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Coffee intake and the effects on intestinal inflammation, metabolic homeostasis and intestinal barrier function in mice

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ABSTRACT

Epidemiological studies have associated coffee consumption with beneficial effects including reduced risk of metabolic diseases such as type 2 diabetes. Conclusive results from experimental studies remain yet to be defined, suggesting a need for further effort. The work conducted in this thesis was part of a project which aimed to investigate the effects of coffee on intestinal inflammation, metabolic homeostasis and intestinal barrier function in mice. The project included a dietary intervention in mice fed a high-fat diet supplemented with coffee in three different doses equivalent to 1, 5 and 10 cups per day, with non-supplemented high-fat and low-fat diets as controls.

We aimed to examine the possible effects of coffee to prevent high-fat-induced weight gain, as increased weight is a well-known risk factor for metabolic diseases. We also wanted to investigate glucose homeostasis *in vivo* to evaluate whether coffee could have a positive influence on the development of insulin resistance. Moreover, reduced intestinal barrier function is thought to occur in situations of subclinical inflammation, as with type 2 diabetes. We investigated whether coffee could have an impact on intestinal barrier function by conducting an intestinal permeability assay. The intestinal inflammatory tone is regulated by several mechanisms and functions. Thus, we wanted to examine the effects of coffee on intestinal inflammatory regulation by analyzing gene expression of cytokines in the bowel and evaluate regulatory T cell populations in the small intestinal lamina propria.

The results showed that coffee in higher doses might have a beneficial effect on weight control, but coffee did not display a significant impact on glucose tolerance. However, the results demonstrated a tendency to poorer glucose tolerance with coffee consumption. Although not statistically significant, findings revealed a tendency for increased permeability in the animals fed coffee. The assessment of intestinal inflammatory regulation showed no significant differences in gene expression, but coffee in the highest dosage had fewer regulatory T cells than the high-fat controls, although not statistically significant.

This study provided new insights to the effects of coffee on intestinal inflammatory tone, barrier function and metabolic homeostasis. The findings in this thesis should provide a basis to further explore the mechanisms behind the observed effects.

SAMMENDRAG

Flere epidemiologiske studier har funnet assosiasjoner mellom kaffe og dens fordelaktige effekter på metabolske sykdommer, deriblant diabetes type 2. Eksperimentelle studier har enda til gode å komme med entydige resultater, noe som tilsier at videre forskning er nødvendig. Arbeidet som ble gjort i denne oppgaven var en del av et større prosjekt som hadde som mål å undersøke effektene av kaffe på faktorer som intestinal inflammasjon, metabolsk regulering og tarmbarrierefunksjon. I prosjektet inngikk det en kostintervensjon på mus hvor de ble fôret en høyfettkost tilsatt kaffe i doser tilsvarende 1, 5 og 10 kopper per dag. En gruppe med kun høyfettkost og en gruppe med lavfettkost ble inkludert som kontrollgrupper.

Vi hadde som mål å undersøke om kaffe kunne forhindre vektoppgang fremmet av høyfettkost, siden økt vekt er en velkjent risikofaktor for utvikling av metabolsk sykdom. Vi ønsket også å undersøke regulering av glukose *in vivo*, for å kunne evaluere om kaffe kan virke positivt på utvikling av insulinresistens. Redusert tarmbarrierefunksjon er ofte funnet å opptre sammen med inflammasjonstilstander av subklinisk karakter, slik som ved diabetes type 2. Vi undersøkte om kaffe kunne påvirke tarmbarrierefunksjonen ved å utføre en intestinal permeabilitets test. Videre, inflammatorisk status i tarm er regulert av flere mekanismer og funksjoner. Derfor ønsket vi å studere effektene av kaffe på inflammatorisk regulering i tarm ved å analysere genekspresjon av utvalgte cytokiner i tarmen, og evaluere andelen T-regulatoriske celler i tynntarmens lamina propria.

Resultatene viste at kaffe i høyere doser kan ha en gunstig effekt på vektkontroll, men kaffe viste ingen statistisk signifikant påvirkning på toleranse av glukose. Likevel, resultatene viste en tendens til dårligere glukose toleranse ved inntak av kaffe. Resultatene fra studien viste også at musene som ble gitt kaffe hadde en tendens til høyere permeabilitet i tarmen. Vurderingen av inflammatorisk regulering i tarm viste ingen signifikante resultater fra genekspresjonsanalysen. Likevel viste det seg at kaffe gitt i den høyeste dosen førte til en markant, dog ikke signifikant, reduksjon i andelen T regulatoriske celler sammenliknet med høyfettkontrollene.

Denne studien har gitt nye empiri omkring effektene av kaffe på inflammatorisk status i tarm, barrierefunksjon og metabolsk regulering. Funnene fra denne oppgaven bør brukes som et utgangspunkt for videre forskning på mekanismene bak de observerte effektene.

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LIST OF ABBREVIATIONS

AUC	Area under the curve
BA	Bile acid
BW	Body weight
CGA	Chlorogenic acid
DC	Dendritic cell
DIO	Diet-induced obesity
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FD4	FITC-dextran 4 kDa
FITC-dextran	Fluorescein isothiocyanate-dextran
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide 1
HFD	High-fat diet
IAP	Intestinal alkaline phosphatase
IDF	International Diabetes Federation
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IL-1β/6/10/17/22	Interleukin-1β/6/10/17/22
ILC	Innate lymphoid cell
IpITT	Intraperitoneal insulin tolerance test
LF	Low-fat
LP	Lamina propria
LPS	Lipopolysaccharide
MetS	Metabolic syndrome
mRNA	Messenger RNA
NF-κB	Nuclear factor kappa B
OGTT	Oral glucose tolerance test
PBA	Primary bile acid
PBS	Phosphate-buffered saline
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RIN	RNA integrity number
RT	Reverse transcriptase
SBA	Secondary bile acid
SFA	Saturated fatty acid
sIgA	Secretory immunoglobulin A
T2D	Type 2 diabetes
TGF-β	Transforming growth factor β
Th17	T helper 17 cell
TJ	Tight junction
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
Treg	Regulatory T-cell
WHO	World Health Organization
ZO-1	Zonula occludens-1

1.0 INTRODUCTION

Epidemiological findings links coffee consumption to potential health benefits including prevention of chronic diseases such as type 2 diabetes. Evidence suggests that long-term coffee intake improves weight control, glucose tolerance, insulin sensitivity and subclinical inflammation. Coffee is a rich source of bioactive components, which substantiates these possible properties. However, experimental studies show inconclusive results regarding coffee and its beneficial effects, thus creating the need for further exploration.

1.1 OBESITY, TYPE 2 DIABETES AND THE GUT

Obesity and associated metabolic disorders are becoming progressively prevalent and constitutes a significant health problem worldwide. The World Health Organization (WHO) estimates that more than half a billion adults worldwide are classified as obese (Mendis 2015), and the global prevalence of the metabolic syndrome (MetS) ranges from <10% to as much as 84% between populations (Desroches & Lamarche 2007; Kolovou et al. 2007). In general, the International Diabetes Federation (IDF) estimates that 25% of the world's adult population are affected by MetS (Zimmet 2010). The metabolic syndrome is a cluster condition and the diagnostic criteria for MetS vary slightly, but the main characteristics include an increase in abdominal fat and impaired insulin action (Alberti et al. 2006). MetS represent a major risk factor for type 2 diabetes (T2D) characterized by high blood glucose levels, which is a direct result of reduced systemic sensitivity to insulin (Alberti & Zimmet 1998).

It is widely accepted that chronic low-grade inflammation resulting from obesity is an important factor in the etiology of obesity-related diseases, including insulin resistance and T2D. Obesity-induced systemic inflammation is thought to originate predominantly in adipose tissue, however, emerging evidence suggests that the intestine also contributes to the development of metabolic disease (De Wit et al. 2008; Ding et al. 2010). The key events in the development of a chronic inflammation and insulin resistance are characterized by abnormal infiltration of macrophages into peripheral tissue with the activation of other immune cells and subsequent increased production of inflammatory mediators (Guilherme et al. 2008; Hotamisligil 2010). The prominent types of such mediators are the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β (Lumeng et al. 2007; Weisberg et al. 2003). Insulin resistance is then promoted by interferences from these inflammatory mediators in the signaling pathways connected to the insulin receptor.

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The characterization of both insulin resistance and low-grade inflammation in obesity and diabetes indicates that immune responses and metabolic regulation are highly integrated. Consumption of diets high in saturated fat, or the typical *Western diet*, has repeatedly been associated with the development of low-grade chronic inflammation and related metabolic diseases. The Western diet is identified by high content of fats, particularly saturated fats, refined carbohydrates and low in dietary fiber.

Although the root cause of obesity is excess caloric intake compared with energy expenditure, the gut microbiome-host interactions are likely to be of significance. Several studies in experimental animals have demonstrated important roles of the intestinal microflora, also known as gut microbiota, for regulating energy homeostasis and inflammation. The first evidence for this notion stems from the observation that germ-free mice do not develop obesity or insulin resistance when put on a high-fat diet (HFD) (Bäckhed et al. 2004; Bäckhed et al. 2007). Another pioneer study in mice demonstrated that obesity can be associated with an altered gut microbiota (Ley et al. 2005). Metagenomic studies in human and mice have shown that the two most abundant bacterial phyla in the gut are Bacteroidetes and Firmicutes (Gill et al. 2006). Lean individuals maintain a relative balance between these two phyla, and many studies have shown that obese mice have an increased ratio of Firmicutes to Bacteroidetes (Bäckhed et al. 2004; Ley et al. 2005; Murphy et al. 2010; Turnbaugh et al. 2006; Turnbaugh et al. 2009). However, Carvalho et al. (2012) and Schwiertz et al. (2010) found that the ratio changed in the favor of the Bacteroidetes in rodent and human models, respectively. Moreover, Duncan et al. (2008) found no changes in the ratio in obese humans, suggesting that this issue is not fully resolved. Turnbaugh et al. (2006) also showed that microbiota in obese mice produced additional enzymes that degrade nutrients, proposing that microbiota of obese individuals extract energy more efficiently from the diet than lean individuals.

In addition to its effect on microbiota, a diet high in saturated fat may also have adverse effects on the integrity of intestinal epithelium. Several studies have demonstrated that high fat diet-induced obese (DIO) mice have elevated levels of endotoxins and other bacterial products in plasma (Cani et al. 2007; Cani et al. 2008; Carvalho et al. 2012; Everard et al. 2012; Kim et al. 2012), an indication of increased permeability of the gut epithelium. Endotoxins, such as lipopolysaccharide (LPS) from Gram negative bacteria are potent stimulators of inflammatory reactions by activating inflammatory pathways in immune cells through the binding of Toll-like receptors (TLRs). It has therefore been proposed that low but chronic elevated levels of bacterial

endotoxins in plasma can lead to both promotion and maintenance of systemic low-grade inflammation and development of obesity related metabolic disorders such as T2D (Cani et al. 2007; Cani et al. 2008). Furthermore, it has been shown in several studies that a high fat diet can alter the distribution and activity of immune cells in the intestinal tissue in a manner that leads to changes in inflammatory status (Garidou et al. 2015; Luck et al. 2015; Ma et al. 2008). High fat diet therefore appears to impact the intestine in multiple manners, that potentially could lead to adverse systemic effects.

Research suggests that dietary components prevent or attenuate the adverse effects seen with high-fat diets. This applies for instance to groups of secondary metabolites from plants such as polyphenols, which were shown to alter both bacterial composition in the gut and strengthen the intestinal barrier in two recent studies (Anhê et al. 2015; Heyman-Lindén et al. 2016). Coffee is a rich source of related phenolic compounds. Further, coffee intake appears to reduce risk of diabetes type 2 development both in humans and in experimental animals (Andersen et al. 2006; Rosengren et al. 2004; Salazar-Martinez et al. 2004; Tuomilehto et al. 2004; Van Dam et al. 2004; Van Dam & Hu 2005). We therefore hypothesized that experimental mice fed coffee in doses relevant to human ingestion, would reduce some of the adverse effects seen with high fat diet on intestinal barrier function and inflammation in the intestinal tissue, and hence to provide mechanistic insight to the putative health promoting role of coffee.

1.2 INTESTINAL BARRIER FUNCTION

To understand the mechanisms by which DIO and other dietary factors may be associated with impairments of barrier function, it is important to identify the structural components and functional properties of the barrier. The intestinal barrier is a complex structure that separates the internal milieu from the luminal environment. It is comprised of the epithelium, immune cells in lamina propria (LP) and Peyer's patches as cellular components, and a mucus layer as an extracellular defense. The feature as a selective permeable barrier allows the absorption of nutrients, electrolytes and water, while maintaining an effective defense against antigens, invading microbes, and intraluminal toxins.

1.2.1 Extracellular components of the barrier

The viscous mucus layer that covers the mucosal surface function act as a barrier against luminal contents and prevents large molecules (e.g. bacteria) from coming into direct contact with

the epithelial layer. Its properties are assigned to the mucin glycoproteins secreted by specialized epithelial cells, such as goblet cells (Deplancke & Gaskins 2001). Mucus also contains secreted antimicrobial peptides that helps avoiding contact between bacteria and the epithelial layer. Commensal bacteria residing in the outermost layer cooperates with the mucus by limiting colonization of pathogenic bacteria by occupying space (Neish 2009). The mucus layer also functions as a residence for secretory immunoglobulin A (sIgA), which binds to antigenic substances.

1.2.2 Cellular components of the barrier

The epithelial cell lining plays a significant role in regulating traffic across the intestinal barrier. Both the transcellular and the paracellular pathway are possible entry routes for luminal molecules and inhabitants. Therefore, selective transporters control the transcellular permeability, whilst tight junctions (TJs) control the paracellular pathway by keeping the epithelial cells together. These junctional complexes consist of both transmembrane and intracellular proteins, such as occludin and zonula occludens-1 (ZO-1) (Anderson & Van Itallie 1995).

The intestinal epithelial cells (IECs) form a specialized biochemical and physical barrier that maintains separation between the luminal microbiota and the mucosal immune system. Goblet cells secrete mucus, and the Paneth cells secrete antimicrobial peptides to promote the rejection of bacteria from the epithelial surface. Both microfold cells (M cells) and goblet cells facilitate transport of luminal antigens and live bacteria across the epithelial barrier to dendritic cells (DCs) located in the lamina propria (LP) (McDole et al. 2012). The LP DCs monitors the gut lumen content and activates lymphocytes. Collectively, the diverse functions of IECs result in a dynamic barrier to the environment.

Interspersed among the IECs are the specialized intraepithelial lymphocytes (IELs). They are important as a first line of defense as well as in tissue maintenance, barrier integrity and by preventing bacterial translocation across the epithelium (Honda & Littman 2012; Li et al. 2011).

Other important innate immune cells in the LP are the macrophages, eosinophils and innate lymphoid cells (ILCs). Gut macrophages regulate inflammatory responses to bacteria and antigens that breach the epithelium, protect the mucosa against harmful pathogens, and scavenge dead cells and foreign debris (Smith et al. 2011). Eosinophils have multiple purposes, yet their primary job is to aid the macrophages in protecting the body. These cells are thought to play a significant role in

gut immune homeostasis, including induction of plasma cell differentiation and maintenance (Chu et al. 2014), and preservation of tissue integrity (Lee et al. 2010). The ILCs can be divided into subgroups, with the ILC3s being the most important in this context. ILC3s are defined by their capacity to produce IL-17 and/or IL-22, and is because of this critically involved in maintaining the barrier function (Spits et al. 2013).

In the adaptive immune system, CD4+ T cells along with CD8+ T cells make up the majority of T-lymphocytes in the intestine. The CD4+ T cells in intestinal mucosa include significant numbers of IL-17 expressing cells (Th17 cells) and fork-head box P3 (Fox-P3)-expressing regulatory T cells (Tregs). Th17 cells play a vital role in the homeostasis of the epithelium and in the regulation of host defense against various extracellular pathogens (Korn et al. 2009). Tregs possess different immunosuppressive functions and play an important role in regulating the intestinal immune system. The gut LP is particular abundant in Tregs, in comparison to other organs and tissues (Hall et al. 2008). Both the initiation and maintenance of differentiation and function of Tregs are critically dependent on the transcription factor FoxP3 (Zheng & Rudensky 2007). Their regulatory effects take place through the production of immunosuppressive cytokines such as IL-10 and TGF-β (Sakaguchi et al. 2008), and through exerting inhibition in a cell contact-dependent matter (Maloy et al. 2003; Sakaguchi et al. 2008). Moreover, both number and function of mucosal Tregs are thought to be affected by the presence of intestinal bacteria (Arpaia et al. 2013; Lathrop et al. 2011; Round & Mazmanian 2010). Tregs are essential for intestinal tolerance toward dietary antigens and the microbiota, and exhibits an important role in modulating gut homeostasis and thus attending gut barrier integrity.

1.3 THE EFFECTS OF A HIGH-SATURATED-FAT DIET ON GUT BARRIER FUNCTION AND INFLAMMATION

The gastrointestinal tract is the first organ to be exposed to dietary components, such as saturated fat. Dietary fats are dissolved in micelles by bile salts in the upper parts of gastrointestinal tract and subsequently taken up by enterocytes. After absorption, the fatty acids are packed into chylomicrons and released into the lymph, before going into circulation for distribution to tissues.

High-saturated-fat diets consists primarily of triglycerides of palmitic and stearic acid. The saturated fatty acids (SFAs) may influence gut microbiota composition, which then modulate intestinal permeability and adipose inflammation through TLR interactions (Bleau et al. 2014; Murphy et al. 2010; Schwiertz et al. 2010; Winer et al. 2016).

1.3.1 Gut barrier impairment

Several mechanisms have been proposed as to how saturated fat can impair the intestinal barrier. Diets rich in fat are known to promote an increase in the secretion of bile salts into the intestine because the absorption of fat requires an increase in bile flow. Increasing concentrations of bile salts have been shown to cause loss of epithelial barrier function with a consequent increase in mucosal permeability (Freel et al. 1983; Henrikson et al. 1989; Keating & Keely 2009). Primary bile acids (PBAs) are synthesized from cholesterol and secreted into the duodenum, before they are absorbed in the distal small intestine and transported to the liver. Some PBAs escape absorption and are then converted to secondary bile acids (SBAs) in the distal small intestine and colon. These BAs are able to interact with intestinal epithelial cells and influence functions including tight junction barrier functions (Raimondi et al. 2008). The amount of SBAs in the intestinal lumen are increased with high-fat feeding, suggesting an association with metabolic dysfunctions. The connection between excess intake of dietary fats (saturated fatty acids), increased bile production and increased intestinal permeability have also been demonstrated in newer studies. Suzuki and Hara (2010) and Murakami et al. (2016) used oral administration of probes (phenolsulfonphthalein and FITC-dextran, respectively) to rodents for the assessment of intestinal permeability. Both groups showed that HF feeding increased the permeability of the probes together with an increase in BAs. Stenman et al. (2012) reported similar results for jejunum and colon in mice with the use of a Ussing chamber system.

Feeding studies in mice/rats show that certain tight junction proteins are down-regulated by high fat diet, which substantiates the link between excess fat intake and increased intestinal permeability (Brun et al. 2007; Cani et al. 2008; Cani et al. 2009; de La Serre et al. 2010; Kim et al. 2012; Lam et al. 2012). Down-regulation of TJ proteins has also been associated with an increase in LPS in circulation, suggesting increased paracellular transport with high fat intake (Cani et al. 2008; Cani et al. 2009; de La Serre et al. 2010; Kim et al. 2012). Other studies have also proposed that increased LPS translocation is not only dependent on paracellular transport. Goshal and colleagues (2009) showed that LPS could translocate by a transcellular manner with the help of chylomicrons. Another transcellular pathway for LPS translocation might be trough M-cells, due to the cells' preference to LPS-covered Gram-negative bacteria (Lapthorne et al. 2012). Dietary fat might thus promote LPS uptake from the intestine. However, it is also suggested that high-fat diets and/or obesity can cause metabolic alterations and inflammation independent of any changes in gut

barrier function. A recent study in mice found that high-fat feeding lead to metabolic impairments without apparent plasma LPS increase or gut barrier dysfunctions (Kless et al. 2015). They assessed the permeability of both the small and large intestine *ex vivo* with the help of a Ussing chamber system. The study concluded that the increased inflammatory activation of adipose tissue cannot be triggered by metabolic endotoxemia, thus challenging previous reports on HFD-induced impairment of gut barrier integrity. Additional explorations have proposed that barrier impairment in mice fed a HFD depend on housing conditions, i.e. the microbial status of an animal facility appeared to explain some of the results (Müller et al. 2016). The researchers suggest further that these findings may explain why different in outcomes of high-fat feeding are seen in different laboratories.

1.3.2 Intestinal inflammation in high-fat feeding

Low-grade inflammation in adipose tissue and other organs such as liver and muscle, have been extensively investigated in the last decades. However, only recently have inflammatory and immune cell changes in the small and large intestine been linked to obesity and insulin resistance. The status of the inflammatory tone in tissues is closely related to the expression of cytokines. Studies have reported both up-regulation and down-regulation of pro-inflammatory cytokines, whilst some have shown no changes in these cytokines with HF feeding (De Wit et al. 2008; Johnson et al. 2015).

Results from Ding and coworkers (2010) showed that a HFD increased TNF-α mRNA levels in the distal part of the small intestine (ileum) in mice within 2 to 6 weeks of HFD. These findings correlated with weight gain, adiposity, and plasma insulin and glucose levels, suggesting an increase in the inflammatory status. However, the researchers did not find detectable increases in plasma TNF-α or other cytokines. Evidence for increased ileal inflammation was also found by de La Serre and colleagues (2010) in a study done in obesity-prone rats. The results showed a decrease in intestinal alkaline phosphatase (IAP) activity and an increase in TLR4 activation in the ileal gut wall. IAP has many important functions in the gut such as detoxifying LPS and prevent bacterial invasion across the gut mucosal barrier (Goldberg et al. 2008). Both Hamilton et al. (2015) and Garidou et al. (2015) found that HFD feeding reduced IL-10 mRNA levels in ileum after 1 week and 30 days, respectively. In contrast to previous reports (Bleau et al. 2014; Lam et al. 2012), Garidou and colleagues (2015) found that HFD for 10- and 30 days did not increase levels of TNF-

α and IL-6 in adipose tissue. A new study in obese humans showed increased levels of the pro-inflammatory cytokine IL-1β in duodenum of insulin-resistant subjects (Veilleux et al. 2015). High levels of IL-1β was also found in the proximal colon of mice fed a café-inspired diet consisting of high-fat foods compared to lean mice fed regular chow (Li et al. 2008). Overall, even though a substantial amount of data indicate that high-fat feeding alters general cytokine expression in the small intestine and colon, this hypothesis is not yet definite.

Intestinal inflammation has also been linked to changes in intestinal immune cell populations, including cells of both the innate and the adaptive immune systems. (Luck et al. 2015) showed a reduction in ILC3s in lamina propria of HFD-fed mice compared to lean mice, which correlated with increased serum LPS. Changes in the proportions and functions of distinct subsets of macrophages and DCs present in the gut related to intestinal inflammatory state have been examined (Ding et al. 2010; Garidou et al. 2015; Johnson et al. 2015), but the significance is not yet fully understood. High-fat feeding has also been shown to alter the composition of adaptive immune cells in the LP of the colon and distal part of the small intestine. One cell type of special interest is the immunosuppressive Tregs. Ma and coworkers (2008) showed in a study in HF-fed mice, decreased levels if colonic Tregs. These findings were later supported by Luck et al. (2015), which after three weeks of HF feeding in mice found a reduction of the percentage of Tregs in the colon. The same study also showed a corresponding decrease of Tregs in both small intestine and colon after 12 weeks on a high-fat diet. Similarly, Garidou et al. (2015) showed that Tregs decreased in both proportion and number in the small intestine after 30 days of HF feeding in mice. Results from Everard and colleagues (2014) also supported findings that HF feeding alters Tregs in the small intestine. They found decreased *FoxP3* mRNA expression in the jejunum of mice fed a high-fat diet for 8 weeks. However, they did not find any changes in colon.

1.4 COFFEE AND EFFECTS ON T2D AND THE INTESTINE

1.4.1 What is coffee?

Coffee is among the most widely consumed beverage worldwide. Due to the broad consumption of coffee, several studies have examined the possibility of an association between coffee intake and health. Among these associations are the antioxidant effect and the inverse relationship of coffee consumption and type 2 diabetes.

INTRODUCTION

Coffee is a complex mixture of thousands of compounds, with the major bioactive compounds being caffeine, the diterpenes cafestol and kahweol, chlorogenic acids, soluble dietary fiber and micronutrients such as magnesium and potassium.

Coffee is a rich source of dietary phenolic phytochemicals, with the total content ranging from 200 mg to 550 mg per cup (Bravo 1998). Phenolic phytochemicals are the largest category of phytochemicals and the most widely distributed in the plant kingdom and they have received much interest due their putative health promoting properties. Coffee is particularly rich in phenolic acids, with chlorogenic acids (CGA) being the most abundant member. CGAs are quinic esters of caffeic acid belonging to the hydroxycinnamic acid group (Crozier et al. 2011). During roasting of coffee CGAs are progressively degraded, but despite this the daily intake of CGAs in coffee drinkers is still substantial, ranging from 0.5 to 1.0 g per day. Dietary phenols are substrates for several enzymes located in the small intestine, colon and in the liver (Yeh & Yen 2006; Zhao et al. 2004). As only 5-10% of the total polyphenol intake are absorbed in the small intestine, the colon might be considered as an active site for metabolism of these phenolic compounds (Cardona et al. 2013; Manach et al. 2005). Furthermore, polyphenolic compounds and metabolites do not necessarily need to be absorbed to mediate effects, for example on the gut microbiota composition. Melanoidins is another compound that could alter gut microbiota. Melanoidins are complex polymers formed during the roasting process of coffee beans via the so-called Maillard reaction. Melanoidins are not by definition dietary fibers, but there is evidence that they behave as dietary fiber in vivo (Ludwig et al. 2014). since they are fermented by the gut microbiota, leading to production of large amounts of acetate and propionate, typical of dietary fibers. As coffee brew is one of the main sources of melanoidins in the human diet, biological activities of coffee melanoidins and their health implications are of great interest. Coffee is also a major dietary source of the natural alkaloid caffeine (Barone & Roberts 1996), with each cup containing 50-300 mg (Crozier et al. 2012). Caffeine is rapidly and completely absorbed in the upper part of the intestines before being metabolized in the liver (Blanchard & Sawers 1983; Nawrot et al. 2003). Systemic effects of caffeine include an acute increase in blood pressure and stimulation of the release of substances such as epinephrine (Keijzers et al. 2002).

1.4.2 Effects of coffee on consumption on T2D and associated risk factors

Convincing epidemiological data and meta-analyses shows an inverse relationship between long-term intake of coffee and the development of T2D and associated risk factors (Andersen et al. 2006; Huxley et al. 2009; Rosengren et al. 2004; Salazar-Martinez et al. 2004; Tuomilehto et al. 2004; Van Dam et al. 2004; Van Dam & Feskens 2002; Van Dam & Hu 2005). Many of the compounds in coffee have biologically active effects that may play a role in glucose metabolism and inflammation with the potential to affect the development of diabetes.

The coffee compounds caffeine (Greer et al. 2001; Keijzers et al. 2002), CGA (de Sotillo & Hadley 2002) and magnesium (de Valk 1999; Rodríguez-Morán & Guerrero-Romero 2003) have been shown to affect glucose metabolism and insulin sensitivity in both animal and metabolic studies. The effects of coffee on glucose homeostasis are somewhat complex. The acute short-term effects on glucose homeostasis differs from the long-term effects seen with coffee intake. These differences are likely to be assigned to caffeine.

Several studies have investigated the short- and long-term administration of caffeine on glucose tolerance. Results have shown that caffeine acutely decreases sensitivity to insulin (Johnston et al. 2003; Könner & Brüning 2011), but it has been hypothesized that a physiological tolerance to caffeine can develop as a result of habitual consumption (Keijzers et al. 2002). This hypothesis is consistent with the suggestion that caffeine acutely inhibits insulin sensitivity and glucose tolerance primarily by increasing epinephrine in humans (Keijzers et al. 2002), but that the effects of coffee on circulating levels of epinephrine disappear within a few days of caffeine consumption (Robertson et al. 1981). In contrast to acute administration of caffeine, long-term exposure seems to increase the insulin sensitivity (Greer et al. 2001; Heckman et al. 2010; Salazar-Martinez et al. 2004). The negative effect of caffeine on insulin sensitivity and glucose tolerance seen in the short term studies may also be modified when caffeine is consumed within a complex mixture such as coffee. However, since decaffeinated coffee is reported to have many of the similar effects on development of T2D, it is unlikely that caffeine plays a role in the negative association for development of T2D.

CGA has for some time been known to be a potent inhibitor of glucose-6-phosphate (G-6-P), which exerts effects in reducing levels of plasma glucose levels and hepatic glucose production (Arion et al. 1997; de Sotillo & Hadley 2002). CGA has also been shown to increase peripheral uptake of glucose (Van Dam et al. 2004), in addition to inhibit sodium-dependent glucose

transporters at the intestinal stage (Natella & Scaccini 2012; Tuomilehto et al. 2004). A forth mechanism is also proposed to CGA; as modulator of gastrointestinal peptides. CGA may influence the secretion of glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP), which both are known for their glucose lowering effects (Johnston et al. 2003; Tuomilehto et al. 2004).

Magnesium is an essential cofactor for multiple enzymes that are involved in the metabolism of glucose (Belin & He 2007). This suggests that magnesium may play an important role in regulation of glucose homeostasis and insulin sensitivity. The fact that magnesium is an important micronutrient of coffee with each cup containing about 7 mg shows that coffee can contribute greatly to daily magnesium intake (Paolisso et al. 1990). Studies in long-term consumption of coffee supports the important role of magnesium by showing that habitual coffee intake increases the serum magnesium levels with reduced risk for T2D (Lopez-Ridaura et al. 2004; Salazar-Martinez et al. 2004; Salmeron et al. 1997). An intervention trial by Kempf et al (2010) tested whether coffee ingestion after 4 weeks of abstinence could affect glucose metabolism. They observed no effects of coffee on fasting and oral glucose tolerance test-derived markers of glucose metabolism.

Coffee contains multiple substances that may impact inflammatory markers. The caffeine, CGA and trigonelline found in coffee are thought to have significant potential as antioxidants and free radical scavengers. A study in rats showed that regular intake of coffee significantly decreased the levels of pro-inflammatory TNF-α in both liver and serum, and increased hepatic levels of the anti-inflammatory cytokine IL-10 (Vitaglione et al. 2010). Studies in cell cultures have showed that CGA may prevent the activation of various pro-inflammatory biomarkers through inhibitory effects on NF-κB (Chu et al. 2011; Johnston et al. 2003). A cross-sectional survey found contradictory results, that regular coffee consumption was related to higher plasma concentrations of IL-6 and TNF-α (Zampelas et al. 2004). It should be noted that unfiltered coffee was included when coffee consumption was measured. A crossover study in healthy men investigated the acute effects of coffee and did not find significant change in IL-6 levels in serum (Gavrieli et al. 2011), this was also the case in a study in habitual coffee drinkers (Kempf et al. 2010).

1.4.3 Effects of coffee on gut microbiota

Beyond being metabolized by the gut microbiota, CGA and related coffee compounds also have the potential to modify the gut microbiome. Studies on the direct effects of coffee and on gut

microbiota modulation are scarce and consists of only smaller studies. Some of the few studies available shows that coffee melanoidins may promoted the selective increase of probiotic bacteria, behaving as soluble fiber (Gniechwitz et al. 2007; Reichardt et al. 2009). Other studies have also shown these prebiotic effects, but could not distinguish the potent components (Jaquet et al. 2009; Nakayama & Oishi 2013). A recent report on a metagenomics analysis presented that coffee consumption was associated with increased microbial diversity in the intestine (Zhernakova et al. 2016). Despite the few existing reports on the relationship between coffee and gut bacteria, this is thought to be an area of great interest for the future.

1.5 AIM OF THE STUDY

This study forms part of a larger project in the Animal Research group at NMBU, where the goal is to explore the effects and mechanisms of coffee on intestinal barrier function and the development of the metabolic syndrome and diabetes in mice.

The main objective of this study is to examine whether coffee consumption may counteract the adverse effects seen with a diet high on saturated fat on features of intestinal inflammation, metabolic homeostasis and intestinal barrier function. In order to reach these objectives, four groups of mice were fed a high-fat diet and one group was fed a low fat control diet and tested for the following 1) Insulin sensitivity by the oral glucose tolerance test and the intraperitoneal insulin tolerance test. 2) Intestinal permeability by oral delivery of FITC-dextran subsequently measured in plasma, 3) Analysis of gene expression of cytokines involved in intestinal inflammatory regulation and 4) Analysis of T-cells in the lamina propria of the small intestine by the use of flow cytometry.

The specific aims were:

I. To examine whether coffee consumption may prevent high-fat diet-induced weight gain, as this is a major risk factor for insulin resistance and development of metabolic diseases.
II. To investigate if coffee supplementation could improve glucose tolerance and insulin sensitivity when given a high-fat diet.
III. To study whether coffee have a beneficial impact on intestinal permeability, as impaired barrier function is thought to play an important part in the development of chronic low-grade inflammation.

IV. To examine the effects coffee consumption on markers of intestinal inflammation; expression of cytokines, and evaluation of T-cell populations

2.0 MATERIALS

2.1 SOLUTIONS FOR LAMINA PROPRIA LEUKOCYTE ISOLATION

cRPMI solution	1x RPMI-1640 w/25 mM HEPES (0.92mL/100mL)
	5 % FBS (0.46mL 100% stock solution/100mL)
	2 mM Ala-Gln (0.1mL 200mM stock solution/100mL)
	1 mM Na-Pyruvate (0.1mL 100mM stock solution/100mL)
	1x NEAA (0.1mL 100x stock solution/100mL)
	1x Pen/Strep (0.1mL 100x stock solution(100mL)
EDTA solution	1x HBSS w/o Ca^{+} and Mg^{+} (96mL/100mL)
	5% FBS (0.96mL 100% stock solution/100mL)
	10 mM HEPES pH 7.4 (0.96mL 1M stock solution/100mL)
	5 mM EDTA (0.96mL 0.5M stock solution/100mL)
	1x Pen/Strep (0.96mL 100x stock solution/100mL)
	1x Entrofloxacin (0.1mL 1000x stock solution/100mL)
	1x Polymyxin B (0.1mL 1000x stock solution/100mL)
DTT solution	1x EDTA solution
	5 mM DTT (500 µL 200x stock solution/100mL)
Pre-digestion solution	1x HBSS w/Ca^{+} and Mg^{+} (98mL/100mL)
	10 mM HEPES pH 7.4 (0.1mL 1M stock solution/100mL)
	1x Pen/Strep (0.1mL 100x stock solution/100mL)
	1x Entrofloxacin (0.1mL 1000x stock solution/100mL)
	1x Polymyxin B (0.1mL 1000x stock solution/100mL)
Digestion solution	1x Pre-digestion solution
	0.2 WüU/mL Liberase (1.4mL 13 WüU/mL stock solution/100mL)
	4000 KuU/mL DNase (1.82mL 10^4 KuU/mL stock solution/100mL)

2.2 GENERAL BUFFERS

PEB buffer	PBS pH 7.2 0.2 mM EDTA 0.5% Bovine calf serum
PE buffer	PBS pH 7.2 0.2 mM EDTA

2.3 ANESTHETICS

ZR cocktail	Zoletil Forte (7.5mL 250mg/mL stock solution/100mL) Rompun (2.26mL 20mg/mL stock solution/100mL) Sterile isotone NaCl (90.24 mL/100mL)

2.4 DIET COMPOSITIONS

Table 2.1. Diet compositions

	LF		HF		HFC1		HFC5		HFC10	
Product #	**D12450J**		**D12492**		**D15092201**		**D15092202**		**D15092203**	
	gram%	**kcal%**	**gram%**	**kcal%**	**gram%**	**kcal%**	**gram%**	**kcal%**	**gram%**	**kcal%**
Protein	19.2	20	26	20	26	20	26	20	25	20
Carbohydrate	67.3	70	26	20	26	20	26	20	25	20
Fat	4.3	10	35	60	35	60	34	60	33	60
Total		100		100		100		100		100
kcal/gram	3.85		5.2		5.2		5.1		5.0	
Ingredient	**gram**	**kcal**	**gram**	**kcal**	**gram**	**kcal**	**gram**	**kcal**	**gram**	**kcal**
Casein	200	800	200	800	200	800	200	800	200	800
L-Cysteine	3	12	3	12	3	12	3	12	3	12
Corn Starch	506.2	2024.8	0	0	0	0	0	0	0	0
Maltodextrin	125	500	125	500	125	500	125	500	125	500
Sucrose	68.8	275.2	68.8	275.2	68.8	275.2	68.8	275.2	68.8	275.2
Cellulose	50	0	50	0	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225	25	225	25	225
Lard	20	180	245	2205	245	2205	245	2205	245	2205
Mineral mix	10	0	10	0	10	0	10	0	10	0
Dicalcium Phosphate	13	0	13	0	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin mix	10	40	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0	2	0
Coffee, Nestle Gold	0	0	0	0	3.9	0	20	0	41	0
Yellow Dye	0.04	0	0	0	0.025	0	0.025	0	0	0
Red Dye	0	0	0	0	0.025	0	0	0	0.025	0
Blue Dye	0.01	0	0.05	0	0	0	0.025	0	0.025	0
Total	**1055.05**	**4057**	**773.85**	**4057**	**777.75**	**4057**	**793.85**	**4057**	**814.85**	**4057**

3.0 METHODS

3.1 EXPERIMENTAL DESIGN

3.1.1 Animals and housing conditions

The animals included in the experiments were 60 male C57BL/6J bought from Envigo, The Netherlands at the age of five weeks. After arrival at the facility, the animals were put on a low-fat diet free from phytochemicals (D12540J). The mice were acclimatized with their new diet for approximately four weeks before the start of the experiment. This allowed the animals to be free of any potential effect of phytochemicals from the previous diet.

The animals were housed in individually ventilated cages (IVC) (Innovive, San Diego, CA). Temperature was monitored daily and ranged from 23 to 25ºC. Relative humidity was kept at 45-55%, controlled by a local humidifier (Condair CP3mini, Qviller, Norway). Room light was following a 12h-light-dark cycle. Mice were given *ad libitum* access to food and water. Cage bedding was changed every second week. The cages were initially equipped with running wheels, but these were removed from all cages at the start of the experiment to exclude physical activity as a possible confounder.

The C57BL/6J mouse strain is lean and insulin sensitive when fed a standard diet. However, when fed a high-fat diet, C57BL/6J mice develop obesity and glucose intolerance, and this mouse model is used frequently in studies of obesity related disease including diabetes.

3.1.2 Ethical aspects

The Norwegian Animal Research Authority (Mattilsynet) approved the animal experiment and the following procedures were performed in compliance with the current guidelines for the care and use of laboratory animals set forth by European Commission guidelines.

3.1.3 Experimental setup

Sixty male C57BL/6J mice were divided into five different groups (n=12); low-fat diet (LF), high-fat diet (HF), high-fat diet supplemented with coffee equivalent to 1 cup/day (HFC1), 5 cups/day (HFC5) or 10 cups/day (HFC10). Four mice were housed per cage. The conversion of estimated cups/day into actual amount of coffee in feed was done by relating intake to body mass/body surface area (kg/m^2). The mice were grouped randomly and fed their respective diets

for approximately 18 weeks until day of termination. The termination of the animals was carried out over seven days with 5 to 10 animals per day.

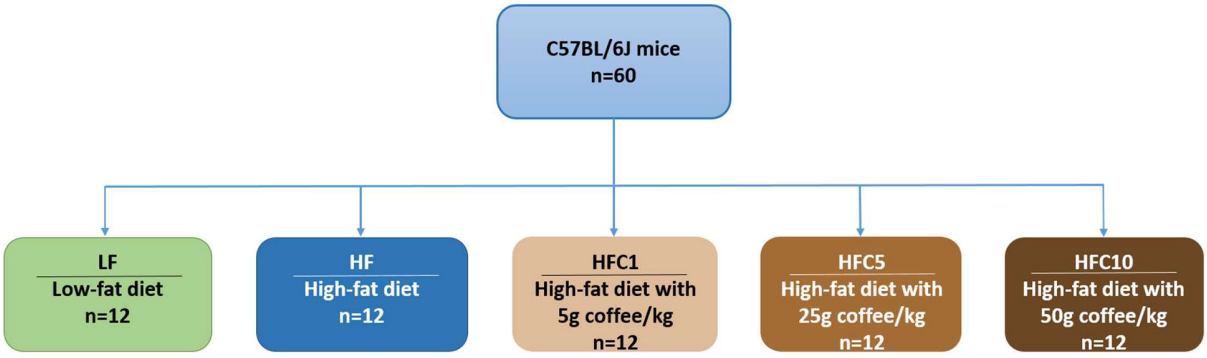


Figure 3.1. Division of the experimental groups

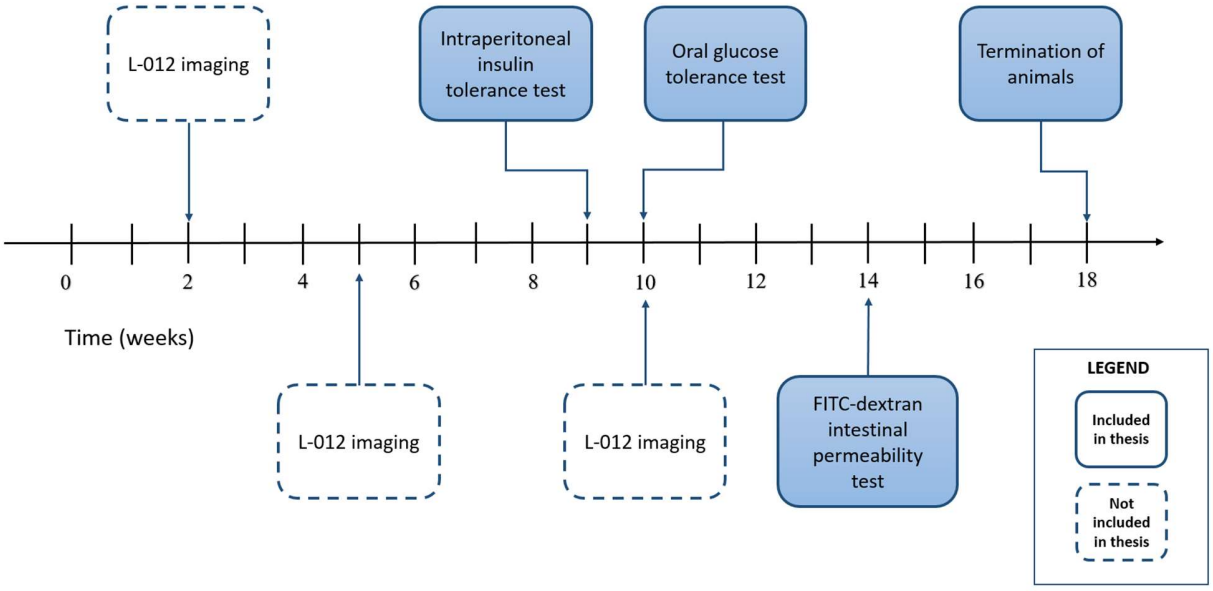


Figure 3.1. Experimental timeline. This study included the results from three *in vivo* experiments: glucose tolerance test, insulin tolerance test, and FITC-dextran intestinal permeability assay. The results from the *in vivo* imaging with L-012 was not included due to format limitations.

3.1.4 Preparation of diets

The diets were obtained from Research Diets (New Brunswick, NJ, USA) with D12492 as the basis for the high fat diets (control and coffee) (60 E% fat), and D12450J as the low-fat control diet (10 E% fat). Details regarding the composition of the diets are shown in Table 2.1. The freeze dried coffee was of the brand Nescafe Gold. The coffee was purchased from a local grocery store

on the same day and was of the same production batch. The coffee was shipped to Research Diets for mixing into the diets. The coffee was added to the D12492 diet in the concentrations of 5 g/kg (HFC1), 25 g/kg (HFC5) and 50 g/kg (HFC10). The high-fat diets were stored at -20ºC, and the LF diet at 4ºC to avoid rancidity.

3.1.5 Food intake and weight development

Mice and food were weighed once per week to assess weight development and to estimate the food intake during the feeding experiment. Food intake per mouse was estimated by dividing the amount of consumed food in the respective cage with the number of animals in the cage.

3.2 INTESTINAL PERMEABILITY ASSAY WITH FITC-DEXTRAN

To evaluate the effect of the different diets on intestinal permeability, fluorescein isothiocyanate (FITC) dextran was used. FITC dextran (FD4) was given orally, and its concentration measured in plasma. The protocol was adopted from Johnson et al. (2015), and performed 14 weeks after start of the diet intervention.

FD4 (Sigma-Aldrich) had an average molecular weight of 4 kDa, and was prepared by dissolving the powder in PBS to 100 mg/mL. Each mouse was orally gavaged with a fixed dose of 650 mg FD4, which was equivalent to 19.5 mg/mouse when related to average weight of 30 g/mouse. The initiation of the experiment started in the morning with 4 hours of fasting before gavaging each animal with FD4. Blood was collected twice from the saphenous vein 1.5 hours after FD4 administration into EDTA-coated tubes and put on ice and shielded from light. Blood was centrifuged at 10 000g for 6 min to prepare plasma, and stored at -20ºC in new tubes until the day of analysis.

Concentrations of FITC dextran in plasma were determined by fluorescence spectroscopy. The plasma samples were diluted at a ratio of 1:5 in PBS, and analysis of each sample was done in duplicates. Fluorescence was measured with excitation and emission wavelengths at 490 nm and 520 nm, respectively. Calculations of FD4 in blood (per mL) were done by relating fluorescence intensity in samples to a linear standard curve using five different concentrations of FD4 in plasma from untreated mice.

3.2.1 Optimization steps

- We tested whether water intake during fasting impacted the stability of the results. Two groups of untreated mice (n=4) were either given water or not during the 4h fasting. We found no significant differences in variation between the groups (data not included).
- The increase in FD4 in plasma after administration is dynamic, and the literature is inconsistent regarding the optimal time points for blood sampling after FD4 administration. We therefore evaluated the best time point for blood sampling; at 1.5, 3 and 5 hours post oral FD4 administration. The results showed no significant differences (data not included). We therefore chose the time point 1.5 h.
- In the literature, many protocols suggest to use dilute plasma 1:2 with PBS when examine the fluorescence intensity. This would require a large amount of plasma from each animal, which may create difficulties when running duplicates. We evaluated if 1:5 or 1:10 dilutions were sufficient. The dilutions of 1:5 gave the best sample-to-background ratio, and was used in the optimized protocol (data not included).

3.3 MEASUREMENTS OF GLUCOSE HOMEOSTASIS

The animals were subjected to both an intraperitoneal insulin tolerance test and an oral glucose tolerance test to determine possible development of reduced insulin sensitivity or insulin resistance.

3.3.1 Intraperitoneal insulin tolerance test

The intraperitoneal insulin tolerance test (IpITT) was included to evaluate the insulin sensitivity, i.e. the ability to remove glucose from the blood after an injection of insulin, in the different feeding groups. The IpITT went over two days (28 mice day 1 and 32 mice on day 2). The test was initiated by 4 hours of fasting, starting at 8 AM. Water, bedding and shelter were not altered during the test. Initiation of fasting, injections and sampling were done in intervals of 4 min per cage with 4 animals, to fit into a time schedule of 30 minutes for each step in the test.

Baseline blood glucose levels were measured twice (15 min apart). Blood (3-5 μL) was obtained by making small cuts on the tail with a scalpel blade. Blood glucose was measured by the use of a glucometer and test strips from Accu-Chek (Roche Diagnostics). Approximately 50 min after baseline blood was sampled, insulin was injected intraperitoneally in a fixed dose of 0.025 in

250 μL, related to mean weight of mice in the low-fat control group. Blood glucose levels were measured at three time points: 30, 60 and 120 minutes after the insulin injection.

Preparation of insulin was done by diluting human insulin from Sigma-Aldrich (27.5 U/mg), with 0.01 M HCl and PBS (pH 7.1) to a final concentration of solution of 0.1 U/mL. Adjustments of pH was done by adding 1 M NaOH to a pH level of 7.4. The aliquots with the standard solution were stored at -20ºC until the day of use.

3.3.2 Oral glucose tolerance test

The oral glucose tolerance test (OGTT) was done to determine the dynamics of glucose uptake and removal from the blood following administration of glucose. It is commonly employed to evaluate degree of insulin sensitivity and pre-diabetes/diabetes. The test extended for two days as described for the IpITT. Further subdivision of cages where done on each day into two additional groups (group 1 and 2). This sectioning enabled a time-efficient way for handling 28 and 32 animals a day, respectively. On the day of testing, mice in both group 1 and 2 were fasted in the morning in pre-set intervals. To prevent the animals from consuming feces and bedding during fasting, the bottom of the cages was complemented with a metal grid to avoid this concern.

To reduce stress and unwanted fluctuations of glucose from the experimental handling, in the days prior to the test, we increased the time spent handling the animals and placed the metal grids in the cages for some hours.

Baseline glucose levels were measured once prior to glucose administration for all animals from group 1, before immediately administering glucose to the very same group. The glucose load was administered orally after 6 hours of fasting with a feeding needle of 21 gauge. The glucose solution was prepared in advance in the working concentration of 20% glucose. The glucose used for this experiment was D-glucose from Sigma-Aldrich dissolved in PBS. Each animal received a fixed dose of 300 μL/mouse related to average weight of LF animals (2 g/kg in 30 g/mouse).

Blood collection and glucose measurements were done as described for the IpITT. Blood glucose levels were measured at four time points (15, 30, 60 and 120 minutes) after glucose administration. Baseline glucose levels for mice of group 2 were measured in between the 30 and 60 minutes' measurement of group 1.

3.4 TISSUE HARVESTING

Animals were terminated 18 weeks after start of intervention, and intestinal tissues was collected for analysis of mRNA expression in different segments, and immune cells in lamina propria of the small intestine. In addition, blood, liver, adipose tissue and fecal content were collected for other analyses outside the scope of this thesis. The complete procedure of tissue harvesting is displayed in Figure 2.3.

Thorax region of the mice was shaved and swabbed with ethanol before termination to avoid the contamination of skin bacteria in samples. Animals were anesthetized by subcutaneous injection of ZR-cocktail (100 μL/30 g mouse). The anesthetic cocktail consisted of the active substances zolazepam, tiletamin and xylazine, which together works to sedate, relieve pain and let the animals enter full anesthesia.

The intestines were rapidly dissected out and immediately rinsed in fresh cold PBS. Harvesting of mucosa samples for mRNA analyses was done by opening 0.5-2 cm long intestinal pieces longitudinally and scraping off mucosa using blunted microscope slides. The samples were preserved immediately in 500 μL RNA*later*. The mucosa samples were taken from three different segments in the small intestine: proximal, middle and distal part representing the duodenum, jejunum and ileum, respectively. Samples from colon were taken from the middle-to-proximal part.

The remaining intestine was prepared for immune cell isolation from the lamina propria by washing in cold cRPMI solution and removing visible Peyer's patches and fat. The intestinal contents were gently squeezed out by using the forceps before opening the intestines longitudinally. The intestines were cut into 0.5 cm long pieces, transferred into 50 mL Falcon tubes with 13-15 mL of cRMPI solution, and manually shaken for a few seconds to remove residual feces. The samples were stored on ice until start of isolation.

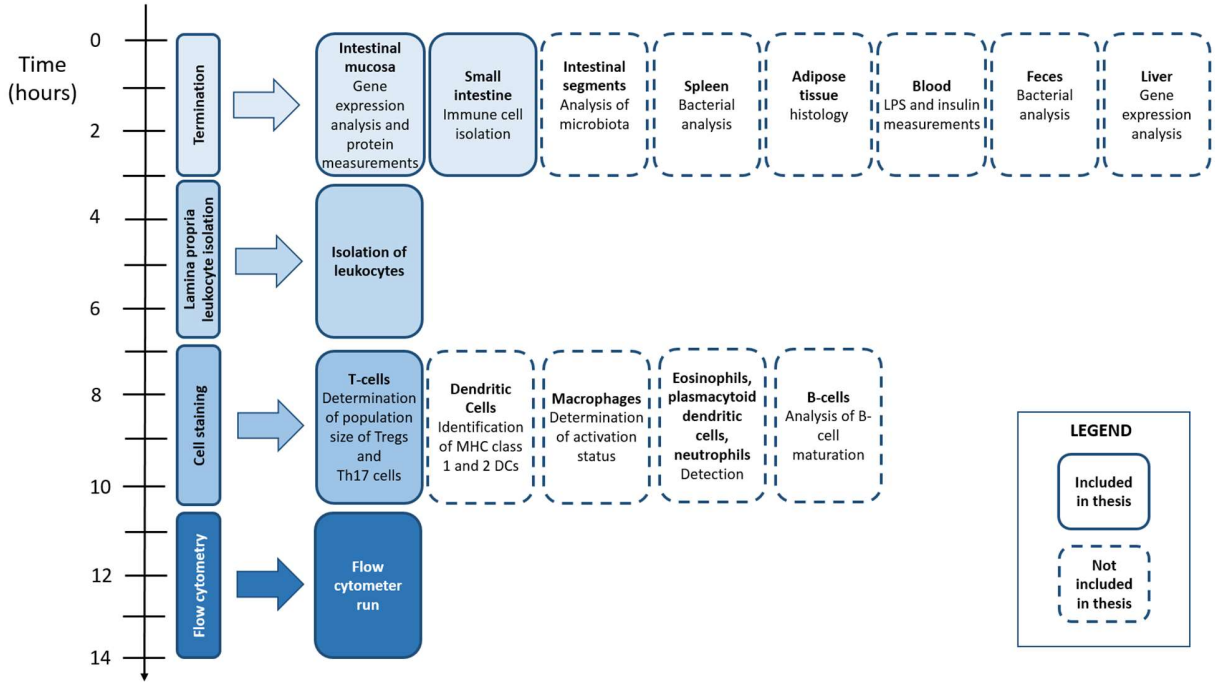


Figure 2.3. Complete procedure plan for each termination day. The procedures shown in dashed lines were not included in this study.

3.5 ISOLATION OF LAMINA PROPRIA LEUKOCYTES

The protocol for isolation of immune cells from lamina propria in the small intestine was derived from Goodyear and coworkers (2014). Changes were made to this protocol through several optimization steps to improve cell yield and reduce death. The purpose and volume of each solution are shown in Table.

Table 3.1. Purpose and volumes of solutions used in lamina propria leukocyte isolation

Solution	Volume per intestine (mL)	Purpose
cRMPI medium	50	Washing of intestines, short term storage and to stop digestion of collagenase
DTT solution	30	Removal of mucus
EDTA solution	70	Removal of epithelial cells
Pre-digestion solution	10	Removal of FBS and EDTA to avoid inhibition of the enzymatic digestion
Digestion solution	7	Membrane digestion
PEB buffer	11	Washing of cells

After dissection and cleaning of the small intestines, the tissues were ready to undergo isolation of lamina propria immune cells. The first step of isolation was to remove the cRMPI solution by pouring the solution through a crude sieve and transferring the small intestines into clean Falcon tubes. Mucus was removed by adding 30 mL of preheated (37ºC) DTT solution with freshly added dithiothreitol (DTT), a reducing agent that is able to split the disulphide bonds present in mucus. The intestines were incubated at 37ºC for 20 min at 175 RPM. The supernatant was discarded using a crude sieve and transferred to clean Falcon tubes.

Epithelium was removed by adding 30 mL of preheated (37ºC) EDTA solution and incubate at 37ºC for 15 min at 175 RPM. This washing step was performed additionally two times with 20 mL EDTA solution. After discarding the supernatant, the tissues were transferred to 10 mL of Pre-digestion solution. The tubes were incubated for 10 min at 37ºC and 175 RPM before discarding the supernatant through a sieve.

The digestion step was included to remove the loose connective tissue of the lamina propria that surrounds the leukocytes. The digestion step was performed by transferring the tissues to clean Falcon tubes with 7 mL Digestion solution containing collagenases (Liberase, Sigma-Aldrich) and DNase. The tissues were incubated at 37ºC and 175 RPM for 15 min.

The digestion was stopped by adding 10 mL cRPMI solution containing serum, and samples were immediately put on ice. The supernatant was transferred to new Falcon tubes by pouring through a 40 μm cell strainer. The strainers were washed with 10 mL cRPMI solution to collect as many cells as possible. The cell solution was centrifuged at 294g for 5 min, and the supernatant removed by decanting. The cells were resuspended and washed in 10 mL PEB buffer before centrifugation at 294g for 5 min. The supernatant was removed by decanting and the pellet resuspended in 1 mL PEB buffer and stored on ice. Cell counting was done by staining with trypan blue and using disposable slides with a hemocytometer counting grid.

3.5.1 Optimization steps

- The digestion step in the initial protocol was performed in petri dishes containing 6.65 mL/g Digestion solution w/o enzymes. The tissues were minced into small pieces with scissors before 350 μL 20x Liberase TM + DNase was added. The minced tissues were incubated at 37ºC for 30 min and stirred every 10 min. We tested the necessity of mincing,

but the results showed no differences in total cell outcome or cell viability between mincing and no mincing (data not included). Mincing step in petri dishes was excluded and digestion was executed in Falcon tubes with continuous shaking instead.

- The initial protocol included trituration of the tissue three times through an 18G needle. Testing showed no differences in total cell outcome or cell viability between trituration and brief vortexing in Falcon tubes of the solution (data not included). We also tested whether treatment of the tissues between digestion and pouring into cell strainer could be skipped altogether. The results showed that vortexing for 5, 10 or 20 sec gave no better outcome on total cell outcome or cell viability than no vortexing (data not included). This step was excluded from the protocol to save time.
- We tested whether different time and force in the centrifugation steps could influence cell viability. The tests included centrifugation at 294-500g and 5-10 min, concluding with 294g and 5 min per centrifugation step as the chosen one (data not included).

3.6 RNA EXTRACTION

Mucosa samples were placed in RNAlater after harvesting to preserve RNA integrity during storage and homogenization. The samples were first kept at 4ºC for 24 hours to allow thorough penetration of the solution into the tissue as the protocol advises (RNAlater, Thermo Fischer), and then stored at -20ºC until time of extraction. The extraction was performed using the NucleoSpin RNA/Protein Purification kit (Macherey-Nagel). This kit allows extraction of both RNA and protein from the very same sample. The protein extraction steps are excluded as only RNA was used for further experiments in this thesis.

The samples were weighted to fit into the recommended size range (10-30 μg). Prior to start, 10 μL of the reducing agent β-mercaptoethanol was added for every 1 mL of RP1 buffer. The mucosa samples were transferred to 350 μL RP1 buffer for cell lysis. The lysis buffer rapidly inactivates enzymes responsible for degrading RNA and protein.

The homogenization was performed by using a 25-gauge needle and 1 mL syringe until complete destruction of visible pieces. Filtration using the NucleoSpin filter was included to reduce viscosity. The samples were centrifuges at 11 000g for 1 min. The filter was discarded after centrifugation and 350 μl ethanol (70%) was added and mixed thoroughly with the homogenized lysate to adjust binding conditions. The lysate mixture was loaded onto the NucleoSpin

RNA/Protein column and centrifuged at 11 000g for 2 min, with the purpose of binding the nucleic acids to the column membrane.

Preparation for the DNase treatment step by desalting of the silica membrane was performed using 350 µL of the desalting buffer (MDB). The reaction mixture (95 µL), with recombinant DNase (rDNase), was applied directly onto the silica membrane of the column and incubated at room temperature for 15 min to ensure complete degradation of DNA. The columns were washed to inactivate the rDNase as well as drying the silica membrane. The first washing was done by adding 200 µL RA2 and centrifuge at 11 000g for 1 min. The second washing step included 600 µL RA3 and equal centrifugation conditions. The finial washing step was done by adding 250 µL of the RA3 buffer and centrifuge at 11 000g for 2 min. To end the RNA extraction, pure RNA was eluted in 50 µL RNase-free H_2O and stored at -80ºC.

3.7 RNA QUALITY AND QUANTIFICATION

Nano-Drop spectrophotometer was used to determine RNA concentration and purity. Eluted RNA was used to determine the concentration by measuring the A260 absorbance. Purity was assessed by calculating the absorbance ratios, 260/280 and 260/230. Values outside the accepted range may indicate the presence of contaminants (e.g. proteins for the A 260/230, and aromatic compounds for the A260/230 ratio). Significant amounts of these contaminants may interfere with downstream applications such as the reverse transcription reaction. A 260/280 nm ratio of approximately 2 was regarded as acceptable for the gene expression study (manufacturer information). Nuclease-free water was used as blanks.

RNA integrity was assessed through capillary electrophoresis with Agilent 2100 Bioanalyzer. The Agilent Bioanalyzer valuates the ratio between the 18S and the 28S ribosomal subunits, as well as the presence of degraded short fragments. This is used to calculate a RIN value, which is an evaluation of the integrity of the RNA in the sample. The degree of degradation is based on a numbering system from 1 to 10, with one being the most degraded profile and ten the most intact. The integrity of total RNA samples was analyzed by the use of the Total RNA 600 Nano Chip.

3.8 GENE EXPRESSION ANALYSIS WITH QUANTITATIVE REAL TIME PCR

Gene expression analysis of selected cytokines involved in inflammatory regulation was conducted in order to determine if coffee affected the inflammatory changes seen in the intestine with HF feeding. Gene expression analysis using quantitative real time PCR (qRT-PCR) offers great sensitivity, dynamic range and reproducibility. In contrast to the traditional PCR, the quantitative real time PCR (qRT-PCR) is both qualitative and quantitative. The principle of qRT-PCR is the same as for the traditional PCR, but the amplified DNA is detected after each round of amplification instead of detection of the amplified DNA only at the end of the reaction. mRNA analysis with qRT-PCR requires the reverse transcription of mRNA transcripts into complementary DNA (cDNA) before amplification and subsequent analysis.

3.8.1 cDNA synthesis

cDNA was synthesized using reverse transcriptase (RT) from the kit iScript cDNA Synthesis Kit (Bio-Rad). The RT in this kit is a modified MMLV-derived RT that is RNase H+. RT is an enzyme that translates RNA to DNA. This creates one DNA (cDNA) strand complementary to the RNA strand in a DNA-RNA hybrid. The RNA is then degraded by RNase H, leaving single stranded cDNA ready for PCR. In this case, total RNA was isolated from mucosa samples and used as template for cDNA synthesis. Like polymerase, RT needs primers to start the DNA synthesis. The iScript reaction mix consists of both oligo(dT) and random hexamer primers. This provides favorable setting for the production of shorter target lengths (<1 kb). The reagents and volumes used for the master mix is shown in Table 2.2.

Table 2.2. Master mix for cDNA-synthesis (1x reaction)

Reagent	Volume (µL)
5x iScript reaction mix	4
iScript reverse transcriptase	1
Nuclease-free water	11
RNA template (200 ng/ul)	4

cDNA synthesis was performed on individual mucosa samples, comprising of samples from four segments from each of the 20 animals (n=4): duodenum, jejunum, ileum and colon. Equal amounts of RNA (800 ng) from each sample was converted. The reverse transcriptase reagents and

the samples were thawed on ice, and kept on a cooling block for the entire procedure. A "no amplification control" (NAC) was produced for each batch of cDNA synthesis (8-12 samples) by pooling the samples and excluding the enzyme in the reaction. The thermal cycle used in the cDNA synthesis is described in Table 3.3. Following the thermal cycle, cDNA was stored at -20°C until start of qRT-PCR.

Table 3.3. Thermal cycle of the cDNA-synthesis

Step	Temperature (°C)	Duration
Primer annealing	25	5 min
cDNA synthesis	42	30 min
cDNA synthesis termination	85	5 min
Hold	4	+∞

3.8.2 Primer design and amplification efficiency

A limited number of relevant genes were selected for analysis due to time limitation. The target genes of interest were the anti-inflammatory cytokine TGF-β, and the pro-inflammatory cytokines TNF-α and IL-6. Primers amplifying a segment of the gene encoding the multifunctional enzyme Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as reference gene and internal control, as GAPDH is constitutively expressed by most tissues and cells. To verify this, we did preliminary tests using RNA samples from the four segments of intestines; duodenum, jejunum, ileum and colon (data not included).

Primer pairs included in this experiment were designed using the free online software Primer-BLAST (NCBI). Quantitative prediction of the efficiency of the primers was done using the primer analysis software NetPrimer (Premier Biosoft). Estimation of primer-dimer probability of the primers was performed using the Multiple Primer Analyzer (Thermo Fisher). Location of exons and introns were detected with the help of the software Spidey (NCBI).

A standard curve was constructed to calculate the gene-specific PCR efficiencies from a 10-fold series dilution of cDNA template for each primer pair. Each gene had a selection of at least two primer pairs to test for the best efficiency. The correlation coefficients (R^2) and slope values was obtained from the standard curve, and the efficiency (E) of PCR was calculated according to

the equation: $E = 10^{-\frac{10}{slope}-1}$. The sequences of the primers, product length and efficiency are shown in Table 3.4.

Table 3.4. Sequence, product length and efficiency of the primers used for PCR analysis

Gene	Primer	Sequence	bp	Eff. (%)
Reference gene				
GAPDH	Forward	5'-CTTCAACAGCAACTCCCACTCTT-3'	103	95
	Reverse	5'-GCCGTATTCATTGTCATACCAGG-3'		
Target genes				
TGF-β	Forward	5'-GCCGTATTCATTGTCATACCAGG-3'	77	95
	Reverse	5'-CGTGGAGTTTGTTATCTTTGCTG-3'		
TNF-α	Forward	5'-CTGTCTACTGAACTTCGGGGTGAT-3'	88	94
	Reverse	5'-GGTCTGGGCCATAGAACTGATG-3'		
IL-6	Forward	5'-GCAGCTGGAGAGTGTGGAT-3'	97	101
	Reverse	5'-AAACTCCACTTTGCTCTTGACTT-3'		

3.8.3 qRT-PCR

Quantitative detection of the amplicon was performed using the 5x HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne). EvaGreen dye is a non-specific fluorescent intercalating double stranded DNA (dsDNA) binding dye. The dye exerts its function by increasing the fluorescence with each PCR cycle as the DNA amplifies. The reagents and volumes used for the master mix is shown in Table 2.5.

Table 3.5. Master mix for qRT-PCR amplification (1x reaction)

Reagent	Volume (µl)
5x HOT FIREPol EvaGreen qPCR Supermix	2
Primer forward (10 pmol/µL)	0.2
Primer forward (10 pmol/µL)	0.2
DNA template (200 ng/µL)	3
H_2O PCR grade	4.6

The set-up of the reactions was carried out in a clean room dedicated to PCR work. All samples and items for the master mix were kept on ice and plates were kept on cooling blocks while working. Each sample were conducted in duplicates except the internal control, which was run in quadruplicates. cDNA was diluted 1 to 10 in RNase/DNase free water prior to use.

PCR runs were performed on the LightCycler 480 Instrument II (Roche Applied Science). The PCR reactions were carried out in a total volume of 10 µL. The amplification process is shown in Table 3.6. Generation of a melting curve was done at the end of the amplification process. The melting steps included 1 min 95ºC, 1 min at 40°C, then a continuous step at 60-90°C with 25 acquisitions per ºC (0.02°C/sec). The melting curve was used to control that the correct DNA sequence was amplified.

Table 3.6. Amplification process of qRT-PCR

Step		Duration	Temperature (°C)
Initial denaturation		15 min	95
Denaturation	Repeated 40x	15 sec	95
Annealing		20 sec	61
Elongation		20 sec	72

3.8.4 Analysis of gene expression

Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. The value at which fluorescence in the sample is detected for the first time is called cycle quantification (Cq) value and is expressed as a cycle number. Thus, the smaller the Cq value for a target sequence, the more of it was present in the original sample and the higher expression level of this target gene was in the specimen.

The Cq values of the internal control samples were used as reference to set the cycle thresholds (CT) for each plate. The mean of Cq values of duplicate wells was calculated and the reference gene was used as marker to which the target genes were normalized to. Relative gene expression was calculated using the delta Cq-method, with GAPDH as reference gene. The method calculates the ratio between the target gene and the reference. The fold value for each individual

sample was calculated by dividing the individual delta Cq value with the average delta Cq value for the control group (HF).

3.9 FLOW CYTOMETRY

Flow cytometry is a fluidic- and laser-based method used for multiparameter analysis of single cells in suspension. Parameters measured can be cell size (forward scatter), cell morphology/complexity (side scatter), and fluorescence to record expression of various intracellular and extracellular molecules with the use of fluorochrome-conjugated antibodies.

The flow cytometer was used to examine differences in populations of Tregs cells between the experimental groups. This was done by staining specific cell surface markers for T-cells and the intracellular transcription factors FoxP3. Evaluation of these T-cell populations was done on all 60 animals in the experiment, and was carried out immediately after finishing the isolation of lamina propria immune cells.

The T-cell panel of antibodies shown in Table 3.7 was used when staining for the T-cells of interest, with the exception of day 3-7 in which CD45 was excluded due to shortage of the antibody.

Table 3.7. Antibodies used for extra- and intracellular staining

Antibody	Purpose	Conjugate	Channel	Conc. (µg/mill cells)
Extracellular staining				
CD3	Gate for T-helper cells	APCvio770	R2	0.076
CD4	Gate for leukocytes	PE	B2	0.15
CD8	Gate for cytotoxic T-cells	PEvio770	B2	0.3
CD45	Gate for leukocytes	PerCPvio700	B3	0.15
Intracellular staining				
FoxP3	Gate for Tregs	APC	R1	0.3
Mouse IgG1	Isotype control for FoxP3	APC	R1	0.3
Other				
FcR-Block	Blocking non-specific binding	-	-	-

3.9.1 Extracellular antigen staining

Freshly isolated immune cells from intestinal lamina propria were resuspended in PEB buffer (16.7 mill cells/mL). Furthermore, $0.5x10^6$ cells/30 µL were added to each well in untreated, straight 96-well plates. The plates were always kept on ice, unless otherwise specified. Non-specific binding of antibodies to Fc-receptors (FcR) was prevented by incubating cells with FcR-Block for 10 minutes at 4ºC. Antibodies in the preferred concentration were added into their respective wells. PBS was added to reach a solution volume of 60 µL in each well, and the cells with antibodies were incubated at 4ºC for 10-15 minutes. Unstained cells were included for later calibration of the flow cytometer. The cells were washed twice by adding 200 µL PE-buffer to each well and centrifuged at 340g for 5 min.

3.9.2 Live/dead staining

The number of live and dead cells were determined by using a fluorescent reagent that penetrated dead, but not live cells. The LIVE/DEAD Fixable Violet Stain kit (Thermo Fisher) was selected based on its fluorescent properties to minimize compensation between the other dyes used with the relevant antibodies.

The live/dead stock solution was prepared in advance by diluting one vial in 50 µL dimethyl sulfoxide (DMSO) and stored at -80ºC in black Eppendorf tubes to protect against light. The working solution was prepared by diluting in PE-buffer (1:750).

The live/dead staining was done by adding 100 µL to each well and incubating for 45 min at 4ºC. The cells were washed twice with 200 µL PE-buffer after incubation and centrifuged at 340g for 5 min.

3.9.3 Intracellular staining using FoxP3 Buffers

The intracellular transcription factor-assay allows the detection of specific subsets of immune cells such as populations of Tregs. After staining for live/dead cells, the cells were subjected to fixation and permeabilization by adding 200 µL of Fixation/Permeabilization solution (Fix/Perm) solution to each well and incubate for 45 min at 4ºC. The Fix/Perm solution was prepared fresh by mixing Solution 1 and 2 from the FoxP3/Transcription Factor Staining Buffer Set (Miltenyi Biotec) in the ratio of 1 to 3, respectively. The cells were centrifuged at 340g for 5 min after incubation. Cells were washed once with 200 µL cold PEB-Buffer and centrifuged at

340g for 5 min. Two additional washing steps was included with 200 µL Permeabilization (Perm)-Buffer and centrifugation at 340g for 5 min. The Perm-Buffer was supplemented in the staining buffer set and prepared fresh by diluting in dH_2O (1:10).

Cells were blocked by adding 10 µL FcR-Block with Perm-Buffer (1:4), and incubated for 5 min at 4C. The antibodies and isotype controls were added to their respective wells to stain for transcription factors for 30 mins at 4ºC. Cells were washed twice in 200 µL Perm-Buffer and centrifuged at 340g for 5 min. Cells were resuspended in 140 µL Running Buffer (Miltenyi Biotec) and stored at 4ºC until flow cytometer analysis.

3.9.4 Flow cytometric equipment setup

Data was acquired on a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec). The cell suspensions were analyzed for their fluorescence, size and granularity. The most basic information provided by a flow cytometer is the forward scatter (FSC) and the side scatter (SSC). The FSC is generated when the light beam hits the cell and is deviated slightly. The FSC is roughly proportional to the size of the cells. The SSC is caused by intracellular bodies; the more granular the cell, the higher the SSC. The fluorophores coupled to the antibodies which stain the cells are excited by lasers in the machine and emits fluorescence at specific wavelengths which is measured by the cytometer. The fluorescence intensity generates a voltage pulse which has three measurable features: the height of the signal (H), the width of the signal (W), or the area under the signal (A). The height signal represents maximum fluorescence intensity, pulse width is indicative of particle transit time, and area represents total fluorescence of particle. The combinations of fluorescence picked up by the channels allow the experimenter to discover the possible identity of the cell type as illustrated in Figure 3..

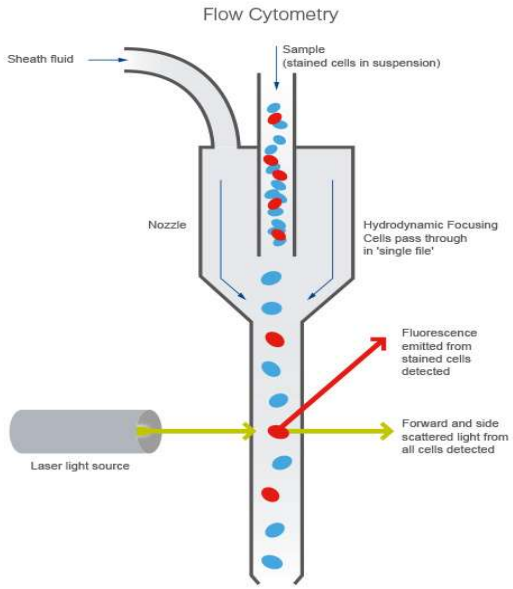


Figure 3.4. Overview of the flow cytometer. Cells pass through a laser beam one cell at a time. Forward and side scattered light is detected, as well as fluorescence emitted from stained cells. Forward scatter is proportional to the cell size. Side scatter is a measure of cell granularity. Figure obtained from www.abcam.com.

Routine calibration was performed daily to enable the adjustments of the voltage settings. Fluorescence spillover was minimized using a process called compensation. The machine was compensated using beads with the same antibodies as the cell samples. For compensation, stained beads were run and compensation was altered for each channel by subtracting the spillover from the channels other than its own.

3.9.5 Flow cytometry analysis

Results were analyzed using MACSQuantify software from Miltenyi Biotec. The gates signify the cells considered for further analysis, and the percentage of the cells within or outside the gate can then be calculated. After the exclusion of all unwanted events by gating them out of the initial population, the data from the remaining cells was used for cell cycle analysis. Isotype controls were used to define no-specific background staining, and cells were gated to eliminate this.

I. Gating for singlets was performed by plotting SSC-H against SSC-A. This allows to detect disproportions between cell size vs. cell signal. Therefore, all singlet events will be presented in a more diagonal display than doublets.
II. To exclude dead cells, the channel for live/dead staining was plotted against the PE channel.

III. FSC-A was plotted against SSC-A for gating of lymphocytes, and exclude other cell types and debris based on size differences and granularity.

IV. CD45 was plotted against SSC-A for additional discrimination of lymphocytes.

V. CD4 was plotted against CD3 to differentiate between T helper cells ($CD4^+$) and other types of T-cells ($CD4^-$).

VI. Separation of $FoxP3^+$ $CD4^+$ T cells from $FoxP3^-$ $CD4^+$ T cells was done by plotting FoxP3 against CD4.

3.10 STATISTICS

Areas under the glucose and insulin curves were calculated using the trapezoidal rule. Comparisons between groups were done using One-Way ANOVA followed by post-hoc Tukey's HSD (honest significant difference) test. Comparisons were considered statistically significant with a p-value of <0.05. Data are expressed as mean ± SEM.

4.0 RESULTS

4.1 BODY WEIGHT DEVELOPMENT AND FEED INTAKE

To explore the effect of coffee and high-fat feeding on the development of weight and food intake, we weighed mice and food each week during the 18-week intervention. We also evaluated if changes in body weight (BW) was due to differences in energy intake.

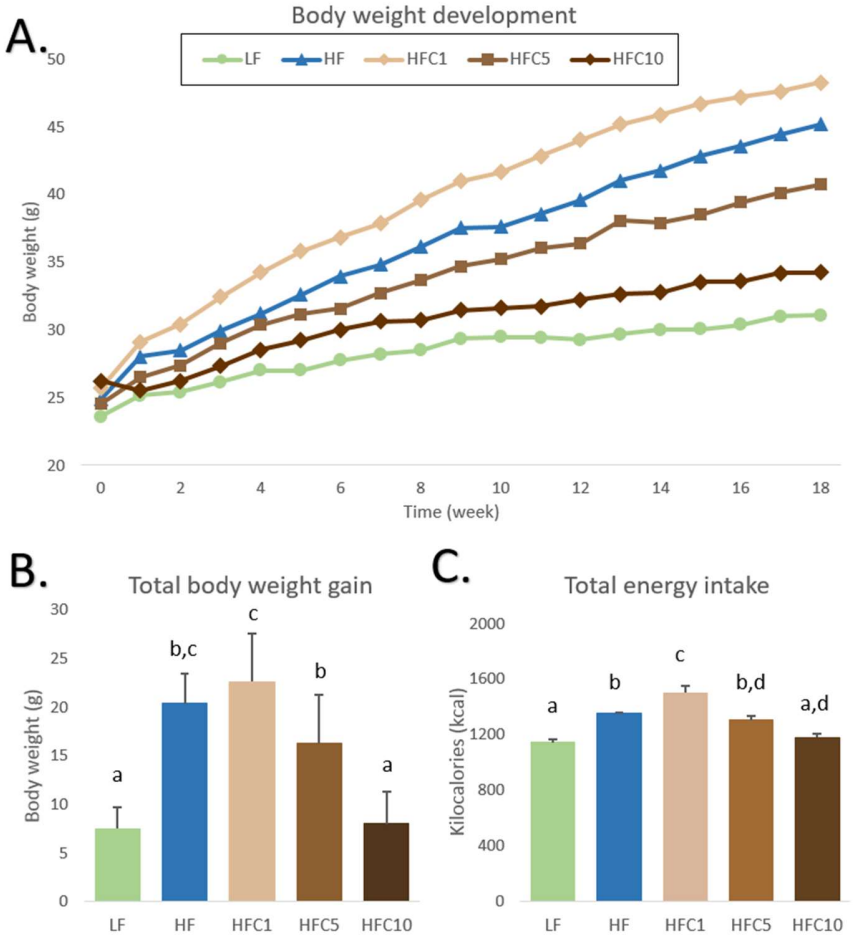


Figure 4.1. Body weight gain and feed intake. A: Weekly body weight (g) recording in all diet groups during 18 weeks of feeding. B: Total weight gain (g) during 18 weeks of feeding. C: Total energy intake (kcal) per mouse during feeding trial. n=12 mice. All values are expressed as mean ± SEM. Mean values without a common letter differ at $p < 0.05$.

As shown in Figure 4.1A, all mice were essentially of same weight in the start of the intervention. Not surprisingly, the LF mice had the lowest mean weight during the entire experiment. Interestingly, the total weight gain of LF mice did not significantly differ from the

HFC10 mice (P=0.997) (see Fig.4.1B). Mice in the HFC1 group gained the most weight during the experiment, but the increase in weight was not significantly higher compared to the HF group (P=0.622).

Following the trends of the weight development and total weight gain, the total energy intake was greatest for the HFC1 mouse (1501 ± 80 kcal), and differed significantly from the other groups ($P<0.05$) (see Fig.4.1C). The HFC5 mice were not significant different from the HF (P=0.791) and HFC10 (P=0.073) group. Mice in the LF group had a lower energy intake compared to the other groups, except from the HFC10 mice (P=0.913).

In summary, the LF and HFC10 mice were the leanest throughout the experiment. The HF, HFC1 and HFC5 mice showed similar development in body weight gain and development. The HFC1 mice distinguished themselves by eating the most kilocalories compared to the other groups.

4.2 EFFECTS OF COFFEE ON GLUCOSE TOLERANCE IN MICE

We next explored to what extent glucose tolerance was affected by coffee and high-fat feeding using an oral glucose tolerance test. Mice were fasted for 6 hours and given a fixed oral dose of glucose. We measured blood glucose at baseline and 15, 30, 60 and 120 minutes after ingestion of glucose.

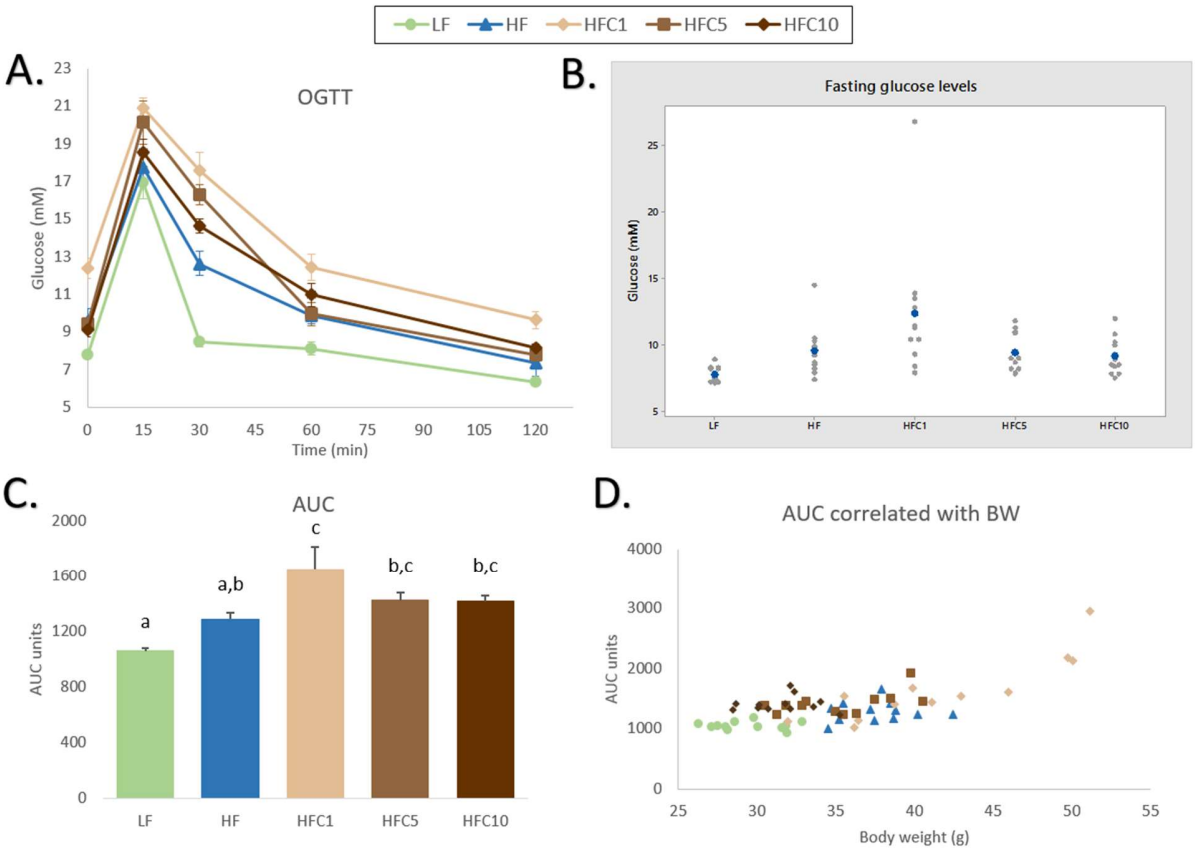


Figure 4.2. OGTT performed after 10 weeks of dietary intervention. A: Levels of blood glucose at baseline, 15 min, 30 min, 60 min, 120 min after glucose administration, in all groups. B: Dispersion of fasting blood glucose levels. C: Area under the curve. D. Area under the curve correlated to body weight of the individual animals. n=12 mice. All values are expressed as mean ± SEM. Mean values without a common letter differ at $p < 0.05$.

As shown in Figure 4.2A, all groups exhibited a normal response with a peak in blood glucose 15 min after glucose administration followed by normalization of glucose levels after 2 hours. We also assessed fasting glucose levels (baseline values), which gives an indication of glucose intolerance (ses Fig. 4.2B). The HFC1 mice had the highest fasting glucose levels, and differed significantly from all groups (P<0.05), except the HF group (P=0.060).

To evaluate glucose clearance between the different diet groups, area under the curve (AUC) was calculated (see Fig. 4.2C). The results from the AUC calculation showed that the LF mice differed significantly from all groups ($P<0.05$) apart from the HF mice ($P=0.271$). Despite showing no significantly differences in body weight gain, the HFC10 mice displayed significantly higher AUC values than the LF mice ($P=0.021$). As for the fasting glucose levels, the HFC1 mice showed the highest AUC values, but differed significantly only from HF and LF mice ($P=0.018$ and $P<0.001$, respectively). Interestingly, when excluding the three animals in HFC1 with the highest AUC values (see Fig. 4.2D), there is no significantly differences between HFC1 and HF group ($P=0.587$). Although not significant, the coffee groups had a tendency to higher glucose AUC values in comparison with the HF mice.

In summary, the results from the OGTT indicate that there is an association between high BW, high fasting blood glucose levels and lowered glucose tolerance. However, we found little or no significant differences between the coffee groups and the non-supplemented high-fat fed mice.

4.3 EFFECTS OF COFFEE ON INSULIN TOLERANCE IN MICE

The IpITT is designed to determine the sensitivity of insulin receptors in tissue by measuring blood glucose levels before and at different time points after an intraperitoneal insulin administration. Insulin was administered after 4 hours of fasting and glucose in the blood was measured at baseline, 30, 60 and 120 minutes post injection. The effects of the different diets on insulin sensitivity are shown in Figure 4.3.

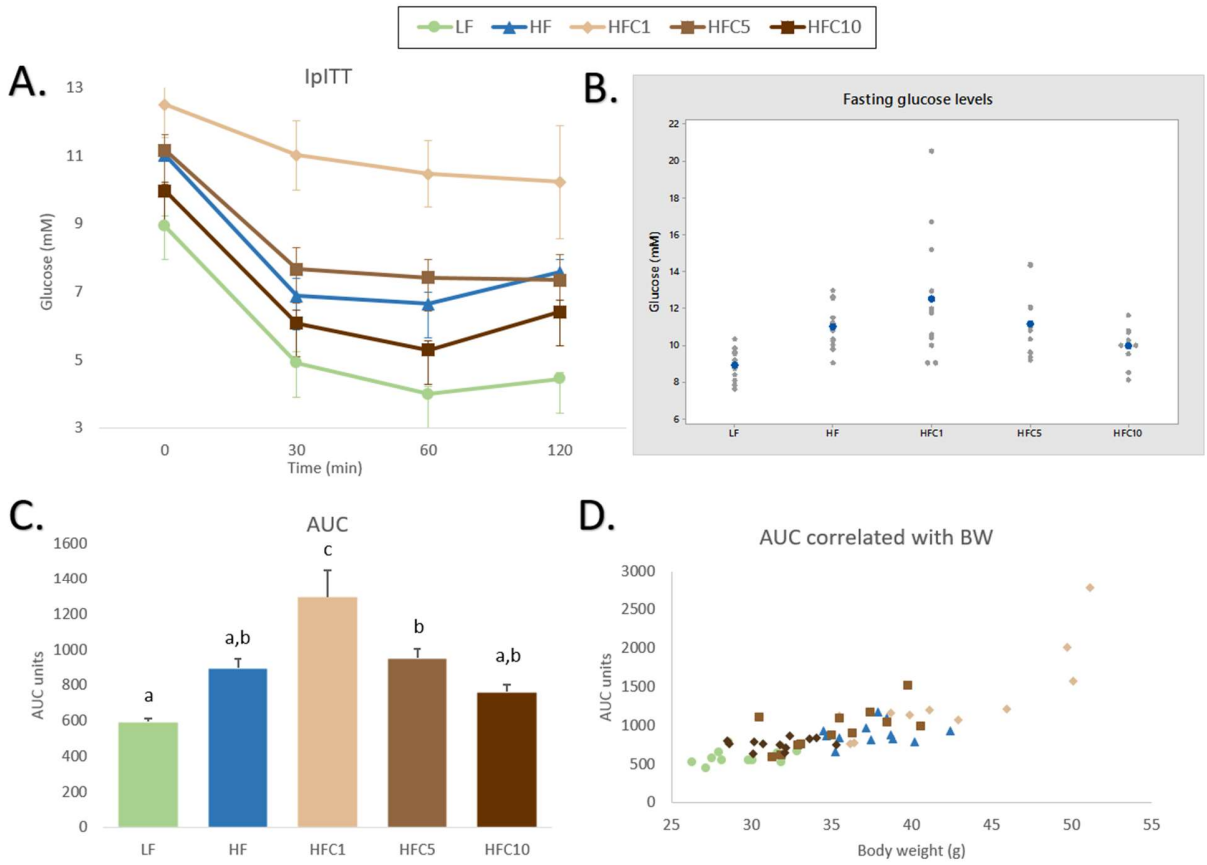


Figure 4.3. IpITT performed after 9 weeks of diet intervention. A: The development of blood glucose levels after insulin administration. B. Dispersion of fasting glucose levels. C: Area under the curve. D. Area under the curve correlated to body weight of the individual animals. n=12 mice. All values are expressed as mean ± SEM. Mean values without a common letter differ at $p < 0.05$.

Figure 4.3A shows that the HFC1 mice had the lowest response to insulin, which implies that their insulin sensitivity is clearly impaired. The other groups showed a more prominent response to insulin, with the LF mice showing the lowest glucose levels at every time point, closely followed by the HFC10 mice. As for the OGTT, fasting blood glucose levels prior to the insulin injection showed that HFC1 mice had the highest glucose levels (see Fig. 4.3B). However, fasting

glucose levels for HFC1 were only significantly different compared to the LF and HFC10 group ($P<0.001$ and $P<0.05$, respectively).

Assessment of the AUC values showed that the HFC1 mice differed significantly from the other groups ($P<0.05$) (see Fig. 4.3C). The only other statistical difference was between the LF and the HFC5 and HFC10 mice ($P=0.037$ and $P=0.021$, respectively). However, when excluding the same three outliers in the HFC1 group as for the OGTT AUC analysis (see Fig. 4.3D), the HFC1 become only statistical different from the LF mice ($P=0.011$).

In summary, The HFC1 mice were the only group that significantly distinguished themselves from the other groups when including all animals. The results from the IpITT also demonstrated an association between high BW and impaired insulin sensitivity. The response to insulin might indicate that coffee ingestion have lesser impact on insulin sensitivity that for example BW.

4.4 ANALYSIS OF INTESTINAL PERMEABILITY

Intestinal integrity was determined by measuring the permeability of FITC-dextran (FD4) in blood 1.5 hours following an oral dose of FD4. The mice were fasted for 4 hours before given a fixed oral dose of 650 mg FD4. Two outliers, one from each group of LF and HFC10, were omitted from the data set as they proved to deviate markedly from the other data points (see Fig. 4.4A)

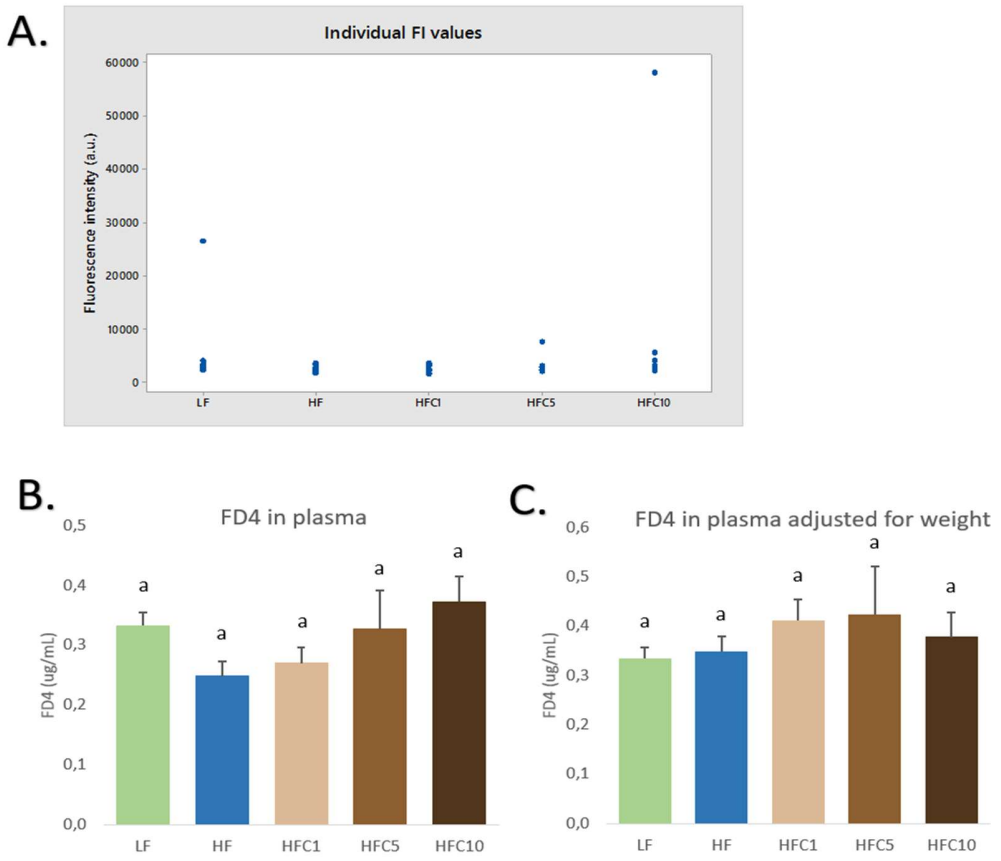


Figure 4.4. Intestinal permeability assay with FITC-dextran. A: Dispersion of fluorescence intensity values. B: FD4 concentrations in plasma. C: FD4 concentration in plasma adjusted for weight by multiplying individual FD4 plasma concentrations with the ratio between individual body weights and average lean control mice. n=12 mice. All values are expressed as mean ± SEM.

As shown in Figure 4.4B, the results from the FITC dextran concentrations in plasma showed that FD4 levels was higher for the mice in the LF, HFC5 and HFC10 groups compared to the HF and HFC1 mice, though not statistical significant. These results appeared to be inversely correlated to the weight of the mice. We adjusted for weight of the animals (see Fig. 4.4C). We found no significant differences in FD4 between any of diet groups. However, although not significant, the results show a trend towards the coffee groups displaying higher FD4 plasma values in comparison with the HF mice.

4.5 DETECTION OF SMALL INTESTINAL TREGS

Small intestinal leukocytes were stained and analyzed to evaluate the relative population of Tregs in the different diet groups.

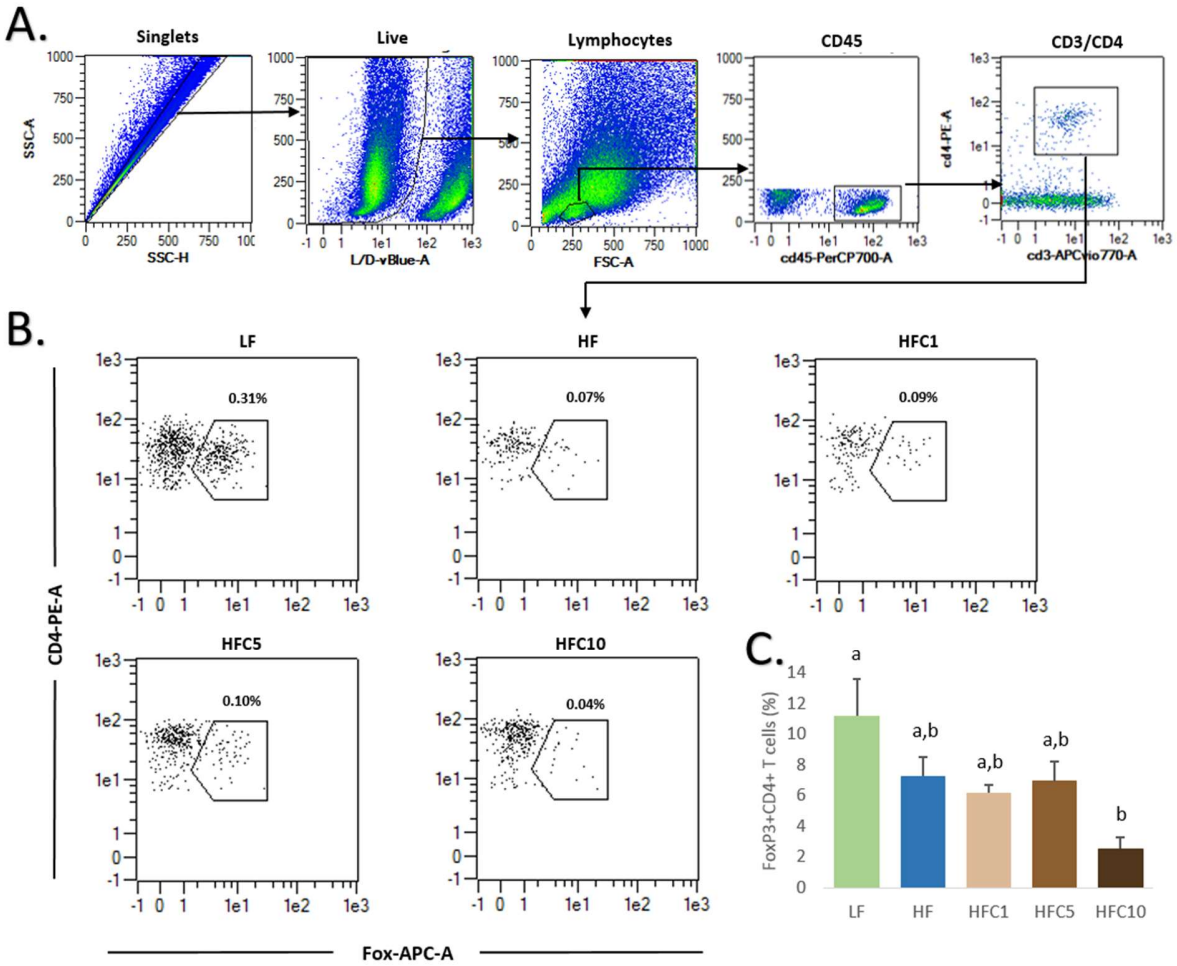


Figure 4.5. Flow cytometric analysis of Tregs by intracellular staining of FoxP3. A. Gating scheme for FoxP3+CD4+ T cell intracellular cytokine cytometry. Black polygons indicate gated cell subsets. B. Demonstration of FoxP3+ gating for one animal from each group. Numbers are the proportion (%) of cells expressing the indicated patterns of cytokines of the total amount of live/singlet cells. C. Proportion (%) of FoxP3+CD4+ T cells of total CD4+ gated cells. n=11-12 mice. All values are expressed as mean ± SEM.

The results from the flow analysis show that the LF mice displayed the highest proportion of FoxP3+CD4+ cells (Tregs) of the diet groups (see Fig. 4.5C), but was only significantly different from the HFC10 group ($P<0.01$). Although not statistically different, the results show a trend towards lower frequencies of Tregs in the coffee groups, especially HFC1 and HFC10.

4.6 RELATIVE GENE EXPRESSION OF CYTOKINES INVOLVED IN INFLAMMATORY REGULATION

Most studies have demonstrated that HF feeding causes increased expression of genes involved in inflammation. We hypothesized that inclusion of coffee in the high fat diet would lead to a reduction in expression pro-inflammatory genes. To investigate the effect of supplementation of coffee we measured the expression of the well characterized pro-inflammatory genes IL-6 and TNF-α, and the anti-inflammatory gene TGF-β in four different mucosal segments along the intestine (duodenum, jejunum, ileum and colon).

Table 4.3. Relative gene expression of TGF-β, TNF-α and IL-6 in different intestinal segments. The HF group is used as control. n=4 mice. *P<0.05.

	TGF-β		TNF-α		IL-6	
	Fold change	*P* values	Fold change	*P* values	Fold change	*P* values
Duodenum						
HF vs. LF	1,06	0,999	0,91	1,000	1,17	0,993
HF vs. HFC1	1,05	1,000	1,63	0,652	0,98	1,000
HF vs. HFC5	0,87	0,986	0,68	0,954	0,68	0,935
HF vs. HFC10	0,88	0,990	0,65	0,936	1,15	0,996
Jejunum						
HF vs. LF	1,72	0,041*	1,45	0,958	2,46	0,483
HF vs. HFC1	1,09	0,993	1,31	0,989	1,68	0,934
HF vs. HFC5	1,18	0,925	0,73	0,993	1,52	0,974
HF vs. HFC10	1,25	0,795	1,45	0,956	1,66	0,940
Ileum						
HF vs. LF	0,85	0,963	0,82	0,994	1,77	0,945
HF vs. HFC1	1,28	0,752	1,15	0,997	2,34	0,710
HF vs. HFC5	0,98	1,000	1,31	0,957	1,97	0,886
HF vs. HFC10	0,69	0,677	0,96	1,000	1,59	0,979
Colon						
HF vs. LF	0,86	0,988	0,86	0,999	3,06	0,757
HF vs. HFC1	1,32	0,791	1,92	0,498	3,73	0,531
HF vs. HFC5	1,05	1,000	0,79	0,996	1,20	1,000
HF vs. HFC10	0,98	1,000	0,70	0,982	2,38	0,927

As shown in Table 4.3 we found statistical difference for the relative expression of the *TGF-β* gene in jejunum for the LF mice compared to the HF mice (P=0.041). We found no statistical differences in the relative expression of the *IL6* gene or *TNF* gene between groups in any of the intestinal segments.

In summary, the analysis showed no significant differences in the relative expression of TGF-β, TNF-α or IL-6 in the small intestine between the high-fat diets.

5.0 DISCUSSION

The purpose of this study was to investigate the potential effects of coffee on development of obesity and type 2 diabetes, intestinal permeability and the inflammatory status in the intestine. Obesity have reached epidemic proportions and is closely accompanied by metabolic disorders such as type 2 diabetes. Identifying foods that could improve metabolic status is of interest to reduce the risk of developing metabolic disease. Moreover, it has been reported that coffee is associated with a reduced risk of insulin resistance and the development of type 2 diabetes.

In this study, it was demonstrated that a diet high in saturated fat caused greater weight gain and induced insulin resistance in mice compared to a low-fat diet. Supplementation of coffee in higher doses (10 cups/day) might display some adverse effects of a high-fat diet on these endpoints. On the contrary, a significant reduction in anti-inflammatory Tregs was found in mice with the highest intake of coffee compared to the other diet groups. The study did not find evidence for any additional effect of coffee ingestion on parameters of intestinal permeability or relative expression of genes involved in inflammatory regulation in the small and large intestine when fed a high-fat diet.

5.1 EFFECTS OF COFFEE AND HIGH-FAT DIET ON WEIGHT AND FOOD INTAKE

This study demonstrated that diets high in saturated fat appears to cause mice to eat more calories and gain more weight compared to low-fat diet. This outcome was expected and confirms what others have already stated (Winzell & Ahrén 2004). More interestingly, supplementation of coffee in dosage equivalent to 10 cups/day may counteract the effects of a high-fat diet on weight gain. Mice in the LF and HFC10 group were noticeably the leanest animals during the experiment and also had the lowest energy intake. The fact that the feed in the HFC10 groups was identical to the other high-fat diets only with the exception of coffee quantity, suggests that coffee in this quantity might have an influence on weight and food intake. This is consistent with other studies showing that coffee promotes weight control (Greenberg et al. 2006). Caffeinated coffee and/or caffeine also exerts properties as a metabolic stimulant and increases motor activity (Acheson et al. 1980; Dulloo et al. 1989; Snyder et al. 1981). The increased metabolic rate and motor activity seen with higher caffeine intake might partly explain why the HFC10 mice were leaner compared to the other high-fat fed groups.

Fairly unexpected, the HFC1 group displayed the highest mean body weight and food intake throughout the experiment. The reason for the increased weight gain in this group might be explained by the effect of caffeinated coffee as an "addictive" stimulant, resulting in increased food intake when the concentration of coffee in the feed is lower. At the same time indicating that the food intake of the groups with higher coffee quantity was naturally limited due to the physical effects of the coffee. Another possible explanation might be that the lower quantities of coffee provided an enjoyable taste to the food despite the bitterness known to coffee, leading to excess eating.

Collectively, coffee ingestion in higher doses (10 cups/day) might be beneficial for weight control when given a high-fat diet.

5.2 EFFECTS OF COFFEE AND HIGH-FAT DIET ON GLUCOSE HOMEOSTASIS

As reduced glucose tolerance and insulin sensitivity is related to the degree of adiposity (Kahn & Flier 2000; Kahn et al. 2006), it was expected to see better outcomes for the leaner animals. Our results showed that mice in the HFC10 group exhibited relatively high sensitivity to insulin in the IpITT compared to the other high-fat groups, but presented a tendency to lower glucose tolerance compared to the HF mice. Consequently, the results related to the HFC10 mice did not fully coincide with the hypothesis that leaner animals show greater insulin sensitivity, suggesting a possible influence by coffee.

Moreover, when excluding the three heaviest animals (>50g) in the study, the calculated glucose AUC from the OGTT showed no differences between the high-fat diet groups. These results may imply that: 1) levels of glucose tolerance do not change considerable until the high-fat fed animals reach a cut-off weight of about 50 grams and 2) coffee have none or little additional effect on glucose tolerance when fed a high-fat diet. Addressing the former, this situation might be due to animals below this cut-off weight are still in the compensated phase of insulin resistance, i.e. insulin levels are higher and blood glucose levels are still maintained. Animals exceeding the weight limit are probable more likely to experience severe impaired glucose tolerance as compensatory insulin secretion fails. The latter implication is in contrast to the general perception from epidemiological studies, which shows that long-term and habitual use of coffee may help maintain normal glucose tolerance and improve insulin sensitivity (e.g. see reviews by Akash 2014, van Dam 2002).

In the present study, both glucose and insulin were given to the animals in a fixed dose, independent of body weight. The standard approach in mice models is to base the dose of glucose and insulin on the weight of the mouse (McGuinness et al. 2009; Muniyappa et al. 2008). A number of studies evaluating the effects of high-fat diets have followed this standard approach (Anhê et al. 2015; Cani et al. 2007; Cani et al. 2008; Everard et al. 2011; Luck et al. 2015; Winer et al. 2009). However, normalizing glucose and insulin dose to body weight during tolerance tests creates problems. Liver, skeletal muscle and brain are the main sites of glucose disposal, and obesity is primarily caused by an increase in fat mass. A heavier mouse will thus receive higher insulin and glucose doses, although the amount of lean tissue may be the same. Administering glucose and insulin on a per body weight basis will therefore increase the glucose and insulin dose disproportionally in relation to tissue utilization. A study assessing the impact of obesity on glucose tolerance in C57BL/6 mice supported this hypothesis by showing that glucose intolerance in obese mice was markedly less pronounced when a fixed dose was administered compared to a weight dependent doses (Andrikopoulos et al. 2008).

In summary, coffee supplementation shows no markedly improvements on glucose tolerance compared to non-supplemented HF mice. Although not significant, coffee might display somewhat poorer glucose tolerance in comparison with the HF control mice. Though, it is possible that coffee in the highest doses (10 cup/day) might display less impairment to insulin compared to the other high-fat groups.

5.3 EFFECTS OF COFFEE AND HIGH-FAT DIET ON INTESTINAL PERMEABILITY

In this study, we used FITC dextran as an indicator of permeability in the small intestine. The results from the assay showed no significant differences between the diet groups. When assessing the graphs, the groups with the highest mean weight (HF and HFC1) displayed the lowest FD4 values. This is contradictory to the results seen by others where the heavier animals showed the highest values (Cani et al. 2008; Everard et al. 2011; Luck et al. 2015). The reason behind these different outcomes might be explained by the choice of FD4 dosage. In this study, we chose a conservative approach and gave the mice a fixed dose related to the mean weight of the animals in the LF group. Others have given mice FD4 related to their individual body weight (Cani et al. 2008; Everard et al. 2011; Johnson et al. 2015; Luck et al. 2015). The normalization of dosage in relation to body weight creates issues parallel to the ones discussed for glucose and insulin administration. The increase in body weight constitutes mostly of an increase in fat mass, thus limiting the increase

in extracellular fluid in which FD4 is dissolved in. Hence, normalizing FD4 dose directly to body weight will overestimate FD4 plasma levels in heavier animals compared to leaner ones. On the contrary, not including any additional corrections to the heavier animals may underestimate the actual plasma levels. In an attempt to compensate for the modest increase in extracellular fluid with weight gain, FD4 plasma levels were multiplied with the ratio between individual body weights and mean weight of lean control mice. Even though the data adjustments did not lead to significant results, we can speculate whether this approach gives a more accurate representation of the dietary influences on intestinal permeability.

In summary, our data propose that there is no difference in intestinal permeability regardless of body weight. Moreover, coffee had no additional significant effect on the results, but might display a tendency to higher intestinal permeability compared to the HF control mice.

5.4 EFFECTS OF COFFEE AND HIGH-FAT DIET ON EXPRESSION OF CYTOKINES INVOLVED IN INTESTINAL INFLAMMATORY REGULATION

The results from the gene expression analysis showed no significant differences in the relative expression of TGF-β, TNF-α or IL-6 in the small intestine between the coffee groups and the non-supplemented high-fat mice. However, it is possible that the large variances within the groups due to the small selection of animals included in the analysis (n=4) influenced on the outcome.

Revisiting the literature, epidemiological studies have shown both inverse (Lopez-Garcia et al. 2006; Williams et al. 2008) and positive (Zampelas et al. 2004) associations between coffee consumption and markers of inflammation. Various results on the effect of coffee on inflammatory markers are also seen with experimental studies in both animals and humans. These experimental studies have investigated changes in biomarkers in tissues other than the intestine such as liver, adipose tissue or in circulation (see chapter 1.6.2). To the best of our knowledge, no other study has investigated the effect of coffee on inflammatory markers in the different segments of the intestine. Some studies on high-fat feeding and intestinal inflammation have reported increases in expression of pro-inflammatory cytokines such as TNF-α in both the small and large intestine with high-fat diets (Ding et al. 2010; Lam et al. 2012; Li et al. 2008), while others have not found such increases (Johnson et al. 2015).

In summary, our data showed no significant effect of coffee supplementation compared to non-supplemented high-fat feeding. Due to the small sample size, it is problematic to draw any final conclusion regarding the gene expression results.

5.5 EFFECTS OF COFFEE AND HIGH-FAT DIET ON SMALL INTESTINAL TREG POPULATION

Tregs are known to be central in the maintenance of immune homeostasis and tolerance in the intestines, but only a few studies have reported on the effects of a high fat diet on intestinal Tregs. Even so, we hypothesized that the Treg population would decrease in the high-fat fed mice compared to the low-fat fed mice, as this phenomenon is previously reported by others in spleen (Wang et al. 2014), adipose tissue (Deiuliis et al. 2011), in addition to the intestine (Everard et al. 2014; Garidou et al. 2015; Luck et al. 2015; Ma et al. 2008). The results from the flow analysis supported this hypothesis as the largest population of Tregs was found in the small intestinal lamina propria of LF mice. The results showed that the HF, HFC1 and HFC5 groups displayed a similar reduction of population size of Tregs. Interestingly, the HFC10 mice showed a drastic reduction of Tregs compared to the other diet groups.

We ought to examine additional literature in an attempt to explain the possible reasons for this unfavorable effect of coffee. Unfortunately, studies investigating the effects of coffee and/or dietary compounds with biological activity on modulation of Tregs are lacking. Despite the scarcity of larger high-quality studies in this area, findings from some minor studies suggests a possibility for modulation of immune function through dietary strategies. Some studies have shown that the green tea polyphenol epigallocathecin (EGCG) might induce Treg numbers in a number of tissues (Pae et al. 2010; Wong et al. 2011; Yun et al. 2010). Vitamin B3 (niacin), which coffee is rich on, has been suggested to do the same (Zeng & Chi 2015). Moreover, diet-derived SCFA, especially butyrate, have showed an important positive influence on the generation of Tregs (Furusawa et al. 2013; Obata et al. 2014). This is interesting as SCFA, among them butyrate, were the fermentation products formed from coffee fiber (Gniechwitz et al. 2007). To the best of our knowledge, published studies thus only point towards a possible beneficial connection between biological active dietary compounds and Treg modulation. Therefore, the present study might be the first to suggest an adverse effect of coffee ingestion on Treg populations in the small intestine.

5.6 LIMITATIONS

The format of a thesis restricts the breadth of the experimental part. To further explore the significant of the results in this study, additional measurements and tests might have been advantageous to include. Additional assessment of intestinal permeability could have been done with measurements of LPS in plasma or analyses of albumin concentration in feces. For the OGTT, insulin measurement in plasma would have given the possibility to calculate the HOMA-IR (homeostasis model assessment of insulin resistance) index to further assess insulin resistance.

In the present study, mice were selected as preferred study objects due to a number of reasons. Mice are the most commonly used animal model for studying human disease, as mice are a cost-effective and efficient research tool. Although laboratory murine studies can often predict human health effects, the results of laboratory animals are not always transferrable to humans. When translating such results from mice models to humans, caution need to be taken when interpreting the results.

5.7 FUTURE PERSPECTIVES

As stated previously, the association between gut microbiota, obesity and diabetes are increasingly recognized (Cani et al. 2012; Flint et al. 2012; Musso et al. 2010; Patterson et al. 2016; Sommer & Bäckhed 2013). A key factor in determining gut microbiota composition and diversity is diet (Cani & Everard 2015; Graf et al. 2015). An interesting continuation of this present study would be to examine whether the microbial flora changes with coffee supplementation in high-fat feeding. Findings of little or no changes would support the absence of distinct results related to coffee ingestion.

To the best of our knowledge, there is only other one published study in high-fat feeding and coffee where coffee in its entirety is administered through the food (Fukushima et al. 2009). Only a few other animal studies have included coffee as a complete mixture when supplemented to a high-fat diet, most of them administering coffee through drinking water rather than food (Matsuda et al. 2011; Nakayama & Oishi 2013; Panchal et al. 2012; Rustenbeck et al. 2014; Vitaglione et al. 2010). Administering of coffee through the diet is thus a relatively unexplored area in the field of research. It would be preferable to further explore whether combining the two may facilitate a greater weight gain compared to administering coffee independent on feed.

Our study presented results proposing that coffee may exhibit neutral or possible adverse effects regarding the intestinal inflammatory tone. As scientific evidence regarding the anti-

inflammatory properties of coffee is mixed, these findings were not entirely unexpected. Still, further experimental work examining possible mechanisms for these effects could be useful. This could for instance include further exploration of cytokine profiling, or use gene knockout models to study immune responses during the dietary intervention.

6.0 CONCLUSION

The current study presents several findings. Primarily, we observed that coffee in higher dosages might help to prevent weight gain, but do not substantially counteract the adverse effects on glucose homeostasis seen with high-fat feeding.

Secondly, intestinal permeability was not improved when supplementing a high-fat diet with coffee. In fact, coffee consumption may even prove to increase permeability in comparison with non-supplemented high-fat diet.

Moreover, coffee might further aggravate the clear reduction of Treg populations seen with high-fat feeding. Indicating a more inflammatory intestinal environment in mice fed coffee.

Collectively, our findings, together with a share of earlier publications, presents a less welcoming theory that coffee might have an adverse impact on some important properties linked to the development of metabolic disease. Further research is necessary to establish whether these findings have an impact on human nutrition.

REFERENCES

Acheson, K. J., Zahorska-Markiewicz, B., Pittet, P., Anantharaman, K. & Jéquier, E. (1980). Caffeine and coffee: their influence on metabolic rate and substrate utilization in normal weight and obese individuals. *The American journal of clinical nutrition*, 33 (5): 989-997.

Alberti, K. G. M. M. & Zimmet, P. f. (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation. *Diabetic medicine*, 15 (7): 539-553.

Alberti, K. G. M. M., Zimmet, P. & Shaw, J. (2006). Metabolic syndrome—a new world-wide definition. A consensus statement from the international diabetes federation. *Diabetic medicine*, 23 (5): 469-480.

Andersen, L. F., Jacobs, D. R., Jr., Carlsen, M. H. & Blomhoff, R. (2006). Consumption of coffee is associated with reduced risk of death attributed to inflammatory and cardiovascular diseases in the Iowa Women's Health Study. *Am J Clin Nutr*, 83 (5): 1039-46.

Anderson, J. & Van Itallie, C. (1995). Tight junctions and the molecular basis for regulation of paracellular permeability. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 269 (4): G467-G475.

Andrikopoulos, S., Blair, A. R., Deluca, N., Fam, B. C. & Proietto, J. (2008). Evaluating the glucose tolerance test in mice. *American Journal of Physiology-Endocrinology and Metabolism*, 295 (6): E1323-E1332.

Anhê, F. F., Roy, D., Pilon, G., Dudonné, S., Matamoros, S., Varin, T. V., Garofalo, C., Moine, Q., Desjardins, Y. & Levy, E. (2015). A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice. *Gut*, 64 (6): 872-883.

Arion, W. J., Canfield, W. K., Ramos, F. C., Schindler, P. W., Burger, H.-J., Hemmerle, H., Schubert, G., Below, P. & Herling, A. W. (1997). Chlorogenic acid and hydroxynitrobenzaldehyde: new inhibitors of hepatic glucose 6-phosphatase. *Archives of biochemistry and biophysics*, 339 (2): 315-322.

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., Liu, H., Cross, J. R., Pfeffer, K., Coffer, P. J. & Rudensky, A. Y. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature*, 504 (7480): 451-455.

Barone, J. & Roberts, H. (1996). Caffeine consumption. *Food and Chemical Toxicology*, 34 (1): 119-129.

Belin, R. J. & He, K. (2007). Magnesium physiology and pathogenic mechanisms that contribute to the development of the metabolic syndrome. *Magnesium Research*, 20 (2): 107-129.

Blanchard, J. & Sawers, S. (1983). The absolute bioavailability of caffeine in man. *European journal of clinical pharmacology*, 24 (1): 93-98.

Bleau, C., Karelis, A. D., St-Pierre, D. H. & Lamontagne, L. (2014). Crosstalk between intestinal microbiota, adipose tissue and skeletal muscle as an early event in systemic low-grade inflammation and the development of obesity and diabetes. *Diabetes/metabolism research and reviews*.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition reviews*, 56 (11): 317-333.

Brun, P., Castagliuolo, I., Di Leo, V., Buda, A., Pinzani, M., Palù, G. & Martines, D. (2007). Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 292 (2): G518-G525.

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., Semenkovich, C. F. & Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*, 101 (44): 15718-15723.

Bäckhed, F., Manchester, J. K., Semenkovich, C. F. & Gordon, J. I. (2007). Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proceedings of the National Academy of Sciences*, 104 (3): 979-984.

Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A. M., Fava, F., Tuohy, K. M. & Chabo, C. (2007). Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes*, 56 (7): 1761-1772.

Cani, P. D., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A. M., Delzenne, N. M. & Burcelin, R. (2008). Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet–induced obesity and diabetes in mice. *Diabetes*, 57 (6): 1470-1481.

Cani, P. D., Possemiers, S., Van de Wiele, T., Guiot, Y., Everard, A., Rottier, O., Geurts, L., Naslain, D., Neyrinck, A. & Lambert, D. M. (2009). Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut*, 58 (8): 1091-1103.

Cani, P. D., Osto, M., Geurts, L. & Everard, A. (2012). Involvement of gut microbiota in the development of low-grade inflammation and type 2 diabetes associated with obesity. *Gut microbes*, 3 (4): 279-288.

Cani, P. D. & Everard, A. (2015). Talking microbes: when gut bacteria interact with diet and host organs. *Molecular nutrition & food research*.

Cardona, F., Andres-Lacueva, C., Tulipani, S., Tinahones, F. J. & Queipo-Ortuno, M. I. (2013). Benefits of polyphenols on gut microbiota and implications in human health. *J Nutr Biochem*, 24 (8): 1415-22.

Carvalho, B., Guadagnini, D., Tsukumo, D., Schenka, A., Latuf-Filho, P., Vassallo, J., Dias, J., Kubota, L., Carvalheira, J. & Saad, M. (2012). Modulation of gut microbiota by antibiotics improves insulin signalling in high-fat fed mice. *Diabetologia*, 55 (10): 2823-2834.

Chu, V. T., Beller, A., Rausch, S., Strandmark, J., Zänker, M., Arbach, O., Kruglov, A. & Berek, C. (2014). Eosinophils promote generation and maintenance of immunoglobulin-A-expressing plasma cells and contribute to gut immune homeostasis. *Immunity*, 40 (4): 582-593.

Chu, Y.-F., Chen, Y., Black, R. M., Brown, P. H., Lyle, B. J., Liu, R. H. & Ou, B. (2011). Type 2 diabetes-related bioactivities of coffee: Assessment of antioxidant activity, NF-κB inhibition, and stimulation of glucose uptake. *Food Chemistry*, 124 (3): 914-920.

Crozier, A., Ashihara, H. & Tomás-Barbéran, F. (2011). *Teas, cocoa and coffee: plant secondary metabolites and health*: John Wiley & Sons.

Crozier, T. W., Stalmach, A., Lean, M. E. & Crozier, A. (2012). Espresso coffees, caffeine and chlorogenic acid intake: potential health implications. *Food & function*, 3 (1): 30-33.

de La Serre, C. B., Ellis, C. L., Lee, J., Hartman, A. L., Rutledge, J. C. & Raybould, H. E. (2010). Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 299 (2): G440-G448.

de Sotillo, D. V. R. & Hadley, M. (2002). Chlorogenic acid modifies plasma and liver concentrations of: cholesterol, triacylglycerol, and minerals in (fa/fa) Zucker rats. *The Journal of nutritional biochemistry*, 13 (12): 717-726.

de Valk, H. W. (1999). Magnesium in diabetes mellitus. *The Netherlands journal of medicine*, 54 (4): 139-146.

De Wit, N. J., Bosch-Vermeulen, H., de Groot, P. J., Hooiveld, G. J., Bromhaar, M. M., Jansen, J., Müller, M. & van der Meer, R. (2008). The role of the small intestine in the development of dietary fat-induced obesity and insulin resistance in C57BL/6J mice. *BMC medical genomics*, 1 (1): 14.

Deiuliis, J., Shah, Z., Shah, N., Needleman, B., Mikami, D., Narula, V., Perry, K., Hazey, J., Kampfrath, T. & Kollengode, M. (2011). Visceral adipose inflammation in obesity is associated with critical alterations in tregulatory cell numbers. *PloS one*, 6 (1): e16376.

Deplancke, B. & Gaskins, H. R. (2001). Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *The American journal of clinical nutrition*, 73 (6): 1131S-1141S.

Desroches, S. & Lamarche, B. (2007). The evolving definitions and increasing prevalence of the metabolic syndrome. *Applied Physiology, Nutrition, and Metabolism*, 32 (1): 23-32.

Ding, S., Chi, M. M., Scull, B. P., Rigby, R., Schwerbrock, N. M., Magness, S., Jobin, C. & Lund, P. K. (2010). High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse.

Dulloo, A., Geissler, C., Horton, T., Collins, A. & Miller, D. (1989). Normal caffeine consumption: influence on thermogenesis and daily energy expenditure in lean and postobese human volunteers. *The American journal of clinical nutrition*, 49 (1): 44-50.

Duncan, S. H., Lobley, G., Holtrop, G., Ince, J., Johnstone, A., Louis, P. & Flint, H. (2008). Human colonic microbiota associated with diet, obesity and weight loss. *International journal of obesity*, 32 (11): 1720-1724.

Everard, A., Lazarevic, V., Derrien, M., Girard, M., Muccioli, G. G., Neyrinck, A. M., Possemiers, S., Van Holle, A., François, P. & de Vos, W. M. (2011). Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. *diabetes*, 60 (11): 2775-2786.

Everard, A., Geurts, L., Van Roye, M., Delzenne, N. M. & Cani, P. D. (2012). Tetrahydro iso-alpha acids from hops improve glucose homeostasis and reduce body weight gain and metabolic endotoxemia in high-fat diet-fed mice. *PLoS One*, 7 (3): e33858.

Everard, A., Geurts, L., Caesar, R., Van Hul, M., Matamoros, S., Duparc, T., Denis, R. G., Cochez, P., Pierard, F. & Castel, J. (2014). Intestinal epithelial MyD88 is a sensor switching host metabolism towards obesity according to nutritional status. *Nature communications*, 5.

Flint, H. J., Scott, K. P., Louis, P. & Duncan, S. H. (2012). The role of the gut microbiota in nutrition and health. *Nature Reviews Gastroenterology and Hepatology*, 9 (10): 577-589.

Freel, R. W., Hatch, M., Earnest, D. & Goldner, A. (1983). Role of tight-junctional pathways in bile salt-induced increases in colonic permeability. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 245 (6): G816-G823.

Fukushima, Y., Kasuga, M., Nakao, K., Shimomura, I. & Matsuzawa, Y. (2009). Effects of coffee on inflammatory cytokine gene expression in mice fed high-fat diets. *Journal of agricultural and food chemistry*, 57 (23): 11100-11105.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K. & Kato, T. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*, 504 (7480): 446-450.

Garidou, L., Pomié, C., Klopp, P., Waget, A., Charpentier, J., Aloulou, M., Giry, A., Serino, M., Stenman, L. & Lahtinen, S. (2015). The Gut Microbiota Regulates Intestinal CD4 T Cells Expressing RORγt and Controls Metabolic Disease. *Cell metabolism*, 22 (1): 100-112.

Gavrieli, A., Yannakoulia, M., Fragopoulou, E., Margaritopoulos, D., Chamberland, J. P., Kaisari, P., Kavouras, S. A. & Mantzoros, C. S. (2011). Caffeinated coffee does not acutely affect energy intake, appetite, or inflammation but prevents serum cortisol concentrations from falling in healthy men. *The Journal of nutrition*, 141 (4): 703-707.

Ghoshal, S., Witta, J., Zhong, J., De Villiers, W. & Eckhardt, E. (2009). Chylomicrons promote intestinal absorption of lipopolysaccharides. *Journal of lipid research*, 50 (1): 90-97.

Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M. & Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. *science*, 312 (5778): 1355-1359.

Gniechwitz, D., Reichardt, N., Blaut, M., Steinhart, H. & Bunzel, M. (2007). Dietary fiber from coffee beverage: degradation by human fecal microbiota. *Journal of agricultural and food chemistry*, 55 (17): 6989-6996.

Goldberg, R. F., Austen, W. G., Zhang, X., Munene, G., Mostafa, G., Biswas, S., McCormack, M., Eberlin, K. R., Nguyen, J. T. & Tatlidede, H. S. (2008). Intestinal alkaline phosphatase is a gut mucosal defense factor maintained by enteral nutrition. *Proceedings of the National Academy of Sciences*, 105 (9): 3551-3556.

Goodyear, A. W., Kumar, A., Dow, S. & Ryan, E. P. (2014). Optimization of murine small intestine leukocyte isolation for global immune phenotype analysis. *J Immunol Methods*, 405: 97-108.

Graf, D., Di Cagno, R., Fåk, F., Flint, H. J., Nyman, M., Saarela, M. & Watzl, B. (2015). Contribution of diet to the composition of the human gut microbiota. *Microbial ecology in health and disease*, 26.

Greenberg, J. A., Boozer, C. N. & Geliebter, A. (2006). Coffee, diabetes, and weight control. *The American journal of clinical nutrition*, 84 (4): 682-693.

Greer, F., Hudson, R., Ross, R. & Graham, T. (2001). Caffeine ingestion decreases glucose disposal during a hyperinsulinemic-euglycemic clamp in sedentary humans. *Diabetes*, 50 (10): 2349-2354.

Guilherme, A., Virbasius, J. V., Puri, V. & Czech, M. P. (2008). Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nature reviews Molecular cell biology*, 9 (5): 367-377.

Hall, J. A., Bouladoux, N., Sun, C. M., Wohlfert, E. A., Blank, R. B., Zhu, Q., Grigg, M. E., Berzofsky, J. A. & Belkaid, Y. (2008). Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. *Immunity*, 29 (4): 637-649.

Hamilton, M. K., Boudry, G., Lemay, D. G. & Raybould, H. E. (2015). Changes in intestinal barrier function and gut microbiota in high-fat diet-fed rats are dynamic and region dependent. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 308 (10): G840-G851.

Heckman, M. A., Weil, J., Mejia, D. & Gonzalez, E. (2010). Caffeine (1, 3, 7-trimethylxanthine) in foods: a comprehensive review on consumption, functionality, safety, and regulatory matters. *Journal of Food Science*, 75 (3): R77-R87.

Henrikson, C., Argenzio, R., Liacos, J. & Khosla, J. (1989). Morphologic and functional effects of bile salt on the porcine colon during injury and repair. *Laboratory investigation; a journal of technical methods and pathology*, 60 (1): 72-87.

Heyman-Lindén, L., Kotowska, D., Sand, E., Bjursell, M., Plaza, M., Turner, C., Holm, C., Fåk, F. & Berger, K. (2016). Lingonberries alter the gut microbiota and prevent low-grade inflammation in high-fat diet fed mice. *Food & Nutrition Research*, 60.

Honda, K. & Littman, D. R. (2012). The microbiome in infectious disease and inflammation. *Annual review of immunology*, 30: 759.

Hotamisligil, G. S. (2010). Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell*, 140 (6): 900-917.

Huxley, R., Lee, C. M. Y., Barzi, F., Timmermeister, L., Czernichow, S., Perkovic, V., Grobbee, D. E., Batty, D. & Woodward, M. (2009). Coffee, decaffeinated coffee, and tea consumption in relation to incident type 2 diabetes mellitus: a systematic review with meta-analysis. *Archives of internal medicine*, 169 (22): 2053-2063.

Jaquet, M., Rochat, I., Moulin, J., Cavin, C. & Bibiloni, R. (2009). Impact of coffee consumption on the gut microbiota: a human volunteer study. *International journal of food microbiology*, 130 (2): 117-121.

Johnson, A. M., Costanzo, A., Gareau, M. G., Armando, A. M., Quehenberger, O., Jameson, J. M. & Olefsky, J. M. (2015). High Fat Diet Causes Depletion of Intestinal Eosinophils Associated with Intestinal Permeability. *PloS one*, 10 (4): e0122195.

Johnston, K. L., Clifford, M. N. & Morgan, L. M. (2003). Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine. *The American journal of clinical nutrition*, 78 (4): 728-733.

Kahn, B. B. & Flier, J. S. (2000). Obesity and insulin resistance. *The Journal of clinical investigation*, 106 (4): 473-481.

Kahn, S. E., Hull, R. L. & Utzschneider, K. M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*, 444 (7121): 840-846.

Keating, N. & Keely, S. J. (2009). Bile acids in regulation of intestinal physiology. *Current gastroenterology reports*, 11 (5): 375-382.

Keijzers, G. B., De Galan, B. E., Tack, C. J. & Smits, P. (2002). Caffeine can decrease insulin sensitivity in humans. *Diabetes care*, 25 (2): 364-369.

Kempf, K., Herder, C., Erlund, I., Kolb, H., Martin, S., Carstensen, M., Koenig, W., Sundvall, J., Bidel, S. & Kuha, S. (2010). Effects of coffee consumption on subclinical inflammation and other risk factors for type 2 diabetes: a clinical trial. *The American journal of clinical nutrition*, 91 (4): 950-957.

Kim, K.-A., Gu, W., Lee, I.-A., Joh, E.-H. & Kim, D.-H. (2012). High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PloS one*, 7 (10): e47713.

Kless, C., Müller, V. M., Schüppel, V. L., Lichtenegger, M., Rychlik, M., Daniel, H., Klingenspor, M. & Haller, D. (2015). Diet-induced obesity causes metabolic impairment independent of alterations in gut barrier integrity. *Molecular nutrition & food research*, 59 (5): 968-978.

Kolovou, G. D., Anagnostopoulou, K. K., Salpea, K. D. & Mikhailidis, D. P. (2007). The prevalence of metabolic syndrome in various populations. *The American journal of the medical sciences*, 333 (6): 362-371.

Korn, T., Bettelli, E., Oukka, M. & Kuchroo, V. K. (2009). IL-17 and Th17 Cells. *Annual review of immunology*, 27: 485-517.

Könner, A. C. & Brüning, J. C. (2011). Toll-like receptors: linking inflammation to metabolism. *Trends in Endocrinology & Metabolism*, 22 (1): 16-23.

Lam, Y. Y., Ha, C. W., Campbell, C. R., Mitchell, A. J., Dinudom, A., Oscarsson, J., Cook, D. I., Hunt, N. H., Caterson, I. D. & Holmes, A. J. (2012). Increased gut permeability and microbiota change associate with mesenteric fat inflammation and metabolic dysfunction in diet-induced obese mice. *PloS one*, 7 (3): e34233.

Lapthorne, S., MacSharry, J., Scully, P., Nally, K. & Shanahan, F. (2012). Differential intestinal M-cell gene expression response to gut commensals. *Immunology*, 136 (3): 312-324.

Lathrop, S. K., Bloom, S. M., Rao, S. M., Nutsch, K., Lio, C.-W., Santacruz, N., Peterson, D. A., Stappenbeck, T. S. & Hsieh, C.-S. (2011). Peripheral education of the immune system by colonic commensal microbiota. *Nature*, 478 (7368): 250-254.

Lee, J. J., Jacobsen, E. A., McGarry, M. P., Schleimer, R. P. & Lee, N. A. (2010). Eosinophils in health and disease: the LIAR hypothesis. *Clinical & Experimental Allergy*, 40 (4): 563-575.

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. & Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America*, 102 (31): 11070-11075.

Li, H., Lelliott, C., Håkansson, P., Ploj, K., Tuneld, A., Verolin-Johansson, M., Benthem, L., Carlsson, B., Storlien, L. & Michaëlsson, E. (2008). Intestinal, adipose, and liver inflammation in diet-induced obese mice. *Metabolism*, 57 (12): 1704-1710.

Li, Y., Innocentin, S., Withers, D. R., Roberts, N. A., Gallagher, A. R., Grigorieva, E. F., Wilhelm, C. & Veldhoen, M. (2011). Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. *Cell*, 147 (3): 629-640.

Lopez-Garcia, E., van Dam, R. M., Qi, L. & Hu, F. B. (2006). Coffee consumption and markers of inflammation and endothelial dysfunction in healthy and diabetic women. *The American journal of clinical nutrition*, 84 (4): 888-893.

Lopez-Ridaura, R., Willett, W. C., Rimm, E. B., Liu, S., Stampfer, M. J., Manson, J. E. & Hu, F. B. (2004). Magnesium intake and risk of type 2 diabetes in men and women. *Diabetes care*, 27 (1): 134-140.

Luck, H., Tsai, S., Chung, J., Clemente-Casares, X., Ghazarian, M., Revelo, X. S., Lei, H., Luk, C. T., Shi, S. Y. & Surendra, A. (2015). Regulation of obesity-related insulin resistance with gut anti-inflammatory agents. *Cell metabolism*, 21 (4): 527-542.

Ludwig, I. A., Clifford, M. N., Lean, M. E., Ashihara, H. & Crozier, A. (2014). Coffee: biochemistry and potential impact on health. *Food & function*, 5 (8): 1695-1717.

Lumeng, C. N., Bodzin, J. L. & Saltiel, A. R. (2007). Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *The Journal of clinical investigation*, 117 (1): 175-184.

Ma, X., Torbenson, M., Hamad, A., Soloski, M. & Li, Z. (2008). High-fat diet modulates non-CD1d-restricted natural killer T cells and regulatory T cells in mouse colon and exacerbates experimental colitis. *Clinical & Experimental Immunology*, 151 (1): 130-138.

Maloy, K. J., Salaun, L., Cahill, R., Dougan, G., Saunders, N. J. & Powrie, F. (2003). CD4+ CD25+ TR cells suppress innate immune pathology through cytokine-dependent mechanisms. *The Journal of experimental medicine*, 197 (1): 111-119.

Manach, C., Williamson, G., Morand, C., Scalbert, A. & Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American journal of clinical nutrition*, 81 (1): 230S-242S.

Matsuda, Y., Kobayashi, M., Yamauchi, R., Ojika, M., Hiramitsu, M., Inoue, T., Katagiri, T., Murai, A. & Horio, F. (2011). Coffee and caffeine improve insulin sensitivity and glucose tolerance in C57BL/6J mice fed a high-fat diet. *Bioscience, biotechnology, and biochemistry*, 75 (12): 2309-2315.

McDole, J. R., Wheeler, L. W., McDonald, K. G., Wang, B., Konjufca, V., Knoop, K. A., Newberry, R. D. & Miller, M. J. (2012). Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature*, 483 (7389): 345-349.

McGuinness, O. P., Ayala, J. E., Laughlin, M. R. & Wasserman, D. H. (2009). NIH experiment in centralized mouse phenotyping: the Vanderbilt experience and recommendations for evaluating glucose homeostasis in the mouse. *American Journal of Physiology-Endocrinology and Metabolism*, 297 (4): E849-E855.

Mendis, S. (2015). *Global status report on noncommunicable diseases 2014: World Health Organization*.

Muniyappa, R., Lee, S., Chen, H. & Quon, M. J. (2008). Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. *American Journal of Physiology-Endocrinology And Metabolism*, 294 (1): E15-E26.

Murakami, Y., Tanabe, S. & Suzuki, T. (2016). High-fat Diet-induced Intestinal Hyperpermeability is Associated with Increased Bile Acids in the Large Intestine of Mice. *Journal of food science*, 81 (1): H216-H222.

Murphy, E., Cotter, P., Healy, S., Marques, T., O'sullivan, O., Fouhy, F., Clarke, S., O'toole, P., Quigley, E. M. & Stanton, C. (2010). Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut*, 59 (12): 1635-1642.

Musso, G., Gambino, R. & Cassader, M. (2010). Obesity, diabetes, and gut microbiota the hygiene hypothesis expanded? *Diabetes care*, 33 (10): 2277-2284.

Müller, V. M., Zietek, T., Rohm, F., Fiamoncini, J., Lagkouvardos, I., Haller, D., Clavel, T. & Daniel, H. (2016). Gut barrier impairment by high-fat diet in mice depends on housing conditions. *Molecular nutrition & food research*.

Nakayama, T. & Oishi, K. (2013). Influence of coffee (Coffea arabica) and galacto-oligosaccharide consumption on intestinal microbiota and the host responses. *FEMS microbiology letters*, 343 (2): 161-168.

Natella, F. & Scaccini, C. (2012). Role of coffee in modulation of diabetes risk. *Nutrition reviews*, 70 (4): 207-217.

Nawrot, P., Jordan, S., Eastwood, J., Rotstein, J., Hugenholtz, A. & Feeley, M. (2003). Effects of caffeine on human health. *Food Additives & Contaminants*, 20 (1): 1-30.

Neish, A. S. (2009). Microbes in gastrointestinal health and disease. *Gastroenterology*, 136 (1): 65-80.

Obata, Y., Furusawa, Y., Endo, T. A., Sharif, J., Takahashi, D., Atarashi, K., Nakayama, M., Onawa, S., Fujimura, Y. & Takahashi, M. (2014). The epigenetic regulator Uhrf1 facilitates the proliferation and maturation of colonic regulatory T cells. *Nature immunology*, 15 (6): 571-579.

Pae, M., Ren, Z., Meydani, S. N. & Wu, D. (2010). Green tea EGCG suppresses specific antigen driven T cell response and affects CD4+ T cell differentiation. *The FASEB Journal*, 24 (1_MeetingAbstracts): 326.8.

Panchal, S. K., Poudyal, H., Waanders, J. & Brown, L. (2012). Coffee extract attenuates changes in cardiovascular and hepatic structure and function without decreasing obesity in high-carbohydrate, high-fat diet-fed male rats. *The Journal of nutrition*, 142 (4): 690-697.

Paolisso, G., Scheen, A., d'Onofrio, F. & Lefebvre, P. (1990). Magnesium and glucose homeostasis. *Diabetologia*, 33 (9): 511-514.

Patterson, E., Ryan, P. M., Cryan, J. F., Dinan, T. G., Ross, R. P., Fitzgerald, G. F. & Stanton, C. (2016). Gut microbiota, obesity and diabetes. *Postgraduate medical journal*: postgradmedj-2015-133285.

Raimondi, F., Santoro, P., Barone, M. V., Pappacoda, S., Barretta, M. L., Nanayakkara, M., Apicella, C., Capasso, L. & Paludetto, R. (2008). Bile acids modulate tight junction structure and barrier function of Caco-2 monolayers via EGFR activation. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 294 (4): G906-G913.

Reichardt, N., Gniechwitz, D., Steinhart, H., Bunzel, M. & Blaut, M. (2009). Characterization of high molecular weight coffee fractions and their fermentation by human intestinal microbiota. *Molecular nutrition & food research*, 53 (2): 287-299.

Robertson, D., Wade, D., Workman, R., Woosley, R. L. & Oates, J. (1981). Tolerance to the humoral and hemodynamic effects of caffeine in man. *Journal of Clinical Investigation*, 67 (4): 1111.

Rodríguez-Morán, M. & Guerrero-Romero, F. (2003). Oral Magnesium Supplementation Improves Insulin Sensitivity and Metabolic Control in Type 2 Diabetic Subjects A randomized double-blind controlled trial. *Diabetes care*, 26 (4): 1147-1152.

Rosengren, A., Dotevall, A., Wilhelmsen, L., Thelle, D. & Johansson, S. (2004). Coffee and incidence of diabetes in Swedish women: a prospective 18-year follow-up study. *Journal of internal medicine*, 255 (1): 89-95.

Round, J. L. & Mazmanian, S. K. (2010). Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proceedings of the National Academy of Sciences*, 107 (27): 12204-12209.

Rustenbeck, I., Lier-Glaubitz, V., Willenborg, M., Eggert, F., Engelhardt, U. & Jörns, A. (2014). Effect of chronic coffee consumption on weight gain and glycaemia in a mouse model of obesity and type 2 diabetes. *Nutrition & diabetes*, 4 (6): e123.

Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. (2008). Regulatory T cells and immune tolerance. *Cell*, 133 (5): 775-787.

Salazar-Martinez, E., Willett, W. C., Ascherio, A., Manson, J. E., Leitzmann, M. F., Stampfer, M. J. & Hu, F. B. (2004). Coffee consumption and risk for type 2 diabetes mellitus. *Annals of internal medicine*, 140 (1): 1-8.

Salmeron, J., Manson, J. E., Stampfer, M. J., Colditz, G. A., Wing, A. L. & Willett, W. C. (1997). Dietary fiber, glycemic load, and risk of non—insulin-dependent diabetes mellitus in women. *Jama*, 277 (6): 472-477.

Schwiertz, A., Taras, D., Schäfer, K., Beijer, S., Bos, N. A., Donus, C. & Hardt, P. D. (2010). Microbiota and SCFA in lean and overweight healthy subjects. *Obesity*, 18 (1): 190-195.

Smith, P., Smythies, L., Shen, R., Greenwell-Wild, T., Gliozzi, M. & Wahl, S. (2011). Intestinal macrophages and response to microbial encroachment. *Mucosal immunology*, 4 (1): 31-42.

Snyder, S. H., Katims, J. J., Annau, Z., Bruns, R. F. & Daly, J. W. (1981). Adenosine receptors and behavioral actions of methylxanthines. *Proceedings of the National Academy of Sciences*, 78 (5): 3260-3264.

Sommer, F. & Bäckhed, F. (2013). The gut microbiota—masters of host development and physiology. *Nature Reviews Microbiology*, 11 (4): 227-238.

Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J. P., Eberl, G., Koyasu, S., Locksley, R. M., McKenzie, A. N. & Mebius, R. E. (2013). Innate lymphoid cells—a proposal for uniform nomenclature. *Nature Reviews Immunology*, 13 (2): 145-149.

Stenman, L. K., Holma, R. & Korpela, R. (2012). High-fat-induced intestinal permeability dysfunction associated with altered fecal bile acids. *World J Gastroenterol*, 18 (9): 923-929.

Suzuki, T. & Hara, H. (2010). Dietary fat and bile juice, but not obesity, are responsible for the increase in small intestinal permeability induced through the suppression of tight junction protein expression in LETO and OLETF rats. *Nutrition & metabolism*, 7 (1): 1.

Tuomilehto, J., Hu, G., Bidel, S., Lindström, J. & Jousilahti, P. (2004). Coffee consumption and risk of type 2 diabetes mellitus among middle-aged Finnish men and women. *Jama*, 291 (10): 1213-1219.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R. & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *nature*, 444 (7122): 1027-131.

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R. & Gordon, J. I. (2009). The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Science translational medicine*, 1 (6): 6ra14-6ra14.

Van Dam, R., Dekker, J., Nijpels, G., Stehouwer, C., Bouter, L. & Heine, R. (2004). Coffee consumption and incidence of impaired fasting glucose, impaired glucose tolerance, and type 2 diabetes: the Hoorn Study. *Diabetologia*, 47 (12): 2152-2159.

Van Dam, R. M. & Feskens, E. J. (2002). Coffee consumption and risk of type 2 diabetes mellitus. *The Lancet*, 360 (9344): 1477-1478.

Van Dam, R. M. & Hu, F. B. (2005). Coffee consumption and risk of type 2 diabetes: a systematic review. *Jama*, 294 (1): 97-104.

Veilleux, A., Mayeur, S., Bérubé, J.-C., Beaulieu, J.-F., Tremblay, E., Hould, F.-S., Bossé, Y., Richard, D. & Levy, E. (2015). Altered intestinal functions and increased local inflammation in insulin-resistant obese subjects: a gene-expression profile analysis. *BMC gastroenterology*, 15 (1): 1.

Vitaglione, P., Morisco, F., Mazzone, G., Amoruso, D. C., Ribecco, M. T., Romano, A., Fogliano, V., Caporaso, N. & D'Argenio, G. (2010). Coffee reduces liver damage in a rat model of steatohepatitis: the underlying mechanisms and the role of polyphenols and melanoidins. *Hepatology*, 52 (5): 1652-1661.

Wang, X., Ota, N., Manzanillo, P., Kates, L., Zavala-Solorio, J., Eidenschenk, C., Zhang, J., Lesch, J., Lee, W. P. & Ross, J. (2014). Interleukin-22 alleviates metabolic disorders and restores mucosal immunity in diabetes. *Nature*, 514 (7521): 237-241.

Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L. & Ferrante, A. W. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *The Journal of clinical investigation*, 112 (12): 1796-1808.

Williams, C. J., Fargnoli, J. L., Hwang, J. J., Van Dam, R. M., Blackburn, G. L., Hu, F. B. & Mantzoros, C. S. (2008). Coffee Consumption Is Associated With Higher Plasma Adiponectin Concentrations in Women With or Without Type 2 Diabetes A prospective cohort study. *Diabetes care*, 31 (3): 504-507.

Winer, D. A., Luck, H., Tsai, S. & Winer, S. (2016). The Intestinal Immune System in Obesity and Insulin Resistance. *Cell Metab*.

Winer, S., Chan, Y., Paltser, G., Truong, D., Tsui, H., Bahrami, J., Dorfman, R., Wang, Y., Zielenski, J. & Mastronardi, F. (2009). Normalization of obesity-associated insulin resistance through immunotherapy. *Nature medicine*, 15 (8): 921-929.

Winzell, M. S. & Ahrén, B. (2004). The high-fat diet–fed mouse a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes*, 53 (suppl 3): S215-S219.

Wong, C. P., Nguyen, L. P., Noh, S. K., Bray, T. M., Bruno, R. S. & Ho, E. (2011). Induction of regulatory T cells by green tea polyphenol EGCG. *Immunology letters*, 139 (1): 7-13.

Yeh, C.-T. & Yen, G.-C. (2006). Induction of hepatic antioxidant enzymes by phenolic acids in rats is accompanied by increased levels of multidrug resistance–associated protein 3 mRNA expression. *The Journal of nutrition*, 136 (1): 11-15.

Yun, J.-M., Jialal, I. & Devaraj, S. (2010). Effects of epigallocatechin gallate on regulatory T cell number and function in obese v. lean volunteers. *British journal of nutrition*, 103 (12): 1771-1777.

Zampelas, A., Panagiotakos, D. B., Pitsavos, C., Chrysohoou, C. & Stefanadis, C. (2004). Associations between coffee consumption and inflammatory markers in healthy persons: the ATTICA study. *The American journal of clinical nutrition*, 80 (4): 862-867.

Zeng, H. & Chi, H. (2015). Metabolic control of regulatory T cell development and function. *Trends in immunology*, 36 (1): 3-12.

Zhao, Z., Egashira, Y. & Sanada, H. (2004). Ferulic acid is quickly absorbed from rat stomach as the free form and then conjugated mainly in liver. *The Journal of nutrition*, 134 (11): 3083-3088.

Zheng, Y. & Rudensky, A. Y. (2007). Foxp3 in control of the regulatory T cell lineage. *Nature immunology*, 8 (5): 457-462.

Zhernakova, A., Kurilshikov, A., Bonder, M. J., Tigchelaar, E. F., Schirmer, M., Vatanen, T., Mujagic, Z., Vila, A. V., Falony, G. & Vieira-Silva, S. (2016). Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. *Science*, 352 (6285): 565-569.

Zimmet, P. Z. (2010). *International Diabetes Federation: The IDF concensus worldwide definition of the metabolic syndrome*. Available at: http://www.idf.org/metabolic-syndrome.

APPENDICES

A. CHEMICALS AND REAGENTS

Chemicals/reagents product name	Cat. No.	Supplier
CD25 VioBrightFITC, clone 7D4	130-104-275	Miltenyi Biotec, Lund, Sweden
CD3 APCvio770, clone 145-2C11	130-102-306	Miltenyi Biotec, Lund, Sweden
CD4 PE, clone GK1.5	130-102-619	Miltenyi Biotec, Lund, Sweden
CD8 PEvio770 clone 53-6.7	130-102-358	Miltenyi Biotec, Lund, Sweden
Delbrecco's phosphate-buffered saline (PBS)	D8537	Sigma-Aldrich, St. Louis, MI, USA
Deoxyribonuclease I from bovine pancreas (DNase)	D5025	Sigma-Aldrich, St. Louis, MI, USA
D-Glucose	G8270	Sigma-Aldrich, St. Louis, MI, USA
DL-Dithiothreitol (DTT)	D9779	Sigma-Aldrich, St. Louis, MI, USA
Enrofloxacin	17849	Sigma-Aldrich, St. Louis, MI, USA
Ethanol 96% GPR Rectapur	20824.365	VWR Chemicals, Radnor, PA, USA
Ethylenediaminetetraacetic acid (EDTA)	03690	Sigma-Aldrich, St. Louis, MI, USA
FITC-dextran MW4000	FD4-1G	Sigma-Aldrich, St. Louis, MI, USA
FoxP3 APC, clone 3G3	130-093-013	Miltenyi Biotec, Lund, Sweden
Hank's Balanced Salt Solution (HBSS) w/Ca+ and Mg+	H9269	Sigma-Aldrich, St. Louis, MI, USA
Hank's Balanced Salt Solution (HBSS) w/o Ca+ and Mg+	H9394	Sigma-Aldrich, St. Louis, MI, USA
HEPES pH 7.4	H0887	Sigma-Aldrich, St. Louis, MI, USA
Insulin, human 50 mg	I2643	Sigma-Aldrich, St. Louis, MI, USA
L-Alanine-L-Glutamine (Ala-Gln)	G8541	Sigma-Aldrich, St. Louis, MI, USA
Liberase	LIBTM-RO	Sigma-Aldrich, St. Louis, MI, USA
MACSQuant Running Buffer	130-092-747	Miltenyi Biotec, Bergisch Gladbach, Germany
Mouse IgG1 APC	130-092-214	Miltenyi Biotec, Lund, Sweden
Non-essential amino acid solution (NEAA)	M7145	Sigma-Aldrich, St. Louis, MI, USA
Penicillin-Streptomycin (Pen/Strep)	P0781	Sigma-Aldrich, St. Louis, MI, USA
Polymyxin B sulfate salt	P4932	Sigma-Aldrich, St. Louis, MI, USA
REA control APC	130-104-615	Miltenyi Biotec, Lund, Sweden
Rompun 20 mg/mL	-	Bayer Animal Health, Germany
RORgt APC, clone REA278	130-103-838	Miltenyi Biotec, Lund, Sweden
RPMI-1640 w/HEPES	R5886	Sigma-Aldrich, St. Louis, MI, USA
Zoletil Forte 250 mg/mL	-	Virbac, Sollihøgda, Norway
β-mercaptoethanol	M7522	Sigma-Aldrich, St. Louis, MI, USA

B. KITS

Kit product name	Supplier
5x HOT FIREPol EvaGreen qPCR Supermix	Solis BioDyne, Tartu, Estonia
Agilent RNA 6000 Nano kit	Agilent Technologies, Santa Clara, CA, USA
FoxP3 Staining Buffer Set, 130-093-142	Miltenyi Biotec, Bergisch Gladbach, Germany
iScript cDNA Synthesis kit	Bio-Rad Laboratories, Hercules, CA, USA
LIVE/DEAD Fixable Violet Dead Cell Stain kit, L34955	Thermo Fisher Scientific
NucleoSpin RNA/Protein Purification Kit	Macherey-Nagel, Düren, Germany

C. EQUIPMENT

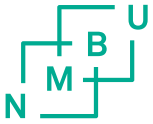
Equipment	Supplier
Accu-Chek Aviva glucometer	Roche Diagnostics, Mannheim, Germany
Agilent 2100 Bioanalyzer	Agilent Technologies, Santa Clara, CA, USA
LightCycler 480 Instrument II	Roche Applied Science, Indianapolis, IN, USA
MACQuant Analyzer 10 Flow Cytometer	Miltenyi Biotec, Bergisch Gladbach, Germany
NanoDrop 2000	NanoDrop Technologies, Wilmington, DE, USA
Synergy H4 Hybrid Microplate Reader	Bio Tek Instruments, Winooski, VT, USA
PTC-200 Thermal Cycler	MJ research, Waltham, MA, USA

D. WEBSITES

Website	URL
Multiple Primer Analyzer – Thermo Fisher	https://www.thermofisher.com/no/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html
NetPrimer – Premier Biosoft	http://www.premierbiosoft.com/netprimer/
Primer-BLAST – NCBI	http://www.ncbi.nlm.nih.gov/tools/primer-blast/
Spidey – NCBI	http://www.ncbi.nlm.nih.gov/spidey/

E. OTHER

Other	Supplier
Costar Assay Plate 96w, white, 3632	Corning Lifesciences, Kennebunk, ME, USA
LightCycler 480 Multiwell Plate 96w, white	Life science Roche
RNA*later* Tissue Collection: RNA Stabilization Solution	Thermo Fisher Scientific, Waltham, MA, USA
Untreated Plate 96w, straight	Thermo Scientific, Roskilde, Denmark



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