyme Activity Assay

HDAC enzyme activity was measured in PBMC nuclear protein extracts using the Active Motif fluorescent HDAC Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's recommendations. The fluorescent HDAC assay uses a short peptide substrate (BOC-(Ac)Lys-AMC) containing an acetylated lysine residue which can by deacetylated by select HDAC enzymes (i.e. Class I, IIB and IV HDAC enzymes) present within the experimental sample. Once deacetylation of the substrate has occurred, the lysine residue reacts with the developing solution and a fluorescent product (fluorophore) is released from the substrate which can be measured at 340-360nm excitation and 440-465 emission. The amount of HDAC activity is then calculated using a standard curve prepared from the non-acetylated version of the peptide substrate BOC-Lys-AMC.

All reagents required to perform the assay were provided within the kit, and were prepared as recommended. Briefly, 10µL of diluted (1:20) substrate, 3ug of total nuclear protein extract and HDAC assay buffer (40µL buffer - XµL nuclear extract) was added to each sample well in duplicate. A standard curve ranging from 0µM to 20µM was prepared using serial dilutions performed in Eppendorf tubes. 50µL of the prepared standards were pipetted into the appropriate wells in duplicate. The plate was then covered, and incubated at 37^{o}C for 60min. Following incubation, 50µL of diluted (1:100) developer solution was added to each sample well, and the plate was incubated for a further 15mins at room temperature. Fluorescence measured at 360nm excitation

and 460nm emission (FLUOstar Optima, BMG Labtech, Durham, NC). HDAC enzyme activity (uM/ug) was calculated using the standard curve.

3.6 Histone Acetyltransferase (HAT) Enzyme Activity Assay

Measurement of HAT enzyme activity in PBMC nuclear protein extracts was performed using the Active Motif fluorescent HAT Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's recommendations, with some modifications. The fluorescent HAT assay uses either a histone H3 or histone H4 substrate peptide which is incubated with acetyl-CoA and the experimental sample containing active HAT enzymes. The HAT enzymes in the experimental sample catalyse the enzymatic transfer of acetyl groups from the acetyl-CoA to the histone peptide. This produces Coenzyme A (CoA-SH) and acetylated histone peptide. Once the reaction is stopped by adding stop solution, developing solution is added which reacts with the free sulfhydryl groups on the CoA-SH producing a fluorescent reading of acetyltransferase activity which can be measured at 360-390nm excitation and 450-470nm emission. The amount of HAT activity is then calculated using a standard curve prepared with either CoA-SH or β-mercaptoethanol.

All reagents, with the exception of β-mercaptoethanol, were provided within the kit and were prepared as recommended. Briefly, for each experimental sample 1ug of total nuclear extracts, 1X HAT assay buffer (50µL buffer - XµL nuclear extract) and 12.5µL acetyl-CoA was mixed thoroughly in individual Eppendorfs. A standard curve of β-mercaptoethanol ranging from 0µM to 14µM was prepared in Eppendorf tubes. 50µL of each standard and 30µL of each sample preparation was pipetted into the appropriate wells of a 96-well plate in duplicate. 20µL of histone H3 substrate peptide was added to each sample well, and the plate was covered and incubated for 20min at

room temperature. Following incubation, 50µL of stop solution was added to each sample well followed by 100µL of developing solution. The plate was covered and incubated for another 15min at room temperature away from light. Fluorescence was measured at 360 nm excitation and 460 nm emission (FLUOstar Optima, BMG Labtech, Durham, NC, USA). The standard curve was used to quantify HAT activity expressed as (uM/ug).

3.7 Gene expression analysis

3.7.1 RNA extraction

RNA was extracted from PBMCs and sputum cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase digestion using a RNase-Free DNase set (Qiagen, Hilden, Germany. All reagents were provided within the kits, and were prepared as recommended. Extraction was performed on the QIAcube using automated methods, as per the manufacturer's instructions. Briefly, an equal volume of 70% ethanol was added to each sample and mixed thoroughly. The mixtures were then transferred to RNeasy mini spin columns which were centrifuged for 15sec at 10 000rpm, and the eluate discarded. Prior to performing the on-column DNase digestion, the spin column membranes were washed by adding 350µL Buffer RWI and centrifuging for 15sec at 10 000rpm. 80µL of DNaseI was then added directly to the spin column membrane and incubated at 30^{o}C for 15min. Following incubation, 350µL Buffer RWI was added to the spin column and centrifuged for 15sec at 10 000rpm. The flow-through was discarded, and the spin column was transferred to a 2ml collection tube. 500µL of Buffer RPE was added to the spin column and centrifuged for a further 2min at 14 000rpm. After transferring the spin column to a collection tube, 30µL of RNase-free water was added and the tubes were centrifuged for 1min at 10 000rpm to elute the RNA. The RNA was then stored immediately at -80 ^{o}C

3.7.2 RNA quantification

RNA was quantified using the Quant-iT Ribogreen RNA Assay Kit (Invitrogen, Paisley, United Kingdom), following the manufacturer's instructions. All reagents required to perform the assay were provided within the kit and were prepared as recommended. Briefly, a standard curve ranging from 0 to 200ng/uL was prepared using the provided rRNA standard and 1X TE buffer. 99µL of 1X TE buffer was pipetted into each sample well, along with 1µL of each sample to be quantified. Each standard and sample was run in duplicate. 100µL of diluted Quant-iT Ribogreen RNA reagent was then added to each well, and fluorescence was read at 485 excitation and 520 emission (FLUOstar Optima, BMG Labtech, Durham, NC, USA). The standard curve was used to quantify RNA concentration.

3.7.3 Reverse Transcriptase -Polymerase Chain Reaction (RT-PCR)

RNA was reverse transcribed to complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, United States of America), following the manufacturer's instructions. All reagents needed were provided within the kit. Briefly, to determine the quantity of sample needed the following equation was used: 200ng/RNA concentration (ng/µL) = volume of RNA to be added in µL. If the volume of RNA required was >10µL, 10µL of the sample was used. If the volume was <10µL, RNase/DNase free water was used to make the sample up to 10µL. The following master mix (**Table 3-1)** was then prepared and 10µL of master mix was added to each sample.

Table 3-1: Master mix components

Component	Volume (µL)/reaction
10X RT buffer	2.0
25X dNTP mix (100mM)	0.8
10X RT random primers	2.0
Multiscribe^{TM} Reverse Transcriptase	1.0
RNase inhibitor	1.0
Nuclease-free H_2O	3.2
Total volume per reaction	***10.0***

The samples were loaded into the ThermoHybaid PX2 thermocycler, and the thermocycler programed as follows: 25ºC for 10min, 37ºC for 120min, 85ºC for 5min and cooled to 4ºC. The samples were then stored at -20ºC.

3.7.4 Real time-PCR/quantitative-PCR (qPCR)

Expression of the target genes (**Table 3-2**) were measured using TaqMan reagents with either the ABI7500 Real Time PCR system (used for sputum cell gene expression) and the Eppendorf RealPlex PCR System (used for PBMC gene expression), relative to the housekeeping gene 18S (223).

Briefly, a master mix for the PCR reaction was prepared as detailed in **Table 3-3**.11.5µL of master mix and 1µL of each cDNA sample in duplicate was pipetted into the specified wells of an Optical 96-well reaction plate. The reaction plate was then sealed with optical caps, centrifuged to eliminate air bubbles, and loaded onto the appropriate PCR system. The cycle parameters outlined in **Table 3-4** were used**.** The analysis was performed on the change in cycle threshold (ΔCt) between the target gene compared with housekeeping gene (18S rRNA), calculated using $2^{-\Delta\Delta Ct}$ relative to 18S **(224)**.

Table 3-2: Gene targets

Gene	Assay ID
18S (housekeeping gene)	
HDAC1	Hs02621185_s1
HDAC2	Hs00231032_m1
HDAC3	Hs00187320_m1
HDAC4	Hs01041648_m1
HDAC5	Hs00608351_m1
HDAC6	Hs00997427_m1
HDAC7	Hs00231032_m1
HDAC8	Hs00954353_g1
HDAC9	Hs01081558_m1
HDAC10	Hs00368899_m1
HDAC11	Hs00227335_m1
FFAR2 (GPR43)	Hs00271142_s1
FFAR3 (GPR41)	Hs02519193_g1

Table 3-3: PCR master mix

Components	Volume (µL) per reaction
2X TaqMan® Gene Expression PCR Master Mix	6.25
20X TaqMan target gene primer/probe or 18s housekeeping primer/probe (Table 10)	0.625
Nuclease-free H_2O	4.625
Total volume per reaction	**11.5**

Table 3-4: PCR cycling parameters

	Initial Steps		Each 40 cycles	
Stage	Hold	Hold	Melt	Anneal/Extend
Temperature (^oC)	50	95	95	60
Time (min)	2:00	10:00	00:15	1:00

Chapter 4: Effects of soluble fibre supplementation, with and without probiotics, in adults with asthma - A randomised, placebo-controlled, three-way crossover trial

4.1 Abstract

Background: Soluble fibres are fermented by bacteria in the colon to short chain fatty acids (SCFAs). Animal and human studies have suggested that anti-inflammatory effects of SCFAs occur via activation of G protein coupled receptor's (GPRs) 41 and 43, and the inhibition of histone deacetylase enzyme (HDAC) activity. We have previously shown that in asthma, a single dose of soluble fibre improved lung function, reduced airway eosinophils, and upregulated airway gene expression of GPR41 and GPR43. This study investigated the effects of soluble fibre (inulin) supplementation with and without a probiotic in asthmatic adults, and the potential anti-inflammatory mechanisms involved.

Methods: A randomised, placebo controlled 3-way crossover study in 17 stable asthmatics of 7 days inulin (12g/day), inulin+probiotic (multi-strain >25 billion CFU) and placebo supplementation was performed. Lung function and asthma control were assessed using spirometry and the Juniper Asthma Control Questionnaire (ACQ), respectively. Sputum was induced during a hypertonic saline challenge, mucus plugs were selected and dispersed using dithiothreitol and cytospins prepared for differential cell counts. Asthma inflammatory phenotype was classified according to sputum eosinophil counts (eosinophilic asthma ≥3% eosinophils, non-eosinophilic asthma <3% eosinophils). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll density gradient. RNA was extracted from PBMCs and sputum, and gene expression was measured by qPCR. Changes following supplementation were analysed using the Wilcoxon signed-rank test, and comparisons between asthma inflammatory phenotypes were conducted using the Wilcoxon rank-sum test. Associations were examined using Spearman's correlations.

Results: Following 7 days of inulin supplementation, we observed a reduction in airway eosinophils (Δ -1.0 [-2.5, 0.0], p=0.006), an improvement in asthma control (Δ -0.35 [-0.50, -0.13], p=0.006) and a trend towards improvement in lung function (Δ 1 [-0.5, 4], p=0.131), in adults with stable asthma. No significant changes in clinical outcomes or airway inflammation were observed with inulin+probiotic supplementation. Following inulin supplementation, gene expression of HDAC9 in sputum was significantly decreased (p=0.008), with sputum HDAC9 expression correlated with sputum %eosinophils (R_s=0.888, p<0.001).

Conclusion: Short term inulin supplementation improves asthma control and airway inflammation in asthma. Furthermore, down regulation of sputum HDAC9 gene expression (p=0.008) was identified as a potential anti-inflammatory mechanism of soluble fibre in asthma. Soluble fibre supplementation warrants further investigation as a potential non-pharmacological addition to current asthma management strategies, particularly in those with eosinophilic airway inflammation.

4.2 Introduction

Asthma is a chronic inflammatory disease with manifestations both within the lungs and systemically (14). It is estimated that asthma effects approximately 300 million people worldwide, with 346 000 deaths attributed to asthma per year (15). Interestingly, prevalence rates vary across countries ranging from 1 to 16% of the population, with asthma prevalence reported to be higher in Westernised countries such as the UK, USA, Canada, and Australia, compared to non-westernised countries (15).

There is increasing evidence that a western style diet, which is typically high in fat and low in fibre, is associated with increased risk of asthma (49, 225-229). Furthermore, evidence from animal models suggests that increasing dietary fibre intake is protective against allergic airway inflammation (9). Dietary fibre intake has been linked with reduced systemic inflammation in both healthy (230-232) and diseased adults (91, 232). In asthma, an observational study showed an inverse association between dietary fibre intake and airway inflammation (airway eosinophils) and a positive association between dietary fibre intake and lung function (8). Furthermore, we have previously reported an improvement in lung function and a significant decrease in airway inflammation in adults with asthma following a single dose of soluble fibre (3.5g inulin) (140).

The anti-inflammatory benefits of dietary fibre intake have been ascribed to the effects of fermentable soluble fibres. Soluble fibres resist digestion in the small intestine and are fermented by beneficial gut bacteria in the colon, generating biologically active by-products, known as short chain fatty acids (SCFA). In addition to providing substrates for fermentation, some soluble fibres act as prebiotics and enhance SCFA production by preferentially stimulating the growth of SCFA-producing bacteria

species such as *Bifidobacterium* and *Lactobacillus* (233). The composition of the gut microbiome, including the abundance of these SCFA-producing bacteria, can also be temporarily altered with the use of probiotics, defined as *"live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*" (104). Indeed there is evidence that probiotic supplementation leads to an increase in SCFA levels (234-237). Probiotics, most commonly strains of *Bifidobacterium* and *Lactobacillus* (105), are typically delivered to the host either in supplemental form (tablet/powder) or via functional foods (e.g. dairy products such as yogurts and cultured drinks).

The SCFA, namely acetate, butyrate and propionate have attracted significant interest in recent years, due to their anti-inflammatory properties. Mechanisms proposed to be mediated by SCFA include activation of G protein-coupled receptor (GPR) 41 and GPR43 (238) also known as free fatty acid receptors (FFAR), and epigenetic regulation via inhibition of histone deacetylase (HDAC) enzyme activity (12). SCFAs have a range of effects on both innate and adaptive immunity including: reduced neutrophil migration, reduced production of pro-inflammatory cytokines and expression of adhesion molecules in neutrophils and endothelial cells, reduced pro-inflammatory mediator release by macrophages and monocytes, enhanced production of regulatory T cells (Treg) (10) and enhanced production of dendritic cell precursors from bone marrow(9). This highlights the potential for anti-inflammatory dietary nutrients such as soluble fibre to provide a non-pharmacological addition to asthma management.

Supplementation with both prebiotic soluble fibre and probiotics is hypothesised to have a greater effect on inflammation than prebiotics alone. This is due to their ability to increase SCFA producing bacterial numbers by improving their survival and

implantation in the gut (107), as well as provide substrates for fermentation (108). However, to our knowledge there have been no studies conducted to date which have examined the effect of prebiotic soluble fibre supplementation, with and without a probiotic, in asthma.

4.3 Hypothesis and aims

4.3.1 Hypotheses:

1. Soluble fibre supplementation will improve clinical outcomes (i.e. asthma control and lung function), reduce airway inflammation and increase plasma and faecal SCFA levels in adults with asthma, with a greater anti-inflammatory effect observed with the addition of a probiotic.
2. Soluble fibre intake will reduce inflammation in asthma via inhibition of HDAC activity and activation of G protein-coupled receptors (GPR41/43).

4.3.2 Aims

1. To examine the effect of soluble fibre supplementation, with and without a probiotic, on clinical outcomes (i.e. asthma control and lung function), airway inflammation, and plasma and faecal SCFA levels in adults with asthma.
2. To examine molecular mechanisms that are modulated by soluble fibre supplementation in asthma, including HDAC enzyme activity in PBMCs and GPR41/43 and HDAC1-11 gene expression in PBMC and sputum cells.

4.4 Methods

4.4.1 Study participants

Adults with stable asthma were recruited through Hunter Medical Research Institute (HMRI) volunteer databases and by advertisement, commencing May 2015. All study visits were completed by March 2016. Asthma diagnosis was confirmed based on doctor's diagnosis of asthma (ever) and current (past 12 months) respiratory symptoms or asthma medication use. Asthma stability was confirmed at study entry, defined as no exacerbation, respiratory tract infection, antibiotics or oral corticosteroids within four weeks. Exclusion criteria included: other respiratory conditions, current smoking (within 6 months), pregnancy or breastfeeding, diagnosed bowel or intestinal disorders, current use of nutritional, fibre or probiotic supplements (within 4 weeks), use of medications known to influence inflammation or participation in a research study within previous 4 weeks. This study was conducted at the HMRI, Newcastle, Australia according to the guidelines in the Declaration of Helsinki. All procedures involving human subjects were approved by the Hunter New England Human Research Ethics Committee (15/03/18/3.03). Written informed consent was obtained from all subjects. The trial was registered with the Australian and New Zealand Clinical Trials Registry, number: ACTRN12615000368538, prior to the study commencing.

4.4.2 Study design

The study was conducted as a double-blind, randomised, placebo-controlled cross-over trial, using inulin, inulin+probiotic or placebo for 7 days with a 2 week run-in and 2 week wash-out periods between treatments (**Figure 4-1**).

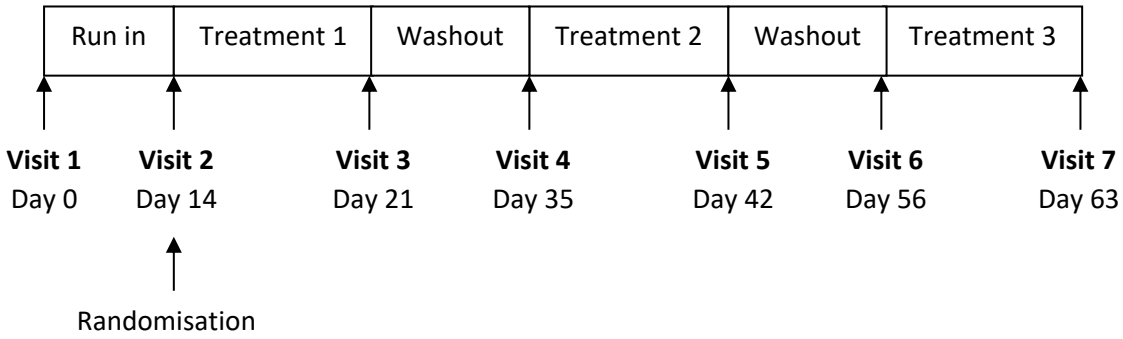

Figure 4-1: Study Schema. Following screening at visit 1, eligible participants commenced a fibre-controlled background diet which was maintained for the duration of the study. After the initial 14 days on the fibre-controlled diet, participantss commenced a randomized cross-over supplementation trial, which included 3 treatment arms [inulin, inulin+probiotic or placebo]. Each treatment was used for 7 days, with a 14 day wash out period between each treatment. All outcomes were measured, and blood, sputum and faecal samples were collect at visits 2-7.

Following a screening visit to confirm eligibility (visit 1), participants were instructed by a dietitian to consume a fibre controlled background diet for 2 weeks prior to the study commencing, and for the entire duration of the study. This was achieved by avoidance of soluble fibre-rich foods including oats, oat bran, beans, seeds and limiting fruit and vegetable intake to no more than 2 serves/ day. Participants were instructed to consume ½ cup of bran-based cereal (45g serve contained 1.5g soluble and 11.8g insoluble fibre) daily to ensure adequate total fibre intake. Participants were also instructed to avoid sources of probiotics such as yoghurt and fermented milk drinks and sources of inulin such as asparagus and artichokes.

Treatment order was randomly allocated via unique randomisation codes using a 3x3 latin square method (239) by an independent statistician. All study staff were blinded to the treatment order. The inulin treatment consisted of 6 grams of inulin twice daily (Frutafit® CLR, Sensus, The Netherlands) and a placebo capsule daily (microcrystalline cellulose). The inulin+probiotic treatment consisted of 6 grams of inulin twice daily and a multi-strain probiotic capsule daily, (Caruso's Natural Health,

Sydney, NSW, Australia) containing *Lactobacillus acidophilus* LA-5 (7.5 billion colony forming units (CFU)), *Lactobacillus rhamnosus* GG (8.75 billion CFU) and *Bifidobacterium animalis* subspecies *lactis* BB-12 (8.75 billion CFU). The placebo treatment consisted of 6 grams of maltodextrin powder twice daily (Bulk Nutrients, Grove, Tasmania, Australia) and a placebo capsule. Both inulin and placebo had similar appearance and taste and were identically packaged. Participants were instructed to mix powder sachets (inulin or placebo) with water and drink immediately morning and night, and to take the capsule in the morning only, both before food. Participants were provided with a study diary to record treatment adherence and adverse effects. Adherence to the intervention was monitored at each visit by 24-h food recall, pill/sachet countback and diary assessment. No changes to asthma medications were made during the study.

4.4.3 Clinical methods

Information including smoking history and medication usage were collected at baseline. Before and after each 7 day treatment, participants attended the HMRI clinic following a 12hr fast. Participants withheld their asthma medications (short acting β2-agonists for 6hrs, long-acting β2-agonists for 24hrs, combination inhalers for 24hrs and anti-cholinergics for 6hrs). 24-hour dietary recalls were administered by a dietitian to assess adherence to the intervention. Participants were asked to recall the amount and types of food and beverages that had been consumed in the previous 24hrs. Participants also provided a stool sample (collected within 12 hours of the clinic visit) which was passed into a collection tray, transferred to a sterile specimen jar, then frozen immediately and stored at -20°C until analysis.

Blood collection and processing:

Blood was collected following ≥12hour fast into EDTA tubes via venepuncture by a trained phlebotomist (up to 27ml), and processed as described in detail in **Chapter 3.**

Anthropometry:

Body weight was measured in light clothing without shoes, using a digital scale to the nearest 100g (NU WEIGH LOG842 scales, NU Weigh Scales Inc, MI). Height was measured to the nearest 0.1cm without shoes, using the stretch stature method and a wall-mounted stadiometer. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in metres (kg/m^2).

Spirometry

Dynamic lung function was measured using a Med Graphics spirometer (CPFS/D and BreezeSuite software, Med Graphics, Minnesota, USA). Using *National Health and Nutrition Examination Survey (*NHANES) III data (240), which accounts for age, gender and height; predicted values for forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were calculated. FEV_1 and FVC values were then be expressed as a percentage of the predicted values (FEV_1 %predicted and FVC %predicted respectively). Prior to conducting the assessment, the room temperature, barometric pressure and humidity were recorded, and the spirometer was calibrated. The participant's height, weight, age and gender were also entered into software.

Each participant wore a nose clip and was seated whilst performing the manoeuvre. A new disposable mouthpiece was attached to the spirometer, and after completing 1-2 tidal breaths, the participant was instructed to inhale to total lung capacity, then forcefully exhale to residual volume. This was repeated until three reproducible and

technically correct results were obtained, with a maximum of eight attempts performed.

Exhaled Nitric Oxide

The fraction of exhaled nitric oxide (FeNO) was measured at each visit according to the ATS/ERS criteria (241) using Ecomedics chemiluminescent detector unit (CLD 88sp FENO, Ecomedics, Switzerland), and reported in parts per billion (ppb). Ecomedics measures FeNO from the exhaled breath of humans, which can be used as a marker of eosinophilic airway inflammation. The participant was instructed to empty their breath away from the mouthpiece, take a normal size breath in through the mouthpiece, and exhale in one single breath at a constant and steady rate. Three technically acceptable attempts were recorded, and an average of the three readings was calculated. FeNO was always measured prior to spirometry as forced breathing manoeuvres may alter FeNO results.

Sputum induction and assessment of airway hyper responsiveness

Sputum induction coupled with bronchial provocation challenge (to assess airway hyper responsiveness (AHR)) was also performed at each visit over a standardised 15.5 minutes nebulisation protocol (30s, 1min, 2mins, 4mins, 4mins, 4mins). To determine AHR, the participant was exposed to a mist of hypertonic (4.5%) saline created by a nebulizer (ULTRA-NEB™ ultrasonic nebulizer, DeVilbiss, Model 2000) (242). Prior to commencing this test, the participant underwent spirometry to determine their best FEV_1 from 3 attempts. The participant was then instructed to inhale the saline aerosol through the mouthpiece for incremental time periods in doubling doses, for no longer than 15.5 mins. FEV_1 was measured after each period of saline inhalation and compared to the baseline value. If the participant's FEV_1 dropped

≥15% below their initial baseline value, the challenge was concluded, as this is indicative of AHR. Participants with AHR were administered 400mcg salbutamol via a spacer, and their lung function assessed after 15min.

During the challenge participants were encouraged to cough and clear their throat after each period of saline inhalation. This helped to dislodge the sputum from their chest wall. Any contents in the participant's mouth were then emptied into a specimen jar and processed within 30 minutes. Sputum induction was continued until the completion of 15.5 minutes of inhalation. If the participants FEV_1 dropped ≥15% again during the test, or if the participant did not wish to continue, testing was ceased.

Asthma Classification and assessment of asthma control

Asthma was classified according to the Global Initiative for Asthma (GINA) guidelines as intermittent, mild persistent, moderate persistent or severe persistent, based on symptoms such as episodic wheezing, breathlessness, cough and chest tightness (14).

To assess asthma control the validated Juniper Asthma Control Questionnaire (ACQ) (243) was administered. The ACQ measures asthma control over the previous week and is comprised of seven items which are equally weighted. Participants were asked to respond to the first six items (five of which record symptoms and one of which assesses rescue short-acting β2-agonist use) using a seven point scale (0 = no impairment, 6 = maximum impairment). The final item provides a score for lung function (FEV_1) based on spirometry results. The overall ACQ score, which was between 0 (totally controlled asthma) and 7 (severely uncontrolled asthma), was calculated as the mean of the seven item scores. A score of 0-0.75 indicates well-controlled asthma, >0.75-1.5 indicates partially controlled asthma and a score of >1.5

indicates poorly controlled asthma. An ACQ change of ≥0.5 is considered clinically significant (244). The ACQ-6 score was also calculated as the mean of the first 6 items, to allow assessment of asthma control without consideration of lung function.

Assessment of gastrointestinal symptoms:

To assess changes in gastrointestinal symptoms throughout the study, the validated Gastrointestinal Symptom Rating Scale (GSRS) questionnaire was completed at each study visit (245). The GSRS is a 15-item questionnaire which is used to verify the presence and intensity of gastrointestinal symptoms which are categorised into 5 symptom clusters; indigestion, reflux, diarrhoea, abdominal pain and constipation.

4.4.4 Laboratory methods

Sputum induction and analysis

Sputum collected by induction during hypertonic saline challenge was processed within 30 minutes of collection. Following dispersion of selected mucus plugs using 0.1% dithiothreitol, total cell counts (TCC) and cell viability (using trypan blue exclusion) were performed. Cytospins were prepared and stained (May-Grunwald Giemsa) for differential cell counts, which were performed on 400 non-squamous cells as previously described (142). Using standard morphological criteria, cells were classified as neutrophils, eosinophil, columnar epithelial, squamous epithelial, macrophages and lymphocytes. For molecular testing, 100μL of sputum plugs were homogenised and stored at -80˚C in buffer RLT (Qiagen, Hilden, Germany) for RNA extraction and gene expression analysis.

Sputum cell counts of neutrophils and eosinophils were used to classify participants by asthma inflammatory phenotype (**Figure 4-2**) (142). This was done by converting

the counts to a percentage of total cells in the sample. The following classifications were used:

- Eosinophilic asthma: Sputum eosinophils >3.0%
- Neutrophilic asthma: Sputum neutrophils >61%
- Paucigranulocytic asthma: Sputum eosinophils <3.0% and sputum neutrophils <61%
- Mixed granulocytic asthma: Sputum eosinophils >3.0% and sputum neutrophils >61%

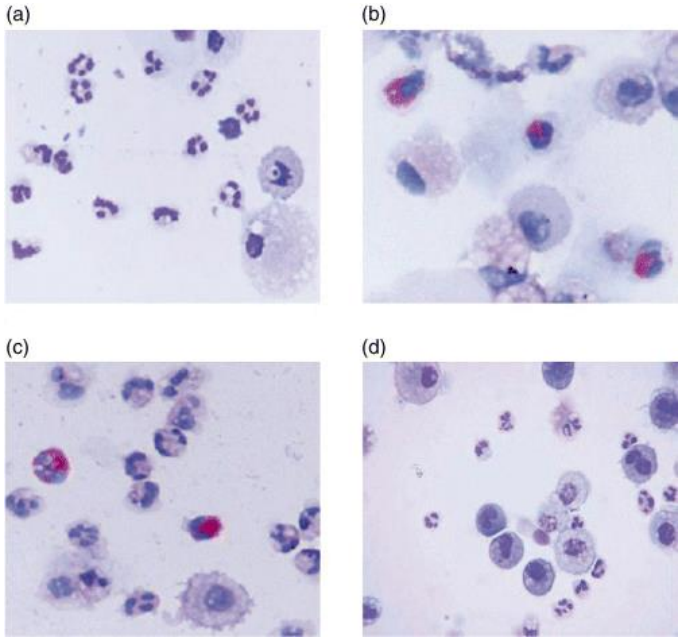

Figure 4-2: Sputum cytospins showing the four inflammatory subtypes of asthma: (a) neutrophilic asthma; (b) eosinophilic asthma; (c) mixed granulocytic asthma; (d) paucigranulocytic asthma. *Data from Simpson et al 2006* (142).

Cell isolations and preparation of nuclear extracts

Blood was collected in EDTA tubes, centrifuged at 20^{o}C, 3000 rpm, for 10 minutes and plasma was stored at -80^{o}C. Remaining blood cells were then used for isolation of PBMCs using the Ficoll-Paque PLUS (GE healthcare, Sydney, Australia) density

gradient method (121), as per the manufacturer's recommendations (described in **Chapter 3**). Cell counts and viability tests with trypan blue staining were performed. Isolated PBMCs were stored in buffer RLT for RNA extraction and gene expression analysis. Nuclear proteins were extracted from PBMCs using the Active Motif Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) and quantified by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), as per the manufacturers protocol (described in **Chapter 3**).

HDAC enzyme activity assay

HDAC enzyme activity was measured in PBMC nuclear protein extracts using the Active Motif fluorescent HDAC Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's recommendations (described in **Chapter 3**). Briefly, substrate was incubated with 3ug of total nuclear protein extracts and incubated for 60mins. Following incubation, developer solution was added and fluorescence measured at 360nm excitation and 460nm emission (FLUOstar Optima, BMG Labtech, Durham, NC). All samples and standards were measured in duplicate. A deacetylated HDAC standard curve was used to quantify HDAC activity (uM/ug).

HAT enzyme activity assay

Measurement of HAT enzyme activity in PBMC nuclear protein extracts was performed using the Active Motif fluorescent HAT Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's recommendations (described in **Chapter 3**). Briefly, 0.5ug of total nuclear protein extracts was incubated with acetyl-CoA and histone H3 substrate peptide for 20min. Developer solution was then added to react with the free sulfhydryl groups on the CoA-SH, producing a fluorescent reading of acetyltransferase activity. Fluorescence was measured at 360 nm excitation and 460 nm emission (FLUOstar Optima, BMG Labtech, Durham, NC, USA). All samples and

standards were measured in duplicate. A standard curve of β-mercaptoethanol was used to quantify HAT activity (uM/ug).

HDAC subtype protein assays

To determine protein levels of select HDAC subtypes (HDAC2 and HDAC7) in nuclear protein extracts, EpiQuik colorimetric assay kits (Epigentek) were used as per the manufacturer's instructions. These colorimetric assays work as an ELISA-like reaction, and utilise similar principles and procedures. HDAC2/7 proteins present in the experimental nuclear protein bind to the unique HDAC affinity substrate that is stably coated on the strip wells. The bound HDAC2/7 is then recognised with a high-affinity HDAC2/7-specific antibody, and the amount of HDAC2/7 is colorimetrically quantified through an ELISA-like reaction following the addition of a secondary detection antibody. The amount of HDAC2/7 is proportional to the intensity of the colour development.

EpiQuik HDAC2 assay kit

All reagents were provided within the kit, and were prepared as recommended. Briefly, a standard curve was prepared using the provided HDAC2 control (ranging from 0µM to 20µM). 10µL of each standard and experimental nuclear protein extract (4-10µg) was spread over the bottom of the appropriate strip wells in duplicate by pipetting up and down. The strip wells were then incubated at 37^{o}C for 90min to allow the wells to completely dry. 150µL of blocking buffer was added to the dried strip wells, and incubated for a further 37^{o}C for 45min. Following incubation, the strip wells were aspirated and washed three times with 150µL of diluted (1:10) wash buffer. 50µL of diluted (1:200) capture antibody was then added to each strip well, and incubated at room temperature for 60min on an orbital shaker.

The strip wells were aspirated and washed another four times with 150µL of diluted (1:10) wash buffer. To each well 50µL of diluted (1:1000) detection antibody was then added and the strip wells were covered and incubated at room temperature for 30min. The wells were again aspirated and washed four times with 150µL of diluted (1:10) wash buffer. After this wash step, 100µL of developing solution was added to each well, followed by a 2-10min incubation at room temperature away from light. Colour development was monitored during this incubation, and once the samples and standard wells began to turn medium blue, 50µL of stop solution was added to each well and absorbance was read at 450nm (FLUOstar Optima, BMG Labtech, Durham, NC, USA). The standard curve was used to quantify HDAC2 protein.

EpiQuik HDAC7 assay kit

All reagents were provided within the kit, and were prepared as recommended. Briefly, a standard curve was prepared using the provided HDAC7 control (ranging from 0µM to 20µM). 100µL of each standard and experimental nuclear protein extract (4-10µg) was added to the appropriate strip wells in duplicate. The strip wells were then covered with an aluminium sealing film and incubated at 37^{o}C for 120min. Following incubation the wells were aspirated, 150µL of blocking buffer was added to each well, and the strip wells were covered and incubated at 37oC for a further 30min. The strip wells were again aspirated and washed three times with 150µL of 1X wash buffer. 50µL of diluted (1:200) of capture antibody was added to each strip well, and the wells were covered for a 60min incubation at room temperature.

Following incubation, the wells underwent another 3 washes with 150µL of 1X wash buffer. After this wash step, 50µL of diluted (1:2000) of detection antibody was added to each strip well, and the wells were covered for another 30min incubation at room

temperature. The wells were aspirated and washed four times with 150µL of 1X wash buffer. 50µL of diluted (1:5000) enhancer solution was then added to each well, and covered for a 30min incubation at room temperature. The enhancer solution was aspirated and the cells were washed five times with 150µL of 1X wash buffer. 100µL of developing solution was then added to each well and incubated at room temperature away from light for 1-10min. Colour development was monitored during this incubation, and once the samples and standard wells began to turn medium blue, 100µL of stop solution was added to each well and absorbance was read at 655nm (FLUOstar Optima, BMG Labtech, Durham, NC, USA). The standard curve was used to quantify HDAC7 protein.

Gene expression analysis

RNA was extracted from sputum cells and PBMCs using RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantitated using the Quant-iT RiboGreen RNA Assay Kit (Molecular Probes Inc, Invitrogen, Eugene, OR, USA) as per manufacturer's instructions (described in **Chapter 3**). RNA was then converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) via standard Taqman methods (described in **Chapter 3**). Taqman qPCR primer and probes for HDAC subtypes (1-11), GPR43 and GPR41 were combined with cDNA Taqman gene expression master mix as per manufacturer's instructions in duplicate real-time PCR reactions (7500 Real Time PCR System: Applied Biosystems) (described in **Chapter 3**). Analysis was performed on the change in cycle threshold (ΔCt) between the target gene compared with the housekeeping gene 18S rRNA, calculated using $2^{-\Delta\Delta Ct}$ relative to 18S (224).

Plasma SCFA

SCFAs were measured in plasma by gas chromatography (GC) at CSIRO (Adelaide, Australia). Plasma samples (200 uL) and heptanoic acid (internal standard, 100 nmole) were extracted with ether (3 mL). The ether layer was transferred to a clean tube containing 0.2 M sodium hydroxide (50 uL). The ether was removed and the aqueous solution washed again with ether (3ml). The aqueous solution was transferred to a GC vial and acidified by addition of 1 M phosphoric acid (30 uL). The mixture was injected onto a GC column (Zebron FFAP 30 m x 530 um x 1.0 um) and SCFAs (acetate, propionate and butyrate) were quantified against calibration mixtures extracted in the same way.

Faecal SCFA

Faecal SCFA concentrations were determined by flame ionisation detection on an Agilent Technologies 7890A gas chromatograph fitted with a flame-ionisation detector at Flinders Analytical (Flinders University, Australia) using nitrogen as the carrier gas (246). Approximately 200 µl of unbuffered distilled water was added to 100 mg of faecal samples. Supernatant was collected after centrifugation at 13,000 x g for 10 mins. A 500 µl aliquot of the faecal supernatant was acidified to below pH 2.0 with 85% orthophosphoric acid, and 11 mM 4-methylvalerate was used as internal standard. Samples (1 µl) were separated on a SCE Analytical BP21 column (15 m x 0.25 mm I.D., with a 0.25 µm film thickness). Acetate, propionate, and butyrate were quantified using a standard curve with 4-methyl valerate as an internal standard.

4.4.5 Statistical analysis

The primary outcome of the study was plasma SCFA. The secondary outcomes included clinical asthma outcomes (asthma control and lung function), biomarkers of

airway inflammation (sputum cell counts and FeNO), faecal SCFA and microbiome changes, epigenetic (HAT/HDAC) activity in PBMCs, and gene expression of GPR41/43 and HDAC subtypes in sputum and PBMC samples. Based on previous studies (247), we hypothesised that we would observe a change in plasma acetate of ~1SD. With n=15 participants, we would have 90% power to detect a difference between groups, using alpha=0.025 (to avoid type 1 error when comparing 3 groups). Allowing for 10% dropouts, we aimed to recruit n=17 participants.

Data were analysed with STATA 11 (StataCorp, College Station, Texas, USA) and reported as mean ± standard deviation or median [interquartile range]. Within group comparisons of outcomes compared to baseline were conducted using the student t-test or Wilcoxon signed-rank tests. Per protocol analysis of mean difference in outcomes between groups was tested using mixed model regression, accounting for repeated measures. An independent ANOVA of period totals by treatment sequence was used to test the assumption of no carry over effects. Associations were assessed using Spearman's correlations. Significance was accepted if $p<0.05$.

4.5 Results

4.5.1 Participant characteristics

Of the 88 individuals assessed for eligibility, 17 (n=9 females, n=8 males) were randomised (**Figure 4-3)**. One participant withdrew during intervention phase 2, only completing the inulin treatment. Two participants withdrew after the second intervention phase; one participant failed to complete the inulin treatment and the other failed to complete the placebo treatment. Two withdrawals were due to participants not having time to attend clinic visits and one participant was lost to follow up. 88% of participants adhered to the background diet and consumed no more than 2 serves of fruit and vegetables during all treatment arms.

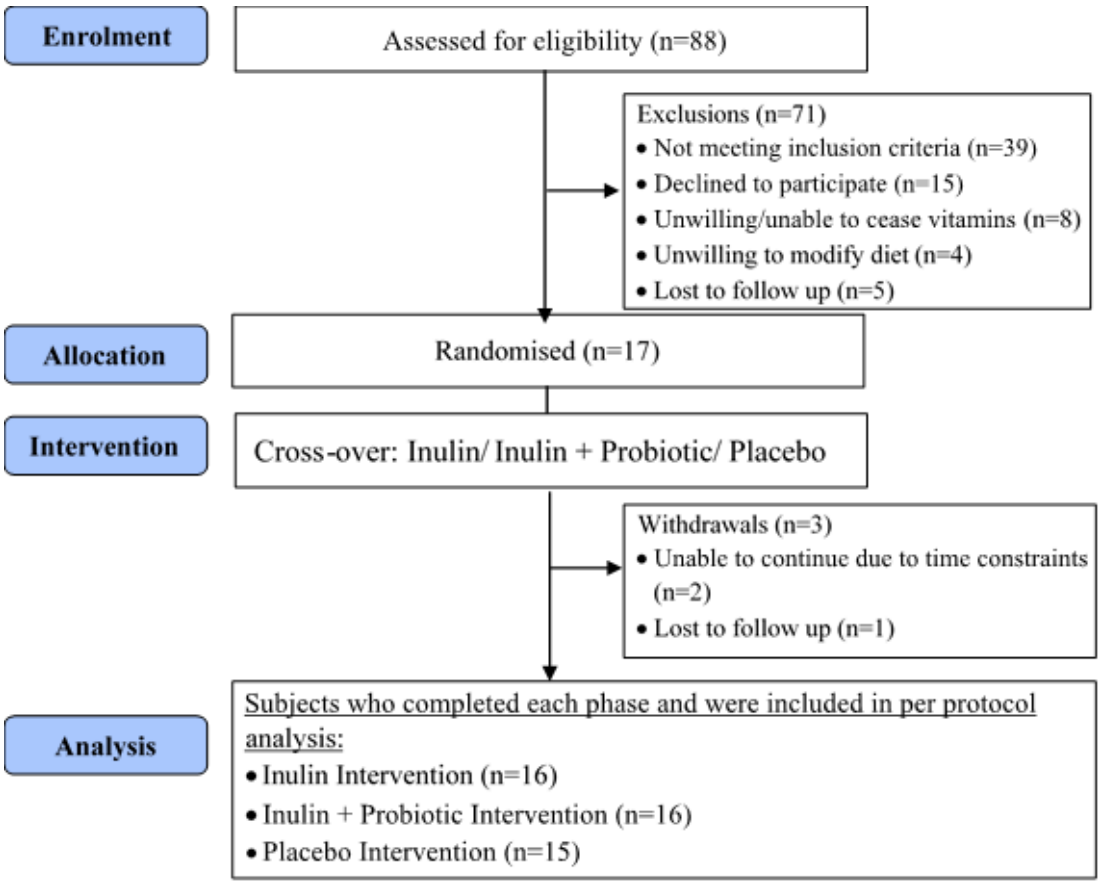

Figure 4-3: Subject flow diagram

Baseline characteristics are summarised in **Table 4-1**. Participants had a median age of 43 (19-82) years, and mean BMI of 30.4±5.7 kg/m^2. Approximately half of the participants (n=9) had an ACQ score ≥0.75, indicating partial-poor asthma control.

Table 4-1: Baseline characteristics

n	17
Age years (range)	43 (19-82)
Weight kg	87.2 ± 19.6
BMI kg/m^{2*}	30.4 ± 5.7
Male n (%)	8 (47)
Female n (%)	9 (53)
Ex-smokers n (%)	5 (29)
Smoking history (pack years)	1 (0, 15)
Asthma Pattern (Intermittent/ Mild/ Moderate/ Severe Persistent)	1/11/4/1
Asthma Pattern + ICS (Intermittent/ Mild/ Moderate/ Severe Persistent)	0/2/5/10
FEV$_1$ % predicted*	76.1 ± 17.8
FVC % predicted*	76.2 ± 12.7
$^{\#}$**FEV$_1$/FVC %***	70 ± 12.2
PD15 (mL)*‡ (n=9)	4.3 ± 3.4
DRS (%fall FEV$_1$/ mL saline)†	1.8 (0.6, 5.6)
ACQ-7*	1.2 ± 0.5
ACQ-6*	0.9 ± 0.5
SABA n (%)	16 (94)
Maintenance ICS n (%)	15 (88)
Maintenance ICS (µg beclomethasone equivalents/day)†	500 (400, 1000)
Oral steroids in 12 months n (%)	2 (12)

BMI, body mass index; FEV$_1$, forced expiratory volume in 1 second; FVC, forced vital capacity; DRS, Dose response slope; ACQ, Asthma Control Questionnaire(248); SABA, short acting β^2-agonist; ICS, inhaled corticosteroids; *Data are normally distributed and presented as mean ± SEM; †Data are non-parametric and presented as median (quartile 1, quartile 3); ‡PD15, provocation dose resulting in 15% fall in baseline FEV$_1$.

4.5.2 Clinical outcomes

Following inulin supplementation there was a significant improvement in asthma control as indicated by a decrease in ACQ-6 score (Δ-0.35(-0.50,-0.13), p=0.006) (**Figure 4-4, Table 4-2)**. Furthermore, 100% of participants with partially-poorly controlled asthma (ACQ-6 >0.75) prior to inulin supplementation (n=8) had an improvement in asthma control following supplementation, with 62.5% (n=5) reaching the minimum clinically important difference (MCID, 0.5). There were no significant changes in lung function following any of the treatment phases (**Table 4-2**). However, following inulin supplementation there was a trend for improved FEV_1 (p=0.131) **(Figure 4-4, Table 4-2)**.

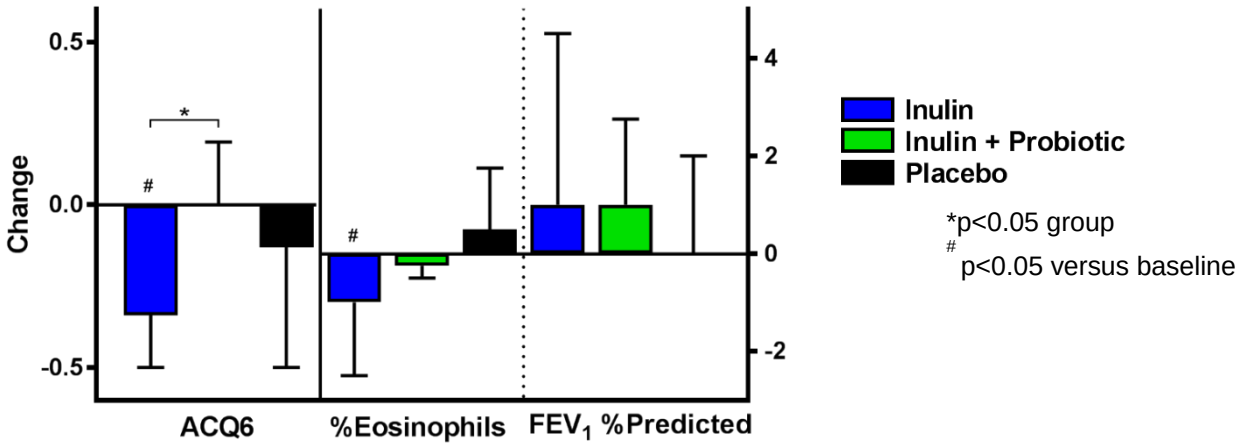

Figure 4-4: Change in Asthma Control Questionnaire (ACQ) score, sputum %eosinophils and FEV_1 %predicted following inulin, inulin+probiotic and placebo treatments.

Table 4-2: Lung function, asthma control scores and airway inflammatory markers before and after treatment

Outcome	Pre[^]	Post	Change	P[#]	P[†]
FEV_1 % Predicted					
Inulin	74 (65, 83)	77 (71, 87)	1 (-0.5, 4)	0.131	0.4
Inulin+Probiotic	74 (66, 84)	80 (67, 88)	1 (-2, 2.5)	0.533	
Placebo	80 (64, 93)	80 (71, 92)	0 (-2, 2)	0.836	
FVC % Predicted					
Inulin	87 (79, 92)	91 (81, 95)	17.5 (2, 27)	0.102	0.3
Inulin+Probiotic	87 (78, 92)	87 (78, 94)	8 (3, 17)	0.938	
Placebo	91 (77, 102)	89 (83, 93)	6 (-1, 20)	0.549	
FEV_1/FVC %					
Inulin	67 (58, 80)	73 (59, 80)	0.5 (-2, 3)	0.497	0.3
Inulin+Probiotic	73 (60, 80)	74 (58, 80)	0 (-1, 2)	0.957	
Placebo	72 (57, 80)	75 (56, 80)	0 (-2, 1)	0.586	
ACQ-6					
Inulin	**0.8 (0.5, 1.2)**	**0.3 (0.3, 0.8)**	**-0.3 (-0.5, -0.1)**	**0.006**	0.06
Inulin+Probiotic	0.8 (0.5, 0.8)	0.7 (0.3, 1.3)	0 (-0.2, 0.2)	0.874	
Placebo	1.0 (0.5, 1.3)	0.5 (0.3, 1.2)	-0.1 (-0.5, 0.2)	0.163	
Total cell count					
Inulin	4.1 (2.4, 8.8)	5.7 (3.3, 9.3)	0.9 (-1.2, 1.5)	0.300	0.57
Inulin+Probiotic	6.0 (2.5, 7.3)	5.7 (3.0, 9.5)	-1.3 (-5.4, 2.0)	0.379	
Placebo	5.5 (3.2, 9.5)	5.4 (3.3, 8.3)	-0.1 (-1.5, 1.7)	1.000	
Eosinophils %					
Inulin	**2.3 (1.3, 5.1)**	**1.5 (0.5, 2.8)**	**-1.0 (-2.5, 0)**	**0.006**	0.31
Inulin+Probiotic	1.0 (1, 4.5)	1.3 (0.5, 3.8)	-0.3 (-0.5, 0.8)	0.548	
Placebo	1.3 (0.3, 6)	2.0 (0.8, 5)	0.5 (-2.0, 1.8)	0.820	
Neutrophils %					
Inulin	52.7 (21.8, 67.4)	46.8 (26.0, 71.3)	1.3 (-11.5, 7.8)	0.798	0.96
Inulin+Probiotic	49.0 (19.8, 59.0)	31.3 (17.3, 62.0)	1.5 (-24.3, 12.8)	0.820	
Placebo	52.3 (13.8, 61.8)	47.5 (25.0, 63.3)	1.3 (-17.0, 11.0)	0.776	
Macrophages %					
Inulin	40.0 (20.1, 61.5)	48.0 (24.0, 69.5)	2.5 (-4.8, 17.8)	0.349	0.83
Inulin+Probiotic	41.5 (33.3, 72.5)	54.0 (31.8, 75.3)	-0.3 (-13.0, 16.8)	0.842	
Placebo	42.8 (28.5, 66.5)	44.8 (31.5, 67.0)	-0.5 (-7.5, 20.0)	0.776	
Lymphocytes %					
Inulin	1.6 (0.8, 2.5)	1.8 (1.0, 2.5)	0.3 (-1.3, 1.5)	0.568	0.54
Inulin+Probiotic	1.5 (0.8, 4.0)	1.8 (0.3, 3.0)	0 (-2.3, 2.0)	0.798	
Placebo	0.8 (0.3, 1.3)[^]	1.5 (0.3, 3.8)	0.5 (-0.3, 2.5)	0.105	
FeNO ppb					
Inulin	11.0 (5.5, 18.0)	11.8 (6.2, 16.9)	-0.6 (-2.0, 2.2)	0.950	0.54
Inulin+Probiotic	11.0 (6.8, 18.2)	13.6 (6.1, 17.3)	0.1 (-2.4, 3.3)	0.796	
Placebo	**11.2 (6.1, 16.4)**	**8.4 (7.0, 19.6)**	**2.3 (0.7, 4.3)**	**0.047**	

[#]Change within group at end of treatment versus baseline analysed by Wilcoxon signed rank

†Difference in variable change between groups analysed by mixed model regression accounting for repeated measures, [^]No difference in pre intervention values for all variables

Gastrointestinal symptoms experienced during each treatment phase were also assessed (**Table 4-3**). Following inulin supplementation, participants reported significantly more discomfort from indigestion and flatulence, compared to placebo. For each of the GSRS symptom domains, median scores did not exceed a rating of 2, indicating slight discomfort.

Table 4-3: Gastrointestinal symptoms rating scale scores (GSRS) following each treatment

GSRS DOMAIN	Inulin (n=16)	Inulin+Probiotic (n=16)	Placebo (n=15)	P[†]
Abdominal	1.3 (1.0, 2.2)	1.3 (1.0, 1.5)	1.3 (1.0, 1.7)	0.487
Reflux	1 (1.0, 1.0)	1 (1.0, 1.0)	1 (1.0, 1.0)	0.313
Diarrhoea	1 (1.0, 1.3)	1.3 (1.0, 1.7)	1 (1.0, 1.3)	0.726
Indigestion	**1.3 (1.0, 2.5)[#]**	1.5 (1.3, 2.0)	1 (1.0, 1.5)	0.028
Flatulence	**2 (1.0, 3.5) [#]**	**2 (1.0, 3.0)**	**1 (1.0, 2.0)**	**0.006**
Constipation	1 (1.0, 1.7)	1 (1.0 1.3)	1 (1.0, 1.7)	0.166
All domains	1.3 (1.1, 1.8)	1.4 (1.1, 1.5)	1.2 (1.1, 1.5)	0.183

[†]Mixed model regression accounting for repeated measures. GSRS scoring: 1=no discomfort, 2=slight discomfort, 3=mild discomfort, 4=moderate discomfort. [#]Significantly different versus placebo.

4.5.3 Airway inflammation

Sputum %eosinophils significantly decreased (Δ-1.0(-2.5, 0.0), p=0.006) following inulin supplementation (**Figure 4-4, Table 4-2)**. No changes were observed in induced sputum TCC, percentage or absolute cell count of airway neutrophils, macrophages and lymphocytes, following any of the treatment phases (**Table 4-2**). Change in FeNO did not significantly differ between treatment phases, despite a significant increase in FeNO following the placebo phase (Δ2.3(0.7, 4.3), p=0.047).

4.5.4 Faecal and plasma short chain fatty acids

Following both inulin and inulin+probiotic treatments there was a trend towards an increase in total and individual faecal and plasma SCFA (acetate, propionate and butyrate) levels compared to baseline, however these changes did not reach significance (**Figure 4-5** and **Figure 4-6** respectively**)** .

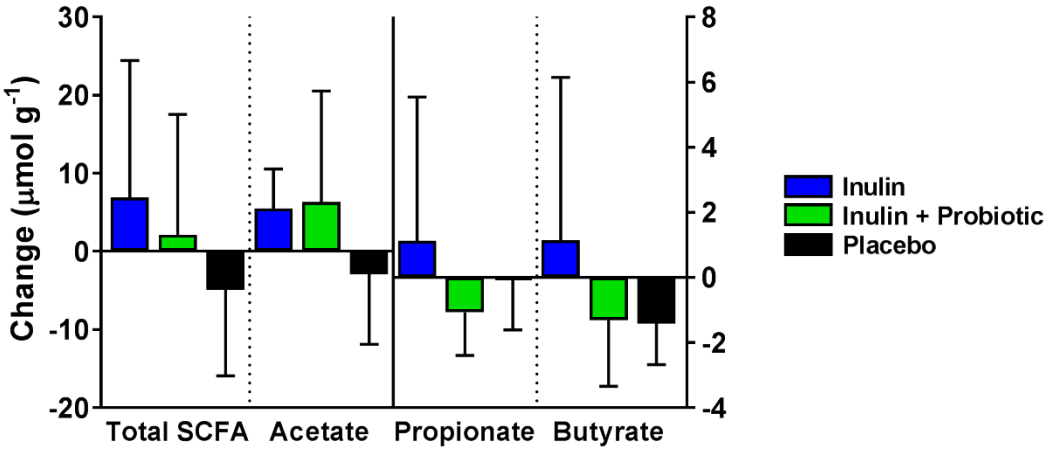

Figure 4-5: Change in faecal total SCFA, acetate, propionate and butyrate following inulin, inulin+probiotic and placebo treatments. Changes following treatments were not significant.

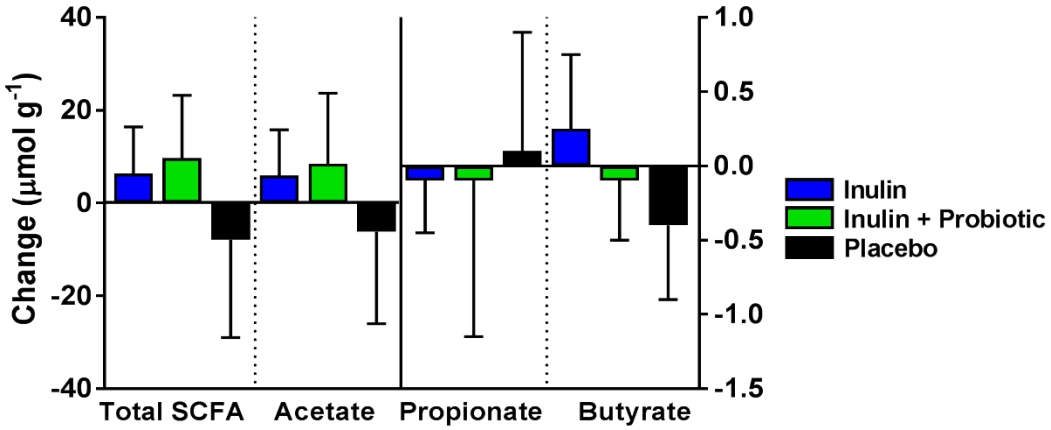

Figure 4-6: Change in plasma total SCFA, acetate, propionate and butyrate following inulin, inulin+probiotic and placebo treatments. Changes following treatments were not significant.

4.5.5 Histone deacetylase (HDAC) and Histone acetyltransferase (HAT) enzyme activity

Total HDAC and HAT enzyme activity in PBMCs did not significantly change following inulin or inulin+probiotic treatment, and as such the HAT/HDAC ratio remained unchanged following each treatment phase (**Table 4-4**).

Table 4-4: HDAC and HAT activity in Peripheral Blood Mononuclear Cells

	N	Pre	Post	Change	P[#]	P[†]
HDAC Activity (µM/µg)						
Inulin	16	3.43 (3.03, 4.28)	3.82 (3.47, 4.58)	0.34 (-0.26, 0.69)	0.098	
Inulin+Probiotic	16	4.35 (3.54, 4.72)	3.78 (3.24, 4.54)	-0.10 (-0.72, 0.48)	0.469	0.09
Placebo	15	3.63 (3.03, 4.35)	3.90 (3.70, 4.46)	0.27 (-0.48, 0.91)	0.307	
HAT Activity (µM/µg)						
Inulin	14	5.65 (3.01, 10.39)	4.26 (2.47, 10.06)	0.35 (-1.82, 4.21)	0.730	
Inulin+Probiotic	16	4.93 (1.88, 9.57)	4.33 (1.83, 8.99)	-0.65 (-2.58, 2.88)	0.836	0.95
Placebo	14	5.51 (2.86, 9.25)	5.65 (2.59, 7.41)	0.44 (-4.05, 1.90)	0.778	
HAT:HDAC ratio						
Inulin	14	1.55 (0.64, 2.82)	0.97 (0.60, 2.97)	0.13 (-0.70, 1.09)	0.975	
Inulin+Probiotic	16	1.12 (0.47, 2.67)	1.06 (0.40, 2.19)	0.32 (-0.51, 0.68)	0.918	0.87
Placebo	14	1.11 (0.75, 3.07)	1.25 (0.66, 1.85)	-0.26 (-1.37, 0.83)	0.433	

[#]Change within group at end of treatment versus baseline analysed by Wilcoxon signed rank.

†Difference in variable change between groups analysed by mixed model regression accounting for repeated measures.

4.5.6 Histone deacetylase subtype protein levels

Protein levels of HDAC2 and HDAC7 in nuclear extracts from isolated PBMCs were too low to be detected using Epigentek colorimetric assays (data not reported).

4.5.7 Gene expression of GPR41/43 and HDAC subtypes in PBMC and sputum cells

Gene expression analysis was conducted in the subset of participants from whom adequate sample was obtained, as indicated in tables 5 and 6. In PBMCs, there were

no significant within-group or between group differences in the expression of GPR41/43 or HDAC subtypes (**Table 4-5)** following any of the treatment phases.

In sputum cells, gene expression of HDAC9 was significantly decreased in the inulin only phase (p=0.0079). (**Table 4-6).** There were no significant between group differences in the sputum cell gene expression of GPR41/43 and other HDAC subtypes.

Table 4-5: Gene expression in Peripheral Blood Mononuclear Cells

	N	Pre	Post	Fold change	P[#]	P[†]
GPR43 (FFAR2)						
Inulin	15	22.65(4.07, 35.92)	15.21(9.46, 31.22)	0.8 (0.39, 4.06)	0.733	
Inulin+Probiotic	14	9.56(4.83, 43.01)	22.81(12.67, 56.95)	1.36 (0.75, 4.26)	0.594	0.29
Placebo	15	24.79(11.56, 120.39)	28.87(14.94, 49.06)	0.72 (0.36, 1.98)	0.394	
GPR41 (FFAR3)						
Inulin	15	4.55(1.15, 7.47)	2.63(1.18, 7.39)	0.55 (0.48, 2.59)	0.307	
Inulin+Probiotic	14	2.39(1.35, 4.95)	3.70(2.23, 5.83)	1.22 (0.79, 3.82)	0.638	0.40
Placebo	15	5.60(2.32, 25.84)	5.92(3.74, 10.17)	0..67 (0.41, 1.36)	0.088	
HDAC1						
Inulin	16	161.52(82.95, 245.77)	186.74(115.55, 313.65)	1.05(0.59, 3.30)	0.623	
Inulin+Probiotic	16	146.54(80.56, 226.09)	203.60(128.13, 405.04)	1.07(0.69, 2.93)	0.408	0.48
Placebo	15	214.76(138.77, 795.97)	274.67(188.91, 465.16)	0.76(0.44, 1.62)	0.609	
HDAC2						
Inulin	15	36.80(15.96, 51.68)	32.17(22.58, 52.10)	1.07 (0.58, 2.56)	0.955	
Inulin+Probiotic	14	27.20(16.70, 87.52)	35.20(24.25, 54.03)	1.09 (0.70, 2.11)	0.778	0.94
Placebo	15	48.22(22.19, 84.25)	48.22(27.41, 71.83)	0.99 (0.41, 1.66)	0.443	
HDAC3						
Inulin	16	57.21(23.15, 112.91)	58.46(43.95, 114.70)	0.98(0.50, 2.99)	0.959	
Inulin+Probiotic	16	54.82(21.13, 81.66)	86.46(43.23, 135.11)	1.31(0.79, 3.52)	0.364	0.47
Placebo	15	110.78(38.23, 229.38)	105.90(52.95, 163.89)	0.70(0.53, 1.74)	0.691	
HDAC4						
Inulin	16	44.87(18.18, 78.67)	51.14(31.31, 87.27)	1.02(0.62, 4.31)	0.756	
Inulin+Probiotic	16	33.32(18.13, 77.99)	74.41(38.03, 104.49)	1.24(0.86, 2.94)	0.134	0.54
Placebo	15	73.09(23.78, 185.02)	77.79(44.07, 127.25)	0.76(0.45, 2.06)	0.733	
HDAC5						
Inulin	16	82.07(31.07, 149.79)	89.61(42.06, 150.36)	1.10(0.51, 3.17)	0.756	
Inulin+Probiotic	16	61.04(37.33, 121.44)	115.64(58.24, 212.29)	1.07(0.72, 3.65)	0.469	0.45
Placebo	15	113.11(44.22, 417.77)	141.20(60.61, 268.09)	0.76(0.41, 1.72)	0.609	
HDAC6						
Inulin	14	172.95(68.97, 332.76)	128.70(83.09, 1.99.68)	0.55(0.30, 1.16)	0.330	
Inulin+Probiotic	15	89.06(53.97, 231.61)	172.63(89.98, 345.27)	1.07(0.64, 3.43)	0.820	0.64
Placebo	15	264.40(140.71, 790.47)	196.25(132.20, 419.22)	0.62(0.36, 1.87)	0.280	
HDAC7						
Inulin	14	316.06(192.88, 446.21)	296.95(183.45, 375.96)	0.85 (0.47, 2.09)	0.638	
Inulin+Probiotic	14	217.79(141.69, 697.75)	322.79(218.34, 597.19)	1.12 (0.56, 2.87)	0.638	0.80
Placebo	15	528.80(196.25, 1516.54)	429.52(278.51, 722.36)	0.84 (0.41, 1.83)	0.307	
HDAC8						
Inulin	16	56.47(18.84, 92.45)	50.01(30.92, 85.51)	0.95(0.47, 3.42)	0.836	
Inulin+Probiotic	16	35.13(18.33, 65.21)	64.71(32.72, 130.84)	1.21(0.82, 4.03)	0.255	0.45
Placebo	15	78.06(29.79, 29.79)	84.83(41.40, 147.70)	0.76(0.41, 1.60)	0.460	
HDAC9						
Inulin	15	38.23(28.28, 69.87)	46.16(31.01, 66.63)	0.90 (0.50, 2.77)	0.955	
Inulin+Probiotic	14	47.52(25.93, 119.56)	45.56(31.63, 80.40)	0.99 (0.73, 1.55)	0.778	0.98
Placebo	15	45.30(40.83, 173.83)	62.75(45.62, 92.83)	1.16 (0.33, 1.48)	0.733	
HDAC10						
Inulin	14	370.05(172.63, 639.84)	258.56(168.50, 476.58)	0.70(0.44, 2.72)	0.553	
Inulin+Probiotic	14	236.64(123.35, 397.98)	372.53(270.89, 1021.57)	1.27(0.76, 2.71)	0.249	0.59
Placebo	15	485.27(291.34, 2909.53)	468.39(216.25, 1137.44)	0.74(0.40, 2.08)	0.594	
HDAC11						
Inulin	15	7.12(3.49, 15.63)	8.32(4.58, 19.25)	0.92(0.58, 2.30)	0.826	
Inulin+Probiotic	16	6.98(5.14, 12.33)	9.88(6.16, 21.70)	1.02(0.73, 3.11)	0.469	0.53
Placebo	15	14.74(6.11, 33.86)	14.29(7.84, 35.79)	0.85(0.44, 1.88)	0.609	

[#]Change within group at end of treatment versus baseline analysed by Wilcoxon signed rank. †Difference in variable change between groups analysed by mixed model regression accounting for repeated measures.

Table 4-6: Gene expression in Sputum Cells

	N	Pre	Post	Fold change	P[#]	P[†]
GPR43 (FFAR2)						
Inulin	11	36.14(21.20, 129.61)	28.48(19.05, 75.72)	1.16 (0.49, 1.26)	0.657	
Inulin+Probiotic	12	46.46(7.80, 110.90)	61.11(15.41, 124.81)	1.10 (0.57, 2.64)	0.638	0.29
Placebo	7	58.29(47.92, 116.15)	66.22(40.73, 130.71)	1.01 (0.38, 2.34)	0.866	
GPR41 (FFAR3)						
Inulin	11	10.79(3.48, 34.45)	10.61(7.03, 13.25)	0.80 (0.33, 2.45)	0.722	
Inulin+Probiotic	12	10.01(4.74, 15.23)	6.57(4.83, 13.85)	0.87 (0.24, 1.41)	0.583	0.75
Placebo	7	7.59(5.35, 30.28)	11.83(5.44, 25.7)	1.21 (0.39, 2.98)	1.000	
HDAC1						
Inulin	11	5.24(3.24, 6.85)	3.92(2.35, 4.19)	0.87 (0.42, 1.27)	0.109	
Inulin+Probiotic	12	2.78(2.42, 5.12)	3.33(2.93, 4.15)	1.21 (0.80, 1.44)	0.480	0.29
Placebo	7	4.57(3.57, 7.51)	4.66(2.14, 6.21)	0.75 (0.44, 1.60)	0.398	
HDAC2						
Inulin	11	2.22(1.77, 3.24)	2.24(0.85, 2.68)	0.65 (0.45, 1.20)	0.213	
Inulin+Probiotic	12	2.10(1.62, 2.33)	1.90(1.39, 2.95)	1.08 (0.66, 1.57)	0.814	0.46
Placebo	7	2.04(1.36, 3.48)	2.27(1.36, 2.35)	0.73 (0.67, 1.73)	0.398	
HDAC3						
Inulin	11	7.94(5.50, 13.74)	5.61(3.62, 9.18)	0.82 (0.34, 1.06)	0.131	
Inulin+Probiotic	12	8.21(4.90, 13.74)	5.87(4.43, 6.70)	0.77 (0.50, 1.26)	0.182	0.85
Placebo	**7**	8.21(5.37, 10.87)	5.87(4.43, 6.70)	0.87 (0.77, 0.95)	0.058	
HDAC4						
Inulin	11	3.10(1.87, 5.55)	3.47(1.99, 3.73)	0.88 (0.67, 1.19)	0.374	
Inulin+Probiotic	12	3.20(1.99, 6.16)	3.10(1.86, 4.70)	0.76 (0.48, 1.87)	0.814	0.17
Placebo	7	2.29(2.05, 4.96)	4.97(4.06, 7.11)	1.94 (1.05, 2.17)	0.091	
HDAC5						
Inulin	11	8.51(4.45, 11.07)	5.84(4.29, 10.84)	1.08 (0.47, 1.31)	0.286	
Inulin+Probiotic	12	6.60(4.68, 14.56)	6.29(4.33, 11.70)	0.90 (0.61, 1.21)	0.583	0.77
Placebo	7	7.35(6.01, 10.99)	8.23(5.19, 9.90)	1.30 (0.48, 1.86)	0.866	
HDAC6						
Inulin	11	9.33(5.21, 16.38)	7.29(5.90, 11.64)	0.91(0.44, 1.30)	0.424	
Inulin+Probiotic	12	6.88(4.75, 15.77)	7.36(6.14, 10.63)	1.10(0.74, 1.48)	0.875	0.29
Placebo	7	6.20(3.65, 9.62)	9.69(4.47, 13.78)	1.48(1.22, 1.89)	0.091	
HDAC7						
Inulin	11	7.53(4.51, 11.08)	5.11(2.68, 9.62)	0.76 (0.37, 1.15)	0.075	
Inulin+Probiotic	12	6.52(3.79, 12.29)	4.83(2.79, 10.50)	0.77 (0.52, 1.30)	0.308	0.12
Placebo	7	7.74(3.58, 8.40)	6.66(4.80, 15.84)	1.81 (0.58, 2.16)	0.237	
HDAC8						
Inulin	11	4.10(2.28, 6.84)	2.49(1.45, 4.49)	0.74 (0.32, 1.10)	0.155	
Inulin+Probiotic	12	3.00(2.11, 6.05)	2.43(2.30, 3.09)	0.80 (0.54, 1.16)	0.084	0.24
Placebo	7	2.40(1.70, 4.70)	2.69(2.23, 4.75)	1.15 (1.01, 1.10)	0.237	
HDAC9						
Inulin	**11**	**1.12(0.5, 2.07)**	**0.40(0.36, 1.06)**	**0.54 (0.29, 0.73)**	**0.008**	
Inulin+Probiotic	12	1.07(0.43, 1.33)	0.72(0.51, 1.31)	0.91 (0.42, 2.70)	1.000	0.07
Placebo	7	0.71(0.45, 1.19)	0.99(0.52, 1.43)	1.46 (0.70, 2.01)	0.311	
HDAC10						
Inulin	11	19.73(14.95, 26.90)	11.82(8.17, 19.46)	0.62(0.34, 1.10)	0.155	
Inulin+Probiotic	12	13.59(10.16, 26.38)	14.41(11.82, 15.75)	0.94(0.50, 1.49)	0.432	0.21
Placebo	7	16.01(7.10, 18.93)	15.46(11.60, 34.85)	0.91(0.79, 2.91)	0.612	
HDAC11						
Inulin	11	0.76(0.46, 0.90)	0.55(0.22, 0.74)	0.76 (0.44, 1.09)	0.110	
Inulin+Probiotic	12	0.64(0.29, 0.93)	0.56(0.34, 0.67)	0.93 (0.70, 1.24)	0.583	0.34
Placebo	7	0.66(0.36, 0.98)	0.54(0.41, 0.68)	0.96 (0.55, 1.17)	0.237	

[#]Change within group at end of treatment versus baseline analysed by Wilcoxon signed rank. †Difference in variable change between groups analysed by mixed model regression accounting for repeated measures.

4.5.8 Correlations

Following inulin treatment, change in FEV_1 was positively correlated with change in total faecal SCFAs (R_s=0.54, p=0.04), faecal butyrate (R_s=0.66, p=0.01) and faecal propionate (R_s=0.64, p=0.01) (**Figure 4-7)**.

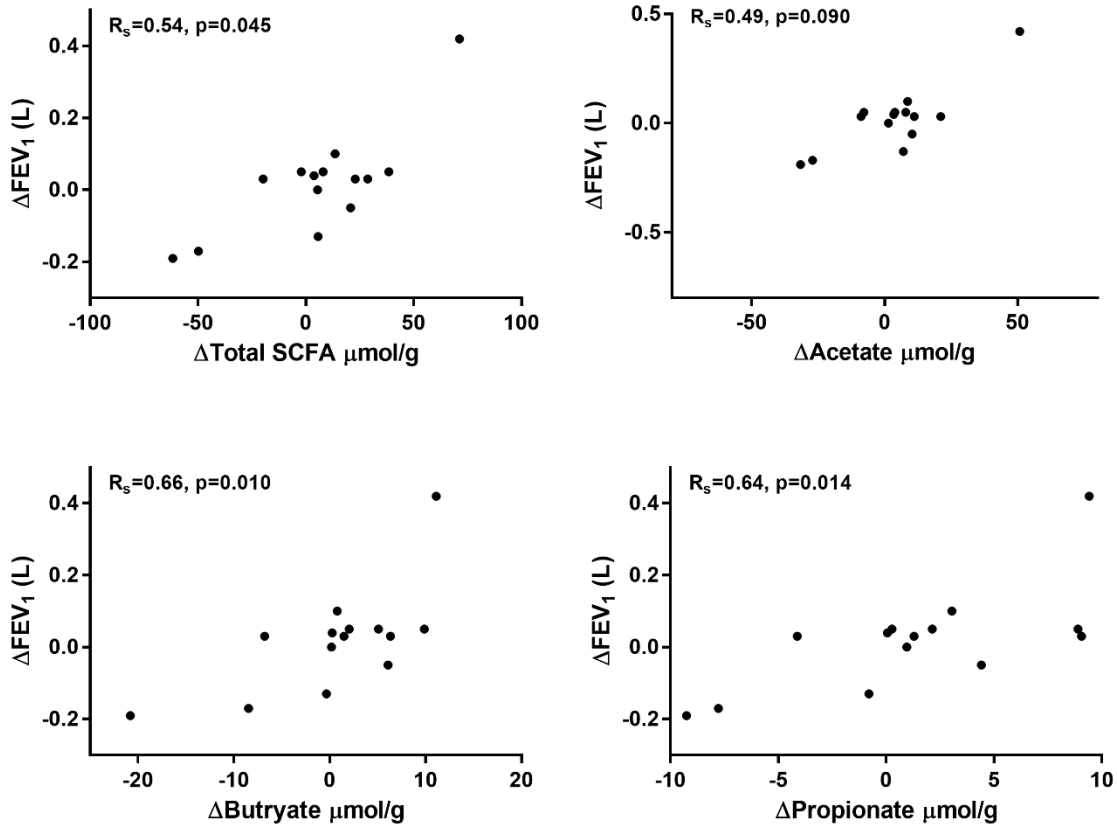

Figure 4-7: Relationship between the change in lung function and the change in faecal SCFA following inulin treatment. Analysed using Spearman's rank correlation.

Furthermore, following inulin treatment there was a positive correlation between plasma butyrate and both GPR41 and GPR43 gene expression in PBMCs (R_s=0.65, p=0.006 and R_s=0.61, p=0.012 respectively) (**Figure 4-8)**, and sputum %eosinophils were positively correlated with sputum HDAC9 gene expression (R_s=0.89, p<0.001) **(Figure 4-9)**.

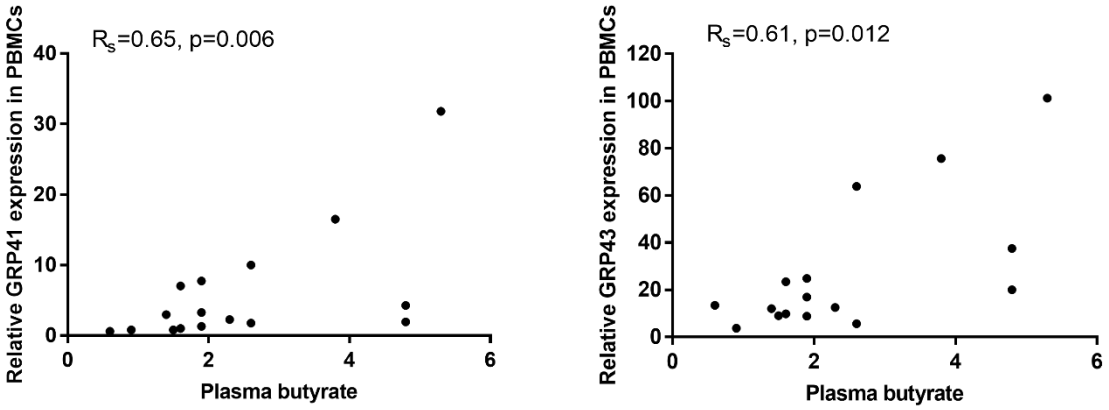

Figure 4-8: Relationship between plasma butyrate and PBMC GPR41/43 gene expression following inulin supplementation. Analysed using Spearman's rank correlation.

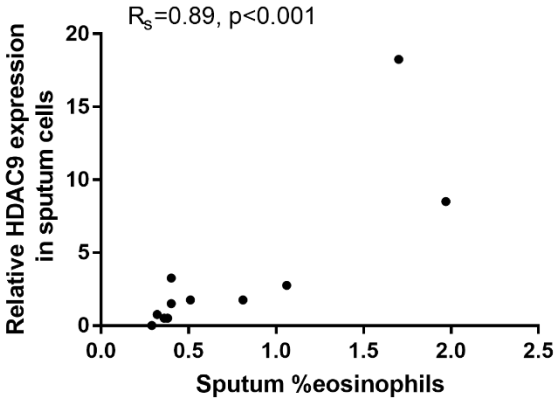

Figure 4-9: Relationship between sputum %eosinophil and sputum HDAC9 gene expression following inulin supplementation. Analysed using Spearman's rank correlation.

4.5.9 Subgroup analysis by asthma inflammatory phenotype (inulin only phase)

Participants were classified as having either eosinophilic asthma or non-eosinophilic asthma, using induced sputum cell counts. Subgroup analysis by these asthma inflammatory phenotypes revealed significant differences in several outcomes. Prior to inulin treatment those with eosinophilic asthma had significantly worse asthma control than those with non-eosinophilic asthma (p=0.033) (**Figure 4-10**), with significant and clinically important improvements in asthma control observed in those with eosinophilic asthma (n=7, Δ-0.5(-0.5, -0.13)(med(IQR) p=0.018), but not in those with non-eosinophilic asthma (n=9, Δ-0.17(-0.34, 0)(med(IQR) p=0.153) **(Figure 4-10)**.

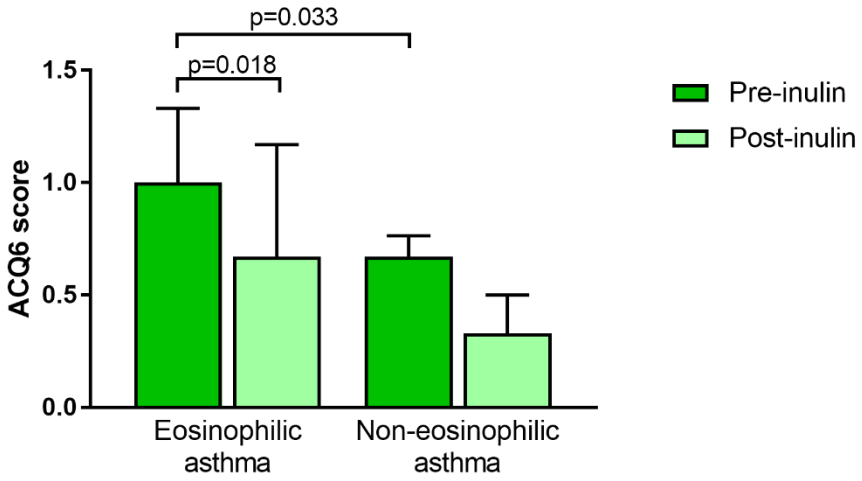

Figure 4-10: Comparison of asthma control (ACQ6 score) between eosinophilic and non-eosinophilic asthma before and after inulin supplementation.

Furthermore, prior to inulin treatment, individuals with eosinophilic asthma (n=7) had significantly higher sputum cell HDAC9 expression than those with non-eosinophilic asthma (n=9, p=0.028) (**Figure 4-11)**. Following inulin treatment sputum cell HDAC9 expression significantly decreased in those with eosinophilic asthma (n=5, Δ-0.60(-

0.63, -0.45)(med(IQR), p=0.043), whereas there was no significant change in those with non-eosinophilic asthma (n=6, Δ-0.38(-0.83,-0.16)(med(IQR), p=0.116) (**Figure 4-11)**. Gene expression of all other HDACs (HDAC1-8, 10 & 11) did not differ according to asthma inflammatory phenotype.

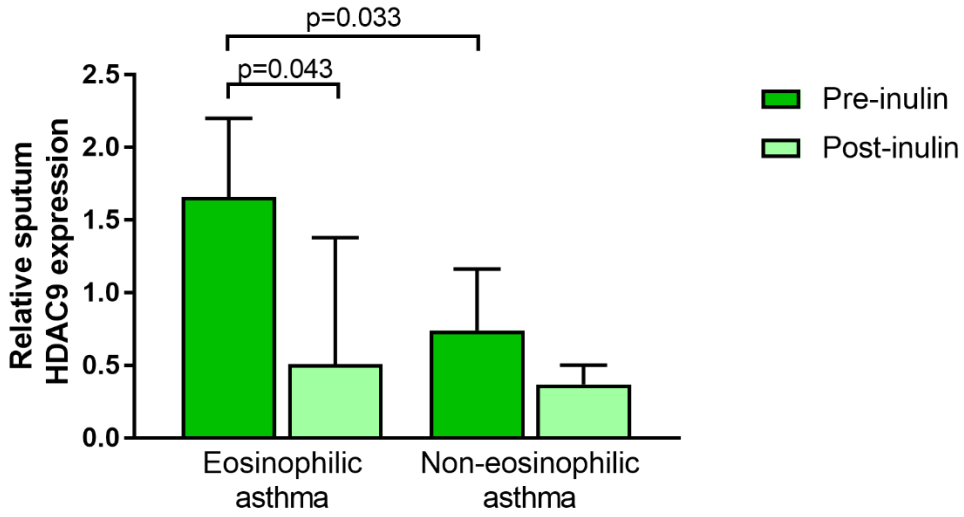

Figure 4-11: Comparison of HDAC9 gene expression in sputum cells between eosinophilic and non-eosinophilic asthma before and after inulin supplementation.

4.6 Discussion

This study aimed to investigate the effect of supplementation with the soluble fibre inulin, with and without a probiotic, on clinical outcomes and airway inflammation in asthma. In addition, SCFA production and potential anti-inflammatory mechanisms of soluble fibre in asthma were examined. Following 7 days of inulin supplementation, we observed a reduction in airway eosinophils and a trend for improvement in lung function in adults with stable asthma. Asthma control also significantly improved with inulin supplementation, with the greatest improvements observed in participants who had poorly controlled asthma at baseline, despite being on inhaled corticosteroids. No significant changes in clinical outcomes or airway inflammation were observed following the inulin+probiotic or placebo phase. Upon exploring potential anti-inflammatory mechanisms of soluble fibre in asthma, we found a significant decrease in HDAC9 gene expression in sputum cells following inulin supplementation, which corresponded with the decrease seen in %sputum eosinophils.

This study provides evidence of anti-inflammatory effects of soluble fibre in adults with asthma, a finding supported by previous studies conducted in both animals and humans. In one animal study, mice fed a diet high in soluble fibre (pectin) were found to be protected against allergic inflammation in the lungs and showed improved airway hyper responsiveness (9). In another animal study using a house-dust mite model of allergic airways disease (AAD), which simulates many features of human asthma, it was observed that a high-fibre diet (high amylose maize resistant starch) protected against the development of AAD in adult mice (124). In an observational study, we have previously shown that dietary fibre intake is inversely associated with eosinophilic airway inflammation and positively associated with lung function in adults with asthma (8). Further, in an acute meal challenge model, we demonstrated

that a single dose of soluble fibre (inulin) was effective in reducing airway inflammation and improving lung function in adults with asthma (140). While we observed a trend for improvement in lung function following inulin supplementation in the current study, this did not reach significance. It should be considered however, that this study was not powered to assess changes in clinical outcomes such as lung function. Therefore these trends should be interpreted with caution, with a larger, adequately powered study needed to see the true effect of supplementation on lung function.

In our intervention, inulin supplementation also significantly improved asthma control, with the greatest improvements observed in those whose asthma was not well-controlled prior to supplementation, despite being on inhaled corticosteroids. Of these individuals, >50% had a clinically important improvement in asthma control, which is defined as a change in ACQ ≥0.5 (243). Interestingly, our results also demonstrate that those with eosinophilic asthma had worse asthma control than those with non-eosinophilic asthma prior to inulin supplementation, with significant and clinically important improvements in asthma control following inulin supplementation observed in individuals with eosinophilic asthma, but not in those with non-eosinophilic asthma. Evidence from both cross-sectional (249-252) and longitudinal studies (253) suggests that patients with more severe disease, or poorly controlled asthma, have higher levels of sputum eosinophils, with a recent study conducted in a real clinical setting demonstrating that asthma control was associated with fluctuations in sputum eosinophilic inflammation (254). These findings suggest that the decrease in sputum eosinophils we observed following inulin supplementation is likely to have contributed to the improvement in asthma control. This further highlights the importance of the anti-inflammatory effects of soluble intake in asthma.

The anti-inflammatory benefits of soluble fibre intake have been ascribed to the by-products of its fermentation, the SCFAs. Soluble fibre is partially fermented by beneficial bacteria in the colon, which increases their number and diversity, and generates physiologically active by-products including the SCFAs: acetate, propionate, and butyrate (94); which are proposed to have systemic anti-inflammatory effects in the body (9). Butyrate is primarily absorbed by colonocytes, being their major energy source; propionate is mostly absorbed by the liver; and acetate is the primary SCFA that enters the circulation (97), which may be most relevant to diseases of the peripheral organs, such as asthma. We were unable to determine in our study whether SCFAs reached the lungs. One previous animal study was unable to detect SCFAs in lung tissue following soluble fibre supplementation, despite increases in serum and cecum SCFA levels being observed (9). It is however suggested that irrespective of whether SCFA enter the airways, the immunomodulatory effects of SCFAs in the airways initially occur systemically (9) .

Following both inulin and inulin+probiotic treatments, we observed a non-significant trend towards an increase in total plasma SCFAs compared to baseline. We speculate that the lack of significance of this result was due to the timing of venous blood sampling. There is evidence that plasma SCFA levels significantly increase 2 hours after soluble fibre consumption, reaching peak levels at around 6 hours (255). In the current study, SCFAs were measured in blood collected following a 12 hour fast. Therefore it is possible that most of the SCFAs had already been cleared from circulation, explaining why we did not see a significant increase following supplementation. Nonetheless, these trends should be interpreted with caution, with addition research needed to explore these findings. In future studies, it would be

recommended to measure plasma SCFAs 4-6 hours post supplementation to more accurately determine changes in SCFA production.

Measurement of faecal SCFAs is another indirect method used to estimate SCFA production in humans, in whom measurement of colonic fermentation is difficult (256). Similar to our plasma SCFA results, we saw a trend towards an increase in total faecal SCFAs following both inulin and inulin+probiotic supplementation, however this did not reach statistical significance. In animal studies it has been demonstrated that prebiotic soluble fibres indisputably increase faecal SCFA concentrations and decrease luminal pH (256). However mixed results have been reported by human intervention studies, with a number of factors suggested to be important to consider when estimating SCFA production using faecal analysis in humans. The dose and composition of the supplement, as well as colonic transit time and the rate of SCFA absorption in the colon can affect faecal SCFA quantification (256). Nonetheless, in this study participants reported significantly more discomfort from flatulence following inulin supplementation compared to placebo, which is consistent with altered colonic fermentation, even in the absence of any significant change in faecal SCFAs. Although we did not see a significant increase in faecal SCFAs following inulin supplementation, change in FEV_1 (a measure of lung function) was found to be positively correlated with change in total faecal SCFA, faecal butyrate and faecal propionate. This is consistent with our previous study, which reported improvements in lung function following inulin supplementation (140), and provides further evidence regarding the beneficial effects of soluble fibre supplementation in asthma.

SCFAs are suggested to have anti-inflammatory effects via mechanisms including the activation of GPR41/43 in immune cells (10, 11). Several animal models have

demonstrated that SCFA stimulated GPR41/43 activity is necessary for the resolution of airway inflammation (9, 112). One such study using an allergic airway inflammation model in mice, demonstrated that mice deficient in GPR43 had more severe inflammation, higher levels of eosinophil peroxidase activity and inflammatory cells in lung tissue and increased numbers of inflammatory cells present in the lung lining fluid (112). In another mouse model of AAD (9), a diet rich in fibre was found to increase circulating SCFA levels, and attenuate airway inflammation with dendritic cells having an impaired ability to activate Th2 effector cells in the lung (9); effects which were found to be dependent on GPR41, but not GPR43 (9).

In humans, we have also previously observed an increase in GPR41 and GPR43 expression in airway immune cells in adults with asthma following a single dose of soluble fibre, which corresponded with a decrease in airway inflammation (140). However, in the current study, no significant changes in GPR41 or GPR43 were observed following inulin or inulin+probiotic supplementation compared to placebo. Nonetheless, our data did show a positive correlation between plasma butyrate levels and GPR41/43 gene expression in PBMCs. Hence, further research is needed to explore this as a potential anti-inflammatory mechanism of soluble fibre in asthma.

SCFAs are also proposed to modulate inflammation by inhibiting the activity of HDAC enzymes, which together with HAT enzymes, are involved in the epigenetic regulation of gene expression (13). Although we did not observe any significant changes in total HDAC/HAT enzyme activity, following 7 days of inulin supplementation we observed a significant decrease in HDAC9 gene expression in sputum cells which corresponded with a decrease in sputum %eosinophils, and a trend towards a decrease in HDAC7 gene expression in sputum cells. There is evidence that HDAC9 gene expression is elevated in individuals with asthma compared to those

without asthma (123). Furthermore, in one study, HDAC9 knockout mice were found to be resistant to the development of AAD (124). Indeed HDAC9 knockout mice have also been found to have increased regulatory T cells (Treg cells) (257), which are known to suppress the development of AAD (258). Treg cells play an important role in asthma, and it is well documented that asthmatics have a lower number and less functional Treg cells compared to their non-asthmatic counterparts, (124). Optimal Treg function requires acetylation of several lysines in the forkhead domain of *Foxp3.* In mice, HDAC inhibition has been found to increase *Foxp3* gene expression as well as the production and suppressive function of Treg cells, with HDAC9 proven to be particularly important in regulating *Foxp3*-dependent suppression (257, 259). Furthermore, studies have shown that gene deletion or knockout of HDAC7 and HDAC9 can increase the suppression function of Treg cells both *in vitro* and *in vivo* (260-262). Linking these observations with our intervention, a study conducted in mice by Thornburn et al demonstrated that a high-fibre diet, via the production of acetate, primes *Foxp3*-mediated protection against asthma, likely through the inhibition of HDAC9 (124).

Treg cells have been shown to play an important role in limiting Th2-type immune responses (e.g. Gata3- and IL-13 expressing CD4+ T cells and eosinophilic influx into the airways) (263). Prior to supplementation individuals with eosinophilic asthma had significantly higher sputum HDAC9 expression than those with non-eosinophilic asthma, with a significant decrease in HDAC9 expression following inulin supplementation observed in those with eosinophilic asthma only. Furthermore, following inulin supplementation sputum HDAC9 gene expression was positively correlated with sputum %eosinophils. It is possible that down regulating HDAC9 gene expression increased the function of Treg cells, which in turn suppressed eosinophilic

influx into the airways. Further research is warranted to explore HDAC9 as a potential therapeutic target for asthmatics, particularly those with eosinophilic airway inflammation.

Interestingly, our data do not support an additive or synergistic effect of inulin+probiotic supplementation, with no significant changes in clinical outcomes or airway inflammation observed. Supplementation with combined prebiotics and probiotics (i.e. synbiotics) is hypothesised to have a greater effect on inflammation than prebiotics alone, due to their superior ability to increase SCFA-producing bacterial numbers by improving their survival and implantation in the gut (107), as well as providing substrates for fermentation (108). Yet evidence in the literature regarding their effectiveness in reducing inflammation is conflicting. In our recent systematic review, detailed in **Chapter 2**, only 50% of studies examining the anti-inflammatory effects of synbiotics reported a significant decrease in systemic inflammation following supplementation (232). Furthermore, only 43% of included studies which compared prebiotics and synbiotics reported greater anti-inflammatory effects with synbiotic versus prebiotic supplementation (232). While we cannot be certain of the reason for the lack of effect of inulin+probiotic supplementation, we hypothesise that differential changes in bacterial profile may have been induced by inulin+probiotic supplementation compared to inulin supplementation alone. Alternatively, given that prebiotics act as a food source for probiotics (107), it is possible that the amount of inulin available for SCFA production was reduced by the simultaneous delivery of the probiotic. While this study provides evidence regarding the benefits of prebiotic supplementation alone, further research is needed to investigate the benefits of combined prebiotic and probiotic supplementation in adults with asthma.

A limitation of this study is the sub-optimal time point at which we measured plasma SCFAs (following a 12 hour fast). As mentioned above, there is evidence in the literature which suggests that plasma SCFA levels significantly increase 2 hours after soluble fibre consumption, reaching peak levels at around 6 hours (255). Although we observed a trend towards an increase in plasma SCFAs at 12hours after supplementation, more informative data would have been obtained if samples were collected at an earlier time point. This should be taken into consideration when designing future studies. Another important area for future work is to examine changes in gut microflora following inulin and inulin+probiotic supplementation. Nonetheless, the prebiotic dose that we used (12g per day) has previously been shown to modulate the microbiome (135). Furthermore, there is evidence that our treatment duration (7 days) is adequate to achieve significant and maximum improvements in gut microflora, with the washout period between treatment arms (2 weeks) sufficient to allow the microflora to return to baseline composition (264). It should also be considered that the study was not powered to assess the inflammatory and clinical outcomes measured, and therefore larger, appropriately powered studies should be conducted.

4.6.1 Conclusion

In summary, this study provides promising evidence regarding the benefits of soluble fibre in asthma, with improvements in asthma control and airway inflammation observed following 7 days of inulin supplementation. Furthermore, our data suggest that individuals with not well-controlled asthma and eosinophilic airway inflammation, despite the use of inhaled corticosteroids, are most likely to benefit from inulin supplementation. Being a non-pharmacological treatment, soluble fibre supplementation has the potential to be widely adopted and accepted as a strategy for assisting in the management of asthma. Nonetheless, larger and longer duration

clinical trials are needed to confirm these clinical findings and to further explore HDAC9 as a potential therapeutic target for asthmatics, particularly those with eosinophilic airway inflammation.

Chapter 5: Investigation of the effects of modulating soluble fibre intake (via fruit and vegetable intake) on inflammation in children with asthma

5.1 Abstract

Background: Epidemiological studies show that fruit and vegetable (F&V) intake is inversely associated with wheeze and asthma risk, and positively associated with lung function. Furthermore, in adults with asthma we have previously demonstrated that consumption of a low fruit and vegetable diet worsens lung function, with time to exacerbation significantly greater in those allocated to a high versus low fruit and vegetable diet (≥7 vs. <3 serves per day) for 14 weeks. One potential mechanism by which a high F&V diet may be protective in asthma is via increased soluble fibre intake. Soluble fibres are fermented by bacteria in the colon to short chain fatty acids (SCFAs). Animal and human studies have suggested that anti-inflammatory effects of SCFAs occur via activation of G protein coupled receptor's (GPRs) 41 and 43, and the inhibition of histone deacetylase enzyme (HDAC) activity. This study investigated the effect of soluble fibre intake (via F&V intake) in children with asthma, and the potential anti-inflammatory mechanisms involved.

Methods: Children aged 3-11 years with asthma (n=47) were randomly assigned to either a high (n=22) or low (n=25) F&V diet for 6 months. Lung function was measured by spirometry and impulse oscillometry, Systemic inflammation (plasma high-sensitivity TNF-α, CRP, and IL-6) was measured by ELISA. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll density gradient, and RNA and nuclear proteins were extracted and quantified. Gene expression was measured by qPCR, and HDAC enzyme activity was measured using fluorescent assays.

Results: After 6 months, improvements in lung function, as indicated by a decrease in airway resistance (R_5-R_{20}, p=0.04) and improvement in airway reactance (X_5 p=0.038 and A_x p=0.001) were observed in the high F&V group. In the low F&V group,

systemic inflammation (hsCRP) significantly increased (Δ 0.37 [-0.04, 1.58], p=0.04), with a decrease in GRP41 gene expression (Δ -0.17 [-3.64, 0.47], p=0.01), and an increase in HDAC enzyme activity observed after 6 months (Δ 0.80 [-0.26, 1.71], p<0.01). Change in F&V consumption was found to be inversely associated with change in hsCRP (R_s= -0.32, p=0.037) and HDAC enzyme activity (R_s=-0.36, p=0.018), with change in HDAC activity positively correlated with change in hsCRP (R_s=0.37, p=0.016)

Conclusion: This study provides promising evidence regarding the beneficial effects of increasing fruit and vegetable intake in children with asthma, with significant improvements in lung function observed. Furthermore, these observations suggest that a high F&V diet may be protective against an increase in systemic inflammation in children with asthma. Additional research is needed to confirm the effects of a higher fruit and vegetable diet on clinical findings, and to provide further evidence for the anti-inflammatory mechanisms of fruit and vegetables, in particular the effect on HDAC activity.

5.2 Introduction

Asthma is the most common chronic childhood disease, affecting almost 10% of Australian children (18). Children with asthma suffer from intermittent exacerbations, triggered by respiratory virus infections in >80% of cases (23). Exacerbations can be life threatening, which causes great anxiety for patients and carers, and incurs significant health care costs (265). Asthma is usually managed using inhaled corticosteroids (ICS); however, ICS are ineffective in viral-induced exacerbations and have cost, side effect and non-compliance issues (20). Hence, new interventions are needed to reduce the burden of asthma in children.

Epidemiological studies show that fruit and vegetable intake is related to improved asthma outcomes, with intake inversely associated with wheeze and asthma risk and positively associated with lung function (69, 70). Furthermore, in our previous RCT conducted in 137 adults with asthma, those randomised to a low versus a high fruit and vegetable diet (<3 vs. ≥7 serves of fruit and vegetables per day) for 14 weeks had a 2.26-fold increased risk of an asthma exacerbation (72). It was recently suggested by the European Academy of Allergy and Clinical Immunology (EAACI) that the current available literature supports recommendations in clinical practice to increase the intake of fruits and vegetables as a way of reducing asthma risk, particularly in children (266). However, as most of this evidence comes from observational studies, it is evident there is a need for more RCTs to investigate the mechanisms responsible for the proposed clinical benefits of increased fruit and vegetable consumption in asthma.

One potential mechanism by which a high fruit and vegetable diet may reduce the risk of asthma exacerbations is via increased antioxidant intake. It is reported that there is an oxidant-antioxidant imbalance in asthma (63) and as such oxidative stress is

elevated, with further increases occurring during acute asthma exacerbations (58, 63, 64). Fruit and vegetables are a rich source of antioxidant and anti-inflammatory phytochemicals including carotenoids (e.g. lutein, lycopene, β-cryptoxanthin, α-carotene and β-carotene), flavonoids (e.g. anthocyanins), organosulfur compounds, indoles and isothiocyanates, and vitamins such as vitamin E and vitamin C (66). These antioxidants are purported to decrease airway inflammation in asthma by protecting the airways from oxidants generated both exogenously (e.g. air pollution and cigarette smoke) and endogenously (e.g. produced by activated immune cells) (73). It is suggested that dietary antioxidants scavenge reactive oxygen species, thus inhibiting NF-κB-mediated inflammation (34).

We have previously demonstrated that circulating carotenoid levels are low in adults with asthma (267), and carotenoid supplementation can reduce airway inflammation in adults with asthma (268). Furthermore we have shown *in vitro* that the carotenoid lycopene protects against virus infection (269), the primary cause of asthma exacerbations in children (23). Pre-incubation of epithelial cells with carotenoids decreased virus-induced inflammation (IL-6, IP-10) and viral replication was reduced by 85% (tissue culture infectious dose, TCID50) (269).

Another potential mechanism by which a high fruit and vegetable diet may be protective in asthma is via increased soluble fibre intake. Dietary fibre intake has been linked to reduced systemic inflammation in both healthy (230-232) and diseased adults (91, 232). Furthermore, we have previously observed that dietary fibre is inversely associated with airway inflammation and positively associated with lung function in asthma (8). Indeed, our RCT in adults with asthma demonstrated a significant increase in dietary fibre, vitamin C & β-carotene intake in participants who consumed the high

versus low fruit and vegetable diet (72). Notably, after 14 weeks airway and systemic inflammation increased in the low, but not the high fruit and vegetable group (72).

The soluble fibre from fruit and vegetables may also have beneficial effects in asthma. Soluble fibre is fermented by beneficial gut bacteria producing physiologically active by-products such as short chain fatty acids (SCFAs), which are proposed to have anti-inflammatory effects (9). SCFAs, namely acetate, butyrate and propionate, have a range of effects on the immune system including: reducing inflammatory mediator release by monocytes and macrophages, reducing neutrophil migration, inducing production of regulatory T cells (Treg cells), modulating cytokine release and expression of adhesion molecules on endothelial cells and neutrophils, and enhancing production of dendritic cell precursors from bone marrow (9).

SCFAs are proposed to modulate immune responses by binding to cell surface receptors such as G-protein coupled receptor (GPR) 41 and GPR43 (9, 112) also known as free fatty acid receptors (FFARs). In animal models, SCFA activation of GPR43/41 reduces systemic and airway inflammation (9, 112). SCFAs also induce epigenetic changes by inhibiting histone deacetylase (HDAC) enzyme activity (12, 13). This process reduces inflammation, by suppressing NF-kB activity and reducing pro-inflammatory cytokine production (126). In animal models, HDAC inhibition has been shown to alleviate airway inflammation, by suppressing airway levels of eosinophils, lymphocytes, IL-4 and IL-5 (125).

In summary, we have previously shown that increasing fruit and vegetable intake reduces risk of asthma exacerbation in adults. We have also demonstrated that the carotenoids found in fruit and vegetables are associated with reduced airway inflammation and virus replication in adults. Furthermore, soluble fibre, also found in

fruit and vegetables, reduces airway inflammation in adults with asthma. Hence, increasing fruit and vegetable intake in children warrants investigation as a potential strategy for improving asthma outcomes.

5.3 Hypothesis and aims

5.3.1 Hypotheses:

1) A high fruit and vegetable diet will reduce the risk of exacerbations requiring medical intervention and improve lung function compared with a control (low fruit and vegetable) diet, in children with asthma.
2) A high fruit and vegetable diet will reduce airway and systemic inflammation in children with asthma, via inhibition of HDAC activity and activation of G protein-coupled receptors (GPR41/43).

5.3.2 Aims:

1) To examine the ability of a high fruit and vegetable diet, versus a control diet, to increase the time to first asthma exacerbation requiring medical intervention and improve lung function, in children with asthma.
2) To examine the ability of a high fruit and vegetable diet, versus a control diet, to decrease systemic and airway inflammation.
3) To examine molecular mechanisms that are modulated by a high fruit and vegetable diet in asthma, including HDAC activity in PBMCs and GPR41/43 and HDAC1-11 gene expression in PBMCs.

5.4 Methods

5.4.1 Study participants

Children with asthma (n=49), aged 3-11years, were recruited via attendance to the emergency department or admission to the John Hunter Hospital and Maitland Hospital following an exacerbation of asthma. Recruitment commenced in September 2015, and all study visits were completed by July 2018. Inclusion criteria included: physician diagnosis of asthma; history of ≥1 exacerbation in the past 6 months or ≥2 exacerbations in the past 12 months that resulted in medical intervention (an unscheduled GP visit, presentation at a hospital emergency department, hospitalisation or rescue oral corticosteroids); stable asthma at the baseline visit (defined as no change in usual asthma medication, hospitalisation or unscheduled doctor's visit, use of oral corticosteroids or antibiotics in the past 4 weeks); consuming ≤3 serves of fruit and vegetables per day (assessed over past week); willingness and ability to attend clinic appointments; willingness to comply with proposed dietary changes; and agreement to the collection of a blood sample for research purposes at the 3-monthly clinic appointments.

Exclusion criteria included: presence of respiratory conditions other than asthma; presence of clinically important medical illness likely to affect participation (including psychiatric or behavioural disorders and chronic conditions requiring regular treatment with medications); chronic use of anti-inflammatory medications (e.g. low-dose systemic corticosteroids); unwilling or unable to modify diet; family member primarily involved in child's food preparation unwilling to adapt methods/recipes/food patterns as needed; diagnosed intestinal disorders (including irritable bowel syndrome, inflammatory bowel disease and coeliac disease); condition requiring specialised diet plan (i.e. diabetes), use of nutritional supplements within the past 4 weeks (including

multivitamins and pre/probiotics), use of any medications within the past 4 weeks known to significantly influence inflammatory biomarkers (such as oral corticosteroids and antibiotics, but excluding maintenance asthma therapies); and a change to maintenance asthma therapies in the past 4 weeks (i.e. change in strength, dose or category of medication).

This study was approved by the HNEH Ethics Committee (15/06/17/4.03) and registered with the UON Human Research Ethics Committee. Written informed parental consent, and child assent (where applicable), was obtained prior to the collection of any data.

5.4.2 Study Design

The study was conducted at the Hunter Medical Research Institute, Newcastle, Australia as a randomised controlled trial examining the effects of a high, versus low fruit and vegetable diet on children with asthma, over a 6 month period (**Figure 5-1**). Following telephone screening to confirm eligibility, the participant and their carer attended the clinic (baseline) where the participant was randomly assigned to either the intervention (high fruit and vegetable diet) or control (standard/low fruit and vegetable diet) group, in a 1:1 allocation ratio.

Parents of participants in the intervention group were telephoned fortnightly and provided with dietetic counselling to assist the child in achieving the recommended number of fruit and vegetable serves/day for the participant's age group as per the National Health and Medical Research Council (NHMRC) Australian Dietary Guidelines **(Table 5-1)** (68). White potatoes and juices were not counted towards the participant's total fruit and vegetable serves.

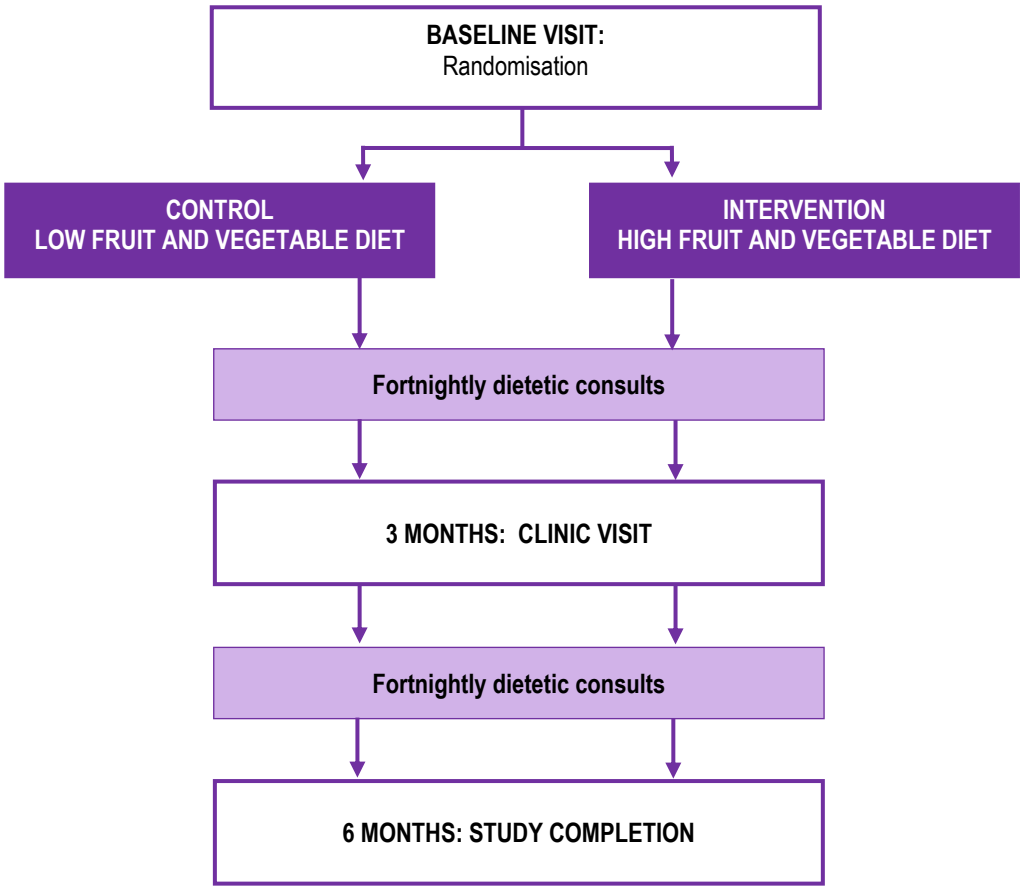

Figure 5-1: Study design for randomised controlled trial examining the effects of a high versus low fruit and vegetable diet in children with asthma.

Table 5-1: Recommended daily fruit and vegetables serves by age group, as per the Australian Dietary Guidelines (67)

Age	Fruit	Vegetables
3 years old	1 serve	2.5 serves
4-8 years old	1.5 serves	4.5 serves
9-11 years old	2 serves	5 serves

Participants in the control group were instructed to continue their usual food consumption (i.e. ≤3 serves of fruit and vegetables per day). Participants in both groups were advised to consume no more than ½ cup of fruit juice per day, and were instructed to avoid the use of vitamins, nutritional supplements and probiotics. All participants

were provided with fortnightly food hampers according to their group allocation. The hampers were tailored to the participant's preferences, within the requirements of the dietary intervention. To assess compliance with the study diets, 24 hour food recalls were collected by a dietitian during face-to-face consults with participants and their carer's at baseline, 3 months and 6 months. 24 hour food recalls were also collected during the fortnightly dietetic counselling telephone calls between weeks 2 and 26.

Randomisation was carried out using a computerised random number generator, in variable block sizes, stratified by age and gender. The randomisation service was managed by an independent statistician at the Hunter Medical Research Institute. Participants in the control group and their carer's, were blinded to the hypothesis of the study.

5.4.3 Clinical methods

Participants attended the clinic at baseline, 3 months and 6 months following a 12hr fast, and received fortnightly telephone consults. At baseline, participant information including medical and family history, exposure to tobacco smoke and medication use was collected, as well as medication and healthcare usage in the previous 12 months. At each of the 3 visits, the following clinical assessments were undertaken as detailed below: venous blood collection, anthropometric measures (height and weight), dietary intake assessment, Impulse Oscillometry (IOS), spirometry (children ≥6 years) and measurement of exhaled nitric oxide. Pulmonary function tests were always completed in order with IOS first, followed by spirometry if appropriate. During each telephone consult and clinic visit, a semi-structured interview was performed to collect information relating to illness, the occurrence of asthma exacerbations/increase in asthma symptoms, healthcare and medication usage.

Anthropometry:

Body weight was measured in light clothing without shoes, using a digital scale to the nearest 100g (NU WEIGH LOG842 scales, NU Weigh Scales Inc, MI). Height was measured to the nearest 0.1cm without shoes, using the stretch stature method and a wall-mounted stadiometer. BMI z-scores were calculated with reference to the Centre for Disease Control and Prevention (CDC) 2000 Growth Charts, using an online calculator (https://reference.medscape.com/guide/medical-calculators#examination_protocols).

Blood collection and processing:

Blood was collected following ≥12hour fast into EDTA tubes via venepuncture by a trained phlebotomist (up to 30ml depending on the child's weight, with an upper limit of 3ml/kg body weight), and processed as described in detail in **Chapter 3**. When elected by the parent or child, an anaesthetic cream was applied at least 30minutes prior to blood collection.

Dietary intake assessment:

A dietitian administered 24hr food recall was completed with the assistance of the child's carer at each clinic visit and fortnightly telephone consult, to assess compliance with the dietary intervention. The participant and/or their carer were asked to recall the amount and types of food and beverages that had been consumed in the previous 24hrs, and whether they would describe this intake as typical. The number of fruit and vegetable serves consumed was determined from the 24-hour recalls using the serving sizes specified by the Australian Dietary Guidelines (**Table 5-2**) (68).

Table 5-2: Standard serving sizes of fruit and vegetables

Standard serve of fruit (150g or 350kJ):	**Standard serve of vegetables (75g or 100-350kJ):**
- 1 medium piece. E.g. an apple, banana, orange or pear - 2 small pieces. E.g. apricots, kiwi fruits or plums - 1 cup diced or canned fruit (no added sugar) *Or only occasionally:* - 125ml (1/2 cup) fruit juice (no added sugar) - 30g dried fruit (E.g. 1.5 tablespoons of sultanas).	- 1/2 cup green or orange vegetables (E.g. broccoli, spinach, carrots or pumpkin) - 1/2 cup cooked dried or canned beans, peas or lentils - 1 cup green leafy salad vegetables - 1/2 cup sweet corn (1 ear of corn) - 1/2 medium starchy vegetable (E.g. sweet potato) - 1 medium tomato

Impulse Oscillometry (IOS)

While spirometry is considered the gold standard for measuring lung function, the success rate of children <6 years of age achieving the correct technique is at most 50% (270). Therefore, impulse oscillometry (IOS) was conducted in all children as an additional measure of lung function using a MasterScreen Jaeger IOS system with SentrySuite software (Carefusion, Hoechberg, Germany), similar to the one in **Figure 5-2a**.

IOS is a non-invasive method which is performed during normal tidal breathing and requires minimal participant cooperation. Lung function is evaluated using IOS by measuring pulmonary impedance (Z) which comprises of both airway reactance (X) and airway resistance (R) (271). Airway reactance relates to the amount of recoil that is generated in response to a pressure wave (i.e. the elasticity of the airways), whereas airway resistance is the amount of energy required to move the pressure wave through the airways (271). IOS can also be used to assess response to therapeutics such as bronchodilators (i.e. salbutamol) (271).

The IOS apparatus generates small pressure oscillations at the mouth at a fixed (square wave) frequency of 5 Hz, from which all other frequencies of interest are derived (271). These pressure impulses are generated by a loudspeaker and travel superimposed on normal tidal breathing (271). Inspiratory and expiratory pressure and flow are measured by a pressure and flow transducer in the apparatus, with signal filtering used to separate out the participants tidal breathing pattern (271).

Low-frequency signals (5 Hz) travel the furthest, penetrating the lung periphery before returning, whereas high-frequency signals (20Hz) reflect back to the mouth from the large/central airways (271). As such resistance measured at 5Hz (R_5) provides information regarding both central/proximal and distal/peripheral airway obstruction (i.e. total airway resistance), whereas resistance measured at higher-frequency oscillations, such as 20Hz, provides information primarily concerning the central airways (272). The difference between R_5 and R_{20} (R_5-R_{20}) is proposed to reflect distal/peripheral airway obstruction only (272). A decrease in airway resistance indicates a decrease in airway obstruction (271).

Reactance at 5Hz (X_5) reflects the elasticity of the peripheral airways as the lungs ability to store capacitative energy is primarily manifested in the small airways (272). Resonance frequency (Fres) is the frequency where reactance equals zero, A_x (area under the reactance curve) is the total reactance at all frequencies between 5Hz and Fres, and reflects peripheral airway obstruction (271). An improvement in airway reactance is indicated by an increase in reactance (X) and a decrease in A_x (271).

Prior to conducting the assessment, the room temperature, barometric pressure and humidity were recorded and the instrument was calibrated according to the manufacturer's specifications. Each participant wore a nose clip and was seated whilst

performing the manoeuvre (**Figure 2b**). A new disposable filter with mouthpiece was attached to the pneumotach, and the participant was instructed to inhale and exhale normally through the mouthpiece until completion of the test (30 seconds). To compensate for the compliance of the cheeks which expand when pressure oscillations are generated, the research officer firmly supported the participant's cheeks throughout the duration of the test. This was repeated until three reproducible and technically correct results were obtained, with a maximum of eight attempts performed. Results are reported as the mean value of at least 2 reproducible tests (coefficient of variation of at least two tests <10%) with coherence values >0.8 at 5Hz and between 0.9-1.0 at 20Hz. Reference values from Dencker and Malmberg et al (2006) were used to generate z-scores (273).

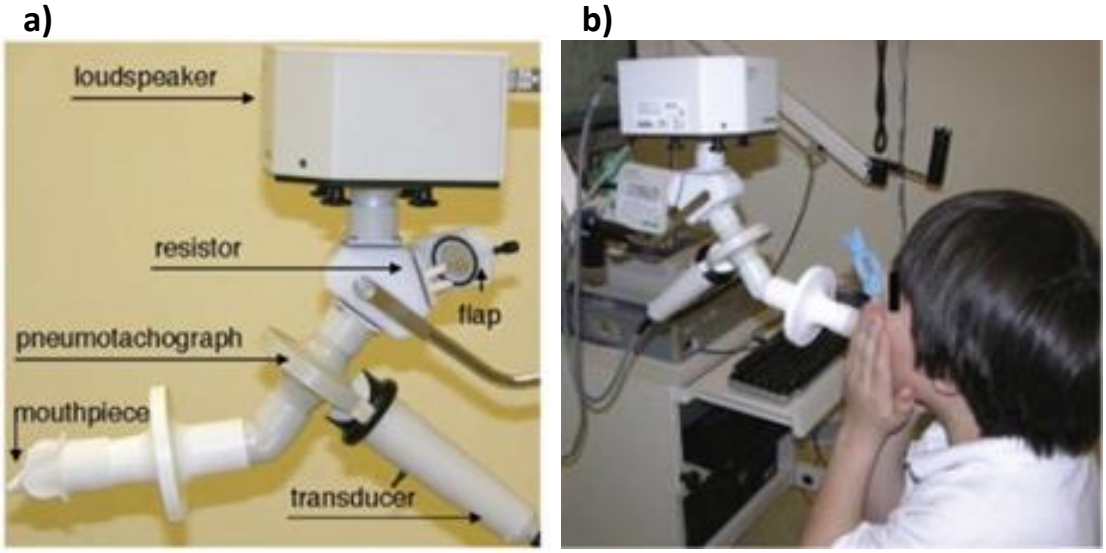

Figure 5-2: The impulse oscillometry (IOS) apparatus. **a)** Components of the apparatus. A pressure signal generated in the loudspeaker reaching both the (terminal) resister proximally and the pneumotachography more distally is transmitted into the airways through the mouthpiece. Pressure and flow signals from tidal breathing and oscillations pass through the pneumotachograph and are measured by the transducer. **b)** Eight-year old boy performing IOS. Patient is using a nose-clip, supporting the cheeks, and making a tight seal with the lips at the mouthpiece of the IOS apparatus. The results of IOS testing are graphically displayed on the computer monitor. *Figure from Komarow et al, 2011* (31).

Spirometry

Dynamic lung function was measured in participants ≥6 years using a MasterScreen Jaeger IOS system with SentrySuite software (Carefusion, Hoechberg, Germany). Predicted values for forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were calculated using reference values developed by Zapletal *et al* (1987) (274). FEV_1 and FVC values were then expressed as a percentage of the predicted values (FEV_1 %predicted and FVC %predicted respectively). Prior to conducting the assessment, the room temperature, barometric pressure and humidity were recorded, and the spirometer was calibrated. The participant's height, weight, age and gender were also entered into software.

Each participant wore a nose clip and was standing whilst performing the manoeuvre. A new disposable filter with mouthpiece was attached to the spirometer, and after completing 1-2 tidal breaths, the participant was instructed to inhale to total lung capacity, then forcefully exhale to residual volume. Visual performance incentives were used to optimise performance. This was repeated until at least two reproducible (within 0.15 L) and technically correct results were obtained as per the ATS/ERS criteria (275), with a maximum of eight attempts performed.

Exhaled Nitric Oxide

The fraction of exhaled nitric oxide (FeNO) was measured online during spontaneous tidal breathing at each visit using Ecomedics chemiluminescent detector unit (CLD 88sp FENO, Ecomedics, Switzerland), and reported in parts per billion (ppb). Ecomedics measures FeNO from the exhaled breath of humans, which can be used as a marker of eosinophilic airway inflammation. The participant was instructed to exhale away from the mouthpiece, then inhale and exhale 10 times through the mouthpiece,

slowly and continuously. Three technically acceptable attempts were recorded, and an average of the three readings calculated.

Assessment of asthma control

Asthma control was assessed using a validated self-completed weekly asthma questionnaire (https://asthmatracker.utah.edu/public/index.php/index/about-asthma-tracker) (276), completed by the child's' parent/guardian. The questionnaire contained 10 questions which asked about the child's asthma control over the past week. The first question assessed how often the child's asthma symptoms interfered with their daily activities during the past week (1. All of the time, 2. Most of the time, 3. Some of the time, 4. A little of the time or 5. None of the time). Question two assessed how often the child experienced shortness of breath (1. More than once a day, 2. Once a day, 3. Three to six times a day, 4. Once or twice, 5. Not at all). Question three assessed how often the child's asthma symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) woke them up during the night or earlier than usual in the morning (1. Four or more nights, 2. Three nights, 3. Two nights, 4. One night, 5. Not at all). Question four assessed how often the child used their quick-relief inhaler or nebuliser medication over the past week (1. Three or more times per day, 2. One or two times per day, 3. Two or three times during the week, 4. Once this week, 5. Not at all). For question five, the parent/guardian was asked to rate the child's overall asthma control (1. Not controlled at all, 2. Poorly controlled, 3. Somewhat controlled, 4. Well controlled, 5. Completed controlled). The final five questions assessed the number of days in the past week that the child used asthma medications (controller medications and oral steroids), whether additional non-prescription medications were used to ease asthma symptoms, and if the child had any unscheduled visits to a general practitioner or the hospital. Based on the responses and sum of the scores of the first 5 questions,

the child's asthma was rated "well-controlled" (>19), "not well-controlled" (15-18), or "poorly controlled" (<15).

5.4.4 Laboratory methods

Cell isolations and preparation of nuclear extracts

Blood was collected in EDTA tubes, centrifuged at 20^{o}C, 3000 rpm, for 10 minutes and plasma was stored at -80^{o}C. Remaining blood cells were then used for isolation of peripheral blood mononuclear cells (PBMCs) using the Ficoll-Paque PLUS (GE Healthcare, Sydney, Australia) density gradient method (121), as per the manufacturer's recommendations (described in **Chapter 3**). Cell counts and viability tests with trypan blue staining were performed. Isolated PBMCs were stored in buffer RLT for RNA extraction and gene expression analysis. Nuclear proteins were extracted from PBMCs using the Active Motif Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) and quantified by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), as per the manufacturers protocol (described in **Chapter 3**).

Carotenoid analysis

High performance liquid chromatography (HPLC) was used to determine carotenoid concentrations (lutein, β-cryptoxanthin, lycopene, α-carotene and β-carotene) in plasma, using validated methodology as described previously (267, 277). A solution comprised of ethanol: ethyl acetate (1:1) containing canthaxanthin was added to the plasma sample and vortexed. The sample was then centrifuged and the supernatant collected. This process was repeated three times, with ethyl acetate added twice to the pellet, followed by hexane. Milli-Q water was added to the pooled supernatant and the mixture was vortexed and centrifuged. After the supernatant was decanted, nitrogen was used to evaporate the solvents. Dichloromethane:methanol (1:2 v/v) was then added to reconstitute the sample.

HPLC was performed on a Hypersil Octadecylsilane (ODS) column (100 mm × 2.1 mm × 5 um) with a flow rate of 0.3 mL/min. A mobile phase of acetonitrile:dichloromethane:methanol 0.05% ammonium acetate (85:10:5 v/v) and a diode array detector (470 and 297 nm) were used to analyse the carotenoids. Sample carotenoid peaks were identified by comparing their retention times with a standard mixture of carotenoids, and quantified using Agilent 1200 Series High Performance Liquid Chromatograph with Chemstations software (Agilent Corporation, Waldbronn, Germany).

Systemic Inflammatory Markers

High-sensitivity C-reactive protein (hsCRP)

Plasma CRP was analysed using a high-sensitivity commercial ELISA assay (MP-Biomedicals, Santa Ana, California, USA) as per the manufacturer's instructions. The sensitivity for this assay was 0.1mg/L. All samples were tested in duplicate.

High-sensitivity Interleukin-6 (hsIL-6)

Plasma IL-6 was analysed using a high-sensitivity commercial ELISA assay (R&D Systems, Minneapolis MN USA) as per the manufacturer's instructions. The sensitivity for this assay was 0.7pg/ml. All samples were tested in duplicate.

High-sensitivity Tumour Necrosis Factor-alpha (hsTNF-α)

Plasma TNF-α was analysed using a high-sensitivity commercial ELISA assay (R&D Systems, Minneapolis MN USA) as per the manufacturer's instructions. The sensitivity for this assay was 5.5pg/ml. All samples were tested in duplicate.

HDAC enzyme activity assay

HDAC enzyme activity was measured in PBMC nuclear protein extracts using the Active Motif fluorescent HDAC Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's recommendations (described in **Chapter 3**).

HAT enzyme activity assay

Measurement of HAT enzyme activity in PBMC nuclear protein extracts was performed using the Active Motif fluorescent HAT Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's recommendations (described in **Chapter 3**).

Gene expression analysis

RNA was extracted from PBMCs using RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantitated using the Quant-iT RiboGreen RNA Assay Kit (Molecular Probes Inc, Invitrogen, Eugene, OR, USA) as per manufacturer's instructions (described in **Chapter 3**). RNA was then converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) via standard Taqman methods (described in **Chapter 3**). Taqman qPCR primer and probes for HDAC subtypes (1-11), GPR43 and GPR41 were combined with cDNA Taqman gene expression master mix as per manufacturer's instructions in duplicate real-time PCR reactions (7500 Real Time PCR System: Applied Biosystems) (described in **Chapter 3**). Analysis was performed on the change in cycle threshold (ΔCt) between the target gene compared with the housekeeping gene 18S rRNA, calculated using $2^{-\Delta\Delta Ct}$ relative to 18S and the mean of the baseline value (224).

5.4.5 Primary and secondary outcomes

The primary outcome of the study was the time to first asthma exacerbation requiring medical intervention (rescue oral corticosteroids, emergency GP visit, emergency department presentation or hospital admission). The secondary outcomes included: asthma control, lung function (measured via spirometry and IOS), airway inflammation (FeNO), biomarkers of systemic inflammation (plasma CRP, TNF-α and IL-6), HAT/HDAC activity in PBMCs and HDAC 1-11 and GRP41/43 gene expression in PBMCs.

5.4.6 Statistical analysis

In our previous study conducted in adults (72), median survival time (time to first exacerbation) on the control treatment was 6 weeks and the true hazard ratio of control participants relative to experimental participants was 2.3. Hence, we required 23 experimental and 23 control participants to complete the trial to be able to reject the null hypothesis that the experimental and control survival curves are equal with power of 80% and alpha=0.05. Statistical analysis was performed by using STATA 15 (StataCorp, College Station, Texas, USA), and reported as mean ± standard deviation or median [interquartile range]. For this proof of concept study, per protocol analysis was reported. Participants in the intervention group were excluded from analysis if they consumed less than the recommended total fruit and vegetable serves (for their age and gender) for >30% of the total study duration.

Group comparisons at baseline were performed by using independent *t* tests (parametric data) or Wilcoxon Rank Sum tests (non-parametric data). Within group changes were analysed using paired *t* tests (parametric data) or Wilcoxon signed-rank tests (non-parametric data). Group differences in change from baseline during the intervention were analysed using linear mixed models (LMMs) with group

(intervention or control) and time (treated as categorical with levels at baseline (0 months), 3 and 6 months). LMMs were adjusted for the number of hospitalisations in the previous 12 months, and random effects were specified for time. LMMs use all data available at each time point; therefore, missing data imputation was not undertaken. To examine differences in time to exacerbation between groups, Kaplan-Meier curves were fitted and *P* values for the difference between groups were calculated by using the log-rank test. Associations were assessed using Spearman's correlations. Significance was accepted if $p<0.05$.

5.5 Results

5.5.1 Participant characteristics

Of the 357 individuals assessed for eligibility, 67 were randomised (n=33 intervention group, n=34 control group) (**Figure 5-3**).

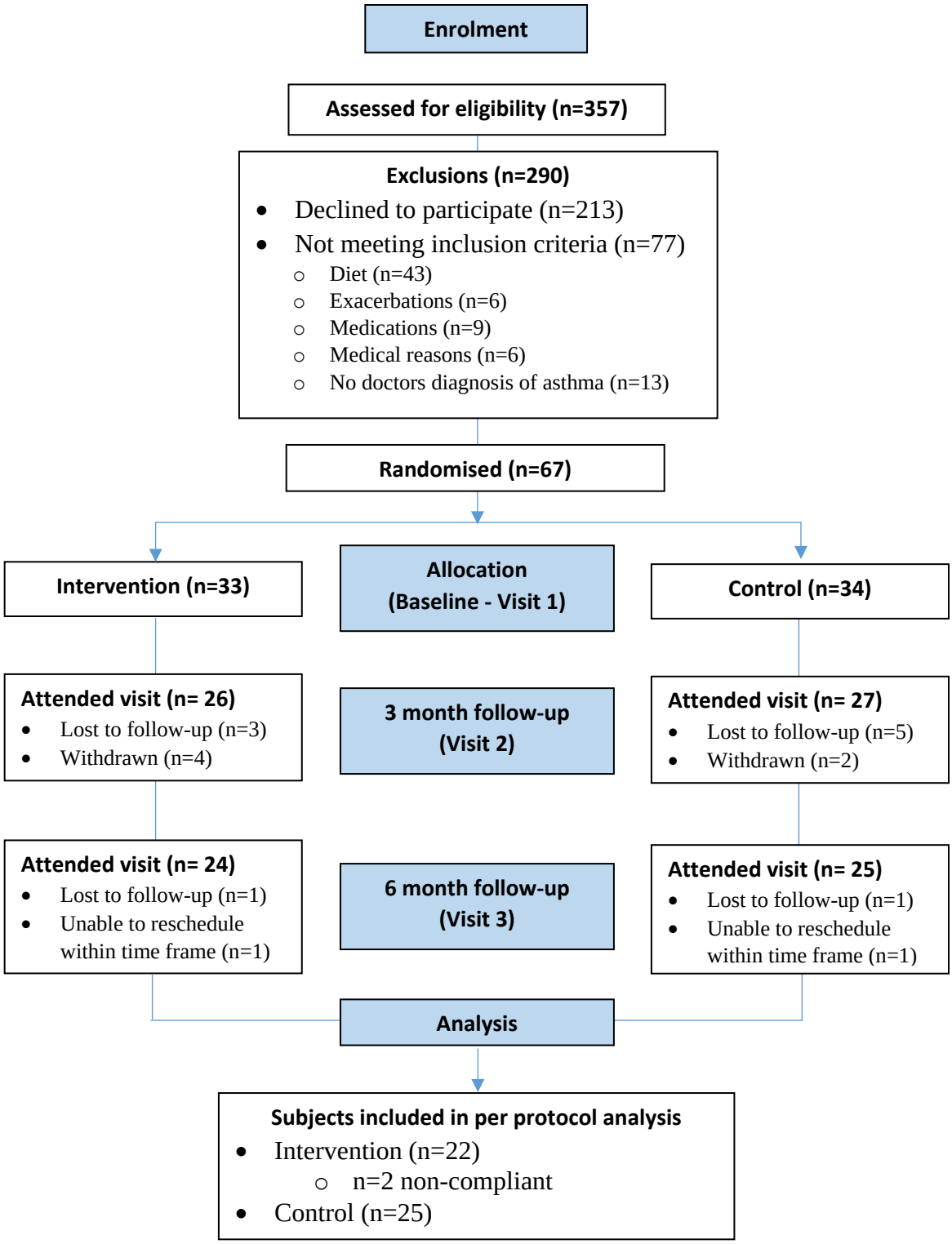

Figure 5-3: Subject flow diagram

Over the course of the study, 10 participants were lost to follow-up (n=4 intervention, n=6 control) and 6 withdrew from the study (n=4 intervention, n=2 control) due to family/work commitments. In addition, 2 participants were unable to attend their final study visit within the required timeframe (n=1 intervention, n=1 control).

The majority (n=47; n=22 intervention, n=25 control) of participants that completed the study were included in the per protocol analysis; n=2 participants in the intervention group were non-compliant with the intervention. Overall, participants had a median age of 5 (3-7) years, and median asthma control score of 24 (21.5, 25), indicating well controlled asthma.

Baseline characteristics were similar between groups (**Table 5-3**), except there were significantly more participants in the intervention group that were exposed to tobacco smoke in-utero (p=0.015), and had ≥1 hospital admissions for asthma in the previous 12 months (p=0.027), compared to the control group. Medication use in the previous 12 months did not significantly differ between groups **(Table 5-4).**

Table 5-3: Baseline characteristics

Variable	Intervention n=22	Control n=25	P value
Age (years), median (range)	5 (3-10)	5 (3-11)	0.827
Age 3-6 years, no. (%)	17 (77)	18 (72)	0.747
Age 7-11 years, no. (%)	5 (23)	7 (28)	
Gender (Male:Female)	15:7	19:6	0.550
Height (cm), mean ± SD	117 ± 14	116 ± 16	0.988
Weight (kg), median (IQR)	21.6 (16.9,25.1)	21.4 (16.9,26.6)	0.664
BMI z-score, mean ± SD	0.1 ± 1.3	0.1 ± 1.4	0.922
BMI percentile, mean ± SD	49.4 ± 32.8	54.7 ± 32.4	0.623
Asthma control score, median (IQR)	(n=18) 23.5 (21, 25)	(n=22) 24.5 (22, 25)	0.539
-Poorly controlled (<15), no. (%)	1 (5)	2 (8)	1.00
-Not well controlled (15-18), no. (%)	2 (9)	2 (8)	
-Well controlled (≥19), no. (%)	19 (86)	21 (84)	
Risk factors, no. (%)			
Current food allergy	6 (27)	5 (20)	0.557
History of Eczema[#]	16 (73)	12 (48)	0.085
History of Hay fever^	16 (73)	12 (48)	0.085
Asthma in first degree relative*	15 (68)	16 (64)	0.592
-Maternal Asthma	7 (32)	8 (32)	0.923
-Paternal Asthma	6 (27)	10 (40)	0.418
Family history of Eczema	11 (52)	17 (68)	0.280
Family history of Hay fever	18 (86)	21 (84)	0.872
In-utero tobacco exposure[†]	5 (23)	0	**0.015**
Passive smoke exposure at home	3 (14)	1 (4)	0.318
Morbidity in previous 12 months			
-ED visits for asthma, mean ± SD	1.7 ± 1.0	1.6 ± 0.9	0.672
-≥1 hospital admission, no (%)	16 (73)	10 (40)	**0.027**
-Hospitalisations, median (IQR)	1 (0,1)	0 (0, 1)	0.142

IQR, interquartile range; BMI, body mass index; ED, emergency department. BMI z-scores and percentiles were calculated with reference to the Centre for Disease Control and Prevention (CDC) 2000 Growth Charts. ED, emergency department. #Based on parental response to "Has your child ever had eczema?"^Based on parental response to "Has your child ever had a problem with sneezing, or a runny or blocked nose when he/she DID NOT have a cold or the flu?" *Based on parental response to "Has anyone in the child's immediate family ever had asthma? (including mother, father direct siblings)". †Based on parental response to ''Does anyone living in the child's immediate home (where the child spends more than half of his/her time) smoke, even if he/ she smokes outside?" Difference between groups analysed by the Wilcoxon Rank sum test (non-parametric data), two-sample t-test (parametric data) or Pearson's Chi-squared test/Fisher's exact test (testing equality of proportions) were appropriate. P<0.05 considered statistically significant.

Table 5-4: Medication use in the previous 12 months

Variable	Intervention n=22	Control n=25	P value
Short courses of OCS, median (IQR)	3 (1, 4)	2 (1, 4)	0.974
≥ 2 Short courses of OCS, no (%)	17 (68)	13 (59)	0.526
ICS or ICS/LABA combination, no (%)	16 (73)	14 (56)	0.234
-ICS intermittent, no (%)*	1 (5)	5 (20)	0.194
-ICS maintenance, no (%)^	13 (59)	8 (32)	0.062
-ICS/LABA maintenance, no (%)^	2 (9)	1 (4)	0.593
-ICS dose, beclomethasone equiv., median (IQR)	400 (200, 400)	400 (200,500)	0.573
-Regular high-dose ICS or ICS/LABA§, no (%)	5 (23)	4 (16)	0.715
Montelukast, no (%)	4 (18)	5 (20)	1.000
SABA only, no (%)	4 (18)	9 (36)	0.207
Intranasal CS, no (%)	3 (14)	3 (12)	1.000

IQR, interquartile range; OCS, oral corticosteroids; ICS, inhaled corticosteroids; LABA, long-acting β2-agonist; SABA; short-acting β2-agonist; CS, corticosteroid. *Reported to have been taken intermittently or on an as-needed basis. ^Reported to have been taken for most of the previous 12 months. §High-dose ICS: greater than 400 mg/d fluticasone propionate or equivalent. Difference between groups analysed by the Wilcoxon Rank sum test (non-parametric data) or Pearson's Chi-squared test/Fisher's exact test (testing equality of proportions) were appropriate. P<0.05 considered statistically significant.

5.5.2 Compliance

Compliance with the allocated fruit and vegetable diet regime (Intervention: high fruit and vegetable diet, Control: Standard/low fruit and vegetable diet) was assessed using 24hour recalls and analysis of plasma carotenoid levels.

Reported fruit and vegetable intake

At baseline there were no significant differences in reported fruit and vegetable intake between groups, with both groups on average consuming <3 serves/day (**Table 5-5**). The change in total fruit and vegetable intake from baseline to 3 and 6 months differed significantly between groups (**Table 5-5, Figure 5-4**); fruit and vegetable intake significantly increased in the intervention group, while no change in fruit and

vegetable intake was observed in the control group. Furthermore, average fruit and vegetable consumption over the duration of the study was significantly higher in the intervention group compared to control ($p<0.001$).

Table 5-5: Dietary fruit and vegetable intake

Variable	Baseline[a]	3months[a]	Adjusted change[b] Coeff. [95% CI]	P value	6months[a]	Adjusted change[b] Coeff. [95% CI]	P value	Average intake	P value
Fruit serves									
Intervention	1.72 ± 0.96	2.57 ± 1.25[c]	0.75 [0.03,1.46]	**0.040**	2.89 ± 1.20[c]	1.47 [0.76,2.19]	**<0.001**	2.55(2.1,3.1)	**<0.001**
Control	1.47 ± 0.79	1.58 ± 0.88			1.17 ± 0.70			1.5(1.1,1.8)	
Vegetable serves									
Intervention	1(0.5,1.5)	2.45(2,3.5)[c]	1.62 [0.98,2.26]	**<0.001**	3(2,4)[c]	2.30 [1.57,3.03]	**<0.001**	2.9(2.2,3.9)	**<0.001**
Control	1(0,1)	1(0,1)			1(0.25,15)			0.8(0.4,1.3)	
Total fruit and vegetable serves									
Intervention	2.75 ± 1.27	5.28 ± 1.65	2.37 [1.43,3.30]	**<0.001**	6.29 ± 1.58	3.77 [2.84,4.71]	**<0.001**	5.35(4.7,5.9)	**<0.001**
Control	2.25 ± 1.14	2.41 ± 1.23			2.01 ± 1.03			2.2(1.7,3.1)	

Intervention (n=22), Control (n=25). [a]Unadjusted mean ± SD or median(IQR) are presented for baseline, 3 month and 6 month measures. [b]Adjusted change means and 95% confidence intervals [CI] are the differences in change from baseline in intervention group compared to control group by linear mixed model adjusted for random effects and hospital admissions 12months prior to recruitment. [c]$p<0.05$ difference within group compared to baseline.

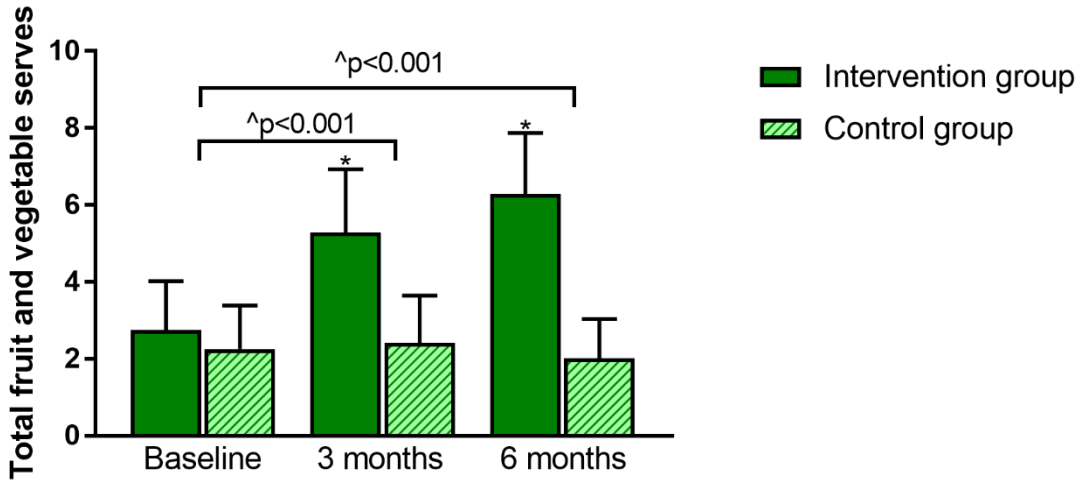

Figure 5-4: Comparison of total fruit and vegetable intake (serves) between groups at baseline, 3 months and 6 months, based on 24hr recall. ^p indicates the difference in change from baseline between groups, analysed by linear mixed model and adjusted for random effects and hospital admissions 12months prior to recruitment. *p<0.05 difference within group compared to baseline.

Plasma carotenoids

Plasma carotenoids were measured as an objective biomarker of fruit and vegetable intake (**Table 5-6).** At baseline there were no significant differences in total or individual plasma carotenoids between groups. After 3 and 6 months, change in plasma carotenoid levels significantly differed between groups; significant increases in total and individual carotenoids (α and β carotene) were observed in the intervention group, with no changes observed in the control group (**Table 5-6, Figure 5-5).** Change in total carotenoids from baseline to 6months was positively correlated with change in reported total fruit and vegetable intake (R_s=0.41, p=0.005) (**Figure 5-6).**

Table 5-6: Biomarkers of fruit and vegetable intake

Variable (μg/mL)	**Baseline[a]**	**3months[a]**	**Adjusted change[b] Coeff. [95% CI]**	**P value**	**6months[a]**	**Adjusted change[b] Coeff. [95% CI]**	**P value**
Lutein							
Intervention	0.13(0.08,0.15)	0.12(0.11,0.22)	0.02 [-0.01,0.04]	0.150	0.14(0.10,0.15)	0.01 [-0.01,0.04]	0.393
Control	0.13(0.09,0.15)	0.11(0.10,0.14)			0.13(0.09,0.15)		
β-cryptoxanthin							
Intervention	0.18(0.14,0.23)	0.18(0.13,0.26)	0.00 [-0.06,-0.06]	0.991	0.18(0.14,0.26)	0.04 [-0.3,0.11]	0.270
Control	0.15(0.11,0.20)	0.14(0.11,0.32)			0.12(0.114,0.188)		
Lycopene							
Intervention	0.37(0.24,0.51)	0.37(0.31,0.44)	0.01 [-0.07,0.09]	0.822	0.41(0.32,0.46)	0.04 [-0.04,0.12]	0.342
Control	0.32(0.24,0.41)	0.34(0.28,0.40)			0.31(0.21,0.44)		
α-carotene							
Intervention	0.06(0.04,0.09)	0.08(0.06,0.12)[c]	0.03 [0.01,0.06]	**0.020**	0.09(0.05,0.12)[c]	0.03 [0.01,0.06]	**0.016**
Control	0.05(0.03,0.08)	0.04(0.03,0.09)			0.05(0.03,0.09)		
β-carotene							
Intervention	0.73(0.56, 0.92)	0.91(0.70,1.08)[c]	0.25 [0.05,0.44]	**0.013**	0.86(0.69,1.33)[c]	0.35[0.14,0.55]	**0.001**
Control	0.50(0.37,0.84)	0.51(0.38,0.85)			0.63(0.35,0.84)		
Total carotenoids							
Intervention	1.5(1.14,1.89)	1.74(1.45,1.93)[c]	0.30 [0.03,0.57]	**0.027**	1.69(1.44,2.48)[c]	0.47[0.20,0.74]	**0.001**
Control	1.07(0.88,1.59)	1.14(1.03,1.69)			1.28(0.73,1.61)		

Intervention (n=22), Control (n=25). [a]Unadjusted median(IQR) are presented for baseline, 3month and 6month measures. [b]Adjusted change means and 95% confidence intervals [CI] are the differences in change from baseline in intervention group compared to control group by linear mixed model adjusted for random effects and hospital admissions 12months prior to recruitment. [c]$p<0.05$ difference within group compared to baseline.

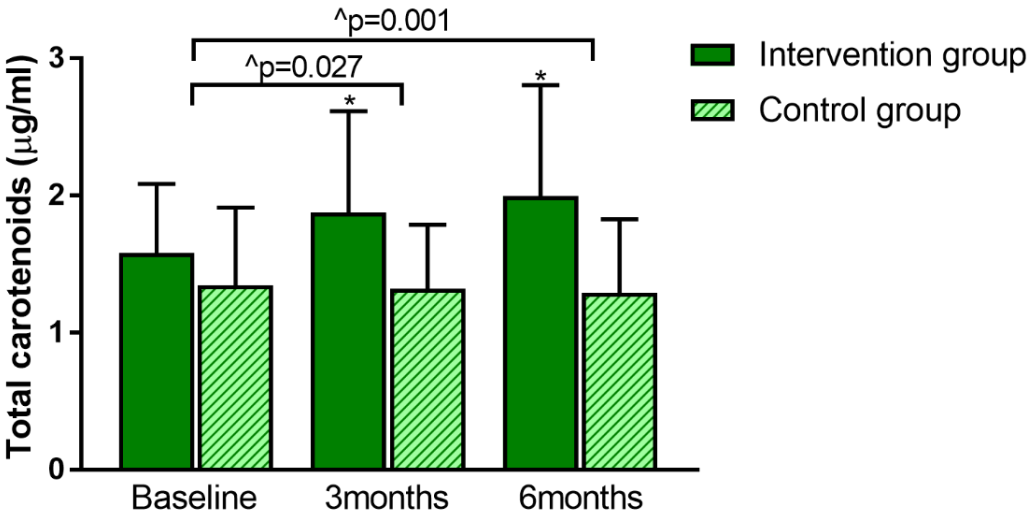

Figure 5-5: Comparison of total plasma carotenoid concentrations (µg/ml) between groups at baseline, 3 months and 6 months, measured by high performance liquid chromatography. ^p indicates the difference in change from baseline between groups, analysed by linear mixed model and adjusted for random effects and hospital admissions 12months prior to recruitment. *p<0.05 difference within group compared to baseline.

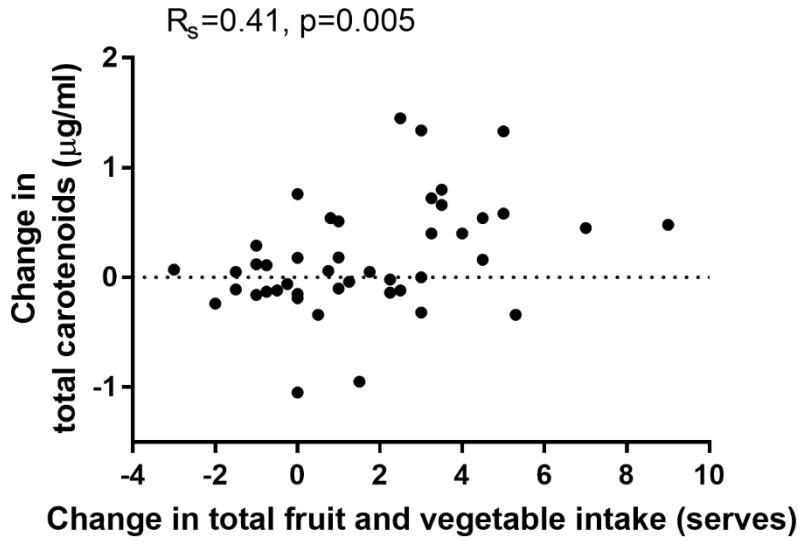

Figure 5-6: Correlation between change in total fruit and vegetable intake (serves) and change in total plasma carotenoids (µg/ml) from baseline to 6months. Analysed by Spearman's rank correlation.

5.5.3 Clinical outcomes

Survival analysis

The time to first asthma exacerbation requiring medical intervention (rescue oral corticosteroids, emergency GP visit, emergency department presentation or hospital admission) did not significantly differ between groups (**Table 5-7, Figure 5-7**). Similarly, there was no significant difference between groups in the occurrence or time to first OCS use, unscheduled visit to GP, emergency department visit or admission to hospital (**Table 5-7).**

Table 5-7: Survival Analysis

	Intervention n=22	**Control n=25**	***P***
Primary Outcome^ occurred n (%)	9 (41)	13 (52)	0.614
Days to Primary Outcome Median (IQR)	33 (12, 82)	78 (36, 131)	
Event - OCS n (%)	7 (32)	12 (48)	0.312
Days to OCS Median (IQR)	67 (12, 89)	74 (32, 107)	
Event – Unscheduled GP Visit n (%)	6 (27)	8 (32)	0.867
Days to Unscheduled GP Visit Median (IQR)	23 (5, 67)	63 (41, 83)	
Event – Emergency Department Visit n (%)	4 (18)	6 (24)	0.660
Days to Emergency Department Visit Median (IQR)	81 (43, 86)	84 (22, 139)	
Event – Hospital Admission n (%)	2 (9)	2 (8)	0.895
Days to Hospital Admission Median (IQR)	47 (5, 89)	22 (8, 36)	
Event – Unscheduled Healthcare Visit† n (%)	7 (32)	10 (40)	0.676
Days to Unscheduled Healthcare Visit Median (IQR)	33 (5, 82)	63 (36, 83)	

^Asthma exacerbation requiring rescue oral corticosteroids, emergency GP visit, emergency department presentation or hospital admission. †Unscheduled visit to GP, emergency department or admission to hospital. Kaplan-Meier curves were fitted to examine differences in time to exacerbation between groups, and *P* values for the difference between groups were calculated by using the log-rank test.

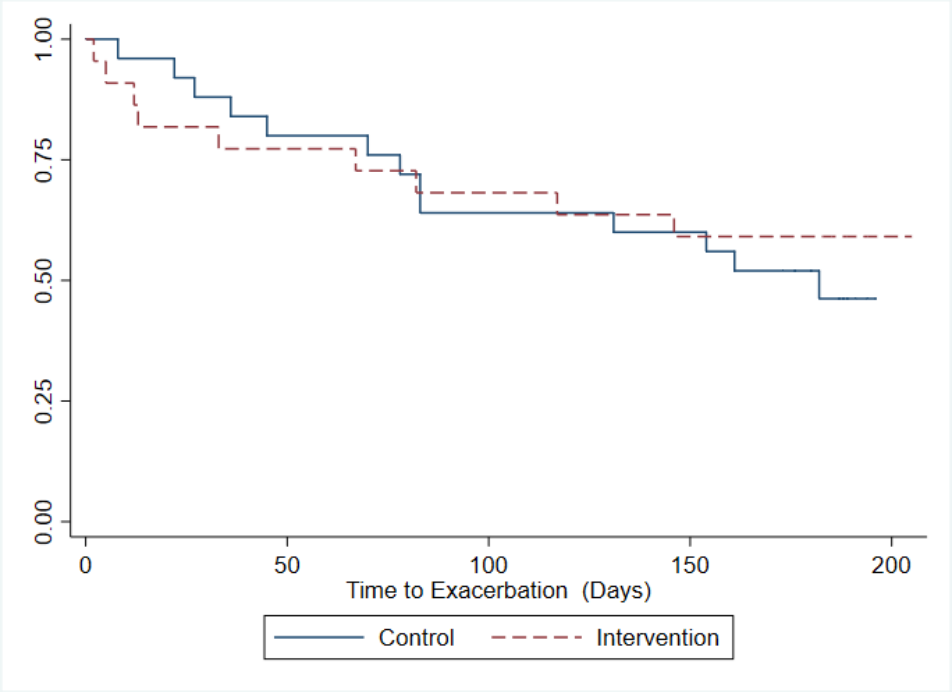

Figure 5-7: Kaplan-Meier survival curve for time to exacerbation in subjects in the control versus intervention group. Kaplan-Meier curves were fitted, and *P* values for the difference between groups were calculated by using the log-rank test

Impulse Oscillometry (IOS)

Z-scores of airway resistance (R), reactance (X) and impedance, measured using Impulse Oscillometry (IOS), are reported in **Table 5-8a** and **Table 5-8b.** Baseline airway resistance, reactance and impedance were comparable between groups, with the exception of central airway resistance at 15Hz (R_{15}) which was significantly higher in the intervention group, and reactance at 5Hz (X_5) which was significantly lower in the intervention group compared to the control group. After 6months, peripheral airway resistance (R_5-R_{20}; difference between resistance at 5Hz and 20Hz) significantly decreased in the intervention group (p=0.040) (**Figure 5-8a)**, however there were no significant changes in z-scores of resistance measured at 5, 10, 15 or 20 Hz within or between groups (**Table 5-8a).**

Change in airway reactance (measured at 5, 15 and 20 Hz) from baseline to 6months significantly differed between groups (**Table 5-8b, Figure 5-8)**, with a significant increase in peripheral airway reactance (X_5 z-score) observed in the intervention group (p=0.038). Furthermore, in the intervention group A_x (area under the reactance curve) significantly decreased from baseline to 6months (p=0.001), with the difference in change between groups approaching significance (p=0.058) **(Table 5-8b, Figure 5-8e)**. There was no significant change in airway impendence (Z_5 z-score) within or between groups (**Table 5-8b).**

Table 5-8a: Respiratory resistance

Variable	Baseline[a]	3months[a]	Adjusted change[b] Coeff. [95% CI]	P value	6months[a]	Adjusted change[b] Coeff. [95% CI]	P value
R_5 Z-Score							
Intervention n=15	0.64 (0.03,1.46)	0.61 (-0.29,1.12)	-0.56 [-1.29,0.16]	0.130	0.66 (-0.14,0.92)	-0.56 [-1.28,0.16]	0.127
Control n=17	-0.07 (-0.27, 0.79)	0.20 (-0.13,0.63)			0.17 (-0.29,1.04)		
R_{10} Z-Score							
Intervention n=15	0.98 (0.05,1.69)	0.91 (-0.05,1.09)	-0.49 [-1.22,0.24]	0.186	0.41 (-0.12,1.3)	-0.61 [-1.33,0.11]	0.099
Control n=17	0.06 (-0.43,0.75)	1.18 (-0.35,0.62)			0.30 (-0.1,0.9)		
R_{15} Z-Score							
Intervention n=15	**0.68 (-0.1,1.61)[d]**	0.28 (-0.22,1.03)	-0.49 [-1.16,0.19]	0.158	0.15 (-0.29,1.09)	-0.58 [-0.52,0.39]	0.090
Control n=17	0.05(-0.66,0.55)	0.12 (-0.39,0.54)			0.26 (-0.22,0.75)		
R_{20} Z-Score							
Intervention n=15	0.52 (-0.21,1.51)	0.15 (-0.18,1.01)	-0.44 [-1.12,0.25]	0.212	0.15 (-0.23,0.94)	-0.46 [-1.14,0.22]	0.185
Control n=17	0.26 (-0.53,0.46)	0.22 (-0.52,0.68)			0.31 (-0.28,0.85)		
R_5- R_{20} Z-Score							
Intervention n=15	0.23 ± 0.86	0.21 ± 0.55	-0.13 [-0.56,0.30]	0.549	**0.11 ± 0.79[c]**	-0.14 [-0.57,0.29]	0.552
Control n=17	0.09 ± 0.86	0.32 ± 0.93			0.10 ± 0.86		

R_5, respiratory resistance at 5 Hertz (Hz); R_{10}, respiratory resistance at 10 Hz; R_{15}, respiratory resistance at 15 Hz; R_{20}, respiratory resistance at 20 Hz. [a]Unadjusted mean ± SD or median(IQR) are presented for baseline, 3month and 6month measures. [b]Adjusted change means and 95% confidence intervals [CI] are the differences in change from baseline in intervention group compared to control group by linear mixed model adjusted for random effects and hospital admissions 12months prior to recruitment. [c]$p<0.05$ difference within group compared to baseline. [d]$p<0.05$ difference between groups at time point specified.

Table 5-8b: Respiratory reactance and impedance

Variable	Baseline[a]	3months[a]	Adjusted change[b] Coeff. [95% CI]	P value	6months[a]	Adjusted change[b] Coeff. [95% CI]	P value
X_5 Z-Score							
Intervention n=15	**-0.51 (- 0.92,0.18)[d]**	-0.40 (-0.84,0.28)	0.71 [-0.01,1.44]	0.054	**-0.23 (-0.67,0.24)[c]**	0.76 [0.05,1.48]	**0.037**
Control n=17	0.07 (-0.73, -0.68)	-0.05 (-0.53,0.20)			-0.01 (-0.75,0.50)		
X_{10} Z-Score							
Intervention n=15	-0.59 (-2.45,0.05)	-0.90 (-1.87, -0.12)	0.61 [-0.21,1.42)	0.145	-1.00 (-1.68,0.34)	0.58 [-0.24,1.39]	0.164
Control n=17	-0.53 (-1.56,0.52)	-0.83 (-2.36, -0.04)			-0.49 (-1.73,0.26)		
X_{15} Z-Score							
Intervention n=15	0.07 (-1.28,0.89)	0.27 (-1.2,0.90)	0.53 [-0.22,1.28]	0.169	0.15 (-0.94,1.40)	0.76 [0.01,1.51]	**0.047**
Control n=17	0.21 (-0.89,1.51)	0.15 (-1.18,1.13)			0.35 (-0.76,1.59)		
X_{20} Z-Score							
Intervention n=15	-2.09 (-2.67, -0.97)	-1.29 (-2.4, -0.65)	0.86 [0.15,1.57]	**0.017**	-1.46 (-2.38, -0.45)	0.94 [0.23,1.64]	**0.009**
Control n=17	-1.07 (-2.63, -0.31)	-1.55 (-2.66, -0.66)			-1.38 (-2.21,0.06)		
Z_5 Z-Score							
Intervention n=19	0.66 (0.02,1.59)	0.49 (-0.22,1.04)	-0.63 [-1.38,0.11]	0.098	0.65 (-0.09,0.83)	-0.63 [-1.37,0.11]	0.094
Control n=22	0.13 (-0.27,0.61)	0.21 (-0.15,0.59)			0.18 (-0.22,0.94)		
A_X (kPaL-^{1})							
Intervention n=21	2.24 (1.46,3.85)	2.02 (1.46, 3.35)	-0.72 [-1.49,0.05]	0.068	**2.07 (1.07,3.39)[c]**	-0.74 [-1.51,0.02]	0.058
Control n=22	1.86 (0.85,2.95)	2.17 (1.17, 4.05)			1.85 (1.01,3.34)		

X_5, respiratory reactance at 5 Hertz (Hz); X_{10}, respiratory reactance at 10 Hz; X_{15}, respiratory reactance at 15 Hz; X_{20}, respiratory reactance at 20 Hz; Z5, respiratory impedance at 5 Hz; A_X, area under the reactance curve. [a]Unadjusted mean ± SD or median(IQR) are presented for baseline, 3month and 6month measures. [b]Adjusted change means and 95% confidence intervals [CI] are the differences in change from baseline in intervention group compared to control group by linear mixed model adjusted for random effects and hospital admissions 12months prior to recruitment. [c]$p<0.05$ difference within group compared to baseline. [d]$p<0.05$ difference between groups at time point specified.

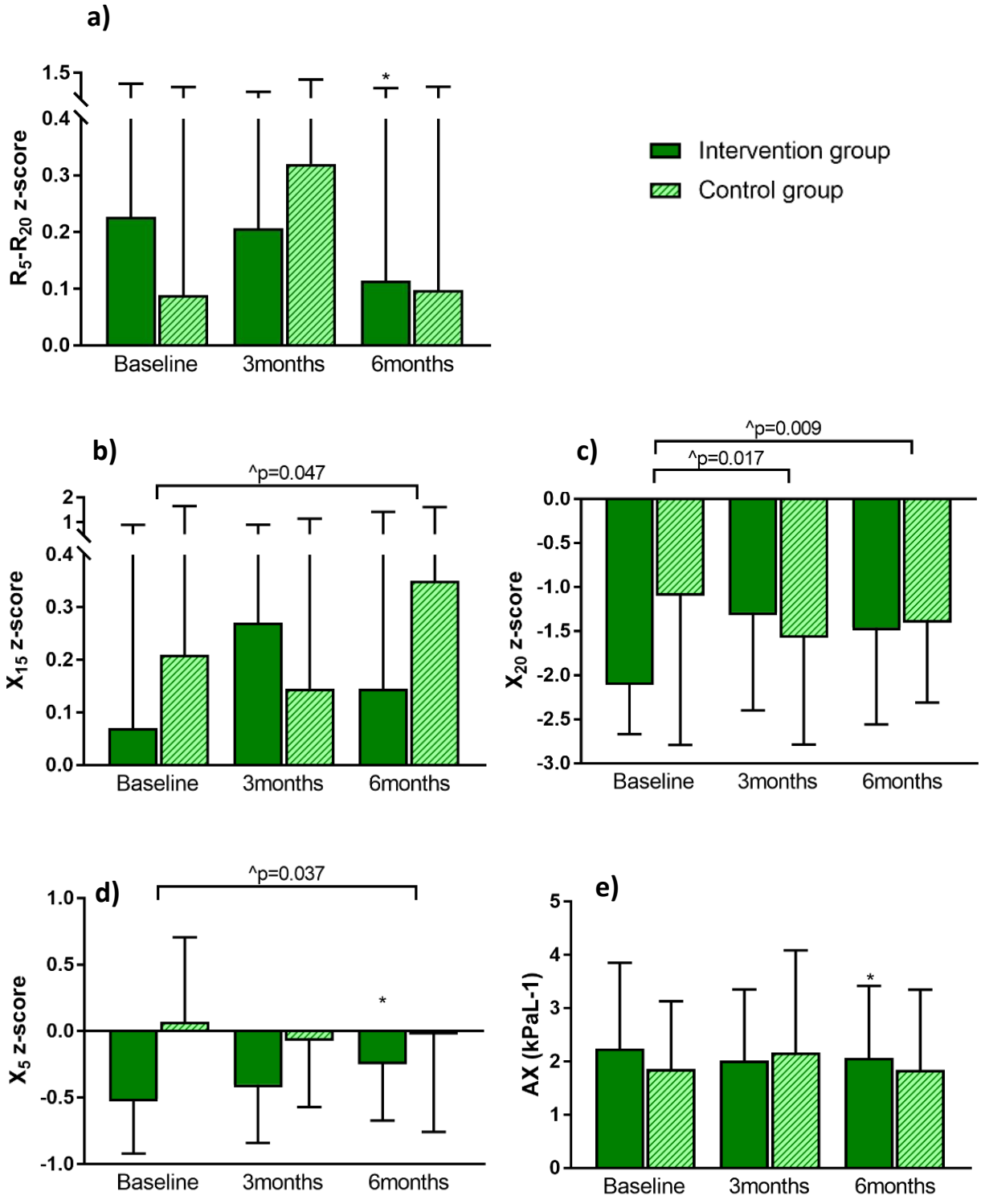

Figure 5-8: Comparison of airway resistance; **a)** R_5-R_{20} z-score, airway reactance; **b)** X_{15} z-score, **c)** X_{20} z-score, **d)** X_5 z-score, and **e)** A_X ($kPaL^{-1}$) area under the reactance curve, between groups at baseline, 3 months and 6 months. ^p indicates the difference in change from baseline between groups, analysed by linear mixed model and adjusted for random effects and hospital admissions 12months prior to recruitment. *p<0.05 difference within group compared to baseline.

Spirometry and airway inflammation

Spirometry was used to measure lung function in participants ≥6 years old, who were able to perform reproducible measurements (n=6 intervention, n=5 control) (**Table 5-9).** FEV_1/FVC ratio was significantly higher in the control group at baseline, however there were no significant changes in lung function within or between groups after 6 months. Similarly, there were no significant within or between group changes in FeNO (measure of airway inflammation) (**Table 5-9**).

Asthma control

There were no significant within or between group changes in asthma control from baseline to 6 months (**Table 5-9).** Similarly, there was no significant difference in asthma control self-reported on a weekly basis, between groups throughout the study (Adjusted change, coefficient (95% CI): 0.16(-0.06, 0.09), p=0.694).

Table 5-9: Lung function, airway inflammation and asthma control

Variable	Baseline[a]	3months[a]	Adjusted change[b] Coeff. [95% CI]	P value	6months[a]	Adjusted change[b] Coeff. [95% CI]	P value
Pre β_2 FEV_1 % predicted							
Intervention n=6	94.8 ± 14.1	95.7 ± 12.1	7.2 [-8.8,23.1]	0.379	93.3 ± 19.2	2.6 [-15.1,20.4]	0.771
Control n=5	100.6 ± 8.6	95.2 ± 9.5			95.8 ±12.1		
Pre β_2 FVC % predicted							
Intervention n=6	102.0 ± 13.1	103.8 ± 11.3	5.3 [-3.1,13.6]	0.215	109.8 ± 7.9	2.4 [-6.5,12.2]	0.554
Control n=5	98.0 ± 11.9	92.8 ± 9.8			92.5 ± 8.1		
Post β_2 FEV_1 % predicted							
Intervention n=5	107.2 ± 7.5	104.4 ± 11.4	3.0 [-7.7,13.6]	0.585	100.9 ± 16.2	-3.2 [-17.1,10.7]	0.651
Control n=6	106.8 ± 7.5	96.9 ± 14.8			98.8 ± 12.4		
Post β_2 FVC % predicted							
Intervention n=5	105.7 ± 11.8	107.2 ± 11.2	1.8 [-5.4,9.1]	0.620	105.1 ± 14.1	0.9 [-6.3,8.2]	0.790
Control n=6	100.2 ± 12.8	94.4 ± 12.7			93.2 ± 10.1		
FEV_1 BDR%[e]							
Intervention n=5	10.9 ± 12.2	9.6 ± 7.5	0.02 [-15.8, 15.8]	0.998	4.8 ± 4.7	-6.6 [-24.2, 11.1]	0.468
Control n=5	2.2 ± 2.4	1.7 ± 7.8			2.2 ± 9.8		
FVC BDR%[e]							
Intervention n=5	4.0 ± 3.2	3.2 ± 2.9	-4.8 [-13.4, 3.9]	0.283	-1.6 ± 6.2	-5.7 [-15.4, 4.0]	0.251
Control n=5	-3.0 ± 3.5	1.3 ± 5.2			-2.9 ± 6.2		
Pre β_2 FEV_1/FVC %							
Intervention n=6	**79.3 ± 10.8[c]**	78.2 ± 9.7	0.68 [-6.1, 7.5]	0.843	71.4 ± 11.6	2.2 [-10.4, 14.9]	0.728
Control n=5	87.5 ± 3.6	87.2 ± 6.9			88.5 ± 6.2		
FEV_1/FVC % BDR%							
Intervention n=5	6.4 ± 8.7	6.2 ± 5.3	6.3 [-3.9, 16.5]	0.229	6.4 ± 2.3	-0.49 [-11.8, 10.8]	0.932
Control n=5	4.9 ± 3.9	0.48 ± 7.7			5.1 ± 3.7		
FeNO (ppb)							
Intervention n=14	2.5 (1.1,5.4)	1.1 (0.8,5.2)	1.5 [-2.8,5.3]	0.539	1.3 (0.5, 3.1)	-0.1 [-4.35,4.08]	0.950
Control n=16	2.7 (1.2,6.3)	2.8 (0.5, 3.8)			1.4 (0.5, 9.8)		
Asthma control							
Intervention n=18	24 (22,25)	25 (23,25)	-1.44 [-4.66,1.79]	0.383	25 (23,25)	1.40 [-1.94,4.75]	0.412
Control n=22	24.5 (22,25)	24 (21,25)			25 (21,25)		

FeNO, exhaled nitrix oxide; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; BDR, bronchodilator response (change following salbutamol 400mcg + 10 minutes), in participants where short acting β_2 agonists were withheld for ≥6 hours and long acting β_2 agonists withheld for ≥24 hours. [a]Unadjusted mean ± SD or median(IQR) are presented for baseline, 3 month and 6 month measures. [b]Adjusted change means and 95% confidence intervals [CI] are the differences in change from baseline in intervention group compared to control group by linear mixed model adjusted for random effects and hospital admissions 12 months prior to recruitment. [c]$p<0.05$ difference between groups at time point specified.

5.5.4 Systemic inflammation

Biomarkers of systemic inflammation were measured at baseline and 6 months (**Table 10)**. In the control group there was a significant increase in plasma hsCRP (**Figure 5-9a),** however there were no significant within or between group changes in plasma hsIL-6 **(Figure 5-9b)** or hsTNF-α (**Figure 5-9c).**

Table 5-10: Markers of systemic inflammation

Variable	Baseline[a]	6months[a]	Adjusted change[b] Coeff. [95% CI]	P value
hsCRP (mg/mL)				
Intervention	0.39(0.25,0.97)	0.48(0.28,1.43)	-0.58 [-1.29,0.14]	0.114
Control	0.71(0.34,1.23)	**1.19(0.55,2.63)[c]**		
hsIL-6 (pg/mL)				
Intervention	0.65(0.53,0.91)	0.73(0.51,1.08)	-0.32 [-1.56,0.93)	0.619
Control	0.87(0.52,1.39)	0.82(0.52,1.66)		
hsTNF-α (pg/mL)				
Intervention	1.81 ± 0.57	1.78 ± 0.68	-0.09 [-0.41,0.23)	0.584
Control	1.76 ± 0.52	1.81 ± 0.81		

Intervention (n=22), Control (n=25). [a]Unadjusted mean (SD) are presented for baseline and 6 month measures. [b]Adjusted change means and 95% confidence intervals [CI] are the differences in change from baseline in intervention group compared to control group by linear mixed model adjusted for random effects and hospital admissions 12months prior to recruitment. [c]$p<0.05$ difference within group compared to baseline.

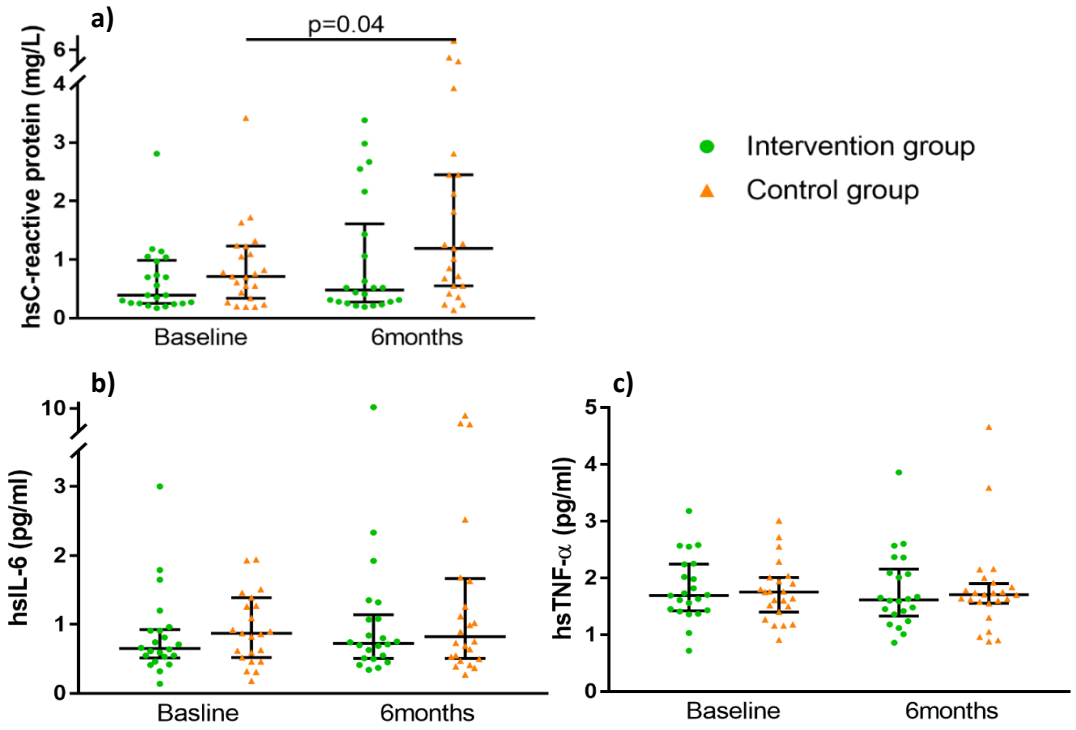

Figure 5-9: Comparison of markers of systemic inflammation between groups at baseline, 3months and 6months; **a)** hsCRP, **b)** hsIL-6 and **c)** hsTNF-α.

5.5.5 Histone deacetylase (HDAC) and Histone acetyltransferase (HAT) enzyme activity

HDAC and HAT enzyme activity was measured in nuclear protein extracted from isolated PBMCs at baseline and 6 months (**Table 5-11)**. HDAC activity significantly increased in the control group, with the difference in HDAC change between groups approaching significance (p=0.061) (**Table 5-11, Figure 5-10b)**. There were no significant within or between group changes in HAT activity, or HAT:HDAC ratio.

Table 5-11: HDAC and HAT enzyme activity

Variable	Baseline[a]	6months[a]	Adjusted change[b] Coeff. [95% CI]	P value
HDAC (µM/µg)				
Intervention	3.12(2.7,3.99)	2.87(2.34,4.45)	-0.66 [-1.34,0.03)	0.061
Control	2.92(2.44,3.4)	**4.09(2.4,4.52)[c]**		
HAT (µM/µg)				
Intervention	5.29(2.72,10.31)	8.27(3.17,15.17)	-1.94 [-10.49,6.61)	0.657
Control	6.80(2.11,9.49)	7.78(3.93,14.81)		
HAT:HDAC ratio (µM/µg)				
Intervention	1.75(0.98,3.18)	1.98(1.06,4.68)	0.15 [-3.82,4.12]	0.940
Control	1.60(0.89,2.80)	1.84 (1.05,4.68)		

Intervention (n=22), Control (n=25). [a]Unadjusted median(IQR) were presented for baseline and 6month measures. [b]Adjusted change means and 95% confidence intervals [CI] are the differences in change from baseline in intervention group compared to control group by linear mixed model adjusted for random effects and hospital admissions 12months prior to recruitment. [c]p<0.05 difference within group compared to baseline.

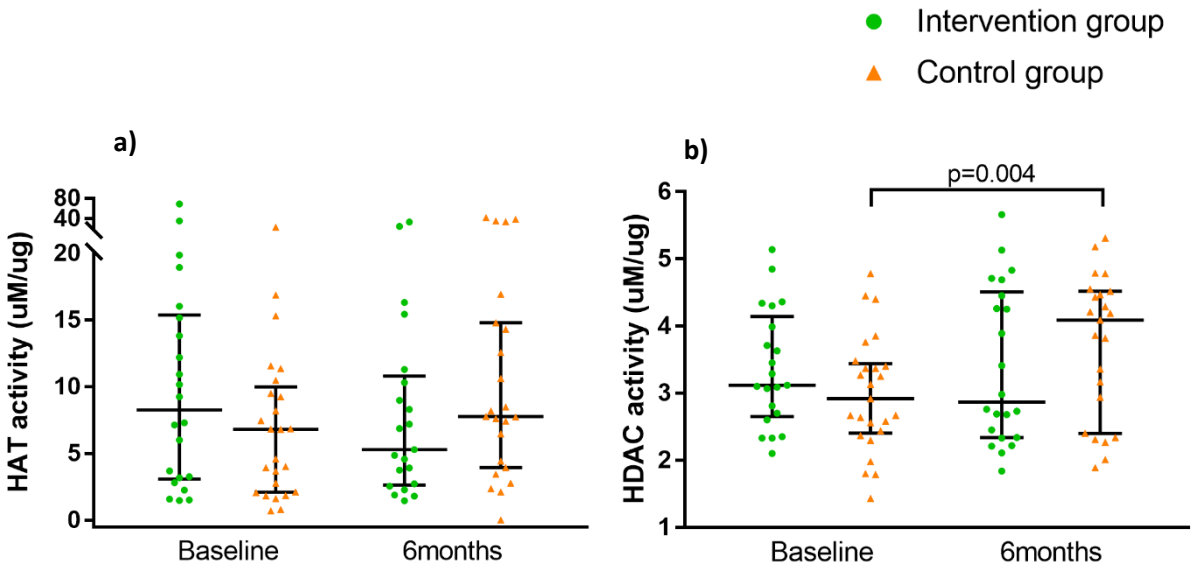

Figure 5-10: Comparison of **a)** HAT activity and **b)** HDAC activity in PBMCs between groups at baseline, 3months and 6months.

5.5.6 Gene expression of GPR41/43 and HDAC subtypes in PBMC cells

Gene expression of G-protein coupled receptors (GPR) and HDAC subtypes were measured in isolated PBMCs at baseline and 6months (**Table 5-12)**. Gene expression of GPR41 (FFAR3) significantly decreased in the control group **(Figure 5-11b)**. There were no significant within or between group changes in GPR43 (FFAR2) or any of the HDAC subtypes.

Table 5-12: Gene expression of GPR41/43 and HDAC subtypes in PBMCs

Variable	Baseline[a]	6months[a]	Adjusted change[b] Coeff. [95% CI]	P value
GPR43(FFAR2)				
Intervention	0.91(0.47,2.12)	1.00(0.61,1.33)	0.51 [-1.29,2.30]	0.580
Control	1.73(0.98,2.94)	1.30(0.70,2.06)		
GPR41(FFAR3)				
Intervention	1.70(1.22,2.95)	1.61(0.89,2.37)	2.51 [-0.48,5.49]	1.00
Control	2.06(1.29,5.31)	**1.61(1.19,2.66)**[c]		
HDAC1				
Intervention	13.44 ± 6.22	12.54 ± 6.89	0.73 [-3.58,5.04]	0.740
Control	13.69 ± 6.07)	12.05 ± 3.69		
HDAC2				
Intervention	5.53 ± 2.85	5.03 ± 3.88	-0.41 [-2.87,2.06]	0.748
Control	5.59 ± 3.01	5.51 ± 2.88		
HDAC3				
Intervention	4.43(3.26,5.25)	3.16(2.89,4.22)	0.00 [-1.58,1.58]	1.00
Control	3.64(3.04, 4.48)	3.57(2.83,3.94)		
HDAC4				
Intervention	3.20(2.08,4.31)	3.24(2.73,4.40)	2.12 [-0.26,4.51]	0.081
Control	3.14(2.64,4.78)	2.94(2.16,4.05)		
HDAC5				
Intervention	6.48 ± 3.29	6.3 ± 5.54	0.13 [-2.59,2.84]	0.928
Control	6.22 ± 2.37	5.94 ± 3.24		
HDAC6				
Intervention	20.52(12.33,23.87)	14.34(12.79,19.78)	-0.55 [-6.67,5.58]	0.861
Control	16.59(12.50,21.55)	16.21(11.32,21.55)		
HDAC7				
Intervention	21.27 ± 8.55	22.71 ± 14.25	6.39 [-1.69,14.46]	0.121
Control	24.72 ± 12.45	19.97 ± 7.91		
HDAC8				
Intervention	2.33(1.94,3.12)	2.42(1.92,2.97)	0.55 [-0.39,1.48]	0.253
Control	2.26(1.81,2.79)	2.20(1.76,2.61)		
HDAC9				
Intervention	6.32(4.06,8.13)	5.72(3.81,7.22)	0.03 [-1.68,1.75]	0.969
Control	4.90(3.98,6.44)	4.42(3.80,5.92)		
HDAC10				
Intervention	19.98 ± 7.36	16.63 ± 10.70	-0.92 [-6.89,5.05]	0.763
Control	19.72 ± 8.68	17.32 ± 7.02		
HDAC11				
Intervention	1.57 ± 0.89	1.79 ± 0.87	0.24 [-0.37,0.85]	0.447
Control	1.35 ± 0.64	1.34 ± 0.43		

Intervention (n=22), Control (n=25). [a]Unadjusted mean ± SD or median (IQR) were presented for baseline and 6month measures. [b]Adjusted change means and 95% confidence intervals [CI] are the differences in change from baseline in intervention group compared to control group by linear mixed model adjusted for random effects and hospital admissions 12months prior to recruitment. [c]$p<0.05$ difference within group compared to baseline.

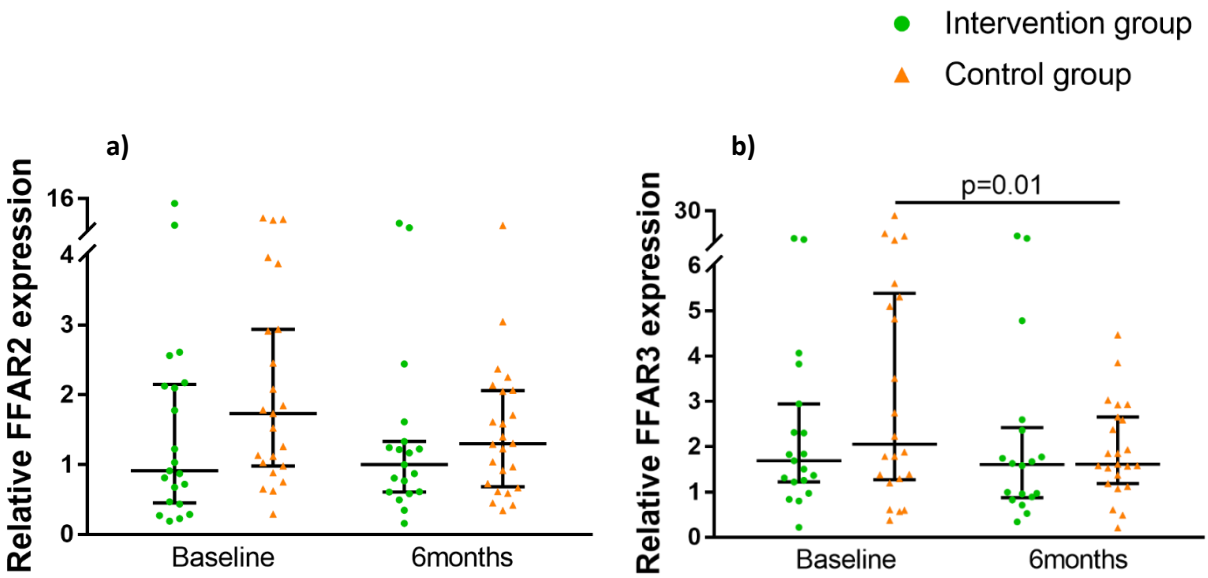

Figure 5-11: Comparison of **a)** relative GPR43 (FFAR2) PBMC gene expression and **b)** relative GPR41 (FFAR3) PBMC gene expression between groups at baseline, 3 months and 6 months.

5.5.7 Associations

Change in total fruit and vegetable intake was positively correlated with change in airway reactance, X_{15} z-score (R_s=0.36, p=0.040) (**Figure 5-12a)**, X_{20} z-score (R_s=0.37, p=0.036) (**Figure 5-12b)**, and inversely correlated with area under the reactance curve, A_X (kPaL-^{1}) (R_s=-0.35, p=0.046) (**Figure 5-12c)**.

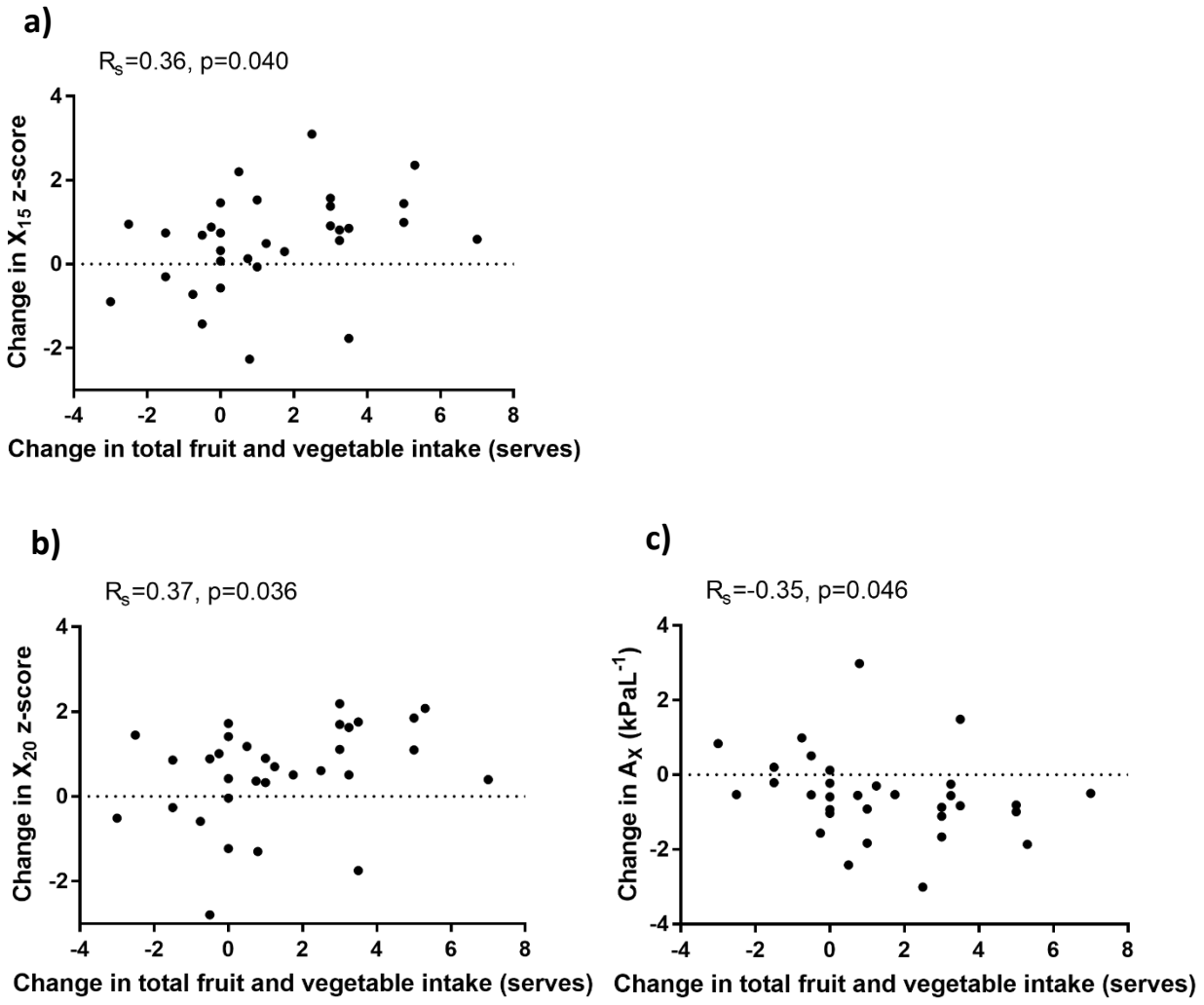

Figure 5-12: Associations between the change in total fruit and vegetable intake (serves) and change in airway reactance; **a)** X_{15} z-score, **b)** X_{20} z-score and **c)** A_X (kPaL-^{1}). Analysed using Spearman's rank correlation.

Furthermore, change in total fruit and vegetable intake was inversely correlated with change in hsCRP (mg/L) (R_s=-0.32, p=0.037) **(Figure 5-13a)** and change in HDAC activity (µM/µg) (R_s=-0.36, p=0.018) (**Figure 5-13b)**, and change in HDAC activity (µM/µg) was positively correlated with change in hsCRP (mg/L) (R_s=0.37, p=0.016) (**Figure 5-14)**. In addition, change in plasma lycopene was inversely associated with change in hsCRP (mg/L) (R_s=-0.41, p=0.007).

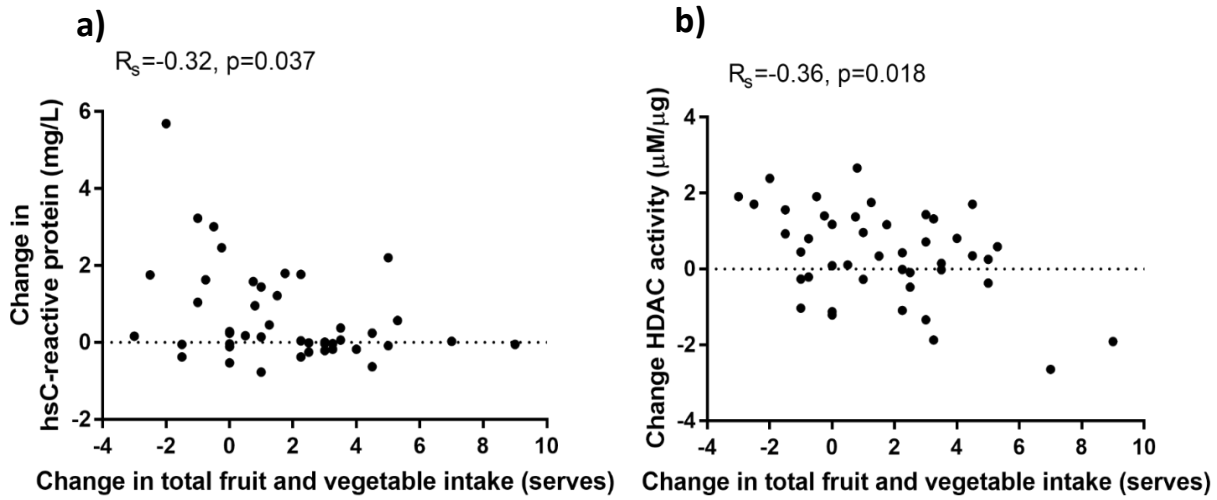

Figure 5-13: Associations between the change in total fruit and vegetable intake (serves) and change in **a)** hsC-reactive protein (mg/L) and **b)** HDAC activity (µM/µg). Analysed using Spearman's rank correlation.

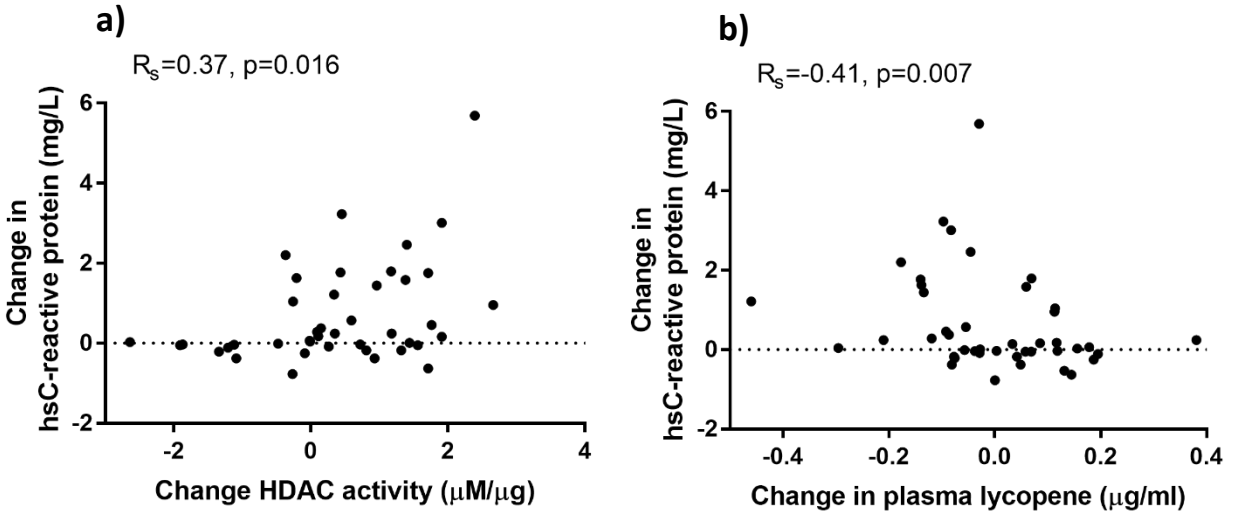

Figure 5-14: Associations between **a)** change in HDAC activity (µM/µg) and **b)** change in plasma lycopene (µg/mL) and change in hsC-reactive protein (mg/L). Analysed using Spearman's rank correlation.

5.6 Discussion

This RCT aimed to investigate the efficacy of a high fruit and vegetable diet in reducing the risk of asthma exacerbations requiring medical intervention, and improving clinical outcomes in children with asthma. In addition, the effect of a high fruit and vegetable diet on airway and systemic inflammation, as well as potential anti-inflammatory mechanisms were examined. While no significant difference in time to exacerbation was observed between groups, the results demonstrate that increasing fruit and vegetable intake in children with asthma can improve lung function, with improvements in airway reactance and resistance observed after 6months. Furthermore, fruit and vegetable consumption was shown to be inversely associated with systemic inflammation (CRP), with a significant increase in hsCRP observed in the control group.

We have previously demonstrated that fruit and vegetable intake can affect clinical outcomes in adults with asthma. In our previous studies, we have shown that consumption of a low fruit and vegetable diet for 10-14 days, worsens lung function (72, 268). Furthermore, in our previous RCT conducted in adults with asthma (72), time to exacerbation was significantly greater in those allocated to a high versus low fruit and vegetable diet (≥7 vs. <3 serves of fruit and vegetables per day) for 14 weeks. In this study in children with asthma, while we didn't see an effect of a high fruit and vegetable diet on time to exacerbation, our data demonstrates that increases in fruit and vegetable intake can positively influence lung function.

In the intervention group (high fruit and vegetable diet), we observed significant improvements in peripheral airway reactance (X_5 and A_X). Furthermore, change in airway reactance (measured at 5, 15 and 20Hz) from baseline to 6 months significantly

differed between groups, with change in fruit and vegetable intake found to be positively associated with change in peripheral airway reactance. Airway reactance relates to the amount of recoil that is generated in response to a pressure wave (i.e. the elasticity of the airways). Reactance measured at 5Hz (X_5) reflects the elasticity of the peripheral airways, whereas Ax is the total reactance at all frequencies between 5Hz and Fres (resonance frequency where reactance equals zero) and reflects peripheral airway obstruction. In the intervention group we also observed a significant decrease in the difference between airway resistance at 5Hz and 20Hz (R_5-R_{20}). Airway resistance is the amount of energy required to move a pressure wave through the airways thus providing information regarding airway obstruction (271). Like A_X, R_5-R_{20} is proposed to reflect peripheral airway obstruction (271, 272). Therefore, these findings suggest that the high fruit and vegetable diet in the intervention group increased peripheral airway elasticity and decreased peripheral airway obstruction. However, whether these changes can be considered clinically significant is unknown, as to our knowledge clinically significant changes in these lung function parameters are yet to be established. Further research is needed in this area.

Decreased airway reactance and increased airway resistance are proposed to be a result of peripheral airway narrowing, which can arise from airway inflammation, airway remodelling and/or local and regional airway-tissue interactions (282). Therefore, it is possible the positive effect of fruit and vegetable consumption on airway reactance and resistance observed in this study may have been due to the anti-inflammatory properties of fruits and vegetables. We have previously demonstrated that both carotenoids and soluble fibre, found in fruit and vegetables, reduce airway inflammation in adults with asthma (140, 268). In the current study, due to the difficulty in obtaining sputum samples from children, our assessment of airway

inflammation was restricted to FeNO, which did not change in either group. Nonetheless, the effect of fruit and vegetables on airway inflammation in asthmatic children is an interesting area for further investigation.

Studies have shown that lung function measured by IOS is correlated with asthma control and clinical outcomes such as health status and dyspnea (278), with peripheral airway function (indicated by R_5-R_{20}, X_5 and A_x) in particular proposed to be associated with asthma control (278-281). In school aged children with asthma, increased peripheral resistance (R_5-R_{20}) and area under the reactance curve (A_X) have been associated with poor disease control (280). Furthermore, these IOS indices have been demonstrated to effectively predict loss of asthma control over the next 2-3months (281). In adults increased R_5-R_{20}, R_{20} and decreased X_5 have also been significantly correlated with poor asthma control (278), with airway reactance alone suggested to be an independent determinant (282).

Linking these observations with the findings from our study, it is possible that in addition to improving lung function, increasing fruit and vegetable intake may have improved asthma control in the children via improved airway reactance. Although we did not observe any significant difference in change in asthma control between groups, measured using a parent-reported asthma control questionnaire, it is possible that this tool did not effectively capture actual changes as there are often discrepancies in perceived symptoms between the child and their parent, with parents found to underestimate asthma severity and overestimate asthma control (283, 284). Alternatively, it is possible that we didn't see a significant change in asthma control as the majority of participants (86% of the intervention group and 84% of the control group) already had well-controlled asthma at baseline, as indicated by a self-completed

asthma control questionnaire score of >19. Nonetheless, further research is needed to explore the effect of fruit and vegetable intake on asthma control in children.

Changes in markers of systemic inflammation were also measured, with a significant increase in hsCRP observed in the low, but not the high, fruit and vegetable group. Furthermore, fruit and vegetable consumption was shown to be inversely associated hsCRP. These observations suggest that in the absence of the protective effect of a high fruit and vegetable diet, over a 6 month period, systemic inflammation will increase in asthmatic children. These findings are consistent with numerous studies conducted in both adults (285, 286) and children (287-290). The high antioxidant and soluble fibre content of fruits and vegetables may in part explain the association between fruit and vegetable intake and hsCRP levels. Antioxidants reduce inflammation via scavenging free radicals, thus suppressing NF-κB mediated inflammation (34). Soluble fibres resist digestion in the small intestine and are fermented by beneficial gut bacteria in the colon generating biologically active by-products, the short chain fatty acids (SCFA). SCFAs are suggested to have anti-inflammatory effects via mechanisms including the activation of GPR41/43 in immune cells (10, 11), and the inhibition of histone deacetylase (HDAC) enzyme activity (12, 13).

In the control (low fruit and vegetable) group, PBMC gene expression of GPR41(FFAR3) significantly decreased. Studies conducted in both humans (291) and animal models (112) have shown that SCFAs have anti-inflammatory effects both systemically (291) (112) and in the airways (9, 112) via activation of GPR43/41. Therefore, it is possible that the lack of soluble fibre in the control diet, has reduced GPR41(FFAR3) activity, which has likely contributed to the increase in hsCRP observed in this group. However, it should be noted that total fruit and vegetable intake

and presumably soluble fibre intake, did not significantly change in the control group. Further research is needed to explore alternate explanations for this finding.

In addition, after 6 months, the low fruit and vegetable intake of the control group, led to an increase in HDAC activity. SCFAs have been shown to reduce inflammation by inhibiting HDAC activity, which in turn suppresses NF-κB mediated production of pro-inflammatory cytokines such as IL-6 (126, 127) which stimulates the production of CRP (292). As such an increase in HDAC activity could be expected to result in increased inflammation. This hypothesis is supported by the findings of our study, with change in HDAC activity found to be positively associated with change in hsCRP. There is evidence that increased systemic CRP levels are associated with respiratory symptoms (293), airway inflammation and airflow obstruction (294), asthma control (295) and bronchial hyperresponsiveness (296). Therefore, these findings provide further evidence of the anti-inflammatory benefits of increasing fruit and vegetable intake in children, with HDAC inhibition a potential anti-inflammatory mechanism.

It is also possible that other nutrients found in fruit and vegetables, such as antioxidant and anti-inflammatory phytochemicals including; carotenoids, flavonoids (e.g. anthocyanins), organosulur compounds, indoles and isothiocynates, vitamins including vitamin E and C, and folate (66, 289, 297, 298), may have contributed to the improvements in lung function observed, and the inverse association between fruit and vegetable intake and hsCRP levels. Indeed, change in plasma lycopene was found to be inversely associated with change in hsCRP. Lycopene has been shown to modify inflammation by supressing NF-κB activity (299), which inhibits the production of pro-inflammatory cytokines such as IL-6 (126, 127) and subsequently CRP synthesis (292). In future studies, measurement of markers of oxidative damaged such as 8-isoprostane, lipid peroxides and protein carbonyls are recommended to assist in

differentiating between the potential antioxidant and anti-inflammatory effects of fruit and vegetable intake, and to investigate whether antioxidant action is important to the improvements we observed. Nonetheless, this highlights the advantage of using a whole food intervention to modulate disease processes, as it is likely that multiple nutrients found in fruits and vegetables are having beneficial, synergistic effects.

The strengths and limitations of this study should be considered. This study was strengthened by the provision of fortnightly dietetic counselling, and the home delivery of fruit and vegetables, which were selected according to patient preferences, which ensured compliance with the intervention. Another strength was the measurement of plasma carotenoids, a biomarker of fruit and vegetable consumption, to objectively confirm compliance. Not only did we observe significant increases in self-reported fruit and vegetable intake in the intervention group, with no change in the control group, but total fruit and vegetable intake was found to be correlated with total plasma carotenoid levels.

Some limitations were introduced due to the difficulty in performing clinical tests and collecting data from a paediatric population. For example, reproducible spirometry was performed in 20% of participants, resulting in an underpowered analysis of spirometric variables. Collection of data on asthma control is also difficult, due to discrepancies in perceived symptoms between the child and their parent, with parents known to underestimate asthma severity and overestimate asthma control (283, 284). Assessment of airway inflammation is also challenging in children. While the single-breath method is considered the gold standard for FeNO measurement in children >4-5 years old (300), several studies have reported that young children often have difficulty performing the method accurately (301). Furthermore, as our study included children 3 years of age, FeNO was measured during spontaneous/tidal breathing which

is recommended for children <4years (301). FeNO measurement during spontaneous breathing however is suggested to be somewhat biased, as the lung volume at which flow rate is measured is not controlled (302). Furthermore, the time within the target flow may not be adequate to allow FeNO to reach equilibrium, which can significantly affect FeNO values (302). In addition to measuring FeNO, induced sputum has emerged as a useful and important method to examine airway inflammation (303). However, as this technique can only be performed in children who can perform adequate spirometry, and due to time constraints during our clinic visits, this was not undertaken in our study. Therefore, this limited our ability to examine changes in airway inflammation between the high and low fruit and vegetable diets.

5.6.1 Conclusion

This study provides promising evidence regarding the beneficial effects of increasing fruit and vegetable intake in children with asthma. Improvements in lung function, as indicated by a decrease in airway resistance and increase in airway reactance, were observed in the high fruit and vegetable group. In addition, fruit and vegetable intake was found to be positively associated with airway reactance, which is demonstrated in the literature to be associated with asthma control. Given that the main goal of asthma treatment is to achieve clinical control (14), increasing fruit and vegetable consumption may prove to be an effective and widely accepted non-pharmacological strategy for assisting in the management of asthma in children. Furthermore, given that the intervention targeted the recommended number of fruit and vegetable serves in the dietary guidelines, and considering the beneficial evidence provided by this study, it would seem prudent to advocate for children with asthma to aim to meet these recommendations. Additional research is needed to confirm the effects of a higher fruit and vegetable diet on clinical findings, and to provide further evidence for the

anti-inflammatory mechanisms of fruit and vegetables, in particular the effect on HDAC activity.

Chapter 6: General discussion and future directions

6.1 Introduction

While genetics play an important role in asthma development and progression, it is apparent that there is an association between asthma prevalence and the westernized lifestyle, including the western diet (5, 6). Westernisation of diets has led to reduced dietary fibre intake (7). This is undesirable as dietary fibre is not only inversely associated with systemic inflammation (80-85), but it is also inversely associated with the severity of airway inflammation and positively associated with lung function in asthma (8). The anti-inflammatory benefits of dietary fibre intake have been ascribed to the intake of fermentable soluble fibres (9). Soluble fibres resist digestion in the small intestine and are fermented by beneficial gut bacteria in the colon generating biologically active by-products, the short chain fatty acids (SCFA); which are proposed to have systemic anti-inflammatory effects (9). Soluble fibres that selectively stimulate the growth and/or activity of beneficial gut bacteria, are referred to as prebiotics (86). Prebiotics have the advantage of both providing substrate for the production of SCFA and enhancing the growth of SCFA-producing bacteria, which further augments SCFA levels. Supplementation with both prebiotics and probiotics (beneficial live bacteria), referred to as synbiotics, are also proposed to be beneficial due to their ability to increase SCFA producing bacterial numbers by improving their survival and implantation in the gut (107), as well as providing substrates for fermentation (108). However, further research is required to examine their effectiveness compare to prebiotic supplementation alone.

This thesis aimed to examine dietary fibre as a modulator of inflammation in asthma. At the time the research in Chapter 2 was conducted, there were no published studies that systematically reviewed the evidence for the effect of SCFAs, prebiotics and synbiotics (prebiotic and probiotic combinations) on inflammation in humans.

Furthermore, there were no studies examining the effects of SCFA, prebiotics and synbiotics in asthma. As such, we conducted the first RCT examining the effect of prebiotic soluble fibre supplementation, with and without a probiotic, in adults with asthma (Chapter 4). Subsequently, we also examined the effect of increasing soluble fibre intake (via increasing fruit and vegetable consumption) in children with asthma (Chapter 5). Potential anti-inflammatory mechanisms of soluble fibre, including the inhibition of HDAC activity and the activation of free fatty acid receptors (GPR41/43), were examined in both clinical trials (Chapter 4 and 5).

6.2 The effect of SCFAs, prebiotics and synbiotics on systemic inflammation

Chapter 2 examined the available evidence for the effect of SCFAs, prebiotics and synbiotics on systemic inflammation in both healthy populations, and those with various inflammatory conditions. The review focussed on systemic inflammation, due to the paucity of human studies examining the effects of these interventions in the airways. This systematic literature review and meta-analysis identified a gap in the literature regarding the effect of SCFA supplementation on systemic inflammation in humans, with only five studies identified. Furthermore, it was revealed that the evidence in the literature regarding the effect of prebiotics and synbiotics on systemic inflammation is conflicting, with approximately 50% of the individual studies reporting a significant decrease in one or more inflammatory biomarker. This was due to heterogeneity in study design, supplement formulation, dosage, duration, and subject population. Nonetheless, results of the meta-analyses of combined studies provide evidence to support the systematic anti-inflammatory of prebiotic and synbiotic supplementation; prebiotics (in particular oligosaccharides) were found to

significantly reduce CRP, whereas CRP and TNF-α were significantly lower following synbiotic supplementation compared with control.

As this is the first systematic literature review to provide a summary of evidence on this topic, it fills an important gap in the literature. Further to this, it highlights the need for more studies to confirm the anti-inflammatory effects of prebiotic and synbiotic supplementation, as well as the most optimal supplement formulation and dosage. Evidence in the asthmatic population in particular was found to be lacking, with only 2 studies conducted in asthma identified at the time this review was conducted. One study demonstrated that while synbiotic supplementation had no effect on bronchial inflammation, it did attenuate increases in systemic IL-5 levels following an allergen challenge (173). Similarly, in the other study, prebiotic supplementation was found to significantly decrease systemic inflammation (TNF-α and CRP), with no direct effect on airway inflammation observed (177). This demonstrates that further research examining the beneficial effect of pre/synbiotic supplementation in asthma is warranted.

6.3 The effect of soluble fibre supplementation, with and without probiotics, in adults with asthma

Using an acute meal challenge model, we have previously demonstrated that a single dose of soluble fibre (inulin) was effective in reducing airway inflammation and improving lung function in adults with asthma (140). In Chapter 4 we extended these findings, and for the first time examined the effect of soluble fibre supplementation, with and without probiotics, in adults with asthma. In keeping with our previous study, following 7 days of soluble fibre supplementation (inulin), we observed a reduction in eosinophilic airway inflammation and a trend for improvement in lung function in adults with stable asthma. Interestingly, no significant changes in clinical outcomes or

airway inflammation were observed following inulin+probiotic supplementation. While we cannot be certain of the reason for lack of effect of the synbiotic, we hypothesise that this may be due to the differential changes in bacterial profile that were induced by the pre- versus synbiotic. Alternatively, given that prebiotics act as a food source for probiotics (107), it is possible that the amount of inulin available for SCFA production was reduced by the simultaneous delivery of the probiotic. This hypothesis requires further investigation.

Asthma control also significantly improved with inulin supplementation, with the greatest improvements observed in participants who had poorly controlled asthma (ACQ6 score >0.75). Interestingly, improvements in asthma control following inulin supplementation were observed in individuals with eosinophilic asthma, but not in those with non-eosinophilic asthma. These findings suggest that the decrease in sputum eosinophils we observed following inulin supplementation have likely contributed to the improvement in asthma control. This highlights the importance of the anti-inflammatory effects of soluble intake in asthma, warranting further investigation.

The anti-inflammatory benefits of soluble fibre intake have been ascribed to the by-products of its fermentation, the SCFAs (acetate, butyrate and propionate). Following both inulin and inulin+probiotic treatments, we observed a non-significant trend towards an increase in total plasma SCFAs compared to control. We speculate that the lack of significance of this result was due to the timing of sampling (following a 12 hour fast), with evidence in the literature suggesting that plasma SCFA levels significantly increase 2 hours after soluble fibre consumption, reaching peak levels at around 6 hours (255). Thus more informative data would have been obtained in future studies if samples are collected at an earlier time point. We also saw a trend towards

an increase in total faecal SCFAs following both inulin and inulin+probiotic supplementation, however this did not reach statistical significance. Nonetheless, change in FEV_1 was positively correlated with change in total and individual faecal SCFAs (butyrate and propionate). This is consistent with our previous study, which reported improvements in lung function following inulin supplementation (140), and provides further evidence regarding the beneficial effects of soluble fibre supplementation in asthma.

Two potential anti-inflammatory mechanisms of SCFAs were examined in Chapter 4; the activation of GPR41/43 and the inhibition of HDAC enzyme activity. In contrast with previous studies, no significant changes in GPR41/43 expression were observed following inulin supplementation. However, our data did show a positive correlation between plasma butyrate and PBMC GPR41/43 gene expression. Hence, further research is needed to explore this as a potential anti-inflammatory mechanism of soluble fibre in asthma.

Following inulin supplementation sputum HDAC9 gene expression significantly decreased. There is evidence that HDAC9 gene expression is elevated in individuals with asthma compared to those without asthma (123). Furthermore, HDAC9 knockout mice have been found to be resistant to the development of allergic airways disease (AAD) (124), with HDAC inhibition demonstrated to increase the production and function of Treg cells, which suppress the development of AAD (258) and play an important role in asthma. Following inulin supplementation, sputum HDAC9 expression was positively correlated with sputum %eosinophils. It is possible that down regulating HDAC9 expression increased Treg cell production and/or function, which in turn suppressed eosinophilic influx into the airways.

Being a non-pharmacological treatment, soluble fibre supplementation has the potential to be widely adopted and accepted as a strategy for assisting in the management of asthma. Nonetheless, larger and longer duration clinical trials are needed to confirm these clinical findings and to further explore HDAC9 as a potential therapeutic target for asthmatics, particularly those with eosinophilic airway inflammation.

6.4 Investigating the effect of soluble fibre intake (via fruit and vegetable intake) in children with asthma

Chapter 5 investigated the effect of soluble fibre intake (via fruit and vegetable intake) in children with asthma. In this RCT, participants were randomized to either a high or low fruit and vegetable diet for 6 months. While we didn't see an effect of a high fruit and vegetable diet on time to exacerbation or airway inflammation, our data demonstrates that increases in fruit and vegetable intake can positively influence lung function, with improvements in airway reactance and resistance observed after 6months. These findings are consistent with the current literature regarding the beneficial effects of fruit and vegetables on lung function (225).

It has been demonstrated in the literature that high airway resistance and low airway reactance is associated with poor asthma control in both children (280) (281) and adults (278) (282), with airway reactance alone suggested to be an independent determinant (282). Linking these observations with the findings of this chapter, it is possible that in addition to improving lung function, increasing fruit and vegetable intake may help to improve asthma control in children via improved airway reactance. Although we did not observe any significant difference in change in asthma control (assessed using a parent completed questionnaire) between groups, further research is

warranted to explore the effect of fruit and vegetable intake on asthma control in children.

Changes in markers of systemic inflammation were also measured, with a significant increase in hsCRP observed in the low, but not the high, fruit and vegetable group. Furthermore, fruit and vegetable consumption was shown to be inversely associated hsCRP. These observations suggest that in the absence of the protective effect of a high fruit and vegetable diet, over a 6 month period, systemic inflammation will increase in asthmatic children. These findings are consistent with numerous studies conducted in both adults (285, 286) and children (287-290). The high antioxidant and soluble fibre content of fruits and vegetables may in part explain the association between fruit and vegetable intake and hsCRP levels.

SCFAs, produced from the fermentation of soluble fibres, are suggested to have anti-inflammatory effects via activation of GPR43/41 (9, 112, 291). We observed a significant decrease in PBMC gene expression of GPR41(FFAR3) in the low fruit and vegetable group. Therefore, it appears that the lack of soluble fibre in the control diet has reduced GPR41(FFAR3) activity, which has likely contributed to the increase in hsCRP observed in this group. Alternatively, this increase in hsCRP may be related to the significant increase in HDAC activity also observed in this group. SCFAs have been shown to reduce inflammation by inhibiting HDAC activity. As such an increase in HDAC activity could be expected to result in increased inflammation. This hypothesis is supported by the findings of our study, with change in HDAC activity found to be positively associated with change in hsCRP. These findings provide further evidence of the anti-inflammatory benefits of increasing fruit and vegetable intake in children, with further investigation into these mechanisms warranted.

The high antioxidant content of fruits and vegetables may have also contributed to the beneficial effects of fruit and vegetable intake on both lung function (72) and systemic inflammation (34, 66, 289, 297, 298). Indeed, change in plasma lycopene was found to be inversely associated with change in hsCRP. This highlights the advantage of using a whole food intervention to modulate disease processes, with multiple nutrients likely to have synergistic effects.

6.5 Strengths and limitations

Clinical trials which involve dietary and behavioural changes can be challenging, particularly in the paediatric population, with adherence paramount to the reliability and accuracy of the results. However, in Chapter 4 and 5 high participant adherence was achieved, which was attributed to the study designs used. In Chapter 4, participants received dietetic counselling at the beginning of the intervention to ensure adherence with the low fibre controlled background diet, and were provided with a study dietary to record supplement compliance. 88% of participants adhered to the background diet, with all participants compliant with the supplement component of the intervention. In Chapter 5, fortnightly dietetic counselling was provided, as well as fortnightly home delivery of fruit and vegetables, which were selected according to patient preferences, which ensured compliance with the intervention. Not only did we observe significant increases in self-reported fruit and vegetable intake in the intervention group, with no change in the control group, but total fruit and vegetable intake was found to be correlated with total plasma carotenoid levels, an objective biomarker of fruit and vegetable intake.

In Chapter 5, several limitations were introduced due to the difficulty in performing clinical tests and collecting data from a paediatric population. For example, collection

of data on asthma control is difficult, due to discrepancies in perceived symptoms between the child and their parent, with parents known to underestimate asthma severity and overestimate asthma control (283, 284). Assessment of airway inflammation is also challenging in children. As our study included children 3 years of age, FeNO was measured during spontaneous/tidal breathing which is recommended for children <4years (301). FeNO measurement during spontaneous breathing however is suggested to be somewhat biased, as the lung volume at which flow rate is measured is not controlled (302), and the time within the target flow may not be adequate to allow FeNO to reach equilibrium (302). Induced sputum has emerged as another useful and important method to examine airway inflammation (303). However, as this technique can only be performed in children over the age of 6 years, and due to time constraints during our clinic visits, this was not undertaken in our study. Therefore, this limited our ability to examine changes in airway inflammation between the high and low fruit and vegetable diets.

6.6 Clinical and scientific implications

Chapters 4 and 5 have demonstrated the benefits of increasing soluble fibre intake in both adults and children with asthma. In Chapter 4 we have demonstrated that soluble fibre supplementation (inulin) for 7 days improves asthma control and airway inflammation in adults with asthma. Of particular clinical interest, the greatest improvements in asthma control were observed in those who had poorly controlled asthma prior to supplementation, despite being on inhaled corticosteroids, with clinically important improvements observed in >50% of these individuals. In addition, significant and clinically important improvements in asthma control following inulin supplementation were observed in individuals with eosinophilic asthma, but not in those with non-eosinophilic asthma. These findings suggest that those with poorly

controlled asthma and eosinophilic airway inflammation are most likely to benefit from inulin supplementation. Being a non-pharmacological treatment, soluble fibre supplementation has the potential to be widely adopted and accepted as a strategy for assisting in the management of asthma.

This thesis has also demonstrated that increasing soluble fibre intake, via increasing fruit and vegetable consumption, has beneficial effects in children with asthma. In Chapter 5 a high fruit and vegetable diet was demonstrated to improve lung function in children with asthma. Furthermore, fruit and vegetable consumption was shown to be inversely associated CRP, with CRP found to significantly increase in children consuming a diet low in fruit and vegetables. These findings suggest that in the absence of the protective effect of a high fruit and vegetable diet, over a 6 month period, systemic inflammation will increase in asthmatic children.

There is evidence that increased systemic CRP levels are associated with respiratory symptoms (293), airway inflammation and airflow obstruction (294), asthma control (295) and bronchial hyperresponsiveness (296). Therefore, given that the intervention targeted the recommended number of fruit and vegetable serves in the dietary guidelines, and considering the protective effects that were induced by that level of intake, it would seem prudent to advocate for children with asthma to aim to meet these dietary recommendations.

This thesis contributes to the evidence base regarding the anti-inflammatory mechanisms of soluble fibre in asthma. In the literature it is currently proposed that SCFAs, produced from the fermentation of soluble fibres, have anti-inflammatory effects via activation of GPR43/41 (9, 112, 291). Indeed, we have previously observed an increase in GPR41 and GPR43 expression in airway immune cells in adults with

asthma following a single dose of soluble fibre, which corresponded with a decrease in airway inflammation (140). In Chapter 4, while no significant changes in GPR41 or GPR43 were observed following inulin supplementation, we did find a positive correlation between plasma butyrate levels and GPR41/43 gene expression in PBMCs. Furthermore in Chapter 5 our results suggest that GPR41 activity is reduced with a diet low in fruit and vegetables, and subsequently soluble fibre. These findings indicate that further research into GPR41/43 activation as a potential anti-inflammatory mechanism of soluble fibre in asthma is warranted.

Findings from this thesis also support the evidence in the literature that soluble fibre reduces inflammation via HDAC inhibition (124) (126, 127). In Chapter 5, low fruit and vegetable intake resulted in an increase in HDAC activity, which was associated with CRP. Furthermore, in Chapter 4 soluble fibre inulin supplementation was found to downregulate sputum HDAC9 gene expression, which was positively associated %sputum eosinophils. This is an important observation which suggests that HDAC9 may be potential therapeutic target for asthmatics, particularly those with eosinophilic airway inflammation.

In addition to being high in soluble fibre, fruit and vegetables are also a rich source of antioxidants which may also have contributed to the association between fruit and vegetable intake and CRP observed in Chapter 5. This highlights the advantage of using a whole food intervention to modulate disease processes, as it is likely that multiple nutrients found in fruits and vegetables are having beneficial, synergistic effects.

6.7 Summary

The research conducted for this thesis has contributed to improving our understanding of the anti-inflammatory effects of SCFAs, prebiotics and synbiotics in humans. As demonstrated by our systematic review and meta-analysis, evidence in the literature regarding the effect of prebiotics and synbiotics on systemic inflammation is conflicting, with approximately 50% of the individual studies reporting a significant decrease in one or more inflammatory biomarker. Furthermore, although results of the meta-analyses of combined studies provide evidence to support the systematic anti-inflammatory of prebiotic and synbiotic supplementation, it is evident that there is a need for more studies to confirm the anti-inflammatory effects of prebiotic and synbiotic supplementation, as well as the most optimal supplement formulation and dosage. This review also highlighted the need for further research in the asthmatic population, with only 2 studies conducted in asthma identified at the time our review was undertaken.

To address this gap in the literature, this thesis has shown that two different methods of increasing soluble fibre intake (i.e. via supplementation or increased fruit and vegetable intake) can markedly improve outcomes in asthma. Soluble fibre supplementation in adults with asthma was found to improve asthma control and airway inflammation, while increasing soluble fibre intake by increasing fruit and vegetable consumption was demonstrated to improve lung function and attenuate increases in systemic inflammation in children with asthma. This highlights the potential of non-pharmacological treatments to assist in the management of asthma. Furthermore, this thesis contributes to the evidence base regarding two potential anti-inflammatory mechanisms of soluble fibre in asthma; activation of GPR41/43 and inhibition of HDAC activity.

6.8 Future directions

This thesis has provided evidence regarding the beneficial effects of increasing soluble fibre intake (either via supplementation or increasing fruit and vegetable intake) in adults and children with asthma, and the potential mechanisms involved. However, this thesis has also provided numerous directions for future research.

While Chapter 4 demonstrated the ability of soluble fibre supplementation (inulin) for 7 days to improve asthma control and airway inflammation in adults with asthma, further trials with a larger sample size are needed to confirm these results, and to investigate longer term supplementation. Dose response studies are also warranted to determine the most optimal supplement dosage, as well as formulation. Furthermore, given the beneficial effects of soluble fibre supplementation observed in adults with asthma, it would be interesting to examine the efficacy and acceptability of soluble fibre supplementation in children with asthma, particularly in those who are resistant to increasing their fruit and vegetable intake.

In addition to providing substrates for fermentation, some soluble fibres including inulin act as prebiotics, selectively stimulating the growth and/or activity of beneficial gut bacteria (86). However, changes in gut microbiota were not investigated in this thesis. Microbial dysbiosis in the gut is increasingly being associated with the incidence and severity of asthma (304), with low gut microbial diversity shown to be an important risk factor for the development of asthma in children (305). Further research is warranted to explore the impact of soluble fibre interventions on microbial diversity in the gut, and how this relates to clinical outcomes in asthma.

In chapter 4, we hypothesised that supplementation with combined prebiotics and probiotics (i.e. synbiotics) would have a greater effect on inflammation than prebiotics

alone, due to their superior ability to increase SCFA-producing bacterial numbers (107), as well as providing substrates for fermentation (108). However, we did not observe any significant changes in clinical outcomes or airway inflammation with inulin+probiotic supplementation. Furthermore, as demonstrated by our systematic review detailed in Chapter 2, evidence in the literature regarding the effectiveness of synbiotics in reducing inflammation is conflicting, as is the evidence that they exert greater anti-inflammatory effects than prebiotic supplementation alone (232). We hypothesis that differential changes in bacterial profile may have been induced by inulin+probiotic supplementation compared to inulin supplementation alone. Alternatively, prebiotics act as a food source for probiotics (107) therefore it is possible that the amount of inulin available for SCFA production was reduced by the simultaneous delivery of the probiotic. Further research is needed to investigate whether the addition of a probiotic supplement has beneficial or detrimental effects in adults with asthma.

In Chapter 5, despite observing improvements in airway reactance with a high fruit and vegetable diet, which is suggested in the literature to be an independent determinant of asthma control (282), we did not observe any significant change in asthma control, measured using a parent-reported asthma control questionnaire. It is possible that this tool did not effectively capture actual changes as there are often discrepancies in perceived symptoms between the child and their parent, with parents found to underestimate asthma severity and overestimate asthma control (283, 284). Given that there is increasing evidence in the literature that IOS may be a reliable and non-invasive method to objectively assess asthma control in children (280), further studies in children with asthma are warranted to establish this as a method of assessing asthma control.

In regards to the potential anti-inflammatory mechanisms of soluble fibre intake in asthma, the positive correlation between plasma butyrate levels and GPR41/43 gene expression in PBMCs in Chapter 4, and the results from Chapter 5 which demonstrated that GPR41 was reduced with a diet low in fruit and vegetables, highlight the need for further research into GPR41/43 activation as a potential anti-inflammatory mechanism in asthma.

Furthermore, this thesis suggests that the anti-inflammatory effects of soluble fibre in asthma relate to modulation of HDAC activity, with HDAC9 in particular suggested to be involved. In the current literature, HDAC2 has been demonstrated to mediate the anti-inflammatory effects of corticosteroids (306), commonly use in the management of asthma, with reduced HDAC2 activity and expression associated with corticosteroid resistance (307). As such, research has focussed on strategies to increase HDAC2 expression in asthma, such as the use of pharmaceutical drugs including theophylline (306). Given the findings of this thesis, further research is warranted to investigate HDAC9 gene expression as an additional target for pharmacological therapy in asthma, particularly in individuals with eosinophilic airway inflammation.

6.9 Final conclusion

This thesis has demonstrated that increasing soluble fibre intake has beneficial effects in both adults and children with asthma. Soluble fibre supplementation in adults with asthma was found to improve asthma control and airway inflammation, while increasing soluble fibre intake by increasing fruit and vegetable consumption was demonstrated to improve lung function and attenuate increases in systemic inflammation in children with asthma. Furthermore, investigation into the potential anti-inflammatory mechanisms of soluble fibre has provided promising evidence

regarding the role of GPR41/43 activation and HDAC inhibition, with HDAC9 in particular identified as a potential therapeutic target.

It is important to note that however, that in addition to being high in soluble fibre, fruit and vegetables are also a rich source of antioxidants. Given that the antioxidant lycopene was found to be inversely associated with change in CRP, this highlights the advantage of using a whole food intervention to modulate disease processes, as it is likely that multiple nutrients found in fruits and vegetables are having beneficial, synergistic effects. Nonetheless, this thesis provides promising evidence regarding the benefits of soluble fibre in asthma. Dietary recommendations to increase soluble fibre, either via supplementation or increasing fruit and vegetable intake, have the potential to be widely adopted and accepted as a strategy for assisting in the management of asthma. Furthermore, this strategy could potentially reduce the amount of inhaled corticosteroids required to achieve asthma control, subsequently reducing the cost of asthma management and the risk of adverse side effects associated with pharmacological treatment.

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