Sex-Based Differences in Obesity, Gut Dysbiosis and Intestinal Inflammation in High-Fat Diet Mice.

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Submitted as total fulfilment of the Master of Research

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Statement of authorship

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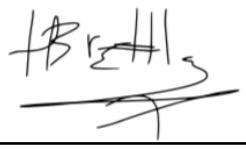
The following individuals also contributed to the work in my thesis:

Name	**Nature of contribution**
Associate Professor Francine Marques	Assisted with 16S rRNA sequencing library preparation and data analysis
Michael Nakai	Assisted with 16S rRNA sequencing library preparation and data analysis
Dr Lisa Mielke	Assisted with project design for intestinal immune cell isolation experiments
Dr Dinesh Raghu	Assisted with project design for intestinal immune cell isolation experiments
LARTF Staff	Maintenance of C57BL/6 mouse colony
Vivian Tran	Assisted with animal work



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Abbreviations

ANOVA	Analysis of variance
ARISA	Automated ribosomal intergenic spacer analysis
BP	Blood pressure
DAMPs	Danger-associated molecular patterns
EDTA	Ethylenediaminetetraacetic acid
FFAs	Free fatty acids
GF	Germ-free
HFD	High-fat diet
HF/HS	High fat/ high sucrose
IELs	Intraepithelial leukocytes
IL-1ß	Interleukin-1ß
IL-6	Interleukin-6
IL-10	Interleukin-10
LARTF	La Trobe Animal Research & Training Facility
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LPLs	Lamina propria leukocytes
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NCD	Normal chow diet
NK	Natural killer
OTU	Operational taxonomic unit
PAMPs	Pathogen-associated molecular patterns

PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PBS	Phosphate buffer solution
PRRs	Pattern recognition receptors
QIIME	Quantitative insights into microbial ecology
SCFAs	Short chain fatty acids
SEM	Standard error of the mean
SOP	Standard operating procedure
T2D	Type II diabetes
TCR	T cell receptor
TNF-α	Tumour necrosis factor alpha
T_{reg}	T regulatory
16S rRNA	16S ribosomal ribonucleic acid

Abstract

The pathophysiology of obesity is largely attributed to the gut microbiota and intestinal immune response. The sexual dimorphism in susceptibility to obesity and its consequences are poorly understood, and thus, this study aimed to characterise intestinal inflammation and gut dysbiosis and determine any sex differences in a mouse model of early obesity. C57BL/6 male and female mice were randomly allocated to either high fat diet (HFD, 42% kcal fat content) or normal chow diet (NCD) at 5-7 weeks of age for 10 weeks (n = 10-12 per sex, per group). During the diet regimen, bodyweight, blood pressure, and glycaemic status were monitored. HFD increased body weight in males from week 6 ($P < 0.0001$) as well as elevating fasting blood glucose and fasting plasma insulin at week 10 ($P = 0.0138$ and 0.0186 respectively). Automated ribosomal intergenic spacer analysis (ARISA) and 16S rRNA sequencing analysis of the faecal DNA revealed sex-specific gut communities, regardless of diet. This sex difference was highlighted in the HFD groups and was demonstrated by an increased Firmicutes: Bacteroidetes ratio, but only in males, with marked differences in specific taxa abundance. The intestinal immune response of the HFD groups in the colon intraepithelial layer and lamina propria layer, assessed by flow cytometry, revealed increased B cell populations in both layers for males but only in the lamina propria layer for females. Moreover, HFD females displayed a stronger immune response in the lamina propria layer for T cell populations, including $CD4^+$, $CD8^+$, $CD4^-/CD8^-$ and $TCR\beta^+$ subpopulations. In conclusion, HFD induced physiological impairments in males in addition to increasing the Firmicutes: Bacteroidetes ratio and producing a marked shift in specific taxa abundance. HFD females showed elevated T cells in the colon LPL, whereas this robust immune response was not seen in males. Understanding the relationship of the gut microbial community and the stronger intestinal immune response seen in females may shed light on how they are protected against obesity.

Chapter 1: Introduction

1.1 Obesity

Obesity is the excessive accumulation of fat that increases the risk of developing metabolic disease and comorbidities such as cardiovascular disease, diabetes, stroke and various cancers (1). Obesity is increasingly prevalent and has reached epidemic proportions worldwide. In Australia, ~2/3 of the population are overweight or obese, costing the economy $58 billion annually in associated health care costs (2). Obesity has many risk factors including poor dietary habits, old age, ethnicity, low income and sex (3). Focusing on sex, in Australia there is a greater prevalence of overweight and obesity in males, 75%, compared to females, 60%, (4). The differing characteristics between the sexes, sexual dimorphism, of obesity, will be discussed in detail in this thesis, with a focus on the intestinal immune response and gut microbiota implicated in the development of the disease (5).

1.1.1 Biological state of obesity

The most common tool used to identify obesity is body mass index, calculated by dividing an individual's weight (kg) by their height (m^2) (6). Overweight and obese individuals are classified as having a body mass index greater than 25 and 30 respectively (6). Obesity occurs as a result of a chronic imbalance of energy intake versus energy expenditure and results in accompanying cardiometabolic disorders including low grade chronic inflammation, metabolic endotoxemia, hypertension, dyslipidaemia and insulin resistance (7). In a state of chronic energy imbalance, the body undergoes specific adaptations and white adipose tissue has been identified as the major initiator of the consequential metabolic disturbances (8). White adipose tissue is made up of a complex network of cells that are constantly regulating metabolism (9). White adipocytes are responsible for hydrolysing triglycerides into free fatty acids (FFAs) and

glycerol and releasing these molecules into circulation to be taken up and used by peripheral tissues (10). However, in the case of excess caloric intake, white adipocytes will store triglycerides until their limit is exceeded and will undergo proliferation and differentiation therefore, resulting in increased adiposity (11). Adipose tissue, now recognised as an important player in endocrine signalling, releases stress signals, in the form of adipokines, into circulation and to peripheral tissues initiating a state of inflammation (Figure 1.1) (12). These secreted proinflammatory factors include cytokines such as tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) (13). Additionally, anti-inflammatory cytokines such as adiponectin are suppressed or downregulated during this time (14).

In response to the stress signals released from adipose tissue, immune cells infiltrate into the tissue and are triggered to alter into their proinflammatory phenotypic expression, also releasing proinflammatory cytokines and thus exacerbating inflammation (15). It is not surprising that in a state of high inflammatory stress as seen in obesity, white adipose tissue and adipocytes provoke the release of high circulating levels of FFAs, resulting in dyslipidaemia and insulin resistance (16). Concomitantly with insulin resistance, is the increase in blood glucose resulting in hyperglycaemia (17). In normal metabolic homeostatic conditions, dietary carbohydrates are broken down into glucose molecules that enter the bloodstream (18). In response to glucose in the blood, insulin facilitates glucose uptake in cells and tissues for energy and to maintain homeostasis (18). However, in a state of obesity and excess energy, both insulin resistance and hyperglycaemia results in the dysfunction of insulin receptor pathways in the muscles and the liver, and a state of glucotoxicity occurs (19). This leads to the dysregulation of whole-body energy homeostasis and increased risk of developing type II diabetes mellitus (T2D) (19).

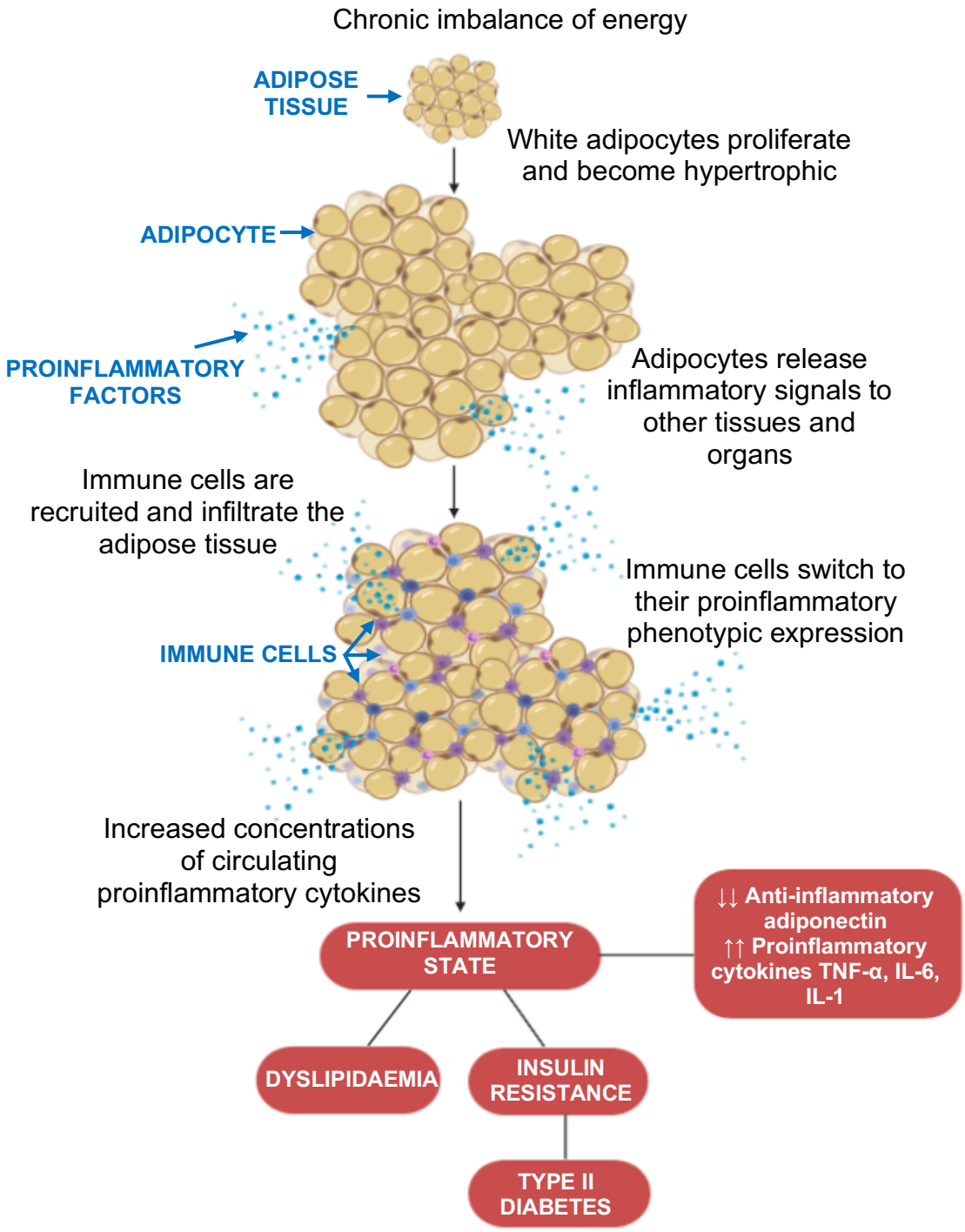

Figure 1.1. The biological development of obesity in white adipose tissue. In a state of chronic energy imbalance, white adipose tissue undergoes proliferation and differentiation, and releases proinflammatory stress signals to other organs and tissues. In response, immune cells such as macrophages, dendritic cells, T cells and B cells infiltrate into the tissue. From there they secrete proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α). In contrast, anti-inflammatory adiponectin production and secretion is suppressed (11, 12, 15).

In addition to increasing the risk of developing T2D, hyperinsulinemia and hyperglycaemia contribute to the pathophysiology of hypertension, clinically diagnosed as persistently high blood pressure (>140 mmHg systolic and/or >90 mmHg diastolic) (20, 21). Vascular dysfunction (impaired vasodilation and increased vasoconstriction) and reduced blood flow to essential organs and tissues are major consequences of hypertension (22). Hypertension can be driven by a number of factors including hyperinsulinemia, but another key disease mechanism is the over-activation of the renin-angiotensin-aldosterone system and retention of salt and water (23). The renin-angiotensin-aldosterone system regulates blood pressure in the instance of low blood volume, by releasing enzymes to ultimately synthesise angiotensin II which constricts blood vessels (23). However, adverse activation of this system, often seen in obese individuals, reduces blood flow to organs such as the heart and brain, increasing chances of cardiovascular disease and stroke (24). The key characteristics of obesity including adipose tissue expansion, hyperinsulinemia, and hyperglycaemia are all recognised to be sexually dimorphic (25).

1.1.2 Sexual dimorphism of obesity

Worldwide, there are differences in the prevalence of obesity in males and females resulting in a disparity of obesity related comorbidities (26). The established sexual dimorphism in obesity-related disorders include differences in the prevalence of diabetes, hypertension, and adipose tissue distribution. For example, the prevalence of obesity-associated T2D in Australia is greater in males compared to females (27). Similarly to T2D, the prevalence of hypertension is greater in males compared to females (28). Interestingly, although prevalence is greater in men, women with T2D and/ or hypertension are at a greater risk of developing cardiovascular disease compared to men, indicating a strong decrease in the protective pathways usually established in females (29). The difference in the prevalence between the sexes in the

concomitant metabolic disorders of obesity, are largely credited to the direct or indirect actions of sex hormones such as estrogen and testosterone found in both males and females (30).

1.1.2.1 The action of sex hormones in obesity

A significant proportion of obesity research has been dedicated to investigating the roles of estrogen and testosterone in the pathophysiology of this condition. Estrogen has three major endogenous forms: estrone, estradiol and estriol (31). Estradiol is the most abundant form and is predominantly synthesised and released by the ovaries however, it can also be synthesised by other tissues such as the brain and adipose tissue (32). Although produced in both sexes, females, and in particular, premenopausal females, have greater levels of total and free estradiol compared to males (33). Testosterone, on the other hand, is the main derivative of androgens, mainly derived from the testes, and produced in males to a much greater extent compared to females (34). Both estradiol and testosterone have been identified as key players in a number of metabolic processes such as energy metabolism, food intake and body weight regulation, and modulation of the immune profile (35, 36). Additionally, these hormones can act on a number of targets including the hypothalamus, adipose tissue, liver, skeletal muscles and gastrointestinal tract (37). For example, estradiol influences the actions of the hypothalamus to decrease food intake, increase energy expenditure and regulate fat distribution (30). In regard to fat distribution, estradiol synthesises with adipose tissue to prevent the accumulation of harmful abdominal visceral fat, often seen in males, and instead, preferentially accumulates gluteofemoral subcutaneous adipose tissue, typically observed in females (38). Although high testosterone levels in males are also associated with beneficial metabolic influence, for example, high levels of testosterone are associated with healthy waist circumference and increased insulin sensitivity, there is a much greater degree of protection offered from estrogens due to the high presence of estrogen receptors on various cells (39, 40).

The role of estrogen has been investigated using obesogenic diet studies, post-menopausal studies and ovariectomised rodent studies. Diet-induced obesity studies have shown beneficial effects of estrogen in both males and females (41, 42). For example, high-fat diet (HFD) male mice supplemented with estradiol had reduced white adipose tissue inflammation and weight gain compared to those without estradiol supplementation (42). Similar associations can be made between the protective effects of estrogen against inflammation in females, with estradiol treatment attenuating weight gain and increasing circulating anti-inflammatory adiponectin levels in obese and HFD females (41). The beneficial effects of estrogen can also be recognized by the detrimental metabolic disorders accompanying menopause, which is characterised by the dramatic reduction in the production of the ovarian hormones, including estrogen (43). The prevalence of obesity and metabolic disorders markedly increases in post-menopausal females, linking the decrease in ovarian hormones as an activating factor (44). The lack of estrogen not only results in a detrimental increase in visceral adipose tissue but also reduces adiponectin levels, increases plasma insulin and glucose, and increases the lipid levels to an unfavourable profile (45, 46). Rodents do not naturally undergo menopause, but this condition can be modelled via ovariectomy. Similarly to post-menopause models, ovariectomy in rodents resulted in increased body weight, blood triglyceride and total cholesterol levels, and decreased high-density lipoprotein cholesterol and glucose intolerance (47, 48). Both post-menopausal and ovariectomised models reveal the importance of ovarian-derived hormones on metabolic homeostasis and thus, the lack of these hormones results in an increased risk of developing cardiovascular disease and diabetes (49). The contribution that sex hormones, in particular estrogen, play in the development of the sexual dimorphism of obesity are well-established however, recent research is now recognising the intestines as a key site for identifying initial differences between the sexes. Therefore, our study addressed the role of the intestinal immune system and the gut microbiota in the sexual dimorphism of obesity.

1.2 The immune system

1.2.1 Innate and adaptive immunity

The immune system is responsible for protecting the host against invading pathogens by initiating a series of chemical signalling pathways (50). It is separated into two divisions; the innate and adaptive immune systems (51). The innate immune system is the first line of defence, that immediately responds to signals of microbial invasion or damage (52). Cellular constituents of the innate system that have the ability to recognise pathogen-associated molecular patterns (PAMPs) and/or danger-associated molecular patterns (DAMPs) are myeloid-derived cells, including macrophages (subsets include "classical" M1 proinflammatory macrophages and "alternative" M2 anti-inflammatory macrophages), dendritic cells and killing cells such as natural killer (NK) cells (Figure 1.2) (50). In sterile inflammation and chronic inflammatory conditions such as obesity, there is an increase in DAMPs (such as cholesterol, reactive oxygen species, high extracellular salt). These "danger signals" are recognised by these innate immune cells by membrane-bound glycoproteins known as pattern recognition receptors (PRRs; i.e. toll-like receptors). This stimulates the release of proinflammatory mediators which recruit additional leukocytes to the site (Figure 1.3) (52).

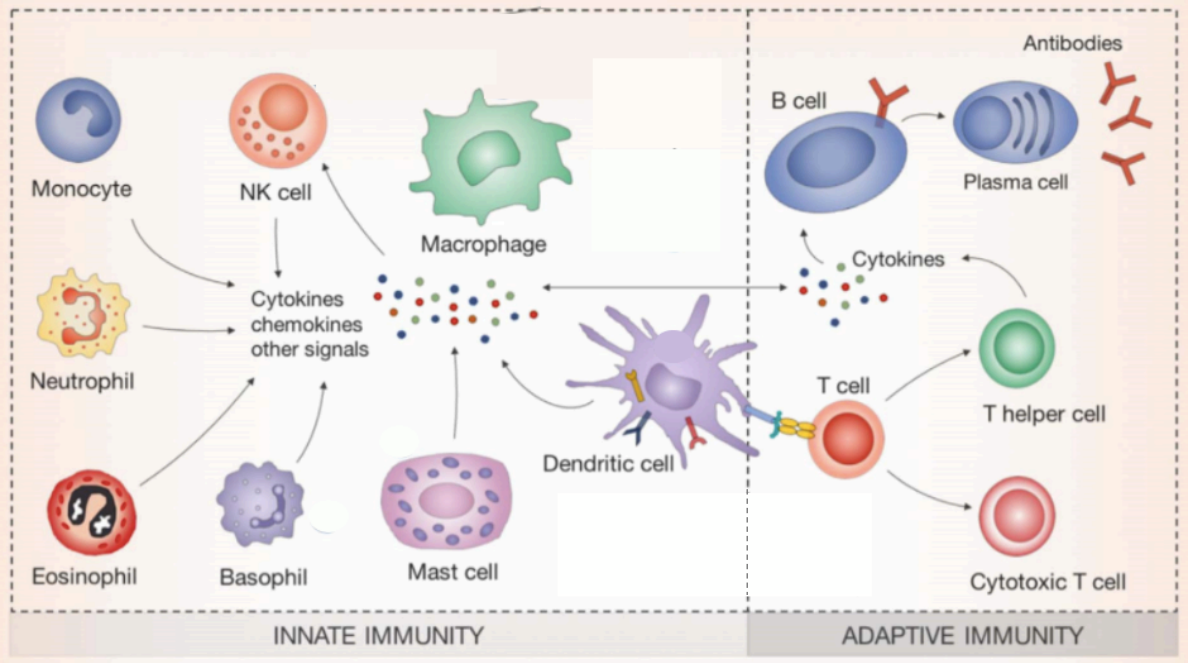

Figure 1.2. Cells of the immune system. The innate immune system consists of myeloid-derived cells including macrophages, monocytes, neutrophils, eosinophils, basophils, mast cells, dendritic cells and natural killer (NK) cells. Innate immune cells recognise pathogen-associated molecular patterns (PAMPs) such as gram-negative bacteria cell wall lipopolysaccharide (LPS) by their pattern recognition receptors (PRRs). In response, they produce cytokines and chemokines to induce an inflammatory state as well as presenting antigens on their major histocompatibility complex (MHC) to activate the adaptive immune system. The adaptive immune system consists of lymphocytes (T and B cells). T cells can be subdivided into cytokine secreting T helper cells and cytotoxic T cells. B cells differentiate into plasma and memory cells to produce antibodies for repeated exposure. Adapted from (53).

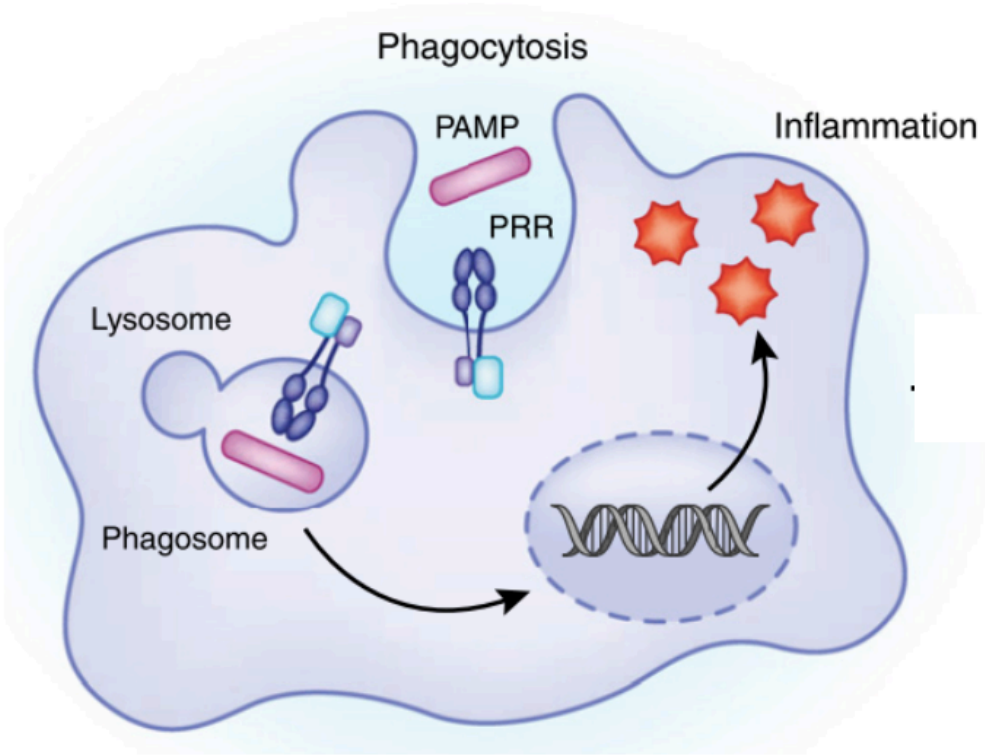

Figure 1.3. Pattern recognition receptors (PRRs) respond to pathogen-associated molecular pattern (PAMPs). Innate immune cells phagocytose the PAMPs and fuse with the lysosome to degrade the pathogen as well as releasing proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) to induce an inflammatory state. Sourced from (52).

Another response from the innate system after recognising PAMPs and/or DAMPs, is the activation of the adaptive immune system (51). The adaptive immune system is the delayed, specialised immune response with memory for repeated pathogen exposure (54). Adaptive immune cells are derived from lymphocytes and are produced in either the bone marrow (B cells) or the thymus (T cells) (Figure 1.2) (53). Antigen-presenting cells, such as macrophages and dendritic cells, have the ability to take up an antigen, and present it on a set of membrane-bound proteins, the major histocompatibility complex class II (MHC II) (51). T cells, using their T cell receptor (TCR), recognise the antigen and bind to the MHC. A co-stimulatory signal is required for activation, and is contributed by the binding of the B7 protein of the antigen-presenting cells to the CD28 protein of the T cell (Figure 1.4) (51).

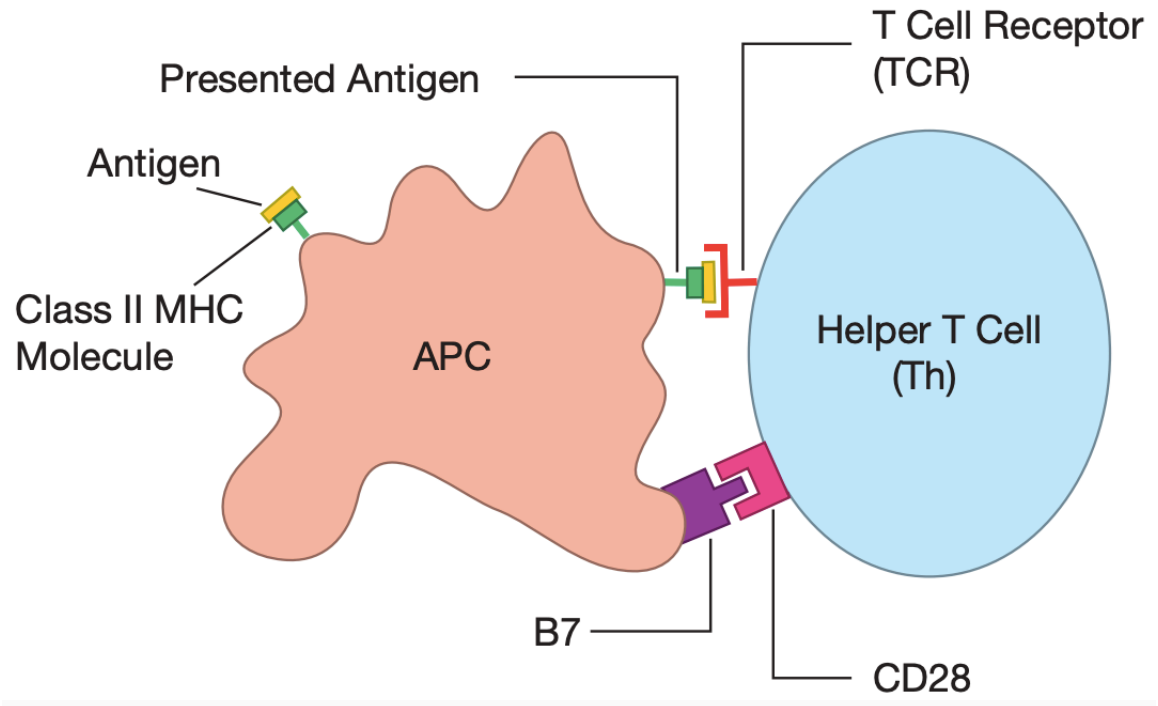

Figure 1.4. Mature antigen-presenting cell activates the adaptive immune system. The antigen is presented on the major histocompatibility complex II (MHC) to the T cell receptor (TCR). A co-stimulant is required for activation and is provided by the B7 protein on the antigen presenting cell and the CD28 receptor on the T helper cell. Sourced from (51).

Activated T cells differentiate into numerous subsets with varying functions. Two major subsets are the cytotoxic ($CD8^+$) T cells and helper ($CD4^+$) T cells. Cytotoxic T cells kill infected cells by inducing apoptosis or releasing cytotoxic proteins such as perforin and granzymes into the target cell (55). Helper T cells, including subsets Th1, Th2 and Th17, have the role of activating various other immune cells, including B cells, and suppressing the immune system (T regulatory cells (T_{reg}) and Th2) (56). The lymphatic B cell is responsible for producing various antibodies to neutralise and phagocytose specific pathogens (57). Both the innate and adaptive immune systems work together to eliminate pathogens and dead or dying host cells, however, inflammatory profiles vary between tissue types and also differ between the sexes.

1.2.2 Sexual dimorphism of the immune system

In targets such as adipose tissue, lymphatic organs, peripheral blood and the intestines, there are immune system differences between the sexes in both the innate and adaptive immune responses (58). Generally, females have a stronger, more intense innate and adaptive immune response compared to males however, this differs based on the site of inflammation (59). In certain circumstances, a greater immune response is beneficial in clearing possible infections however, this stronger immune activation and response, that is often observed in females, may influence the development of inflammatory diseases specifically, autoimmune diseases (60, 61). The sexual dimorphism of immunity can be partially explained by the differing efficiency of the immune cells and the size of the lymphatic site (62, 63). For example, female antigen-presenting cells have been shown to respond to the antigen, take up the foreign substance and express the antigen on its MHC, quicker than male antigen-presenting cells (64). Additionally, differences can be seen in the size of certain lymphatic vessels, where immune cells are generated, undergo differentiation and proliferation, and are stored for future immune response, such as the thymus, Peyer's patches and spleen (63, 65). The thymus, crucial in the development of adaptive immune cells, has been shown to be bigger in males compared to females however, the female thymus harbours more cells than males reflecting their readiness for eliminating possible future pathogenic invasions (63, 66). Flow cytometry analysis on other lymphatic vessels such as the Peyer's patches and spleen also reveal that females have a higher number of T cells but a lower number of the T_{reg} subset, further suggesting a stronger and more robust adaptive response in females (65). The reasons underlaying the sex differences in the immune system, are also a result of sex hormones and the central role that they play due to the expression of estrogen and androgen receptors on many of the immune cells such as lymphocytes, including T and B cells, macrophages, and dendritic cells (63).

1.2.2.1 The role of sex hormones and the immune system

The important role of estrogen has been identified in the pathogenesis of inflammation by studying the female menstrual cycle. In the follicular phase of the cycle, when estrogen levels are low, T_{reg} cells are high, suggesting a suppression of the immune system (67). Opposingly, in the luteal phase of the cycle, when estrogen levels are high, cytotoxic ($CD8^+$) T cells are high, signifying the immunoenhancing role of estrogens (67, 68). Contrastingly to immunoenhancing estrogen, testosterone is seen as immunosuppressive (68). This can be seen in males with testosterone deficiencies, that reveal higher circulating proinflammatory cytokines as well as higher numbers of helper T cells ($CD4^+$) and cytotoxic ($CD8^+$) T cells compared to males with healthy levels of testosterone (62). Studies involving estrogen and testosterone administration to *in vitro* cultured peripheral blood immune cells, strengthen the findings of their role in the immune response. Female cultured cells reveal an increase in both innate and adaptive immunity in response to antigens whereas, testosterone inhibits the proliferation of both lymphocytes and their cytokine secretion (69). The sex difference of the immune system is also seen in the intestinal tissue and in our research, we must consider how the sexual dimorphism of the intestinal immune system contributes to the development of obesity in males and females (70).

1.2.2.2 Obesity and the intestinal immune system

The intestinal barrier is made up of a layer of epithelial cells linked together by tight junction proteins and coated with a mucus layer (71). The intestinal tract harbours the greatest compartment of the systematic innate and adaptive immune systems and therefore, the intestines are crucial in preventing antigens and microbiota from gaining access to underlying tissues (72). In obesity, the increased proinflammatory circulating adipokines and cytokines, as well as neutrophils, dendritic cells and monocytes from visceral adipocytes, are able to easily

disrupt the intestinal epithelial barrier (73). This is due to the close proximity of central visceral adipose tissue to the intestines (73). In addition to the disruption of the intestinal barrier brought on by adipose tissue inflammation, the intestinal immune system is strongly shaped by diet (74). For example, consumption of HFD triggers the proinflammatory pathways in the intestines causing disturbance to the mucus layer coating the intestinal epithelial barrier (75). The inflammatory environment induced by HFD deteriorates mucus production and thickness and thus, increases intestinal permeability (75). Intestinal permeability allows leakage of water, proteins and other endotoxic molecules such as lipopolysaccharide (LPS) into systemic circulation with the ability to reach other organs and tissues (Figure 1.5) (5). High circulating levels of LPS, termed metabolic endotoxemia, promotes further inflammation, weight gain and diabetes in experimental animals and humans (76). For example, endotoxin and circulating plasma cytokine levels (IL-1β, IL-6, and TNF-α) are increased in mice with obesity and diabetes, compared to wildtype mice (77).

To our knowledge, sexual dimorphism of the intestinal immune system as a result of HFD has not been researched. However, studies have identified differences in healthy individuals as well as functional gastrointestinal disorders having a higher prevalence in females (60, 78). For example, analysing the lamina propria layer of the intestinal samples of healthy males and females revealed a higher immune activation and higher $CD4^+$ and $CD8^+$ T cell counts in females (60). The balance of the immune system, and in particular the intestinal immune system, appears to be essential in maintaining gut homeostasis and another contributing factor to this stability is the microbial community residing in the intestines (50, 79).

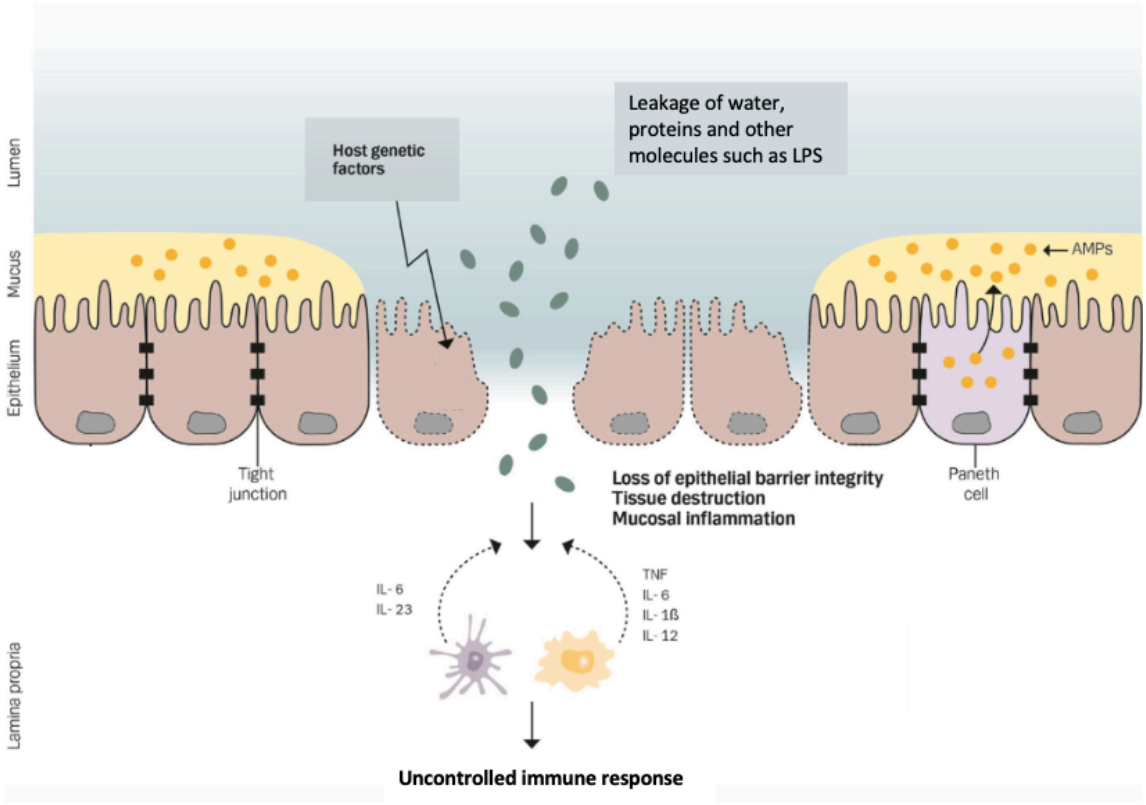

Figure 1.5. Obesity-induced degradation in the gut epithelial barrier. High levels of circulating proinflammatory cytokines induced by obesity disrupts the integrity of the epithelial barrier and tight junctions. This allows access of endotoxic lipopolysaccharides (LPS) and elicits an uncontrolled immune response and increased inflammation. Adapted from (80).

1.3 The gut microbiota

The gut microbiota refers to the trillions of microorganisms living in the human intestine (81). Advances in techniques used to characterise microbial populations has led to a significant interest in analysing communities within the gut and their correlation with health and disease (81). Compared to the 30,000 human genes, the gut microbiota is estimated to contain 22 million genes and thus, is commonly referred to as our second genome (82, 83). Colonisation of the gut occurs immediately after birth and continues to shift and develop as we age (84). Establishment of the first colonisers have long-term effects on an individual and are influenced by factors such as the birth delivery route (vaginal or caesarean), feeding method (breastmilk or powdered formula) and environmental exposures (85, 86). Following colonisation in the

first couple years of life, the gut microbiota composition becomes individually distinct, but may be modified by factors such as diet, medication and health status (86). In a healthy state, the gut microbiota and host have a symbiotic beneficial relationship.

1.3.1 The role of the gut microbiota in health

1.3.1.1 Digestion of dietary components

The gut microbiota and the host work symbiotically with one another to maintain essential metabolic processes (87). The gut microbiota is capable of digesting carbohydrates, proteins and fatty acids (88). For example, their action on indigestible polysaccharides produces by-product metabolites such as short chain fatty acids (SCFAs), primarily acetate, propionate and butyrate (89). Higher abundances of SCFAs, in particular butyrate, has been found to benefit the host by downregulating intestinal inflammation, improving insulin resistance, and reducing dyslipidaemia (90, 91). Moreover, there are specific beneficial, anti-inflammatory bacterial species that respond well to fibre rich diets, such as *Akkermansia muciniphila, Bifidobacterium spp., Prevotella spp.,* and *Veillonella spp.,* forming a favoured environment in terms of functionality and immunity (92). As well as the production of anti-inflammatory SCFAs, the gut microbiota produces other beneficial by-products such as organic metabolites including pyruvic, citric, fumaric and malic acid (93, 94). The production of these organic metabolites is strongly associated with plant-based and vegan diets that are typically rich in fibre and are absorbed by the host aiding in digestion, immunity, and can specifically inhibit the growth of pathogenic bacteria from colonising the gut (59, 95).

1.3.1.2 Preventing the growth of pathogenic bacteria in the intestines

The commensal microbiota existing within the intestine are responsible for eliminating and inhibiting the growth of harmful and pathobiont microbes (96). Commensal microbiota have

evolved a number of mechanisms, including the competition for essential nutrients, the production of harmful molecules and inhibitory by-products, and maintaining intestinal barrier integrity (96, 97). The competition for nutrients is displayed in the action of beneficial bacterial species *Bacteroidetes thetiotaomicron* (98). *B. thetiotaomicron* is able to consume specific carbohydrates that are usually consumed by pathogenic *Citrobacter rodentium* and therefore, limit the pathogen from colonising within the intestine (98). Another mechanism to inhibit pathogenic infection used by the gut microbiota is through the production of small bioactive molecules (96). These bioactive molecules, termed bacteriocins, can range in specificity to target a number of microbes or specifically target one species (99). Examples of the action of microbiota-derived bacteriocin are those targeting harmful, spore-forming *Clostridium difficile* (100). Additionally, to producing harmful bioactive molecules, the gut microbiota generates by-products such as SCFAs that create an inhibitory environment for possible enteric infections. Once more it is butyrate that induces the beneficial mechanisms in its ability to decrease the expression of both *Salmonella enterica* and *Escherichia coli*'s virulence factors (101). Another factor in which the gut microbiota protects its host and the intestinal environment is by maintaining the robustness of the intestinal epithelial barrier. As discussed, the stability of the intestinal barrier is essential in maintaining intestinal homeostasis therefore, the gut microbiota can enhance host protection by upregulating the expression of mucin, as well as occupying specific niches on the epithelial cells in order to prevent barrier degradation (102). Furthermore, some gut microbiota, such as *A. muciniphila*, are able to prevent mechanisms used by pathogens to permeate the barrier and provide further protection (103). In addition to aiding the host in the digestion of foods and offering protection from pathogens, the gut microbiota also plays a key role in the regulation of the intestinal immune system (104).

1.3.1.3 Maintenance of the intestinal immune system

The intestinal immune system heavily shapes the composition and function of the gut microbiota and reciprocally, the gut microbes influence the development of both the innate and adaptive intestinal immune responses (105). For example, bacteria such as *Lactobacillis spp.* and certain strains of *Clostridium* promote the expansion and differentiation of T_{reg} cell populations (106, 107). Inversely, specific innate immune sensors can reduce inflammation by downregulating inflammatory signalling and proinflammatory cytokines to stimulate the growth of protective commensal bacteria (108, 109). The lumen of the gastrointestinal tract is exposed to the external environment thus, much of it is populated with potentially pathogenic bacteria (110). Therefore, the intestinal immune system must be able to distinguish mutualistic commensal microbes from pathogenic microbes and consequentially, develop a tolerant phenotype to those benefitting the host (105). The development of a tolerant phenotypic expression is crucial to avoid overactivated immunity and is established by antigen-presenting cells, such as dendritic cells and macrophages, sampling the gut lumen such as food protein or commensal bacteria (111). From this tolerogenic development, intestinal immune cells can then differentiate harmless from harmful bacteria and produce an appropriate proinflammatory response when needed (112). This role of the gut microbiota in intestinal immunity can be highlighted in germ-free (GF) mice, that display low levels of cytokine-secreting T cells and antibodies due to the lack of pathogen exposure (113). When the gut of a GF mice is then colonised with beneficial bacteria, such as *Morganella morganii* and *B. thetiotaomicron*, the interaction between gut microbiota and the host's immune system results in an activation of T cell and antibody production (102). The gut microbiota also plays a vital role in the development and strength of the epithelial barrier as demonstrated in Peyer's patches in GF mice. Peyer's patches are aggregated lymphoid nodules which appear as elongated thickenings of the intestinal epithelial barrier. Peyer's patches perform immune surveillance to prevent any

pathogenic bacteria from growing (110). Reduced numbers of Peyer's patches are observed in GF mice indicating that the gut microbiota are crucially involved in the development of these structures (Figure 1.6) (110). While the gut microbiota benefits the host in a number of ways, an imbalance in the microbiota, termed dysbiosis, is associated with the aetiology of diseases (Figure 1.7) (110).

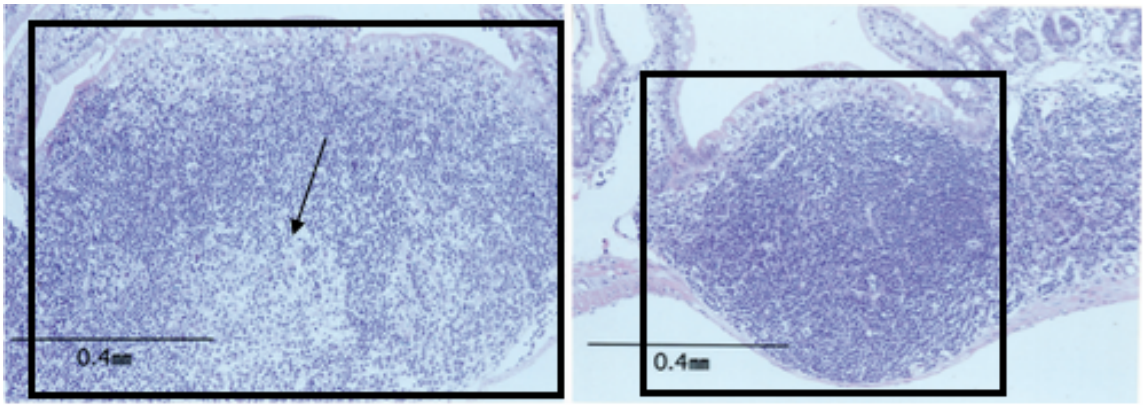

Figure 1.6. Representative microphotographs of Peyer's patches in the small intestine of specific-pathogen free mice (left) compared with germ-free mice (GF) (right). The size of the Peyer's patch (represented by the black outline boxes) is significantly larger in the specific-pathogen free mice compared to the GF mice. A germinal centre (indicated by black arrow) for the proliferation of immune cells, B cells, is seen in the specific-pathogen free mice but not in the GF mice. Hematoxylin and eosin stain. Scale bar 0.4nm (110).

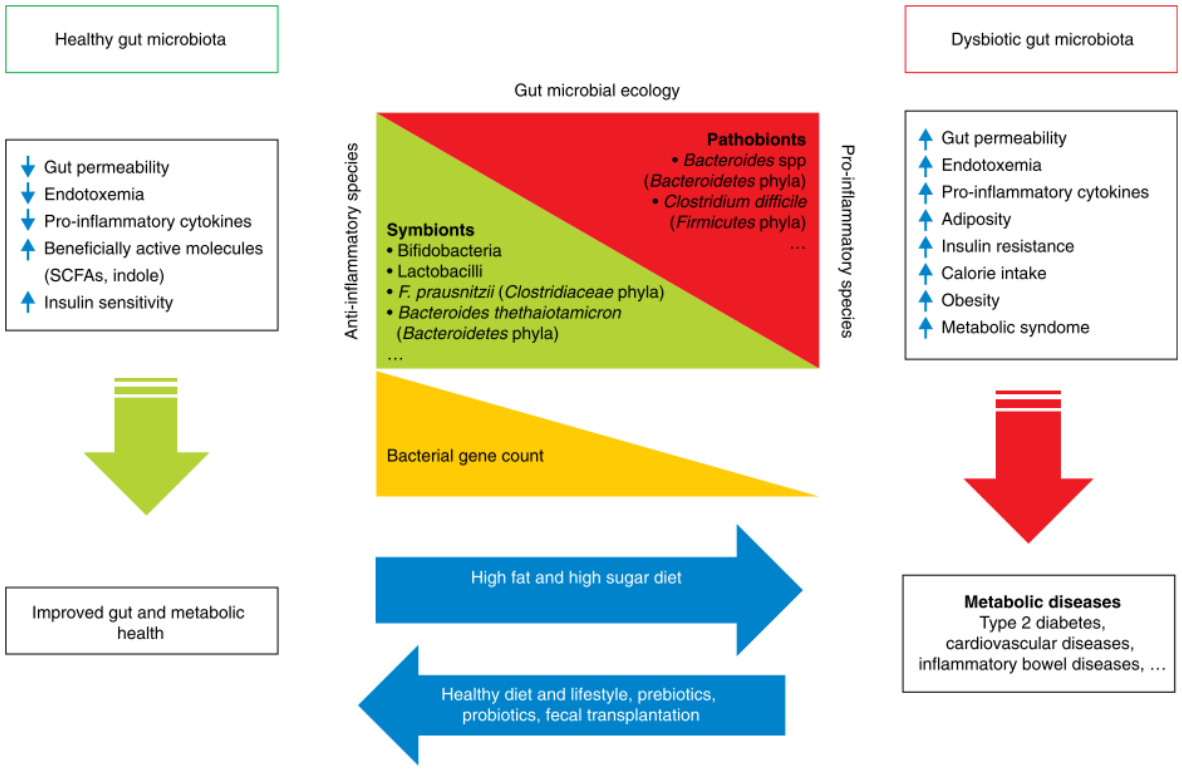

Figure 1.7. The association between the gut microbiota and the metabolic health of an individual. A healthy gut microbiota is composed of a diverse mix of bacterial species that produce beneficial by-products such as short chain fatty acids (SCFAs). The beneficial gut ecosystem enables a strong gut epithelial barrier leading to reduced metabolic endotoxemia and a reduced proinflammatory immune response. In the case of poor diet, high fat and high sugar, dysbiosis begins within the gut microbiota resulting in an increase in harmful bacteria and a loss of diversity as well as negative biological effects such as increased adiposity and insulin resistance, increased gut permeability, increased endotoxemia and an increased proinflammatory immune response leading to metabolic disorders such as obesity and metabolic syndrome. Sourced from (114).

1.3.2 Dysbiosis of the gut microbiota

1.3.2.1 Altered abundance of Firmicutes: Bacteroidetes ratio

Dysbiosis, a significant characteristic of obesity, is defined by the structural and functional imbalance of microorganisms within the intestines and subsequently, leads to metabolic disturbances (115, 116). Generally, dysbiosis can be described by the alteration of dominant phyla, the loss of beneficial microbes, an increase in detrimental microbes, and an overall loss of diversity (117). In a cohort of 300 adults, The Human Microbiome Project Consortium analysed the microbiome of various habitats on the human body, including the gut (118). Two

dominant phyla were identified in the gut microbiota, the Bacteroidetes and the Firmicutes, followed by smaller abundances of Actinobacteria, Proteobacteria and other phyla. Characterisation of both human and animal models indicate that the ratio of Bacteroidetes and Firmicutes in the gut changes with the development of obesity (119). The obese phenotype is associated with an increase in Firmicutes and relative reduction in Bacteroidetes (e.g. (120, 121)). These changes have also been shown to be reversible with weight loss regimens in animals and in humans (120, 122). The shifts in dominant phyla consequentially lead to alterations at the genus, family and species levels which are also associated with disease.

1.3.2.2 The loss of beneficial bacteria

As well as the changes in the Firmicutes: Bacteroidetes ratio, obesity reduces the abundances of beneficial microorganisms (123). Typically, healthy subjects are host to particular species identified for their health benefits on their host such as *A. muciniphila* and the *Bifidobacterium* genus (123, 124). *A. muciniphila* in particular, has a crucial role in providing protection for the intestinal epithelial barrier by enhancing the regulation of mucin expression and occupying binding sites on the epithelial cells (125). Often in obese individuals, both the abundance of *A. muciniphila* and mucus thickness are diminished thus, increasing gut permeability and inflammation (Figure 1.8) (126). In 10-week-old male mice fed HFD, daily *A. muciniphila* oral supplements reduced fat mass, insulin resistance and dyslipidaemia as well as promoted a stronger gut barrier with increased intestinal energy absorption compared to HFD-only fed mice (127). Similarly to *A. muciniphila*, particular strains of *Lactobacillus* and *Bifidobacterium* have also been found to apply their advantageous functions on the intestinal epithelial barrier. In a study following 8-week-old male mice that were fed 8 weeks of high fat/ high sucrose diet (HF/HS), it was found that those supplemented with strains of *Lactobacillus paracasei* and *Bifidobacterium animalis* not only attenuated obesity, visceral fat accumulation and

inflammation but also boosted the gene expression of key intestinal integrity markers (128). Expression of markers such as zonula, occludens 1, and occludin (genes encoded for tight junctions between epithelial cells and responsible for maintaining barrier integrity) as well as markers for mucin 2 and mucin 3 (genes encoded for the major secreting mucins) are all upregulated by these specific strains (128). Additionally, to the loss of specific bacterial strains, there is also a loss of similar functional groups of bacteria. A reduction of butyrate-producing bacteria groups promotes inflammation due to the obvious loss of its anti-inflammatory abilities (129). For example, *Faecalibacterium prausnitzii* and *Roseburia hominis* produce high concentrations of butyrate, which subsequently downregulate proinflammatory cytokines (129). A reduction in butyrate-producing microbes is a characteristic of the gut microbiota in patients suffering from intestinal inflammatory disorders, such as Crohn's disease and inflammatory bowel disorder, as well as obesity (130). In addition to the reduction in beneficial bacteria, harmful proinflammatory microbes are increased in the gut of obese individuals.

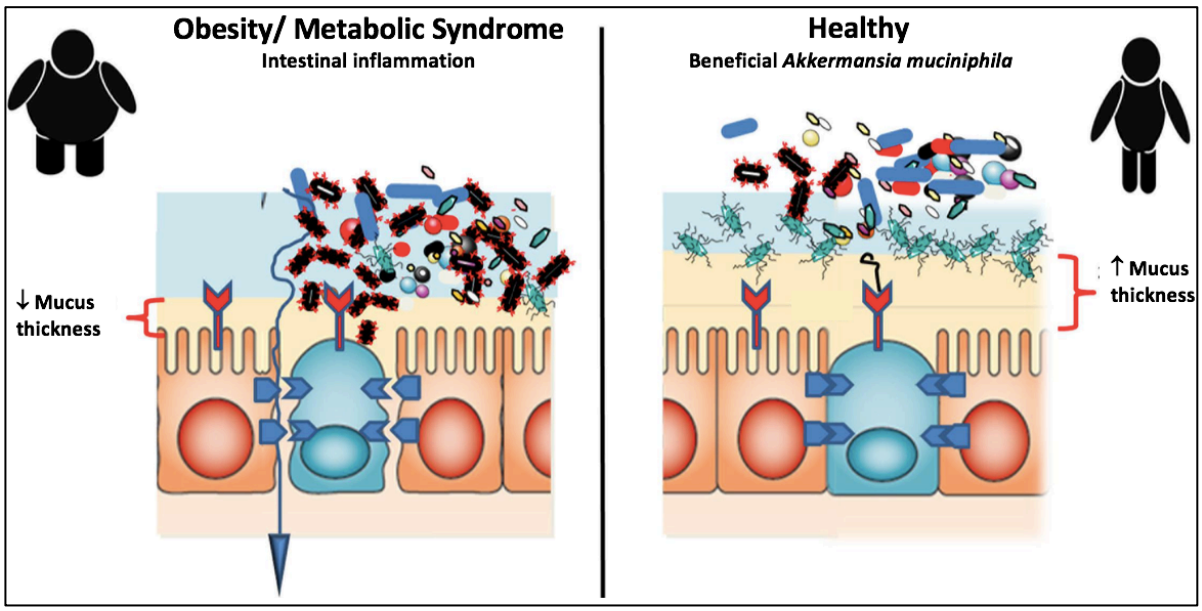

Figure 1.8. The abundance of *Akkermansia muciniphila* as well as mucus thickness is reduced in individuals. Obese individuals (left) exhibit a decreased abundance of *A. muciniphila.* This leads to decreased mucus thickness and increased epithelial permeability, which allows endotoxic molecules and pathogens to reach underlying tissues. On the other hand, healthy individuals (right) have a good abundance of *A. muciniphila* allowing increased mucus thickness and tight epithelial junctions. Adapted from (126).

1.3.2.3 Increased harmful bacteria

Perturbations in the gut microbiota in obesity can also be characterised by the increase in harmful, proinflammatory microorganisms such as those from the *Desulfovibrio*, *Fusobacterium* and *Bilophila* genera (123, 124, 131). These harmful bacteria have evolved in order to perform specific mechanisms that are destructive to the host. For example, members of the *Desulfovibrio* genus reduce sulphate to produce hydrogen sulphide which has cytotoxic effects. This causes damage to the gut epithelial barrier by inducing apoptosis of cells on the intestinal epithelial barrier allowing barrier degradation and therefore, increasing metabolic endotoxemia and inflammation (132, 133). This can be seen in mice with impaired glucose tolerance and increased body fat due to HFD, displaying an increased abundance of bacteria from the *Desulfovibrionaceae* family (134). Likewise, abundance of the *Fusobacterium* genus are increased in obese subjects and these bacteria have the ability to cause destruction to the intestinal epithelial cells (135, 136). It has been discovered that *Fusobacterium* species possess virulence factors such as autotransporters, that allow adhesion and binding to the epithelial cells, and subsequent destruction to invade into the underlying tissue (137). Additionally to the increase of specific species, there is increased abundance of gram-negative bacteria, with endotoxic LPS in their outer membrane (138). LPS gains access into systemic circulation due to the permeability of the epithelial barrier (139). The combination of increased harmful microorganisms, decreased beneficial microorganisms, and increased concentrations of proinflammatory cytokines, causes degradation of the tight junction proteins between cells and apoptosis of epithelial cells, allowing LPS and other molecules into underlying tissues and thus, increasing intestinal inflammation (140).

1.3.2.4 Loss of diversity

Over the past decade in the scope of research performed on the gut microbiota, there has been a high degree of variability in regard to dysbiosis and the alteration of specific microbial species and their abundances in individuals with obesity. However, despite these disparities, obesity reduces microbial compositional and functional diversity in both pre-clinical and clinical studies (141). Obese subjects are found to have lower diversity in their microbial community and is associated with pronounced functional metabolic disturbance. This is demonstrated by marked increased adiposity, a higher predisposition to gain more weight over time, insulin resistance, dyslipidaemia, and increased low-grade inflammation (142, 143). The importance of a strongly diverse gut microbiota is stressed in research as being more resilient, referring to the strength of the ecosystem in handling incoming agitation as well as the ability of the system to restructure in the case of imbalance (144). Additionally, a more diverse ecosystem builds a stronger and more stable immune system especially in terms of developing the tolerogenic phenotype in intestinal immunity (Figure 1.9) (145, 146). Dysbiosis, and the associated reduction in diversity, has been consistently reported in obesity however, dysbiosis is commonly found to vary among individuals depending on many factors including the strong influence of poor diet (147, 148). Moreover, new research is revealing that an individual's response to poor diet is influenced by sex.

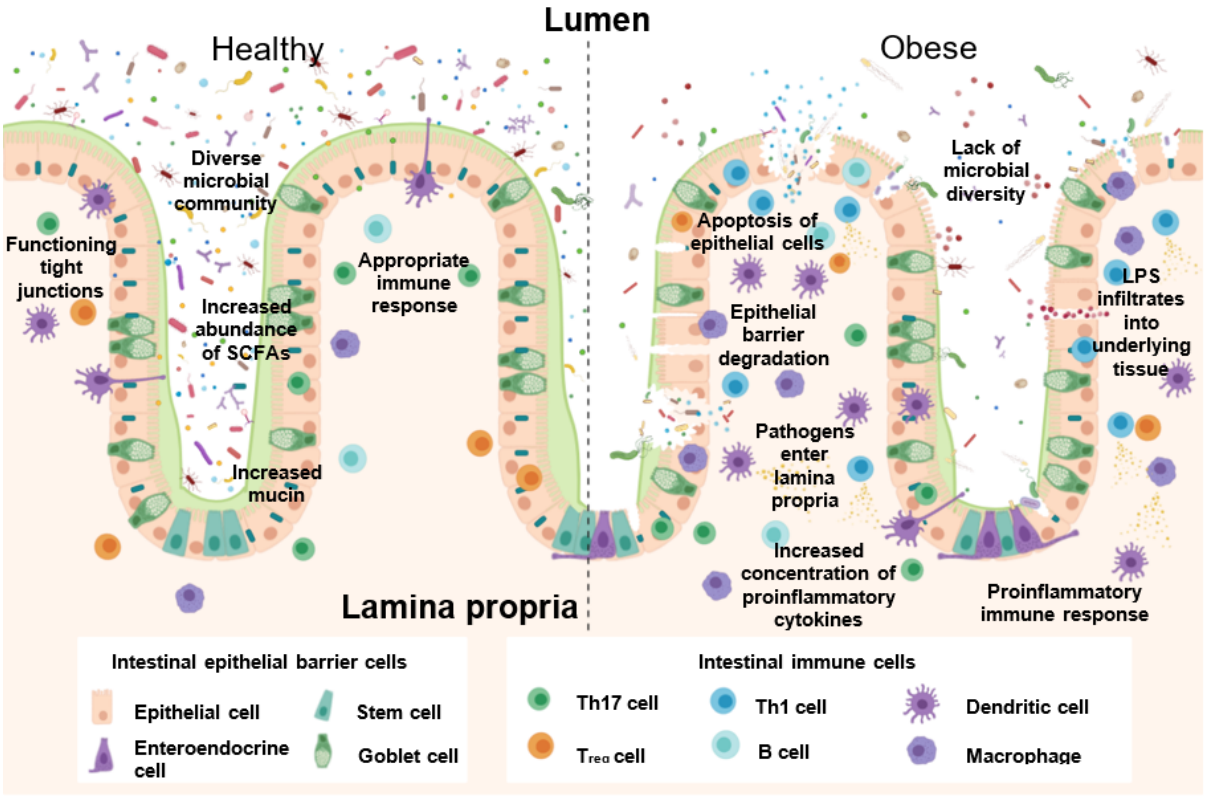

Figure 1.9. Gastrointestinal epithelial barrier. The gut microbiota is confined to the intestinal lumen by the epithelial layer. Typically, in healthy individuals (left), there is a diverse microbial community, a strong abundance of short chain fatty acids (SCFAs), functioning tight junctions between epithelial cells and a thick layer of mucin covering the epithelial cells. Each of these components prevent pathogens from reaching underlying tissue and therefore, an appropriate and tolerogenic intestinal immune response is established. Contrastingly for obese subjects (right), there is a lack of microbial diversity and commonly, a decrease in beneficial bacteria and an increase in harmful bacteria. Additionally, there is a high degree of degradation of the epithelial barrier as a result of decreased mucin expression, poor functioning tight junction proteins and apoptosis of epithelial cells induced by bacteria. This results in pathogens and lipopolysaccharide (LPS) infiltrating into underlying tissue and producing a proinflammatory immune response in both the intraepithelial layer and lamina propria layer demonstrated by increased proinflammatory immune cells and proinflammatory cytokines (145, 146)

1.3.3 Sexual dimorphism and the gut microbiota

Alike the sexual dimorphism associated with the immune system, the gut microbiota also differs between the sexes in both healthy and metabolically disturbed males and females (149, 150). Sequencing of the gut microbial community of healthy subjects revealed that males have a lower species richness and evenness compared to females, as well as differences in abundances of specific bacteria (151, 152). Interestingly, the female gut microbiota closer

resembles that of prepubescent or castrated males, opposed to age-matched males, signifying yet again the importance of sex hormones (153). Higher concentrations of sex hormones, estrogen for females and testosterone for males, are associated with greater gut microbiota diversity as well as having a stronger resilience against dysbiosis (154). For example, estradiol treatment in 11-week-old female mice limits the loss of crucial microbial diversity and promotes the growth of beneficial bacteria such as those from the *Lactobacillaceae* family (155). Moreover, estradiol reduces pathogenic bacterial virulence by inhibiting quorum sensing used by pathogens to communicate and recognise their population abundance (156). Estradiol administration also exerts beneficial effects in the gut microbial community of male mice to reduce susceptibility to gut epithelial permeability, inflammation and weight gain (157). The use of GF mice provides further insight into the interaction between the gut microbiota and the sex specific responses (158, 159). Inoculating male and female GF mice with the same human male's gut microbiota resulted in significantly different microbial compositions as well as a higher bacterial diversity in females compared to males (159). The difference between males and females in the composition and function of the gut microbiota, naturally leads to differences in metabolic developments and therefore, aids in the protection or predisposition to metabolic disturbances such as obesity (160).

Diet-induced obesity in animal models is often used to mimic metabolic disturbances such as obesity and the concomitant gut dysbiosis seen in humans (161, 162). Obesogenic diets contain high compositions and different types of fats and/ or sugars with variations in the length of the diet (162, 163). Typically when investigating obesogenic diets in both sexes, there is a protection or delay in the development of metabolic disorders in females, as well as the observation that the gut microbiota responds differently to diet based on sex (150, 164). For example, a 14 week HF/HS diet given to 8-week-old male and female mice resulted in a slower

development of biologically adverse effects as well as a different composition of the gut microbiota in females (165). Regarding the increase in the Firmicutes to Bacteroidetes ratio that is characteristically seen in the development of obesity, female mice are delayed in their increased in abundance of Firmicutes compared to males (166). Moreover, when looking at specific genera within the microbial community in obese subjects, there is a greater abundance of *Veillonella*, *Methanobrevibacter, Acidaminococcus, Clostridium, Roseburia* and *Faecalibacterium* genera in males compared to higher abundances of *Bilophila, Ruminococcus* and *Bacteroides* were greater in females (7, 116). In the male gut bacteria abundances mentioned above, *Veillonella* genera are found in higher abundances in children with type 1 diabetes however, *Roseburia* genera is found to improve metabolic alterations brought on by HFDs (167, 168). Similarly for females, *Bilophila* genera aggravates metabolic dysfunction however, *Bacteroides* genera has numerous anti-inflammatory health benefits on the host (169, 170). The functions of specific bacteria demonstrate that not only do the abundances of certain bacteria within the host need to be considered but also determining their functionality on the host. In addition to the sex differences in response to obesogenic diets, sexual dimorphism has been demonstrated in response to diet supplementations and treatments (171, 172).

A number of fibre compounds have been investigated for their ability to attenuate the detrimental health outcomes of poor diet as well as assisting in shaping the gut microbiota (123). Digestible prebiotic fibres, such as inulin and oligofructose, reveal significant increases in favourable bacteria including *Bacteroides* and *Bifidobacterium* genera and *A. muciniphila* in females only (173). Probiotic supplementation also adjusts the abundances of specific bacteria differently for males and females (174). For example, probiotic *Lactobacillus reuteri* increases the abundance of the Bacteroidetes phylum and decreases the Firmicutes phylum in female mice but an opposite effect is seen in male mice (174). Phylum differences also incur

significant genus level differences, observed as greater amounts of *Bacteroides, Prevotella* and *Lactobacillus* for females and a higher abundance of C*lostridium* for males (174). These findings illustrate that male and female gut microbiota behave differently in response to diet, supplements and treatments and how they harbour their microbial community. The growing epidemic of obesity and how it differs between sex, reinforces the importance of our research project. In order to reflect the early developmental stages of the obese human condition, that allows the investigation of the initial pathophysiology of obesity in the intestines and identifying possible therapeutic targets to prevent the progression to more severe obesity, we implemented the most appropriate mouse model (175).

1.4 Current mouse models of obesity

1.4.1 Genetically induced mouse models

In this Masters project a mouse model of obesity was used and hence, the discussion of experimental animal models will be confined to this species. Common mouse models for genetically-induced obesity are the leptin-deficient mice (*ob/ob*) and leptin receptor-deficient mice (*db/db*) (175). These mice primarily display obesity, as well as insulin resistance and dyslipidaemia (176). A criticism of these genetically-induced models however, is that they don't recapitulate key underlying causes for the development of obesity such as poor diet, intestinal inflammation and dysbiosis (175). Moreover, some of these mouse models induce far more severe symptoms compared with obese humans (175).

1.4.2 Diet-induced mouse models

As discussed, diets that are high in fat or high in simple sugars are most often used to replicate human obesity (162, 163). For example, a 60% kcal fat diet for 16 weeks induces severe obesity, with insulin resistance and tissue inflammation (177). Mice put on a long-term high

sugar (36% kcal), high butter (48%) diet causes significant weight gain, elevated blood glucose levels and decreased adipose T_{reg} cells (178). Rodent chow with reduced lipid content (42.5% kcal) but increased simple carbohydrate and salt content also appears to be a good model of a "Western diet" since it produces weight gain and commonly associated obesity symptoms seen in humans (179). Another experimental model frequently used to induce obesity in mice is to combine diet intervention (e.g. HFD) with low-dose streptozotocin (toxic agent to insulin-secreting beta cells) treatment (180). The addition of streptozotocin to HFD results in a quick developing and severe model of obesity and diabetes however, complications such as liver and kidney organ damage can occur (181). To avoid such a severe a model of obesity for this project, a milder diet-induced obesity model was used. Based on previous models of obesity, HFD with 42% kcal fat content was used for 10 weeks to replicate the early stages of obesity, allowing the pathophysiology over the diet duration to be tracked as well as determining any sex differences (182, 183).

1.5 Conclusion

Obesity is the excessive accumulation of fat that increases an individual's risk of developing metabolic disease (1). Often overlooked is the sex bias that exists in metabolic disease and must be considered in research in determining the development of obesity as well as looking at possible treatments. In addition to the well-established role of fat distribution and sex hormones, intestinal immunity and the gut microbiota are now regarded as vital influencers of obesity development (158). However, it is still unclear how these factors differ between the sexes in the early pathophysiology of obesity and therefore, I focused on these two key areas in my thesis.

1.6 Masters project

1.6.1 Aims

To characterise sex differences in intestinal inflammation and gut dysbiosis in a mouse model of early obesity.

1.6.2 Hypotheses

High-fat diet will induce obesity in both sexes, but the disease development will be delayed in female mice. Additionally, HFD will drive dysbiosis in the gut microbiota, activating an immune response and detrimentally affecting the intestinal immune profile however, changes to the gut microbiota and intestinal immune responses will be sex specific.

Chapter 2: Methods

2.1 Animals

All animal experiments complied with the National Health and Medical Research Council (NHMRC) of Australia code of practice for the care and use of animals for scientific purposes and were approved by the La Trobe University Animal Ethics Committee (AEC#19009). Male and female C57BL/6 mice (aged 5-7 weeks; n = 22 per sex) were obtained from the La Trobe University AgriBio facility, Bundoora campus. Mice were kept on a 12h light/ dark cycle with *ad libitum* access to food and water. Mice were housed with litter mates (1-3 mice per cage) in individually ventilated Tecniplast cages (Rydalmere, Australia). Following acclimation and collection of baseline measures, mice were randomly assigned to either the high-fat diet (HFD) or normal chow diet (NCD) group. The NCD mice served as a control group; HFD mice were fed a 42% kcal fat, high glycaemic index semi-pure rodent diet, (SF03-030; Specialty Feeds Perth, Western Australia) for 10 weeks (Figure 2.1).

2.2 Study design

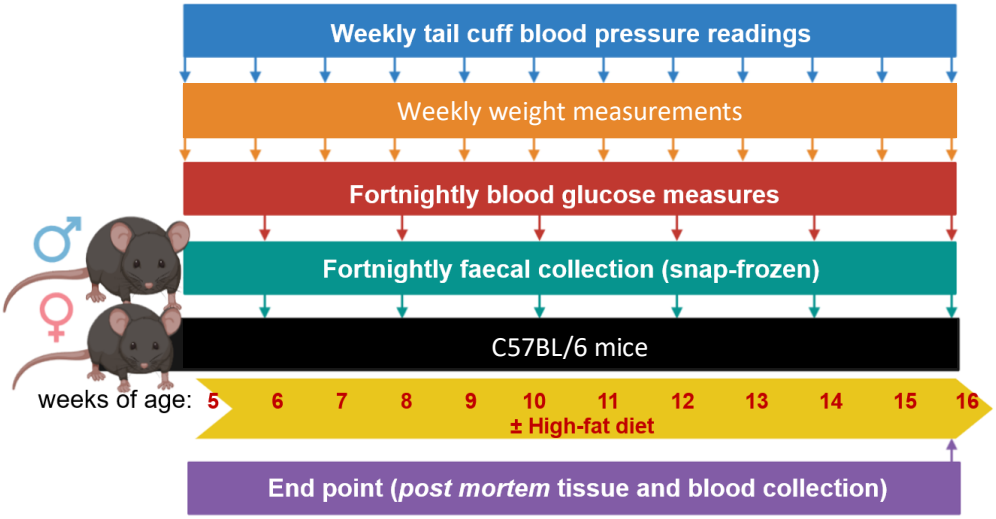

Figure 2.1. Summary of the experimental timeline. Male and female C57BL/6 mice (5-6 weeks of age) were randomly assigned to a high-fat diet or control normal chow diet (n = 10-12 per sex, per diet) for 10 weeks. Blood pressure and body weight were measured weekly with fortnightly fasted blood and faecal collections.

2.3 *In-vivo* measures

2.3.1 Blood pressure and body weight

Systolic blood pressure (BP) was measured in mice using a non-invasive, tail-cuff method our lab has previously described (184) (MC4000 Multi-Channel Blood Pressure Analysis System; Hatteras Instruments, North Carolina, USA) (Figure 2.2A). Briefly, mice were placed in specimen holders and a 0.5 cm occlusion cuff was placed around their tails. The specimen holders were placed onto a platform heated to 37°C to promote vasodilation (i.e., blood flow) of the tail. Upon commencement, the occlusion cuff automatically inflated around the mouse's tail until blood flow occluded and systolic BP was detected and read by the LED sensor. The cuff inflated and deflated in sets of 10 cycles. Mice generally had 3-4 sets of 10 cycles in a single session. Maximum systolic BP inflation was set at 170 mmHg throughout the study. One day prior to recorded measurements commencing, mice were placed on the machine for an acclimation session to familiarise with the machine and reduce stress levels. The acclimation session was run identical to a recorded session. Thereafter, readings were taken weekly from baseline through to week 10 of the diet regimen. The waveform for each cycle was shown on a desktop computer connected to the BP system (Figure 2.2B). Successful readings were those with appropriate occlusion of blood flow and minimal mouse movement. For each animal at each time point, a minimum of 20 successful measures were recorded and averaged for each weekly session. Weekly body weights and fortnightly faecal collections were obtained immediately following BP measurements.

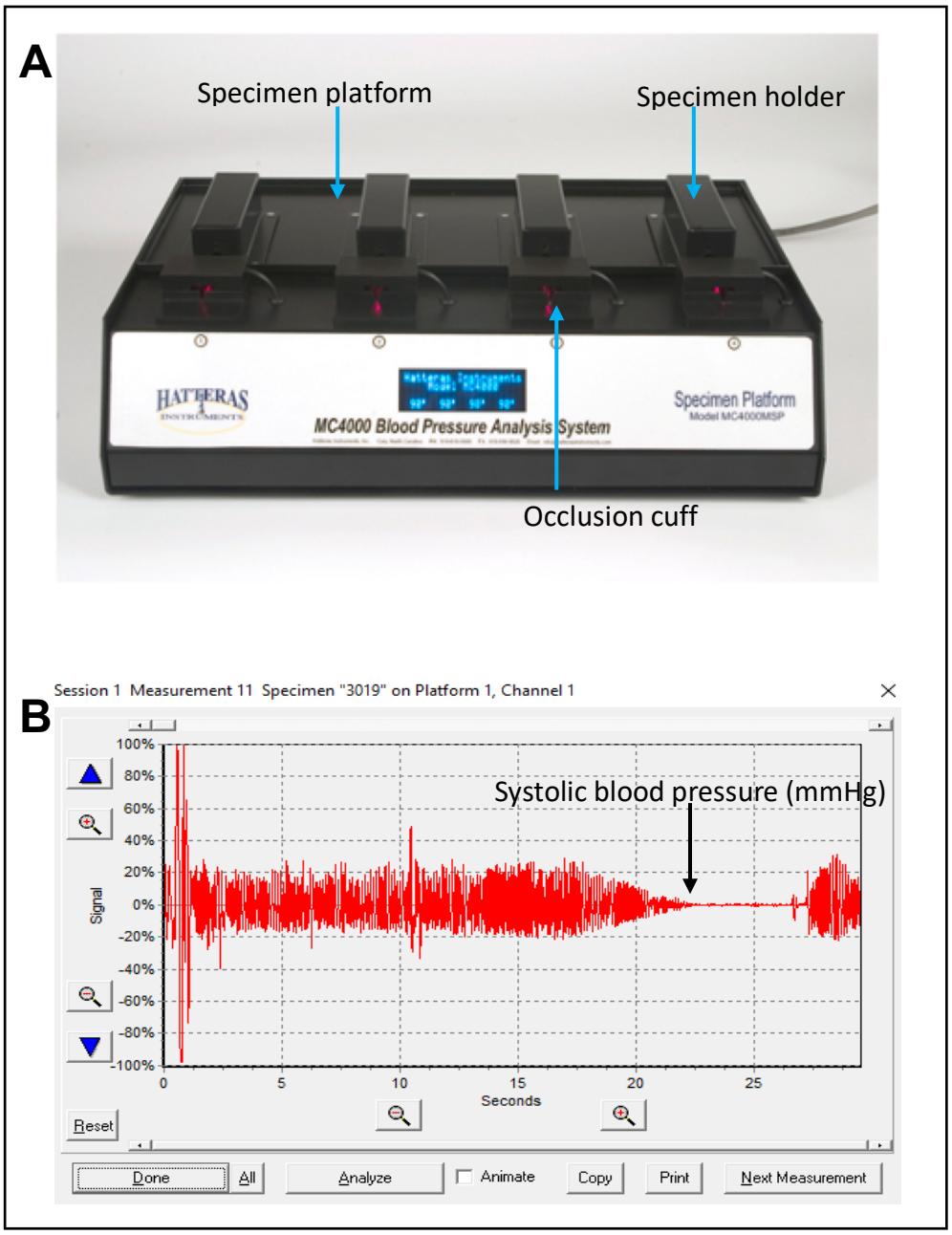

Figure 2.2. Non-invasive tail-cuff blood pressure measurement technology. A photo of the MC4000 Multi-Channel analysis system for measuring systolic blood pressure in mice (A) and a representative image of the waveform of tail blood flow occluding in one mouse as the automated cuff inflated (B). At the point of zero flow, systolic BP measurement was taken as indicated by the black arrow.

2.3.2 Glycaemic status

Fasted blood glucose levels were measured fortnightly from baseline. This involved fasting the mice, during their inactive period in the morning, for 6-8 hours prior to performing a lateral

saphenous vein bleed (as per Standard Operating Procedure (SOP) set by La Trobe Animal Research and Teaching Facility (LARTF)). Briefly, mice were held in an open-ended 50 ml falcon tube. Depilatory cream (Nair, NSW Australia) was applied to the hind leg for up to 1 minute and wiped off with 80% ethanol. After applying a thin layer of Vaseline to the skin to assist in blood drop formation, a 27-gauge needle was used to prick the vein and collect a drop of blood onto the ACCU-CHEK Guide Blood Glucose Monitor test strip (Castle Hill, NSW Australia). An additional 100-120 µl of blood was collected into a heparinised tube and centrifuged at 10,000 rpm, 4°C for 10 minutes (Mikro 2000R, Hettich). Plasma samples were then stored in a -80°C freezer for later analysis of insulin levels. The concentration of plasma mouse insulin was measured using the ALPCO Mouse Ultrasensitive Insulin ELISA (Enzyme linked immunosorbent assay; New Hampshire, USA) following the manufacturer's instructions. Briefly, 5 µl duplicates of each plasma sample from each time point were placed into individual wells on the plate and mixed with 75 µl of conjugate stock. The plate was incubated for 2 hours on a microplate shaker. Following incubation, the samples were washed 6 times with a wash buffer and 100 µl of TMB substrate was added, followed by a further 30-minute incubation. 100 µl of stop solution was added to each sample and the plate was run through the CLARIOstar microplate reader at 450 nm (Ortenberg, Germany).

2.4 Study endpoint measures

2.4.1 Blood collection

After 10 weeks of the diet, mice were euthanised using carbon dioxide asphyxiation (LARTF SOP #0003). An abdominal incision was extended along the chest to expose the heart. To minimise blood clotting, 0.05 ml of Clexane, enoxaparin sodium (400 Units/ml, Sanofi, NSW, Australia) was injected into both the left and right ventricle of the heart and blood (approximately 0.7-1.0 ml) was collected from the right ventricle using the cardiac puncture

method (LARTF SOP #0088). A small incision was then made into the right atria, and PBS was perfused slowly into the left ventricle to remove blood and circulating leukocytes from organs. The digestive tract (from the stomach to the anus) was then removed from the mouse as a whole and placed in ice-cold PBS for further dissection and analysis as per below.

2.5 Flow cytometry

2.5.1 Cleaning of small intestine and colon

Once removed from the mice, the length of the small intestine, caecum and colon were recorded with the caecum used as an anatomical landmark separating the intestinal sections (Figure 2.3). Excess mesenteric adipose and Peyer's patches, harbouring excess immune cells, were removed, and the colon and small intestine were isolated from the gastrointestinal tract. Although caecum lengths and weights were recorded, this tissue was not processed for flow cytometry. Tissues were placed in ice cold phosphate buffer solution (PBS) (for up to 30 minutes) until tissue digestion. A small 0.5 cm section of colon and small intestine were reserved and fixed in 10% neutral buffered formalin (Amber Scientific, Ringwood, Australia) for histological analyses to be performed at a later date.

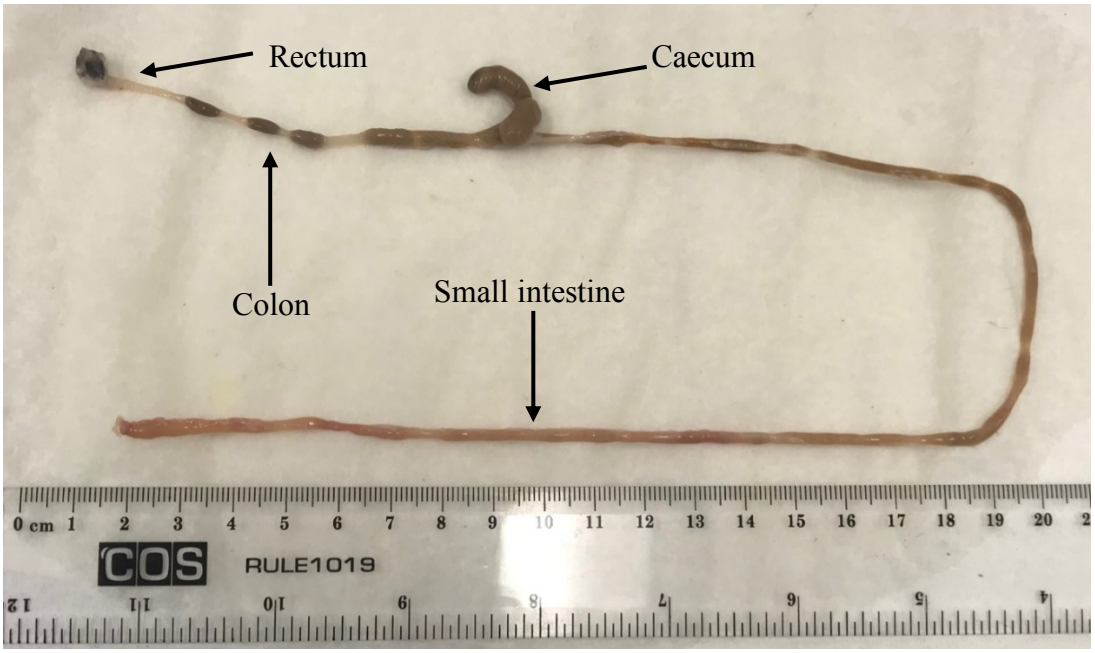

Figure 2.3. Representative image of a dissected male mouse gastrointestinal tract.

2.5.2 Digestion and incubation of gastrointestinal tissue

The colon harbours the greatest population of gut microbiota and therefore, allows us to see the relationship between the immune system and the gut microbiota making it more relevant for this project compared to the small intestine. Intraepithelial leukocytes (IELs) and lamina propria leukocytes (LPLs) from the colon were isolated as previously described (185). Briefly, the colon was separated and cut longitudinally, and excess mucus was lightly scraped out. The tissue was placed into PBS, cut into 0.5 cm pieces and vortexed using IKA Vortex Genius 3 at 2,500 rpm (Selangor, Malaysia). The tissues were strained through a 70 μm filter, placed back into PBS and vortexed. This step was repeated but samples were then placed into 20 ml of dissociation solution (2% foetal calf serum (Gibco, Victoria, Australia) in 1x Hanks' balanced salt solution Ca^{2+} Mg^{2+} free media (Gibco, Victoria, Australia) with 5 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, USA)) and incubated at 37°C for 30 minutes with gentle shaking (190 rpm). Following incubation, the supernatant (containing IELs) was strained through a 70 μm filter and centrifuged at 1700 rpm for 7 minutes.

The remaining gut tissue was placed into a digestion buffer (2% foetal calf serum in RPMI L-Glutamine free media (Gibco, Victoria, Australia), 1-2 mg/ml Collagenase III (ScimaR, Victoria, Australia), 200 μg/ml dispase II (Life Technologies, Victoria, Australia) and 1 μg/ml DNase (Sigma-Aldrich, USA)) and incubated again with gentle shaking (190 rpm) for 45 minutes. Following the second incubation, the supernatant (containing LPLs) was strained through a 70 μm filter and spun with the IELs at 1700 rpm for 7 minutes. The supernatant was discarded, and the pelleted cells were resuspended in 7 ml of 40% isotonic Percoll solution (Sigma-Aldrich, NSW, Australia) and underlaid with 3 ml of 80% isotonic Percoll solution. All samples were centrifuged for 20 minutes at 2200 rpm which separated the immune cells into the interface. This interface was collected, washed and spun down to pellet the cells. The supernatant was discarded, and the pellets were resuspended in the remaining media and placed onto a 96-well plate that was centrifuged at 1500 rpm for 5 minutes to begin staining for surface markers as previously described (184).

2.5.3 Antibody staining

Cells were stained with live/dead fixable aqua dead cell stain for 15 minutes at room temperature (1:1000 dilution, Invitrogen Oregon, USA). 100 μl of MACS buffer was added to each well to stop the reaction, then the plate was spun again (15000 rpm, 5 minutes) and the supernatant was discarded. Cells were stained with antibodies for 20 minutes at room temperature for the following cell surface markers (fluorophores indicated in brackets); CD45 (AF700), CD3 (APC), CD11β (BV421), CD4 (BV605), CD8α (PerCP/Cy5.5), TCRβ (PE/Cy5), F4/80 (APC/Cy7), Ly6G (PE/Cy7), Ly6C (FITC) and B220 (PE). All antibodies were purchased from Biolegend (California, USA) and concentrations, fluorophores, dilution factors and target cells are indicated in Table 2.1. Cells were fixed in 1% formalin overnight

and run through the CytoFLEX S, Beckman Coulter system (Indianapolis, USA) to quantify innate and adaptive immune cells in the various tissue preparations.

Table 2.1. Antibody panel used for flow cytometry of the small intestine and colon.

Antigen	Fluorophore	Clone	Concentration	Dilution factor	Target cell
CD45	A700	30-F11	0.5 mg/ml	1:500	Leukocytes
CD3	APC	17A2	0.2 mg/ml	1:500	T cells
CD11β	BV421	M1/70	0.2 mg/ml	1:500	Myeloid-derived cells
CD4	BV605	RM4-5	0.2 mg/ml	1:500	T helper cells
CD8α	PerCP-Cy5.5	53-6.7	0.2 mg/ml	1:500	Cytotoxic T cells
TCRβ	PE-Cy5	H57-597	0.2 mg/ml	1:500	$TCR\beta^+$ T cells
F4/80	APC-Cy7	BM8	0.2 mg/ml	1:500	Macrophages
Ly6G	PE/Cy7	1A8	0.2 mg/ml	1:1000	Neutrophils
Ly6C	FITC	HK1.4	0.5 mg/ml	1:1000	Monocytes
B220	PE	RA3-6B2	0.2 mg/ml	1:1000	B cells

2.5.4 Gating strategy

FlowJo software was used to analyse data following flow cytometry. The gating strategy used to identify immune cells can be seen in Figure 2.4. Lymphocytes were gated based on forward scatter height and forward scatter area to isolate singlet cells and remove any doublets or higher (Figure 2.4A). By selecting isolated singlets, the cells were plotted on forward scatter area against $CD45^+$ (Figure 2.4B). The separated population was identified as live $CD45^+$ cells and selected to determine B cells, myeloid-derived cells and T cells. B cells were identified by plotting $CD45^+$ against $B220^+$ (Figure 2.4C). Myeloid-derived cells were identified from the live $CD45^+$ cells and plotted for $CD11\beta^+$ against $CD3^+$ (Figure 2.4D). Cells in quadrant 1 ($CD11\beta^+/CD3^-$) were identified as myeloid-derived cells and cells in quadrant 3 ($CD11\beta^-/CD3^+$) were identified as T cells. By selecting total T cells in Q3, we further identified T cell

subsets $CD8^+$, $CD4^+$ and double negative T cells by plotting cells for $CD8^+$ against $CD4^+$. Cells in quadrant 1 ($CD8^+/CD4^-$) were identified as $CD8^+$ T cells, those in quadrant 3 ($CD8^-/CD4^+$) were identified as $CD4^+$ T cells and those in quadrant 4 ($CD8^-/CD4^-$) were identified as double negative T cells (Figure 2.4E). Although antibodies for macrophages, neutrophils and monocytes were used in the staining process (F4/80, Ly6G and Ly6C), we were unable to identify distinct separation of these populations and thus, excluded these from our analyses.

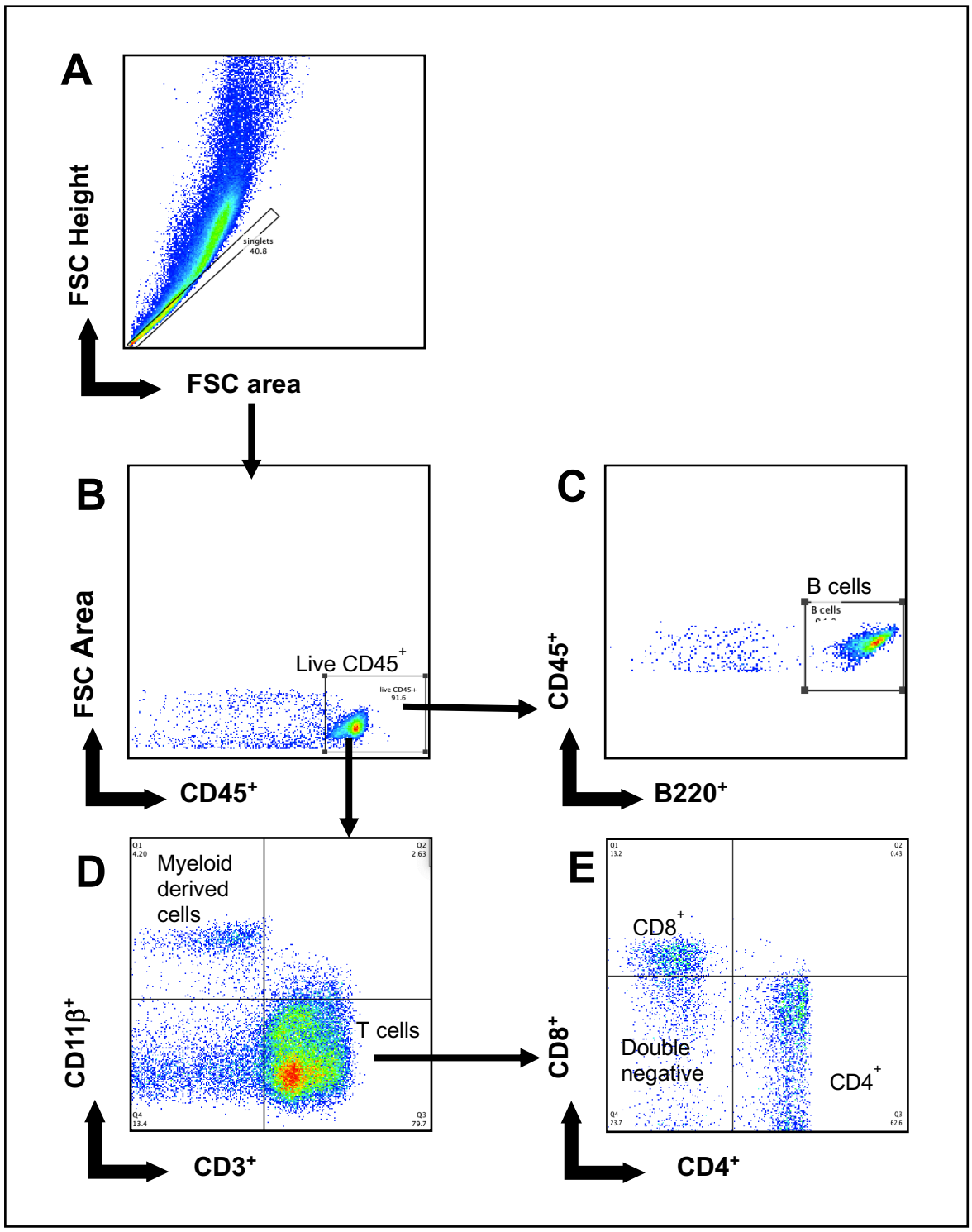

Figure 2.4. Gating strategy used for determining total live cells, B cells, myeloid-derived cells and T cells. Lymphocytes were primarily gated based on the forward scatter height and area to isolate and select the singlets. Next, forward scatter area was plotted against $CD45^+$ to determine live cells. The population of live cells was selected and plotted $CD45^+$ against $B220^+$. The separated population was identified at B cells**.** Live $CD45^+$ cells were also selected and plotted for $CD11\beta^+$ against $CD3^+$. Those in Q1 were identified as myeloid-derived cells and those in Q3 as total T cells. By selecting Q3, total T cells, the population was gated by plotting $CD8^+$ against $CD4^+$. Q1 ($CD8^+/CD4^-$) identified $CD8^+$ T cells, Q3 ($CD4^+/CD8^-$) identified $CD4^+$ T cells and Q4 ($CD4^-/CD8^-$) identified double negative T cells.

2.6 Faecal DNA analysis

2.6.1 DNA extraction

Bacterial DNA was extracted from the faecal samples (weighing between 0.01-0.24 g) using a DNeasy PowerSoil kit (Qiagen, Hilden, Germany) and following the manufacturer's instructions. Pellets were placed into a tube containing Power beads and 60 µl solution C1 (anionic detergent), then vortexed for 10 minutes to homogenise and lyse the sample and break down fats. The tube was centrifuged at room temperature, 10,000 g for 1 minute. The supernatant was transferred to a fresh tube and 250 µl of solution C2 (inhibition removal technology) was added to remove contaminated matter and the sample was incubated at 4°C for 5 minutes then centrifuged (room temperature, 10,000 g, 1 minute). The supernatant was transferred to a fresh tube and solution C3 (inhibition removal technology) was added as another reagent to remove contaminated matter. The sample was incubated and centrifuged again. The supernatant was transferred to a fresh tube containing 1.2 ml of solution C4 (high concentration salt solution) and then transferred to a MB spin column, 675 µl at a time, and centrifuged. The high salt solution allowed the DNA to bind to the silica membrane in the MB spin column and all other contaminated matter to flow through the column. The flow through was discarded and the step was repeated until all of the solution had been processed. 500 µl of solution C5 (ethanol wash) was added to the spin column and the tube was centrifuged to remove further contaminants. The flow through was discarded and the spin column was placed into a fresh tube. Finally, 100 µl of solution C6 (sterile buffer) was placed onto the centre of the silica membrane and the tube was centrifuged to release the DNA from the silica membrane.

2.6.2 Normalisation and polymerase chain reaction

The concentration of DNA was quantified for each sample, using the dsDNA high sensitivity Invitrogen Qubit 3.0 Fluorometer ThermoFisher (Victoria, Australia) and normalised to 5 ng/µl

using Milli-Q water. Concentrations below 5 ng/µl remained neat. For our first analysis, automated ribosomal intergenic spacer analysis, the normalised DNA was amplified by a polymerase chain reaction (PCR) using a CFX96 Real-Time C1000 Touch Thermal Cycler System (Bio-Rad, NSW, Australia) following the polymerase chain reaction (PCR) cycle settings in Table 2.2. Each DNA sample was processed using a TopTaq DNA polymerase kit (Qiagen, Hilden, Germany). Each 20 µl reaction contained 4 µl Q solution, 2 µl TopTaq 10x buffer, 1 µl 10 mM dNPT mix, 1.2 µl $MgCl_2$ (25mM), 0.1 µl TopTaq DNA polymerase as well as 8.7 µl ultrapure distilled water (Invitrogen, New York, USA), 1 µl of 5ng/µl of DNA solution and 1 µl each of 10 µM forward and reverse primer (primer sequences shown in Table 2.3). These universal primers were used to amplify the non-coding DNA fragments between the 16S and 23S rRNA genes.

Table 2.2. Cycle settings for ARISA bacterial DNA amplification.

Step	Temperature (°C)	Time (seconds)	Number of cycles
Initial Denaturation	94	180	1
Denaturation	95	60	33
Annealing	52	60	33
Extension	72	90	33
Final Extension	72	360	1

Table 2.3. Sequences for the forward and reverse primer used in PCR.

Primer name	Sequence (5' – 3')
16S-1392F	-FAM-GYACACACCGCCCGT-
23S-125R	-GGGTTBCCCCATTCRG-

2.6.3 Gel electrophoresis

Following ARISA-PCR, gel electrophoresis was used to confirm successful amplification of the PCR DNA. A gel was made up of 1 g of Agarose, LE, Analytical Grade (Promega, WI, USA) and 100 ml of 1x Tris-Acetate-EDTA buffer and heated until the agarose dissolved. Once cooled, 5 µl of SYBR Safe DNA Gel Stain 10,000x concentrate in dimethyl sulfoxide (Invitrogen, California, USA) was added and the gel was poured into a 20-well mould until hardened. The gel was placed into an Agarose Electrophoresis System (Cleaver Scientific Ltd, Warwickshire, UK) and charged by a PowerPRO 300 Power Supply CS-300V (Cleaver Scientific Ltd, Warwickshire, UK). 2 µl of 5x DNA Loading Buffer Blue (Bioline, NSW, Australia) was added to 8 µl of each PCR sample. 5 µL of Hyperladder II 50bp (Bioline, NSW, Australia) was loaded into the first well on the gel and 10 µl of each PCR samples was loaded into subsequent individual wells. After running the gel at 100 volts for 60 minutes, the gel was visualised by a ChemiDoc MP Imagine System (Bio-Rad, NSW, Australia). 10 µl of each of the amplified PCR products were sent to the Australian Genome Research Facility (Melbourne) for genotyping fragment separation analysis. The returned data was binned using R software (version 3.6.1) and Primer software (version 7.0), to create ordinations of the binned data using non-metric multidimensional scaling with the Bray-Curtis coefficient.

2.6.4 16S ribosomal ribonucleic acid (rRNA) sequencing analysis

2.6.4.1 V4 region amplification

High-throughput sequencing was used as a second analysis to examine the gut microbiota. While the ARISA analysis allows us to determine how separated the gut communities are, 16S rRNA analysis identifies bacterial phylogeny and taxonomy. The V4 region of the 16S gene was used as this is a semi-conserved region and therefore, can precisely identify microorganisms within the community. In order to amplify the V4 region of the 16S rRNA

gene, PCR was used on the normalised extracted faecal DNA using a CFX96 Real-Time C1000 Touch Thermal Cycler System (Bio-Rad, NSW, Australia) following the settings laid out in Table 2.4. To prepare the samples for PCR, the Invitrogen Platinum Hot Start PCR Master Mix kit (Victoria, Australia) was used in addition to specific forward primers and reverse primers with overhang adapters to amplify the 16S rRNA V4 region identified in Table 2.5. Briefly, the PCR master mix was made up of the following components: Platinum Hot Start PCR Master Mix, reverse primer and nuclease water. The PCR mix was loaded into a 96-well plate with 2µl of DNA sample pipetted separately into each well and 0.5 µl of a specific forward primer added to each well. The forward primer was unique to each DNA sample as this primer contains the barcode and sample identifiers. The plate was then run through the thermocycler.

Table 2.4 Cycle settings for 16S rRNA V4 region bacterial DNA amplification

Step	Temperature (°C)	Time (seconds)	Number of cycles
Initial Denaturation	94	180	1
Denaturation	94	60	35
Annealing	50	60	35
Extension	72	105	35
Final Extension	72	600	1

Table 2.5 Sequences for the forward and reverse primers used in PCR.

Primer name	Sequence (5' – 3')
515F	-GTGCCAGCMGCCGCGGTAA-
806R	-GGACTACHVHHHTWTCTAAT-

2.6.4.2 PCR clean-up

The QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) was used in order to remove primers, nucleotides, enzymes, oils, salts and other impurities from our DNA samples

following PCR. PCR clean-up was performed following manufacturer's instructions. Briefly, phosphate buffer (buffer used in clean-up procedures that enables DNA binding to a spin column membrane) was added to the PCR samples at a 5:1 ratio and mixed. Next the samples were placed into a spin column and centrifuged in a microcentrifuge at 13,000 rpm at room temperature for 1 minute to allow the DNA to bind to the membrane. The flow through was discarded and the column was placed back into the tube. 0.75 ml of ethanol wash buffer was added to the column and spun again. The flow through was discarded and the column was placed back into the column and spun again. To remove the DNA from the membrane, 50 µl of elution buffer was placed on the membrane and the tube was centrifuged for 1 minute and the flow through containing the DNA was collected. Following DNA library preparation, DNA samples were sent to the Australian Genome Research Facility where an Illumina MiSeq sequenced the V4 region.

2.6.4.3 Sequence denoising

Following sequencing, the data was represented as text files called FASTQ files. Prior to any downstream analyses, the FASTQ files underwent quality control using Quantitative Insights Into Microbial Ecology (QIIME 2, version 2019.10). The quality filtering process removed low quality sequences and ambiguous sequences as well as sequences that did not pair well (paired sequences must cross-over with at least 20 base pairs). Next, sequences were truncated at specific base positions based on the median quality score. Forward reads were truncated at position 6 and 241 and reverse reads at position 6 and 202 due to lower terminal base quality in the reverse reads. The sequenced base pairs were matched with Greengenes database (version 13.8). Samples were then rarefied to 13,000 reads in order to minimise the effect of sequencing depth.

2.7 Statistical analysis

Statistical analysis was performed using Prism version 8.2.1 (GraphPad Software, San Diego, CA, USA). All data are presented as mean ± standard error of the mean (SEM). For body weight, blood pressure, blood glucose and plasma insulin, the differences between HFD and NCD mice were analysed using a two-way repeated measure analysis of variance (ANOVA). Sidak *post-hoc* analysis was only performed when the F value was significant and there was no variance in homogeneity. For flow cytometry data and intestinal size, a two-way ANOVA with Tukey *post-hoc* was used to determine differences between tissue types and diet regimens. Non-metric multidimensional scaling was employed to create a 3D ordination of the ARISA data set in order to visualise the dissimilarities and similarities between communities and non-parametric statistical test, analysis of similarities was used to determine the significance of the separation between gut microbial communities. For 16S rRNA sequencing, the Faith-pd metric was used to determine diversity within samples (alpha diversity) and unweighted and weighted UniFrac metrics were used to determine the diversity between samples (beta diversity) generated in QIIME 2. Community differences and similarities based on unweighted and weighted UniFrac were visualised using principal coordinates analysis (PCoA) generated with QIIME 2. The significance of the Firmicutes: Bacteroidetes phyla ratio between groups at each time point was determined by a mixed effects 2-way ANOVA for each sex. Differences in order level taxa abundance between groups was identified by linear discriminant analysis (LDA > 2.0) effect size (LEfSe) with significance determined by non-parametric factorial Kruskal-Wallis sum rank test and generated using Huttenhower Galaxy (version 1.0). The level of significance was set at $P < 0.05$ for all statistical analyses.

Chapter 3: Results

3.1 The effect of HFD on physiological measures

HFD significantly increased body weight in males ($F_{(1,39)} = 21.37$, $P < 0.0001$). *Post-hoc* analyses revealed this occurred from week 6 onwards ($P < 0.05$; Figure 3.1A). Fasting blood glucose and plasma insulin levels were significantly increased in HFD males ($F_{(1,39)} = 6.655$, $P = 0.0138$ and $F_{(1,14)} = 7.087$, $P = 0.0186$, respectively). Specifically, this occurred at week 10 only ($P < 0.01$; Figure 3.1B & C). Systolic BP was not affected by HFD in either sex (Figure 3.1D & H). Interestingly, HFD did not significantly alter body weight, fasting blood glucose levels or fasting plasma insulin levels in female mice (Figure 3.1E - G).

There were no significant differences in small intestine length between sex or diet regimen (Figure 3.2A). However, HFD significantly reduced colon length, caecum length and caecum weight ($F_{(1,63)} = 11.91$, $P = 0.001$; $F_{(1,63)} = 32.59$, $P < 0.0001$ and $F_{(1,46)} = 206.2$, $P < 0.0001$ respectively; Figure 3.2B - D). *Post-hoc* analyses revealed that colon length and cecum length were only significantly reduced in HFD females whereas caecum weight was significantly reduced in in both HFD males and females (compared to NCD controls; $P < 0.001$). Sex also significantly affected colon dimensions, whereby females presented with reduced colon length, caecum length and caecum weight ($F_{(1,63)} = 9.10$, $P = 0.0037$; $F_{(1,63)} = 20.51$, $P < 0.0001$ and $F_{(1,63)} = 14.02$, $P = 0.0005$, respectively; Figure 3.2B - D). However, colon and cecum lengths were only significantly reduced in HFD females (compared to HFD males, $P < 0.001$) and conversely, caecum weights only significantly reduced in NCD females (compared to NCD males, $P = 0.0007$).

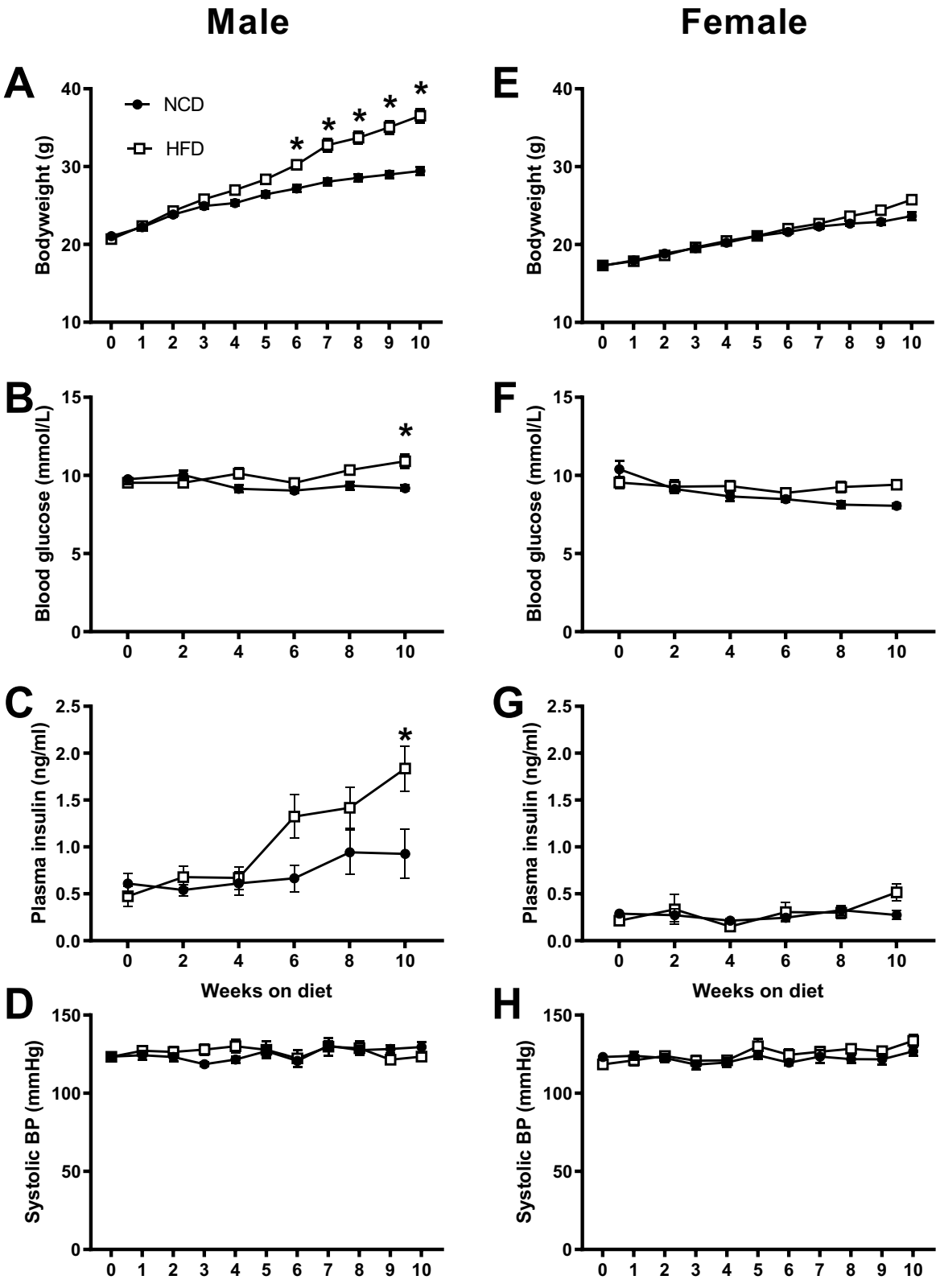

Figure 3.1. High-fat diet increased body weight, fasting blood glucose and fasting plasma insulin in male mice. Body weight (A & E), fasting blood glucose (B & F), fasting plasma insulin (C & G) and systolic blood pressure (BP; D & H) in male (A - D) and female (E - H) normal chow diet (NCD; ●) and high-fat diet (HFD; □) mice. Values are mean ± SEM. **P* < 0.05 vs NCD. n = 19-22 per group for A, B, E & F; n = 7-9 per group for C & G and n = 12-16 per group for D & H.

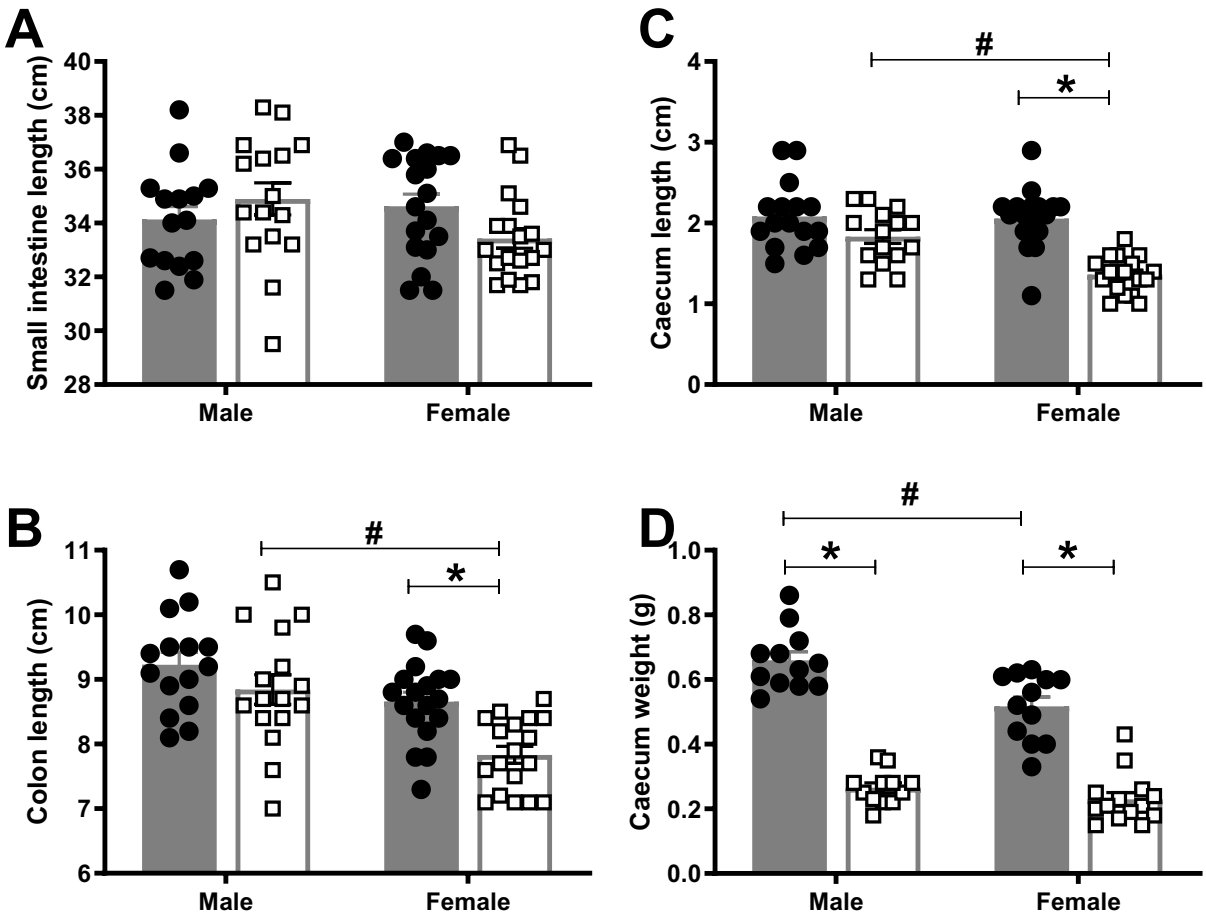

Figure 3.2. High-fat diet mice had smaller caecum sizes and females had shorter colons. Small intestine length (A), colon length (B), caecum length (C) and caecum weight (g; D) in male and female normal chow diet (NCD; ●) and high-fat diet (HFD; ☐) mice. Values are mean ± SEM. **P* < 0.05 vs NCD, #*P* < 0.05 vs opposite sex, n = 12-18 per group.

3.2 The effect of HFD on the gut microbial communities

3.2.1 ARISA

Automated ribosomal intergenic spacer analysis (ARISA) was used to determine the changes in structure and diversity of the gut microbial communities of mice over the duration of the 10-week diet regimens. In a non-metric multidimensional scaling 3D ordination, the dispersion of the points refers to the dissimilarity and the clustering of the points refers to the similarity of the gut microbial community. The R value at baseline for males (R = -0.067, $P = 0.78$; Figure 3.3A) and females (R = 0.006, $P = 0.342$; Figure 3.3B) suggested that the gut microbial communities were similar before the diet regimen began. From week 2, there was a clear significant separation of diversity between diet groups in males (R = 0.97, $P = 0.002$; Figure 3.3A) and females (R = 1, $P = 0.002$; Figure 3.3B) as a result of the HFD. The dysbiosis of the gut microbial communities in the HFD mice continued throughout the diet regimen for male (week 4: R = 1, $P = 0.002$; week 6: R = 0.998, $P = 0.002$; week 8: R = 0.98, $P = 0.002$ and week 10: R = 0.51, $P = 0.002$; Figure 3.3A) and female mice (week 4: R = 0.80, $P = 0.002$; week 6: R = 0.91, $P = 0.002$; week 8: R = 0.90, $P = 0.002$ and week 10: R = 0.73, $P = 0.002$; Figure 3.3B).

Stacked column graphs represent the abundance and distribution of operational taxonomic units (OTUs) in the gut microbial community. The abundance and diversity of OTUs for male (Figure 3.4A) and female (Figure 3.4B) mice at baseline represent the similarity of the gut microbial community before the diet began. Unlike the HFD communities, NCD of both sexes remained similar in OTU diversity and abundance throughout the 10-week regimen. The HFD communities changed significantly from week 2 for male and female mice and did not return to the baseline state. HFD increased the abundance of specific OTUs within the communities as well as reducing, almost to a total loss, of other specific OTUs (Figure 3.4). There was a

significant difference in the OTU diversity of the gut microbiota of mice on the HFD compared to NCD from week 2 in males ($t = 7.16$, $P < 0.0001$) and females ($t = 5.80$, $P < 0.0001$).

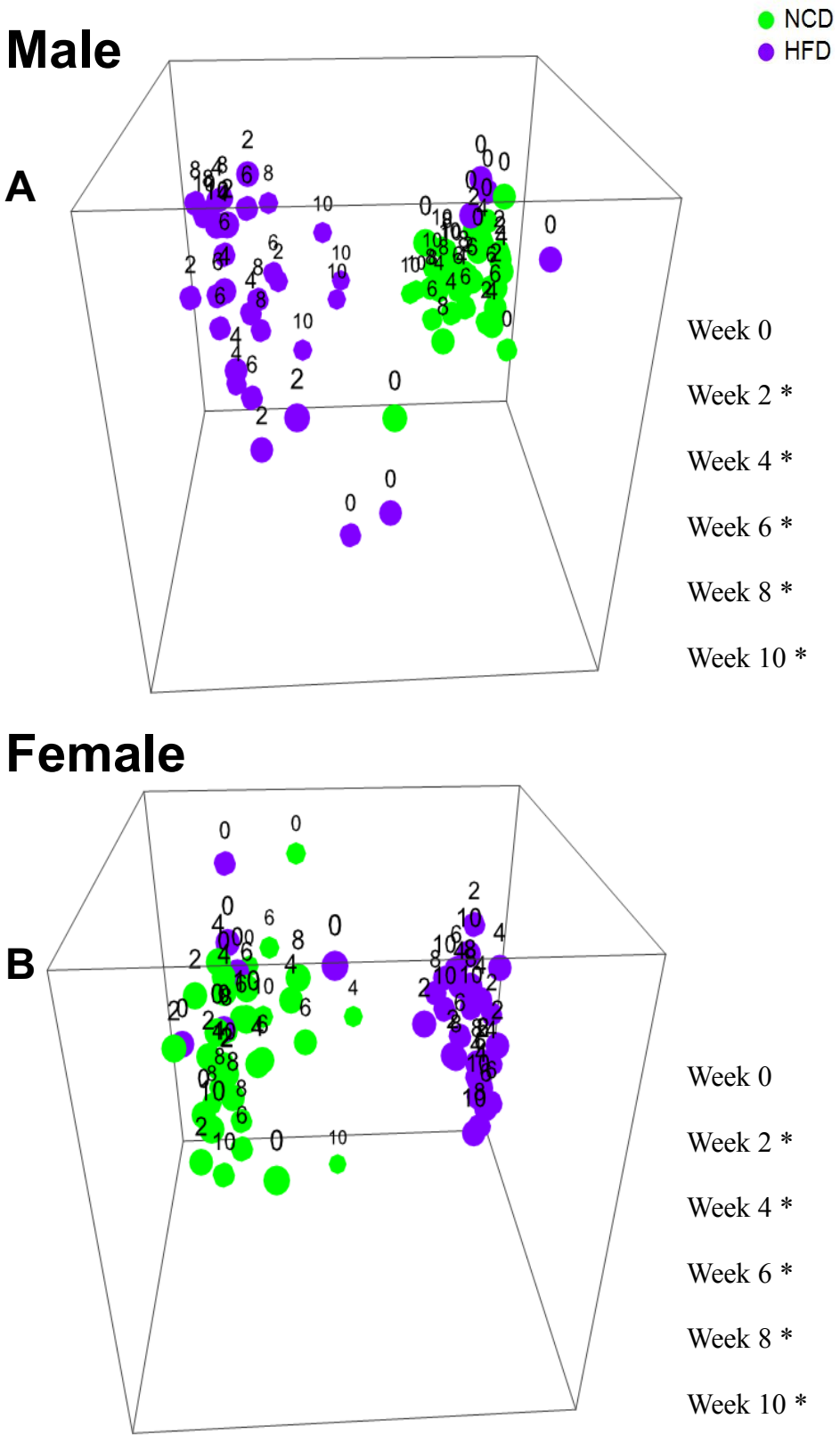

Figure 3.3. High-fat diet caused alterations in community structure and diversity from week 2 in male and female mice analysed by an automated ribosomal intergenic spacer analysis (ARISA). 3D ordination representing the diversity of the gut microbial communities of male (A; 3D stress level = 0.09) and female (B; 3D stress level = 0.1) mice receiving either a normal chow diet (green) or high-fat diet (purple) at baseline (0), week 2, 4, 6, 8 and 10 plotted using non-metric multidimensional scaling (resemblance: S17 Bray-Curtis similarity), *$P < 0.05$ HFD vs NCD, n = 6 per group. The dispersion of the points refers to the dissimilarity/ similarity of the communities.

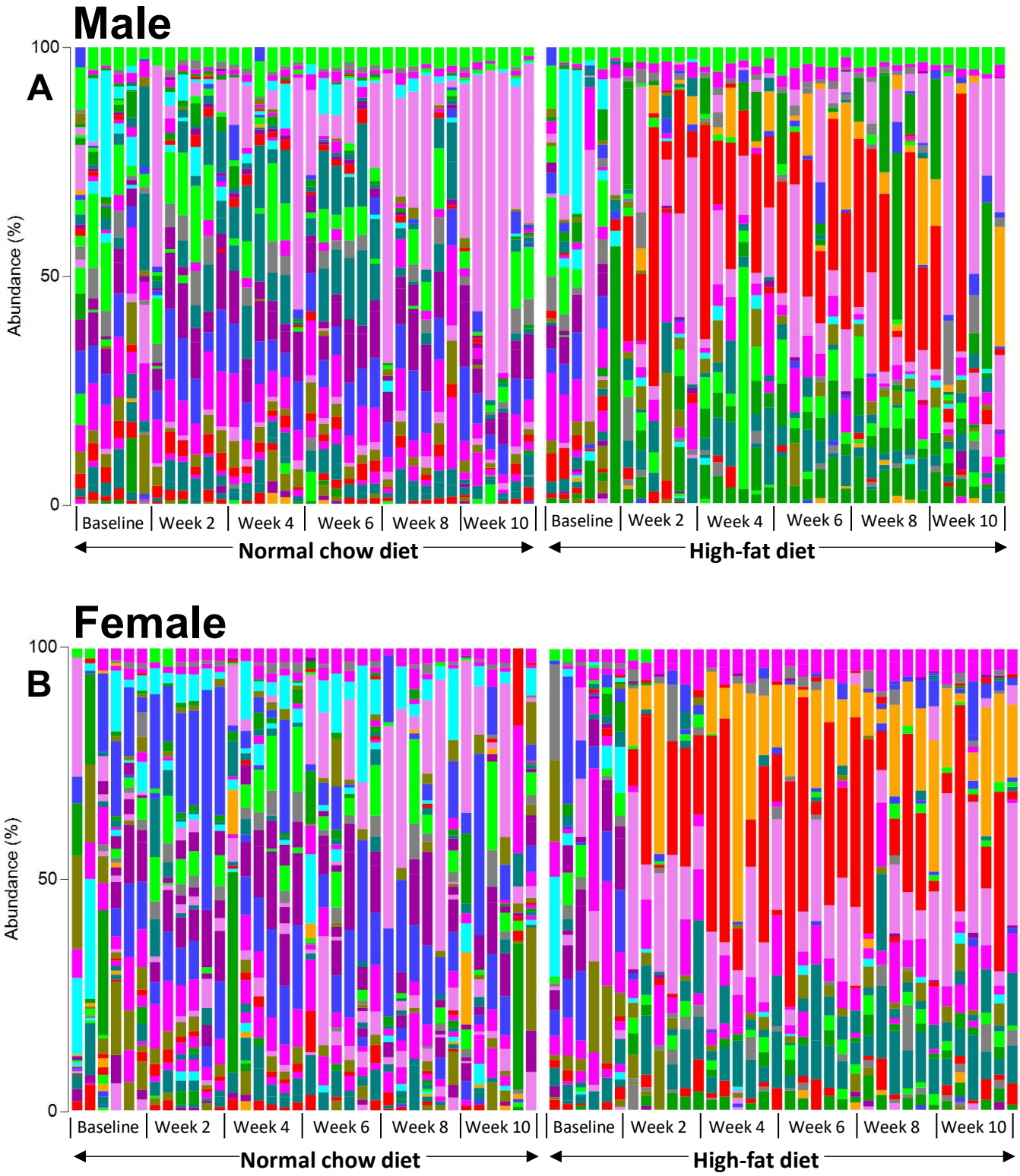

Figure 3.4. High-fat diet reduced the diversity of the gut microbial community in male and female mice. Stacked column graphs representing the diversity and abundance of the gut microbial community, analysed by an automated ribosomal intergenic spacer analysis (ARISA), in male (A) and female (B) mice fed either a normal chow diet (left) or high-fat diet (right) from baseline through to week 10 of the respective diet, n = 6 per group. Each colour, automatically generated using the Primer 6 program, represents an operational taxonomic unit (OTU) with the size corresponding to the percentage abundance within the community. OTUs are depicted in the same colour for both males and females.

3.2.2 16S rRNA sequencing

16S rRNA sequencing (of the V4 region) of faecal samples was used to detect and identify specific microbial populations within the gut community of mice on HFD, and to determine how this community changes over the duration of the 10-week diet. Beta diversity measured by unweighted UniFrac (based on the presence or absence of organisms) and weighted UniFrac (based on the presence or absence of organisms whilst accounting for the abundance of those organisms) did not reveal any significant differences between male and female mice at baseline prior to commencing the respective diets (unweighted UniFrac $P = 0.156$; weighted UniFrac $P = 0.488$). Multidimensional scaling using PCoA was used to visualise the similarities and dissimilarities of the weighted UniFrac distances of male and female mice at baseline (Figure 3.5A). PCoA did not demonstrate separate clustering of the different sexes indicating similarity of gut microbial communities in male and female mice at baseline. Alpha diversity, according to faith-pd metric, was also not significant between sexes at baseline ($P = 0.110$). After 10 weeks of the diet regimen, there were significant differences between the sexes, regardless of diet, in alpha diversity (faith p-d: $P = 0.044$) and beta diversity (unweighted UniFrac $P = 0.016$), however weighted UniFrac was not significant ($P = 0.51$; Figure 3.5D). These diversity differences between the sexes demonstrated that the gut microbial communities of males and females respond differently independent of diet.

Supporting the findings from the ARISA, no significant differences were found in the analyses of alpha and beta diversity between diet groups at baseline, regardless of sex, revealing the similarity of gut microbial communities prior to the commencement of HFD regimen (faith p-d: $P = 0.406$, unweighted UniFrac: $P = 0.457$ and unweighted UniFrac: $P = 0.554$). Following 10 weeks of the respective diet regimens, LEfSe identified the greatest taxa abundance differences, to the order level, between NCD and HFD mice, regardless of sex (Figure 3.5B &

E). Taxa abundances of mice fed NCD showed the promotion of microbes from the Bacteroidetes phylum, such as Bacteroidetes, Bacteroidia and Bacteroidales, compared to HFD mice which revealed greater abundances of taxa belonging to the Proteobacteria and Firmicutes phyla such as Gammaproteobacteria, Deltaproteobacteria Desulfovibrionales, Enterobacteriales, Proteobacteria, Firmicutes, Clostridiales and Clostridia. Diet differences at week 10, revealed borderline alpha diversity differences (faith p-d: $P = 0.088$) and significant beta diversity differences (unweighted UniFrac: $P = 0.001$; weighted UniFrac: $P = 0.001$). Beta diversity was also significant when separating the sexes at week 10 and showed strong separation between diets in PCoA plots (male unweighted and weighted UniFrac: $P = 0.001$ (Figure 3.5C); female unweighted UniFrac: $P = 0.001$; weighted UniFrac: $P = 0.006$ (Figure 3.5F)).

Given the strong separation of diet groups in both male and female mice (Figure 3.5C & F), we then examined the sex differences within diet groups at week 10. Analysis within the NCD group, revealed no alpha diversity differences however, there was a significant difference in beta diversity between males and females for unweighted UniFrac only (unweighted UniFrac: $P = 0.006$; weighted UniFrac: $P = 0.22$). Within the HFD groups, significant alpha diversity (faith-pd: $P = 0.027$) and significant beta diversity in the unweighted UniFrac only was found between males and females (unweighted UniFrac: $P = 0.007$; weighted UniFrac: $P = 0.092$ (Figure 3.6A). Taking into consideration the beta diversity differences between HFD male and female mice, we next investigated both the changes in the Firmicutes: Bacteroidetes ratio as well as changes in taxa abundances. The counts of major bacterial phylum Firmicutes were divided by the counts of Bacteroidetes to calculate the Firmicutes: Bacteroidetes ratio. The Firmicutes: Bacteroidetes ratio was significantly increased by the HFD in both male ($F_{(5, 68)} = 3.354$, $P = 0.0091$) and female mice ($F_{(5, 59)} = 3.627$, $P = 0.0063$). *Post-hoc* analyses revealed

that in males this occurred from week 4 onwards ($P < 0.05$, Figure 3.6B), but only occurred at week 4 in females ($P = 0.0228$, Figure 3.6C).

Given that the significant changes in Firmicutes: Bacteroidetes ratios in HFD mice, predominantly in male mice, LEfSe was performed to identify any sex differences in specific microbial abundances at each time point. The LDA score of each taxon indicated the degree of abundance difference between HFD males and females, regardless of positivity and negativity, and only LDA scores >2 were plotted. At baseline, a number of taxa were significantly increased for both sexes, however, these taxa did not appear at any other time point with the exception of *Ruminococcus* and *Lachnospiraceae* in HFD males which also had significantly greater abundance at weeks 6, 8, and 10 (Figure 3.6D, F - H). Taxa that only appeared at baseline and were not seen in subsequent time points were established as random fluctuations. There were no significant differences between sexes in taxa abundances at week 2. At week 4, *Enterobacteriaceae*, *Proteus*, Gammaproteobacteria and Enterobacteriales were significantly more abundant in HFD males (compared to HFD females) which persisted at each time point through to week 10 (Figure 3.6E - H). In HFD females, 1 unknown taxon and 3 taxa belonging to the Firmicutes phylum were significantly more abundant than in HFD males, however, these taxa did not appear in greater abundance at any other time points (Figure 3.6E). A significant shift in microbial composition occurred in HFD males at week 6, with 14 taxa appearing in greater abundance compared to HFD females (Figure 3.6F). In particular, Synergistia, *Eubacterium*, Synergistes and Synergistales were significantly more abundant in HFD males compared to HFD females from week 6 through to week 10 (Figure 3.6F - H). Firmicutes was also seen in significantly greater abundance at weeks 6 and 10 in HFD males (Figure 3.6F & H). HFD females showed greater abundance of *S24-7* taxon compared to HFD males at weeks 6 and 8 (Figure 3.6F & G). HFD females had greater abundances of 9 taxa compared to HFD

males in week 8. These were from the Proteobacteria and Bacteroidetes phyla, however, these appear to be random as they did not persist through to week 10 (Figure 3.6G). Finally at week 10, HFD males had significantly greater abundances of 18 taxa compared to HFD females (Figure 3.6H). Most of these 18 taxa were seen at earlier time points with the addition of Actinobacteria, Actinomycetales, *Micrococcaceae* and *Micrococcus*. LEfSe measurements were also performed at each time point for NCD mice to determine any differences between the sexes (Figure 3.7). The taxa that were in greater abundances for the mice following NCD fluctuated randomly throughout the regimen with no sex-by-diet interactions. For example, specific taxa, such as *Barnesiellaceae*, emerging in greater abundance for females at week 6 (Figure 3.7C) then became more abundant in males at week 10 (Figure 3.7E) strengthening our conclusion that the taxa in greater abundance in NCD mice were randomly fluctuating.

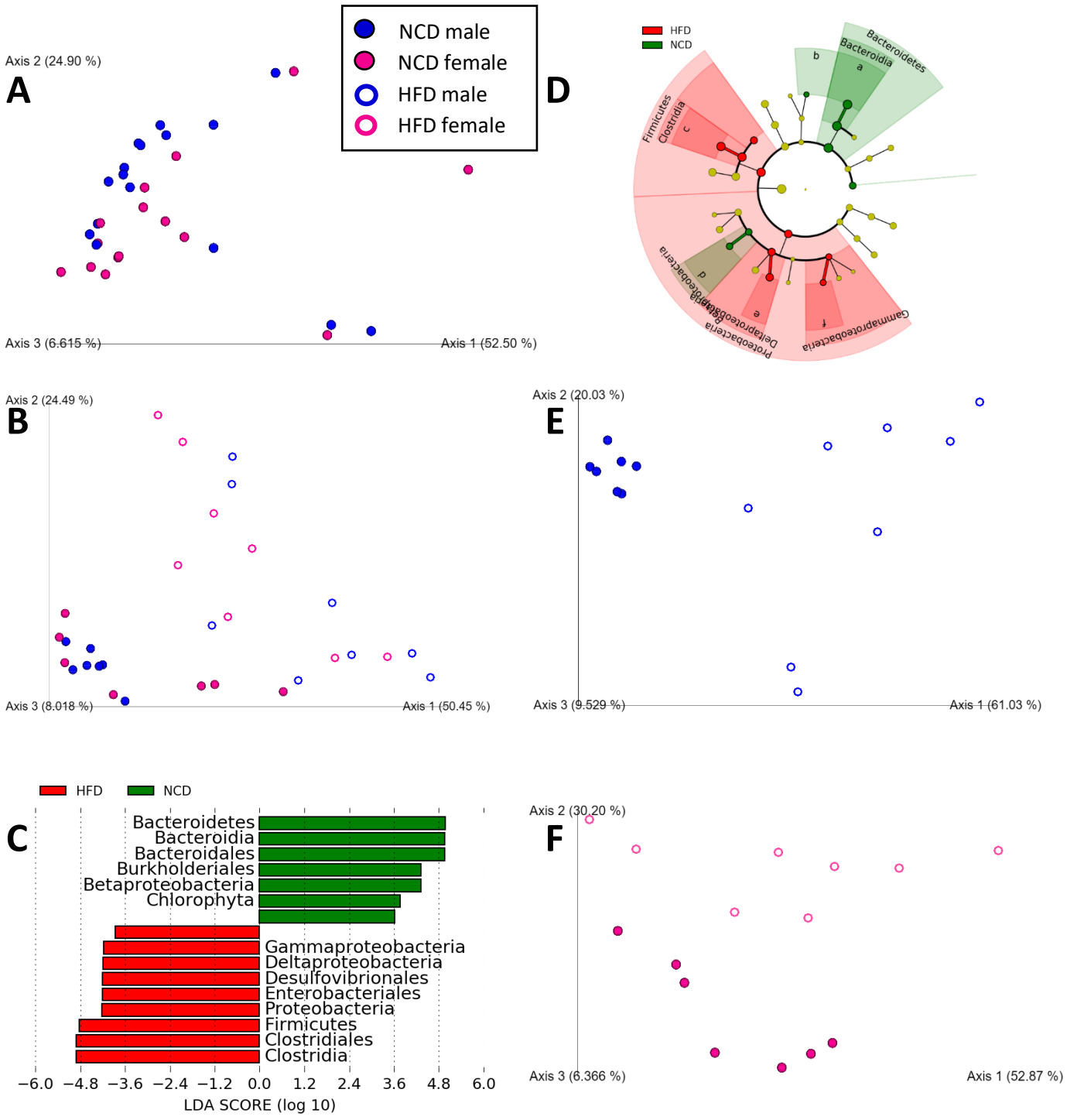

Figure 3.5. High-fat diet significantly separates gut microbial communities at week 10 analysed by 16S rRNA sequencing. PCoA of weighted UniFrac distances of male and females on a normal chow diet (NCD; ●) or high-fat diet (HFD; o) (males represented in blue and females in pink) at baseline (A) and week 10 (D). Linear discriminant analysis (LDA) effect size (LEfSe) (B & E) identified the greatest taxa abundance differences, to the order level, between diet groups with those enriched by HFD in red and those enriched by NCD in green, regardless of sex. LDA scores of each diet group indicate the degree of difference between groups regardless of positivity and negativity and only taxa with an LDA score greater than 2 are shown. PCoA of weighted UniFrac distances at week 10 of male (C) and female mice (F). Axes represent the variation percentages explained by each coordinate's dimensions.

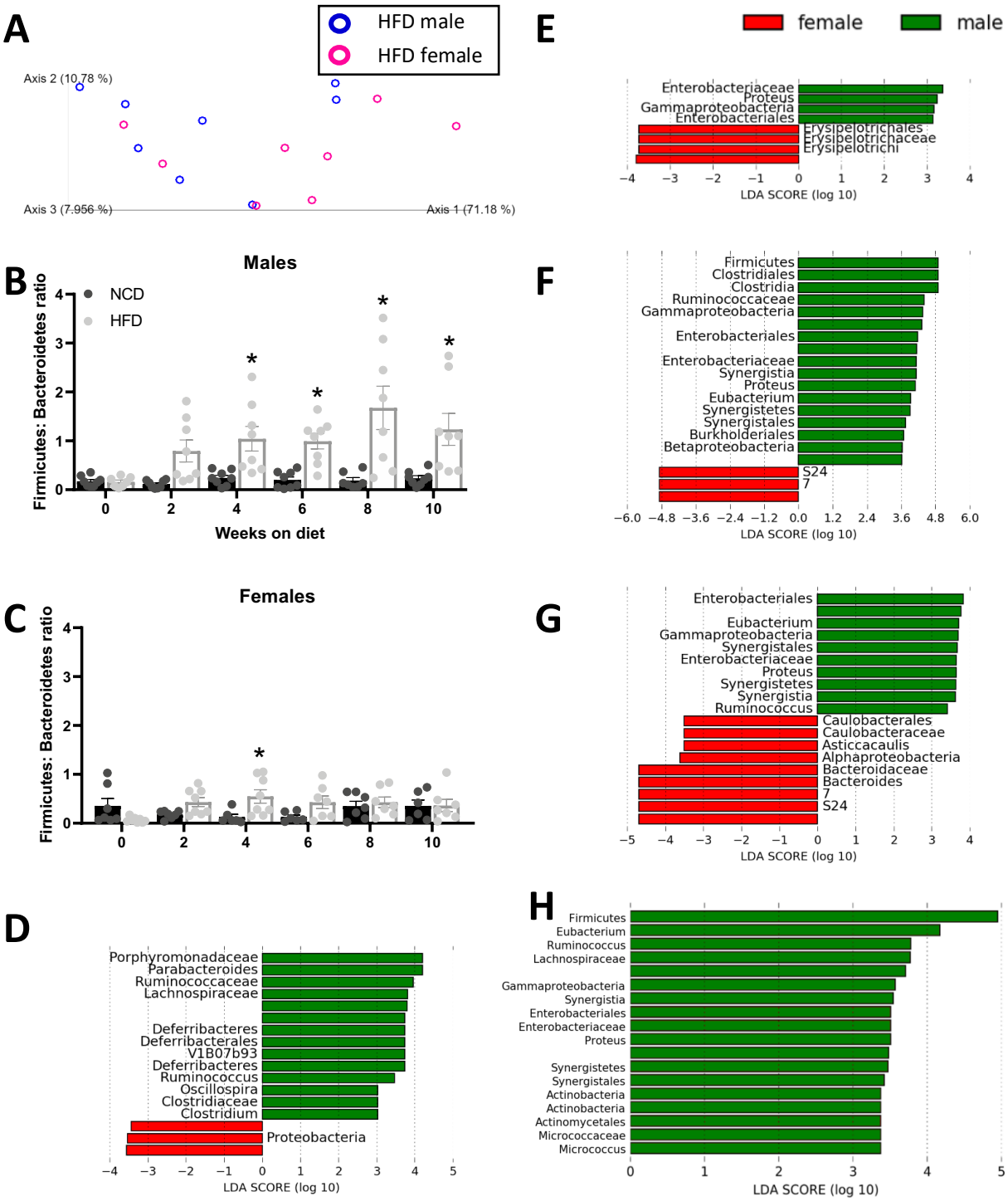

Figure 3.6. High-fat diet significantly altered taxa abundance in male and female mice analysed by 16S rRNA sequencing. Principle coordinates analysis (PCoA) (A) of weighted UniFrac distances of high-fat diet male and female mice at week 10. Axes represent the variation percentages explained by each coordinate's dimensions. Ratio of Firmicutes to Bacteroidetes in males (B) and females (C) from baseline to week 10. Values are mean ± SEM. *$P < 0.05$ vs NCD, n = 7-8 per group. Linear discriminant analysis (LDA) effect size (LEfSe) (D - H) of changes in the gut microbiota following HFD. LEfSe identified the greatest taxa abundance differences between female (represented by red) and male mice (represented by green) at baseline (D), week 4 (E), week 6 (F), week 8 (G), and week 10 (H) of the HFD regimen. LDA scores of each sex group indicate the degree of difference between groups regardless of positivity and negativity and only taxa with an LDA score greater than 2 are shown.

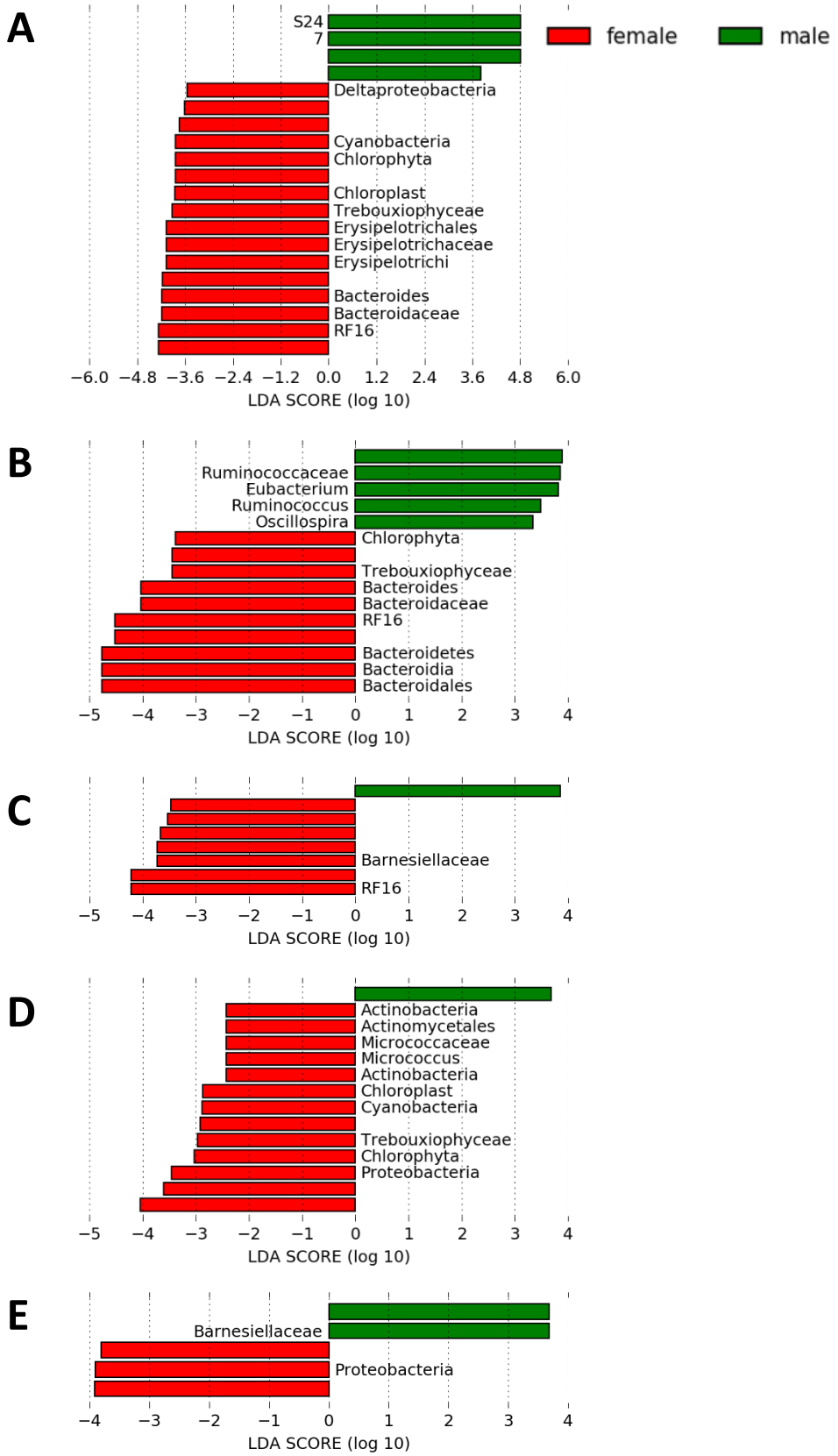

Figure 3.7. Normal chow diet randomly increased taxa abundance in male and female mice analysed by 16S rRNA sequencing. Linear discriminant analysis (LDA) effect size (LEfSe) (A - E) of changes in the gut microbiota following a normal chow diet (NCD). LEfSe identified the greatest taxa abundance differences between female (represented by red) and male mice (represented by green) at week 2 (A), week 4 (B), week 6 (C), week 8 (D) and week 10 (E) of the NCD regimen, n = 7-8 per group. LDA scores of each sex group indicate the degree of difference between groups regardless of positivity and negativity and only taxa with an LDA score greater than 2 are shown.

3.3 The effect of HFD on intestinal inflammation

Flow cytometry was used to characterise the immune cells within the intestines, and to investigate the effects of HFD and sex on colon immune response. Only the colon was used to identify intestinal inflammation as the colon harbours the greatest population of gut microbiota making it more relevant to the faecal ARISA and 16SrRNA sequencing data. The colon was split into two layers, the intraepithelial layer (Figure 3.8) and the lamina propria layer (Figure 3.9). Colon intraepithelial leukocytes (IELs), myeloid-derived cells, T cells, $CD8^+$ T cells, $CD4^+$ T cells, double negative T cells, and $TCR\beta^+$ T cells were not significantly altered by diet nor sex (Figure 3.8A - G). HFD significantly increased B cell populations in the colon intraepithelial layer ($F_{(1, 46)}$ = 14.10) and *post-hoc* analyses identified that this was only occurred in male mice (P=0.0054; Figure 3.8H), but not females. Despite this sex bias, sex did not significantly affect B cell populations in the colon intraepithelial layer.

Both HFD and sex had greater implications in the immune cell populations within the colon lamina propria layer (Figure 3.9). HFD significantly increased total leukocytes ($F_{(1, 49)}$= 17.67, P = 0.0001) and *post-hoc* analyses revealed that this was only significant in females (P = 0.0016; Figure 3.9A). T cells overall ($CD3^+$ cells) in the colon lamina propria layer were significantly altered by both diet ($F_{(1, 49)}$ = 19.14, P < 0.0001) and sex ($F_{(1, 49)}$ = 8.585, P = 0.0051). *Post-hoc* analyses showed that HFD significantly increased T cells in females only (P = 0.0006) and that HFD females had significantly more T cells compared to HFD males (P = 0.0154, Figure 3.9C). Investigating further into the T cell subpopulations, HFD also significantly increased $CD8^+$ T cells ($F_{(1, 46)}$ = 10.79, P = 0.002), $CD4^+$ T cells ($F_{(1, 48)}$ = 8.497, P = 0.0054), double negative T cells ($F_{(1, 46)}$ = 22.44, P < 0.0001), and $TCR\beta^+$ T cells ($F_{(1,50)}$ = 20.27, P < 0.0001). *Post-hoc* analyses revealed that this occurred in female mice only (P = 0.00116, P = 0.011, P < 0.0001, P = 0.0002 respectively, Figure 3.9D - G). Additionally, sex

differences were present in double negative and TCRβ$^+$ T cells ($F_{(1, 46)} = 26.89$, $P < 0.0001$ and $F_{(1, 50)} = 10.04$ $P = 0.0026$, respectively). Specifically, HFD females had significantly increased double negative and TCRβ$^+$ T cells compared to HFD males ($P < 0.01$, Figure 3.9F & G). Finally, HFD significantly increased B cells ($F_{(1, 47)} = 21.57$, $P < 0.0001$) in both male and female mice ($P < 0.05$, Figure 3.9H).

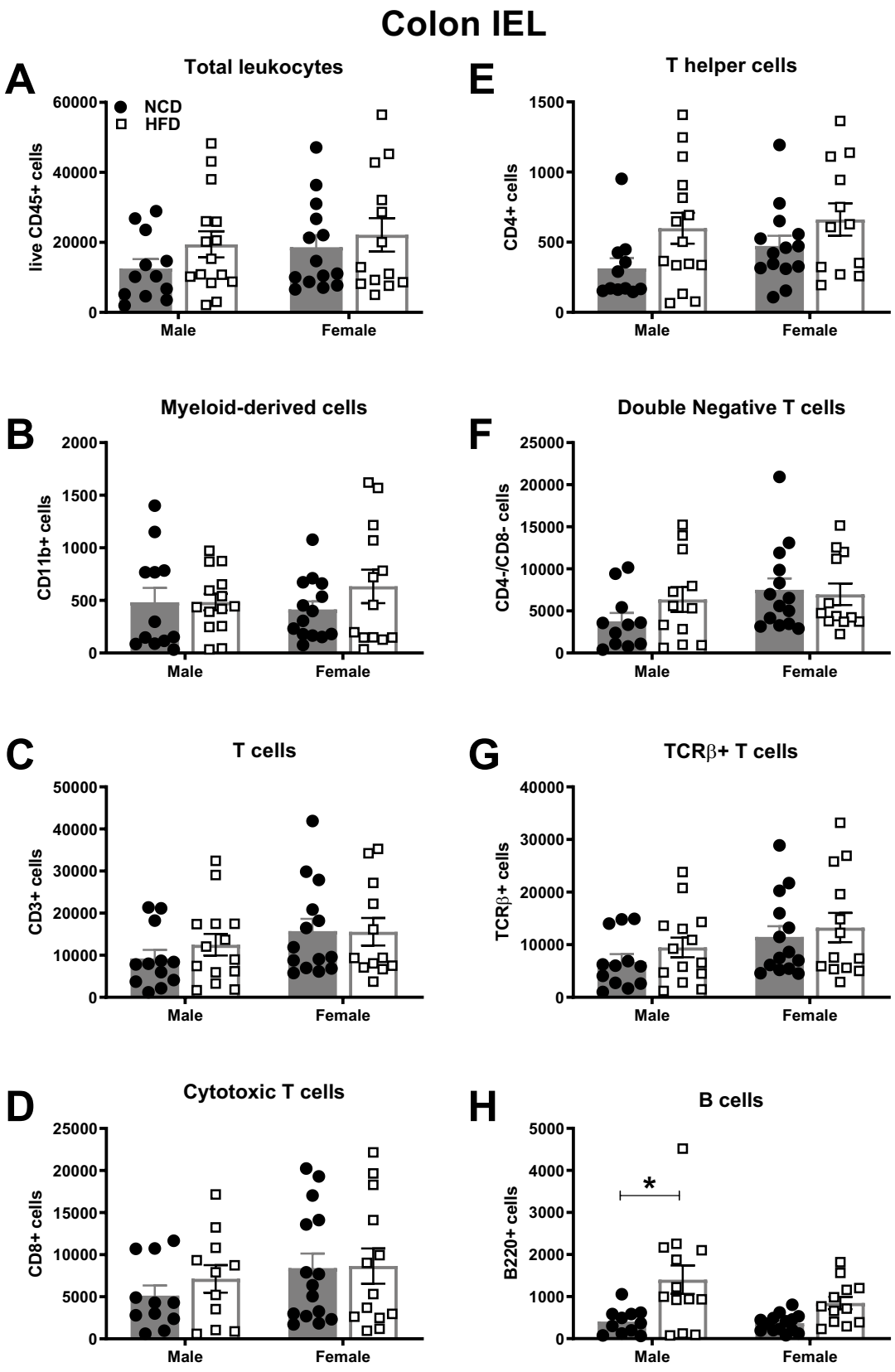

Figure 3.8. High fat diet increased the B cell population in males in the colon intraepithelial layer. Total cells in the colon intraepithelial layer for male and female mice; A: total leukocytes ($CD45^+$), B: myeloid-derived cells ($CD11\beta^+$), C: total T cells ($CD3^+$), D: $CD8^+$ T cells ($CD8^+$), E: $CD4^+$ T cells ($CD4^+$), F: double negative T cells ($CD4^-$ /$CD8^-$ cells), G: $TCR\beta^+$ cells ($TCR\beta^+$) and H: B cells ($B220^+$). Normal chow diet (NCD; ●) and high-fat diet (HFD; □) mice were analysed. Detected using flow cytometry. Values are mean ± SEM. *$P < 0.05$ vs NCD, #$P < 0.05$ vs opposite sex, n = 11-15 per group.

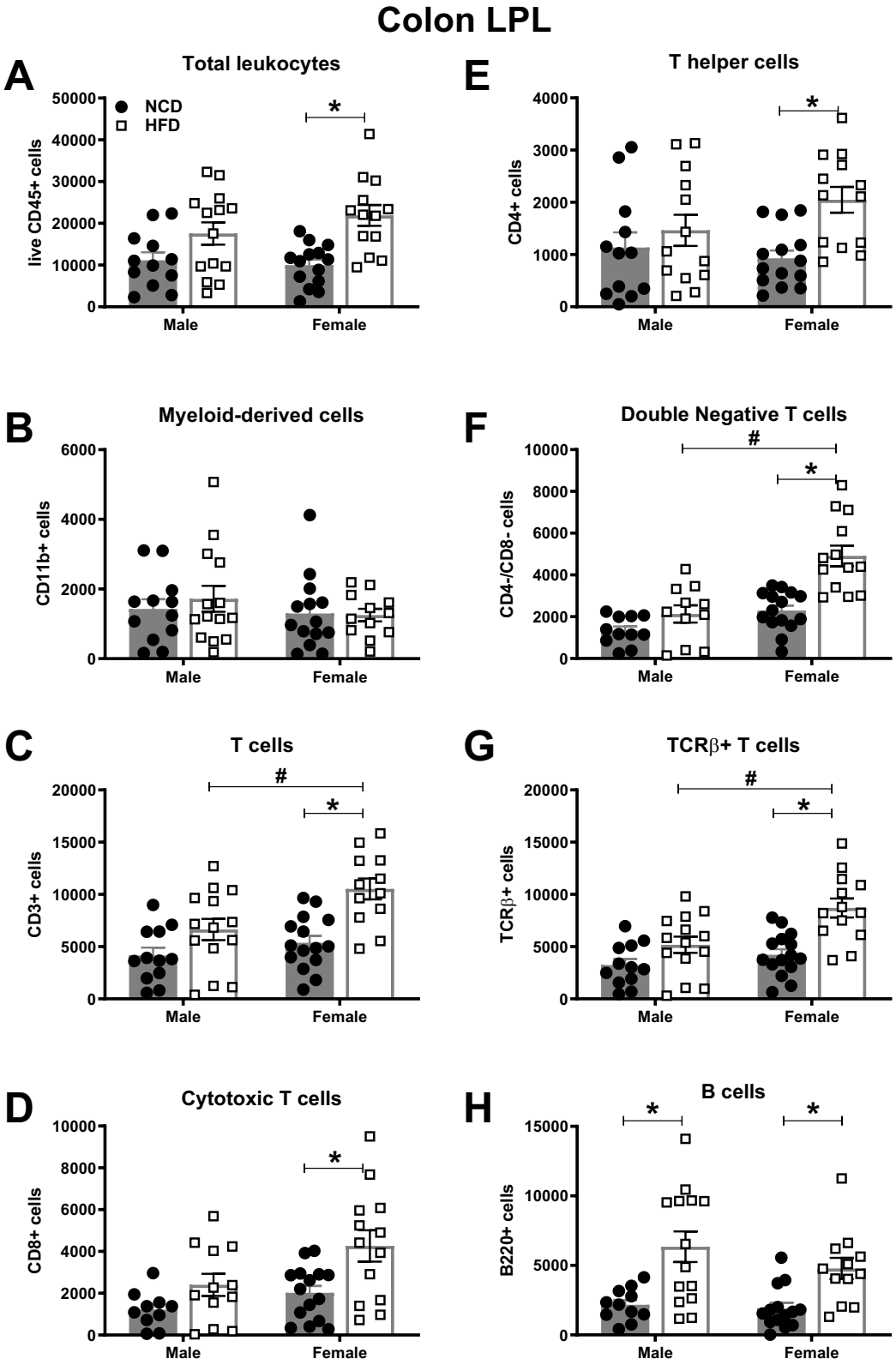

Figure 3.9. High fat diet female mice had a significantly increased immune response in T cells, double negative T cells and TCRβ$^+$ cells compared to high fat diet males. Total cells in the colon lamina propria layer for male and female; A: total leukocytes (CD45$^+$), B: myeloid-derived cells (CD11β$^+$), C: total T cells (CD3$^+$), D: CD8$^+$ T cells (CD8$^+$), E: CD4$^+$ T cells (CD4$^+$), F: double negative T cells (CD4$^-$/CD8$^-$ cells), G: TCRβ$^+$ cells (TCRβ$^+$) and H: B cells (B220$^+$). Normal chow diet (NCD; ●) and high-fat diet (HFD; □) mice were analysed. Detected using flow cytometry. Values are mean ± SEM. *$P < 0.05$ vs NCD, #$P < 0.05$ vs opposite sex, n = 11-15 per group.

Chapter 4: Discussion

The present study demonstrated that HFD produced obesity, hyperglycaemia and hyperinsulinemia in males, but not females. Importantly, HFD shaped the structure and diversity of gut microbiota however, the gut of males and females harboured their microorganisms differently. In particular, marked differences between the sexes in the major phyla abundance, Firmicutes and Bacteroidetes, as well as alterations in specific taxa abundance were displayed. Moreover, after identifying and quantifying the immune cells of the colon, further differences between the sexes were detected with females indicating a stronger, more robust immune response in the gut. Therefore, the underlying causes for sexual dimorphism of obesity, and the clear protection offered to females, can be partially explained by the discrepancies in both gut microbiota composition and the intestinal immune response.

4.1 HFD induces obesity, hyperglycaemia and hyperinsulinemia in males

Numerous HFD mouse models exist with variations in the fat calorie content and length of the diet, and demonstrate symptoms such as obesity, insulin resistance, hyperglycaemia, and hypertension (186-188). In our present study, body weight was altered by HFD from week 6 to induce obesity in males, but not females. Our findings of increased body weight, match those of similar studies using HFDs with comparable fat contents (189, 190). Sex-based differences in weight gain have also been previously reported. Male mice fed HFD (60% kcal fat content) were overweight by week 5 of the diet however, it took a further 5 weeks of the diet for female mice to reach the same benchmark, signifying that male mice respond faster to poor diet compared to female mice (166). Studies have provided evidence that this phenomenon is present even with similar energy intake between sexes (191). Unfortunately in our study, due to the crumbly nature of the HFD food, we were unable to measure food intake and therefore, corroborate this finding however, it should be considered for future work. Although the present

HFD diet model induced obesity in male mice, the opportunity to make conclusions about this phenotype in the female mice are limited as obesity was not evident at the study endpoint. Therefore, a suggestion for future work arising from this study, is combining HFD with added challenges, such as added fructose and/or salt to the drinking water or lengthening the diet regimen, in order to induce a more representative model of human obesity in females.

Commonly accompanying obesity is a prediabetic or diabetic state, and this has been demonstrated by impaired glucose and insulin levels in HFD male mice (192). In the present study, 10 weeks of high fat feeding in male mice significantly increased both fasting blood glucose and fasting plasma insulin levels, correlating with previous findings (193). Similar to the benign effect of HFD on body weight, female mice were also resistant to developing glucose and/or insulin impairment (193). Of note, glucose levels and insulin levels at week 10 in HFD female mice showed higher measures than NCD female mice however, they were not significant. In addition to the role of the gut microbiota and intestinal immune system, which will be discussed shortly, this sexual dimorphism can likely be partially explained as a result of the mechanisms surrounding adipose tissue. For example, female adipose tissue has been demonstrated to have a greater degree of expandability compared to males, permitting energy intake to match storage capacity and preventing lipid accumulation in other tissues (194, 195). Therefore, the increased expandability of female adipose tissue provides increased protection against insulin resistance and better maintenance of metabolic processes for both NCD and HFD diet (196). Additionally, B cells have been shown to play a significant role in the development of insulin resistance and our intestinal immune data, showing that colonic B cells are more readily upregulated in males (which will be discussed shortly), supports this finding.

4.2 HFD atrophies the intestines in both sexes

Another outcome of our HFD model, was the reduction in colon and caecum length in female mice and the reduction in caecum weight in both sexes. The shortening of the intestines, along with intestinal damage and inflammation, has been described as a common characteristic of obesity and diets lacking fibre (197). The changes in morphology are accompanied by intestinal functional disturbances such as decreased intestinal permeability and alterations in the distribution of tight junction proteins leading to an influx of pathogens and LPS into underlying tissue (198). This infiltration of pathogenic organisms and molecules has been shown to produce a proinflammatory immune response similar to our findings of a greater immune response in HFD mice particularly in the lamina propria, albeit not significant in every immune cell population (199). To my knowledge, the comparison of sexes with the shortening of their intestines due to HFD is not well documented. However, previous studies have shown that inducing intestinal inflammation by exposing mice to dextran sulphate sodium demonstrates that male mice are more susceptible to intestinal atrophy and inflammation (200, 201). Our finding that only female mice, show a significant reduction in colon and caecum length as a result of HFD, contrasts with this data as females produced a greater colonic immune response to the HFD.

Additionally, in our study we observed atrophy of the caecum in both male and female mice which correlates with previous reports of reduced caecum size following reductions in dietary fibre (202, 203). In this model, the HFD has 2% fibre from cellulose which is significantly lower than the 15% fibre content of the NCD. Lack of fibre, especially fermentable fibre, in the diet not only promotes obesity, but also decreases gut epithelial proliferation and atrophy (204). HFD enriched with fibre, stimulated enterocyte proliferation and strengthened the epithelial barrier as well as increasing the production of antibacterial proteins in male mice

(204). Another responsibility of the caecum, is to harbour a large population of gut microbiota (202). The gut microbial community was significantly altered in the present HFD mice. Therefore, the atrophy of the caecum and colon could also be due to reductions of diversity in the gut microbial community.

4.3 HFD changed the microbial populations in male and female mice

4.3.1 Automated ribosomal intergenic spacer analysis (ARISA)

Two analyses were used in this study to examine the gut microbial community. The first, automated ribosomal intergenic spacer analysis (ARISA), determined changes to the structure and diversity of the gut microbial communities and how they changed over time. The second analysis, 16S rRNA sequencing which is a more thorough analysis, identified and quantified microorganisms within the samples at each time point. Both analyses were applied across fortnightly intervals from baseline through to week-10 of the diet to observe how the community changed as a result of diet. According to the ARISA, prior to the commencement of the respective diet regimens, the microbial communities were comparable between mice for both males and females. HFD resulted in a clear separation in the structure and diversity from week 2 for both sexes, well before obesity was apparent, and this segregation of the NCD and HFD communities continued through to the end of the diet regimen. Our findings from the ARISA, that the alterations in the community structure and diversity precedes obesity and other accompanying comorbidities, is supported by previous literature (166). Changes to the diversity and composition of the gut microbiota community influenced by HFD can be initiated within one day (205). Therefore, it would be valuable for future work to collect faecal samples every day within the first week of commencing HFD to determine the exact time point, prior to week 2, that gut community changes occur and use a thorough analysis, such as 16S rRNA sequencing to determine the population alterations.

The ARISA data generated in this study also confirmed that the relative diversity and abundance of OTUs was significantly changed as a result of the HFD from week 2, selecting for specific OTUs within the community. There was an increased abundance of particular OTUs in the HFD gut microbial communities that were only seen in small quantities, or were otherwise absent, in the NCD counterparts. Additionally, specific OTUs represented in the NCD were reduced or completely abolished in the HFD communities, with a resultant decrease in the overall diversity in both male and female mice. The reduction in microbial diversity has been demonstrated as detrimental to host health in functions such as resisting pathogenic bacteria and establishing a strong and stable intestinal immune system (145, 206). Subjects with greater microbial richness have also demonstrated a superior metabolic status in terms of fasting blood glucose and body fat distribution compared to those with low microbial richness (206). This correlates with our data, especially in HFD male mice exhibiting obesity, hyperglycaemia and hyperinsulinemia where diversity was markedly reduced (207). Although reduced diversity was also indicated in HFD females, they did not exhibit detrimental metabolic characteristics. Under the influence of HFD, it is known that the environment of the gut community changes throughout the intestines however, the ARISA technique does not have the capacity to determine the severity of the gut microbial changes nor does it reveal taxonomic detail of the changes in the gut microbial diversity of each diet group. Additionally, it also does not allow conclusions to be drawn in regard to the sexual dimorphism of the gut microbiota hence, the importance of our second analysis using 16S rRNA sequencing.

4.3.2 16S rRNA sequencing analysis

The ARISA results revealed that there was no separation of diversity and composition between NCD and HFD groups at baseline. Findings from the 16S rRNA analysis supported this, with an additional discovery that sexes were also similar prior to the start of the experiment. This

allowed us to eliminate the possibility that any differences that arose over the course of the 10 weeks of the respective diets were not due to pre-existing differences between diet groups or between sexes. This finding has been supported by previous work stating that there is no sex diversity differences in the gut microbiota of prepubescent mice (149). In order to determine the influence of HFD on the gut microbiota after 10 weeks, we first analysed the differences between NCD and HFD, regardless of sex.

4.3.2.1 Diet differences

Mice in their respective diet groups at week 10 demonstrated a clear separation from one another. We were able to identify taxa that were in greater abundance in one diet group compared to the other. LEfSe measurements revealed that HFD promotes bacteria from the Proteobacteria and Firmicutes phyla as well as the Clostridium order whereas, NCD promotes those in the Bacteroidetes phylum. The change in the relative abundance of Firmicutes and Bacteroidetes is commonly proposed as a biomarker of disease due to their predominance in the gut (208). Typically obese subjects, or those undergoing HFD feeding (both humans and mice), demonstrate an increased abundance of Firmicutes and a relative decrease in Bacteroidetes compared to normal-weighted subjects (209, 210). For example, the gut microbial richness and diversity of obese children, aged 9-11, revealed lower abundances of Bacteroidetes compared to the normal-weighted children (211). Additionally, and supporting our findings, was the overrepresentation of Proteobacteria in these obese children (211). The same occurrence is commonly reported in HFD (ranging from 21-60% kcal fat content) mice, displaying higher proportions of Firmicutes, Proteobacteria and Clostridium (212, 213). After confirming the differences as a result of diet, we next analysed the gut microbial differences as a result of sex.

4.3.2.2 Sex-specific diversity differences

In addition to the diet-induced changes to the gut microbiota, analyses also revealed both male and female mice responded differently to diet. A previous study, with similar fat content and diet length, has demonstrated that the composition of the gut microbial community in male mice is markedly different to that of female mice (214). This study and others, have also established that these sex differences are independent of diet which resembles our findings of significant sex differences at week 10, regardless of diet, however, this depends on the diversity metric (158, 214). Sex-dependent responses to diet has been previously demonstrated in both animal models and humans. These sex differences in the gut microbiota have been established in various human research cohorts such as healthy Japanese subjects consuming differing levels of yoghurt as well as metabolic syndrome-affected adults following a low-fat diet (151, 215). Additionally, this sexual dimorphism is present in mouse models for example, gnotobiotic female mice inoculated with faecal bacteria from a male vegetarian human, displayed greater diversity than matched-males as well as being significantly separated in composition (159). The significant separation in gut microbiota composition and diversity of male and female mice, in healthy mice following NCD and metabolically affected mice on HFD, at week 10 provides a possible explanation for the differences observed in physiological parameters, intestinal morphology and gut immune response in this present study, but further studies are required to confirm this (as mentioned in section 4.6 below).

4.3.2.3 Sex-specific compositional differences

The sex differences detected at week 10 are a result of the substantial alteration at the phylum level of the Firmicutes to Bacteroidetes ratio. The increase in Firmicutes: Bacteroidetes ratio has been consistently reported as a clinical indicator of disease as a result of poor diet however, here we demonstrate that this ratio differs in a sex specific manner in response to HFD.

Notably, from week 4 through to week 10, HFD males had a marked increase in their Firmicutes: Bacteroidetes ratio compared to NCD males, whereas females only demonstrated this increase at week 4. The influence of diet on the Firmicutes: Bacteroidetes ratio is well documented (216). Previous data established that the Firmicutes phylum are more effective in consuming calories from food and have a negative influence of glucose metabolism thus, an increased abundance leads to weight gain and glucose impairment (208, 217). Conversely, Bacteroidetes are generally considered beneficial to host health due to their abilities in adapting to their environment and the availability of nutrients, as well as their capacity to influence the immune system (218, 219). Despite research indicating the harmful effects of Firmicutes and the beneficial effects of Bacteroidetes, these phyla properties are not intransigent and can also act upon the host in the opposing way (208). In terms of obesity and metabolic disorders however, a positive correlation between Firmicutes: Bacteroidetes ratio and the increasing severity of disease is established (220). In our study, we demonstrated that not only does HFD increase the Firmicutes: Bacteroidetes ratio but sex also has a strong influence. To our knowledge, we are the first to demonstrate the resilience of HFD female's gut microbiota in maintaining the Firmicutes: Bacteroidetes ratio across the diet regimen. In a similar study examining the sexually dimorphic response to HFD, the contribution from beneficial microbes was associated with the resistance in female mice against metabolic disorders (214). Therefore, in our study we deduce that the ability in HFD females to preserve the Firmicutes: Bacteroidetes ratio strongly influences their protection against developing obesity and other comorbidities such as hyperglycaemia and hyperinsulinemia as demonstrated in HFD males.

Following on from the sex differences present in the Firmicutes: Bacteroidetes ratio, we investigated the specific taxa abundance differences at lower taxonomic ranks, to the order level, between HFD males and females. Despite the lack of separation between the sexes at

baseline, several taxa were significantly more abundant in one sex compared to the other. Of the 14 significantly abundant taxa in males and 3 significantly greater abundant taxa in females, only 2, *Lachnospiraceae* and *Ruminococcaceae*, were seen again at subsequent time points for males and the remaining 15 taxa appeared to be random fluctuations which commonly occurs with the gut microbiota as demonstrated in mice and humans (221, 222). *Lachnospiraceae*, resurfacing again at week 10, and members of the *Ruminococcaceae* family, reappearing in greater abundance at week 6, 8 and 10, were in greater abundance in male mice compared to female mice at baseline. Members of the *Lachnospiraceae* and *Ruminococcaceae* families have been associated with beneficial effects such as demonstrating high production of anti-inflammatory butyrate (223). However, they have also been linked with harmful effects and strongly associated with metabolic disorders, and interestingly, these taxa are commonly more prevalent in males compared to females (224, 225). Thus, prior to the diet regimen, it is possible that males already possess certain microbial populations predisposing them to disease development.

Four weeks into the HFD regimen, both male and female mice had 4 taxa in greater abundance compared to the opposite sex. In the HFD females, these taxa belonged to the Firmicutes phylum which coincides with the increase in the Firmicutes: Bacteroidetes ratio however, in the same manner that the increased ratio was not seen in subsequent weeks, these 4 taxa also did not resurface. Contrastingly, in HFD males, 4 taxa from the Proteobacteria phylum, *Enterobacteriaceae, Proteus*, Gammaproteobacteria and Enterobacteriales, that were found in greater abundance at week 4, persisted at each time point through to week 10. Given the strong associations with increased Proteobacteria abundance and individuals suffering from various intestinal and metabolic diseases, the Proteobacteria phylum has been proposed as a potential diagnostic biomarker for disease (226, 227). Given that the increased abundance of

Enterobacteriaceae, Proteus, Gammaproteobacteria and Enterobacteriales were identified in the HFD males prior to the detection of significant obesity, hyperglycaemia and hyperinsulinemia, this also suggests that these taxa could be used as potential biomarkers for metabolic diseases.

At week 6, HFD males showed marked physiological differences demonstrated by their significantly increased body weight compared to their NCD counterparts. This correlates positively with our LEfSe measurements at this time point, revealing a large shift in the overrepresentation of 17 taxa in the HFD males compared to HFD females. Within these 17 taxa, *Enterobacteriaceae, Proteus*, Gammaproteobacteria and Enterobacteriales persisted from week 4, with an additional 2 taxa from the Proteobacteria phylum, Burkholderiales and Betaproteobacteria. Additionally, 3 of the 17 taxa belonged to the Synergistetes phylum which persisted through to week 10. The Synergistetes phylum is commonly found in oral sites however, its increased abundance within the gut has been reported in response to HFD (228). Despite their increased population within the gut community in response to diet, the function of this phylum is poorly described however, given that it is more commonly found in oral sites, I speculate that it may act as an opportunistic microorganism when it colonises the gut (229). Furthermore, at this time point coinciding with the increased Firmicutes: Bacteroidetes ratio, was a significantly greater abundance of 5 taxa belonging to the Firmicutes phylum in HFD males. Of interest was the *Eubacterium* genus and Clostridia class which have been stated to be inversely correlated with dietary fructose and largely associated with the intake of dietary fibres which contrasts with our low fibre/ high fat dietary regimen (230, 231). *Eubacterium* consists of various diverse species that are known for their butyrate-producing abilities and thus, their anti-inflammatory effects on the gut (232). Despite the disparity in how the increased abundance of this bacterium occurs, the anti-inflammatory modulating effects it possesses,

coincides with our intestinal immune data. Given that *Eubacterium* persisted through to week 10 when the intestinal immune data was collected, which revealed that males do not display the same intense and robust immune response as females, it is likely that this genus had a strong influence on suppressing intestinal inflammation.

At the week 8 time point, *Enterobacteriaceae, Proteus*, Gammaproteobacteria, Enterobacteriales, *Eubacterium*, Synergistetes, Synergistales, Synergistia, and *Ruminococcus* persisted in greater abundance in HFD males as seen in previous time points. Conversely, HFD females exhibited a greater abundance of 9 taxa at week 8, 6 of which had not been observed previously, belonging to both the Proteobacteria and Bacteroidetes phyla. It is important to note that although these newly observed taxa were not seen at the final subsequent time point, it is possible that their presence influenced the response of the intestinal immune system at week 10 which will be discussed shortly. Following 10 weeks of HFD, males developed severe obesity as well as exhibiting hyperglycaemia and hyperinsulinemia. Accordingly, their gut microbiota exhibited a large shift in composition, displaying 18 taxa in greater abundance compared to HFD females. As discussed, 11 of these taxa were seen at previous time points and therefore, are regarded as possible obesity biomarkers as well as accounting for the sexual dimorphism in response to HFD. In addition to the persistent taxa, 4 from the Actinobacteria phylum emerged in greater abundance. In accordance with these findings, a previous study observing the sex differences in microbiota composition, found the Actinobacteria phylum in greater abundance in males compared to females when following a HF/HS diet for 8 weeks (158). When reviewing the collective abundance differences over the course of the diet regimen, it is clear that a sex-specific gut microbiota exists in response to HFD and are associated with the protection or susceptibility to developing obesity and accompanying comorbidities. Whilst we infer that HFD females are largely protected from obesity due to their

gut microbial composition, what is unknown from our study is why their microbial community differs given that both sexes were fed the same diet. Previous data suggests that it is the influence of estrogen in females, and the key role that it has in preventing obesity and shaping the composition of the gut microbiota (47).

Copious amounts of evidence demonstrate that estrogen regulates metabolism and influences the gut microbiota (233). Studies into pre- and post-menopause subjects as well as ovariectomised and hormone treatment models have identified the protective effects of estrogen in preventing obesity as well as its influence in shaping the gut microbiota (233). For example, in a cohort of women grouped into low, medium and high levels of serum estradiol, those with distinguished high estradiol serum levels displayed greater diversity in their gut microbiota compared to the lower serum level groups (154). Moreover, women with greater amounts of estradiol also exhibited a greater abundance of Bacteroidetes and a relative decrease in Firmicutes. Similarly in HFD rodent models, the administration of estradiol slowed the increase in the Firmicutes: Bacteroidetes ratio compared to HFD mice not treated with estradiol (164). These results offer clarification for the differences observed between males and females and in particular, the difference in the ability to maintain the Firmicutes: Bacteroidetes ratio and the prevalence of specific taxa within the community. Therefore, it would be valuable in future work to encompass the effects of estradiol with the use of hormone supplementation or ovariectomising the mice prior to the beginning of the diet (discussed in further detail in section 4.6). Another known influencer of the gut microbiota, and thus can provide further explanation behind the sexual dimorphisms observed in HFD mice, is the intestinal immune system (234).

4.4 HFD females illicit a stronger immune response in the colon lamina propria

Obesity is known to generate a proinflammatory shift in a number of immune cell populations within the intestines however, relatively unknown is how this shift differs between males and females (235). In order to comprehensively characterise the intestinal immune response to HFD in our project, the layers of the colon were separated into the intraepithelial leukocytes (IELs) and lamina propria leukocytes (LPLs). The IELs consist mainly of adaptive immune cells with majority of this layer made up of T cells (236). The essential qualities of the immune cells within this layer, due to the close proximity of microbial and dietary antigens, are high and rapid expression of activation markers (e.g. proinflammatory cytokines), cytotoxic-related mechanisms, and antigen-specific memory (237, 238). The populations isolated in the present study correlated with these previous reports, namely, T cells dominated in the intraepithelial layer. Looking at diet-dependent differences, B cells were significantly higher for HFD males but not in HFD females. The significant elevation of B cells correlates with the hyperglycaemia and hyperinsulinemia in HFD male mice, which is fitting as B cells are known drivers of this prediabetic state (239). Commonly, patients with T2D display increased levels of B cells as well as changes in cell function (239). Functional alterations include increased secretion of proinflammatory cytokines and pathogenic antibodies, increasing their antigen-presenting abilities to T cells, and decreasing the production of IL-10, a known insulin sensitivity promoting cytokine (239, 240). The detrimental effects of B cells are exhibited in mice deficient in B cells (μ heavy chain knockout mice) as well as B cell-depleted mice (CD20-treated mice) who are protected against HFD-induced obesity as well as displaying a stronger ability to maintain glucose homeostasis (241). Therefore, the upregulation of B cells in HFD males explains their inability to resist developing a prediabetic state, unlike HFD females.

Conversely to IELs, the LPLs are comprised of both innate and adaptive immune cells (234). Innate immune cells such as macrophages, dendritic cells, eosinophils, mast cells as well as adaptive T cells and B cells can be found in the lamina propria (242). In the colon lamina propria layer of our study, both diet-derived differences and sex differences were detected. Similarly to the intraepithelial layer, B cells were upregulated in the HFD males compared to NCD males. Interestingly, they were also upregulated in the HFD females which could explain the trending increase in blood glucose and blood insulin in females at week 10. Additionally, the total leukocyte population within the lamina propria layer was significantly greater in HFD females compared to NCD females. This was represented by the significant increase in T cells and all subsets analysed (incl. cytotoxic T cells, T helper/regulatory cells, double negative T cells and TCRβ^+ T cells subsets). Moreover, when comparing sexes, HFD females had a significantly stronger immune response compared to HFD males. The total T cell population and double negative T cell and TCRβ^+ T cell population subsets were all increased in HFD females (compared to HFD males). To our knowledge, this study is one of the first to highlight the sex differences in intestinal immunity in response to HFD. In human studies investigating the role of the intestinal immune system using biopsies of the small intestine, immune cell activation was higher in both healthy and obese women compared to men (60, 243). Additionally, Peyer's patches and spleens from healthy female mice contain a higher proportion of T cells than in males (65). Our findings of a greater colonic immune response in females corroborates and contributes to previous research. Additionally, past studies demonstrate that the higher immune activation in the female intestines is multifactorial accounted to the influence of estrogen and the gut microbiota.

In addition to the effect that estrogen has on both metabolism and the gut microbiota, unsurprisingly, it also acts on the immune system, which could partially explain the sex

differences observed in colonic immune cell populations in response to HFD (244). In terms of intestinal inflammatory disorders, such as Crohn's disease and irritable bowel syndrome, the prevalence of these disorders is indeed higher in females compared to males, in part due to the role of estrogen on the intestinal tract (245). Typically, estrogen produces immunoenhancing effects via its actions on estrogen receptors (mainly estrogen receptor-α), which are located on most immune cells (246). For instance, in a previous study involving a mouse model of colitis, the deletion of estrogen receptor-α expression on T cells decreased inflammation within the colon and lowered the risk of pathogenic comorbidities in intestinal autoimmune disorders (247). Despite the commonly reported enhancing effect of estrogen on the immune system, it can also act in an opposing manner based on the concentration (245). For example, during pregnancy when circulating estradiol levels are high, estrogen acts in an anti-inflammatory approach by suppressing the production of proinflammatory cytokines (TNF-α and IL-1β) and bolstering anti-inflammatory immune cells such as Th2 and T_{reg} cells in the peripheral blood (248). However, in lower circulating estradiol conditions, the opposite effects are observed (increased TNF-α, IL-1β, NK cells and B cells) (248). Therefore, it could be worthwhile for our study, to determine any associations between increased immune cell activation and plasma estradiol levels. Estrogen is a well-established mediator of the immune system and provides explanation for the sex differences observed in our study however, another crucial influential factor is the gut microbiota.

4.5 The association between intestinal immunity and gut microbiota

Due to the close proximity of the gut microbiota to the intestines, intestinal microorganisms are widely acknowledged in shaping the development and tolerance of the intestinal immune system (249). GF mice, lacking gut microbiota, highlight this relationship, demonstrating poorly developed lymphatic tissue and immune cell populations (250). Interestingly, the sex

differences in intestinal immunity (or autoimmune diseases) are abolished in GF mice, suggesting that the sexual bias in immunity is driven by the microbiome rather than sex hormones (149, 153). As previously mentioned, studies demonstrate a stronger intestinal immune response in females compared to males however, the difference in the gut microbiota between the sexes is often overlooked (244). Therefore, from our study we infer that the strongly separated gut communities and in particular, the Firmicutes: Bacteroidetes phyla and taxa abundance differences likely drive the discrepancy of the intestinal immune system seen in male and female mice.

Previous studies that have investigated the relationship between the gut microbiota and the intestinal immune system, have stated that by identifying microbial species within the gut and their associated function can begin to explain how they influence intestinal immunity (251). For example, molecular metabolites derived from the metabolism of dietary components, such as SCFAs, produced by certain bacterial species can influence both arms of the immune system and generate both proinflammatory and anti-inflammatory responses (251). Interestingly, bacteria from the Firmicutes phylum are the predominant producers of butyrate, and those from the Bacteroidetes phylum produce most of the acetate and propionate present in the gut (252). Butyrate in particular, is a known anti-inflammatory metabolite involved in downregulating inflammatory pathways and promoting T_{reg} cell production in the colon (253). Pertinent to our data, HFD males showed increased abundances of Firmicutes throughout the diet regimen, compared to HFD females that retained their Firmicutes: Bacteroidetes ratio. Therefore, the increased abundance of Firmicutes in male mice, and the resultant elevated concentration of butyrate, could be responsible for downregulating the intestinal immune system in response to HFD seen at week 10. Moreover, males possessed a greater abundance of specific taxa (compared to females) that are known for their anti-inflammatory functions and thus, their

presence in the male microbiota could explain the weaker intestinal immune response. *Eubacterium* and *Lachnospiraceae* in particular, were present in greater abundances throughout the diet regimen for HFD males and have known anti-inflammatory effects. For example, members of the *Eubacterium* genus promote the expression of anti-inflammatory IL-10 cytokine *in vitro* (254). Moreover, in IL-10-deficient mice, supplementation with prebiotics reduces colonic inflammation and substantially increases *Lachnospiraceae* (255).

In addition to the involvement of molecular metabolites derived from dietary components and the presence of anti-inflammatory bacteria, the increased presence of bacterial-derived structural components such as LPS also influences intestinal immunity (256). LPS is a major outer membrane structural component of gram-negative bacteria and increases in LPS is strongly linked with elevated intestinal inflammation (199). Interestingly, bacteria in the Bacteroidetes phylum, including the *Bacteroides* genus and *S24-7* family, as well as the *Asticcacaulis* genus, *Caulobacteraceae* family and Alphaproteobacteria class (all in greater abundance for HFD females at week 8) are gram-negative bacteria. Thus, increased abundance of these taxa (and subsequent greater concentration of LPS) correlates with the stronger intestinal immunity observed in HFD females at week 10. Despite HFD female mice in this study having a stronger intestinal immune response, we speculate that this response acted in a beneficial way. For example, females are known to be superior in eliminating pathogenic and opportunistic bacteria present in the gut, therefore, by eliciting a robust immune response they were able to prevent harmful bacteria, and possibly obesity-related bacteria, from colonising the gut and thus protecting them from developing obesity (245). In the opposing manner, HFD males did not produce the same immune response allowing the manifestation of deleterious microorganisms and thus, permitting the disease development of obesity.

4.6 Future Directions

Unfortunately, in my study, I did not look at changes to adiposity in response to HFD-induced weight gain. However, it is important to note that in men, increased weight gain, as seen in our male mice, is generally associated with increased accumulation of visceral adipose tissue. In women, weight gain increases adiposity more predominantly in subcutaneous depots (257). This has also been demonstrated in animal studies, whereby 8 weeks of HFD-feeding promotes adipogenesis of visceral adipose fat in males, but accumulation of adipose tissue distributed over both visceral and subcutaneous depots in females. Visceral adipose is known to be more detrimental than subcutaneous adipose, and thus obese (or HFD-fed) females have a decreased threat to their health, compared to males (258). Moreover, other studies highlight the metabolic influence that estrogen has on females, demonstrating that in ovariectomised mice and post-menopause studies, when this hormone decreases, there is regressed protection (1). Estrogen can regulate the distribution of adipose tissue, resulting in visceral and subcutaneous fat differences in males and females and therefore, differences in the susceptibilities of disease (30). Due to the clinical relevance of abdominal visceral adipose accumulation, it is worthwhile to measure both subcutaneous and visceral adipose tissue accumulation in future studies. Additionally, measuring estrogen and testosterone levels would allow conclusions to be drawn in regard to the influence of sex hormones over metabolic processes. Both estrogen and visceral adipose tissue have positive correlations with hypertension, coronary atherosclerosis and importantly for our findings, they have strong associations with hyperglycaemia and insulin resistance (259).

Throughout this discussion (and based on previous studies) we have assumed that it is an abundance of estrogen rather than reduced testosterone protecting the female HFD mice from adverse effects on their gut microbiota and metabolic status. Others have highlighted the

metabolic influence estrogen has on females, demonstrating that in ovariectomised mice and post-menopause studies, when this hormone decreases, there is regressed protection and increased metabolic disturbances (32). However measuring estrogen and testosterone levels throughout this study would allow conclusions to be drawn in regard to the influence of sex hormones over metabolic processes. Future studies could also manipulate the effect of estrogen and testosterone with the use of ovariectomy or hormone replacement therapies. For example, if we were to ovariectomise mice prior to the commencement of HFD and the same protection was observed, we would be able to confirm that it is not simply a consequence of estrogen. Alternatively, administering estrogen to males throughout the diet regimen would also allow conclusions to be drawn surrounding the protection observed in females. Moreover, administering testosterone in females would also provide insight into the potentially detrimental effects of this male hormone on the gut microbiota and colonic inflammation in obesity.

In addition to manipulating the action of estrogen, future studies could incorporate the use of antibiotics or faecal microbial transplants to determine whether modifications in the gut microbiota alters physiological responses to HFD. For example, one could test whether or not the administration of antibiotics to HFD females, which would remove the influence of changes to the microbial communities, would result in regressed or altered protection against obesity. This would confirm the influence of the gut microbiota in obesity. Furthermore, in this current study, although no differences were observed in the beta diversity at baseline, males possessed greater abundances of specific taxa compared to females, possibly predisposing them to obesity development. As discussed, bacteria from the *Ruminococcaceae* and *Lachnospiraceae* families were present in significantly greater abundance in males at baseline. Given that both of these families have been linked with metabolic disorders, eliminating these prior to HFD

commencement with antibiotics or with the use of GF mice would allow for more comparable gut microbial communities between sexes from the beginning. This again could alter the effects of HFD on the gut microbiota and inflammation.

4.7 Conclusion

To summarise, my study demonstrates that both the gut microbiota and intestinal immunity play a vital role in the development of sexual dimorphisms in obesity. While it is already well-established that the gut microbiota is highly sensitive to dietary factors, my work reveals that in the gut of HFD male and HFD female mice, microorganisms are harboured differently. This leads to differences in the ratio of Firmicutes to Bacteroidetes as well as significantly different abundances of specific taxa throughout the diet regimen. This sex-specific gut microbiota composition likely contributes to differences in the protection or susceptibility of developing obesity and accompanying comorbidities. Additionally, a number of microorganisms were identified in this study that could be used as biomarkers for obesity development (such as those exhibited in male mice). This study also established the importance of maintaining a balanced intestinal immune system. For example, HFD male mice exhibited increased B cell populations in both layers of the colon, which possibly contributed to hyperglycaemia and hyperinsulinemia. Conversely, the strong immune response elicited in the colon lamina propria layer in HFD females is likely beneficial and aids in clearing pathogenic and opportunistic bacteria whilst preventing the development of obesity (Figure 4.1). The protection in females could also be a result of estrogen, in metabolic, microbial and immunological capacities. Overall, the present study improves our understanding of sex differences in the gut microbiota and intestinal immunity, whilst identifying possible biomarkers that indicate the increased likelihood of developing obesity.

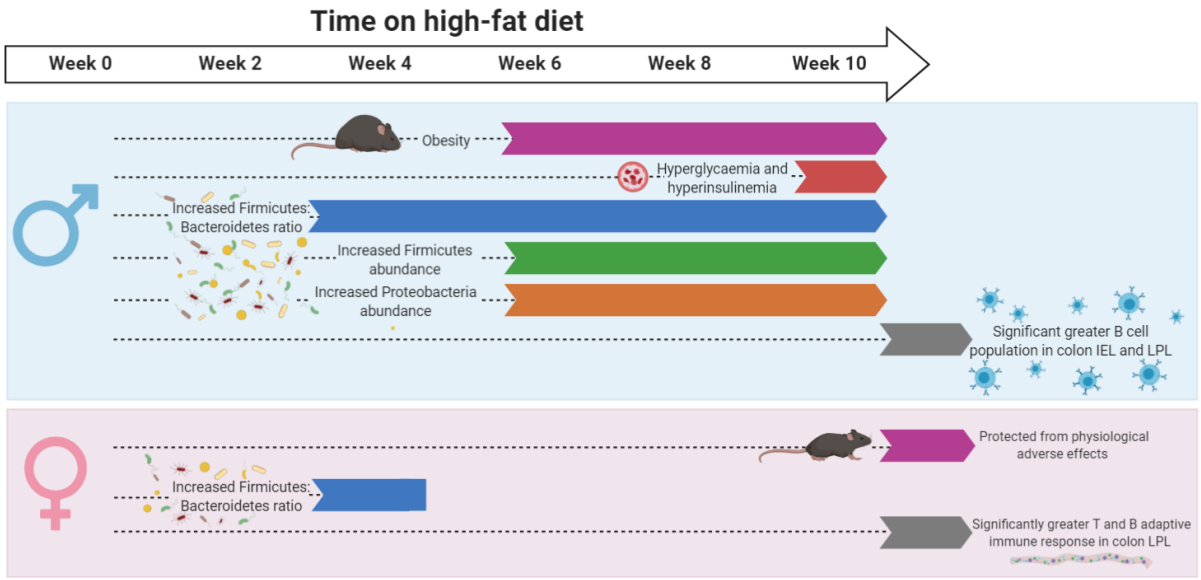

Figure 4.1. Summary schema of 10 week high-fat diet regimen on the gut microbiota and intestinal immunity in male and female mice. Male mice developed obesity from week 6, and hyperglycaemia and hyperinsulinemia at week 10 whereas, female mice are protected throughout the diet regimen. Male mice also exhibited higher abundances of microbial populations known for their involvement in obesity (i.e. Firmicutes and Proteobacteria phyla). Female mice displayed their resilience in maintaining a favourable gut microbiota as demonstrated by the conservation of their Firmicutes: Bacteroidetes ratio. Moreover, males demonstrated dysregulation of their B cell populations in the colon associated with a prediabetic state whereas, the stronger immune response elicited in the female mice appeared to be beneficial in preventing the development of obesity and accompanying comorbidities.

Chapter 5: References

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