Impact of hookworms and their secreted proteins on the microbiota and subsequent development of type 2 diabetes in mice

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Statement of contributions

This thesis contains my original research, and contains no material previously published or written by another person except where due reference has been made in the text.

I have clearly stated the contribution of others throughout the thesis, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice and any other original research work I used or reported in my thesis.

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Abstract

In the past few decades there has been a significant increase in the number of people with type 2 diabetes (T2D) worldwide. Socioeconomic developments have led to changes in diet, lifestyle, and environmental factors, which have been associated with complex changes in disease patterns, evident from the increasing prevalence of non-communicable diseases, including T2D. Cumulative evidence suggests that long term activation of pro-inflammatory immune responses contribute to the development of T2D. This phenomenon has piqued the interest of researchers in the potential of targeting inflammation to prevent and/or treat T2D. Despite the strong evidence that T2D is associated with an increased risk of infectious diseases, recent evidence suggests that helminths and their secreted proteins can modulate the host's immune system by activating regulatory pathways which can control inflammatory immune responses associated with T2D. Moreover, the gut microbiota is another substantial player in host metabolism, physiology, nutrition, and immune function, and impacts on T2D. On the other hand, helminths have been shown to alter the composition of the gut microbiota by promoting species that correlate with good gut health. These helminth-driven changes to immunity and microbiota have a major impact on inflammatory metabolic disorders such as diabetes.

This thesis aimed to examine the potential of *Nippostrongylus brasiliensis* infections and their excretory/secretory (ES) proteins as therapeutics in mouse models of T2D, via providing an anti-inflammatory milieu by normalising gut microbiota composition and restoring/maintaining metabolic homeostasis. Firstly, I showed that *N. brasiliensis* infection is associated with changes in local and systemic immune cell populations, and induction of Th2 immune responses measured by *IL-4*, *Rentla* and *Chil3*, and might be responsible for the improved glucose metabolism and reduced weight gain observed. I next assessed whether administration of *N. brasiliensis* ES products induces a similar phenotype in mice by regulating glucose metabolism. Similar to the results observed with *N. brasiliensis* infection, treatment with adult ES products (AES) or infective third-stage larvae ES products (L3ES) from *N. brasiliensis* improved glucose tolerance and reduced body weight gain in mice fed a high glycaemic index (HGI) diet as a model of T2D. In addition, I found for the first time that treatment of mice with *N. brasiliensis* ES was associated with type 2 immune responses measured by increased numbers of eosinophils and M2 macrophages and IL-5 in peripheral tissues, and a corresponding decrease in the levels of IL-6 in adipose tissue. Thus, helminth infections and their ES products are important for suppressing immune responses that drive metabolic dysfunction and T2D. Lastly, I investigated for the first time the role of *N. brasiliensis* infection and their ES products in modulation the gut microbiota composition in normal control (NC) mice and in mice fed both high-fat (HF) and HGI diets to induce T2D. I found modest shifts in species diversity in the intestinal

microbial communities of mice with *N. brasiliensis* infection and mice treated with ES products. Infection with *N. brasiliensis* or treatment with their ES products resulted in non-significant changes in α−diversity. However, infection with *N. brasiliensis* or treatment with L3ES or AES resulted in significant compositional changes in the gut microbiota that occurred at both the phylum and order levels. Abundance of the phyla Actinobacteria, Verrucomicrobia, Proteobacteria and TM7, and orders Clostridiales, Desulfovibrionales, Burkholderiales, Verrucomicrobiales and Coriobacteriales were the most affected by infection or treatment with ES products. Whether these microbial changes are essential components in maintenance of glucose metabolism and T2D is yet to be confirmed.

This project sheds light on the mechanisms by which parasitic helminths and their ES products can manipulate their host's immune and metabolic networks and reveals novel mechanisms by which gastrointestinal nematodes and their ES proteins can be harnessed as a source of next generation therapeutics for inflammatory and metabolic diseases.

Table of Contents

List of Figures

List of Abbreviations

A

AAMs = Alternatively activated macrophages

AcES = ES of adult hookworm *Ancylostoma caninum*

ADA = American diabetes association

ALDEx = Anova-like differential expression analysis

AT = Adipose tissue

APC = Antigen presenting cells

Arg1 = Arginase-1

C

CAMs = Classically activated macrophages

CD = Crohn's disease

CDKAL1 = CDK5 Regulatory Subunit Associated Protein 1-Like 1

CDKN2A/B = Cyclin-dependent kinase inhibitor 2A

CTLA-4 = Cytotoxic T-lymphocyte-associated protein 4

CeD = Celiac disease

CIA = Collagen-induced arthritis

D

DCs = dendritic cells

DM = Diabetes mellitus

DSS = Dextran Sodium Sulfate

E

IEC = Intestinal epithelial cell

ES = Excretory/secretory

ES-62 = Secreted phosphorylcholine-containing glycoprotein

F

FBG = Fasting Blood Glucose

FDR = False discovery rate

FFAs = Free fatty acids

FHES = *Fasciola hepatica* ES products

Foxp3$^+$ = Forkhead box P3

G

GAD = Glutamic Acid Decarboxylase

GF = Germ free

H

HbA1c = Glycosylated Haemoglobin

HDL = High density lipoprotein

HES = *Heligmosomoides polygyrus* ES products

HF = High fat diet

HFN = High fat diet naïve group

HFNb = High fat diet *N. brasiliensis* infected group

HGI = High glycemic index

HGIAES = High glycaemic index diet group treated with adult excretory/secretory

HGIL3ES = High glycaemic index diet group treated with larvae 3 excretory/secretory

HGIN = High glycaemic index naïve group

HGINb = High glycaemic diet *N. brasiliensis* infected group

HLA = Human leukocyte antigen

HOMA-IR = Homeostatic model assessment for insulin resistance

I

IA-2 = Islet antibody

IAA = Insulin autoantibody

IBD = Inflammatory bowel disease

IDDM = Insulin dependent diabetes mellitus

IDF = International Diabetes Federation

IGF1 = Insulin-like growth factor 1

IGT = Impaired glucose tolerance

IFN-γ = Interferon-gamma

IL-1β = Interleukin-1 beta

IL-2 = Interleukin-2

IL2RA = Interleukin 2 receptor alpha

IL-4 = Interleukin-4

IL4Ra = Interleukin-4 receptor alpha

IL-6 = Interleukin-6

IL-10 = Interleukin-10

IL-13 = Interleukin-13

IL-23 = Interleukin-23

IR = Insulin resistance

IRS1 = Insulin Receptor Substrate 1

K

KCNJ11 = Potassium Channel, Inwardly Rectifying Subfamily J, Member 11

L

LDL = Low density lipoprotein

LMWM-ESP = Low molecular weight metabolites derived from somatic extract ES products

LMWM-SE = Low molecular weight metabolites derived from ES products

M

MACs = Macrophages

M1 = Classically activated macrophages

M2 = Alternatively activated macrophages

MCP-1 = Monocyte Chemoattractant Protein-1

MS = Multiple sclerosis

MUC = Mucin

N

NC = Normal control diet

NCAES = Normal control diet group treated with adult excretory/secretory

NCL3ES = Normal control diet group treated with larvae 3 excretory/secretory

NCN = Normal control diet naïve group

NCNb = Normal control diet *N. brasiliensis* infected group

NIDDM = Non-insulin dependent diabetes mellitus

NOD = Non-obese diabetic

O

OGTT = Oral Glucose Tolerance Test

P

PPARγ = Peroxisome proliferator-activated receptors

PTPN22 = Protein tyrosine phosphatase non-receptor type 22

R

RA = Rheumatoid arthritis

RDA = Multivariate redundancy analysis

RegIII-γ = Regenerating islet-derived protein III-gamma

rNB-Cys = Recombinant protein cysteine protease inhibitor Nippocystatin

S

SEA = *Schistosoma mansoni*-soluble egg antigens

SPF = Specific pathogen free

T

T1D = Type 1 diabetes

T2D = Type 2 diabetes

TCF7L2 = Transcription Factor 7-like

TG = Triglycerides

Th = T helper 1

Th2 = T helper2

Th17 = T helper 17

Th22 = T helper 22

TLR= Toll like receptor

TNBS = 2,4,6-trinitrobenzene sulfonic acid

TNF-α = Tumor necrosis factor alpha

Treg = Regulatory T cell

TsES = *Trichinella spiralis* ES products

TSLP = Thymic stromal lymphopoietin

U

UC = Ulcerative colitis

V

VAT = Visceral adipose tissue

W

WAT = White adipose tissue

CHAPTER 1

INTRODUCTION

Chapter 1- Introduction

1.1. Diabetes Mellitus

1.1.1. Definition and Prevalence

Diabetes mellitus (DM) is a metabolic disorder characterised by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism due to an absence of, or deficiency in, insulin secretion, insulin action or both. This condition is associated with long-term dysfunction and failure of many organs such as the eyes, kidneys, nerves, heart, and blood vessels (1). DM is divided into two main classes. Type 1 diabetes (T1D), also called insulin-dependent diabetes mellitus (IDDM), represents around 10% of all cases of diabetes, and is the result of autoimmune destruction of beta cells in the pancreas that leads to an absolute deficiency of insulin secretion. Type 2 diabetes (T2D) or non-insulin-dependent diabetes mellitus (NIDDM) represents around 90% of all diabetes, and is the result of abnormalities in carbohydrate, fat and protein metabolism that results in resistance to insulin action on target tissues, leading to a relative deficiency in insulin secretion (1).

The prevalence of DM is increasing worldwide in both genders, and in all ethnic groups (2). The trend of developing diabetes is increasing towards younger people (3). According to International Diabetes Federation (IDF), DM is the fourth leading cause of non-communicable disease deaths, and is responsible for about 4 million deaths, and accounted for USD 727 billion of the world's health expenditure in 2017 (3).

IDF estimated that the number of people with all forms of diabetes worldwide will increase from 424.9 million people in 2017 to 628.6 million people in 2045 (3). There are more people with all forms of diabetes living in urban (279.2 million), than in rural (145.7 million) areas, and this gap is predicted to widen to 472.6 million people living in urban areas and 156 million people in rural areas by 2045 (3). There are however wide variations in prevalence worldwide. The Western Pacific region has the highest number of people with all forms of diabetes, with 159 million people, followed by South-East Asia (82 million people), Europe (58 million people) and North America (46 million people), respectively (Fig.1). The incidence of T1D is increasing, particularly in the age groups under 20 years, with 1,106,500 cases recorded in 2017 worldwide and approximately 132,600 newly diagnosed T1D cases annually (3). T2D occurs in youth, with the highest rates observed in people aged 15–19 (4). Moreover, it has been estimated that 352.1 million people have impaired glucose tolerance (IGT) worldwide in 2017 (almost half of them under the age of 50), and nearly 212 million people remain undiagnosed (3).

Figure 1.1. Number of people with all forms of diabetes as determined by the International Diabetes Federation, 2017 (3).

According to the IDF 2017, 425 million people have diabetes mellitus. The majority of cases can be found in the Pacific region.

1.1.2. Aetiology

DM is a multifactorial disease where genetic, environmental, and immunological factors interplay in the development of the disease.

1.1.2.1. Genetics

DM is a multigenic disease. A strong association between family history, and the risk of development of diabetes has been documented (5). In addition, family history has been shown to affect the phenotype of patients with DM (6). Individuals with first degree relatives who are diabetic are at risk of developing diabetes (1). Indeed, the risk of developing T2D increases by 40% if individuals have one parent with T2D, and 70% if both parents are affected. Moreover, in monozygotic twins the concordance of T2D increases by 70% compared with 20-30% in dizygotic twins (5, 7). Similarly, the risk of developing T1D increases by 6% if individuals have one parent with T1D and by >30% if both parents are affected. In monozygotic twins the concordance of T1D increases by 50% compared with 8% in dizygotic twins (8). Many studies have focused on the analysis of the genetic factors associated with DM, and over 60 genes have been shown to be associated with this disease. The human leukocyte antigen HLA class II region on chromosome 6p21 is the primary region that contributes to approximately 40–50% of the heritable risk for T1D (9). In addition, the insulin gene on chromosome 11p15, the cytotoxic T-lymphocyte-associated protein 4 (*CTLA-4*) gene on chromosome 2q33, a protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), the gene on chromosome 1p13 and the interleukin 2 receptor alpha (IL2RA) gene on chromosome 10p15 have also been found to be associated with T1D (8, 9). Genome-wide association studies have identified a number of genetic loci on chromosomes 3q, 4q, 7q, 9q, 10q, 11q 13q, 15q and 17q that contribute to the susceptibility to T2D. In European and Asian populations, 45 and 29 loci were identified respectively (10). The Transcription Factor 7-like (*TCF7L2*) gene is the most susceptible gene found to be associated with T2D (11). Moreover, genes like Potassium Channel, Inwardly Rectifying Subfamily J, Member 11 (*KCNJ11*) and peroxisome proliferator-activated receptors (*PPARγ*) which are targets for anti-diabetic medications have been shown to affect insulin sensitivity (5). Insulin Receptor Substrate 1 (*IRS1*) and Insulin-like growth factor 1 (*IGF1*) genes are associated with fasting insulin and homeostatic model assessment for insulin resistance (HOMA-IR), and deletion of these genes results in insulin resistance (IR) (11). Furthermore, proteins like CDK5 Regulatory Subunit Associated Protein 1-Like 1 (*CDKAL1*), cyclin-dependent kinase inhibitor 2A (*CDKN2A/B*) and Insulin-Like Growth Factor 2 may have an effect on β -cell function (10, 12).

1.1.2.2. Environmental factors

1.1.2.2.1. Lifestyle factors

Several environmental factors influence the development of DM. Lifestyle factors, such as obesity, dietary patterns (western diet), and sedentary lifestyle have been found to associate with increased incidence of T2D (13). Accumulating evidence suggests that obesity is associated with increased risk of metabolic and cardiovascular diseases (14). Excessive caloric intake, in particular higher consumption of trans-fats and sugars, causes increased body weight and central adiposity, which have a significant impact on T2D (15). High intake of saturated and trans fats, refined cereals and sweetened beverages leads to rapid increases in blood glucose levels, which is accompanied by an increase in the level of insulin. Overproduction of insulin for long periods of time leads to the release of free radicals from pancreatic β-cells, causing deficiency in insulin secretion (15). Similarly, accumulation of lipids in the liver and adipose tissue (AT), releases free fatty acids that have a deleterious effect on the insulin signaling pathway, leading to IR (13). On the other hand, increased fibre, fruit, and vegetable consumption and physical activity, and reduced intake of fat and sweetened beverages can reduce body fat mass, improve lipid metabolism, and increase glucose uptake in muscle (13), all of which have a positive effect on reducing the risk of developing T2D (16).

1.1.2.3. Inflammation

Antigen presenting cells (APCs) such as macrophages (MACs), dendritic cells (DCs), antigen-presenting B-cells, Kupffer cells in liver, adipocytes, and intestinal epithelial cells (IECs) are the main components of the innate immune system that detect environmental threats (eg. chemicals and pathogens). These threatening materials interact with the pattern recognition receptors such as toll-like receptors (TLRs), nod-like receptors (NLRs) on or inside these cells leading to activation of signaling pathways that induce inflammatory responses (17). Accumulating evidence suggests that inflammation and induction of innate immunity are associated with T1D and T2D (18).

Pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and chemokines such as Monocyte Chemoattractant Protein-1 (MCP-1) play crucial roles in the destruction of pancreatic β-cells (19).

In T1D, Glutamic Acid Decarboxylase (GAD)-65, tyrosine-phosphate like protein IA-2 (islet antibody), and insulin autoantibody (IAA) are the common auto-antigens that mediate destruction of pancreatic β-cells (20). MACs and DCs that reside in the pancreatic islets can activate $CD4^+$ and $CD8^+$ T cells, leading to the infiltration of more immune cells into islets. This in turn, activates autoimmune processes, and induction of β-cell destruction (21). During this process, cytokines such as IL-1β, interferon-gamma (IFN-γ), TNF-α and MCP-1 are secreted (20).

MAC polarization has been shown to have a key role in systemic insulin resistance, glucose tolerance and the development of metabolic disorders, and T2D (22, 23). MACs can differentiate into two major effector cells: M1 (pro-inflammatory), and M2 (anti-inflammatory) (24). Activation of M1 MACs, or classically activated macrophages, requires two signals: firstly, IFN-γ through the IFN-γ receptor, and secondly, TNF-α associated with production of IL-1 β, IL-6 and additional TNF-α (25, 26). On the other hand, activation of M2 MACs, or alternatively activated macrophages requires interaction with Th2 cytokines such as interleukin-4 (IL-4), interleukin-13 (IL-13), interleukin-10 (IL-10), transforming growth factor-beta (TGF-β) and galectin-3 (26).

Obesity is the main cause of insulin resistance and plays a key role in T2D (23). Liver, AT, and muscle are the major players in maintaining glucose homeostasis. Liver maintains glucose homeostasis between meals via two processes: glycogenolysis and gluconeogenesis. However, AT may regulate glucose homeostasis after a meal indirectly by regulating lipid homeostasis (27). Accumulation of lipid in AT, releases free fatty acids (FFAs) that induce the production of adipokines such as leptin and the activation of pro-inflammatory cytokines such as IL-1 β, IL-6 and TNF-α. M1 MACs are also recruited into AT, inducing the secretion of pro-inflammatory cytokines (28). On the other hand, resident M2 MACs are responsible for maintaining tissue homeostasis in metabolic organs (AT, pancreas, liver, and skeletal muscles) (29). Obesity induces local inflammation and recruitment of M1 MACs, leading to an imbalance between M1/M2 MACs and causing impaired glucose tolerance in adipocytes, hepatocytes, and myocytes (23, 30). M1 MAC numbers were found to be elevated in the islets of humans with T2D, and in a mouse model of T2D. However, in normal conditions the islets exhibited large numbers of resident M2 MACs (25, 31). Moreover, the M2 MACs comprise 10% of total cells in lean white adipose tissue (WAT) and are associated with the maintenance of insulin sensitivity (32).

Eosinophils play important roles in the induction of Th2 immunity via production of many Th2 cytokines such as, IL-4, IL-10, IL-13, and TGF-β that participate in anti-inflammatory immune responses (27). In a human study, it was found that elevated eosinophil numbers were associated with a decreased risk of T2D (33). Interestingly, it has been demonstrated that eosinophils might play an important role in the polarisation of MACs towards the M2 phenotype, which in turn allows maintenance of metabolic homeostasis, and glucose tolerance (34, 35). In particular, Wu and colleagues have highlighted the role of residential eosinophils in AT as a major IL-4-expressing cell type that might sustain production of M2 MACs (36, 37). Eosinophil numbers correlated inversely with body mass in mice on a high fat diet (HFD). Obesity was associated with decreased AT eosinophil numbers, and increased eosinophil expression in IL-5 transgenic mice improved obesity-induced insulin resistance (37). Interestingly, distinct groups of innate lymphoid cells (ILCs), a

recently discovered innate immune cell type, have been shown to be involved in regulating obesity (38). Group 2 innate lymphoid cells (ILC2s) are considered as anti-obese immune regulators, and they are involved in the browning of AT that induce the lean phenotype. These cells participate in the anti-inflammatory immune response deriving the secretion of Th2 cytokines IL-4, IL-5, and IL-13, the accumulation of eosinophils in AT, and the polarisation of MACs into the M2 phenotype (38, 39). On the other hand, group 1 innate lymphoid cells (ILC1s) have been found to participate in tissue inflammation via induction of IFN-γ, and TNF-α, which drives the polarisation of MACs into the M1 phenotype, in the obese state (38). Administration of the alarmin IL-33 was shown to reduce adiposity and fasting blood glucose (FBG), and to improve glucose and insulin tolerance (40). Signaling via IL-33 was required for ILC2-induced IL-5 production for induction of visceral adipose tissue (VAT) eosinophils, and polarization of VAT MACs towards an M2 phenotype (39-41).

Figure 1.2 Changes in the environment of resident immune cells in adipose tissue as a result of obesity (31).
Resident immune cells of lean individuals provide an anti-inflammatory milieu to maintain glucose homeostasis, however adiposity induces pro-inflammatory immune responses that are an important trigger of insulin resistance.

Recently, new subpopulations of $CD4^+$ T cells such as T helper 17 (Th17) and T helper 22 (Th22) cells have been associated with the pathogenesis of T1D. Interleukin-23 (IL-23), which is mainly secreted by MACs, is responsible for the expansion of Th17 cells and is considered to be one of the most important cell populations involved in the development of T1D (28). $CD4^+$ T cells skewed toward a Th17/Th22 phenotype in AT of obese subjects with insulin resistance and had a greater percentage of AT cells producing IL-17 and IL-22 (42). However, IL-22 was found to protect endothelial cells from glucose, and lysophosphatidylcholine-induced injury. Blocking the IL-22

receptor-1 (IL-22R1) diminished the protective role of IL-22, suggesting a double function of IL-22 in T2D (43). Moreover, mice deficient in IL-22R1 were prone to develop metabolic disorders when fed a HFD. Administration of IL-22 to genetically obese leptin receptor deficient mice (db/db) or HF mice had beneficial effects in improving insulin sensitivity, and regulating lipid metabolism in liver and AT (44). Many studies have revealed that $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Tregs) producing IL-10, IL-4 and IL-13 are involved in the suppression of pro-inflammatory Th1/17 responses (45). In addition, the prospective role of Treg cells in the pathogenesis and treatment of T1D and T2D has been highlighted. In a human study, Tregs have been shown to induce M2 MACs, maintaining tissue homeostasis (45). Furthermore, in non-obese diabetic (NOD) mice, T1D progression is associated with a progressive loss of Treg cells in the inflamed islets and treatment of mice with interleukin-2 (IL-2), which mediates the induction of CD25, promoted Treg cell survival and subsequently prevented the onset of diabetes (46). Moreover, depletion of Treg cells accelerates the development of T1D (47). A study by Feuerer and colleagues demonstrated higher numbers of $CD4^+$ $Foxp3^+$ Treg cells in the AT of mice fed a normal diet in comparison with those fed a HFD (48). Moreover, transferring Tregs from healthy donor mice to db/db mice improved insulin sensitivity (22).

1.1.3. Diagnosis

Diabetes is diagnosed by a blood glucose test from venous blood. The biochemical tests that are considered for diagnosis of diabetes are: FBG, two hours Oral Glucose Tolerance Test (OGTT) and Glycosylated Haemoglobin (HbA1c). The FBG test measures the level of glucose in the blood at a single time point, usually after overnight fasting for at least eight hours. The diagnostic level of blood glucose for diabetes in this test is ≥ 126 mg/dL (7.0 mmol/L). The OGTT test measures the level of glucose in the blood at two time points. This test is also performed after overnight fasting. The patient is given glucose solution containing the equivalent of 75 g glucose, and the blood glucose is measured at baseline (before the glucose load) and two hours after the glucose load. The diagnostic value in this test is blood glucose ≥ 200 mg/dL (11.1 mmol/L). The HbA1c test is commonly used to diagnose diabetes in individuals with risk factors and measures the average level of blood glucose over a period of three months. The diagnostic cut-off point of diabetes in this test is ≥ 6.5% (1).

1.1.4. Treatment

Diabetes is a complex, chronic illness, and thus, long-term medical care and treatment are needed to help control blood sugar levels and prevent disease complications. Nutritional therapy includes managing the amount and type of food taken, weight loss and regular exercise (1). Indeed,

150 min/week of moderate exercise or 75 min/week of vigorous aerobic physical activity have been shown to improve blood glucose control, reduce cardiovascular risk factors by an increase in high density lipoprotein (HDL) cholesterol, decrease in triglycerides (TG), and decrease in blood pressure (1). Furthermore, regular exercise may prevent the risk of developing T2D in high risk individuals (1). However, for many patients, these methods alone are insufficient in managing the disease. Thus, pharmacological therapy is also used to manage insulin secretion and blood glucose levels. Patients with T1D require lifelong insulin therapy, usually twice or more daily, with doses adjusted on the basis of self-monitoring of blood glucose levels (49). Metformin, sulphonylureas, thiazolidinediones, and other drugs are the first line therapy for T2D patients (50), however treatment with insulin is sometimes needed. Despite the wide use of these medications in the treatment of T2D, and their proven efficacy, these medications can have side effects including hypoglycaemia, weight gain, and increased low density lipoprotein (LDL) and TG levels (51).

1.2. The Hygiene hypothesis

The transition of nations from primarily agricultural to industrial societies has been associated with a rapid rise in the incidence of many immune diseases, including inflammatory bowel disease (IBD), multiple sclerosis (MS), rheumatoid arthritis (RA), and T1D (52). The hygiene hypothesis, formulated by David Strachan in 1989 (53), proposed that changes in human environment such as changed dietary habits, a cleaner environment with improved sanitation, vaccination programmes and excessive antibiotic use have reduced our exposure to many infectious agents (53), and symbiotic microorganisms (including helminths and gut microbiota) that had an evolutionary relationship with humans (54). The coevolution of humans and infectious agents established an immunological interaction that ensured the development of regulatory pathways to keep inflammation in check, thereby reducing inappropriate immune responses, which are considered the key mediators in many immune disorders (54).

At first, the imbalance between Th1 (pro-inflammatory)/Th2 (anti-inflammatory) responses was suggested to explain the epidemiology underlying the hygiene hypothesis, whereby reduced exposure to microbial products that stimulate Th1 responses resulted in excessive Th2 responses, culminating in an increase in the incidence of Th2-mediated allergic diseases (55). However, it soon became apparent that this theory of opposing Th1 and Th2 responses was insufficient to explain the increased incidence of Th1/Th17 mediated autoimmune diseases in addition to Th2-mediated allergic conditions in developed countries (56).

As the important role of Treg cells in controlling inappropriate inflammation was revealed, the importance of pathogen exposure on the subsequent development of a functional regulatory immune response became apparent (57). Indeed, the failure of immunoregulatory mechanisms (primarily Treg

responses) can lead to increased immunopathology (58). In DM, mutation in Foxp3, a transcription factor that plays a crucial role in the development and function of Treg cells is associated with T1D (59). Moreover, the depletion of Treg cells leads to the development of various autoimmune diseases, such as gastritis, T1D, and IBD (60). Hence, the balance between Tregs, Th1, Th2, and Th17 responses is essential to prime immunoregulation and provide protection against diseases that result from a dysfunctional immune system.

1.2.1. Studies supporting the hygiene hypothesis

The biota that inhabits the mammalian body is a major driving force in shaping the mammalian immune system (61). The mode of child delivery (Cesarian section) and feeding (formula vs. breast feeding), as well as antibiotic therapy, have a strong effect on the early distribution of gut microbiota. These, in turn, affect the susceptibility of individuals to many inflammatory diseases and allergic disorders (61-63). Helminth parasites are another important element of the human biome, and they have co-evolved over millennia, resulting in "pathogen tolerance" through the induction of regulatory immune responses (64). Many epidemiological studies have demonstrated the importance of an early exposure to different pathogens on the development of the immune system. Absence of early exposure to pathogens is thought to increase susceptibility to allergic and autoimmune diseases like, asthma, IBD, and T1D (65). This phenomenon is also supported by many studies showing a higher incidence of allergic conditions such as asthma (66), atopy (67), and IBD (68) in urban areas compared to rural areas. Similarly, other studies have found an increase in the prevalence of allergy and MS in the developed world compared to helminth-endemic areas or regions with poor sanitation (69, 70). Moreover, studies have found an increase in allergen skin reactivity after anthelmintic treatment (71). Birth mode, breast feeding, and antibiotic usage have an effect on the composition of gut microbiota and have been associated with the development of DM and other metabolic diseases. For instance, babies born via cesarean section harbored bacterial communities dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. However, vaginally delivered babies harbored bacterial communities dominated by *Lactobacillus*, *Prevotella*, or *Sneathia spp.* (72). Cesarean delivery and short term of breast feeding positively associated with atopic dermatitis (73). Moreover, a meta-analysis found a 20% increase in the risk of asthma and T1D in children delivered by cesarean section compared to vaginal delivery (74, 75). In a cohort study of 1,650 German school children, cesarean delivery resulted in a more than two-fold increase in childhood T1D risk compared to vaginal delivery (76). The use of antibiotics early in life has been associated with paediatric IBD (77), and increases the risk of asthma in early childhood (78). Moreover, antibiotic exposure during the first 6 months of age has been associated with an increase in body mass (63).

1.3. Helminth infection

Nematodes (also known as roundworms), trematodes (also known as flatworms), and cestodes (also known as tapeworms) are the three main groups of parasitic helminths (79). The major gastrointestinal (GI) nematodes of humans, often referred to as soil-transmitted helminths (STH), are the cause of one of the most important neglected tropical diseases, and include the ascarids (eg. *Ascaris lumbricoides*), hookworms (eg. *Necator americanus*, *Ancylostoma sp.*), threadworms (eg. *Strongyloides stercoralis*) and whipworms (eg. *Trichuris trichiura*) (80). Successful parasites employ different strategies to maintain a harmonious relationship with their host in order to survive (64). It is well established that helminth infections induce a profound Th2 response but without the hallmark features of serious allergy such as urticaria or anaphylaxis, and this phenomenon is attributed to the ability of helminths to promote regulatory networks that prevent excessive inflammation (81). Induction of a Th2 immune response is associated with an increase in the levels of IL-4, IL-5, IL-9, IL-13 and IL-21 as well as expansion of specific effector cells, such as eosinophils, MACs, mast cells, neutrophils, basophils and ILCs (82). M2 MACs have been shown to play an important role in host protective immunity against helminth infections (83). IL-4 and IL-13 induce M2 MACs by signalling through the IL-4 receptor alpha/ signal transducer, and activator of transcription 6 (IL-4Rα /STAT6). These cells are multi-faceted and orchestrate diverse processes such as immune regulation, tissue repair, and worm expulsion and resistance (64, 83). The protective effect of M2 MACs depends on the expression of arginase-1 (Arg1), chitinase-like 3 (Ym1) and Resistin-like molecules (RELMα and β) (83). Eosinophil numbers are increased in the blood following helminth infection and they are rapidly recruited to the site of infection where they participate in worm killing through the secretion of toxic mediators such as major basic protein, eosinophil peroxidase and eosinophil neurotoxin, in addition to the production of various chemokines, and cytokines such as, IL-4, IL-5, TGF-β (82, 84). Moreover, a rapid rise in $Foxp3^+$ Tregs mediated by IL-10 and TGF-β are reported after helminth infections. This response plays an instrumental role in regulating inflammation in response to helminth infection. Depletion of these cells has an influence on both pathology and resistance to infection (reviewed in ref (85)). Generally, the infective stage of STHs enter their host either orally or via skin penetration, and mature to become adult worms which inhabit different niches within the intestine (86). *N. brasiliensis* is a GI hookworm-like nematode of rats but has been studied extensively in the mouse (87). The lifecycle of this parasite involves two phases; a free-living phase in the external environment, and parasitic phase that takes place inside the definitive rodent host. This latter phase commences when infective third-stage larvae (L3) penetrate the skin and enter the circulation to reach the lung vasculature. In the lungs they moult to (L4), break through the alveoli and are then coughed up and swallowed as they enter the digestive system to reach the small intestine. In the small intestine,

they moult to L5 then become dioecious adults which mate. The female worm starts releasing eggs in the host faeces, which then hatch into L1 then L2 and finally L3 in the soil, before the L3 penetrates the tissues of a new host (88). Following GI nematode infections, APCs such as DCs, MACs, basophils and ILC2s play different roles in response to infection through influencing the induction of both Th2 and Treg immune responses (reviewed in ref (86)). Eosinophils (89), neutrophils, MACs (90, 91), and ILCs (92, 93) are essential in maintaining protection against *N. brasiliensis* infection in mice through orchestrating Th2 immune responses (94, 95). Both eosinophils and M2 MACs accumulate at sites of *N. brasiliensis* infection where they participate in worm killing and expulsion, and tissue remodelling. Activation of STAT6 by IL-4 and IL-13 play an important role in this process. Resistance to *N. brasiliensis* infection was impaired in eosinophil-deficient mice, and mice lacking IL-5 (89, 96, 97), STAT6, IL-4Rα and IL-13 (96). Eosinophils in these mice were recruited (skin, lung, small intestine) in very small numbers, more larvae migrated to the lungs, and adult worms displayed prolonged production of eggs compared to control mice (89). Rapid upregulation of YM1, RELM-α, and Arg1 were found in the lungs of mice infected with *N. brasiliensis* with increased IL-4 and IL-13, indicating the role of M2 alveolar MACs in the induction of host immune responses to the infection (91, 98). By day 8 post-infection, these cells suppressed inflammation caused by larval migration through the pulmonary environment (99).

1.3.1. Helminths as a therapeutic modality for immune-mediated inflammation

1.3.1.1. Evidence from human studies

Epidemiological studies from helminth-endemic areas show an inverse relationship between helminth infection and inflammatory diseases. For instance, there is an inverse relationship between the frequency of *S. stercoralis* infection and the incidence of autoimmune liver disease (100). Different clinical trials have highlighted the therapeutic roles of helminths in immune mediated diseases. To assess the immunosuppressive properties of *N. americanus* in coeliac disease (CeD), two clinical trials have been conducted where CeD patients were infected with low numbers of live *N. americanus* L3. Hookworm infection induced strong systemic and mucosal Th2 (IL-4, IL-5, IL-9 and IL-13) and regulatory (IL-10 and TGF- β) cytokines, supressed the production of IFN-γ and IL-17A and increased the numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in duodenal biopsy cultures (105). Moreover, suppression of mucosal IL-23 and upregulation of IL-22 (which promotes mucous production) of hookworm-infected participants with CeD after gluten challenge was observed. In these studies, treatment with *N. americanus* appears to be safe and hookworm-infected mucosa retained a healthy appearance (101-105). Most importantly, moderate gluten challenge of hookworm-infected patients did not induce any immunopathology in the gut (villous height to crypt depth ratio) and anti-tissue transglutaminase levels remained unchanged (105).

In two different studies, infection with live *N. americanus* was safe for crohn's disease (CD) patients, and resulted in a reduced CD activity index score (106). In the second study on seven IBD patients (four with active CD and three with UC), administration of eggs of the porcine GI whipworm *Trichuris suis* was shown to be safe and patients showed improvement in the common clinical indices of the disease (107). Another study on 29 patients with CD who received *T. suis* eggs revealed a significant reduction in symptoms (108). Furthermore, a randomized, double blind, placebo-controlled trial including 54 patients with active colitis showed improvement in 43.3% of the patients that received *T. suis* compared with 16.7% that received placebo treatment (109).

Moreover, in a cohort study, 12 patients with MS infected with different GI nematodes showed significantly lower relapse frequency than uninfected patients, accompanied by an increase in IL-10- and TGF-β-secreting cells, and a decrease in IL-12- and IFN-γ-producing cells compared with non-infected patients (110). When these infected patients received anthelmintic treatment, a significant decrease in IL-10- and TGF-β-secreting cells, and a significant increase in IFN-γ- and IL-12-producing cells was observed (110). Also, a clinical trial of 4 MS patients using experimental *T. suis* therapy revealed a downregulation of Th1 responses (particularly IL-2 and IFN-γ) and an increase in Th2 associated IL-4 (111). A survey of self-treatment with helminths (*T. suis* ova, *T. trichiura*, *N. americanus* and tapeworm *Hymenolepis diminuta*) revealed that helminth therapy was effective for many people in reducing a variety of inflammatory diseases including IBD, allergies, and autoimmune diseases (112). All of these trials have revealed the potential use of helminths as therapeutic agents in a wide range of inflammatory mediated diseases. You are mostly reporting the positive outcomes. There are many papers that show tht it doesn't work very well too. You might cite a few of them to keep the balance. For example, phase 2 trials of TSO in CD and UC failed to meet clinical endpoints. See attached review paper of Stephanie's that is in press at PLoS Path. If I forget to send it to you, remind me.

1.3.1.2. Evidence from animal models

A variety of helminths and their products have been tested in different mouse strains to assess their roles in the prevention of immune mediated diseases. For instance, in a mouse model of asthma, infection with *N. brasiliensis* suppresses the development of allergen-induced airway eosinophilia via the production of IL-10. Interestingly, *N. brasiliensis* infection alone (in the absence of allergen challenge) induced airway and blood eosinophilia (113). Restimulating the MLNs and spleen cells of infected mice with anti-CD3 and IL-2 led to increased amounts of IL-4, IL-5, IL-10 and IL-13 in comparison to uninfected mice. However, when *N. brasiliensis* infected mice were challenged with OVA allergen, a significant decrease in airway eosinophilia was observed, accompanied by reduced levels of eotaxin in the broncho-alveolar lavage fluid of these mice in comparison to uninfected mice

challenged with OVA. This effect was possibly dependent on IL-10, as the suppressive effect was not observed in mice deficient in IL-10 (113). Infection with the GI nematode of mice *Heligmosomoides polygyrus* showed reduction of airway eosinophilia, and neutrophilia after OVA challenge. In addition, elevation in Tregs and regulatory B cells (Bregs), and increased expression of TGF-β and IL-10 were also detected in MLNs after challenge (114, 115).

In two different model of arthritis, *N. brasiliensis* infection suppressed inflammatory arthritis through induction of Th2 immune response. The anti-arthritis effect was dependent on the activation of the STAT6 pathway by IL-4/IL-13. Eosinophil numbers increased in the joints of infected mice and neutrophil numbers decreased. Expression markers of M2 MACs were increased while expression markers of M1 MACs decreased in the joints of infected mice (116). In another model of arthritis, mice infected with either *N. brasiliensis* or *H. polygyrus* also showed reduced arthritis severity that was associated with increased levels of IL-4 (117).

In a mouse model of dinitrobenzene sulfonic acid (DNBS)-induced colitis, infection with the GI nematode *Trichinella spiralis* reduced the severity of colitis. This was accompanied by a significant reduction in IL-12 levels and elevated production of IL-4 and IL-13 (118). In addition, *H. polygyrus* infection of Rag IL-10$^{-/-}$ mice was protective in the T cell transfer model of colitis, and was accompanied by alterations in mucosal DC function and reduction in the capacity of intestinal T cells to produce IFN-γ and IL-17 (119).

In a mouse model of MS, rats infected with *T. spiralis* showed reduction in the clinical score of the disease, which was associated with increased levels of IL-4 and IL-10 and decreased levels of IFN-γ and IL-17 (120). Likewise, mice infected with *Trichinella pseudospiralis* showed amelioration in the clinical score of the disease with reductions in the levels of IL-17, IL-6, IL-1β, IFN-γ and TNF-α (121). Infection with *H. polygyrus* also showed beneficial effects on disease severity, which is associated with increased levels of IL-10, TGF-β, and IL-6 in the cerebrospinal fluid and in the serum, and decreased IL-17A and IL-2 levels in the serum (122).

1.3.2. Helminths as a therapy for diabetes mellitus

Recent epidemiological studies in indigenous communities of north-west Australia, Indonesia, rural China and India revealed an inverse correlation between helminth infection and T2D (123-126). Additionally, infection with *S. stercoralis* in Australian Aboriginal communities seems to protect against the onset of T2D (127). Moreover, there is an inverse relationship between the prevalence of lymphatic filariasis and T1D in southern India (128). NOD mice are widely used as a model to study human T1D. In this model of DM, Th1 responses drive the development of inflammation. Many studies have investigated the protective role of nematodes (*T. spiralis, H. polygyrus*, and *Litomosoides sigmodontis* (filarial nematode)) in the treatment of T1D in NOD mice. The data show

increased levels of IL-4, IL-5, IL-10, Foxp3, IgG1 and IgE; and decreased levels of IFN-γ and IL-12 as well as a reduction in $CD8^+$ lymphocyte infiltrate in the pancreas (129-132). However, in IL-4 deficient mice the protection against diabetes does not depend on the Th2 shift, and requires TGF-β and IL-10, which suggests that other regulatory mechanisms might be involved in diabetes prevention (129, 130). These findings provide evidence that GI nematodes in particular can induce a mixed Th2/Treg response that holds potential for treating T1D (133).

In the context of T2D, diabetic mice infected with *H. polygyrus* had improved glucose tolerance, decreased HOMA-IR and body weight gain with decreased fat accumulation and fatty acid synthase gene expression in the liver compared to uninfected diabetic mice (134, 135). The infection also increased the expression of uncoupling protein 1 (UCP1), the M2 MAC markers, *RELMα*, *Arg1*, and *Ym1* and resulted in increased levels of IL-4, IL-13, and IL-10 in the small intestine and MLNs (134, 135). This shift towards a Th2 environment was accompanied by increased expression of *GATA3*, and *Foxp3*$^+$ and decreased IFN-γ and IL-17 in the MLNs (134). Similarly, in a mouse model of obesity, infection with *N. brasiliensis* resulted in reduced body weight gain, decreased adipose tissue, and liver masses with decreased levels of hepatic triglycerides. This was accompanied by improved glucose homeostasis and expression of M2 MAC markers *Arg1* and *Ym1* and Th2 cytokines IL-4 and IL-5 in AT and liver (136). Two additional studies showed that *N. brasiliensis* infection improved insulin sensitivity through induction of IL-33, which mediates activation of resident VAT, ILC2 producing IL-5- and IL-13-dependent accumulation of VAT, and eosinophilia (37, 41). Additionally, mice infected with the filarial nematode *L. sigmodontis* also showed improved glucose tolerance which was associated with increased numbers of eosinophils, M2 MACs, and $CD4^+$ T cells in the AT (137). C57BL/6 mice fed a HFD and infected with the blood fluke trematode *Schistosoma mansoni* showed an increase in WAT eosinophils, and M2 MACs. This was associated with metabolic homeostasis, and improved insulin sensitivity (138). Moreover, in a mouse model of atherosclerosis, treatment with *Schistosoma* eggs reduced total serum cholesterol and triacylglycerol and cholesteryl ester lipids in the liver (139).

1.4. Helminth-derived products

Helminths release soluble mediators that interact with host immune cells. These helminth-derived molecules, referred to as excretory/secretory (ES) products, play important roles in host immune modulation and represent the molecular interface between host and parasite. Several helminth-derived molecules that interfere with host immune processes have been described (140). These molecules include proteases, protease inhibitors, cytokine homologues, anti-oxidants, various esterases, proteins of unknown function, and glycans and lipids (140). Numerous schistosome soluble egg antigens (SEA) direct DCs to drive Th2 responses *in vitro* and *in vivo* (141). For instance, Lacto-

N-fucopentaose III (LNFPIII) was capable of inducing Th2 immune responses, with decreased levels of IFN-γ, and increased levels of IL-4, IL-5 and IL-10 (142). Additionally, recombinant alpha-1 induces basophils to produce IL-4 (143), and omega-1 induces Th2 responses by driving production of IL-4 (144). Likewise, recombinant thioredoxin peroxidase from the liver fluke *Fasciola hepatica* has been shown to induce the recruitment of M2 MACs, which is associated with the induction of Arg1, and high levels of IL-10 (145). Parasitic nematode ES products also drive regulatory immune responses. One of the best known examples is ES-62, a phosphorylcholine-bearing glycoprotein from *Acanthocheilonema viteae* that promotes the differentiation of DCs and MACs towards an anti-inflammatory phenotype (146). ES products of *T. spiralis* have been shown to modulate the function of MACs via inhibiting the production of TNF-α and IL-6 (147) and inducing type 2 cytokine responses via increased production of IL-4 and IL-10 (148). *H. polygyrus* ES (HES) products have been reported to inhibit T cell proliferation, and HES-exposed DCs can induce differentiation of IL-10-producing $CD4^+$ Tregs (149). Moreover, hookworms express molecules with immunosuppressive properties, including neutrophil inhibitory factor, anticoagulant peptides and protease inhibitors (150). ES products of adult *N. americanus* bind selectively to NK cells and induce IFN-γ production in the presence of both IL-2 and IL-12 (151). Proteases secreted by *N. americanus* have been shown to induce type 2 cytokine production by basophils, and this was associated with increased levels of IL-4, IL-5 and IL-13 but not IFN-γ (152). Importantly from the perspective of my PhD project, ES products of *N. brasiliensis* (NES) have been shown to stimulate maturation of DCs towards a Th2 phenotype (153-156).

Furthermore, helminths were recently described to secrete particles known as extracellular vesicles (EVs). These EVs might have a role in parasite-host interaction and modulation of the host immune response (157). Researchers have described EVs from *F. hepatica* (158), *Schistosoma* spp. (159-161), and *N. brasiliensis* (162, 163), among others (164, 165), and highlighted their roles in parasite-host interactions (161), including both suppression of inflammation and enhanced inflammatory responses that predispose to cancer (158). For instance, EVs from *S. japonicum* increase numbers of host peripheral monocytes *in vivo* (166) and skew MACs towards the M1 phenotype accompanied by increased levels of TNF-α and IL-12 *in vitro* (167). Internalization of *H. polygyrus* EVs by MACs caused downregulation of Th1 and Th2 responses which was associated with suppressed expression of *IL-6*, *TNF*, *Ym1*, *RELMα*, and expression of the IL-33 receptor subunit *ST2* (168).

1.4.1. Helminth-derived products as therapies for immune-mediated inflammation

Many studies have indicated the therapeutic potential of helminth ES products for treating a diverse array of inflammatory conditions. ES products of the hookworm *A. caninum* (AcES) suppressed intestinal pathology in a dextran sulphate sodium (DSS) mouse model of colitis, and was associated with the upregulation of Th2/anti-inflammatory cytokines IL-4, IL-5 and IL-10, and downregulation of the pro-inflammatory cytokines IL-6, IL-17 and IFN-γ (169, 170). NES of *N. brasiliensis* suppressed asthma by inducing a Th2 immune response that was associated with increased IL-4 and IL-5-producing $CD4^+$ T cells that simultaneously inhibited the development of OVA-specific allergic responses (171, 172). ES products of adult *T. spiralis* attenuated the severity of DSS colitis in mice, characterised by a significant reduction in IL-17 levels in the colon and MLNs (173). Additionally, rats administered with crude *T. spiralis* larval (L1) ES products exhibited improved clinical scores in experimental autoimmune encephalomyelitis (EAE) as a model of human MS. This was associated with increased levels of IL-4, IL-10 and TGF-β, and decreased levels of IFN-γ and IL-17 with increases in the proportion of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells at the systemic level, and in target organs (spleen and spinal cord) (174). In another study, soluble products of both *T. suis* and *T. spiralis* significantly reduced the clinical signs of experimental EAE in mice (175). Administration of EVs from *H. polygyrus*, and *L. sigmodontis* reduced eosinophil numbers and IL-5, IL-13 and IL-33 levels in bronchoalveolar lavage, expression induced by allergen *Alternaria* (176). In a more recent study, EVs from *N. brasiliensis* protected against chemically-induced colitis inflammation in mice, which was associated with the suppression of pro-inflammatory cytokines such as IL-6, IL-1β, IFNγ and IL-17a and increase of the anti-inflammatory cytokine IL-10 in EV-treated groups compared to control groups (163). *H. diminuta* antigen-pulsed DCs were found to be protective in di-nitrobenzene sulphonic acid (DNBS)-induced colitis, and required IL-4Rα and the capacity to secrete IL-10 (177, 178). However, ES from helminth is very complex and difficult to produce in large scale, hence recombinant Es proteins would be better to produce as new therapeutic modality. *S. mansoni* recombinant protein, 28-kDa glutathione S-transferase (P28GST) prevented inflammation in the TNBS model of colitis in both mice and rats, characterised by decreased expression of pro-inflammatory cytokines and increased expression of Th2 and anti-inflammatory cytokines (179). Recently, one of the most abundant AcES proteins, AIP-2, was produced in recombinant form and shown to suppress airway inflammation in a mouse model of asthma via induction of tolerogenic DCs, and $Foxp3^+$ Tregs (180). Recombinant filarial cystatin was also found to suppress allergic airway inflammation via induction of IL-10-producing MACs that inhibited eosinophil recruitment, reduced levels of total IgE and downregulated IL-4 production (181). Recombinant *T. spiralis* Ts-specific 53-kDa glycoprotein (TsP53) also protected mice from colitis, typified by a reduced mucosal damage and a reduced inflammatory cell infiltration in the colonic

mucosa and submucosa (182). A systematic review reported that treatment of mice with multiple helminth recombinant proteins including *A. viteae* ES-62 - and its small molecule analogue SMA-12b, 11a and PC- as well as recombinant SJMHE1 from *S. japonicum* resulted in significant reduction in clinical arthritis score. In all of these studies the treatment with these helminth recombinant proteins was associated with a significant decrease in the pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1β, IFNγ and IL-17, and with a significant increase in IL-10 (183).

1.4.2. Helminth-derived products as therapeutics for diabetes mellitus

T1D being an autoimmune inflammatory condition could potentially be treated with helminth ES products. ES of *F. hepatica* liver flukes prevented T1D in NOD mice through induction of Bregs and Tregs, and was associated with induction of M2 MACs and increased IL-10 and TGF-β levels (184). Schistosome crude SEA also prevented T1D in the same mouse model, inducing Th2 and Treg responses which involved induction of DCs and M2 MACs and increased levels of IL-4, IL-10 and TGF-β (185). SEA was also protective in an atherosclerosis model of mice. In this model low dose exposure to SEA promoted Th2 responses and reduced the levels of total cholesterol and LDL (186). Likewise, mice treated with SEA had lower plasma cholesterol levels, mainly LDL and VLDL, which had an effect on reducing the size of atherosclerotic plaques. SEA treatment resulted in reduced circulating neutrophils and M1 MACs and TNF-α expression, and increases in M2 MACs producing IL-10 (187). The LNFPIII glycan found in schistosome SEA (also found in human breast milk) has anti-inflammatory properties via induction of IL-10 from DCs and $Arg1^+$ M2 MACs. HFD mice treated with LNFPIII had improved insulin sensitivity and reduced liver TG (188). In two different studies the researchers found that ES products from *L. sigmosoides* and *S. mansoni* increased the number of eosinophils and M2 MACs in AT and were able to improve metabolic homeostasis, and insulin sensitivity in murine obesity models (137, 138).

1.5. The microbiota, mucous and inflammation

The mammalian body is inhabited by a microbial community that is essential for the formation and maintenance of a balanced immune system with functioning effector and regulatory capacity (60). Mode of birth and feeding, antibiotic use, dietary habits, and helminth infection can all have an impact on the composition and diversity of the intestinal microbiota (189-191). The gut microbiota plays an important role in host metabolism, physiology, nutrition, and immune function. In order to maintain a homeostatic relationship with these microbes, the intestinal immune system has to reduce direct contact with resident bacteria, and limit their exposure to immune system components (60). The intestine is protected by mucous secreted mostly by goblet cells. Mucous consists of mucins, which are glycoproteins that have more than 50% of their mass as *O*-glycans. The mucin (Muc) family includes members Muc1 to Muc12 (192). Defects in the mucous layer allow resident gut bacteria to

be in direct contact with the epithelium, leading to induction of inflammatory responses as demonstrated by increased permeability and bacterial adherence to the epithelial cell surface in Muc2-deficient mice (193), and their progression to develop colitis (194). RELMβ upregulates Muc2 secretion in human goblet-like cells in culture as well as in mouse goblets cells *in vivo* where it reduces the colonic damage in a mouse model of inducible colitis (195). Moreover, germ-free (GF) mice showed a dramatic reduction in RELMβ, however, enhanced expression and robust secretion of RELMβ were found following colonisation by commensal bacteria (196). Th2 immune-mediated responses can activate *RELMβ* gene transcription in a STAT6-dependent manner (196). Interestingly, induction of Th2 cytokines by GI nematode infections induces *RELMβ* expression by IECs that is essential for helminth expulsion (197). IL-4/IL-13–dependent goblet cell hyperplasia drives *N. brasiliensis* expulsion through production of mucous-containing RELMβ that can encapsulate worms and inhibit their ability to sense their source of nutrition (198). In this context, IEC may have an immunoregulatory function in addition to their role in maintenance of mucosal barrier integrity. Paneth cells are also specialised cells in the intestinal epithelium that secrete antimicrobial molecules such as regenerating islet-derived protein III (RegIII-β, RegIII-γ), which is capable of killing Gram positive bacteria, limiting the microbial penetration of the epithelial surface and preventing them from damaging the epithelium. This homeostatic relationship with gut microbes stimulates a highly regulated innate and adaptive immune response (199).

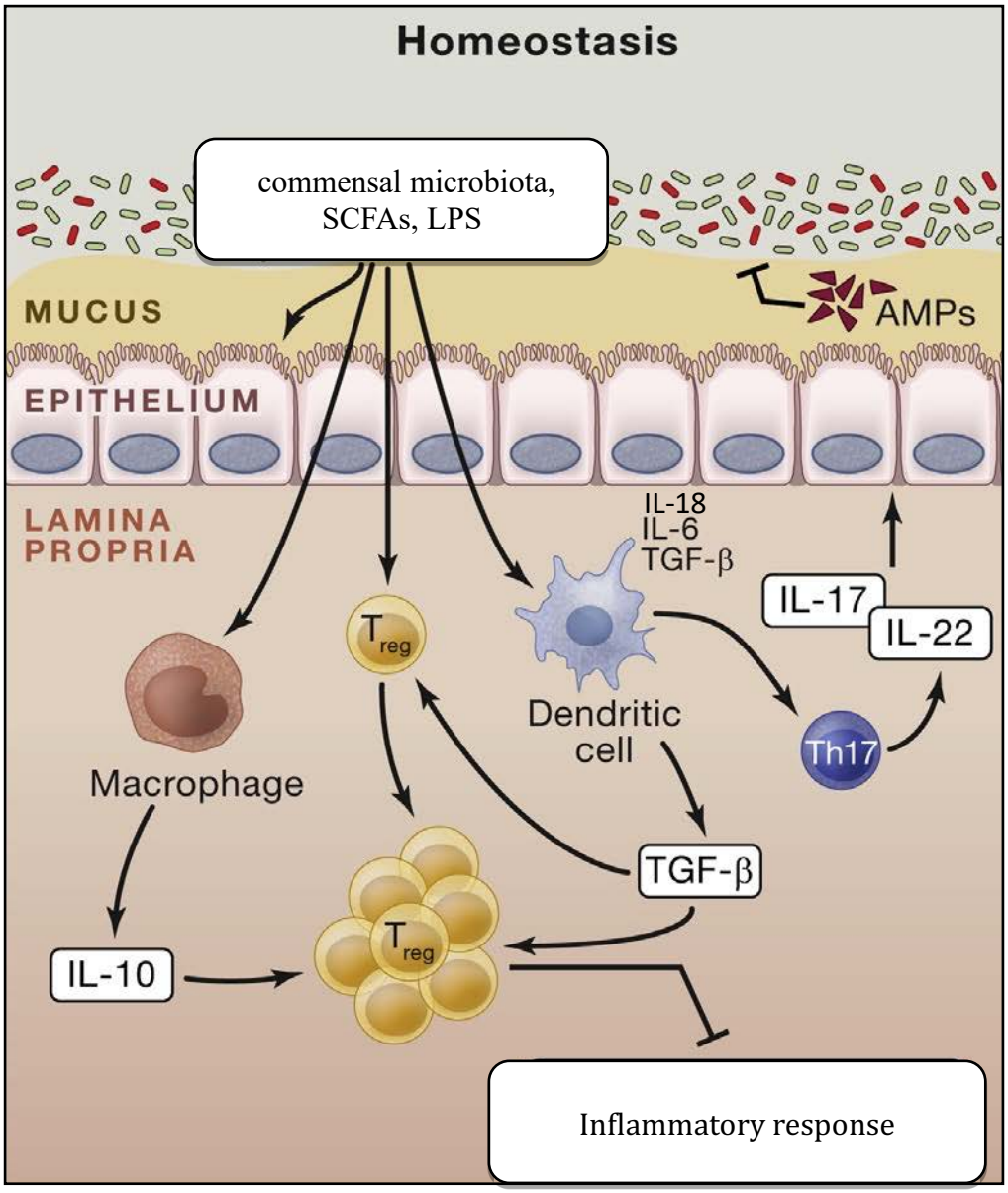

Figure 1.3. The gut microbiota and host immune responses in the steady state (modified from (199)).

Microbiota and their associated molecular patterns such as short chain fatty acids (SCFAs) and LPS induce immune cells such as MACs, DCs and Tregs to provide an anti-inflammatory milieu that maintains immune homeostasis.

The resident M2 MACs and DCs in the intestinal lamina propria have developed mechanisms to downregulate pro-inflammatory responses (200). Interaction of intestinal DCs with B and T cells results in IgA production, preventing gut microbes from penetrating systemic secondary lymphoid tissues (60). Intestinal MACs through MyD88 signaling downregulate pro-inflammatory cytokines, resulting in wound healing and tolerance to these commensal bacteria (200). Moreover, stimulation of resident ILCs in the lamina propria results in production of IL-22, which plays an important role in preventing the spread of bacteria to systemic sites (60). IL-22 is required for protection during bacterial infection through direct induction of antimicrobial proteins, RegIII-β, and RegIII-γ (201).

IL-22 enhanced activation of STAT3-mediated enhancement of mucous-associated proteins (Muc1, Muc3, Muc13) within colonic epithelial cells, leading to attenuation of local intestinal inflammation (202). Th17 cells also contribute to host defence via producing IL-22. Smaller Peyers patches, lymph nodes, and spleens of GF mice have an impact on the maturation of the immune system. These mice show decreased (or a complete absence) intestinal Th17 cell development, and decreased production of mucosal IgA (203). Interestingly, infection with *N. brasiliensis* increases IL-22, which directly increased goblet cell numbers and RELMβ, Muc1, Muc2 and Muc3 production in the small intestine, and which was shown to be essential for helminth expulsion (204). It has been shown that IL-33-indcued IgA production is important for maintaining microbial homeostasis in the intestine and confers protection against colitis. However, IL-33 deficient mice exhibit microbial dysbiosis and are prone to develop colitis (205). Noteworthy, in *N. brasiliensis* infection, IL-33 induced ILC2-producing IL-13.

In the healthy state, a number of commensals have been found to induce $CD4^{+}Foxp3^{+}$ Tregs that secrete IL-10 and TGF- β (206). However, there is a significant decrease in these cells with a reduction in IL-10 levels in GF mice (207). Microbial-associated molecular patterns such as bacterial LPS and SCFAs such as, acetate, propionate and butyrate, which are the main end-products of the bacterial fermentation of non-digestible dietary fibres, also participate in modulation of host immune responses (208). In the homeostatic state, sensing of LPS molecules occurs directly by host protein lipid A-binding protein (LBP). LBP binds to LPS forming LBP/LPS complexes which then bind to CD14. The latter LPS/LBP/CD14 complex signals through TLR4, leading to downstream inflammatory responses (209). SCFAs, apart from their role as a source of energy for IECs, also play an important role in the homeostasis of the intestinal epithelium. These bacterial metabolites induce a tolerogenic and anti-inflammatory response, and under some conditions they may also induce Th1 and Th17 responses through activation of the host G-protein- coupled receptors Gpr-41 and -43 (210). The immune mechanisms by which they do this involves an increase in the expression of antimicrobial peptides and IL-18 (a key cytokine for the repair, and maintenance of epithelium homeostasis) by IECs, and regulation of the differentiation and activation of immune cells such as neutrophils, DCs, MACs and T lymphocytes which is most likely associated with activation of $FoxP3^{+}$ Tregs cells, and increased production of IL-10 (208). Moreover, depending on the cytokine milieu, these metabolites can promote the differentiation of naïve T cells into T cells producing IL-17 and IFN-γ (208, 210, 211). Thus, the shift in the composition or density of gut microbiota leads to changes in the inflammatory state associated with imbalance in intestinal and systemic immune homeostasis (199).

1.5.1. Role of microbiota in immune mediated diseases

There is substantial evidence highlighting the beneficial role of gut microbiota in shaping the mammalian immune response. Disturbance of the intestinal microbial community leads to altered immune responses that can result in various human inflammatory disorders (212). Firmicutes (Gram positive) and Bacteroidetes (Gram negative) represent the largest phyla in the human and mouse microbiota, and a shift in the ratio of these phyla has been associated with many disease conditions (213). Studies using GF mice have revealed the crucial role of the microbiota in the pathogenesis of many immune-mediated diseases.

A recent study demonstrated that OVA-sensitized GF mice subjected to an OVA aerosol challenge showed increased allergic airway inflammation. Recolonisation of these mice with a complex specific pathogen free (SPF) microbiota for 3 to 4 weeks protected mice from increased allergic airway inflammation. This was associated with a decrease in regulatory DCs and alveolar M2 MACs and an increase in proallergic basophils in the lung tissue and airways of GF mice compared with SPF mice (214). Administration of SCFAs to mice attenuated the severity of allergic airway inflammation. This was associated with increased production of DC and MAC precursors in the bone marrow with high phagocytic activity, and reduced expression of MHCII that were less effective at inducing Th2 effector cells in the lung. Alteration in the ratio of Bacteroidetes to Firmicutes was detected in the gut and the lungs of SCFA-treated mice (215). Administration of DSS to GF mice caused severe colitis and led to death of mice on day three (216). Mono-colonisation of GF mice with *Bacteroides fragilis* (BF) significantly ameliorated DSS-induced colitis and increased animal survival by 40%. BF-DSS mice exhibited decreased production of the pro-inflammatory cytokine TNF-α and increased production of the anti-inflammatory cytokine IL-10 (217). Moreover, administration of SCFAs (butyrate) ameliorated the development of colitis-mediated induction of functional Tregs in the colonic mucosa (218).

In human studies, differences in the gut flora of healthy infants in comparison with allergic infants were observed. Increases in *Clostridia* and decreases in *Bifidobacteria* were shown to predispose infants to allergy, and early colonisation with *B. fragilis* at 3 weeks of age is an early indicator of possible asthma later in life (219). Moreover, RA patients showed a reduction in the abundance of *Bacteroides* with an increase in the abundance of *Prevotella* (220), and an increased number and community diversity of *Lactobacillus* spp. compared to controls. These changes may also represent the outcome of disease progression (221). Furthermore, IBD has been associated with an alteration in the abundance of Firmicutes and *Enterobacteriaceae* (222). Different studies have reported a reduction in Firmicutes including *Clostridium*, *Ruminococcus* and *Lactobacillus* with a decrease or increase of Bacteroidetes in UC patients compared with healthy controls (223). However, treatment with SCFAs attenuated the severity of UC (224). Moreover, several studies have

highlighted the beneficial effect of SCFAs on human health and their role in the attenuation of the severity of different inflammatory diseases (225). These studies highlight the important role of the microbiota in the aetiology and pathogenesis of inflammatory diseases.

1.5.2. Role of microbiota in diabetes mellitus

The connection between gut microbiota and regulation of host homeostasis and inflammation in obesity, metabolic syndrome and DM has been demonstrated (226). The intestine is the primary site of nutrient absorption in the body, thus, the immune system and commensal microbiota are sensitive to changes in diet. The first evidence came from studies on GF mice, and revealed that GF mice administered a Western diet (high-fat, high-carbohydrate) were resistant to obesity-associated insulin resistance, hepatic steatosis, dyslipidemia, and elevated systemic TNF-α (227). Conventionally raised mice have a 40% increase in body fat content compared to GF mice, and when the latter group were colonised with microbiota from conventionally raised mice they experienced a 57% increase in total body fat, a 2.3-fold increase in hepatic triglycerides and a dramatic increase in IR (226). Changes in the composition and number of gut microbiota were observed in HFD mice compared to mice fed a normal chow diet. HFD mice had more Firmicutes and a 75% decrease in *Lactobacillus*, 279% increase in *Oscillibacter* and fewer Bacteroidetes. These changes were associated with weight gain and increased expression of TNF-α in the colon, mesenteric fat and liver, and IL-6 in mesenteric fat and liver (228). A reduction in Bacteroidetes and an increase in Firmicutes have also been observed in genetically obese *ob*/*ob* mice (229). Consistent with the animal studies, a study in obese children showed an elevation in the Firmicutes to Bacteroidetes ratio compared with lean children (230). Notably, several human studies have reported a decrease in the abundance of Bacteroidetes in obese individuals (231) but an increase in the abundance of Firmicutes (232). Others have found a decrease in the abundance of Bacteroidetes and an increase in the abundance of Actinobacteria, with no differences in Firmicutes (232). However, four studies reported reductions in Firmicutes with an increase, decrease or no change in Bacteroidetes (233).

In the context of diabetes, different studies have associated changes in the intestinal microbiota with T2D (234, 235). Reduction in the abundance of Firmicutes, and increase in the proportion of Bacteroidetes was observed in diabetic patients (236). Increased levels of *Lactobacillus spp.*, and decreased levels of *Clostridium spp.* have also been observed in T2D patients (230).

Alteration in the composition of gut microbiota has also been observed in T1D patients. Four studies with children reported an increase in Bacteroides with T1D, whereas Firmicutes levels were higher in healthy children. Furthermore, a large increase in *Bacteroides dorei* was observed in autoimmune-prone children (237). These results suggest a role of gut microbiota dysbiosis in diabetic pathology. Different mechanisms have been proposed linking the effect of dysbiosis in the microbiota

on glucose metabolism, and their link to diabetes. Changes in the amount or the types of Gram positive bacteria (which lack LPS) vs. Gram negative bacteria (contain LPS) led to increases in gut permeability and increases in systemic LPS which is a key mediator in the induction of low grade chronic inflammation leading to T2D. Changes in the gut microbiota also lead to changes in the concentration of SCFAs which have an effect on the regulation of the intestinal hormones glucagon-like peptide-1 and the anorectic hormone peptide YY, and stimulating fatty acid oxidation and inhibiting *de novo* lipogenesis and lipolysis. The effect of microbial dysbiosis also extends to influencing the concentration of bile acids in the liver and circulating branched chain amino acids which have been found to play a role in glucose homeostasis (reviewed in (238, 239).

1.6. Helminth-microbiota interactions

Both helminths and gut microbiota play a role in the modulation of the host's immune system (240, 241), and helminth presence has been linked with microbiota diversity and composition (242). One of the first positive effects of this interaction was observed in a model of *T. muris* infection where the gut microflora was shown to be essential for *T. muris* eggs to hatch in the gut and establish an infection (243). Chronic infection of C57BL/6 mice with *T. muris* demonstrated a reduction in microbial α-diversity and led to decreased diversity and abundance of Bacteroidetes, specifically *Prevotella* and Parabacteroides, and increased abundance of Firmicutes (244). This was associated with decreases in IL-10 and Treg cells and increases in IFN-γ, T-bet$^+$ cells and TCR β$^+$ cells (245). *N. brasiliensis* infection reduced the total bacterial load of Firmicutes and increased the total load of Bacteroidetes and Actinobacteria in the ileum of infected mice. At a family level increases in the abundance of family *Lactobacillaceae* with decreases in the abundance of *Peptostreptococcaceae*, *Clostridiaceae* and *Turicibacteraceae*. This was associated with increased expression of antimicrobial peptides, *RELMβ*, *Muc2* and mucins and increased expression of the M2 MAC expression markers *YM1* and *RELMα*, and decreased *IL-17* expression in the ileum (246). Additionally, *H. polygyrus* infection resulted in a significant increase in the numbers of γ-Proteobacteria/ *Enterobacteriaceae* and members of the *Bacteroides/Prevotella* in the cecum. Higher loads of Gram-positive bacteria such as *Lactobacillus* and *Clostridium* were detected in the small intestine. Alteration in gut microbiota composition was independent of IL-4/IL-13/STAT6 pathway as similar increases in the bacterial load were also observed in infected IL-4 KO mice (247), particularly an abundance of *Lactobacillus* species in the duodenum and ileum of infected mice, with increases in Foxp3$^+$ cells and IL-17 levels in the MLNs (248, 249). Infection with *H. polygyrus* altered the intestinal bacterial communities, which led to attenuation of allergic airway inflammation in mice inoculated with house dust mite (HDM), increased production of SCFAs and decreased eosinophil numbers in the airways of the infected group. On the other hand, in antibiotic-treated mice helminth

infection did not attenuate the severity of inflammation. Noteworthy, when antibiotic-treated mice were cohoused with helminth infected mice they exhibited the same protective effect (250). Nucleotide-binding and oligomerization domain containing protein 2 $(NOD2)^{-/-}$ mice showed increases in *Bacteroides vulgatus*, whereas infection with the GI nematodes *T. muris* or *H. polygyrus* completely inhibited growth of *B. vulgatus*, and was accompanied by a significant decrease in *Prevotella* and *Bacteroides*, and significant increases in the *Lachnospiraceae* family of the order Clostridiales. The $NOD2^{-/-}$ infected mice showed increased IL-4 and IL-13 with decreased IFN-γ levels. Administration of a mixture of Clostridiales strains or cohousing of the $NOD2^{-/-}$ uninfected mice with $NOD2^{-/-}$ infected mice both resulted in reduction in *B. vulgatus* levels (251). *H. polygyrus was* also found to have a protective effect against respiratory syncytial virus infection in the lung, and this was dependent on the presence of the microbiota, as GF mice showed no protective effect against viral infection when infected with the helminths. The protective effect was Th2-independent as $Rag^{-/-}$ and $IL\text{-}4^{-/-}$ mice maintained the same effect (252). Colonisation of rats with the tapeworm *H. diminuta* led to a shift in the microbial community. Most of the changes observed within the Firmicutes phylum involved an increase in *Clostridia* and a decrease in *Bacilli* (253). Moreover, infection with *H. polygyrus* suppresses obesity via increased UCP1 expression in AT and serum norepinephrine (NE) concentration. This was associated with an increase in *Firmicutes* and *Proteobacteria* phyla, and mainly in *Bacillus* and *Escherichia* species (254). In another model, infection with *Strongyloides venezuelensis* improved insulin sensitivity in a mouse model of obesity. This was associated with an increase in Firmicutes phylum mainly in *Lactobacillus spp.*, and decrease in Bacteroidetes phylum with a decrease in circulating LPS, and an increase in M2 MACs in the adipose tissue, and circulating IL-10 (255).

Furthermore, in the collagen-induced arthritis model, administration of ES-62 protects against arthritis-dependent modulation of the gut microbiome (256). ES-62 administration normalises the gut microbiota communities (mainly *Clostridiaceae*, *Lachnospiraceae* and *Ruminococcaceae*) and maintains barrier integrity. This was associated with an increase in splenic $IL\text{-}10^{+}$ B cells and serum IL-10 levels and a decrease in serum IL-6 levels. This effect was lost after antibiotic treatment (256).

In human studies, infection with *S. stercoralis* has been associated with a shift in the abundance of faecal microbiota, in particular *Leuconostocaceae*, *Ruminococcaceae* and *Paraprevotellaceae*, which were significantly increased in the infected groups while bacteria belonging to the order Turicibacterales were significantly decreased compared to the control groups (257). A significant increase in the intestinal microbial species richness of CeD patients was observed following infection with *N. americanus* with a trends towards increased abundance of species within the Bacteroides phylum (258-260). Moreover, higher microbial diversity was found in helminth-infected subjects

from indigenous communities in Malaysia compared to helminth negative subjects. Additionally, in a comparison between Malaysian and New York residents, Firmicutes were more abundant in the New York subjects while Cyanobacteria, Actinobacteria, Tenericutes and Proteobacteria were more abundant in Malaysian subjects (261).

This highlights the role of helminth-induced microbiota alterations in triggering host immunomodulatory pathways, which contributes to inhibition of inflammation and reduced disease severity (189).

1.7. Hypothesis underpinning this thesis

Microorganisms, including parasitic worms and gut microbiota, have co-evolved with their hosts over millennia. This coevolutionary relationship has established an immunological interaction that is essential for the formation and maintenance of a balanced immune system that is associated with induction of Th2/regulatory immune responses and suppression of Th1/Th17 inflammatory responses. As mentioned previously in this chapter, data from helminth endemic areas have revealed negative associations between helminth infection and incidence of many inflammatory and metabolic diseases such as IBD, T1D and T2D. In addition, experimental studies from mouse models and human trials showed protective effects of helminths and their ES products against different inflammation-mediated diseases. On the other hand, many inflammatory diseases, and in particular T2D, were found to associate with gut microbiota alterations. Moreover, other studies have highlighted the role of helminth infections in altering the composition of the gut microbiota. There is therefore a real need to uncouple this three-way relationship between helminths, the microbiome and inflammatory/metabolic diseases. Induction of Th1 immune responses that increase production of pro-inflammatory cytokines and M1 MACs in AT, liver and pancreas have been associated with T2D. Helminth infections induce eosinophils, which play a key role in maintaining M2 MACs. I hypothesise that induction of Th2 immune responses by *N. brasiliensis* and its L3 and adult ES products, characterised by eosinophilia in particular, will reduce the inflammatory milieu in target tissues associated with T2D and improve insulin sensitivity. Therefore, the major aim of this thesis is to examine the impact of infection with *N. brasiliensis* or administration of their ES products on the development of T2D. Microbial dysbiosis promotes the accumulation of pro-inflammatory Th1 and Th17 cells in the small intestine, and increases production of IL-12, IL-17, IFN-γ and TNF-α. However, in homeostatic conditions IECs promote Tregs, and increase production of RELMβ, Muc2, IL-10 and TGF-β that downregulate Th1/Th17 immune responses. Moreover, induction of Th2 immune responses, expansion of eosinophils, and M2 MACs in the IECs and increased production of Th2/regulatory cytokines such as IL-4, IL-5, IL-13, IL-10, TGF-β, RELMβ and Muc2 are the main features of the host immune response to many GI helminth infections. Targeting the gut microbiota

to counter obesity and metabolic-related diseases is highly topical. Given that helminth infections promote diversity in the composition of the gut microbiota culminating in a phenotype associated with good gut health, improved understanding of these interactions is essential to determine if helminths and their secreted products are to be considered as a novel therapeutic platform. Thus, I hypothesise that helminth infection provides an anti-inflammatory milieu that normalises gut microbiota composition and restores/maintains gut homeostasis. Therefore, in the second aim of this thesis I will investigate the effects of *N. brasiliensis* infection and their ES products on the diversity of the gut microbiota. In the final aim, I will further explore the interaction between helminths and the microbiota by determining whether *N. brasiliensis* and its ES products confer protection from T2D in a manner that is dependent on the alteration of the gut microbiota. Hence, the overall hypothesis of this project is that *N. brasiliensis* and its secreted products restore gut microbial homeostasis and reduce systemic inflammation, which in turn, can be exploited as a novel therapeutic for T2D.

CHAPTER 2

2. Gastrointestinal helminth infection improves insulin sensitivity and decreases systemic inflammation in a mouse model of type 2 diabetes

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2.1. Abstract

Diabetes is a major health problem and is considered one of the top 10 diseases leading to death globally. This disease has been widely associated with systemic and local inflammatory responses, and with alterations in the gut microbiota. Microorganisms, including parasitic worms and gut microbiota, have co-evolved with their hosts over millennia. This co-evolutionary relationship has established an immunological interaction that is essential for the formation and maintenance of a balanced immune system, including suppression of excessive inflammation. Herein we have shown that infection with the parasitic nematode *N. brasiliensis* significantly reduced fasting blood glucose, oral glucose tolerance and body weight in two different mouse models of T2D. We also found that the infection was associated with elevated type 2 immune responses including increased eosinophil numbers in the mesenteric lymph nodes, liver and adipose tissues as well as increased expression of *IL-4* and alternatively activated macrophage marker genes *Rentla*, and *Chil3* in adipose tissue, liver, and gut.

Our findings show that *N. brasiliensis* infection is associated with changes in local and systemic immune cell populations. These changes are associated with a reduction in systemic and local inflammation and might be responsible for the improved insulin sensitivity observed, suggesting that experimental hookworm infection could be a novel therapeutic approach for preventing T2D.

Keywords: Type 2 diabetes, *Nippostrongylus brasiliensis*, hookworm, eosinophils, OGTT oral glucose tolerance test, HGI high glycaemic index diet, HF high fat diet

2.2. Introduction

Diabetes is a metabolic disease resulting from the absence of, or deficiency in, insulin secretion, insulin action or both, leading to an abnormal metabolism of carbohydrates and elevated levels of glucose in the blood (3). The main types of diabetes are T1D, which represents around 10% of all diabetes cases, and T2D, which represents around 90% of all diabetes forms. Diabetes is a fast-growing health problem worldwide. According to the International Diabetes Federation there were 424.9 million people living with diabetes with a further 352.1 million with impaired glucose tolerance in 2017 (3). Diabetes caused 4 million deaths and accounted for 10.7% of global all-cause mortality, and cost USD 727 billion in healthcare spending in 2017 alone (3).

Cumulative evidence suggests that T2D is associated with inflammation. Induction of Th1 immune responses, in particular activation of M1 MACs and increased production of pro-inflammatory cytokines such as IL-1β, IFN-γ, TNF-α and IL-6 play a crucial role in the destruction of pancreatic β-cells, and insulin resistance in AT, liver and muscle (17). In contrast, cells such as ILC2s, eosinophils, and M2 MACs, as well as increased levels of Th2 cytokines such as IL-5, IL-4, and IL-13 have been found to regulate adipose tissue homeostasis (262, 263), liver regeneration (264), and gastrointestinal homeostasis (265), leading to whole body metabolic homeostasis.

Environmental changes such as changes in dietary habits, improved sanitation, vaccination and excessive use of antibiotics has reduced our exposure to various infectious agents and symbiotic microorganisms (including helminths and gut microbiota) that had a co-evolutionary relationship with humans (266). This relationship has established an immunological interaction with highly developed regulatory pathways that serve to dampen inappropriate immune responses, which are considered the key drivers in many immune-mediated disorders including T2D (267). Helminth infections have been found to induce Th2 immune responses by expansion of innate immune cells such as eosinophils, M2 MACs, ILCs, and upregulation of cytokines such as IL-4, IL-5 and IL-13. Furthermore, it has been widely shown that helminth infections promote expansion and/or recruitment of Tregs that play an important role in regulating inflammation (268, 269). Recent experimental evidence in animal models has highlighted the therapeutic role of helminth-mediated induction of Th2- and Treg-mediated immune responses in many inflammatory diseases such as IBD, MS, arthritis, asthma and T1D (270). Likewise, helminth infections also showed promising results in patients with IBD, CeD and MS (104, 105, 271).

In the context of diabetes, epidemiological studies from helminth-endemic areas such as Indonesia, rural China, India and Aboriginal communities from North-West Australia found an inverse relationship between helminth infection and incidence of T2D (123, 125, 126, 272). Additionally, it has been shown that helminth infection of mice with *N. brasiliensis*, *H. polygyrus*, *L. sigmodontis* and *S. mansoni* are associated with significant increases in ILC2s, eosinophils, M2

MACs, and Th2 cytokines that result in restoration of glucose levels and improved insulin sensitivity in mouse models of obesity (134-137, 262, 263).

Diabetes has also been found to associate with alterations in the composition of the gut microbiota. Human studies as well as studies in animal models of obesity and T2D revealed a shift in the abundance of the dominant gut phyla Bacteroidetes and Firmicutes (273-275). Shifts in the abundance of these phyla has also been observed after infection with the gastrointestinal nematodes *N. brasiliensis*, *T. muris* and *H. polygyrus* (244-247, 276, 277), suggesting that helminth infections might have a positive role in maintaining gut homeostasis and preventing the development of T2D (255).

In this study, we found that infection with *N. brasiliensis* maintains glucose homeostasis, probably via induction of Th2 immune responses characterised by increases in eosinophils, M2 MAC markers *Rentla,* and *Chil3*, and *IL-4* levels in lymphoid and non-lymphoid tissues in both high-glycaemic index (HGI), and high fat (HF) diet-induced T2D.

2.3. Materials and Methods

2.3.1. Ethics statement

All procedures were approved by the JCU Animal Ethics Committee, ethics application number A2244. The study protocols were in accordance with the 2007 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the 2001 Queensland Animal Care and Protection Act.

2.3.2. Animals and diet

Male C57BL/6 wild-type (WT) (JCU Townsville) mice were used for all experiments (10 mice per group). At the age of 5 weeks control groups were fed a normal control diet (NC), whereas to induce T2D mice were either fed a HGI diet (SF03-30; Speciality Feeds, Western Australia) or a HF diet (SF07-066; Speciality Feeds, Western Australia).

2.3.3. Helminth infection

N. brasiliensis life cycle was maintained in our laboratory at James Cook University (Cairns). Briefly, faeces from *N. brasiliensis*-infected rats were collected from days 5-9 post-infection. Egg-containing faeces were mixed with an equal amount of water and charcoal, distributed into Petri dish plates and incubated at 26°C. One week after incubation, L3 were collected from the faecal/charcoal culture plate, washed three times with PBS, then all infections with *N. brasiliensis* were performed by inoculating subcutaneously 500 third-stage larvae of *N. brasiliensis* (*Nb*L3) into the skin over the interscapular region. To determine if infection with *N. brasiliensis* could prevent T2D, mice were infected once every month with *N. brasiliensis* starting at 6 weeks of age.

2.3.4. Fasting blood glucose (FBG) and oral glucose tolerance test (OGTT)

The FBG was measured in 6-hour unfed mice. Blood sampling was performed by tail bleeding. Mice were screened for blood glucose levels every 2 weeks using Accu-Check® Performa (Roche). Mice were considered diabetic when glucose levels reached >12.0 mmol/L. For the OGTT, after initial blood collection (time 0) in 6-hour unfed mice, mice were administered D-glucose orally (2 g/kg body weight) by gavage. Blood sampling was performed by tail bleeding at 15, 30, 60, 90, and 120 minutes after administration of glucose.

2.3.5. Blood collection for cytokine analyses

Blood was collected by cardiac puncture for terminal procedures, or submandibular bleeding for non-terminal procedures. Serum was separated, collected and stored at -30°C for further analysis.

2.3.6. Isolation of MLNs, AT and liver

In brief, epididymal fat pads or liver from male mice fed with ND, HGI or HF diet were removed and minced into small pieces. Minced tissues were then transferred to a 50 ml conical tube containing 1 ml DPBS (0.5% BSA) (Sigma) and 3 ml collagenase type II (Life technologies), and incubated in a rotating shaker (200 rpm) at 37°C for 35 minutes The homogenates were filtered through a 70 μm strainer into a new tube and centrifuged at 500 *g* for 10 minutes at 4°C. Following centrifugation, the supernatant was discarded and the pellet was resuspended in 1× red blood cell lysis buffer (Sigma) followed by a washing step with 5 ml FACS buffer, and a final centrifugation at 500 *g* for 10 minutes at 4°C.

MLNs were collected and transferred to a 5 ml tube containing 1 ml of RPMI media (Gibco), then filtered through a 70 μm strainer.

Cell viability was assessed by Trypan Blue and cells were blocked using FcR blocking reagent (BD biosciences) for FACS analysis.

2.3.7. Flow cytometry

Cell surface marker analysis was performed using flow cytometry. Single-cell suspensions prepared from MLNs, AT and liver were collected from mice at the times indicated. Cell surface markers were stained for 30 minutes at 4°C with rat anti-mouse CD3/CD19-CF594 (Clone:145-2C11,1D3) F4/80-APC (Clone: T45-2342), CD11c-FITC (Clone: HL3), CD301-pecy7 (Clone: LOM-14), CD64-PerCp-Cy5.5 (Clone: X45-5/7.1), CD11b-BV650 (Clone: M1/70), Ly6G-efluor700 (Clone: 1A8) and Siglec-F-PE (Clone: E50-2440) (BD Bioscience). All antibody incubations were performed at 4°C for 30 minutes (isotype controls were included). Data were acquired using a BD FACS Aria and analysed using FlowJo software (Tree Star, Inc).

2.3.8. Quantitative real-time PCR

A small piece (<0.5 cm) of AT, liver and small intestine (SI) was collected in a 2 ml Eppendorf tube containing 1 ml TRIzol-reagent (Sigma) and homogenised using a TissueLyzer (QIAGEN). Tissues were homogenised and RNA was extracted using TRIzol-reagent (Sigma) following the manufacturer's protocol. RNA samples were reverse transcribed to cDNA as follows. After RNA quantification, 50-70 ng of each sample was transferred to a 0.2 ml tube and 1 µl of each of oligo(dT) (Qiagen) and 10 mM dNTPs were added, followed by incubation at 65°C for 5 minutes in a Veriti 96-well thermal cycler (Applied Biosystems) followed by incubation on ice for 2 minutes. Four (4) µl of first strand buffer (Qiagen), 1 µl of each of 0.1 M DTT (Qiagen), RNAse out and 0.5 µl of Superscript III were added to the sample (Qiagen). The sample was incubated for 60 minutes at 55°C, then 15 minutes at 70°C. Finally, cDNA was quantified on a Nanodrop 2000 (Thermo scientific).

For qPCR reactions, 100 ng of RNA was mixed with 12.5 µl of SYBR Green and 2.5 µl of each primer of the selected genes in a total volume of 25 µl per sample. A Rotor-Gene Q (QIAGEN) was used for real time thermal cycling. All genes were normalised for levels of transcription relative to the housekeeping gene β-actin.

2.3.9. Data analysis

Data were tested for statistical significance using GraphPad Prism software (version 6). A Mann-Whitney U test was applied to test statistically significant differences between two unpaired groups with non-parametric distribution. Data that were normally distributed were tested for statistical significance using the unpaired t test for comparisons of two groups or the ANOVA test followed by the Holm-Sidack multiple-comparison test to compare more than two groups. Values of $p < 0.05$ were considered statistically significant. Results are expressed as mean with SEM or means ± SD. *$p < 0.05$; **$p < 0.01$.

2.4. Results

2.4.1. Infection with *N. brasiliensis* maintained glucose homeostasis

In order to address the prophylactic and therapeutic effect of infection with *N. brasiliensis* on the outcome of T2D, we used two different models of diet (HGI and HF) to induce T2D in C57BL/6 mice. At week 5 of age, male C57BL/6 mice were divided into groups fed on a NC diet, HGI diet or HF diet for up to 30 weeks to induce T2D. To ascertain the prophylactic effect of the infection on T2D, mice were infected at week 6 and re-infected once every month until the end of the experiment. To ascertain the therapeutic effect, infection with *N. brasiliensis* started at week 24, and continued once every 3 weeks for a total of three infections.

As predicted, mice on either HGI or HF diet had a significant increase in the level of FBG compared to those on NC (Fig. 2.4.1A and B). Prophylactic infection as well as therapeutic infection with *N. brasiliensis* significantly decreased the FBG levels in all groups, compared to their respective uninfected groups (Fig. 2.4.1A and B). A similar result was also observed for the OGTT test. HGI and HF diet infected mice had significantly lower levels of blood glucose than the respective control (uninfected) groups at all time points, both prophylactically and therapeutically (Fig. 2.4.1 C-F). Of note, the blood glucose level of the HGI and HF diet infected groups was also comparable to those mice on a NC diet and infected with *N. brasiliensis* (Fig. 2.4.1 C-F).

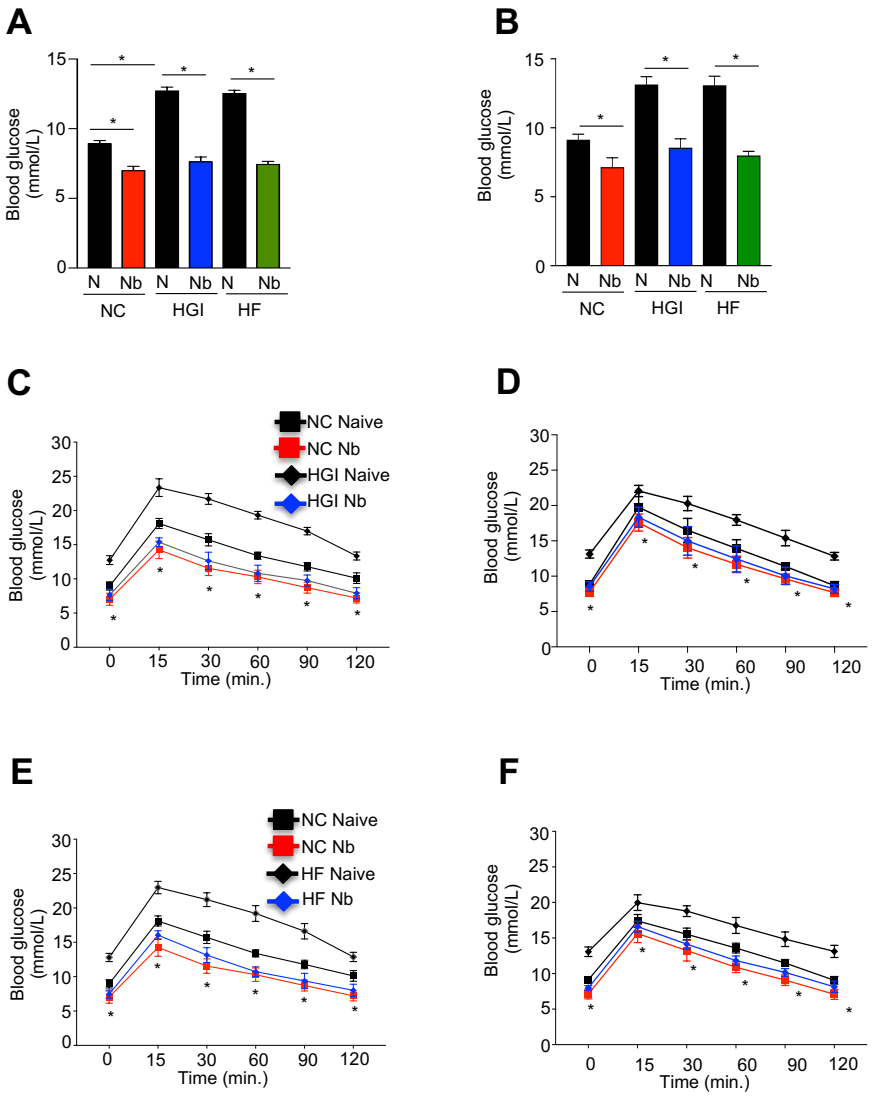

Fig 2.4.1. Infection with *N. brasiliensis* maintained glucose homeostasis.

C57/BL6 mice were fed NC or either HGI or HF diets and frequently infected with 500 *Nippostrongylus brasiliensis* L3 larvae. **(A)** Prophylactic FBG, HGI and HF diets; **(B)** therapeutic FBG, HGI and HF diets; **(C)** prophylactic OGTT, NC and HGI diets; **(D)** therapeutic OGTT, NC and HGI diets; **(E)** prophylactic OGTT, NC and HF diets; **(F)** therapeutic OGTT, NC and HF diets. Statistical significance was determined with Student's t test or Tow-way analysis of variance (ANOVA). Data are expressed as mean ± SEM or as means ± SD and are representative of 2 experiments where n = 5/group. *p < 0.05, **p < 0.01.

2.4.2. *N. brasiliensis* infection slowed weight gain in HGI and HF diet models of T2D

Reduction in the rate of body weight gain was also observed as a result of infection. Mice on either HGI or HF diets gained significantly more weight compared to mice on a NC diet (Fig. 2.4.2); however, infection with *N. brasiliensis* significantly reduced the body weight gain in all infected groups compared to their uninfected counterparts (Fig. 2.4.2).

These data indicate that in response to *N. brasiliensis* infection, HGI and HF diet groups maintained low levels of FBG and displayed improved glucose metabolism compared to uninfected controls.

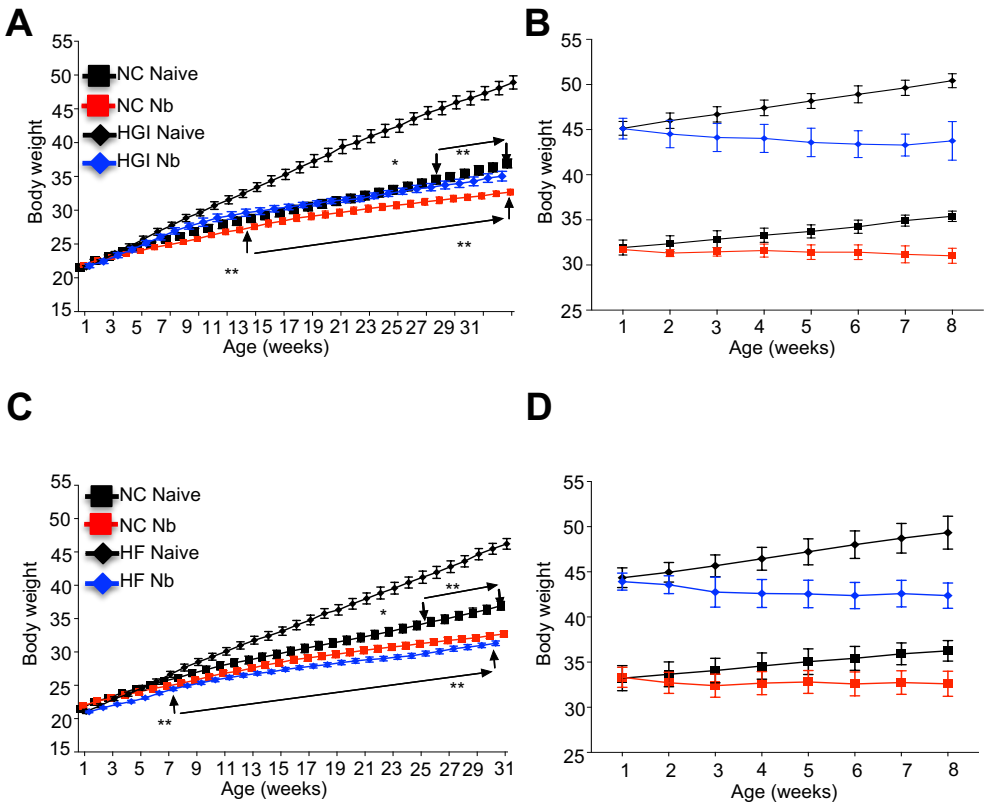

Fig 2.4.2. *N. brasiliensis* infection slowed weight gain in HGI and HF diet models of T2D.

C57/BL6 mice were fed NC or either HGI or HF diets then frequently infected with 500 *Nippostrongylus brasiliensis* L3 larvae. **(A)** Prophylactic body weight, NC and HGI diets. **(B)** Therapeutic Body weight, NC and HGI diets. **(C)** Prophylactic body weight, NC and HF diets. **(D)** Therapeutic Body weight, NC and HF diets. Statistical significance was determined with Tow-way analysis of variance (ANOVA). Data are expressed as means ± SD and are representative of 2 experiments where n = 5/group. *p < 0.05, **p < 0.01.

2.4.3. *N. brasiliensis* infection induces local eosinophilia and Th2 immune responses

To determine whether an increase in eosinophil numbers could induce a potent Th2 cytokine response and alternative activation of MACs in MLNs, AT, liver and gut, mice fed a NC, HGI or HF diet were infected with 500 *N. brasiliensis* L3 and sacrificed at different time points. At the end of the experiment MLNs, AT, liver and SI were collected. MLNs, AT and liver tissue were analysed by flow cytometry for eosinophils. qPCR analysis was performed on AT, liver and SI to assess M2 MAC expression markers. In response to *N. brasiliensis* infection, there was a significant increase in the total number of eosinophils in the MLNs, AT and liver in all groups compared to their uninfected littermates (Fig. 2.4.3A and B).

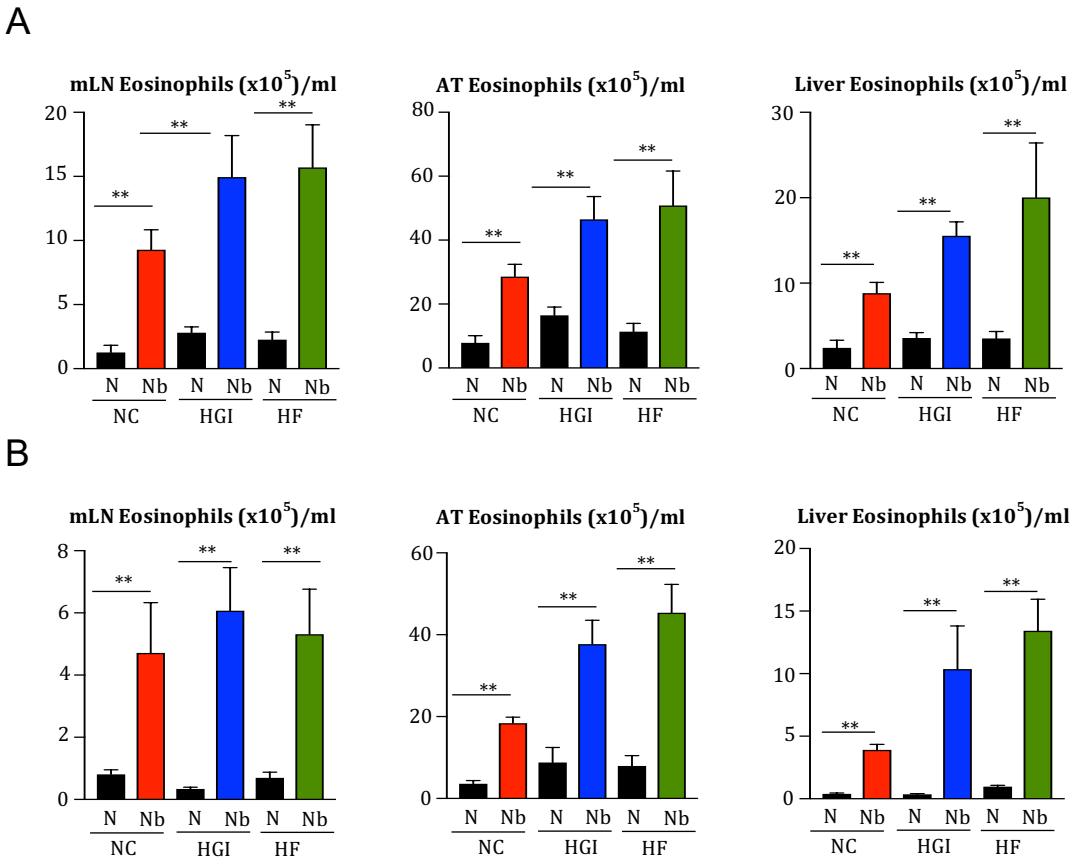

Fig 2.4.3. *N. brasiliensis* infection induces local and systemic eosinophilia.

C57/BL6 mice were fed NC, HGI or HF diets and frequently infected with 500 *Nippostrongylus brasiliensis* L3 larvae. **(A)** Prophylactic eosinophil frequency and total numbers in mLNs, AT and liver; **(B)** therapeutic eosinophil frequency and total numbers in mLNs, AT and liver. Statistical significance was determined with Student's t test. Data are expressed as mean ± SEM and are representative of two different experiments where n = 5/group. *p < 0.05, **p < 0.01.

2.4.4. Increased expression of genes involved in Th2 responses in AT, liver and small intestine of infected groups

N. brasiliensis infection significantly upregulated the transcripts for major Th2 cytokines and associated proteins, including *IL-4, Rentla*, and *Chil3* in AT, liver (Fig. 2.4.4) and SI (Fig. 2.4.5) of all the infected groups compared to their respective naïve groups.

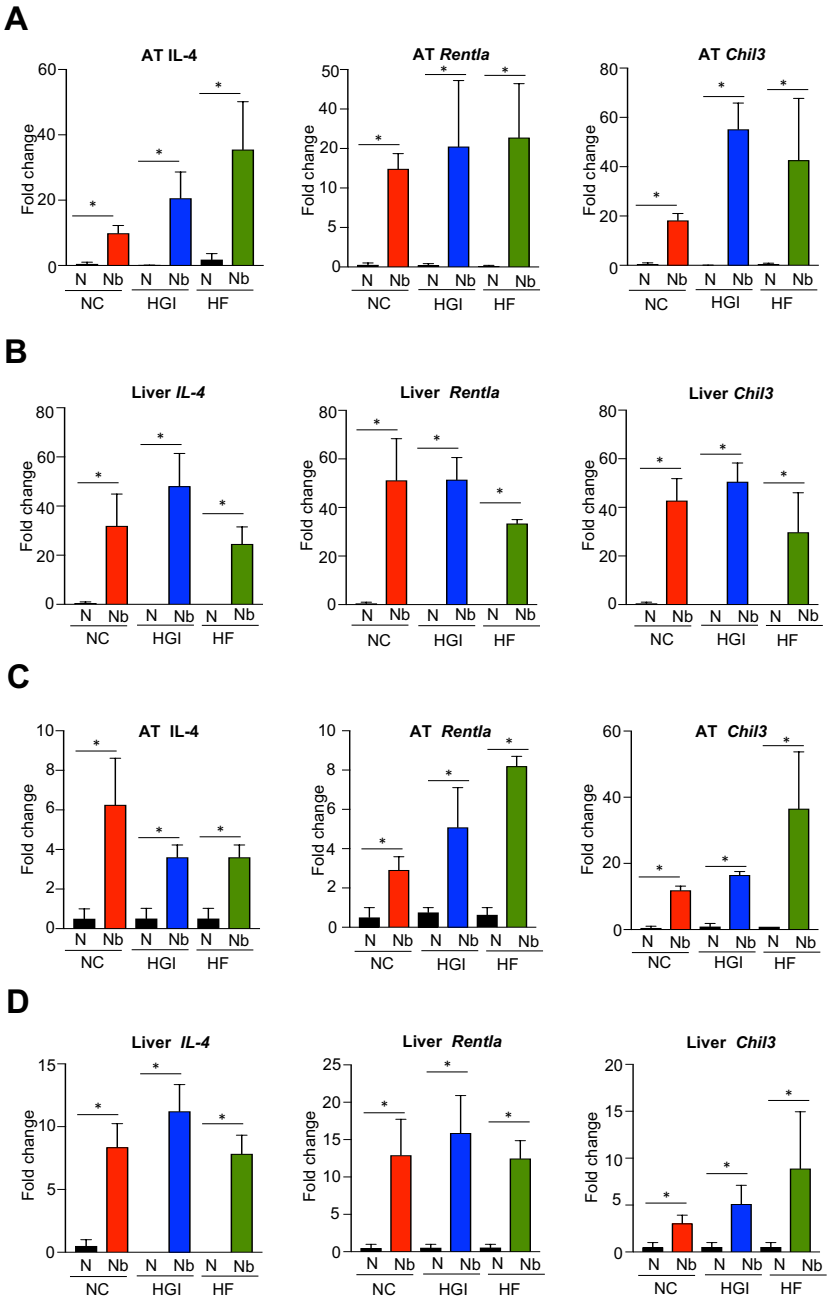

Fig 2.4.4. Increased expression of IL-4, Rentla and Chil3 in AT and liver of infected groups.

C57/BL6 mice were fed NC, HGI or HF diets and frequently infected with 500 *Nippostrongylus brasiliensis* L3 larvae. **(A)** Prophylactic *IL-4*, *Rentla* and *Chil3* levels in the AT; **(B)** prophylactic *IL-4*, *Rentla* and *Chil3* levels in the liver; **(C)** therapeutic *IL-4*, *Rentla* and *Chil3* levels in the AT; **(D)** therapeutic *IL-4*, *Rentla* and *Chil3* levels in the liver. Statistical significance was determined with Student's t test. Data are expressed as mean ± SEM and are representative of two different experiments where n = 5/group. *p < 0.05, **p < 0.01.

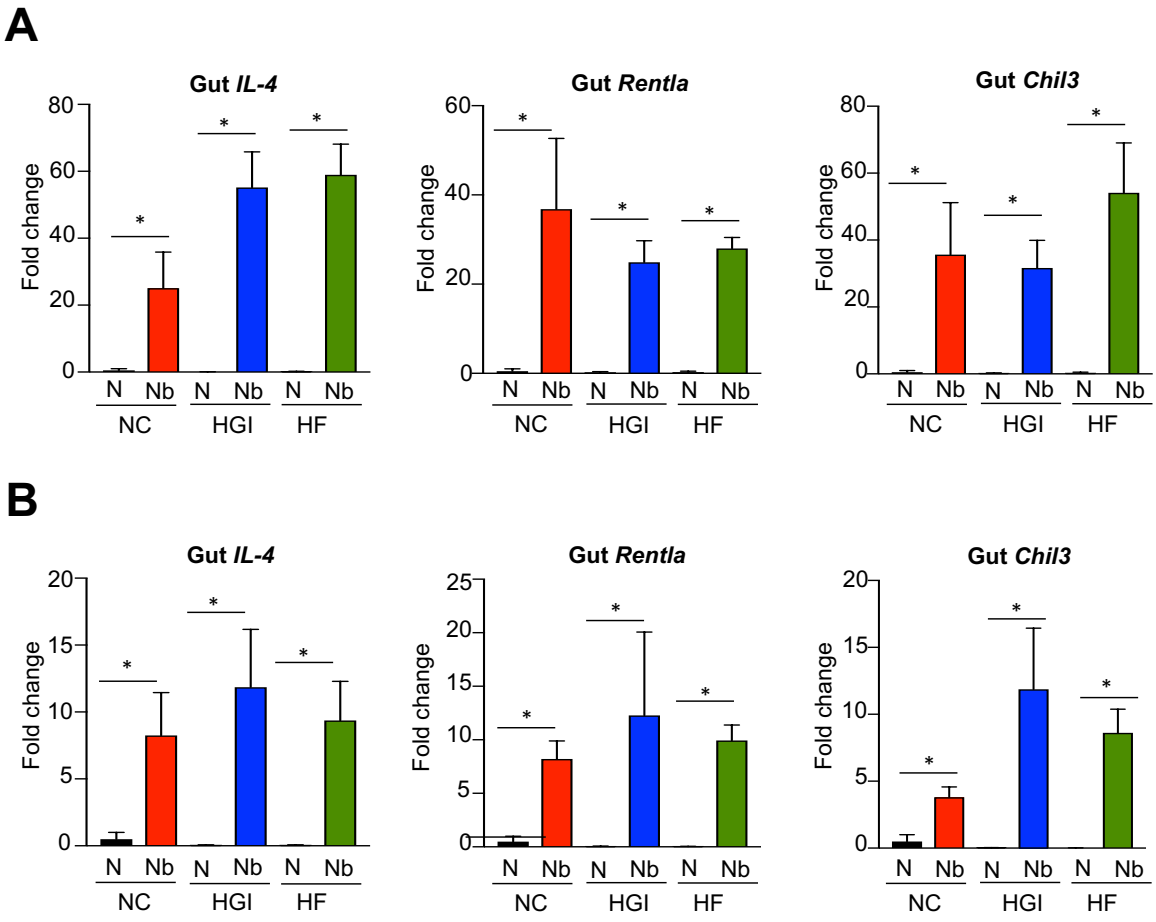

Fig 2.4.5. Increased expression of IL-4, Rentla and Chil3 in gut of infected groups

C57/BL6 mice were fed NC, HGI or HF diets and frequently infected with 500 *Nippostrongylus brasiliensis* L3 larvae. **(A)** Prophylactic *IL-4*, *Rentla* and *Chil3* levels in the gut; **(B)** therapeutic *IL-4*, *Rentla* and *Chil3* levels in the gut. Statistical significance was determined with Student's t test. Data are expressed as mean ± SEM and are representative of two different experiments where n = 5/group. *p < 0.05, **p < 0.01.

2.5. Discussion

Diabetes is recognised as the world's fastest growing chronic condition (3). The number of people with T2D is growing all over the world and its health and socioeconomic importance is indisputable (3). As mentioned previously, helminth infections have been associated with a lower prevalence of T2D, due to their ability to induce type 2 immune responses (123, 125, 126, 272). T2D is associated with Th1 and Th17 immune responses, innate immune cells such as M1 MACs and pro-inflammatory cytokines such as IL-1β, IL-6, IFN-γ, and TNF-α (278). However, in a normal state, resident ILCs, eosinophils and Tregs maintain metabolic homeostasis, favoring the production of Th2 cytokines such as IL-4, IL-5, IL-13, and IL-10 which also drive proliferation of M1 MACs towards an M2 MAC-like state (278, 279).

N. brasiliensis is a strong inducer of type 2 immunity, involving activation and expansion of $CD4^+$ T cells that produce cytokines such as IL-4, IL-5, IL-9, IL-10, and IL-13. This response induces a systemic and localised eosinophilia and activates ILC2 and M2 MACs, which play an important role in generating protective immunity against *N. brasiliensis* (89, 280-285). We therefore set out to investigate the role of helminth-induced type 2 immunity, and the mechanisms underlying protection against the development of T2D-induced insulin resistance. C57BL/6 mice were fed a HGI or HF diet and infected frequently with *N. brasiliensis*. This strain of mice is genetically susceptible to obesity, glucose intolerance, hyperglycaemia and T2D when fed a HF or HGI diet (286, 287). Here, we demonstrate that infection with *N. brasiliensis* had a beneficial effect, both prophylactically and therapeutically against T2D in two different diabetic diets. Our findings are consistent with a role for helminth infection in promoting type 2 immune responses by eliciting eosinophil production in MLNs, AT and liver, with increased levels of *IL-4* and markers of M2 MACs *Rentla* and *Chil3* in AT, liver and SI.

Loss and gain of function studies revealed the involvement of eosinophils, M2 MACs, ILCs and Th2 cytokines in lowering blood glucose levels, improving insulin sensitivity, increasing energy expenditure and decreasing adiposity (262, 263, 288-291). In our work we found an increase in *IL-4* gene expression in the AT, liver and SI of infected groups. Of note, overexpression of *IL-4* has been shown to reduce the adipocyte layer in the skin (292). Moreover, IL-4 restored insulin sensitivity in the 3T3-L1 cultured cell line used for adipocyte differentiation in a dose-dependent manner (293). The phosphoinositide 3-kinase (PI3K)/Protein kinase-B (AKT) pathway is involved in insulin signalling in AT, muscle and liver, mediating cellular functions such as glucose homeostasis, lipid metabolism, protein synthesis and cell proliferation and survival (294). Overexpression of *IL-4* induced glucose tolerance- and insulin sensitivity-associated reductions in body weight gain and fat mass via activation of AKT in a PI3K-dependent manner. IL-4 also regulates adipokines and free fatty acids levels, implicating its role in lipid metabolism (295). Furthermore, immune cell signalling via IL-4/IL-13-mediated activation of STAT6 is required for *N. brasiliensis* expulsion (284). Signalling via this pathway was also found to improve insulin resistance, decrease body weight gain and adiposity, regulate liver metabolism and decrease inflammation in AT (291). Interestingly, infection with *N. brasiliensis* downregulated expression of genes encoding enzymes involved in lipid metabolism in liver and AT, leading to weight loss via an IL-4/IL-13/STAT-6-dependent manner and activation of M2 MAC markers (136, 296).

Recently, eosinophils in particular have been implicated in glucose homeostasis and energy expenditure. These cells play an unexpected role in metabolic homeostasis through maintenance of adipose M2 MACs. Absence of eosinophils resulted in increased body weight gain and impaired

glucose tolerance in mice (262, 263, 290). Moreover, in the absence of eosinophils, mice also exhibit a defect in lipid metabolism in the liver and SI, an increase in the expression of pro-inflammatory IFN-γ and a decrease in the expression of IL-4 and IL-13 in AT (288).

In our work, *N. brasiliensis* induced MLN, AT and liver eosinophilia, and increased gene expression of M2 MAC markers in AT, liver and SI. This was consistent with other studies which showed that infection with *N. brasiliensis* induced adipose eosinophilia and M2 MACs, enhanced glucose tolerance and lipid metabolism and ameliorated body weight gain in different mouse models of obesity (136, 262, 263). These studies have proposed mechanisms by which these cells might influence AT homeostasis. Eosinophils in bone marrow and their recruitment into WAT are largely controlled by IL-5. The main source of adipose IL-5 is a newly recognised population of ILC2s (262). These cells induce the accumulation of eosinophil-derived IL-4 which sustains M2 MACs in the tissue (262, 263, 290). Moreover, the chemokine eotaxin-1 expressed by adipocytes attracts and promotes survival of eosinophils in AT (288). Eosinophils are also implicated in the AT (mainly brown and beige fat) thermogenesis process, which contributes to whole body energy expenditure. Mechanistically, eotaxin-1 induces eosinophils to secrete IL-4/IL-13. This leads to accumulation of M2 MACs and proliferation of adipocytes which increases energy expenditure, reduces weight gain and improves glucose intolerance (297, 298). Loss of eotaxin-1, eosinophils or IL-4/IL-13 impairs the process (297-299).

In line with our results, studies utilising the HF diet model of obesity showed improvements in glucose intolerance of obese mice after infection with the filarial nematode *L. sigmodontis* or administration of soluble adult worm extract. This was associated with increased numbers of $CD4^+$ T cells, eosinophils and M2 MACs, and depletion of eosinophils resulted in impaired glucose tolerance (137). Chronic *S. mansoni* infection and administration of schistosome soluble egg antigens resulted in increased numbers of AT eosinophils, M2 MACs and Th2 cytokines, and a corresponding decrease in body weight gain and improved insulin sensitivity in obese mice (300). Infection with the gastrointestinal nematode *H. polygyrus* also resulted in decreased body weight gain and improved glucose and lipid metabolism, and an associated increase in Th2/Treg immune responses in the MLNs, AT and SI. Moreover, infected mice on a HF diet displayed dysregulated expression of genes and proteins involved in energy expenditure and lipid metabolism in AT and liver (134, 135).

This study has revealed that chronic infection with *N. brasiliensis* protects against metabolic disorders in mouse models of diet-induced T2D. We have established that *N. brasiliensis* reduces body weight gain, promotes peripheral glucose uptake and specific glucose sensitivity. Through analysis of immune cell composition at the cellular level, we show that *N. brasiliensis* infection promotes eosinophil accumulation and Th2 responses in MLNs, AT, liver and SI. Mechanistically,

the increase in eosinophil numbers in mice fed a HGI or HF diet following *N. brasiliensis* infection, may be the result of local and systemic increases in eosinophils and Th2 cytokines, as well as increases in M2 MAC numbers that regulate many key events involved in the control of metabolic homeostasis. It is not yet clear whether the eosinophil-mediated regulation of obesity-induced insulin resistance and AT inflammation is due to the direct and primary effects of eosinophils on insulin resistance or due to secondary effects of eosinophils on changes in body weight and adiposity. Further studies are required to elucidate the functions of helminth-induced eosinophils in terms of their beneficial and detrimental effects in driving metabolic reprogramming, and the therapeutic utility of this phenomenon for treating the global epidemic of metabolic disorders.

Acknowledgments

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CHAPTER 3

3. Immune modulation by hookworm excretory/secretory proteins improves insulin sensitivity in a mouse model of type 2 diabetes

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3.1. Abstract

Diabetes is recognised as the world's fastest growing chronic condition. The number of people with T2D is growing all over the world. Helminth infections have been shown to be associated with a lower prevalence of T2D, mainly due to their ability to induce a type 2 immune response. Therefore, to understand the molecular mechanisms that underlie the development of T2D-induced insulin resistance, we treated mice fed on normal or diabetes-promoting diets with *Nippostrongylus brasiliensis* excretory/secretory products (ES). We demonstrated that treatment with crude adult (AES) or infective L3 ES (L3ES) from *N. brasiliensis* improved glucose tolerance and attenuated body weight gain in mice fed a HGI diet. In addition, we found for the first time that *N. brasiliensis* ES treatment was associated with type 2 immune responses measured by an increase of eosinophils and IL-5 in peripheral tissue but not IL-4 and with a decrease in the level of IL-6 in AT while increase in its level in the liver

These data highlight a role for *N. brasiliensis* ES in modulating the immune response associated with T2D, and suggest that *N. brasiliensis* ES contain molecules with therapeutic potential for treating metabolic syndrome and T2D.

Keywords: *Nippostrongylus brasiliensis*, helminth, diabetes, excretory/secretory products, Th2, eosinophils, M2 MAC

3.2. Introduction

T2D is a metabolic disease resulting from defects in protein, fat and carbohydrate metabolism and elevated levels of glucose in the blood that lead to impairment in insulin action and to relative deficiency in insulin secretion (3). The incidence of T2D is increasing in all regions of the world. According to the International Diabetes Federation (IDF), there were 424.9 million people with diabetes in 2017 and this number is expected to reach 628.6 million people by 2045, with T2D accounting for 90% of these cases (3).

It is well established that helminth infection induces Th2 and Treg immune responses in infected hosts as a survival strategy for these chronic pathogens (301). The resulting immune response suppresses immunopathology induced by infection with these large parasites but also contributes to the overall protection against immune mediated diseases (80). It is also becoming apparent that helminth secretions or excretory/secretory (ES) products include potent factors that can modulate the host's immune response, contributing to the control or prevention of inflammation-mediated diseases (164). Recently, in a proteomic analysis conducted in our lab, almost 200 ES proteins from different developmental stages of the hookworm *Necator americanus* were identified (302). Sperm coating protein-like extracellular proteins (SCP/TAPS) also known as Venom allergen-like (VAL) or Activation-associated Secreted Proteins (ASPs) and different mechanistic classes of proteases were over-represented, and these proteins shared different degrees of homology with proteins from other related nematodes such as *A. caninum*, *H. polygyrus*, and *N. brasiliensis* (302). ES products released by hookworms drive an immunoe-regulatory environment characterised by a polarisation towards the production of anti-inflammatory cytokines such as IL-10 and TGFβ as well as Treg cells, ILC2s, tolerogenic DCs, and M2 MACs (303, 304). Indeed, it has been previously reported that crude ES of the adult hookworm *A. caninum* (*Ac*ES), as well as a recombinant version of the most abundant *Ac*ES protein AIP-1, and *Ac*ES low molecular weight metabolites were all capable of reducing chemically-induced colitis in mice (169, 305-307). This was associated with induction of Th2/anti-inflammatory cytokines such as IL-4, IL-5, IL-10 (169), TGF-β and thymic stromal lymphopoietin (TSLP) (305), and downregulation of Th1/pro-inflammatory cytokines such as IL-6, IL-17, IFN-γ (169), and TNF-α (305), and recruitment of M2 MACs, eosinophils (169) and Treg cells (305) to the site of ES administration (169, 305). It has also been shown that the recombinant protein *Ac*-AIP-2, one of the most abundant *Ac*ES proteins, suppressed airway inflammation in a mouse model of asthma via induction of tolerogenic DCs and FoxP3$^+$ Tregs (180). ES products of another GI nematode, *H. polygyrus* (HES) also impaired DC function and induced Treg cells (308, 309). ES products of *N. brasiliensis* have also been found to modulate DCs, favouring the induction of Th2 immune responses (153). Moreover, in a recent study, extracellular vesicles from *N. brasiliensis* were shown to protect against TNBS-induced colitis in mice. This was associated with suppression of the inflammatory

cytokines IL-6, IL-1β, IFNγ and IL-17a and an increase in the anti-inflammatory cytokine IL-10 (163). Furthermore, ES products from adult *N. brasiliensis* (171), and recombinant forms of the cysteine protease inhibitors Nippocystatin (rNB-Cys) from *N. brasiliensis* and (rHp-CPI) from *H. polygyrus* suppressed antigen-specific antibody production (172) and IFNγ production (310), and simultaneously induced ES-specific Th2 immune responses (171) in ovalbumin-immunised mice (171, 172, 310). It has also been revealed that the filarial secreted protein ES-62 inhibited pro-inflammatory Th1 cytokines (i.e. TNF-α, IL-6 and IFNγ) and suppressed severity and progression of collagen-induced arthritis (CIA) *in vivo* using DBA/1 mice in a murine model of rheumatoid arthritis (RA), and *in vitro* using synovial fluid and peripheral blood mononuclear cells (PBMCs) from RA patients (311). ES-62 administered to mice also induced M2 MACs and IL-10-producing B cells, and decreased the levels of IL-22 in the kidney that suppressed development of proteinuria and protected against kidney damage in a mouse model of systemic lupus erythematosus (312).

In the context of diabetes, the ES products from different parasites have been shown to confer protection against T1D and T2D. For instance, the ES products of *F. hepatica*, and *S. mansoni* SEA prevented T1D in NOD mice via induction of Bregs, Tregs, M2 MACs and DCs, and increased IL-4, IL-10 and TGF-β levels (184, 185). Moreover, treatment with SEA (138), LNFPIII glycan from SEA (also found in human breast milk) (188), SEA T2 RNase recombinant protein-derived ω1 (ω1) (313) or soluble products from *Trichuris suis* (TsSP) (314) improved insulin sensitivity and suppressed liver lipogenesis in HF diet-induced obesity in mice. This was associated with increased numbers of ILC2s, eosinophils and M2 MACs and increased levels of Th2 cytokines and the alarmin IL-33 in AT and liver (138, 188, 313, 314). In another study, administration of somatic extract from the filarial nematode *L. sigmosoides* improved metabolic homeostasis and insulin sensitivity in a murine obesity model. This was also associated with increase in the eosinophils, M2 MACs, ILC2s, and expression of *Arg-1*, *Foxp3*, *Gata3* and *IL-10* (137). In a recent study, using a mouse model of T2D, researchers found that administration of *S. japonicum* SEA protect against T2D via induction of Tregs and Th2 immune responses, which was characterised by an increase in the frequency of $CD25^{+}Foxp3^{+}$ T cells, and the expression of IL-4 and IL-5 in the spleen of the treated groups (315).

In the present study, we describe a role for ES products from the gastrointestinal nematode *N. brasiliensis* in modulating the immune response associated with T2D. Intraperitoneal injection of *N. brasiliensis* ES resulted in reduced glucose levels and reduced body weight gain in a mouse model of T2D, which was mediated by activation of systemic and local eosinophils and IL-5 but not IL-4. *N. brasiliensis* ES administration also decreased the levels of IL-6 in AT but increased them in the liver. Together, these data highlight for the first time the importance of *N. brasiliensis* ES products in modulating the immune response associated with T2D.

3.3. Materials and Methods

3.3.1. Ethics statement

All procedures were approved by the JCU Animal Ethics Committee, ethics application number A2244.

3.3.2. Animals and diet

Male C57BL/6 wild-type (WT) (JCU Townsville) mice were used. At the age of 5 weeks mice were separated into 2 main groups: one group was fed a normal control (NC) diet and the other group was fed a high glycaemic index (HGI) diet (SF03-30; Speciality Feeds, Western Australia) to induce T2D.

3.3.3. ES preparation and administration

For preparation of the crude L3ES proteins, the *N. brasiliensis* life cycle was maintained in our laboratory at James Cook University (Cairns). Briefly, faeces from *N. brasiliensis*-infected rats were collected from days 5-9 post-infection. Egg-containing faeces were mixed with an equal amount of water and charcoal, distributed into Petri dish plates and incubated at 26°C. One week after incubation, L3 were collected from the faecal/charcoal culture plate, washed three times with PBS, then three times with PBS supplemented with 5% antibiotic-antimycotic (AA) (Gibco). L3 were then transferred into a flat bottom 24 well-plate, cultured in serum-free RPMI media (Gibco) supplemented with 2% AA and 1% D-glucose (Sigma) and incubated at 37°C and 5% CO_2 at a density of 500 L3/well. The supernatant was then collected daily from days 1-10, ensuring motility of the worms at all times. Supernatants were stored at -30°C before protein concentration and lipopolysaccharide (LPS) content were determined.

For adult ES (AES) preparation, rats were infected with 3,000 L3 *N. brasiliensis*. At day 6 post-infection rats were euthanized and the small intestine collected and opened in a Petri dish containing RPMI and incubated at 37°C and 5% CO_2 for 2 hours. Adult worms were then collected, washed three times with PBS followed by three washes with PBS supplemented with 5% AA and one wash with RPMI media supplemented with 2% AA. 100 worms/well were then transferred into a flat bottom 24 well-plate and incubated in RPMI media supplemented with 2% AA, 1% D-glucose and 1% Glutamax at 37°C and 5% CO_2. After 24h the supernatant was discarded to minimise contamination with host proteins and replaced with new media. AES was collected at day 2 and replaced subsequently every day until day 10. Supernatants were stored at -30°C before protein concentration and lipopolysaccharide (LPS) content were determined .

Amicon ultrafiltration 3 kDa tubes (Thermo scientific) were used for protein concentration and buffer exchange with PBS. The Pierce BCA protein assay kit (Thermo scientific) was used for

quantification of the protein content following the manufacturer's instructions. The Pierce LAL Chromogenic Endotoxin Quantification Kit (Thermo Scientific) was used for the quantification of LPS in AES and L3ES preps. Firstly, AES and the L3ES were incubated with 1% Triton-114 at 4°C on a rotor (Ratek) overnight with low constant stirring. After that, ES proteins were incubated at 37°C for 10 minutes using a dry bath incubator (Major Science), followed by centrifugation in a Microfuge 22R centrifuge (Beckman Coulter) at 14,000 *g* for 5 minutes. The top layer was collected and prepared for LPS quantification. Briefly, 4 standards were prepared, ranging from 1- 0.1 EU/ml. Then, 50 µl of the standards and ES samples were added in duplicate to a 96 well plate and incubated at 37°C and 5% CO_2 for 5 minutes Subsequently, 50 µl of Limulus Amebocyte Lysate was added and the plate was incubated at 37°C for 10 minutes, followed by addition of 50 µl of Chromogenic Substrate and incubation for 6 minutes at 37°C. Then, 50 µl of the stop solution was added and the results were read on a BMG Polarstar Omega fluorescence microplate reader at 405 nm. The ES proteins were suitable for use when the LPS content was below 5 EU/mg of protein.

The ES products were administered to mice intraperitoneally twice weekly from week 6 until the end of the experiment at a dose of 1 mg/kg. Control groups were administered intraperitoneally with the same volume of PBS.

3.3.4. Fasting Blood Glucose (FBG) and Oral Glucose Tolerance Test (OGTT)

The FBG was measured in 6-hour unfed mice. Blood sampling was performed by tail bleeding using Accu-Check® Performa (Roche). Mice were considered diabetic when glucose levels reached > 12.0 mmol/L. The OGTT was measured in 6-hour unfed mice, after initial blood collection (time 0). Mice were administered D-glucose orally (2 g/kg body weight) by gavage. Blood sampling was performed by tail bleeding at 15, 30, 60, 90, and 120 minutes after administration of glucose.

3.3.5. Tissue collection and cytokine analysis

The AT, liver and small intestine were collected and washed with PBS; one cm of the tissue was combined with 0.5 mL of PBS, homogenised using a TissueLyzer (QIAGEN), centrifuged and supernatants stored at -80°C until use. The ELISA Ready-Set-Go set (eBioscience) for the cytokines IL-4, IL-5, IL-6 was used following the manufacturer's instructions of standard sandwich ELISA protocols. In brief, a 96-well flat bottom plate was coated with 50 µl of capture antibody and incubated overnight at 4°C. The plate was then washed 3 times with washing buffer. Then, 50 µl of blocking reagent was added and the plate was incubated for 1 hour at room temperature. The washing step was performed twice. 50 µl from the samples and from serial dilution of the standards were added to the plate and incubated overnight at 4°C, followed by 3 more washing steps. Subsequently, 50 µl of detection antibody was added, incubated for 30 minutes at room temperature and followed by 3 more washes. Then, 50 µl of substrate solution was added and incubated for 15 minutes at room

temperature. Finally, 50 µl of stop solution (1M HCl) was added and the absorbance was read on a BMG Polarstar Omega at 450 nm. The lower detection limit for all cytokines was ≥ 6 pg/ml.

3.3.6. Isolation of the mesenteric lymph nodes, adipose tissue and liver for flow cytometry analysis

In brief, epididymal fat pads or liver from male mice fed a NC or HGI diet were removed and minced into small pieces, then transferred to 50 ml conical tubes containing 1 ml DPBS (0.5% BSA) (Sigma) and 3 ml collagenase type II (Life Technologies), and incubated on a rotational shaker (200 rpm) at 37°C for 35 minutes The AT or liver homogenates were filtered through a 70 µm strainer into a new tube and centrifuged at 500 *g* for 10 minutes at 4°C. Following centrifugation, the supernatant was discarded and the pellet was resuspended in 1× red blood cell lysis buffer (Sigma), followed by a washing step with 5 ml FACS buffer and centrifugation at 500 *g* for 10 minutes at 4°C (316, 317). MLNs were removed and filtered through a 70 µm strainer. Cell viability was assessed by Trypan Blue and cells were blocked for FACS analysis. Cell surface marker analysis was performed using flow cytometry. Single-cell suspensions prepared from MLNs, AT and liver were collected from mice at the times indicated. Cell surface markers were stained for 30 minutes at 4°C with CD3/CD19-CF594 (Clone:145-2C11,1D3) F4/80-APC (Clone: T45-2342), CD11c-FITC (Clone: HL3), CD301-pecy7 (Clone: LOM-14), CD64-PerCp-Cy5.5 (Clone: X45-5/7.1), CD11b-BV650 (Clone: M1/70), Ly6G-efluor700 (Clone: 1A8) and Siglec-F-PE (Clone: E50-2440) (BD Bioscience). All antibody incubations were performed at 4°C for 30 minutes (isotype controls were included). Data were acquired using a BD FACS Aria with BD FACS DIVA software (BD Bioscience) and analysed using FlowJo software (Tree Star, Inc).

3.3.7. Data analysis

Data were tested for statistical significance using GraphPad Prism software (version 6). The Mann-Whitney U test was applied to test differences between two unpaired groups with nonparametric distribution for statistical significance. Data that were normally distributed were tested for statistical significance using the unpaired t test for comparisons of two groups or the ANOVA test followed by the Holm-Sidack multiple-comparison test to compare more than two groups. Values of $p < 0.05$ were considered statistically significant. Results are expressed as mean with SEM or means ± SD. *$p < 0.05$; **$p < 0.01$.

3.4. Results

3.4.1. Treatment with either AES or L3ES from *N. brasiliensis* improves glucose tolerance and attenuates body weight gain

In order to address the prophylactic effect of *N. brasiliensis* ES on the outcome of T2D, 5 week-old male C57BL/6 mice were used; half the mice were fed NC diet and the other half were fed a HGI diet to induce T2D for up to 30 weeks. Mice were treated twice weekly with *N. brasiliensis* AES or L3ES at a dose of 1 mg/kg starting at week 6 until the end of the experiment. Mice treated with AES or L3ES showed a significant decrease in the level of FBG compared to the control groups (Fig. 3.4.1A). Moreover, results from the OGTT test showed that NC and HGI diet groups treated with AES or L3ES had significantly lower levels of blood glucose at all time points (Fig. 3.4.1B and C). Reduction in body weight gain was also observed as a result of the treatment. Mice on either NC or HGI diet treated with AES or L3ES gained less weight compared to control groups (Fig. 3.4.1D and E).

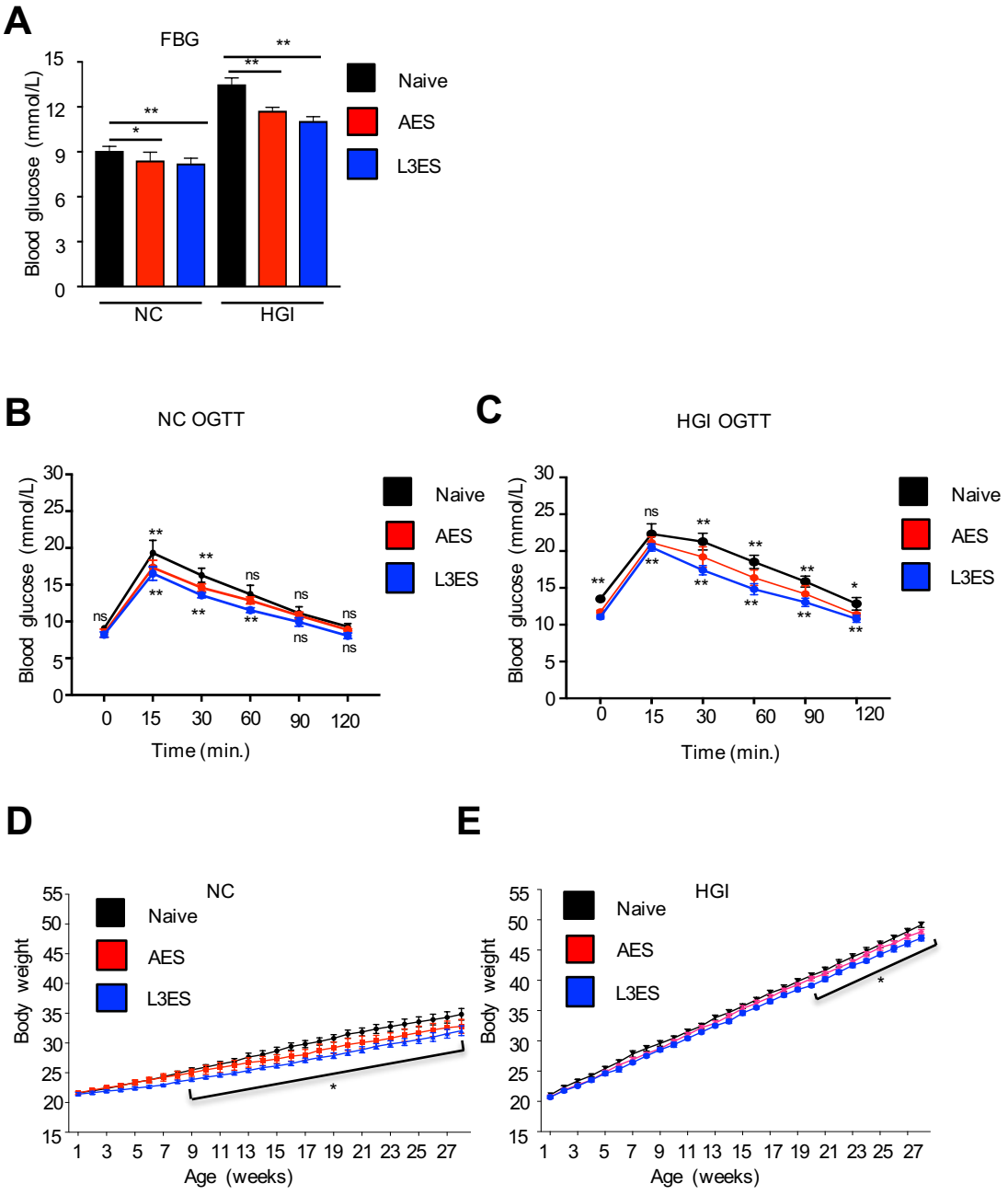

Fig 3.4.1. Treatment with either AES or L3ES from *N. brasiliensis* improves glucose tolerance and attenuates body weight gain.

C57/BL6 mice were fed NC or HGI diet and treated frequently with L3ES or AES of *N. brasiliensis*. **(A)** FBG; **(B)** OGTT, ND; **(C)** OGTT, HGI; **(D)** Weight, NC; **(E)** Weight, HGI. Statistical significance was determined with Student's t test or Tow-way analysis of variance (ANOVA). Data are expressed as mean ± SEM or as means ± SD and are representative of 1 experiment where n = 10/group. *p < 0.05, **p < 0.01.

3.4.2. Treatment with *N. brasiliensis* ES induces tissues eosinophilia and Th2 immune responses

Mice were fed an HGI diet and treated with AES or L3ES of *N. brasiliensis* as described above. At the end of the experiment, various tissues were collected such as MLNs, AT and liver for further characterisation of the Th2 immune response. Interestingly, in response to *N. brasiliensis* AES or L3ES injection, there was a significant increase in the total number of eosinophils in the mLNs, AT and liver of the HGI and NC diet groups compared to the control group (Fig. 3.4.2A).

To assess whether the increase in the eosinophils numbers induced a potent Th2 cytokine response by elevating cytokine levels in AT and liver we analysed the levels of several cytokines by ELISA. Injection of AES or L3ES significantly increased the levels of Th2 cytokines, measured by IL-5 in AT and liver (Fig. 3.4.2B and C) compared to the naïve groups. However, our study showed no significant differences in the levels of IL-4 between treated and un-treated groups (Fig. 3.4.2B, and C). Moreover, groups treated with either AES or L3ES had significantly lower levels of IL-6 in the AT (Fig. 3.4.2B) while this level was higher in the liver of mice treated with AES (Fig. 3.4.2C).

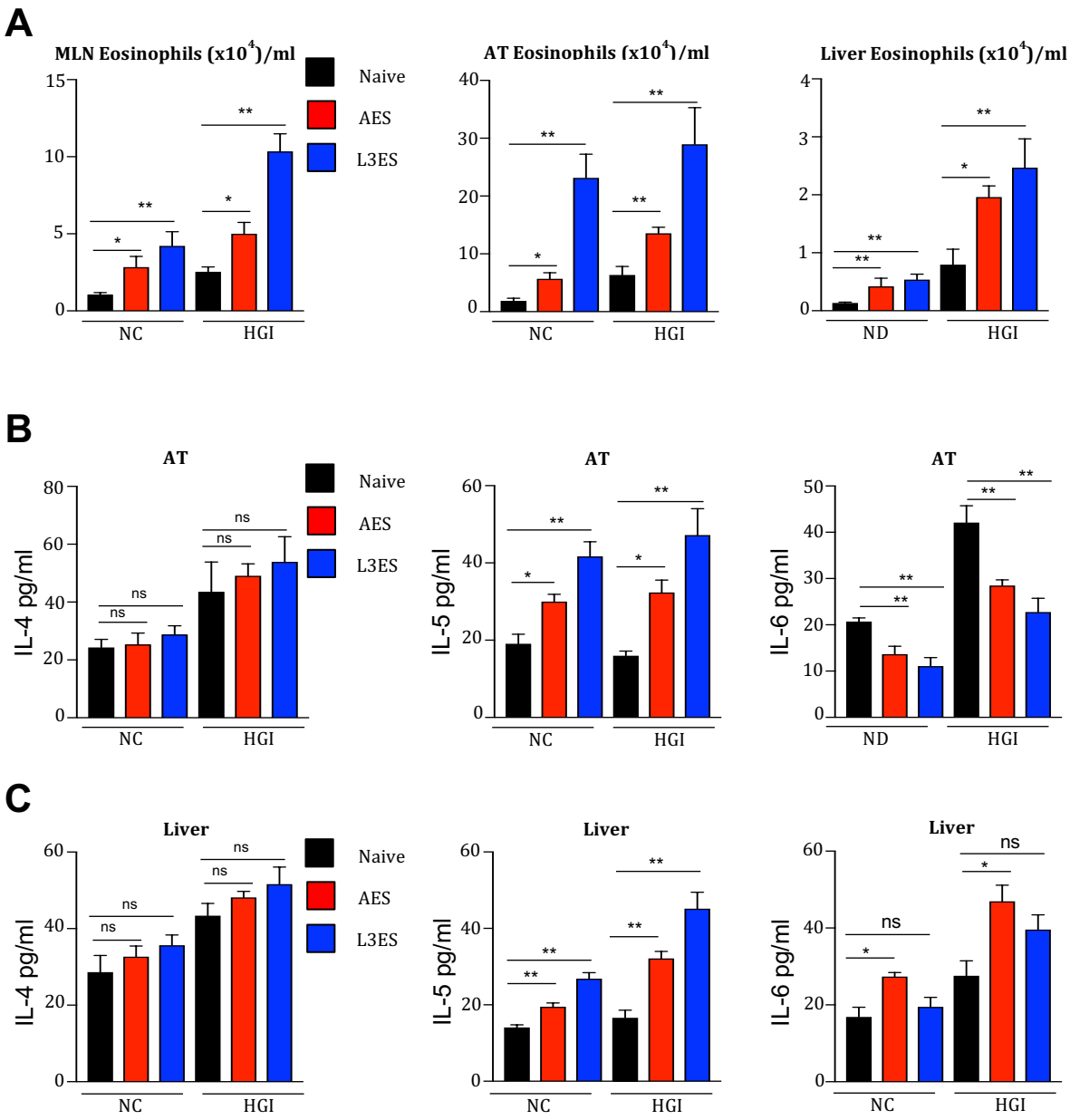

Fig 3.4.2. Treatment with *N. brasiliensis* ES induces tissues eosinophilia and Th2 immune responses.

C57/BL6 mice were fed normal control (NC) or high glycaemic index (HGI) diet and treated twice weekly with L3ES or AES of *N. brasiliensis*. (A) Eosinophil frequency and total numbers in mLNs, AT and Liver. (B) AT IL-4, IL-5 and IL-6 expression. (C) Liver IL-4, IL-5 and IL-6 expression. Statistical significance was determined with Student's t test. Data are expressed as mean ± SEM and are representative of 1 experiment where n = 10/group. *$p < 0.05$, **$p < 0.01$.

3.5. Discussion

Incidence and prevalence of diabetes is on the rise globally, imposing a drastic socio-economic burden on public health (3). Many researchers have reported that helminths and their secretions can modulate the host's immune response, which might have beneficial effects in controlling or preventing the inflammatory responses associated with immune-mediated diseases (164). Understanding the mechanisms by which helminth ES products can skew the immune response away from a T2D-promoting phenotype is critical for the rational design of preventative therapies against this disease. It has been found that *N. brasiliensis* and its secreted products are able to induce a Th2 immune response. This was characterised by an increase in a systemic and localised eosinophilia, ILC2s, M2 MACs, DCs, and increased production of cytokines such as IL-4, IL-5, IL-9, IL-10 and IL-13 (89, 153, 154, 280). In addition, it has been previously reported that administration of SEA from *S. mansoni* or *S. japonicum* (138, 185, 315) or LsAg from the adult filarial nematode *L. sigmodontis* (137) protect against T1D and T2D in mice (185, 315) and improved insulin sensitivity in obese mice (137, 138).

In the present study, we showed that administration of *N. brasiliensis* crude ES from adult and L3 developmental stages attenuated the clinical indicators of T2D in the HGI diet mouse model. Our data showed that AES and L3ES administration ameliorates glucose intolerance and attenuates body weight gain in the treated groups. Previous findings using SEA from *S. japonicum* significantly reduced the level of blood sugar, however the level was still higher than those on a normal diet (315). This was consistent with our results, as we also found that treatment with AES or L3ES of *N. brasiliensis* significantly reduced the blood glucose level in the diabetic group, although they were still higher than those mice fed a normal chow diet.

Our results showed non-significant differences in the levels of IL-4 in the liver and adipose tissue of treated groups compared to their naïve counterparts; however we found a significant increase in the number of eosinophils in the MLNs, AT and liver with a significant increase in the level of IL-5 in the AT and liver. IL-5 is responsible for the release, expansion and survival of eosinophils (318-320), and *N. brasiliensis* infection is characterised by high eosinophil production induced by IL-5 secreted from $CD4^+$ T cells (285). Importantly, it has been demonstrated that AT eosinophils are highly dependent on IL-5 (41) and eosinophils play an unexpected role in metabolic homeostasis through maintenance of adipose M2 MACs (37). As with eosinophil deficiency, IL-5 deficiency in mice impairs eosinophil accumulation in AT, and mice develop increased body fat, impaired glucose tolerance and insulin resistance when fed a HF diet (41). Moreover, ILC2s have been found to play an important role in sustaining AT eosinophils (41). Infection with *N. brasiliensis* induces adipose eosinophilia and enhances glucose tolerance in mice fed with a HF diet via activation of AT ILC2s, which induces IL-5 production that leads to accumulation of eosinophils in the AT (41). Deletion of

IL-5 resulted in the loss of eosinophil activation in AT (41) and impaired glucose tolerance (37). It has been reported that *N. brasiliensis* infection protects against experimental autoimmune encephalomyelitis (EAE) via increased IL-5 levels and subsequent increases in eosinophils and $CD4^+CD25^+$ Tregs. Moreover, anti-IL-5 or anti-CD25, but not anti-IL-4, can abolish the beneficial effect of the parasitic infection (321). Furthermore, treatment of mice with *F. hepatica* ES products attenuates EAE independently of IL-4 and IL-10, but via production of IL-33 and IL-5, which promoted accumulation of eosinophils (322).

In our study, treatment with AES or L3ES of *N. brasiliensis* significantly decreased the expression of IL-6 in AT. However, in the liver IL-6 expression was comparable between treated and untreated groups. In one study, treatment of intestinal pig epithelial cells *in vitro* with *T. suis* ES products increased the level of IL-6 and IL-10, but not IL-4 in both differentiated and undifferentiated cells (323). IL-6 is a multifaceted cytokine that is involved in the induction of insulin resistance and pathogenesis of T2D (324), and secretion of IL6 by AT resulted in the induction of hepatic insulin resistance (325). On the other hand, IL-6 is involved in liver regeneration and maintenance of liver tissue homeostasis (326). Moreover, IL-6 is required for protection against hepatic inflammation and insulin resistance, and lack of IL-6 reversed the effect (327, 328). Short term preincubation with IL-6 of human PBMC stimulated with anti-CD3 and anti-CD28 antibodies led to up-regulation of IL-4 and IL-5. However, the effect on IL-4 production gradually disappeared but the effect on IL-5 became more pronounced in the long term preincubation of these cells with IL-6 (329). Moreover, IL-6 enhanced expansion and survival of antigen-stimulated $CD4^+$ T cells and reduced their apoptosis in the blood, lymph nodes, spleen, liver, and lung *in vivo* and *in vitro* (330). Furthermore, in the presence of eosinophil-derived neurotoxin (EDN), splenocytes of $TLR2^{+/+}$ mice immunized with OVA produced IL-5, IL-6, IL-10 and IL-13, whereas in the presence of LPS they produced IFN-γ (331). One possibility for the increase in liver IL-6 we observed might be the effect of IL-6 as a survival factor in liver tissue-mediated EDN in activation of TLR2 and expansion and survival of $CD4^+$ T cells in the liver.

Here we found that treatment with L3ES or AES from *N. brasiliensis* using a dose of 1 mg/kg twice weekly significantly decreased the level of blood glucose in diabetic mice, although levels were still higher than control group. We also found a significant increase in the number of eosinophils in the MLNs, AT and liver associated with an increase in the levels of IL-5 in AT and liver but no differences in the levels of IL-4 in the same tissues. Moreover, a decrease in the level of IL-6 was observed in AT whereas it was increased in the liver. It must be pointed out, that *N. brasiliensis* ES products induced airway eosinophilia in a dose-dependent manner (156). Moreover, we (data not published) and others (37, 41, 136) have found a robust increase in the systemic and peripheral eosinophil number, IL-4 level and IL-5 level after infection with *N. brasiliensis*. Infection with *N.*

brasiliensis requires parasite development through different larval stages and migration through different tissues, which may induce distinct mechanisms of modulating the host immune response that differ from the administration of its ES proteins. This might explain the differences in the ability of L3ES and AES to suppress T2D. So, it is worth considering the route of infection and the therapeutic/prophylactic dose of ES proteins. As previously mentioned, ES products induce a variety of immune responses that promote innate and adaptive immune cells, and these cells play very different roles in inducing or protecting against metabolic diseases. Understanding the precise immunological mechanism of action that underpins the clinically beneficial effect is now paramount. To our knowledge, this study is the first to uncover a novel effector response of helminth ES product-mediated reduction of insulin resistance in a mouse model of T2D. Future work should focus on defining the bioactive ES components and their recombinant or synthetic production and validation.

Acknowledgments

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CHAPTER 4

4. Role of helminths and their excretory/secretory products on the composition of the gut microbiota in type 2 diabetic mice

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4.1 Abstract

T2D is a highly prevalent metabolic disorder characterised by an imbalance in the blood glucose level. Dysbiosis of the gut microbiome may reshape intestinal barrier functions, host metabolic and signalling pathways, which are directly or indirectly related to insulin resistance in T2D. Recently, a growing body of evidence has highlighted a role for helminth infection and/or their ES products in modulating the composition of the gut microbiota, which in turn might maintain and promote intestinal health. We demonstrated that infection with *N. brasiliensis* or treatment with L3ES or AES were associated with significant compositional changes in the gut microbiota that occurred at both the phylum and order level. Phyla that were particularly affected included Actinobacteria, Verrucomicrobia, Proteobacteria and TM7. Orders that were most impacted by infection or treatment with ES products included Clostridiales, Desulfovibrionales, Burkholderiales, Verrucomicrobiales and Coriobacteriales. The effects on microbiota composition were also impacted by diet. However, whether these microbiota changes are a necessary component for helminth- or -ES product- mediated protection against T2D is unclear and has yet to be investigated. These findings indicate that gastrointestinal nematode ES products hold promise as a novel intervention approach for the treatment of T2D.

Keywords: Type 2 diabetes, Microbiota, *Nippostrongylus brasiliensis*, Excretory/Secretory, Clostridiales

4.2. Introduction

The gut microbiota is not only essential for host metabolism but is also important in the maintenance of immune homeostasis (275, 332). The immune system has largely evolved as a means to maintain a symbiotic relationship with these highly diverse and evolving microbes. When operating optimally, the immune system-microbiota alliance permits the induction of protective responses to pathogens, and the maintenance of regulatory pathways that govern tolerance to innocuous antigens (241). Indeed, a decrease in our exposure to infectious agents such as helminths, and alteration of the gut microbiota composition has resulted in an enhanced inflammatory environment and poorly developed regulatory networks, and has been strongly associated with the increased incidence of immune mediated diseases (333). There is cumulative evidence highlighting the beneficial role of gut microbiota in shaping the mammalian immune system (212).

Disturbance of the intestinal microbial community leads to altered immune responses that can result in various inflammatory disorders (212). Regulation of metabolic homeostasis and inflammation in obesity, metabolic syndrome and DM has been increasingly connected with the gut microbiota (226). The first evidence came from studies on GF mice, as they were resistant to obesity-associated insulin resistance and dyslipidemia when fed a Western HF or HGI diet; however, colonization of these mice with microbiota from conventionally raised mice led to an increase in the total body fat, increased lipids in the liver and a dramatic increase in IR (227, 334, 335). Many studies have demonstrated differences in the composition of gut microbiota between healthy, obese and T2D patients. The ratio of Firmicutes to Bacteroidetes found to be changed with obesity, favoring increase in Bacteroidetes in some reports (336) or decrease in its ratio in others (337) and increase in the Firmicutes (338). Moreover, T2 diabetic subjects had significantly lower proportions of the phylum Firmicutes and class Clostridia compared to control group. Also the ratio of Bacteroidetes to Firmicutes, as well as the ratio of *Bacteroides-Prevotella* to *Clostridium* coccoides-*Eubacterium rectale* were correlated positively and significantly with the plasma glucose concentration (236). Other metagenomics association studies of gut microbiota in T2D subjects found a reduction in the number of Clostridiales bacteria (*Roseburia* species and *Faecalibacterium prausnitzii*) compared to diabetic patients (339, 340).

A growing body of literature has linked helminth presence with microbiota diversity and composition (242). For instance, infection with the roundworm *T. muris* led to a reduction in the microbial α-diversity and the abundance of Bacteroidetes, specifically *Prevotella* and *Parabacteroides* (244), and an increase in the abundance of Proteobacteria (245), Firmicutes (244) and the *Lactobacillus* genus belonging to Firmicutes (245). Infection with *H. polygyrus* increases the members of γ-Proteobacteria/*Enterobacteriaceae* group and members of the *Bacteroides/Prevotella* genera (247), as well as members of Lactobacillaceae family (249). *N. brasiliensis* infection reduces

the abundance of Firmicutes/Peptostreptococcaceae, Clostridiaceae and Turicibacteraceae and increases the abundance of Lactobacillaceae, Bacteroidetes/*S24-7* and Actinobacteria/ Coriobacteriaceae. (246). Human studies also highlighted the role of helminth infection in modulating the composition of gut microbiota between helminth-infected and helminth-naive individuals (257, 258, 261). CeD subjects infected with hookworm *N. americanus* and challenged with gluten showed tolerance to gluten and had increase in the intestinal microbial species richness and a trend towards an increase in the abundance of species within the Bacteroidetes phylum in comparison with the uninfected group (103-105, 258-260). This was also associated with increase in the Th2 and Tregs cytokines (102, 103). Moreover, increase in the abundance of Paraprevotellaceae, *Mollicutes*, Bacteroidales and α-proteobacteria was observed in subjects naturally infected with multiple helminths (*Trichuris spp*., *Ascaris spp*. and hookworm). However, the helminth-negative group showed increased abundance of *Bifidobacterium* (261). Furthermore, people infected with *S. stercoralis* had a higher abundance of the families Leuconostocaceae, Ruminococcaceae, Paraprevotellaceae and the genus *Peptococcus* , while the *S. stercoralis*-negative group showed a significant increase in the order Pseudomonadales/*Pseudomonas* and an unidentified species of the genus *Bacteroides* (257).

In mice studies, others have highlighted a role for helminth-microbiota interaction in the protection against many inflammatory diseases such as allergy (250), IBD (251), arthritis (256), Lupus (341) and obesity (254, 255). $NOD2^{-/-}$ mice develop spontaneous colitis, and infection of these mice with *T. muris* or *H. polygyrus* inhibited growth of *Bacteroides vulgatus* and decreased the abundance of Bacteroidales/*Bacteroides* and *Prevotella* and increased the abundance of Clostridiales/Lachnospiraceae. Administration of a mixture of clusters IV, XIVa and XVIII Clostridiales and Erysipelotrichales strains to $NOD2^{-/-}$ mice can inhibit colonization of *B. vulgatus* (251). Mice infected with *H. polygyrus* then challenged with HDM, this altered the intestinal bacterial communities that attenuated allergic airway inflammation (250). Suppression in obesity was also observed when mice fed HF diet then infected with *H. polygyrus* (254). This was associated with an increase in order Clostridiales (250), *Bacillus* and *Escherichia* species as well as in the Proteobacteria phylum (254). Infection of obese mice with *S. venezuelensis* improved insulin sensitivity and was associated with an increase in Firmicutes/*Lactobacillus spp.* and a decrease in Bacteroidetes (255). Administration of the *A. viteae* immunomodulator ES-62 maintained a healthy microbiome diversity and normalised gut microbiota communities, mainly Clostridiaceae, Lachnospiraceae and Ruminococcaceae, that protect against arthritis (256). The anti-inflamamtory moiety of ES-62 is phosphorylcholine, and treatment with TPC (synthetic tuftsin-phosphorylcholine) induced remission of lupus in mice, and was associated with a decrease in the abundance of *Akkermansia* and an increase

in the abundance of several genera, including *Turicibacter, Bifidobacterium*, unclassified Mogibacteriaceae, unclassified Clostridiaceae, *Adlercreutzia*, *Allobaculum* and *Anaeroplasma* (341).

There is, therefore, a real need to uncouple this three-way relationship between helminths, microbiome and inflammatory/metabolic diseases. Thus, we aimed to investigate whether infection with *N. brasiliensis* has a role in altering the composition of the gut microbiota, and whether this alteration might have an impact on maintenance of gut homeostasis in T2D mice.

4.3. Materials and Methods:

4.3.1 Ethics statement:

All procedures were approved by the JCU Animal Ethics Committee, ethics application number A2244.

4.3.2 Animals and Diet:

Male C57BL/6 wild-type (WT) (JCU Townsville) mice were used. To induce T2D, at the age of 5 weeks, mice were either fed a HGI diet (SF03-30; speciality feeds, Western Australia) or a HF diet (SF07-066; speciality feeds, Western Australia) while animals from the control group were fed a normal control (NC) diet.

4.3.3. Whole worm infection

N. brasiliensis life cycle was maintained in our laboratory at James Cook University (Cairns). Briefly, faeces from *N. brasiliensis*-infected rats were collected from days 5-9 post-infection. Egg-containing faeces were mixed with an equal amount of water and charcoal, distributed into Petri dish plates and incubated at 26°C. One week after incubation, L3 were collected from the faecal/charcoal culture plate, washed three times with PBS, then the mice were injected subcutaneously above the upper thoracic spine with 500 L3 of *N. brasiliensis* using a 21-gauge needle once a month, starting at 6 weeks of age until the end of the experiment (30 weeks).

4.3.4. Administration of AES and L3ES products

For preparation of the crude L3ES proteins, the *N. brasiliensis* life cycle was maintained in our laboratory at James Cook University (Cairns). Briefly, faeces from *N. brasiliensis*-infected rats were collected from days 5-9 post-infection. Egg-containing faeces were mixed with an equal amount of water and charcoal, distributed into Petri dish plates and incubated at 26°C. One week after incubation, L3 were collected from the faecal/charcoal culture plate, washed three times with PBS, then three times with PBS supplemented with 5% antibiotic-antimycotic (AA) (Gibco). L3 were then transferred into a flat bottom 24 well-plate, cultured in serum-free RPMI media (Gibco) supplemented with 2% AA and 1% D-glucose (Sigma) and incubated at 37°C and 5% CO_2 at a density of 500

L3/well. The supernatant was collected daily from days 1-10, ensuring motility of the worms at all times. Supernatants were stored at -30°C before protein concentration and lipopolysaccharide (LPS) content were determined.

For adult ES (AES) preparation, rats were infected with 3,000 L3 *N. brasiliensis*. At day 6 post-infection rats were euthanized and the small intestine collected and opened in a Petri dish containing RPMI and incubated at 37°C and 5% CO_2 for 2 hours. Adult worms were then collected, washed three times with PBS followed by three washes with PBS supplemented with 5% AA and one wash with RPMI media supplemented with 2% AA. 100 worms/well were then transferred into a flat bottom 24 well-plate and incubated in RPMI media supplemented with 2% AA, 1% D-glucose and 1% Glutamax at 37°C and 5% CO_2. After 24h the supernatant was discarded to minimise contamination with host proteins and replaced with new media. AES was collected at day 2 and replaced subsequently every day until day 10. Supernatants were stored at -30°C before protein concentration and lipopolysaccharide (LPS) content were determined.

Amicon ultrafiltration 3 kDa tubes (Thermo scientific) were used for protein concentration and buffer exchange with PBS. The Pierce BCA protein assay kit (Thermo scientific) was used for quantification of the protein content following the manufacturer's instructions. The Pierce LAL Chromogenic Endotoxin Quantification Kit (Thermo Scientific) was used for the quantification of LPS in AES and L3ES preps. Firstly, AES and the L3ES were incubated with 1% Triton-114 at 4°C on a rotor (Ratek) overnight with low constant stirring. After that, ES proteins were incubated at 37°C for 10 minutes using a dry bath incubator (Major Science), followed by centrifugation in a Microfuge 22R centrifuge (Beckman Coulter) at 14,000 *g* for 5 minutes. The top layer was collected and prepared for LPS quantification. Briefly, 4 standards were prepared, ranging from 1- 0.1 EU/ml. Then, 50 µl of the standards and ES samples were added in duplicate to a 96 well plate and incubated at 37°C and 5% CO_2 for 5 minutes Subsequently, 50 µl of Limulus Amebocyte Lysate was added and the plate was incubated at 37°C for 10 minutes, followed by addition of 50 µl of Chromogenic Substrate and incubation for 6 minutes at 37°C. Then, 50 µl of the stop solution was added and the results were read on a BMG Polarstar Omega fluorescence microplate reader at 405 nm. The ES proteins were suitable for use when the LPS content was below 5 EU/mg of protein.

ES products were administered intraperitoneally twice weekly from week 6 until the end of the experiment at a dose of 1 mg/kg. Control groups were administered intraperitoneally with the same volume and frequency of PBS.

4.3.5. DNA extraction and bacterial 16S rRNA Illumina sequencing

Small intestine samples were collected and stored immediately at -80°C for further analysis. DNA extraction and 16s rRNA sequencing were performed by the Australian Centre for Ecogenomics, University of Queensland, Brisbane. In brief, a total of 50-100 mg of tissue sample was disrupted mechanically using a Powerlyzer 24 at 2,000 *g* for 5 minutes. A QIAamp 96 PowerFaecal QIAcube HT Kit was used to process the resulting lysate as per the manufacturer's instructions, and a Qubit assay (Life Technologies) was used for measuring DNA concentration, which was then adjusted to a concentration of 5 ng/µl. The 16S rRNA gene was targeted, using the 803 forward primer (5'- TTAGAKACCCB NGTAGTC -3') and 1392 reverse primer (5'- ACGGGCGGTGWGTRC -3') to cover the V6-V8 regions. Preparation of the 16S library was performed following the protocol outlined in the Illumina guide. In the first stage, 466 bp of the PCR products were amplified. The resulting PCR amplicons were then purified using Agencourt AMPure XP beads (Beckman Coulter). The purified DNA was indexed with unique 8cbp barcodes using the Illumina Nextera XT 384 sample Index Kit A-D (Illumina FC-131-1002). The Qiagen QIAquick Gel Extraction Kit was used for the isolation of the indexed amplicons as per the manufacturer's instructions for the specific band at 450 bp (running at 610 bp with the adaptor sequence). Then, the resulting purified indexed amplicons were pooled together in equimolar concentrations and sequenced on a MiSeq Sequencing System (Illumina) using paired end (2 x 300 bp) sequencing with V3 chemistry in the Australian Centre for Ecogenomics according to the manufacturer's protocol. Passing quality control of resulting sequence is determined as 10,000 raw reads per sample prior to data processing and passing quality control metrics in line with Illumina supplied reagent metrics of overall Q30 for 600cbp reads of >70%.

4.3.6. Bioinformatics and statistical analysis

Raw reads were run through fastqc for quality control, Trimmomatic (342) for adapter trimming and low quality base removal, QIIME (343) for Operational Taxonomic Units (OTUs) generation, and BLAST (344) for OTU identification. Within QIIME, low-quality reads are filtered with all remaining sequences de-multiplexed and chimeric sequences removed using UCHIME (345). Sequences were subsequently clustered into OTUs on the basis of similarity to known bacterial sequences in the Greengenes database (346) (cut-off: 97% sequence similarity) using the UCLUST software (347).

For each biom file, the taxonomic observation and metadata was added using biom API (348) which was next loaded into the R package phyloseq (349). Within phyloseq, the DESeq2 (350) API was called and a list of most differentially expressed bacteria generated for all possible pairings of

conditions (NC and NC infected with *N. brasiliensis*, T2D mice fed HGI and T2D mice fed HGI infected with *N. brasiliensis* or T2D mice fed HF and T2D mice fed HF infected with *N. brasiliensis*)(NC and NC L3ES or NC AES, T2D fed HGI and T2D fed HGI L3ES or T2D fed HGI AES). All subsequent plots were generated using ggplot2 and Calypso online software (version 8.84) (http://cgenome.net/calypso/) (351). Within Calypso, data were normalised by total sum normalisation (TSS) combined with square root transformation. Multivariate redundancy analysis to overall differences in the microbial profile between groups and Adonis based on the Bray-Curtis dissimilarity were used. Differences in bacterial alpha diversity (Shannon diversity) and richness between groups were used. Values of $p < 0.05$ were considered statistically significant following false discovery rate (FDR) correction. Differences in the bacterial taxa abundance between groups were assessed using ANOVA-like differential expression analysis (ALDEx2) and quantitative visualisation of phyla abundance.

4.4. Results

4.4.1. Infection with *N. brasiliensis* resulted in altered microbial profile in normal control and T2D mice

We wanted to address the effect of infection with *N. brasiliensis* on the composition of the gut microbiota in two different models of diet-induced T2D in mice (HGI and HF diet). At week 5 of age, male C57BL/6 mice were kept on a NC diet or were fed either a HGI or a HF diet for up to 30 weeks to induce T2D. Infection with *N. brasiliensis* started at week 6 and continue once every month until the end of the experiment. Small intestine samples were collected at the end of the experiments to determine the differences in the composition of gut microbiota between infected and uninfected groups. Multivariate redundancy analysis (RDA) on OTU level showed a different clustering in the microbial profile of the infected groups compared to the uninfected control groups for all diets. Adonis analysis also confirmed the differences between infected and uninfected groups on the NC, HF and HGI diets based on Bray-Curtis and spearman index (Table 1). The α-diversity and microbial richness were not statistically significant between naïve groups and *N. brasiliensis* infected groups for all three diets (Supplementary Fig. 4.4.1).

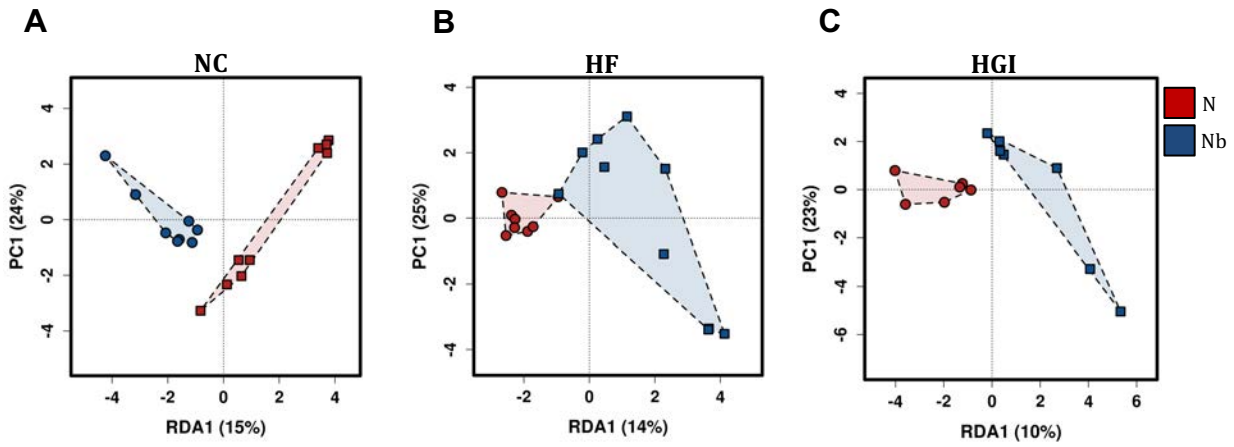


Fig 4.4.1. Infection with *N. brasiliensis* resulted in altered microbial profile in normal control and T2D mice.

C57/BL6 mice were fed a normal control (NC) diet or either a high fat (HF) or high glycaemic index (HGI) diet and infected once monthly with 500 L3 of *Nippostrongylus brasiliensis*. Multivariate redundancy analysis. **(A)** Normal control naïve (N) group and normal control *N. brasiliensis* (Nb) infected group. **(B)** High fat naïve (N) group and high fat *N. brasiliensis* (Nb) infected group. **(C)** High glycaemic index naïve (N) group and high glycaemic index *N. brasiliensis* (Nb) infected group. The data are representative of two different experiments where n = 5/group.

4.4.2. Infection with *N. brasiliensis* alters the abundance of bacterial taxa in different diets

In general, there were no significant differences in the most dominant phyla of the infected groups compare to their uninfected counterparts in all diets used in this study (Fig. 4.4.2). In regard to the NC diet groups, abundance of Actinobacteria phylum was significantly decreased in the *N. brasiliensis* infected (NCNb) group compared to the uninfected naïve group (NCN). The abundance of both Firmicutes and Bacteroidetes phyla was slightly increased in NCNb group compare to their NCN group but were not significantly different and no changes in any other phyla were detected (Fig. 4.4.2A). No significant changes at the phylum level were found between HF diet groups infected with *N. brasiliensis* (HFNb) and the uninfected naïve group (HFN) in all phyla detected (Fig. 4.4.2B). A significant decrease in the abundance of Verrucomicrobia phylum and a significant increase in the abundance of Proteobacteria phylum were detected in the HGI groups infected with *N. brasiliensis* (HGINb) compared to the uninfected naïve group (HGIN) (Fig. 4.4.2C). HGINb also showed a trend towards an increase in the abundance of Actinobacteria phylum while there was a decrease in the TM7 phylum; however, these changes were not statistically significant. No significant differences were detected in any other phyla.

At the order level, *N. brasiliensis* infection significantly increased the abundance of the Clostridiales group, belonging to the Firmicutes phylum, in both the NCNb group and the HFNb group compared to the uninfected naïve groups (Fig. 4.4.3A and 4.4.3B). The abundance of

Bifidobacteriales belonging to the Actinobacteria phylum significantly decreased in the NCNb group compared to the NCN group (Fig. 4.4.3A). *N. brasiliensis* infection also caused a significant elevation in the abundance of Desulfovibrionales and Burkholderiales orders belonging to the Proteobacteria phylum in both the HFNb and HGINb groups, respectively, compared to the uninfected naïve groups (Fig. 4.4.3B and 4.4.3C). The abundance of both the CW040 order belonging to the TM7 phylum and the Verrucomicrobiales order belonging to the Verrucomicrobia phylum significantly decreased in the HGINb group compared to the HGIN group (Fig. 4.4.3C).

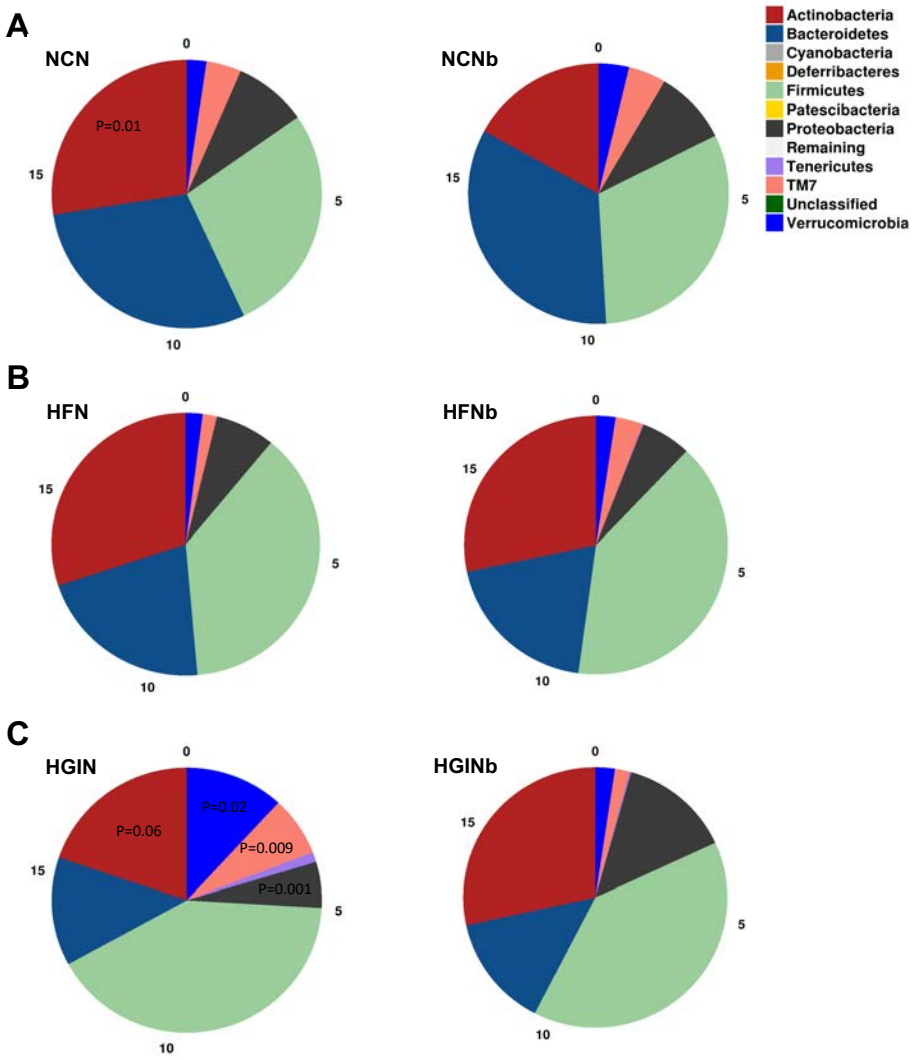


Fig 4.4.2. Infection with *N. brasiliensis* alters the abundance of bacterial phyla in different diets.

C57/BL6 mice were fed either a normal control (NC), high fat (HF) or high glycaemic index (HGI) diet and infected once monthly with 500 *Nippostrongylus brasiliensis* L3. **(A)** Quantitative visualisation of phylum abundance between normal control naïve (NCN) and normal control *N. brasiliensis* (NCNb) infected groups; **(B)** Quantitative visualisation of phylum abundance between high fat naïve (HFN) and high fat *N. brasiliensis* (HFNb) infected groups; **(C)** Quantitative visualisation of phylum abundance between high glycaemic index naïve (HGIN) and high glycaemic index *N. brasiliensis* (HGINb) infected groups. P value based on ANOVA-like differential expression analysis. The data are representative of two different experiments where n = 5/group.

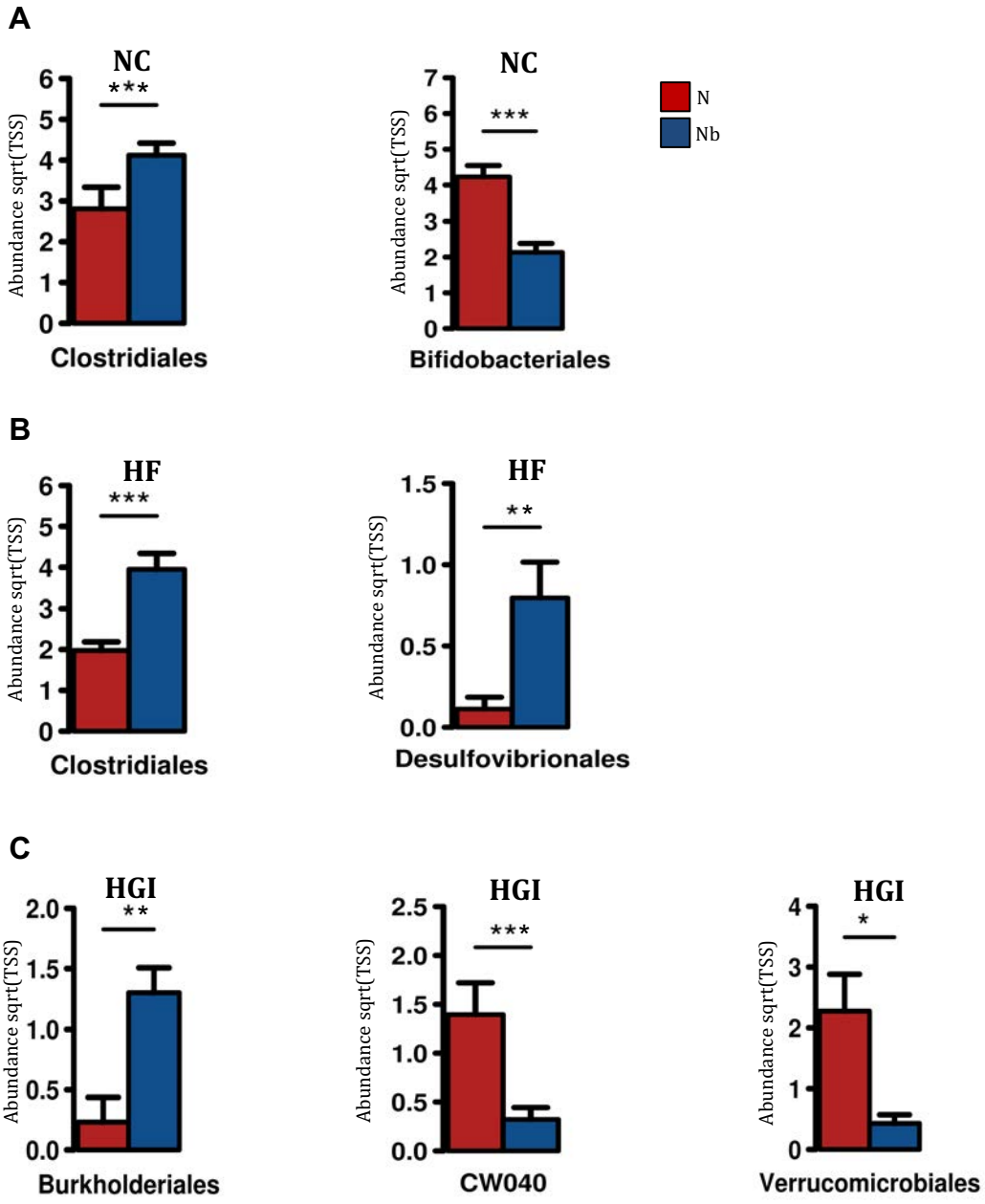


Fig 4.4.3. Infection with *N. brasiliensis* alters the abundance of bacterial order in different diets.

C57/BL6 mice were fed normal a control (NC), high fat (HF) or high glycaemic index (HGI) diet and infected once monthly with 500 L3 from *Nippostrongylus brasiliensis*. **(A)** Bacterial order abundance between normal control naïve (N) and normal control *N. brasiliensis* (Nb) infected groups; **(B)** Bacterial order abundance between high fat naïve (N) and high fat *N. brasiliensis* (Nb) infected groups; **(C)** Bacterial order abundance between high glycaemic index naïve (N) and high glycaemic index *N. brasiliensis* (Nb) infected groups. P value based on ANOVA-like differential expression analysis. The data are representative of two different experiments where n = 5/group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.00$.

4.4.3. Treatment with *N. brasiliensis* L3ES and AES resulted in alternation in the microbial profile of both normal control diet and high glycaemic index diet groups

In order to address the effect of treatment with L3ES or AES of *N. brasiliensis* on the composition of gut microbiota in the HGI model of diet-induced T2D, 5 week old male C57BL/6 mice were fed either a NC or HGI diet to induce T2D. Treatment with *N. brasiliensis* L3ES or AES started at week 6 and continued twice every week until the end of the experiment. Stool samples were collected at the end of the experiment to determine the differences in the composition of gut microbiota. RDA on an OTU level was significantly different in both NC diet and HGI diet groups treated with L3ES or AES compared to their untreated littermates (Fig. 4.4.4A; Fig. 4.4.5A). Adonis analysis, showed significant differences in the L3ES treated groups on both diets compared to their untreated naïve groups based on Bray-Curtis and spearman index (Table 1). The AES treated group fed a HGI diet but not on the NC diet showed significant differences measured by an Adonis test based on Bray-Curtis and spearman index (Table 1). The α-diversity and the richness increased significantly in the HGI diet group treated with L3ES compared to their untreated naïve group (Supplementary Fig. S2). However, the α-diversity and the richness were not statistically significant in any of the HGI diet groups treated with AES or the NC diet group treated with AES and L3ES compared to their naïve littermates (Supplementary Fig. 4.4.3).

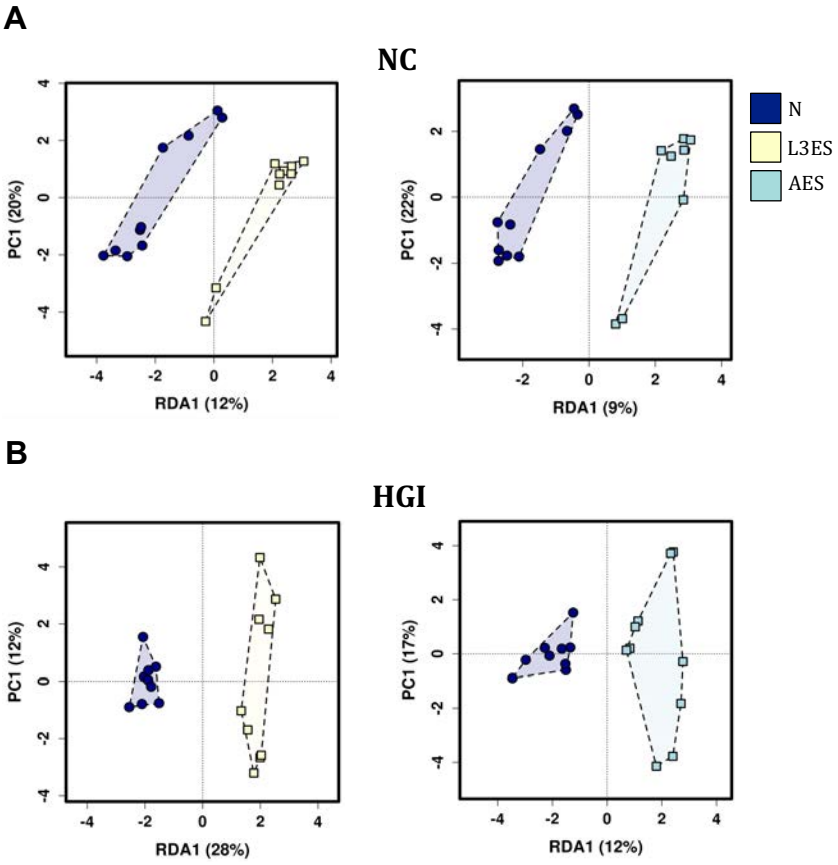


Fig 4.4.4. Treatment with L3ES or AES of *N. brasiliensis* resulted in alternation in the microbial profile of both normal control diet and high glycaemic index diet groups.

C57/BL6 mice were fed normal control (NC) or high glycaemic index (HGI) diets and treated twice weekly with L3ES or AES of *Nippostrongylus brasiliensis* **(A)** Multivariate redundancy analysis between normal control naïve (N) and normal control *N. brasiliensis* (L3ES) or (AES) treated groups **(B)** Multivariate redundancy analysis between high glycaemic naïve (N) and high glycaemic *N. brasiliensis* (L3ES) or (AES) treated groups. The data are representative of one experiment where n = 10/group.

4.4.5. Treatment with L3ES or AES of *N. brasiliensis* alters the abundance of bacterial taxa

L3ES or AES treatment showed no significant changes in the most dominant phyla in comparison with their untreated littermates in both diets (Fig. 4.4.5). There was only a significant increase in the abundance of the Verrucomicrobia phylum of the NC diet group treated with L3ES compared to the naïve un-treated group. No differences in any other phyla were detected (Fig. 4.4.5A). The abundance of the Actinobacteria phylum showed a significant decrease in the HGI diet groups treated with either L3ES or AES compared to their untreated littermates. Furthermore, the abundance of the Patescibacteria phylum significantly increased in the AES group fed on a HGI diet (Fig. 4.4.5B). At the order level, the Coriobacteriales group belonging to Actinobacteria showed a significant decrease in abundance in the NC and HGI diet groups treated with L3ES, as well as in the HGI diet group treated with AES (Fig. 4.4.6A and 4.4.6B). Verrucomicrobiales belonging to Verrucomicrobia significantly increased in the NC and HGI diet groups treated with L3ES or AES, respectively. However, this order showed a significant decrease in abundance in the HGI diet group treated with L3ES (Fig. 4.4.6A and 4.4.6B). Desulfovibrionales, Rhodospirillales and Betaproteobacteriales belonging to the Proteobacteria phylum showed a significant decrease in the NC diet group treated with L3ES, the NC diet group treated with AES and the HGI diet group treated with L3ES, respectively, compared to naïve littermates (Fig. 4.4.6A and 4.4.6B). The abundance of Saccharimonadales belonging to the Patescibacteria phylum and Lactobacillales belonging to the Firmicutes phylum were significantly higher in the HGI group treated with AES compare to their untreated littermates (Fig. 4.4.6B).

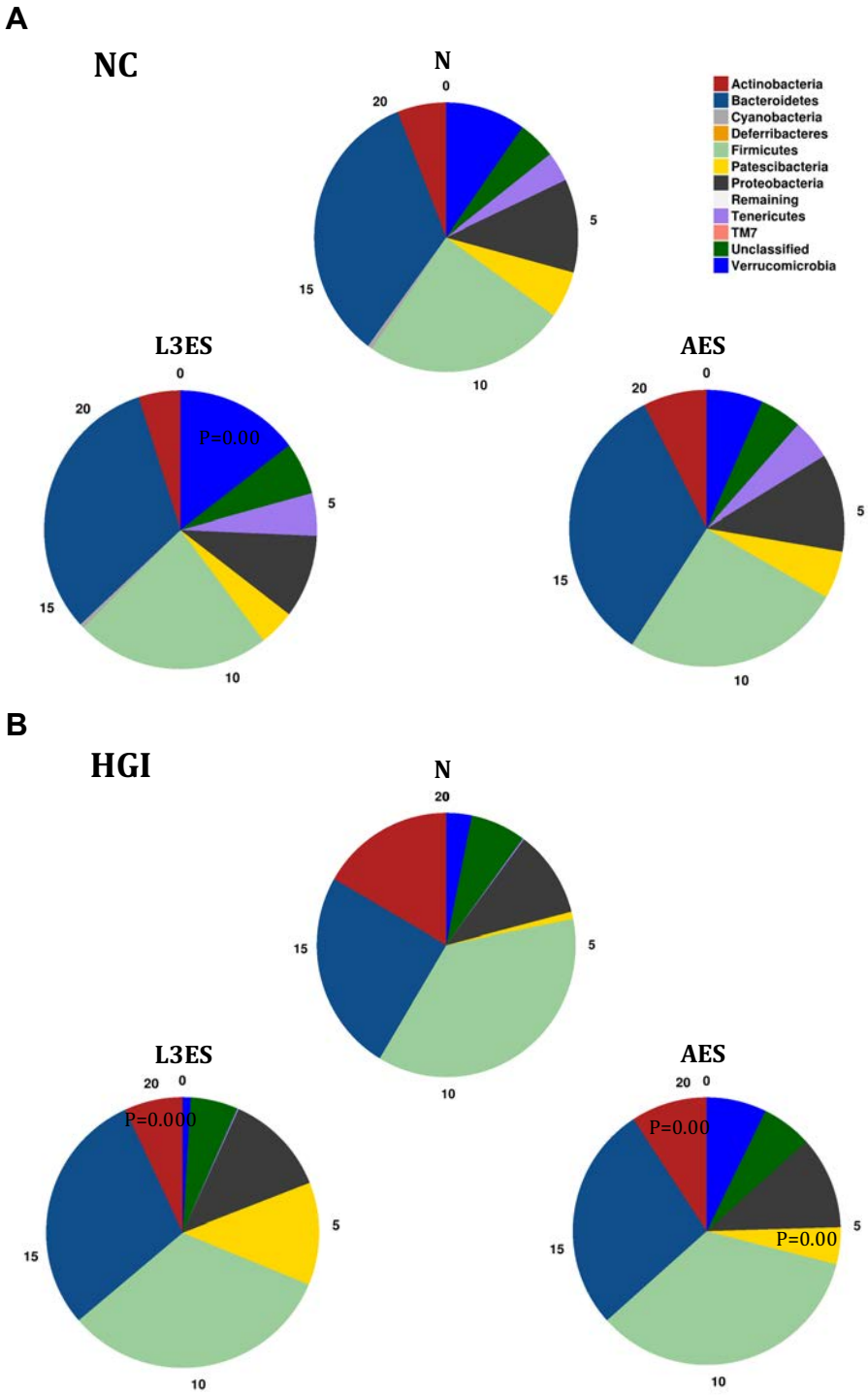


Fig 4.4.5. Treatment with L3ES or AES of *N. brasiliensis* alters the abundance of bacterial phyla.

C57/BL6 mice were fed normal control (NC) or high glycaemic index (HGI) diets and treated twice weekly with L3ES or AES from *Nippostrongylus brasiliensis*. **(A)** Quantitative visualisation of phylum abundance between normal control naïve (N) and normal control *N. brasiliensis* (L3ES) or (AES) treated groups; **(B)** Quantitative visualisation of phylum abundance between high glycaemic index naïve (N) and high glycaemic index *N. brasiliensis* (L3ES) or (AES) treated groups. P value based on ANOVA-like differential expression analysis. The data are representative of one experiment where n = 10/group.

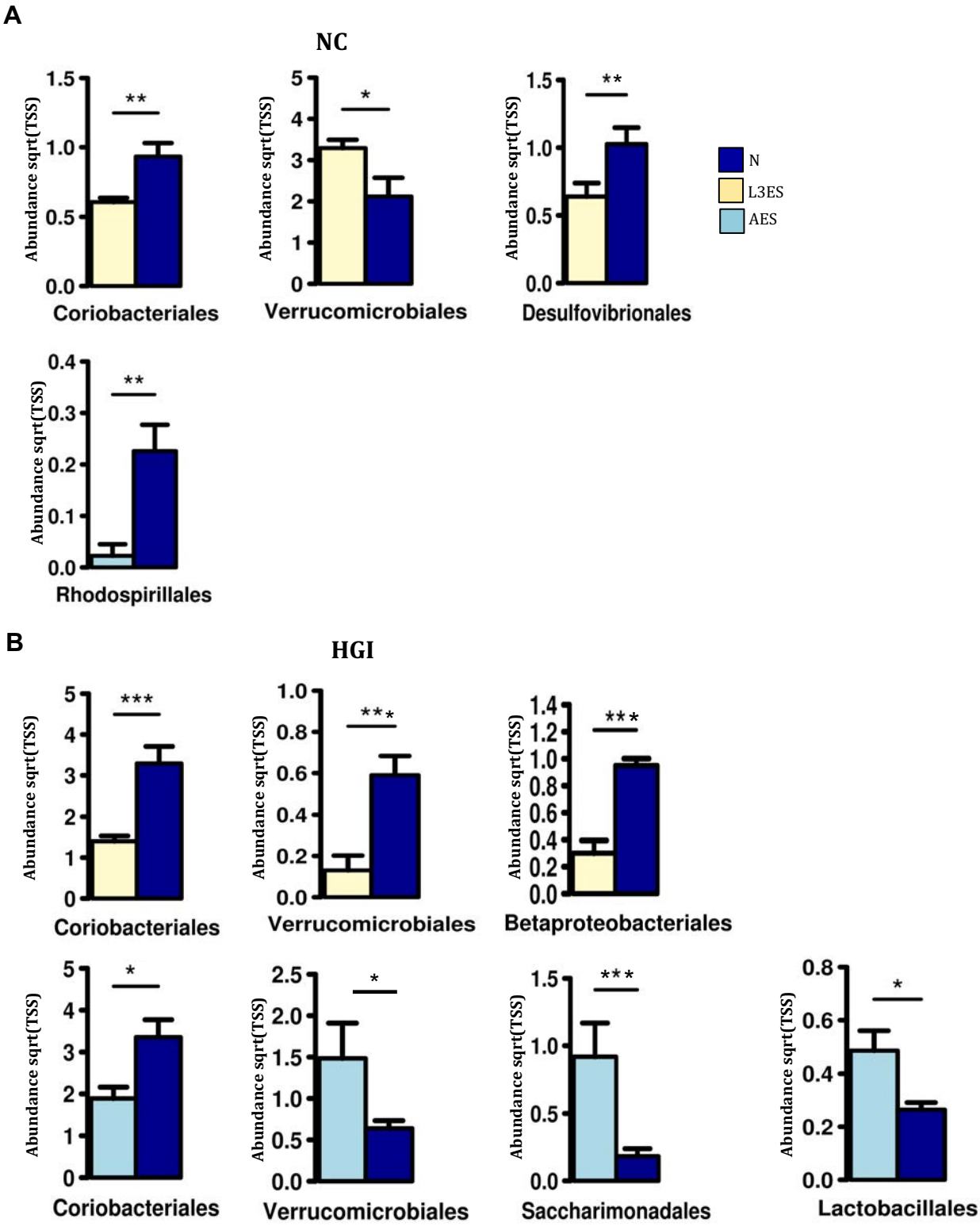


Fig 4.4.6. Treatment with L3ES or AES of *N. brasiliensis* alters the abundance of bacterial order.

C57/BL6 mice were fed normal control (NC) or high glycaemic index (HGI) diets and treated twice weekly with L3ES or AES from *Nippostrongylus brasiliensis*. **(A)** Bacterial order abundance between normal control naïve (N) and normal control *N. brasiliensis* (L3ES) or (AES) treated groups; **(B)** Bacterial order abundance between high glycaemic index naïve (N) and high glycaemic index *N. brasiliensis* (L3ES) or (AES) treated groups. P value based on ANOVA-like differential expression analysis. The data are representative of one experiment where n = 10/group. *p < 0.05; **p < 0.01; ***p < 0.00.

Table:1 Adonis p-value result based on Bray-Curtis and spearman index

Groups	p-value Bray-Curtis	p-value spearman index
NCN vs NCNb	0.002	0.0003
HFN vs HFNb	0.0006	0.005
HGIN vs HGINb	0.06	0.03
NCN vs NCL3ES	0.01	0.004
NCN vs NCAES	0.14	0.1
HGIN vs HGIL3ES	0.0003	0.0003
HGIN vs HGIAES	0.0006	0.0003

4.5 Discussion

Changes in environmental factors such as diet can modulate bacterial composition and metabolic activity (332). These changes can result in a higher intestinal glucose and energy absorption as well as an increase in the absorption of LPS produced by gut microbiota, which can trigger an inflammatory immune response leading to the development of T2D (275, 332). Indeed, there is growing evidence that helminth infection and their ES products alter the composition of the gut microbial community, conferring protection against immune mediated diseases such as allergic inflammation (250), CD (251), obesity (254, 255), arthritis (256, 352) and Lupus (341). Here, firstly we aimed to address the effect of infection with *N. brasiliensis* on the composition of the gut microbiota in mice fed with a normal control diet and in two mouse models of diet induced T2D (HF and HGI). Secondly, we aimed to address the effect of treatment with *N. brasiliensis* L3ES or AES on the composition of the gut microbiota in mice fed a normal control diet or a HGI diet that caused the development of T2D.

No significant changes were observed in α-diversity in response to *N. brasiliensis* infection in all diets studied (NC, HF and HGI diet); however, we still found a significant shift in the microbiota composition at the community level. This was in agreement with other studies with *N. brasiliensis* and *Hymenolepis diminuta* that also found no significant differences in α-diversity between infected and uninfected groups (246, 253), which suggested inter-individual variation in the microbiota composition as a result of infection (246). In the latter study, mice on a NC diet infected with *N. brasiliensis* showed a significant reduction in the Firmicutes phylum and an increase in Bacteroidetes/*S24-7* and Actinobacteria/*Coriobacteriaceae*. However, in our study we found that, in response to infection, the NC group had non-significant increases in both Firmicutes and Bacteroidetes phyla, while Actinobacteria (in particular the Bifidobacteriales group) abundance was significantly lower and abundance of order Clostridiales belonging to Firmicutes phylum was

significantly higher. While the aforementioned study found that among the Firmicutes phylum abundance of *Clostridiaceae*, *Peptostreptococcaceae* and *Turicibacteraceae* were significantly lower, abundance of the *Lactobacillaceae* was significantly higher in the infected group (246). The differences between this study and our own study reported herein might be a result of the differences in the microbiota analysis in terms of 16srRNA amplification and sequencing, as well as differences in the experimental design in terms of infection strategy and time point for both experiments, as studies in humans (353, 354) and mice (355) found changes in the composition of gut microbiota correlating with age. In agreement with our findings, in human studies, individuals with different helminth infections (i.e. *Trichuris spp.*, *Ascaris spp.* and hookworm) had lower abundance of the Bifidobacteriales compared to helminth uninfected individuals (261). Many linked the latter group with health benefits in T2D (356); however, others have reported them as causing infections (357). In mouse studies, infection with *T. muris* or *H. polygyrus* protected against CD in *NOD2*$^{-/-}$ deficient mice (251) and attenuated allergic airway inflammation in mice inoculated with HDM (250), which was associated with an increase in the abundance of the order Clostridiales (250, 251).

In regard to diabetic groups, infection increased the abundance of the Clostridiales group in the HF diet group as well as the abundance of Desulfovibrionales and Burkholderiales belonging to Proteobacteria in the HF and HGI diet groups, respectively. As previously mentioned, in a mouse study, attenuation of allergic airway inflammation was associated with an increase in the abundance of Clostridiales (250). It has also been reported that reduction in the abundance of Clostridia was associated with T2D in humans (236, 339, 340, 358), and an increase in this group was associated with an improvement in glucose and lipid metabolism (359). In one study, the Proteobacteria phylum also increased in *H. polygyrus*-infected mice fed a NC or a HF diet compared to naïve littermates. This phylum is, in part, responsible for regulating weight gain in HF diet fed mice (254). Abundance of Desulfovibrionales order, class and family were increased in mouse faecal samples as a result of infection with *Schistosoma heamatobium* (360), *Ascaris lumbricoides* and *Trichuris trichiura* (361). Moreover, the abundance of Desulfovibrionales and Burkholderiales groups was lower in obese mice (362). Cold exposure attenuated diet-induced obesity in mice, which was associated with an increase in the abundance of *Desulfovibrionaceae* (363). Diabetic rats treated with insulin had higher relative abundance of Burkholderiales; however, another study found lower abundance of the Desulfovibrionales order in diabetic rats when treated with insulin (364). Others have also reported an increase in the abundance of the Desulfovibrionales order and family in HF compared to NC diet fed groups (365, 366). Desulfovibrionales are sulphate-reducing bacteria that use hydrogen or other compounds such as lactate, pyruvate and ethanol as electron donors to produce hydrogen sulphide (H_2S) (367), which is implicated in intestinal disease (368). So, on both sides, many parameters need to be considered, such as the concentration of sulphide inside the intestine and the ability of IECs to

detoxify and to utilise sulphide as an energy source and other mechanisms that might be involved in the signalling pathway of H_2S (368). In our study, the Verrucomicrobiales group belonging to the Verrucomicrobia phylum and the CW040 group belonging to the TM7 phylum were significantly lower in the HGI group infected with *N. brasiliensis* in comparison with uninfected littermates . Verrucomicrobia and TM7 groups were significantly higher in obese compared to non-obese children (369), and mice with leptin deficiency (db/db)-induced T2D showed an increase in the abundance of Verrucomicrobia (370). However, these mice exhibited a decrease in the abundance of Verrucomicrobia when subjected to an intermittent fasting regime which protected against diabetic retinopathy (371).

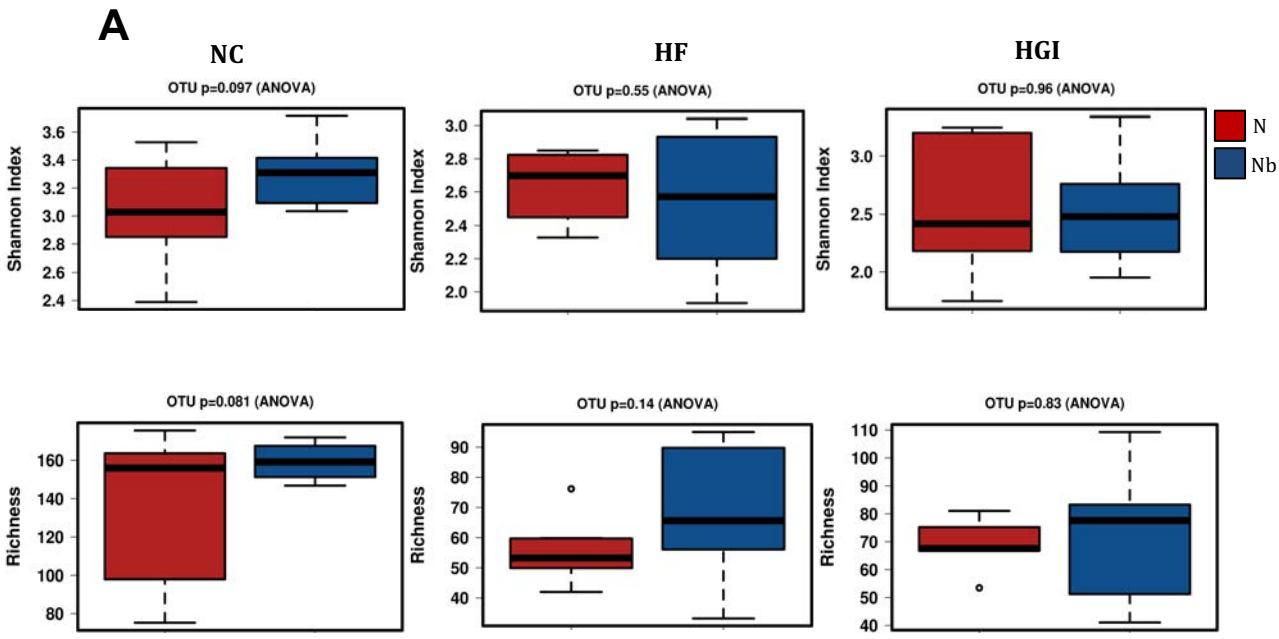
In regard to treatment with *N. brasiliensis* ES products, while mice on a NC diet treated with AES or L3ES and those on a HGI diet treated with AES showed non-significant increases in α-diversity based on Shannon index, those on a HGI diet treated with L3ES had significantly higher α-diversity. The NC diet group treated with L3ES as well as the HGI diet groups treated with L3ES or AES had lower abundance of the Actinobacteria/Coriobacteriales group compared to the untreated controls. The abundance of members of the Coriobacteriaceae family was increased in obese children and adolescents compare to their normal weight groups (372). Patients with T2D had increased abundance of Coriobacteriales in comparison with the non-diabetic and pre-diabetic groups (373). Moreover, T2D patients with cardiovascular complications had higher abundance of the latter group compare to the diabetic patients without cardiovascular complications (374). Rats fed a fructose-rich diet had higher Coriobacteriales/Coriobacteriaceae compared to rats fed a control diet (375), however faecal microbiota transplantation from the latter control group to the high fructose diet group resulted in decreased Coriobacteriaceae abundance (375). In our study, the Verrucomicrobia/ Verrucomicrobiales group showed a different outcome, as NC diet mice treated with L3ES and HGI diet mice treated with AES had a higher abundance of this group, however those mice on a HGI diet treated with L3ES had lower abundance of Verrucomicrobiales than their control groups. In one study, mice treated with helminth derivative phosphorylcholine TPC had lower abundance of Verrucomicrobia but higher abundance of *Clostridiaceae* compared to their control group (341). Furthermore, as we mentioned previously, in obese people (369) as well as in obese mice, (370) researchers reported an increase in the abundance of the Verrucomicrobia group. However, another study found a decrease in the abundance of Verrucomicrobia in both prediabetes and T2D subjects (376). Verrucomicrobia are mucin degrading bacteria (377) implicated in health and disease. In this sense, systematic contribution of mucin degrading bacteria in gut homeostasis and dysbiosis has yet to be investigated (378).

Abundance of Desulfovibrionales, Betaproteobacteriales and Rhodospirillales groups belonging to proteobacteria was lower in NC diet group treated with L3ES, HGI diet group treated

with L3ES and NC diet group treated with AES respectively compare to their untreated littermates. In a previous study, mice fed with a diet that was rich in fat, fructose and cholesterol had a higher abundance of Rhodospirillales compared to the control diet fed group (379). Moreover, pre-diabetic and diabetic subjects had a higher abundance of Betaproteobacteria compared to non-diabetic subjects (236, 376).

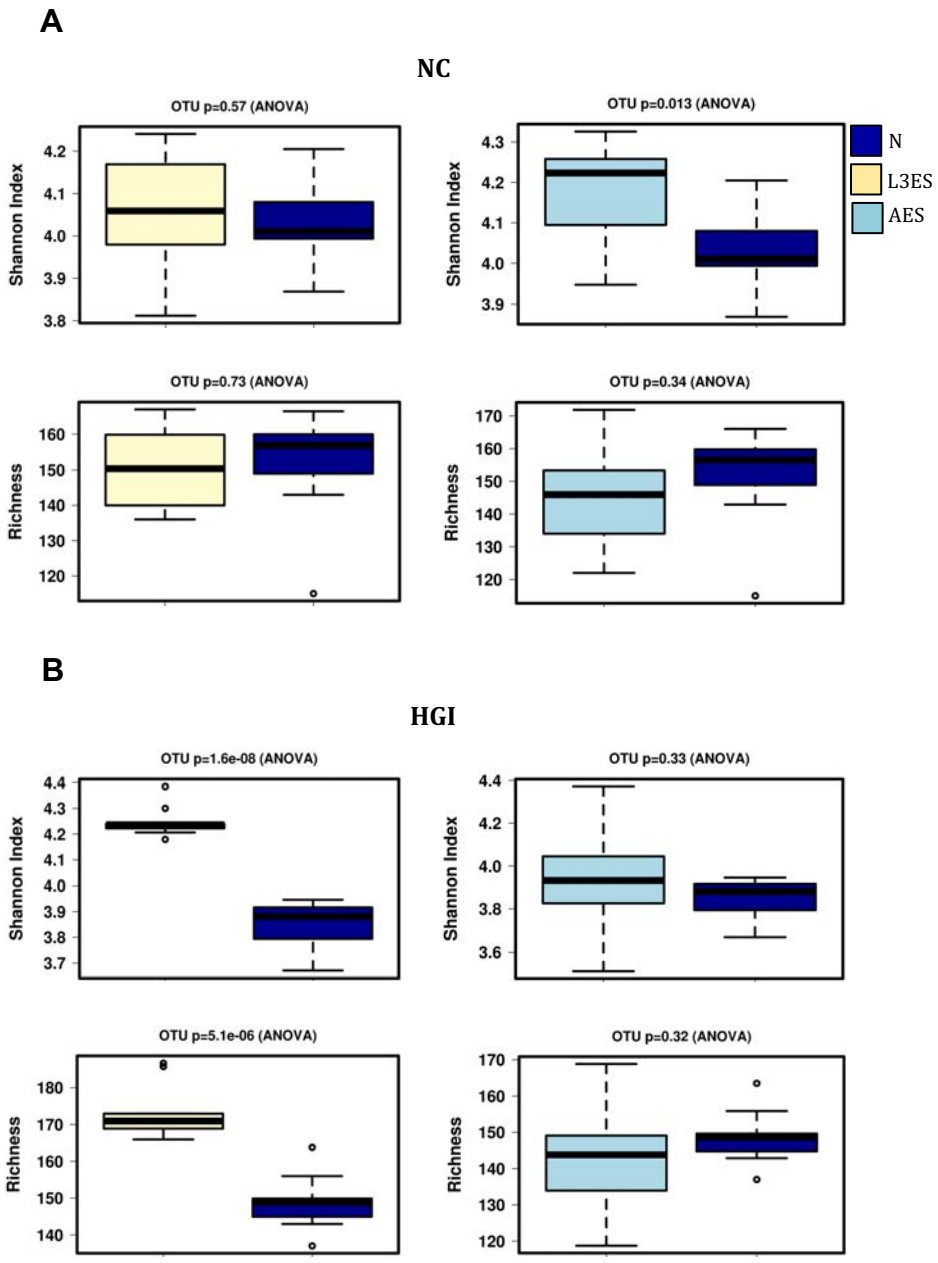
Abundance of Saccharimonadales (belonging to Patescibacteria) and Lactobacillales groups was significantly higher in the HGI diet group treated with AES compare to their untreated group. Patescibacteria are anaerobic fermenters, but this newly identified bacterial group has not been well studied and information on their function is still scarce (380). *Lactobacillus* on the other hand has been widely studied, and different *Lactobacillus* probiotic strains have been reported to improve parameters related to T2D (381).

Many factors may play a role in modulating the abundance of microbial species in the gut. The bacterial composition can vary in the same individual due to variation in the physiology, pH and O_2 tension, digesta flow rates in different anatomical regions, as well as between individuals due to variation in genetic factors, environment and habitual dietary intake (382). These variations can be factors contributing to the onset of many diseases and may have potential therapeutic implications (383). Correlation between helminth presence and changes in the microbial composition have already been mentioned elsewhere throughout, but it is pertinent to note that many studies reported changes in the composition of the gut microbiota as a result of helminth infection and subsequent improvement in the outcome of immune mediated diseases. Whether the changes we found in our study are a direct effect of helminth infection or a consequence of the host's immune response to the infection, and whether these changes are essential to confer protection against T2D are yet to be investigated. A deeper understanding of the interplay between the host-microbiota-helminth triad and other variables may represent a new therapeutic strategy to reverse the pathological effects of T2D.



Supplementary Fig 4.4.1: Infection with *N. brasiliensis* resulted in altered alpha diversity and microbial richness in normal control and T2D mice.

C57/BL6 mice were fed either a normal control (NC), high fat (HF) or high glycaemic index (HGI) diet and infected once monthly with 500 *Nippostrongylus brasiliensis* L3. Microbial alpha diversity and richness in the small intestine of *N. brasiliensis* infected groups (Nb) and uninfected groups (N) in normal control diet (NC) diet, high fat (HF) diet and high glycaemic index (HGI) diet. The data are representative of two different experiments where n = 5/group.



Supplementary Fig 4.4.3: Treatment with *N. brasiliensis* L3ES and AES resulted in alternation in the alpha diversity and species richness of both normal control diet and high glycaemic index diet groups.

C57/BL6 mice were fed normal control (NC) or high glycaemic index (HGI) diets and treated twice weekly with L3ES or AES of *Nippostrongylus brasiliensis*. Microbial alpha diversity and richness in the faecal samples of *N. brasiliensis* (L3ES) and (AES) treated groups and untreated groups (N). **(A)** In normal control diet (NC). **(B)** In high glycemic index diet (HGI). The data are representative of one experiment where n = 10/group.

Acknowledgments

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5. General Discussion

5.1 Overview

DM is widely distributed and considered the fourth leading cause of non-communicable disease deaths. DM and the associated pathologic sequelae are responsible for about 5.1 million deaths per year globally and accounted for USD 727 billion of the world's health expenditure in 2017 (3). It has been reported that the prevalence of diabetes is higher in urban (279.2 million) than in rural (145.7 million) areas and this gap is predicted to widen to 472.6 million people in urban areas and 156 million in rural areas by 2045, with wide variations in this prevalence worldwide (3). For example, the Western Pacific region has the highest number of people with diabetes (159 million people), followed by South-East Asia (82 million), Europe (58 million), and North America (46 million) (3). Further, it has been shown that T2D occurs in youth, with the highest rates observed in people aged 15–19 (384). Moreover, it has been estimated that 352.1 million people have impaired glucose tolerance worldwide and nearly half of those are under the age of 50 (3). T2D is characterised by a persistent elevation in the blood glucose level due to an absolute or relative deficiency in insulin or resistance to its action (385).

Diabetes is a multifaceted disease where genetics, immunological factors and environmental factors have a major role in its pathogenesis (386). Appropriate balance between pro-inflammatory and anti-inflammatory immune responses is essential to maintain whole body homeostasis and to avoid inflammatory diseases (387). Inflammation is a crucial factor contributing to the development of T2D (388). In T2D, levels of certain inflammatory molecules are increased, and the components of the immune system are skewed toward pro-inflammatory subsets (388). It has been proposed that changed dietary habits, a cleaner environment, vaccination programmes and excessive antibiotic use have reduced our exposure to many infectious agents and symbiotic microorganisms (including helminths and gut microbiota) that had an evolutionary relationship with humans, and this relationship helps in the regulation of immune responses and preservation of homeostasis (389). It has been suggested that gut microbiota composition has an effect on the pathogenesis of T2D (390). Lack of exposure to pathogens is thought to increase susceptibility to allergic and autoimmune diseases like, asthma, IBD and T1D (65). Epidemiological studies as well as experimental studies have reported an inverse relationship between the presence of helminths and T2D (391). Moreover, helminth presence has been linked with microbiota diversity and composition which may contribute to beneficial health effects in the host (392). Hence, alteration of the gut microbiome in the presence of helminth parasites might contribute to improved metabolic outcomes observed in T2D.

Data presented in this dissertation provide evidence that infection with *N. brasiliensis* and administration of its ES products from both adult larval stages significantly decreased blood glucose levels and reduced body weight gain in mice using two different models of diet-induced T2D: HGI, HF. Specifically, I have shown that *N. brasiliensis* infection and administration of ES products resulted in significantly increased systemic eosinophil responses in different organs including MLNs, AT, and liver. This was associated with an increase in type 2 immune responses measured by *IL-4*, *Chil3* and *Rentla* in AT, liver and gut tissue and an increase in the levels of IL-4 and IL-5 in AT and liver of ES treated groups. I also found that infection with *N. brasiliensis* or treatment with their ES products showed a significant difference in the microbial profile of those groups compared to naïve littermates. Moreover, there were trends towards an increase in the gut microbial diversity and changes in specific bacterial taxa after helminth infection or treatment with ES products.

5.1.1 N. brasiliensis *infection and ES products improve glucose metabolism and regulate the immune responses associated with T2D.*

Helminth infections play a crucial role in the induction of Th2- and Treg-mediated immune responses in many inflammatory disorders such as IBD, MS, arthritis, asthma and T1D (270). Similarly, it has been reported that helminth infections showed promising results in patients with IBD, celiac disease and MS (104, 105, 271). Further, it has been demonstrated that helminths and their secretions can regulate the host's immune response, which might have beneficial effects in promoting or preventing the inflammatory responses associated with immune-mediated diseases (164).

In our studies, we found that infection with *N. brasiliensis* or treatment with its ES products had a beneficial effect against T2D in two different diabetic models. Infection with *N. brasiliensis* or treatment with their ES products significantly induced peripheral glucose uptake and specific glucose sensitivity and reduced body weight gain (see results from Chapter 2 & 3). In line with my studies, infection with *N. brasiliensis* decreased weight gain and improved glucose metabolism in two different models of obese mice, which was associated with induction of Th2 immune responses (136). Other studies have also reported improvements in glucose tolerance of obese mice after infection with the filarial helminth *L. sigmodontis* or administration of soluble adult worm extract which was associated with an increase in the numbers of $CD4^+$ T cells, eosinophils and AAMs in the AT. Depletion of eosinophils resulted in impaired glucose tolerance (137). Infection with *H. polygyrus* also resulted in decreased body weight gain and improved glucose and lipid metabolism with increases in Th2/Treg immune responses in the MLNs, AT, liver and gut. (134, 135). SEA from *S. japonicum* decreased the level of blood sugar and induced Th2 and Treg immune responses that protected against T2D in mice (315).

5.1.2 Mechanism(s) by which **N. brasiliensis** *and their ES regulate immune responses to T2D.*

In the present study I have identified an important role for helminth infection in the induction of type 2 immune responses by enhancing eosinophil recruitment in MLNs, AT and liver, with increased expression of *IL-4* and markers of M2 MACs *Rentla* and *Chil3* in AT, liver and SI (see results from Chapter 2). Further, I showed that administration of *N. brasiliensis* crude ES from adult and L3 stages also induced eosinophil recruitment in the MLNs, AT and liver, with a significant increase in the level of IL-5 in the AT and liver (see results from Chapter 3). In line with my results, *N. brasiliensis* infectyion resulted in improved insulin sensitivity and lipid metabolism, decrease in adiposity and increase in energy expenditure in mouse models of obesity (37, 41, 136). Infection with *N. brasiliensis* is associated with induction of Th2 immune responses, resulting in the production of IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and IL-33 and induction of a systemic and localized eosinophilia, ILC2s and M2 MACs (94, 280-285, 393-395). Infection with *N. brasiliensis* induces residential ILC2s that secret and maintain IL-5 leading to local eosinophil accumulation (396). Also, *N. brasiliensis* induced ILC2s and $CD^{+}4$ T cells and expansion of IL-4 and/or IL-13 that maintain M2 MACs (280). *N. brasiliensis* infection drives signaling via IL-4/IL-13/STAT-6 axis (282, 284), which has already been reported to be involved in the regulation of lipid metabolism leading to weight loss in obese mice after *N. brasiliensis* infection (136).

Eosinophils are involved in the regulation of homeostasis of many tissues including gastrointestinal tract, lungs, adipose tissue, thymus, uterus and mammary glands (397). Recently, eosinophils have been implicated in the regulation of glucose metabolism and energy expenditure (37, 41, 288, 298, 398). IL-5 promotes eosinophil development, activation and survival (397). IL-5 plays an important role in promoting AT eosinophils that maintain adipose AAMs. Eosinophil accumulation in AT was impaired in IL-5 deficient mice, leading to increased body fat, impaired glucose tolerance and insulin resistance in obese mice (37, 41). An *in vitro* study found that IL-4 restored insulin sensitivity in an experimental IR model of 3T3-L1 adipocytes (293). Moreover, IL-4 decreased blood glucose and body weight, which promotes insulin sensitivity and inhibits accumulation of lipids in AT and reduced the adipocyte layer in the skin *in vivo* (292, 295). Emerging studies implicate different immunological pathways in the regulation of glucose metabolism and energy expenditure. Resident ILC2s in AT increase production of IL-5 and IL-13, which promotes accumulation of eosinophils and M2 MACs. This reduces inflammation in AT and increases insulin sensitivity and beiging of AT (41, 399). Another study has highlighted the role of Metrnl in the recruitment of eosinophils to the AT (398). This induced IL4/IL13, which are required to increase M2 MAC numbers and induction of AT beiging (298, 398). Moreover, the IL-33/ILC2 axis can promote beiging in AT and limit adiposity via increasing caloric expenditure that regulates metabolic homeostasis (290). Signaling via IL-4/ STAT6 regulates nutrient metabolism in the liver and insulin

sensitivity and attenuates body weight and AT inflammation (291). Considering these multiple immunological signaling pathways, a plausible mechanism by which *N. brasiliensis* improves glucose metabolism and decreases adiposity might due to local and systemic eosinophilia that leads to expansion of Th2 cytokines and M2 MACs.

Regarding treatment with *N. brasiliensis* ES products, I have reported that administration of AES or L3ES twice weekly at a dose of 1 mg/kg resulted in significantly decreased blood glucose levels and attenuation of body weight gain. This was associated with an increase in the level of AT and liver IL-5 and a trend towards an increase in the level of IL-4 in these tissues. Expression of IL-6 significantly decreased in AT but increased in the liver. As previously discussed, eosinophils play a role in the increase in levels of IL-5 and/or IL-4 in regulating glucose and lipid metabolism as well as energy expenditure (37, 41, 399). An *in vitro* study revealed that treatment of intestinal pig epithelial cells with *T. suis* ES products resulted in increased levels of IL-6 and IL-10, with no secretion of IL-4 in both differentiated and undifferentiated cells (323). Although an increase in the levels of IL-6 were reported to be involved in the induction of insulin resistance in the AT and liver and pathogenesis of T2D (324, 325), others have reported a beneficial role for IL-6 in liver regeneration and maintenance of liver tissue homeostasis as well as protection against liver inflammation and insulin resistance (326-328). Human PBMC stimulated with α-CD3 and α-CD28 antibodies showed up-regulation in the expression of IL-4 and IL-5 after short term preincubation with IL-6. However, long term preincubation of these cells with IL-6 led to gradual disappearance of its effect on IL-4 production but the effect on IL-5 became more pronounced (329). Moreover, IL-6 enhanced *in vivo* and *in vitro* expansion and survival of Ag-stimulated $CD4^+$ T cells in the blood, lymph nodes, spleen, liver and lung, which reduced the level of apoptosis in these cells (330). Furthermore, splenocytes of $TLR2^{+/+}$ mice immunised with OVA in the presence of eosinophil-derived neurotoxin (EDN) produced IL-5, IL-6, IL-10 and IL-13, whereas in the presence of LPS they produced IFN-γ (331). So, it is more likely that the increase in the levels of IL-6 in the liver that I observed are a consequence of avoiding apoptosis in this tissue, and which might be mediated via EDN activating TLR2 and expansion and survival of $CD4^+$ T cells in the liver. The mechanisms by which IL-6 might enhance a protective effect against T2D has yet to be investigated.

5.1.3 Mechanism(s) by which N. brasiliensis *and host microbiota regulate immune responses and T2D.*

The gut microbiota that inhabits the mammalian body plays an essential role in regulating host metabolism, physiology, nutrition and immune function (400). Changes in environmental factors such as dietary habits, excessive use of antibiotics, sanitation, mode of birth and feeding pattern all can have a devastating impact on the composition and diversity of the intestinal microbiota (401).

Changes in the composition of the gut microbiota as a result of helminth infections have been reported by many researchers (244-247, 249, 257, 261). On the other hand, a growing body of evidence suggests that helminth infections and their ES products can alter the gut microbial community, leading to protection against immune mediated diseases, such as allergic inflammation, colitis, obesity, arthritis and lupus (250, 251, 254-256, 341, 352).

My data showed significant differences in the clustering analysis between *N. brasiliensis* infected groups compared to naïve groups in all diets (NC, HGI and HF diets). The same result was found between the groups treated with L3ES or AES and untreated littermates in both NC and HGI diets. However, no significant differences in α-diversity and richness were detected in the *N. brasiliensis* infected groups compared to the uninfected groups, as well as between L3ES or AES treated groups on a NC diet, or the AES treated group on a HGI diet compared to the untreated groups. Only mice on a HGI diet and treated with L3ES showed significantly higher α-diversity and richness when compared with the naïve group; however, I still found significant changes in the community composition at the phylum and order levels in the infected as well as treated groups compared to naïve littermates, but each of them showed variability among different diets as well as between the different treatments (L3ES and AES). Clostridiales increased while Bifidobacteriales decreased in the NC diet group. Clostridiales and Desulfovibrionales increased in the HF diet group. Burkholderiales increased, while CW040 and Verrucomicrobiales decreased in the HGI diet group. Coriobacteriales and Desulfovibrionales decreased, while Verrucomicrobiales increased in the NC diet group treated with L3ES. Rhodospirillales decreased in the NC diet group treated with AES. Coriobacteriales, Verrucomicrobiales and Betaproteobacteriales decreased in the HGI group treated with L3ES. Coriobacteriales decreased while Verrucomicrobiales, Saccharimonadales and Lactobacillales increased in the HGI diet group treated with AES.

It has been reported that T2D patients had lower abundance of Clostridiales and this group negatively correlated with glucose and lipid metabolism (236, 339, 340, 358, 359). Moreover, the microbiota of diabetic group is represented in lower members of *Bifidobacterium* in comparison with the healthy group (402). These organisms are reported to be associated with health benefits in T2D (356); however, a few studies have reported *Bifidobacterium* to cause infections (357). The Burkholderiales group was lower in obese mice, and treatment of diabetic rats with insulin increased the abundance of Burkholderiales (362, 364). I found a conflicting result regarding Desulfovibrionales and Verrucomicrobiales, although this controversy is also found in the literature. While some groups have reported a decrease, others have reported an increase in the abundance of the Desulfovibrionales group when treating obese and diabetic rats with insulin (362, 364, 365). Furthermore, while some researchers reported an increase in the abundance of the Verrucomicrobia group in obesity and T2D, others found a decrease in the abundance of this group (369-371, 376). In

addition, order CW040 belongs to the TM7 phylum, and this phylum has been found to be higher in obese children and adolescents (369). A fat, fructose and cholesterol-rich diet has been associated with a higher abundance of *Rhodospirillales* (379). Also, a higher abundance of Betaproteobacteriales has been detected in pre-diabetic and diabetic subjects (236, 376). Saccharimonadales belonging to the Patescibacteria phylum are anaerobic fermenters and a newly identified bacterial group from which little is known (380). The effect of many different *Lactobacillus* probiotic strains has been reported in their ability to improve parameters related to T2D (381). My result are in line with other studies conducted in humans and rats that found no changes in α-diversity as a result of infection with different parasitic nematodes, but still reported some changes in the bacterial communities (253, 403, 404). To explain the variability in the microbiota community composition among different diets as a result of infection as well as between the different treatments, one might consider the differences in the dietary substrates. We also should consider the pathways for the metabolism of these substrates as well as the inter-individual variation in the metabolism they can have and its implication on the abundance of different microbial species in the gut (405). In my work, the sample size was small, which allowed greater sampling variability. On the other hand, helminth presence has diverse effects on the composition of the gut microbiota, with some species having minor effects while others have greater effect swith potential to protect against immune-mediated diseases. In summary, infection with *N. brasiliensis* or treatment with their ES products has no effect on microbial α-diversity; however, clustering analysis demonstrates the existence of discrete bacterial communities. Many factors can influence the abundance of microbial species in the gut, such as diet, host physiology and inflammatory immune responses produced as a consequence of helminth invasion; however, whether the changes I found in this study are a cause of direct and/or indirect effect of helminth infection or their ES products or a consequence of host immune response, and whether these changes are essential to confer protection against T2D are yet to be investigated.

5.2 Conclusion

In conclusion, in this thesis I found that infection with *N. brasiliensis* or treatment with AES or L3ES improves glucose metabolism and decreases body weight gain in diet-induced T2D mouse models. This was associated with an increase in systemic and peripheral eosinophilia along with an increase in Th2 immune responses, characterised by an increase in *IL-4, Rentla* and *Chil3* in infected groups and an increase in IL-5 and a trend towards an increase in IL-4 with a decrease in IL-6 in AT but not in liver of AES and L3ES treated groups. I found no changes in α-diversity after infection or ES treatment, but did detect a shift in the microbiota at the community level which needs to be further investigated.

5.3 Future perspective

- In my work I demonstrated a role for eosinophils in the improvement of glucose metabolism and modulating the immune response associated with T2D. Further studies in eosinophil-depleted mice would be important to elucidate if this pathway is important for the beneficial effect.
- I reported an involvement of other immune cells including M2 MACs, ILC2s and Tregs, so these cell types and their roles in protecting against T2D need to be addressed.
- A large-scale study is important to address the role of infection or treatment with ES on the gut microbiota composition. Also, a microbiota depletion study will also be important to address whether the gut microbiota is essential for protection against T2D after helminth infection or treatment with ES products.
- Further work should be aimed at addressing the potential of anti-inflammatory proteins secreted by *N. brasiliensis* as a therapeutics modality for T2D and other inflammatory-mediated diseases.

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