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Can oral administration of lipopolysaccharide (LPS) exaggerate metabolic effects and inflammation imposed by a Western diet in mice?

Vemund Heggen Skogen

Biotechnology, molecular biology

Preface

This master thesis was conducted at the departement of Chemistry, Biotechnology, and Food Science (KBM) during august 2018-september 2019, at the Norwegian university of Life Sciences (NMBU), and accounts for 60 ECTS.

My sincerest thanks to my supervisor Professor Harald Carlsen whom i consider a fount of knowledge. He has been both a guiding and supporting hand during this project. Heartfelt thanks to my co-supervisor PhD student Silje Else Harvei for her unwavering help and contribution to this thesis and for forever teaching me that one can never be too prepared prior to conducting experiments.

I would also like to thank the molecular cell group at KBM, which is host to no end of inspiring individuals. Composed of fine people studying many sorts of avenues within the life sciences, they are no strangers to lending a helping hand, one merely has to ask, and one shall receive. Curiosity and learning teem across the surface of this group. Indeed, the curious will in this group find limitless variety for learning. Thanks to PhD students Sérgio Rocha, and Dimitrios Papoutsis as well as senior engineer Lars Fredrik Moen for your assistance both inside and outside of the lab.

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Vemund Heggen Skogen

Abstract

A Western diet is an energy dense and nutrient poor diet associated with low-grade inflammatory consequences and is implicated in causing a range of non-communicable diseases (NCDs) including obesity and diabetes type 2 (T2DM). A chronic unresolved inflammatory response is recognized in causing chronic diseases. Elevated subclinical blood levels of lipopolysaccharides (LPS) from Gram negative bacteria can lead to metabolic endotoxemia (ME) and is postulated to be a causal factor for the triggering and progression of metabolic inflammation that can lead to NCDs. LPS in blood is mainly derived from the intestine but can originate in the diet or from endogenous Gram-negative bacteria normally residing in the gut. A Western diet is thought to increase blood levels of LPS exaggerated by amounts of dietary fat. Effects of the gastrointestinal tract on the fate of LPS given orally remain unraveled, as most studies have scrutinized systemic administration of LPS.

We wanted to see if the hypotheses and evidence presented by other studies would hold up when LPS is administered orally, and not by systemic injection, with regards to metabolic inflammation. Three experiments were therefore carried out to investigate effects of a Western diet and oral administration of LPS in mice. Our main aim was to investigate to what extent LPS given orally would affect obesity, ME, insulin resistance and low-grade inflammation in mice fed a Western diet. Additionally, we tested if orally ingested LPS reached the small intestine and if intestinal alkaline phosphatase levels and blood levels of LPS-binding protein (LBP) were affected by LPS. Finally, we investigated whether different doses of LPS were sufficient to detect LPS in plasma and induce inflammation.

We found that oral intake of LPS did not affect weight gain, energy intake or glucose homeostasis beyond the effects of a Western diet. We found that a Western diet and LPS upregulated inflammation in the liver and in the intestine, but this was restricted to a short-term effect.

These experiments showed that the gastrointestinal tract influences the fate(s) of LPS and that the chain of events regarding metabolic inflammation is not clear cut when LPS is ingested orally contra systemic delivery of LPS. With regards to inflammation, we demonstrated a limited, but not inconsequential effect of a Western diet and LPS given orally in mice by using NF-κB activity as a marker. Based on these findings, the potential fates and effects of orally ingested LPS should be further investigated.

Sammendrag

Et vestlig kosthold preges av et høyt energiinnhold og er ernæringsmessig av dårlig kvalitet. Et vestlig kosthold assosieres med lavgradig inflammasjon hvis konsekvenser er implisert i å forårsake livsstilssykdommer, henholdsvis fedme og diabetes type 2. Vedvarende inflammasjon er ansett som en årsaksfaktor i å drive kronisk sykdom. En subklinisk økning i blodnivåer av lipopolysakkarid (LPS) fra Gram-negative bakterier er knyttet til metabolsk endotoksemi (ME), en tilstand som ansees som en kausal årsaksfaktor i utvikling av metabolsk inflammasjon som kan føre til livsstilssykdommer. LPS i blod er tatt opp fra tarm men har sitt opphav i mat og i den endogene Gram-negative tarmmikrobiotaen. Et vestlig kosthold impliserer økt inntak av fett fra mat som er videre knyttet til økt opptak av LPS i blodsirkulasjonen. Mulige påvirkninger fordøyelseskanalen kan ha på LPS gitt oralt er ukjent ettersom mange studier har fokusert på å undersøke effektene av LPS administrert systemisk.

Vi ønsket å teste om det var hold i påstandene utledet av andre studier ved oral tilføring av LPS i stedet for systemisk administrering, med hensyn til metabolsk inflammasjon. Tre forsøk ble derfor utført for å undersøke effektene av et vestlig kosthold og oral tilførsel av LPS i mus. Det overordnede målet var å utlede til hvilken grad oral tilførsel av LPS påvirker fedme, ME, insulin resistens og lavgradig inflammasjon i mus gitt et vestlig kosthold. Videre undersøkte vi om LPS gitt oralt var å finne i tarmen og om nivåer av enzymet intestinal alkaline phosphatase samt blodnivåer LPS-binding protein (LBP) ble påvirket av LPS.

Våre funn tyder på at oral tilførsel av LPS ikke påvirker vektendringer, energiinntak eller insulins resistens i større grad enn et vestlig kosthold. Et vestlig kosthold og LPS oppregulerer alikevel kortsiktig inflammasjon i lever og tarm.

Sett under ett viste disse forsøkene at fordøyelseskanalen påvirker omsetningen av LPS og at hendelsesforløpet ikke er entydig når LPS tilføres oralt kontra systemisk, med hensyn til metabolsk inflammasjon. Vi demonstrerte en kortsiktig effekt av LPS gitt oralt i sammenheng med et vestlig kosthold i mus ved å anvende NF-κB aktivitet som en markør for inflammasjon. Mulige omsetningsmekanismer og effekter av LPS gitt oralt kan undersøkes videre med utgangspunkt i disse funnene.

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List of abbreviation

AMP	Anti-microbial peptide
ANOVA	Analysis of variance
AUC	Area under the curve
CD14	Cluster of differentiation 14
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EU	Endotoxin unit
FSA	Food safety authority (*Mattilsynet*)
HEK cells	Human embryonic kidney cells
HFD	High-fat diet
HOMA	Homeostatic model assessement
i.p.	Intraperitnoeal
IAP	Intestinal alkaline phostphatase
IpITT	Intraperitoneal insulin tolerance test
IU	Insulin units
IVIS	*In vivo* imaging system
LBP	LPS-binding protein
LPS	Lipopolysaccharide
MD2	Myeloid differentiation 2
ME	Metabolic endotoxemia
MetS	Metabolic syndrome
MUFA	Monounsaturated fatty acid
NaOH	Sodium hydroxide
NCD	Non-communicable disease
NF-κB	Nuclear factor kappa B
OGTT	Oral glucose tolerance test
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptor
PUFA	Polyunsaturated fatty acid
ROI	Region of interest
rpm	Rotations per minute
SCFA	Short-chain fatty acid
SD	Standard deviation
SEM	Standard error of the mean
SFA	Saturated fatty acid
T2DM	Type 2 diabetes mellitus
TLR4	Toll-like receptor 4
WD	Western diet
WHO	World Health Organization
WT	Wild type

1. Introduction

A Western lifestyle is implicated in causing a range of non-communicable diseases (NCDs) including obesity, diabetes type 2 (T2DM), and metabolic syndrome (MetS) (Calder et al., 2011a)[22]. MetS is a collective term used for describing the simultaneous affliction of multiple metabolic disorders. Diet, genetics, sleep, stress, age, and physical (in)activity among others are paramount to the development and prevention of such disorders. Herein, the role of the Western diet will be of focus which is related to low-grade inflammatory consequences (Zinöcker & Lindseth, 2018)[179]; (Lopez-Garcia et al., 2004)[93]. Relative to advisable diets, a Western diet has in many studies correlated with elevated subclinical blood levels of lipopolysaccharides (LPS) and LPS-binding proteins (LBP) which can lead to a condition termed metabolic endotoxemia (ME). ME is thought to be augmented by diets high in dietary fat (Pendyala et al., 2012)[124]. LPS is postulated to be a causal candidate for the triggering and progression of low-grade inflammation and NCDs through ME (Cani et al., 2007)[24]. This theory has gained considerable traction and since the turn of the century, the number of pubmed entries concerning endotoxemia has increased 6-fold. LPS can provoke a pro-inflammatory immune response and subsequent disease by stimulating Toll like receptors, specific immune receptors found in many different cell types (Mehta et al., 2010)[82]. LPS found in blood mainly originate from the intestine, either through the diet or from abundant intestinal Gram-negative bacteria. (Erridge, 2010b)[43;] Kelly et al., 2012)[81]. LPS is a heterogeneous class and its potency depends on the source, which has implications for the degree of inflammatory contribution of LPS from food contra intestinal bacteria. The gastrointestinal tract may affect the inflammatory potential and fate of intestinal derived LPS, as intestinal enzymes are a part of the barrier defence implicated in depotentiating LPS (Fawley & Gourlay, 2016)[50]. Many studies have looked at systemic administration of LPS, thus, several novel questions arise which instead seeks to elucidate the physiological relevance of a Western diet and oral intake of LPS in physiologically relevant doses and its relationship to systemic chronic low-grade inflammation and metabolic disorders. Proceedingly, topics mentioned here will be elaborated on, ultimately showing how they are affected by a Western diet and low-grade inflammation.

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1.1 Low-grade inflammation: A shared feature of metabolic disorders

Inflammation is a biological response to tissue or cellular damage or events that disrupts homeostasis. The immune system recognizes and responds to disturbances in the normal function of cells and tissues by initiating counter-measures until the event is resolved (Palm & Medzhitov, 2009)[122]. Inflammation has a dual role of being both "friend and foe" as in protecting the host during infections or tissue damage, whilst being associated with NCDs in the context of a continuous low-grade-inflammation. During classical acute inflammation, pro-inflammatory cytokines and other mediators (e.g complement system) recruit immune cells with the intended purpose of fighting undesirable particles or organisms until the unfavorable condition is resolved. However, sustained stimulation of immune receptors, such as during continuous low-grade inflammation (Jialal et al., 2014)[57], can in the long run have adverse effects on host tissues and organs such as the liver, intestine and vasculature (Virtue & Vidal-Puig, 2008)[165].

The role of a Western diet

The prevalence of type 2 diabetes mellitus and impaired glucose tolerance (a pre-diabetic state) is over 285 million individuals worldwide, a number projected to increase to 439 million by 2030 (Chen et al., 2011)[27]. Over 500 million adults worldwide are afflicted with obesity (Mendis, 2015)[104], which is estimated to affect 20% of the world population by 2030 (Kelly et al., 2008)[83]. MetS is estimated to affect a quarter of the world population (Saklayen, 2018)[138]. This compehensive expansion of NCDs worldwide motivates and gives incentive for finding underlying causes so that appropriate dietary strategies with respect to preventive measures can be formulated.

A Western lifestyle is associated with an eating pattern known as a Western diet, a diet characterized by being rich in; saturated fatty acids (SFAs), salts, omega-6 fatty acids, and processed and refined sources of carbohydrates and proteins (Cordain et al., 2005)[29]. A Western diet is low in; whole foods, all classes of dietary fibers, omega-3 fatty acids, monounsaturated fatty acids (MUFAs), micronutrients and other potential favorable plant chemicals such as antioxidants (Zinöcker & Lindseth, 2018)[179]; (Lopez-Garcia et al., 2004)[93]. In other words, a Western diet is a

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diet that is in excess of unfavorable nutrients while also lacking health promoting nutrients. For these reasons and due to the Western diet being a hypercaloric and hyperpalatable diet makes it a health concern when it comes to excess weight gain and development of obesity. This is especially true for individuals that are more often than not in energy surplus (Schenk et al., 2008)[141]. T2DM is characterized by insulin resistance and glucose intolerance, but the exact reason(s) for why this happens is unknown, although the importance of diet-induced obesity and inflammation is recognized. A Western diet (WD) increases adipose tissue mass which is in turn correlated with activation of transcription factors involved in regulating inflammation (Cruz-Teno et al., 2012)[31]. In line with this and central to WD-induced obesity and T2DM involves the hypothesis of chronically activated cytokine-secreting adipose- and immune cells which propagates inflammation and impairs insulin sensitivity (Virtue & Vidal-Puig, 2008)[165]. WD-induced adipose tissue hypertrophy is linked to insulin resistance (Chan & Hsieh, 2017)[26]. The associated inflamed adipose tissue environment is thought to attract and be influenced by chronically activated cytokine-secreting immune cells which in turn impairs insulin signal transduction and glucose homeostasis (Harford et al., 2011)[66]. For instance non-diabetic obese humans and mice (C57BL/6J) on a Western-like diet has been demonstrated to develop inflammation and insulin resistance. Dietary modulation on the other hand reduced inflammation and insulin resistance in the respective studies (Itariu et al., 2012)[76]; (Neuhofer et al., 2013)[119]. WD-induced obesity-related insulin resistance and indeed MetS are all correlated with a state of low-grade inflammation (Nettleton et al., 2010)[82]; (Hotamisligil, 2006)[71]. Additionally, chronic activation of Toll-like receptor 4 (TLR4) has emerged as another mediator of low-grade inflammation. Toll-like receptors represent a class of immune receptors associated with detection of adverse- or pathogen associated molecules, yielding an inflammatory response upon induction. TLR4 has been increasingly investigated as a mediator of subclinical inflammation and chronic disease. Whereas the onset of inflammation due to intake of unfavorable dietary fat was generally assigned to fatty acid immune signalling, TLR4 stimulants became recognized as potential instigators of obesity-related insulin resistance (Könner & Brüning, 2011)[87]. TLR4 stimulants are intestinal derived bioactive metabolites. These stimulants are estimated to be plentiful in certain foodstuff, being present in amounts of 1 ng-1 µg per g of certain foods (Inagawa et al., 2011)[74], and appear to be especially connected to fatty meat, dairy products and processed foods, even if the product is unspoiled (Erridge, 2011b)[45]. Extracts from

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these types of food are restricted to TLR4-induced inflammation as they were unable to cause inflammation in the abscence of TLR4. This is why food associated with a WD is implicated in contributing to T2DM, more so than unprocessed foods with an otherwise identical macronurient profile (Herieka et al., 2016)[69], emphasizing the role of a WD in promoting inflammation and disease through TLR4 stimulants (Fretts et al., 2012)[54]; (Renata Micha et al., 2010)[135]. Furthermore, chopped contra unchopped meat or vegetables stimulates to a larger extent the growth of food borne Gram-negative bacteria containing agonistic TLR4 stimulants with retained bioactivity after both cold storage and cooking (Faraj et al., 2019)[49]; (Erridge, 2011a)[44]. A change in intestinal permeability brought on by WD-induced inflammatory insult may increase uptake of TLR4 stimulants in the circulation which is thought to be augmented by both excessive intake of dietary fat and by the associated intestinal inflammatory environment leading to subsequent disease (Erridge et al., 2010a)[42]. The Western diet is rich in certain SFAs which are particularly, but not exclusively, implicated in TLR4 stimulation (Suganami et al., 2007)[151]. SFAs like palmitate (C16:0), myristic acid (C14:0), lauric acid (C12:0), but also the MUFA oleate (18:1n-9c), have been suggested to stimulate TLR4 (Shi et al., 2006)[147]; (Lee et al., 2010)[90], and are abundant in a Western diet. However, recent experimental evidence indicates that, although dietary fatty acids are implicated in inducing inflammation, they are not direct TLR4 agonists (Lancaster et al., 2018)[88]; (Erridge & Samani, 2009)[41]. The observed TLR4 stimulatory effect and WD-induced insulin resistance may instead come from bacterial molecules contained in lipoproteins which in addition to coming from the diet are also abundant in residential intestinal Gram-negative bacteria (Erridge & Samani, 2009)[41], although studies question the potency of these contained bacterial molecules to invoke TLR4 (Lee et al., 2010)[90]. Nevertheless, exposure to a fat rich and energy dense Western diet is linked to excessive uptake of TLR4 stimulants which in combination with dietary fat intake is thought to exaggerate weight gain, adipocyte hypertrophy, inflammation and insulin resistance in humans and mice (Kelly et al., 2012)[81]; (Shi et al., 2006)[110]; (Moreira et al., 2012)[85]. The potential effects of the gastrointestinal tract on the potency of TLR4 stimulants coming from the diet is unraveled and it is unknown to which degree oral intake of TLR4 stimulants can induce metabolic inflammation. Proceedingly, the gastro-intestinal tract and its role in disease will be elaborated on.

1.2 The gastrointestinal tract

The gastrointestinal tract encompasses the oral cavity, the stomach and the intestines and finally the rectum. It is a hollow passage located outside of the body, and it is where the majority of particles foreign to the host are located. The first and foremost role of the gastrointestinal tract is to ensure digestion and uptake of the nutrients that end up in the intestinal lumen, including potential immunostimulatory molecules derived from the diet and the intestinal bacteria (Haller, 2018)[64]. However, the intestinal layers have mechanisms in place that regulates this process.

1.2.1 Intestinal layers: Anatomy, contents and functions

Uptake of nutrients occurs primarily in the small intestine, owing to its large surface area. The intestinal system consists of several layers and components including the intestinal lumen, mucus, anti-microbial peptides (AMPs), antibodies, the intestinal epithelial cell lining, tight junctions, and immune cells. Mucus is a viscous substance made up of glycoproteins consisting of a network of mucins or branched sugar polymers connected to proteins. This mucosal network limits the ability of organisms foreign to the host to physically adhere and translocate to the intestinal cell lining. The large intestine has a dense double layered mucus layer, owing to a different set of mucosal physiochemcial properties as compensation for its comprehensive microbial habitat. The small intestine has a single, less dense, unattached mucus layer (Johansson et al., 2013)[78] making it more prone to alterations and bacterial infiltration. Bacterial adhesion occurs through the binding of saccharide receptors to saccharide ligands such as glycans, present on the membrane of intestinal cells. Present in the mucus are AMPs and antibodies which aid in keeping foreign organisms at bay and away from intestinal cells (Macpherson et al., 2005)[97]. The epithelial layer consists of different cell types performing different functions such as secretion of mucus, antibodies and AMPs. Enterocytes perform the majority of all uptake of foreign particles from the intestinal lumen such as nutrients, fluids and bacterial products. Transport across the the intestinal epithelium occurs through two routes, namely the transcellular and paracellular routes. Transcellular uptake of larger substances often involves the use of energy and carrier molecules/vesicles, depending on the physiochemical properties of the substance to be transported (Lea, 2015)[89]. Substances are further transported in the portal vein leading directly to the liver which gets a first claim at incoming

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nutrients and non-nutrients. Substances that are not routed to the portal vein are transported in the lymphatic circulation through lymph nodes before entering the general circulation and peripheral tissues (Iqbal & Hussain, 2009)[75]. Paracellular uptake occurs by diffusion between intestinal cells and relates more to hydrophilic substances. This form of uptake is routed through the portal vein (Ghoshal et al., 2009)[57]. Tight junction proteins are located inbetween the cells of the intestinal cell lining which function to adhere neighbouring cells and limit the diffusion of molecules including bacterial products into the circulation (figure 1.1) (Buckley & Turner, 2018)[20]. Beneath the epithelial layer is a section called the lamina propria, which harbors a vast range of immune cells involved in regulating immune responses and inflammation. In total, 2/3rds of the immune cells in the body reside in the intestines, concentrated in the epithelial cell layer and in the lamina propria. The epithelial cell lining relays and translates signals from the intestinal lumen to the immune system present in the lamina propria which dictates if and how the immune system responds (Haller, 2018)[64]. Together, these components; epithelial cells, mucus, and various elements of the immune system, make up the intestinal barrier. The high turnover rate (4-7 days) of epithelial cells is part of the barrier function. The intestinal barrier must limit the entry of pathogens attempting to colonize the host and in addition be able to recognize and separate these from both food and commensal bacteria in order to ensure the right immune response at the right time (Thaiss et al., 2016)[154]; (MacDonald & Monteleone, 2005)[96]. The intestinal layers are continously exposed to foreign objects and must know when to be tolerant and when to be intolerant towards such objects. Barrier integrity has been experimentally shown to be essential in determining the amount of bacterial translocation (Brun et al., 2007)[18]. Loss of barrier function is significantly associated with amounts of intestinal TLR stimulants (Erridge et al., 2010a)[42], dysregulated epithelial cell turnover, increased permeability and bacterial translocation, popularly referred to as having a "leaky gut" (figure 1.2) (Stewart et al., 2017)[150]. It remains to be determined whether TLR stimulants are simply markers or mediators. A trend in leaky gut-related conditions

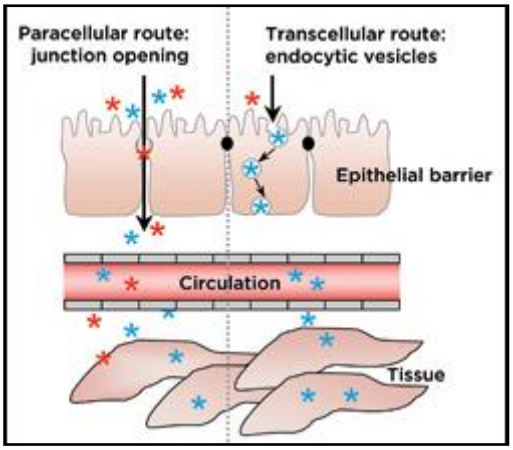

Figure 1.1: Paracellular and transcellular uptake. *Intended transcellular transport of substances. *Unintended leakage of substances.through intestinal junctions. Modified and adopted from Ghaffarian (2011).

is the marked reduction in microbiota diversity, inflammation and a systemically aggravated immune system affecting peripheral tissues (Erridge et al., 2010a)[42]. This process warrants further investigations (Karl et al., 2017)[80]. The degree of bacterial translocation and inflammation depends on the interplay between diet, homeostasis and the conditions in the intestinal lumen (Kelly & Conway, 2005)[82].

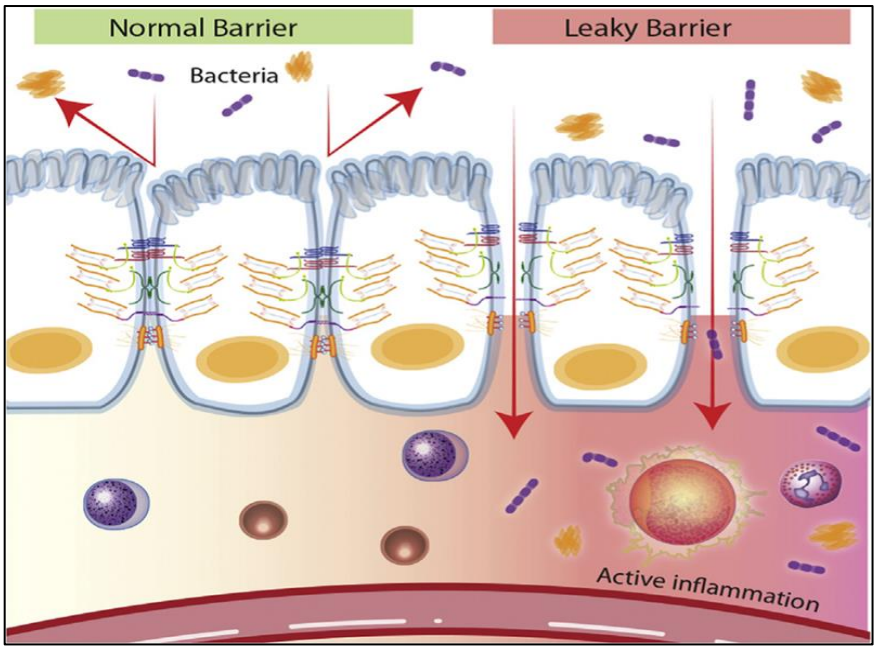

Figure 1.2: A normal barrier function during homeostasis contra a leaky barrier. Disruption of homeostasis and loss of barrier function due to impaired mucosal layer and intestinal junctions, dysbiosis as well as dyregulation of epithelial turnover is thought to cause a leaky barrier. Increased bacterial translocation and TLR stimulation due to a leaky barrier aggravates the immune system, causing an inflammatory response. Modified and adopted from Stewart et al., (2017).

1.2.2 **Microbiota:** Homeostasis and dysbiosis

The human body is home to bacterial communities covering the skin, mouth, lungs, urethra and the digestive tract. Both the small and the large intestine are inhabited by residental bacteria, both commensal and pathogenic which together makes up the microbiota. Bacterial load increases longitudinally in the intestines in conjunction with receeding AMP levels and a potential spillover-effect from the proximal colon. The distal ileal part contains the majority of the bacteria residing in the small intestine.During homeostasis, the host and the microbiota partake in a symbiotic relationship. The host provides a habitat and nutrients to the bacterial inhabitants. The microbiota primarily feeds on nutrients that are not digested by the host such as resistant saccharides, dietary

non-starch polysaccharides or fiber. In turn the bacteria provide the host with energy and potentially beneficial metabolites being the by-products of a complete or partial bacterial fermentation process such as short-chain fatty acids (SCFA). Bacterial metabolites play a role in host satiety, apetite regulation, energy intake and regulates the intestinal barrier, pH, susceptibility for colonization and in turn limits bacterial translocation, immune responses and inflammation (Hooper & Gordon, 2001)[70] (Wang et al., 2012)[168]. A Western diet lacking nutrients fermentable by the bacteria may promote opposite effects, ultimately creating more opportunities for intestinal translocation of bacteria and inflammation (Sivamaruthi et al., 2019)[148]; (Miele et al., 2009)[105]. Commensal bacteria can in this regard aid in degrading immune stimulatory molecules as well as being able to limit mucus degradation (Wang et al., 2006)[169], and colonization of pathogenic bacteria (Guarner & Malagelada, 2003)[61]. However, even commensal bacteria can cause disease in immunocompromised hosts (Delmas, 2015)[34]. Intestinal enzymes also contribute to maintining homeostasis by reducing inflammatory relations between the host and foreign objects which are abundant in the intestinal lumen. This is covered further in chapter 1.3.2.

A diverse microbiota is regarded as being beneficial for human physiology, while changes in the microbiota which reduces overall diversity, a condition known as dysbiosis, gives pathogenic bacteria an advantage which can have consequences on health. With a focus on effects of the diet, evidence suggest that certain foods, particularly those found in a Western diet, facilitate dysbiosis (Martinez et al., 2017)[100]. Long term intake of; dietary saturated fats, trans-fats, cholesterol, excess energy intake and the lack of fiber alters intestinal conditions over time, increasing pH, and production of immune stimulatory molecules (Moreira et al., 2012)[107]. Consequences of dysbiosis include but is not limited to increased host energy harvest, impaired intestinal barrier and increased bacterial translocation and immune stimulation (Turnbaugh et al., 2008)[157]. Early studies and follow-up studies comparing germ-free mice to colonized mice gave insight into the relationship between the diet, the microbiota and host metabolism, showing amongst other the gut bacteria being able to influence host suceptibility for increased or decreased adiposity implicating a role of the microbiota in progression or prevention of obesity (Backhed et al., 2004)[7]. Together, such studies illustrated the importance of diet compostion in determining the influence of the microbiota on metabolism. In particular, the importance of energy dense food, size, texture and the type and origin (plant or animal source) of dietary fat and sugar in determining wether or not the microbiota

plays a protective or detrimental role, although not causative, in inflammation and metabolic disease (Clavel et al., 2014)[28]; (Kübeck et al., 2016)[86]; (Fleissner et al., 2010)[52]; (Backhed et al., 2007)[8]. Taking this information into account, the microbiota can both be a preventer or an enabler of obesity, T2DM and MetS, which goes to show the complexity of host-microbe interactions (Turnbaugh et al., 2006)[156]; (Ussar et al., 2015)[159]. Drawing on this, research has shown an association between the microbial product LPS and metabolic disease, but it is uncertain whether or not this Gram-negative bioactive bacterial product effectively contributes to disease or not. That is, when regarding the complex situation in the gut pertaining to interactions between microbial products and the host. This is indeed a topic the literature requests to expand its vision on (Haller, 2018)[64]. More specificly, to shift focus from the microbiota profile and rather to expand knowledge about the physiological relevance of microbial bioactive products such as LPS, including how LPS may or may not affect host metabolism. On one hand, bacterial molecules such as LPS can reduce inflammation (Erridge, 2010b)[33] and condition the immune system, by ensuring that it responds when deemed necessary and contribute to secretion of intestinal enzymes and maintenance of the intestinal barrier (Haller, 2018)[64]; (Vaishnava et al., 2008)[160]. This can in turn reduce allergic and autoimmune incidences (Vatanen et al., 2016)[163]. On the other hand, exaggerated presence of LPS is associated with dysbiosis.

1.3 Lipopolysaccharide (LPS)

1.3.1 Structure and function

Referred to as endotoxins, LPS is a structural component of the outer cell membrane of Gram-negative bacteria. LPS enhances translocation and adherence to host cells (Neal et al., 2006)[90], and limits influx of polar molecules as well as protecting the bacteria from harmful substances such as antibiotics (Mayer et al., 1985)[102]. LPS, named for its characteristic properties, consists of polysaccharide polymers covalently bound to lipid acyl chains (figure 1.3). The sugar polymers are the peripheral and antigenic part of membrane bound LPS and is the part of LPS which gives rise to different serotpyes, a term used to classify bacteria. The LPS antigen is targeted by human and animal antibodies. The lipid- and most conserved part of LPS, called lipid A, is oriented towards

the intermediate membrane and is bound to glucosamine residues and phosphate groups (Raetz, 1990)[130]. Lipid A is the immune- and inflammatory stimulating moiety of LPS, while the sugar polymers acts as adaptors.

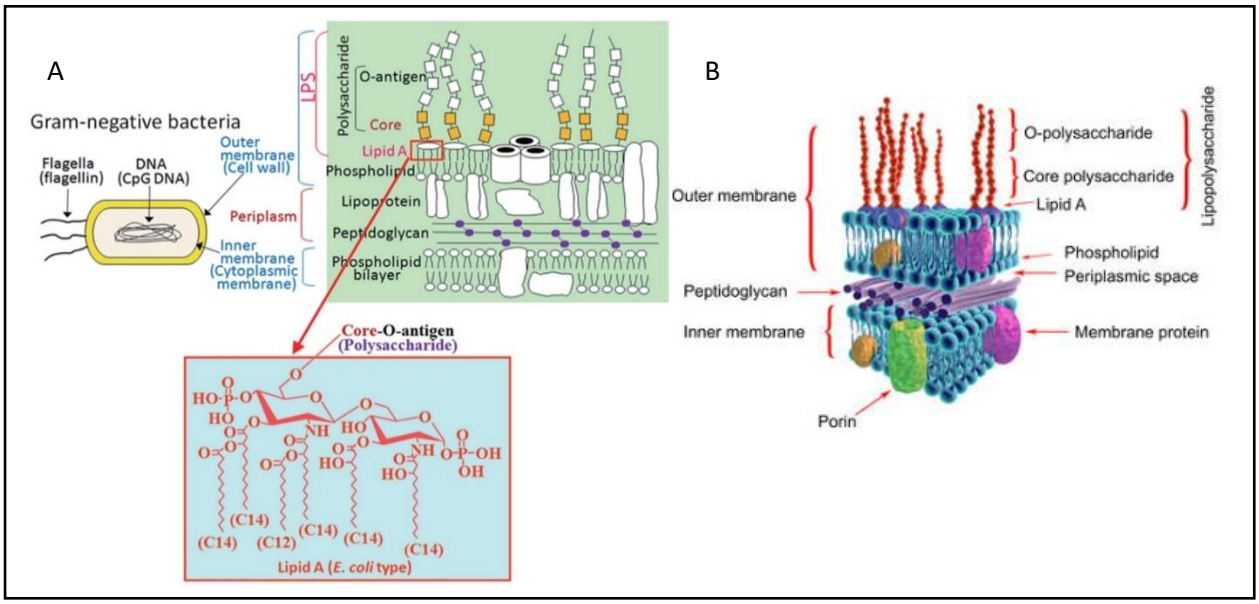

Figure 1.3: Lipopolysaccharide (LPS)
A: Structure and location of LPS as a component anchored in the Gram-negative bacterial outer membrane. LPS is composed of sugar polymers connected to a lipid compound called lipid A which is capable regulating inflammation. Lipid A is oriented towards the intermediate membrane.
B: 3D illustration of LPS anchored in the Gram-negative bacterial membrane.
Modified and adopted from Matsuura (2013).

1.3.2 Inflammatory potency of LPS and activation of NF-κB through TLR4

LPS molecules are only released from the cell membrane during cell division, rejuvenation or cell death. Small amounts (1-2 µg) of systemic LPS can be lethal in humans (Sauter & Wolfensberger, 1980)[139], but LPS is still capable of causing inflammation in picogram doses by binding to and stimulating receptors part of the immune system called Toll-like receptor 4 (TLR4) present on the cell membrane of intestinal cells, adipose cells and immune cells among others (Beutler & Rietschel, 2003b)[11]; (Lu et al., 2008)[95]. LPS from *E. coli* has the greatest capacity for stimulating an immune response (Rietschel et al., 1994)[136]. The heterogeneity of LPS and its immunostimulatory effect depends on its Gram-negative bacterial origin due to differences in LPS-synthesizing enzymes (Wassenaar & Zimmermann, 2018)[171]. Thus, heterogenous LPS derived

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from the intestine can be antagonistic with respect to TLR4 (Erridge et al., 2010a)[32]. Host intestinal enzymes contribute to maintining intestinal homeostasis by reducing inflammatory relations between the host and foreign objects which are abundant in the intestinal lumen. Intestinal alkaline phostphatase (IAP) is one such enzyme, implicated in de-potentating lipids, foregin organisms and bacterial products, such as LPS. IAP is produced and secreted by the intestinal epithelial cell lining into the intestinal lumen in response to the presence of dietary lipids, bacteria and LPS. IAP expression is highest in the duodenum while phosphatase activity is highest in the ileum. Impaired expression or function of IAP due to nutritional effects such as in a Western diet is correlated with dysbiosis and bacterial translocation and inflammation (Fawley & Gourlay, 2016)[50]. To which degree inflammation causes loss of IAP activity or vice versa is unclear. Variations in lipid A bound phosphate groups and acyl chains can yield antagonistic LPS. Phosphate groups on lipid A are cleavable by IAP (Fawley & Gourlay, 2016)[50]. IAP-cleaved LPS will compete with uncleaved/agonistic LPS for binding to TLR4 which may inhibit TLR4 stimuli in the intestine by reducing available binding sites (Faraj et al., 2017)[48]. Dephosphorylation of LPS has shown to result in a 100 fold reduction in *E. coli* LPS activity (Schromm et al., 1998)[142]. IAP and other enzymes have the potential to alter the structure of LPS, as illustrated in figure 1.4, and may influence to which degree LPS can (or cannot) stimulate an inflammatory immune response.

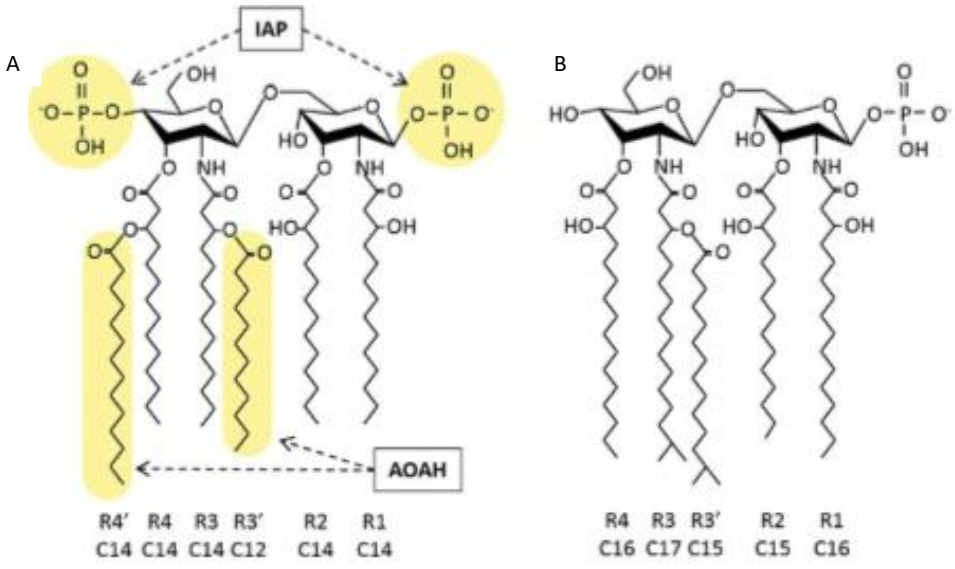

Figure 1.4: LPS- agonist and antagonist
A: Canonical E. coli lipid A structure with its two phsophor groups and short acyl chains, which are the respective enzymatic reaction sites of IAP and other enzymes.
B: Antagonistic LPS structure after 1x dephosphorylation by IAP and altered acyl chains which is thought to account for its lack of TLR4-stimulatory potential. Modified and adopted from Faraj et al., (2017).

NF-κB is a transcripton factor involved in regulating inflammation. NF-κB is stimulated by non-dietary factors (UV, ionizing radiation etc.) as well as dietary factors (fat, LPS), wherein LPS is of focus in this thesis. TLR4 can be stimulated by LPS at a concentration as low as 0.1 ng/mL (Yan et al., 2004)[175], wherein NF-κB ultimately translocates to the cell nucleus transcribing pro-inflammatory cytokines in the affected tissue. Cytokines attracts leukocytes and increases blood supply to the inflamed tissue (Baker et al., 2011)[9]. LPS can bind directly to or be delivered by LBP to TLR4 and its two crucial membrane surface mediators, namely, a macrophage pattern recorgnition receptor (PRR); cluster of differentiation 14 (CD14) and a lymphocyte antigen; myeloid differentiation 2 (MD2) (Abreu et al., 2002)[2]. This forms protein complexes on the extracellular and intracellular sides of TLR4 enabling the downstream signaling components in the LPS recognition cascade (Park & Lee, 2013)[123]. This causes degradation of IκB, a protein a protein responsible for retaining NF-κB dimers in the cytosol, resulting in NF-κB translocation into the cell nucleus. This whole process occurs and is regulated through a series of phosphorylation and ubiquination events (Scheidereit, 2006)[140]. Once in the cell nucleus, NF-κB dimers can bind sequences upstream of its target genes, often proinflammatory, and initiate transcription. Inhibition of the TLR4 complex reduces inflammatory signaling (Cani et al., 2007)[24] indicating the role of TLR4 in mediating inflammation. LPS is metabolized by animal tissues, especially in the liver, but a range of events and mechanisms influence LPS before and during its uptake in the circulation (Scott et al., 2009)[124].

1.3.3 Uptake and circulation of LPS

Bioactive LPS has been suggested to be continuously absorbed in low amounts from the intestine in healthy animals and humans (Nadhazi et al., 2002)[115]; (Ghoshal et al., 2009)[57]; (Ravin et al., 1960)[132]. LPS enters the circulation from the intestinal lumen through or between intestinal cells, representing transcellular or paracellular uptake respectively. LPS is part lipid and will therefore have a certain affinity for lipid transporters such as chylomicrons, LBP and other lipoproteins. Inside the intestinal cell LPS may travel through the portal vein, or through the lymphatic and then systemic circulation, as free LPS or as LPS contained in chylomicrons or LBP (Faraj et al., 2017)[48].

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As an escort of LPS, LBP which is synthesized in liver, adipose and intestinal tissue, is upregulated during inflammation and is used as a marker of LPS (Moreno-Navarrete et al., 2013)[109]. LBP can reduce inflammatory signaling due to its ability to sequester and mask LPS (Erridge, 2010b)[43], by binding to lipid A (Gutsmann et al., 2001)[62]. LPS can also be sequestered in chylomicrons eventually ending up in the general blood circulation after passing through the lymphatic circulation. In contrast, inhibition of chylomicron formation prevented LPS absorption. To which degree orally ingested LPS is able to circulate freely (unbound) is unknown but based on radiolabeling of 1 mg of LPS given orally in rats, 1 - 2 µg of LPS would be expected in blood (Ghoshal et al., 2009)[57]. A Western diet can increase paracellular infiltration or diffusion of LPS, in which LPS ends up in the liver (Miele et al., 2009)[105]. This correlates well with the outcome other studies investigating uptake of LPS (Cani et al., 2009)[25];(Peterson & Artis, 2014)[126]; (Ravin et al., 1960)[132]. However, paracellular transport of LPS has not been directly proven (Caesar et al., 2010)[21]. Mechanisms relating to permeability and influx of LPS seem to exacerbate eachother during inflammation. Either way, around 0.4% of ingested LPS is reported to be absorbed which, as with injected LPS is first detected in liver cells (Freudenberg et al., 1982)[55]; (Nakao et al., 1994)[116];; (Faraj et al., 2019)[49]. However, circulating LPS can be contained in LBP and lipoproteins (Read et al., 1993)[133], avoiding immune detection which can limit an inflammatory response (Levine et al., 1993)[91]. LPS-inhibitors in the circulation such as various proteins (Elsbach & Weiss, 1993)[38]; (Elsbach, 2000)[39]; (Bucki et al., 2005)[19]; (Drago-Serrano et al., 2012)[35; Hampton et al., 1991)[65], and antibodies (Braun et al., 2002)[16] inhibits the immune stimulatory effects of circulating LPS in contrast to proteins that enhance bioactivity of LPS (Youn et al., 2008)[177]. Furthermore, it may take up to a week for LPS to escape the lymphatic circulation, wherein LPS is exposed to a range of enzymes and other interactive components (Yokochi et al., 1989)[176]. It remains unknown to which degree heterogenous LPS retains and exerts bioactive effects before/after it has entered the general circulation (Munford, 2016)[114]. It is currently not possible to distinguish between heterogeneous LPS in the circulation. Lymph drainage, enzymatic reactions, and lipoprotein sequestering are all involved in determining inflammatory potency of circulating LPS (Lu & Munford, 2011)[94]. The majority of LPS is reported to be excreted in bile without structural alteration (Van Bossuyt et al., 1988)[161], indicating masking by lipoproteins to be dominant and not reactions that cause structural alterations of LPS.

1.4 Metabolic endotoxemia

Weight gain and diet composition is causal to the development of obesity and increases the risk for T2DM but the exact cause(s) for the induction of T2DM is unknown. Enter metabolic endotoxemia (ME), a condition defined as elevated subclinical levels of circulating LPS. Epidemiological studies connects ME to WD-induced obesity and incidence of T2DM and MetS (Gomes et al., 2017)[50], wherein low-grade inflammation is a common denominator and plasma LPS and LBP levels increases in accordance with how far MetS has progressed (Pussinen et al., 2011)[128]. LPS-mediated NF-κB activation through TLR4 is linked to inhibited insulin signaling and disrupted glucose homeostasis (Wen et al., 2011)[172]. Furthermore, WD-induced obesity is concurrent with the onset of inflammation and disrupted energy metabolism driven by chronic influx of WD-derived LPS (Erridge, 2011)34; (Kelly et al., 2012)[42]. In spite of the high bacterial load and abundancy of endogenous LPS in the colon, agonistic LPS in processed food may greatly outnumber these estimates both in availability and bioactivity (Faraj et al., 2019)[49]. In contrast to the inconsistent evidence regarding the TLR4 stimulative potential of SFAs, the ability of LPS to invoke the TLR4 signaling cascade is established (Manco et al., 2010)[99]. For these reasons and in addition to the small intestine harboring fewer bacteria and being the main site of uptake of metabolites is why the Western diet is thought to be the main provider of agonistic LPS. Others argue that consumption of animal products does not pose a risk to healthy individuals as LPS is not expected to accumulate in edible tissues (Wallace et al., 2016)[167]. Furhermore, LPS is thought to increase weight gain by disrupting both microbiota related- and unrelated apetite signalling in the digestive tract (de La Serre et al., 2015)[33]. A Western diet facilitates ME by providing agonistic LPS augmented by excessive intake of dietary fat, obesity and by the associated intestinal inflammatory environment which amplifies intestnial growth of the otherwise underrepresented Gram-negative bacteria (Pendyala et al., 2012)[124]; (Umoh et al., 2016)[158]; (Winter et al., 2013)[174]; (Erridge et al., 2010a)[42]. On that note, it is shown in mice that plasma LPS is elevated post-prandially following a high fat meal and after oral intake of oil, not water, in conjunction with LPS. This effect was less pronounced in mice on a high carbohydrate diet. Additionally, a decrease in plasma LPS was observed during fasting, further suggesting that enteric LPS may translocate transcellularly to a degree dependent on the presence of dietary fat (Cani et al., 2007)[24]. This coupled with the fact that increased demand and production of chylomicrons is a digestive response to the intake of dietary

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fat, furthers the notion that a high fat diet and lipid carriers escalates LPS absorption (Amar et al., 2008)[4]; (Moreno-Navarrete et al., 2012)[108]. This is backed up by observations of increased LBP expression in accordance with weight gain and adipocyte hypertrophy and insulin resistance in rats (Hamilton et al., 2015)[55], humans and mice exposed to a Western-like diet and LPS (Moreno-Navarrete et al., 2013)[109]; (Mehta et al., 2010)[103]. On the other hand, LPS and LBP are reported to be reduced following weight loss, which is primarily linked to a reduction in adipose tissue (Moreira et al., 2012)[107] and a fiber based diet reduced blood levels of LPS in T2DM subjects (Ahola et al., 2017)[3]. This highlights the role and importance of adiposity and diet on ME. A striking overlap amongst inflammatory markers is observed when comparing markers expressed during ME (Mehta et al., 2012)[55] and during MetS (Esser et al., 2014)[46]; (Anderson et al., 2007)[5] (Table 1.1). Inflammatory markers including LPS and LBP are notably increased post-prandially, more so in obese and in diabetics than in healthy individuals (Jialal et al., 2014)[57]; (Creely et al., 2007)[24]; (Calder et al., 2011a)[22]. However, circulating LPS levels do not always correlate with inflammatory markers (Hurley et al., 2015)[72]. In rodents, altered IAP activity is associated with a Western-like diet (Sefcikova et al., 2008)[145];(Serre et al., 2010)[146];(Kaliannan et al., 2013)[79].

Table 1.1: Comparison of features and markers exhibited in the circulation during MetS and ME. Features focused on in this thesis are highlighted. Mehta et al., (2012).

Property	MetS	Metabolic endotoxemia
Inflammation	↑ LPS, LBP ↑ Leukocytosis ↑ CRP, SAA, sialic acid	↑ LPS, LBP ↑ Leukocytosis, ↑ CRP, SAA ↑ IAP
Cytokines	↑ IL-6, TNFa ↓ IL-10	↑ IL-6, IL-12, TNFa, IL-1 ↓ IL-10
Lipoproteins	↑ LDL, VLDL, TGs ↓ HDL	↑ LDL, VLDL, TGs ↓ HDL
Glucose homeostasis	↑ Insulin, glucose, FFA	↑ Insulin, glucose, FFA

A chronically over-active immune system can manifest in the form of a cycle of repeated intestinal epithelial cell damage, disruption of intestinal barrier defences, increased permeability, dysbiosis,

proliferation and infiltration of Gram-negative LPS bacteria, damage to pancreatic beta cells (Esser et al., 2014)[46], and tissue specific impaired insulin response (Manco et al., 2010)[36]; (Song et al., 2006)[149]. The information above taken together represents how a vicous cycle of repeated low-grade inflammatory insults arising from LPS and Western diet-induced obeisty can cause T2DM (Kirwan et al., 2017)[64]; (Berg & Scherer, 2005)[10]. A whole food and plant based diet can in contrast to a Western diet, modifiy circulating levels of LPS, inflammation and metabolic disease (Brown, 2017)[17]. Without proper lifestyle and dietary interventions, systemic low-grade inflammation is thought to ensue (Gregor & Hotamisligil, 2011)[60], underpinning common NCDs (Winer et al., 2016)[173]. However, current findings paint a more vivid picture showing that there is more to this than first meets the eye.

1.5 Summary and aim of thesis

Circulating subclinical levels of LPS and LBP represents ME which lays the foundation for the Western diet and LPS as mediators of chronic low-grade systemic inflammation as a cause in initiating obesity, insulin resistance and MetS (Shi et al., 2006)[147]; (Cani et al., 2007)[24]; (Manco, 2009)[98]. It is plausible that LPS is absorbed in the small intestine (Faraj et al., 2017)[48]. However, estimates of TLR4 stimulants in food varies considerably, depending on the method of measurement (Wassenaar & Zimmermann, 2018)[171]. This is also true concerning measurements of LPS in blood which creates uncertainties when comparing studies (Gnauck et al., 2016)[58]. This casts doubt on the estimated capacity of dietary LPS to reach the intestines intact as well as the combined ability of exogenous and endogenous LPS to elevate LPS blood levels, which also varies considerably across studies (Boutagy et al., 2016)[14]. A chronic positive feedback-like loop of systemic low-grade inflammation culminating to disease is illustrated in figure 1.5, summarizing the concepts laid forth in the introduction (Rastelli et al., 2018)[131].

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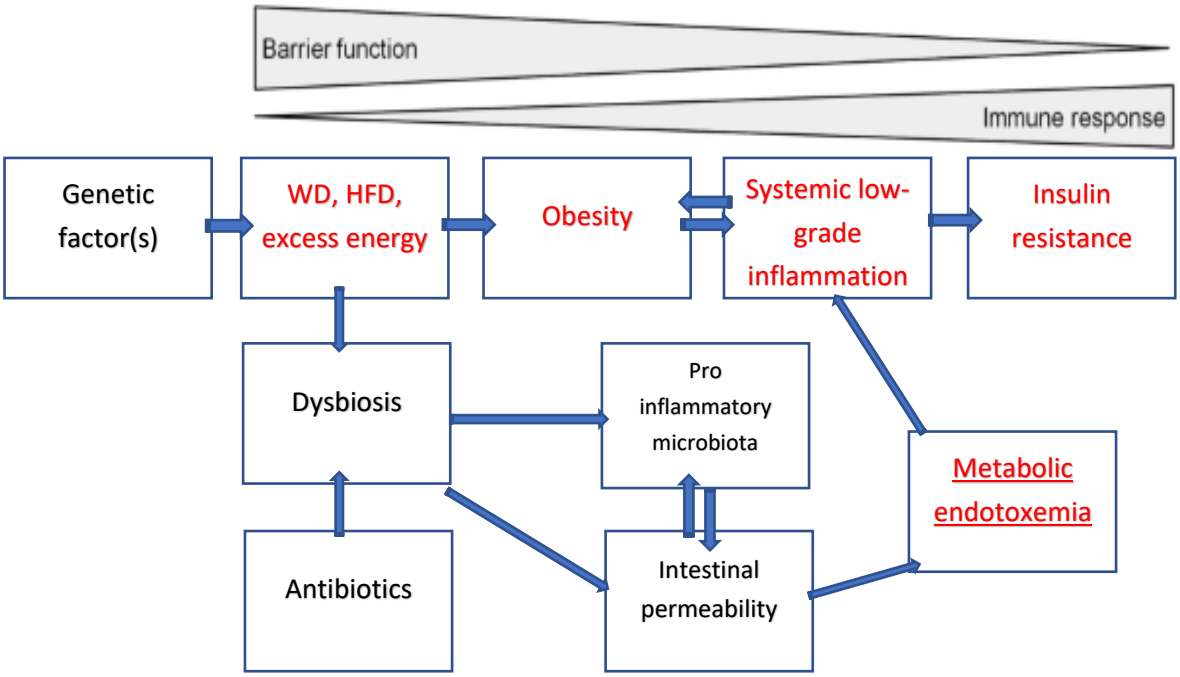

Figure 1.5: Chronic low-grade systemic inflammation. Example of how the interplay between different factors including metabolic endotoxemia, and constituent disorders of MetS can advance inflammation and the developement of insulin resistance. Factors emphasized and investigated in this thesis are highlighted. Figure modified and adopted from Martinez-Medina et al., (2014).

Studies that have looked at the capacity of LPS to initiate low-grade inflammation in humans and animals, *in vitro* and *in vivo*, have done so by administering LPS directly onto cultured cells or into the blood circulation of animals. This method excludes potential interactions and consequences the components of the digestive tract such as IAP and lipoproteins/LBP may have on LPS and the development of low-grade inflammation and how this is conveyed in the body. As such, it is believed that oral delivery of LPS does not produce the same effects as LPS delivered systemically due to the low abundance and absorption of LPS coming from food compared to endogenous LPS in the intestines (Eckburg et al., 2005)[37]. This is also based on a lack of displayed effects when LPS is given in animals (Harper et al., 2011)[67], where some concluded that chronic oral ingestion of LPS is not harmful (Oketani et al., 2001)[121], at neither small doses: 20 µg/mL and 200 µg/mL for 40 and 1 day(s), respectively (Schryvers et al., 1987)[118], nor at higher doses given repeatedly or acutely (Illyes et al., 2008)[73]; (Taniguchi et al., 2009)[153]. Additionally, anti-inflammatory and microbicidal activity is assigned to the effects of LPS (Qu et al., 1996)[129]; (Abreu et al., 2001)[1]; (d'Hennezel et al., 2017)[32]. Cani and co-workers showed principal evidence of experimentally

induced ME causing obesity and insulin resistance in mice receiving systemic infusion of LPS from *E. Coli* (055:B5) (Cani et al., 2007)[24]. In this study, blood glucose levels, both during fasting and after an oral challenge, and fasting insulin levels are elevated in LPS-infused mice on a LFD. Body weight gains were increased to the same extent as HFD fed mice, which was not due to increased energy intake. Although compared to control groups, only the high-fat diet mice showed a significant effect with respect to weight gain. Considering the evidence, oral ingestion and intestinal uptake of LPS represents how a following inflammatory response may affect insulin sensitivity and weight gain which could be augmented by dietary fat. And so we are left wondering; can oral intake of LPS in physiological relevant doses in conjunction with a Western diet overwhelm the intestinal defenses? Can potent LPS reach the circulation unaltered and spread sufficiently enough to cause ME and systemic low-grade inflammation and in turn show signs of metabolic disease?

Main aim:

To what extent will LPS given via the oral route effect obesity, metabolic endotoxemia, insulin resistance and low-grade inflammation in mice fed a Western type diet.

Sub aims:

i. Does ingested LPS in drinking water reach the lumen of the small intestine?

ii. Does long-term exposure to LPS exaggerate obesity and insulin resistance, low-grade inflammation and blood LBP levels imposed by a Western diet?

iii. Does oral intake of LPS affect IAP activity in the intestine?

iv. What is the necessary dose of orally derived LPS to induce inflammation and detectable levels of LPS in blood?

2. Methods

In this thesis, *in vivo* and *in vitro* experiments involving the use of laboratory animals and cells were carried out. Conjunctionally, a range of tests were performed to evaluate the effect of diet and oral LPS supplementation on different variables. This involved different techniques including *in vivo* imaging, insulin resistance tests, enzymatic assays, enzyme linked immunosorbant assays (ELISA), and cell based HEK-blue LPS detection assays. These aspects of the thesis will be further introduced and described on the parameters set by this chapter, in the context of the three individual animal experiments conducted during this project.

2.1 Animal research

The experiments described in this thesis included wild-type (WT) and transgenic reporter mice. 129 mice (70 male; 32 of which were luciferase positive, and 59 females; 40 of which were luciferase positive) of the species Mus musculus bred at the animal facility (NMBU) from female WT C57BL6/J mice and male NF-κB luciferase+/- transgenic reporter mice. The transgenic reporter model hail from surrogate mice (UiO) transferred with pronuclei-inserted luciferase reporter constructs. Mice with a similar age (12-22 weeks) were used in the experiments.

The luciferase gene in the transgenic mice model is a reporter gene whose production reflects NF-κB activity which is quantifiable by an optical imaging device. This model can by proxy reflect LPS-stimulated NF-κB activity. The transgenic mice model is phenotypically equivalent to the WT model.

The mice were housed in individually air ventilated cages (Innovive, USA) in numbers ranging from 2-5 mice per cage and a fed a low-fat Chow diet and water *ad libitum.* The cages were supplied with running wheels, nests and wooden bedding. Cages and equipment were regularly cleaned and changed. The animal facility was temperature- and moisture controlled (23-25°C and relative humidity of 45-55%). The facility followed a 12-hour light and dark cycle. Only certified personnel had access to the animal facility, which reduced unnecessarily stress to the animals.

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Dedicated labwear was used in the animal lab. This minimized the introduction of undesirable particles and organisms into the animal lab, as well as minimizing exposure of personnel to potential allergens. All lab surfaces and equipment used in conjunction with animals was cleaned and disinfected before and after use.

A detailed application regarding the use of animals in experiments was submitted to the Norwegian food safety authority (FSA) for approval. The three R's were emphasized in determining the minimal amount- and use of animals for research purposes as detailed in the text above.

The purpose of the project was to evaluate the role of a Western diet and oral intake of LPS on different physiological parameters and to see if corresponding LPS levels could be detected in the intestines and in blood by TLR4 transfected cells (HEK293). The hypothesis generated from available information in the literature was that a Western diet (WD) and LPS poses a chronic threat to health by disrupting homeostasis and by initiating low-grade inflammation, obesity, and T2DM. In this regard, C57BL6/J mice are a convenient model, as these otherwise healthy mice are prone to develop obesity and diabetes depending on the diet.

2.1.1 Animal laboratory diets

In comparison to the low-fat Chow diet (RM1801151, 7% E from fat), a WD (D12079B, 29% E sucrose, 38% E fat of which 2% is vegetable fat; in total 40%E from fat) is not just a diet that owe more of its energy content to fat (at the cost of energy from carbohydrates), but it is also a diet with considerably less fibre and more energy coming from refined and simple carbohydrates such as sucrose. As depicted in the table below (table 2.1) showing diet compositions, the WD contains indeed more fat and SFAs. Lauric acid (C12:0), myristic acid (C14:0), palmitate (C16:0) and the MUFA oleate (C18:1n-9c) are typically found in a Western diet. These fatty acids have a reputation in the literature due to their inflammatory effects on health, which also includes potential indirect TLR4 stimulating effects following TLR4 activation by LPS as described in the introduction. Additionally, dietary LPS can be present in uknown amounts in different animal laboratory diets

(Lindenberg et al., 2019)[92]. The major sources of fat and protein in the provided Chow diet is soy oil, and whey and soy, respectively.

Table 2.1: Diet compositions: The main protocol of the composition of WD is based on the one provided by Research Diets (RD, New Brunswick, NJ, USA). Chow diet is provided by Special diets services (SDS, Essex, UK).

WD		
RD #	**D12079B**	
Macronutrients	**gm%**	**Kcal%**
Protein	20	17
Carbohydrates	50	43
Fat	21	40
Total		100
Kcal/gm	4.70	
Ingredients	**gm**	**kcal**
Casein	195.00	780.00
Corn starch	50.00	200.00
Maltodextrin	100.00	400.00
Sucrose	341.00	1364.00
Cellulose	70.00	0.00
Milk Fat	200.00	1800.00
Corn Oil	10.00	90.00

Chow		
SDS #	**RM1801151**	
Macronutrients	**gm%**	**Kcal%**
Protein	14.38	17
Carbohydrates	66.38	76
Fat	2.71	7
Total		100
Kcal/g	3.52	
Ingredients	**gm**	**kcal**
Starch	44.97	180.00
Dietary fibre	17.05	0.00
Sugar	4.05	16.00

2.1.2 LPS administration

Purified lyophilized LPS powder from *E. coli* (serotype: O55:B5, Sigma-Aldrich) was dissolved and stirred for > 30 mintues in the drinking water in amounts corresponding to desired concentrations and volumes on a mass/volume basis (µg/mL) for each experiment as depicted in the experimental layout. LPS in solution was stored at 4°C and appeared slightly turbid. The pH of LPS in solution was determined using litmus paper and was not found to deviate from the pH of

the drinking water (pH ~ 7) that the animals are used to. The dose of LPS per kg animal is found by factoring in estimated water intake (5 mL/day) with the reference weight (in kg) set by a reference animal with an average weight of 20 g. The amount of LPS in µg/animal is found by factoring the reference weight with the dose (mg/kg). Injection volume was found by multiplying animal weight by the animal dose and then dividing by the concentration.

2.2 Experimental layout and procedures

Three experiments were carried out to investigate metabolic effects of a Western diet and LPS to see if hypotheses and evidence based on systemic administration of LPS would hold up when LPS is administered orally. Mice have higher conditioning and resistance against LPS than humans (Munford, 2010)[113], (Boes et al., 1998)[13], including higher concentrations of circulating antibodies limiting translocation of LPS (Reid et al., 1997)[134]; (Nys et al., 1990)[120]. While a dose of 1 µg/kg can be lethal in humans, mice can tolerate more than 1000-fold higher doses (Warren et al., 2010)[170]. Oral delivery of LPS was administered in physiological relevant doses by taking this information into account so that a translatable metabolic response could be elicited. In determining the design of the experiments the main points we looked to analyze for were emphasized:

Differences in body weight development, energy intake, and glucose homeostasis. Regional differences in NF-κB activity in the liver and the intestine.

Differences in luminal and plasma LPS and LBP levels, including temporal effects of LPS administered over different durations. Also, regional differences in plasma LPS levels from portal and cardiac blood which relates to the two different uptake routes through the portal vein and the lymphatic duct, implicating the preferred uptake mechanism of LPS.

Differences in duodenal IAP levels.

And any effects these changes may potentially have had on the observed behavior and appearance of mice. Ear markings were used for identifying individual mice in group-labeled cages.

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Experiment 1: Establishing tolerance of mice towards oral ingestion of LPS in the drinking water

The first experiment assessed the tolerance of WT mice (19 females) fed a low-fat Chow diet to orally ingested LPS over three different time intervals. The LPS solution (330 µg/mL, translatable to approx. 1.6-3.3 mg LPS/animal/day with an estimated water intake of 5-10 mL/day) was administered *ad libitum* through the drinking water. The experimental groups (n=4) were terminated one day (LFL1), four days (LFL4) and eight days (LFL8) after initial LPS exposure. The control group (LFW, n=7) received tap water and was terminated independent of a set time interval. Animals were weighed during the experiment in conjunction with visual inspections and marking of changes in the water levels of drinking bottles as a way of evaluating whether mice ingested the solution. Animal behavior was visually evaluated. This was based on if mice deviated from generally observed activites such as actively utilizing the running wheel, continuously consuming food and water and the absence of hunched posture, bloody stool, and other visible impairments. As there are few previous conducted experiments based on the oral delivery of LPS, it is unknown if exogenous LPS reaches the small intestine intact after passing through the first part of the gastrointestinal tract, or to which degree endogenous LPS is detectable and present in the small intestine. Luminal content was therefore sampled from the duodenal and ileal part of the small intestine after cervical dislocation of deeply anesthetized mice. LPS levels were measured in these respective regions with the HEK293 LPS detection assay (covered in 2.3.2). Schematic diagram of experimental design is depicted in figure 2.1.

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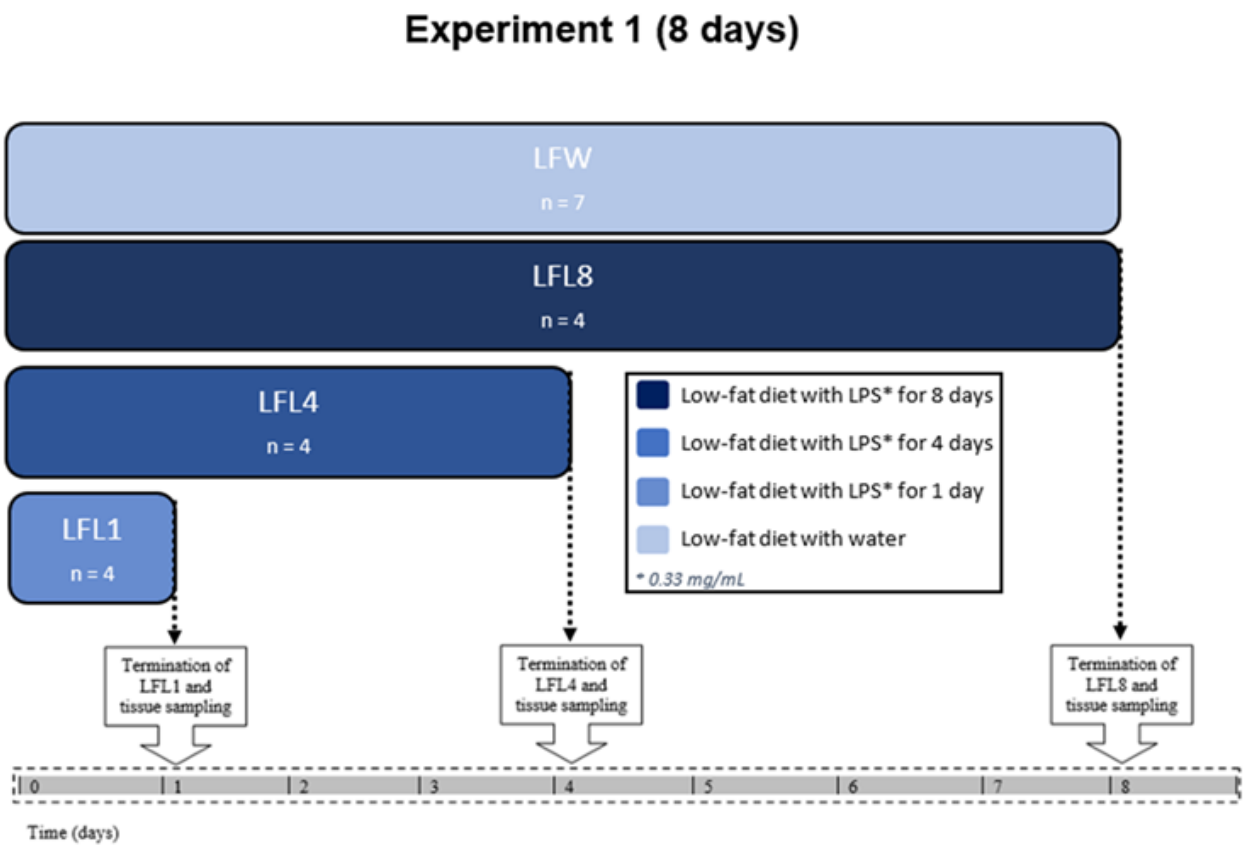

Figure 2.1: Timeline and set-up of experiment 1: Termination intervals for the different groups: LFL1, LFL4 and LFL8 were terminated at days 1, 4 and 8, respectively, followed by collection of tissue samples. Color gradient indicates duration of intervals. LFL represents a low-fat Chow and LPS diet. LFW represents a low-fat Chow and water diet.

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Experiment 2: Evaluating diet and LPS on metabolism and low-grade inflammation

The second experiment sought to explore the effects of diet on parameters of metabolic endotoxemia and low-grade inflammation. 65 male mice were divided into four groups (n=16-17). Two groups received a low-fat Chow diet with (LFL) or without (LFW) LPS in the drinking water. The two other groups received a Western diet with (WDL) or without (WDW) LPS in the drinking water. Non-LPS supplemented groups functioned as controls within the respective diet groups, and the diet groups themselves acting as controls between the two diets. LPS was administered in concentrations of 330 µg/mL (translatable to approx. 1.6-3.3 mg LPS/animal/day with an estimated water intake of 5-10 mL/day). The experiment lasted for 8 weeks, including a 2-week run-in period of the experimental diet feeding before start of LPS administration. To investigate if LPS and a Western diet caused changes in body weight and feed intake, animals and food were both weighed each week for the duration of the experiment.

The objective of the experiment was in part assessed by imaging of NF-κB activity in liver and intestines at certain time points during the experiment. The imaging was performed to observe the development of low-grade inflammation by assessing NF-κB activity through luciferase activation. Each group had eight transgenic luciferase positive mice that were included in the NF-κB imaging. Baseline imaging was done prior to the LPS start-up at day 14 of the experiment. Subsequent imaging was carried out at 1, 7, and 35 days post LPS start-up.

To assess insulin sensitivity, insulin- and glucose tolerance tests were performed at week 5 and 6, respectively. Termination of animals and tissue sampling were done after 8 weeks on the experimental diets. Cardiac blood was sampled prior to cervical dislocation of deeply anesthetized mice for the comparison of circulating LPS and LBP levels between diet groups. Duodenal tissue was sampled for comparison of IAP levels between diet groups. Intestinal lumen content was sampled for comparison of duodenal and ileal LPS levels between diet groups. Schematic diagram of experimental design is depicted in figure 2.2.

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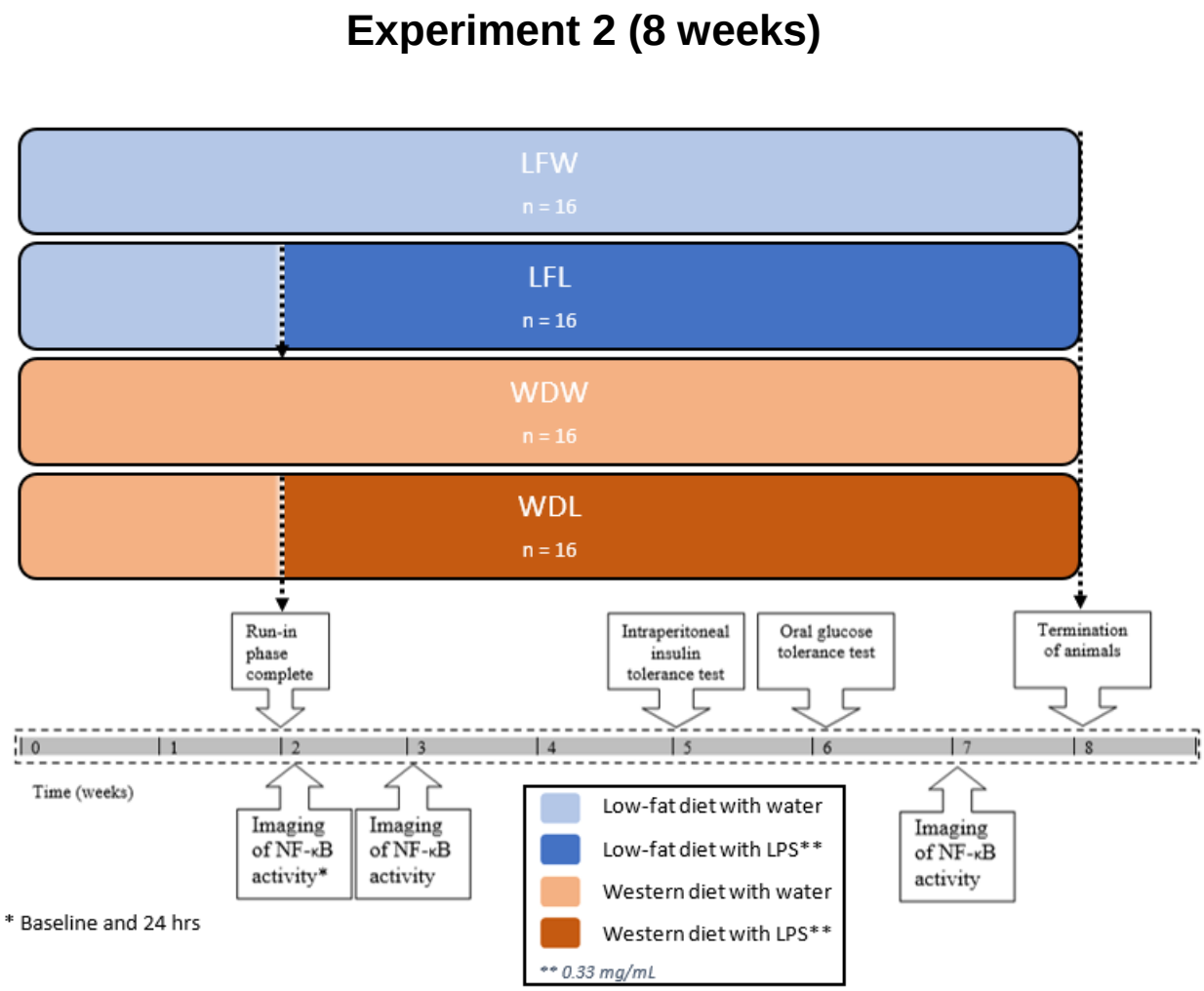

Figure 2.2: Timeline and set-up of experiment 2: Designated groups received LPS after a 2-week run-in phase (dotted line). Color gradient indicates diet. Imaging of NF-kB activity at several time points. Insulin and glucose tolerance tests were carried out during week 5 and 6, separated by one week. Termination of all groups at week 8 followed by collection of tissue samples.

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Experiment 3: Evaluating potential dose-dependent inflammatory effects of LPS

The third experiment sought to examine the capacity of LPS to induce low-grade inflammation in mice in three different doses in conjunction with a Western diet. 20 luciferase positive female mice were divided into three groups (n=5) receiving different doses of LPS in the drinking water: 1000.0 µg/mL (WDL1, 5.0-10.0 mg LPS/animal/day, assuming a water intake of 5-10 mL), 100 µg/mL (WDL01, 0.5-1.0 mg LPS/animal/day) and 10 µg/mL (WDL001, 0.05-0.1 mg LPS/animal/day). A control group (n=5) received a Western diet with tap water (WDW). Imaging of NF-κB activity was carried out at baseline and at day 1, 4, and 8. Animals were terminated, and tissues were sampled at day 8. Cardiac and portal blood was sampled prior to cervical dislocation of deeply anesthetized mice for the comparison of systemic LPS and portal LPS which relates to which of the two intestinal entry routes that is preferred by LPS before entering the circulation as described in the introduction (1.2.1). Schematic diagram of experimental design is depicted in figure 2.3.

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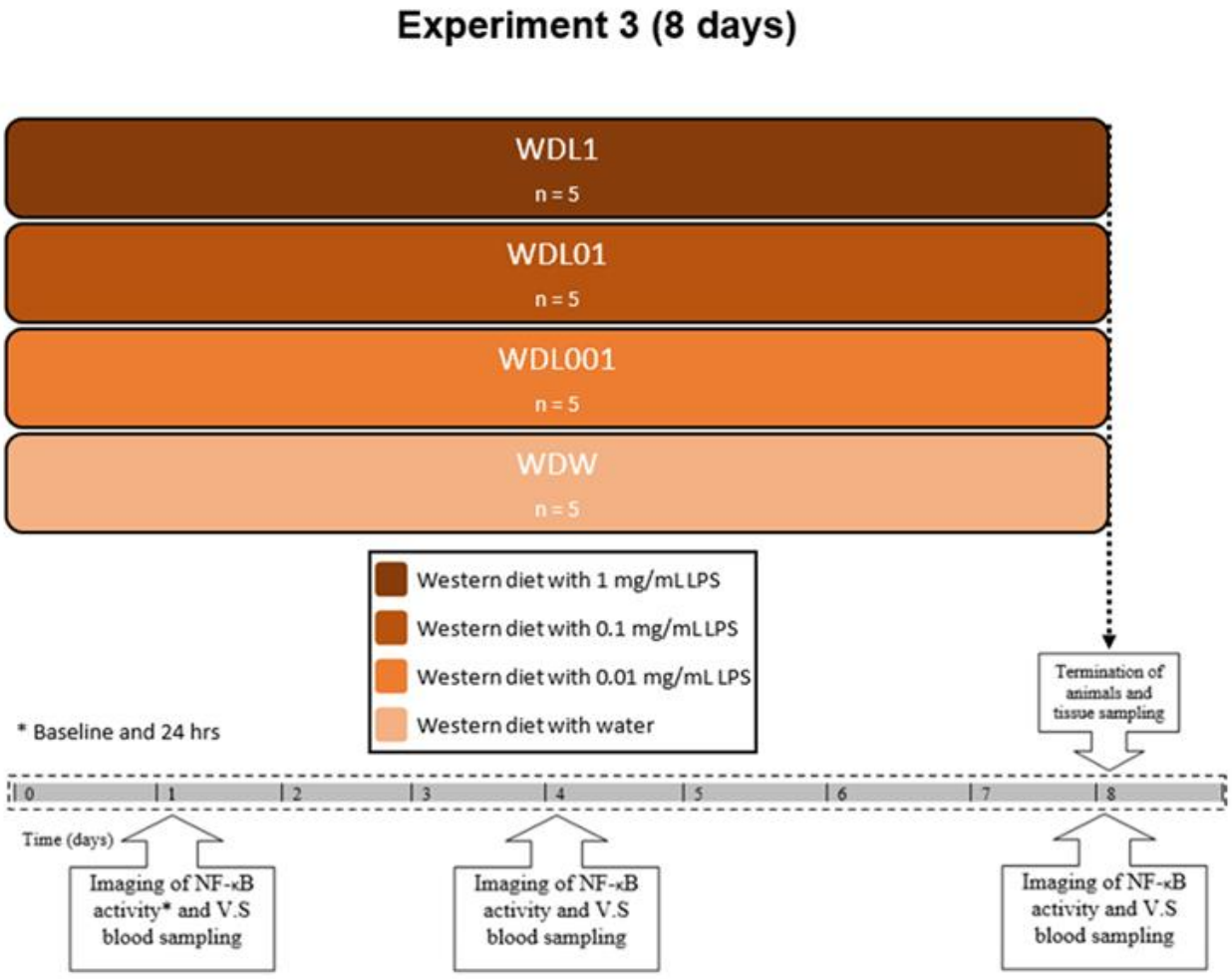

Figure 2.3: Timeline and set-up of experiment 3: Several time points for the imaging of NF-kB activity, as well as blood collection from the vena saphena. This was carried out at days 1, 4 and 8 for all groups. All groups were terminated at day 8 followed by collection of tissue samples. Color gradient indicates different doses of LPS.

2.2.1 Termination and sampling

Terminal blood and tissue samples were collected in order to shed light upon the questions raised by the three individual experiments.

Before conducting any terminal sampling, mice were deeply anesthetized using a ZRF cocktail (zolazepam, tiletamine, xylazine, fentanyl) as an anesthetic agent delivered by intraperitoneal injection (10 µl/g). Each animal was given sufficient time to let the complete effect of anesthesia set in. Complete anesthesia was checked for by confirming the absence of involuntary reflex movements as well as checking for a decline in respiratory rate. During this time period, each animal was kept warm by a heat blanket.

For terminal cardiac blood sampling, the torso of each animal was sprayed with 70% ethanol to avoid bacterial contamination of samples before systemic blood (~1 mL) was extracted in an Eppendorf tube by cardiac puncture of the heart. A 1mL EDTA coated syringe equipped with a 25G needle was utilized for this purpose. For blood sampling of the portal vein we used a 30G needle. Each blood sample was collected in an eppendorf tube which was gently inverted and immediately put on ice to halt coagulation processes. Finally, blood samples were centrifuged (4°C) for 10 minutes at 6000g before collecting blood plasma (upper phase) with a pipette, while avoiding platelets and blood cells (bottom phase). Plasma samples were stored at -20°C.

For blood sampling of the vena saphena, mice were immobilized in a modified 50 mL falcon tube before carefully penetrating the vena saphena with a small needle and collecting blood samples drop-by-drop by repeatedly applying soft pressure movements on the vein. This technique is non-terminal and was performed without anesthetization.

For tissue and lumen content sampling, the small intestine was rinsed in cold PBS after dissection. Luminal LPS content was sampled by using forceps to gently force out the content contained in the first 1-4 cm portion of the proximal duodenum. In accordance with Erridge et al. (2010a), PBS was promptly added to lumen content samples in a 1:4 ratio (weight:volume) before samples were momentarily stored on ice. Finally, lumen content samples were centrifuged (4°C) at 13000g for

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20 minutes before the supernatant was collected and subsequently filtrated with a small (4mm diameter) 0.45µm hydrophilic membrane sterile filter unit (Millex, Merck Millipore Ltd. Ireland). Filtration of lumen content was a meticolous process as filtration units would often clog during the filtration of lumen samples, which resulted in sample loss, but not so much as to hinder subsequent measurements. Lumen samples were centrifuged at 13000g for 5 minutes in an attempt to obtain a more concentrated and less turbid sample for the sake of avoiding clogging of filter units but did not resolve the issue in a notable way.

Filtrated samples were stored at -20°C. Lumen content sampling from the distal ileum and the proximal colon was carried out in the same manner.

Duodenal tissue containing IAP was collected by extracting the first 3 cm portion of the proximal duodenum. Tissue samples were stored at -80°C and were subsequently thawed, weighed and diluted in lysis buffer (8 µl lysis buffer per 1mg tissue) before being homogenized by sonication, in accordance with Serre et al. (2010). Finally, the homogenized tissue samples were centrifuged (4°C) at 10000g for 15 minutes and the supernatant containing IAP was collected and stored at -80°C.

Additional samples were collected but not analyzed in this thesis: Liver tissue samples and mucus tissue samples were collected (by using microscope glass slide coated with RNAse-zap to gently force out mucus content) with intended purpose of analyzing gene expression. Liver and mucus tissue samples were stored in RNAlater at 4°C allowing the solution to seep into the tissue before being stored at -20°C. Bacterial samples from different segments of the colon were collected and stored at -20°C with intended purpose of analyzing microbial profiles. Visceral fat tissue intended for immunohistological analysis was collected and fixated in formalin before dehydrated with 70% ethanol and embedded in paraffin wax and then stored at 4°C.

2.2.2 *In vivo* imaging of NF-κB activity in mice over time

To evaluate if diet and LPS given orally can induce low grade inflammation, NF-κB activity was measured in the intestine and liver of mice by using a non-invasive photon collecting, light proof bioluminescent *in vivo* imaging system (IVIS, Lumina II, Perkin Elmer). Treatment of mice with LPS and the development of an inflammatory response over time has been previously demonstrated to be traceable by real time monitoring of tissue specific areas in mice. Biolmuniescence generated by the reaction between the luciferase enzyme and its substrate D-Luciferin is measured as radiance (photons/second/cm^2/steradian) (Kielland & Carlsen, 2010)[84]. This system provided a non-invasive, quantitative way of recording disease progression with anatomical precision in live (unconscious) mice by detecting luciferase generated photon output at a certain wavelenght (560nm) (Zhu et al., 2017)[178]. This allowed for multiple measurements in the same animals over time so that each animal can function as its own control. This provides data over time wherein NF-κB activity is visualized by the software Living Image (PerkinElmer) with a color gradient reflecting intensity of radiance.

First, the abdominal-thorax region of mice was shaved with a razor before imaging to ensure that photon output could be sufficiently registered by the optical imaging device.
Then, the anesthetic agent Isoflurane was used for immobilization of mice in conjunction with *in vivo* imaging. Mice were anesthetized by isoflurane vapor in a sealed induction chamber connected to a gas delivery system (Xenogen XGI-8, Caliper life sciences) coupled to a gas valve for oxygen flow and an isoflurane vaporizer (Midmark Matrx). The vaporizer and the gas delivery system are also connected to the imaging chamber of the IVIS in addition to the induction chamber. Isoflurane vapor concentrations of 2.5 - 3.0% were used. The anesthesia-inducing delivery system connected to the IVIS imaging chamber, allows for imaging of up to five mice simultaneously. An evacuation pump ventilates isoflurane by directing the gas through an exhaust air system.
Anesthetized mice were then intraperitoneally injected with D-luciferin (15 mg/mL, 10 µL/g mouse) before being transferred from the induction chamber to the imaging chamber.
Luciferin facilitates a reaction with luciferase resulting in light emission which can be registered by the IVIS. Mice were monitored carefully for adverse effects during anesthetization and were momentarily removed from gas exposure if deemed necessary. Regulation of gas delivery settings

were evaluated in such cases. Time of isoflurane exposure in the induction chamber and the imaging chamber was together recorded to be approximately 15 minutes. The imaging software Living Image was used for analyzing bioluminescense intensity of the ventral surface of mice, expressed as photons/s/cm^2/steradian.

Luciferin solutions were prepared in advance by dissolving D-Luciferin powder in PBS and NaOH to a concentration of 15mg/mL before filtrating it through a 0.2µm membrane filter unit and stored at -20°C. Injection was done 10 minutes prior to *in vivo* imaging, giving luciferin time to circulate and be distributed in the body tissue.

2.2.3 Glucose homeostasis

Oral glucose tolerance test (OGTT)

In order to assess potential glucose intolerance, all animals were fasted to reduce variations in base glucose levels. Overnight fasting can enhance insulin stimulated glucose turnover (Heijboer et al., 2005)[68] and is associated with a decrease in metabolic rate of mice (Ayala et al., 2010)[6], and might induce a state of starvation instead of fasting (Bowe et al., 2014)[15]. Thus, for glycogen depletion, a six hour long fast in the morning was elected to better represent evaluation of glucose homeostasis within a physiological context. During fasting and for the duration of the test, the bedding and running wheels were removed from cages and a metal grid was placed inside the cage at an elevated position to allow for excrement to fall to the bottom, avoiding consumption of fecal energy content. A 20% glucose solution was made by dissolving glucose powder (Sigma Aldrich) in PBS. Baseline levels were measured before administering the 20% D-glucose solution. Glucose was administered orally with a 21 G feeding needle after 6 hours of fasting. Mice were weighed at the start of the fasting period and the dose (2 g/kg per 31g/mouse, 100 µL/10 g mouse) was set to the average weight (31g) of the low-fat Chow diet control group. Rate of blood glucose removal was evaluated by measuring glucose levels over time, where the shape of the curve gives information about degree of glucose intolerance. Measurements were carried out by insterting test strips laced with blood (~2.5 µL) into a glucometer (Accu-Check, Roche diagnostics) at four time points (15, 30, 60 and

120 minutes) following baseline measurement and glucose administration. Blood was sampled from the tail vein, by carefully making an incision with a scalpel blade.
Animals were divided in two subgroups for two days and the OGTT was performed with timed transitions that allowed for both subgroups to be tested the same day. Area under the curve (AUC) was computed by the trapezoid rule.

Intraperitoneal insulin tolerance test (IpITT)

Based on the same premise and procedures as the OGTT with minor alterations, the IpITT examines the response in blood glucose concentration by injecting a bolus of insulin in order to determine the sensitivity of insulin-responsive tissues in mice.
For glycogen depletion, animals were fasted for 4 hours. Animals were kept under the same conditions as during the glucose test. Baseline levels were measured 45 minutes prior to intraperitoneally injecting a set dose 240µL of an insulin solution (0.1U/mL, 0.024 U/animal, 75 µL/10 g mouse) by oral gavage. The dose was based on the average weight (32g) of the low-fat Chow diet control group. The insulin stock solution (27,5 U/mg) was diluted in PBS (pH 7.1) and 0.01M HCL to a desired concentration of 0.1U/mL at the day of the test. Glucose levels were then measured with the glucometer at four time points; 15, 30, 60 and 120 minutes following the insulin injection. The ipITT was performed in the same manner as the OGTT with regards to time efficiency.

2.2.4 Enzymatic assay: IAP

Expression of IAP is found to be regulated by the presence of Gram-negative bacteria and bioactive LPS molecules (Fawley & Gourlay, 2016)[50]. IAP activity was therefore measured to see if LPS given orally and a Western diet had an effect on IAP concentration which may in turn affect the abundancy of potent LPS molecules.
The assay we used (SensoLyte ® pNPP Alkaline Phosphatase Assay Kit**)** is based on the enzymatic conversion of a substrate solution (pNPP) wherein enzyme activity in each sample is reflected by

a yellow color development proportinal to its gradient. Enzyme activity was measured by color emission spectrophotometry. Photons are sent through each sample contained in a 96-well microplate, and a prism produces light at a certain wavelength (405nm) based on the properties of the solution in each well. The optical density of each sample is recorded as absorbance and is converted to ng/mg by using the equation given by the standard curve and by factoring in the dilution factor. The standard curve is based on known concentrations ranging from 3.1 to 200 ng/mL. Samples (50 µL) were diluted with a 1X-dilution buffer made from MQ-H_2O, 10X-assay buffer and Triton-X-100. A test run was performed and a dilution factor of 3200 was found to be sufficient. 50 µL pNPP substrate solution was added to each well after diluting all samples, and aluminum sheets were used to cover the microplate due to the light sensitive nature of the substrate solution. After 30 minutes of incubation at room temperature, a stop solution was added (50 µL) to each well, arresting further substrate conversion and color development. Finally, the optical density of samples was measured by a plate reader (Synergy H4, BioTek Instruments, inc., VT, USA). Samples and blank controls (only dilution buffer) were included in duplicate.

2.2.5 ELISA: LBP

LBP is considered a marker for inflammation and metabolic endotoxemia. Plasma LBP levels were therefore measured as a proxy for endotoxin levels.

Antibody coated microplate wells were provided in the assay kit (Biometec ELISA mouse LBP kit). These antibodies bind to LBP (free or LPS-bound) present in a diluted plasma sample (100 µL/well) during one of two incubation periods at room temperature in an orbital shaker (300rpm) for 1 hour. A washing stage preceeded the second incubation period which occured under the same conditions as during the first incubation, but in conjunction with added (100 µL) detection antibodies (HRP-labeled monoclonal mouse LBP antibodies) provided in the assay kit. This was followed by second washing stage. A color development occurred after the addition (100 µL/well) of a substrate solution which is measured colorimetrically (optical density) at 450nm by a plate reader (Spectramax) after 13-15 minutes of incubation time. A stop solution (100 µL/well) was added prior to the measurements. Aluminum sheets were used to cover the microplate due to the light sensitive nature of the substrate solution. Calculation of LBP concentrations (ng/mL) are

based factoring in the dilution factor in conjunction with concentration estimates based on a standard absorbance curve based on known concentrations ranging from 1.56 to 50 ng/mL. Samples and blank controls (only dilution buffer) were included in duplicate. Each washing step was repeated three times using 300 µL of a washing buffer (distilled water, Tween 20 (0.05 %) and PBS). All samples were diluted 1:800 with a dilution buffer (buffer kit solution and PBS) per kit instructions.

2.3 HEK Blue LPS detection assay

Adherent cells of the type HEK-293 (human embryonic kidney cells) transfected with murine TLR4 were stored, thawed and sub-cultivated/passaged according to the protocol of the manufacturer (Invivogen®). Cryogenic cell vials ($6.25x10^6$ cells/mL) were stored in a liquid nitrogen tank (-135°C). Cells were grown in tissue culture flasks (25/75 cm^2) with Dulbeccos modified eagle medium (DMEM). Medium was regularly changed due to build up of cellular debris. Included in the kit were reagents to optimize cell growth and to reduce risk of chemical and biological contamination. Reagents were added to cell medium in the following order: 5mL penicillin-streptomycin, 5mL L-glutamine, and 10% fetal bovine serum (FBS), 1mL of normocin. A 1mL vial of mixed antibiotics (selection vial) was added to half of the aliquoted medium which corresponded to medium used for maintenance of cells, the other half being used during the LPS assay. According to the manufacturer, the selection vial guarantees the continued expression of integrated transgenes present in the HEK blue cells. Cell medium and other solutions (PBS, endotoxin-free water, etc.) were pre-heated (37°C) to avoid cellular shock. Tissue culture flasks containing cells were placed in an incubator (Thermo electron) simulating *in vivo* conditions by maintaining a constant temperature of 37°C and 5% atmospheric CO_2 and a relative humidity of 95-98% to avoid condensation of cell medium. To check for contamination, cell medium flasks and other solutions were regularly examined under visible light while tissue culture flasks were assessed with the microscope. Handling of cells and associated equipment was done aseptically using a 70% ethanol solution and dedicated lab wear.

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HEK-blue cells were centrifuged at 200 g for 5 minutes. Desired cell density (cells/mL) was based on average counts of living cells estimated by the cell counter (Cell countess, invivogen®) using trypan blue. Desired number of cells was determined by dividing the number of desired cells by the average cell density. Table 2.2 shows typical cell density and volumes used.

Table 2.2: Typical numbers of cell density (cells/mL), and volumes used for DMEM and PBS during passaging of cells.

Type of tissue culture flask	Surface area (cm^2)	Cell density at ideal confluency (50-80%) (Cells/mL)	Cell seeding density used for sub-cultivation (Cells/mL)	Volume of cell medium (DMEM) added (mL)	Volume of PBS used (mL)
T25	25	$5.0x10^5$ - $8.0x10^6$	$2.0x10^5$ - $4.0x10^5$	3.0-10.0	1.0-2.0
T75	75	$8.0x10^5$ - $2.0x10^6$	$4.0x10^5$ - $1.0x10^6$	10.0-20.0	7.0-10.0
T175	175	$6.0x10^6$ - $1.0x10^7$	$3.0x10^6$ – $5.0x10^6$	20.0-35.0	10.0-20.0

To see if corresponding LPS levels could be detected in the intestines (in the duodenum, ileum and distal colon) and in plasma, an LPS detection assay (Invivogen HEK-Blue™ LPS Detection Kit2) based on TLR4 transfected cells (HEK293) was used. Higher endotoxin values are expected in the intestines, especially in the colon due to the great abundance of Gram-negative bacteria.

Endotoxin activity in murine plasma samples or stored and pre-prepared lumen samples were determined by colorimetric quantification of biological LPS activity. Upon the binding of LPS to TLR4 present on the cell membrane of TLR4 transfected HEK293 cells, a cellular signal cascade is generated which stimulates the NF-κB pathway resulting in nuclear translocation of the transcription factor NF-κB from the cell cytosol. Once in the cell nucleus, NF-κB will stimulate the transcription of targeted pro-inflammatory genes (TNFα, IL-1β, IL-6, IL-12, IFN-β) and a secreted embryonic alkaline phosphatase (SEAP) reporter gene. The transcribed gene (SEAP) reacts with the detection medium Quanti-blue (QB) and the resulting blue/pink/purple color development and change in optical density can be measured with a spectrophotometer at a wavelength of 620-655nm. The recorded absorbance is proportionally related to the amount of LPS

in a sample, and the concentration of LPS can be calculated as endotoxin units (EU) by using a standard curve based on endotoxin standard samples of known concentrations ranging from 0.004 EU/mL to 1 EU/mL. The provided endotoxin standard was vortexed for several minutes before use, as endotoxin adheres to labware. 1 EU is approximately equal to 0.1 to 0.2 ng/mL LPS.

After thawing and diluting samples, they are transferred (20 µL) in duplicate to a 96-well microplate. Additionally, 20 µL endotoxin free water (EFW) and 20 µL endotoxin standard spike solution (ESSS, 0.1 EU/mL) is transferred to separate sample wells in duplicate which represents unspiked and spiked samples, respectively. Spiked samples are used for estimating assay inhibiton. Blank samples containing only EFW (40 µL) were included. Next, HEK-293 TLR4 cells are transferred (160 µL) at a density of $1.0x10^5$-$5.0x10^5$ cells/mL to all wells. 7 mL of PBS was used for detatchement of cells cultured in tissue flasks (T75) after aspiration of cell medium per kit instructions. 10 mL of PBS is used for washing the cell monolayer prior to detachement. Lastly, the microplate is incubated for 18-24 hours before 20 µL of supernatant from each well is transferred to a new microplate together with pre-warmed (37°C, 30 minutes) QB (180 µL). After an incubation period of 3 hours (37°C), the absorbance of samples is measured by a microplate reader (Synergy H4) at 620-655nm. Aluminum sheets were used to cover the microplate during the final incubation period due to the light sensitive nature QB.

Transfer of cells was carried out in the same manner as during cell passaging (see passaging section above). Estimation of cell density was estimated by using a cell counter (Cell countess, invivogen®). A QB solution is prepared by dissolving QB powder into a sterile flask together with 100 mL EFW which is warmed at 37°C for 30 minutes before being filtrated (0.2 µm membrane unit) and stored at -20°C.

Method refinement:

Two issues were consistent with the LPS detection assay. Negative control samples (groups receiving only water/PBS) would have lower absorbance values than blank samples which yields negative endotoxin concentration values. Negative controls are thought to have normal (low) levels of circulating LPS. This was also the case in plasma samples of groups receiving LPS orally.

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Furthermore, we recorded high variations in measured absorbances in the same spiked-unspiked samples ranging from high to very high for indicating that there was so much substrate present that the TLR4 units are completely saturated and the true endotoxin value remains unclear as long as this variation in absorbance is high and outside the range range of the standard curve.

Due to the inability to detect plasma LPS in our samples of mice receiving LPS orally, the validity of the assay kit was questioned. Mice (4 WT female) were therefore divided in two groups, injected intraperitoneally with either 100 µL LPS (500 µg/mL) or PBS to serve as positive and negative controls. This was done to identify if said issues were related to the assay kit or to the oral intake of LPS. The dose (2.5 mg/kg) set to reference animal at 20 g. Cardiac blood was sampled 5 minutes post LPS/PBS administration of all subjects, in mice anesthetized with ZRF (10 µL)/g).

Attempts were made to circumvent or minimize assay inhibition. One approach was to heat treat samples for 10-45 minutes prior to running the assay in order to prevent inhibition. Attempts at heat treating samples at 45, 75 and 100°C did not notably improve assay inhibition. The manufacturer defined the threshold for assay inhibition as samples with an absorbance equal to the absorbance of the spike ± 25%. Due to the high variation and absorbance of samples, the estimated inhibition would be very high in either directions. Another approach was to let the cells be incubated in the microplate one day in advance before running the assay, in case the issue is related to cell stress.

As all attempts at avoiding inhibition was insufficient and due to the true absorbance value remaining unclear, we found it necessary to specify a dilution factor for individual samples, which depending upon the absorbance value of the sample could be imply a dilution anywhere from a factor of 1:10 to 1:2000.

2.4 Statistical analyses

Statistical analyses offered by the software Graphpad Prism 8 were employed in determining statistically significance. Significance threshold (alpha) is commonly limited to a level of 0.05 in biological sciences, i.e. we accept that 5% of the times results may be due to chance, or in other words, 95% of the time we may have the correct inferral. The statistical test to be used depends on several conditions, such as distribution of data, sample size, and variability of data (SD). Hence, we first evaluated if recorded data followed a normal distribution. This refers to data having a certain frequency of having a value close to the mean. SD refers to the tendency of data values to deviate in either directions from the mean value. Analysis of variance (ANOVA) models are used for comparison of more than two groups for normally distributed or non-normally distributed data. A post-hoc multiple comparisons test was used for precise group-group comparison of differences between all groups. This sets a stricter criterion for claiming statistical significance by employing a maximum allowed threshold of how much group means can vary from each other. In the case of non-normal distributions, and as not to jump the gun on non-parametric tests, extreme outliers were evaluated in addition to logarithmic transformation of data values. These are ways to compensate for asymmetric data. This assumes that other criteria are met such as similarity in SD across groups (no more than ~4 times the SD is suggested) (Motulsky, 2015)[111]. Suffice to say that the mathematics of logarithms can allow for more equally spacing of the transformed data (Motulsky, 2009)[110]. Alpha level was in all cases set to 0.05. All data are presented as mean ± standard error of the mean (SEM), unless specified otherwise. Significant results are stated with P-value.

3. Results

3.1 Mice tolerate LPS given orally in the drinking water for eight days and LPS reaches the small intestine

As a preparation for a long-term experiment with LPS in the drinking water, we first tested whether mice would tolerate the chosen dose of 330 µg/mL, which theoretically will give a dose of 1.5 – 3 mg of LPS per day per mouse assuming a water intake of 5-10 mL/day. This dose was chosen because the content of TLR4 stimulants in food can range from 1 ng-1 µg per gram food, surpassing that of endogenous TLR4 stimulants present in the small intestine. Furthermore, 0.4% of orally ingested LPS has been reported to be absorbed into the portal circulation (Faraj et al., 2019)[49]. Based on this, the chosen dose corresponds to 6.6 µg of absorbed LPS which is equivalent to eating 660 g of food containing 10 ng/g LPS. This is correlatable to humans, as mice are less suceptible to effects of LPS by a factor of 1000 and more, meaning, it should be comparable to the pg-ng subclinical range in humans. Mice on a low-fat Chow diet were divided in three groups exposed to LPS orally in the drinking water for one day (LFL1), four days (LFL4) or eight days (LFL8). The control group (LFW) received only water.

Intake of LPS was evaluated by visually observing the water level in bottles at day 2, 4 and 8. From these observations we concluded that water intake was similar in mice receiving LPS and only water. To exclude any impact of general well-being of the mice, we both weighed the mice and monitored them for behavioral abnormalities. Weight development was not affected (data not shown). Mice in all groups were visually observed to be active, in the form of being continously; curious and alert, consuming food and water, and using the running wheel without any visual impariments. Thus, mice tolerated oral consumption of LPS in the drinking water.

We next measured the luminal levels of bioactive TLR4 stimulating LPS in both duodenum and ileum (Figure 3.1). LPS treated mice displayed higher luminal LPS levels than controls (LFW) in both duodoenum and ileum. LPS treated mice displayed higher luminal LPS levels in the duodenum at day 4 ($P = 0.02$) and 8 ($P = 0.69$).

RESULTS

In the ileum, LPS treated mice displayed higher luminal LPS at day 1 ($P < 0.0001$), 4 ($P = 0.01$) and 8 ($P = 0.003$). Thus, LPS given orally in the drinking water reaches the small intestine.

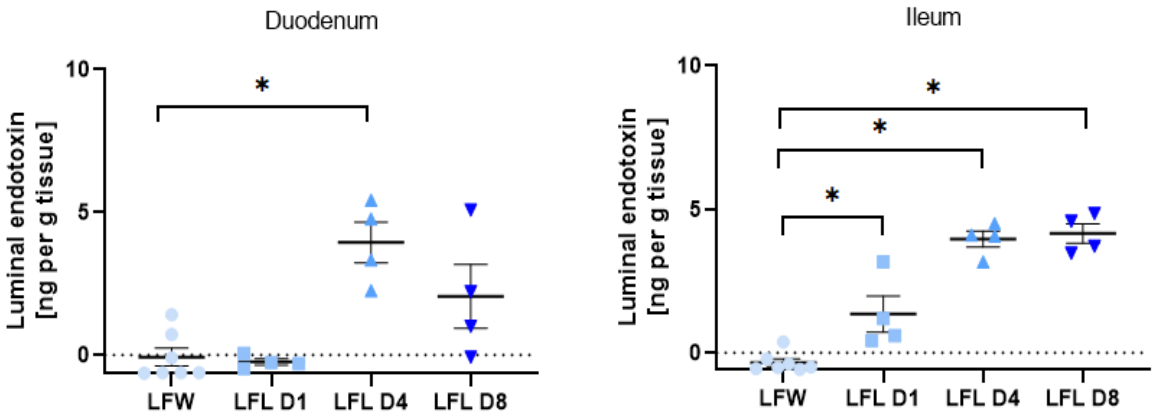

Figure 3.1: Oral administration of LPS reaches the small intestine (duodenum and ileum respectively) and elevates endotoxin levels (ng/g). Endotoxin concentration measured at day one, day four, and day eight after initial LPS exposure (n= 4,4,4). The control group (LFW, n=7) was given tap water and terminated independent of a set time interval. Data are expressed as means (± SEM). Analyzed by two-tailed t-test between control (LFW) and each intervention group. * = $P < 0.05$.

RESULTS

3.2 Effect of orally administered LPS in mice fed a western diet for eight weeks

Body weight and food intake is not affected by LPS

Since we established that the dose of LPS (330 µg/mL) was tolerated and gave appreciable high levels of LPS in the small intestine, we fed mice with Western diet or regular Chow (low fat) supplemented with or without LPS in the drinking water for eight weeks. The weight of mice and food intake were recorded each week for the duration of the experiment. As expected, both groups of mice fed WD gained significantly more weight than the corresponding Chow fed mice (P = 0.0001). However, LPS was not found to influence weight gain in either WD fed or Chow fed mice compared with corresponding control mice given water (P = 0.99) (Figure 3.2 A). In accordance with the weight gain, WD fed mice displayed similar and the highest mean energy intake throughout most of the experiment although only differing statistically between low-fat Chow diet mice and the WDW group the first week (P = 0.014) (Figure 3.2 B). No statistical effect of LPS was found within respective diets with respect to: Changes in body weight, total body weight gain (data not shown), and weekly and total energy intake (data not shown).

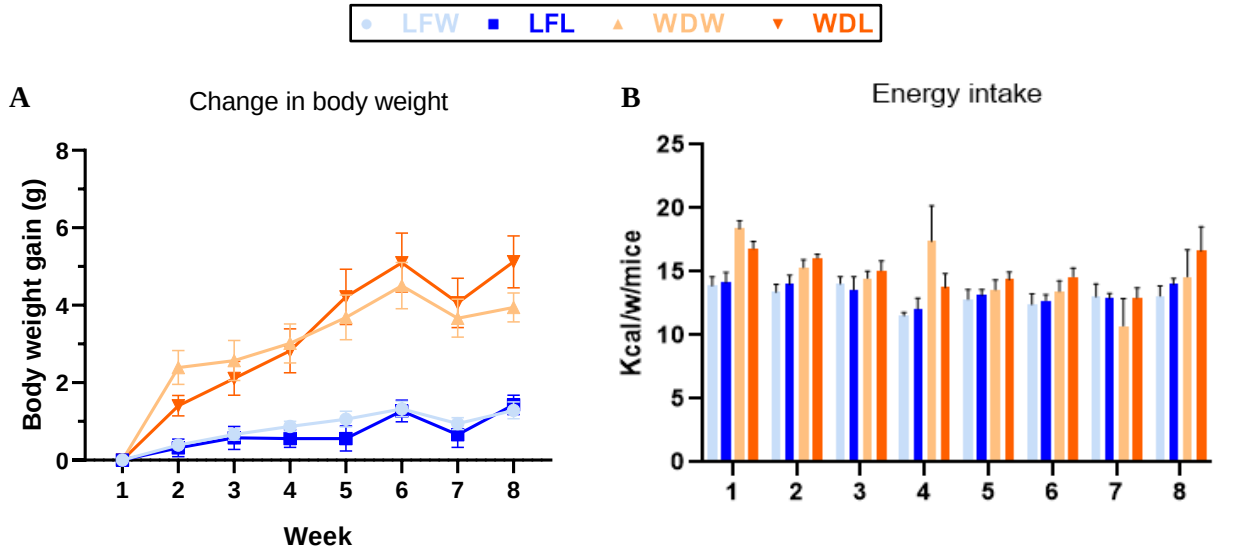

Figure 3.2: Change in body weight and energy intake.
A: Weekly change in body weight (g) corrected for uneven initial weight between groups. **B:** energy intake (kcal/w/mice) of all groups after 8 weeks. n = 16-17. Data are means (± SEM). Analyzed by two-way ANOVA with Tukey's multiple comparisons test and mixed effects model, respectively.

RESULTS

Oral glucose tolerance test (OGTT) and i.p. insulin tolerance test (ipITT) showed no effect of LPS

We next evaluated if the LPS supplementation affected glucose homeostasis and insulin sensitivity using OGTT and ipITT as viable tests.

During OGTT, all groups experienced an inital spike in blood glucose levels before returning to baseline values (Figure 3.3. A). There were no significant difference in blood glucose levels between or within diets and intervention groups ($P = 0.22$). There were no differences in AUC levels across diets and intervention groups ($P = 0.44$) (Figure 3.3. B).

Based on the same premise as the glucose tolerance test, the insulin tolerance test examines the response in blood glucose conentration after the mice were injected with a bolus of insulin (ipITT). Curiously, we found a significant difference in fasting blood glucose levels between WD-fed mice and LF-fed mice ($P \leq 0.015$; Figure 3.4 A), which was not found when we used the same mice in the OGTT-procedure. However, when assessing the the whole time period from baseline to two hours post insulin injection (AUC), we could not find significant differences between any of the groups with respect to insulin tolerance ($P \geq 0.07$; figure 3.4 B).

RESULTS

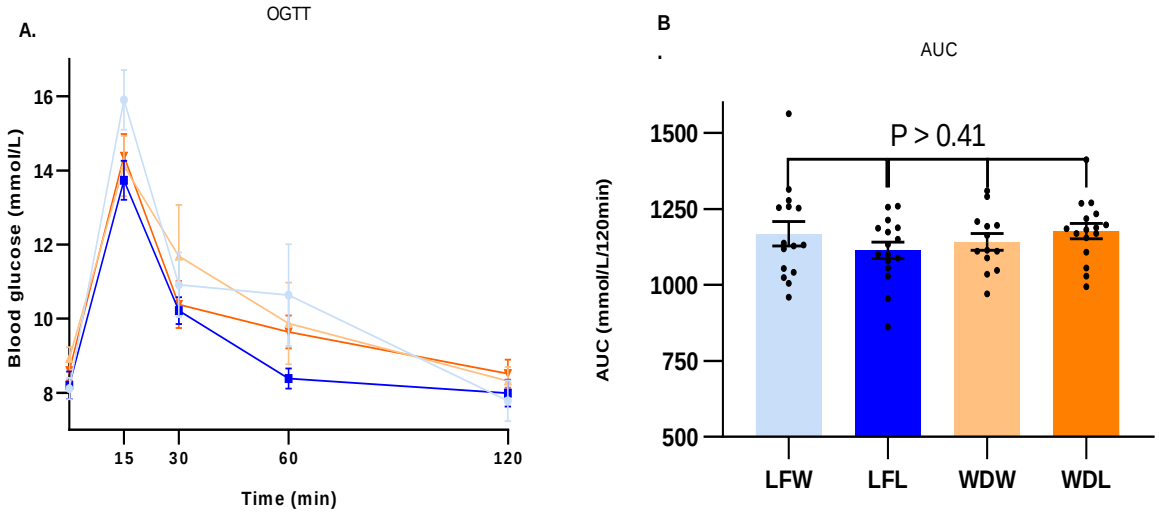

Figure 3.3: OGTT: **A:** Blood glucose levels (mmol/l) at different time points (baseline, 15, 30, 60, 120 minutes after glucose administration by oral gavage, 2g/kg) of all groups during OGTT. Analyzed by two-way ANOVA. **B:** Area under the curve (AUC). n = 16-17. Analyzed by one-way ANOVA. Data are means (± SEM). Rout's test (Q = 1%) were used for identifying outliers.

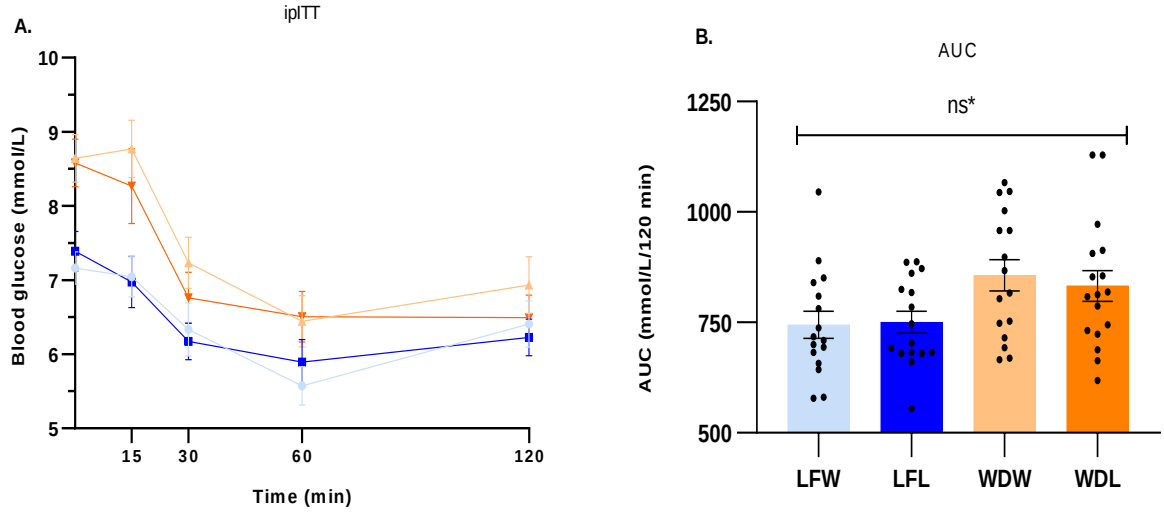

Figure 3.4: IpITT: **A:** Blood glucose levels (mmol/L) at different time points: baseline, 15, 30, 60, and 120 minutes after insulin administration by oral gavage (0.75IU/kg). **B:** Area under the curve (AUC). n= 16-17 mice. Data are means (± SEM). Analyzed by two-way ANOVA with Tukey's multiple comparisons test, and one-way ANOVA, respectively. *A significant difference (P = 0.032) was found in mean between all groups but not within or between any specific groups.

RESULTS

Inflammation and NF-κB activity is upregulated short term by LPS and a WD

As we could find no effect of LPS given orally on the parameters of MetS, we investigated the low-grade inflammatory inductive capacities of a Western diet and LPS (330 µg/mL) given through the drinking water. NF-κB is a transcription factor and has a key role here as a marker of inflammation. TLR4 can be stimulated by LPS causing a transfer of intracellular signals leading to activation of NF-κB. Transgenic luc+ mice express luciferase under the the control of binding sites for NF-kB. Hence increased luciferase will reflect NF-kB activity. Luciferase reacts with injected luciferin (15 mg/mL, 10 µg/g), which is oxidized leading to emission of light that can be detected by bioluminescence imaging. To assess NF-κB activity during the whole period of eight weeks, we imaged the mice at baseline, day one, day seven and day 35. Emission of light from the intestinal and liver regions were assessed by counting photons from selected regions of interest (ROIs; expressed as photons/second/cm^2/steradian, figure 3.5). A Western diet and LPS given orally significantly elevates NF-κB activity 1 day post administration (Figure 3.6), and in contrast to the intestine, this effect is seen in the liver to be between Western diet fed mice with and without LPS. Intriguingly, we found a significant upregulation of NF-kB activity from the liver region in mice fed WD + LPS compared with WD and water. When we compared the same in Chow fed mice, no significant effect of LPS was found.

Oral administration of LPS (330 µg/mL) was not sufficient to differentially elevate NF-κB activity long term. WD mice displayed slightly higher NF-κB activity than low-fat Chow diet mice, mainly the first day in both the liver and the intestine, before declining. In the intestine a difference in NF-κB activity was found at day 1 between LFW and WD fed mice ($P \leq 0.03$) and between LFL and WDL ($P < 0.0001$) and at day 35 between LFL and WDW ($P = 0.003$). In the liver, a statistical difference ($P \leq 0.002$) was found at day 1 between WDL and all other groups.

RESULTS

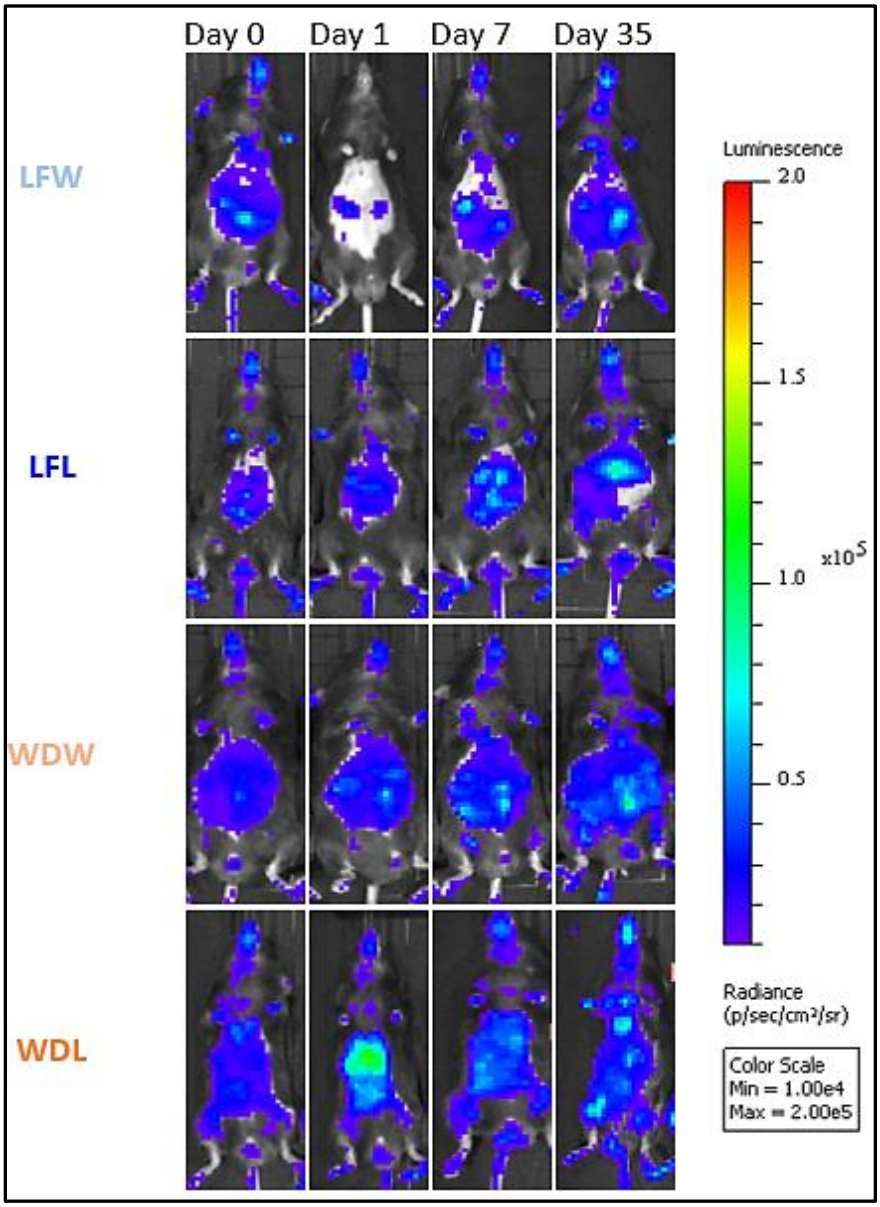

Figure 3.5: In vivo imaging of NF-κB activity during 35 days of LPS administration in the drinking water. Representative images of one animal/group. Baseline (D0) acts as control for each animal. Intensity of luciferase induced bioluminescence is indicated by the color bar measured as radiance (photons/sec/cm2/sr). Photon emission in region of finterests (ROIs) represents NF-κB activity in the intestinal region and liver region.

RESULTS

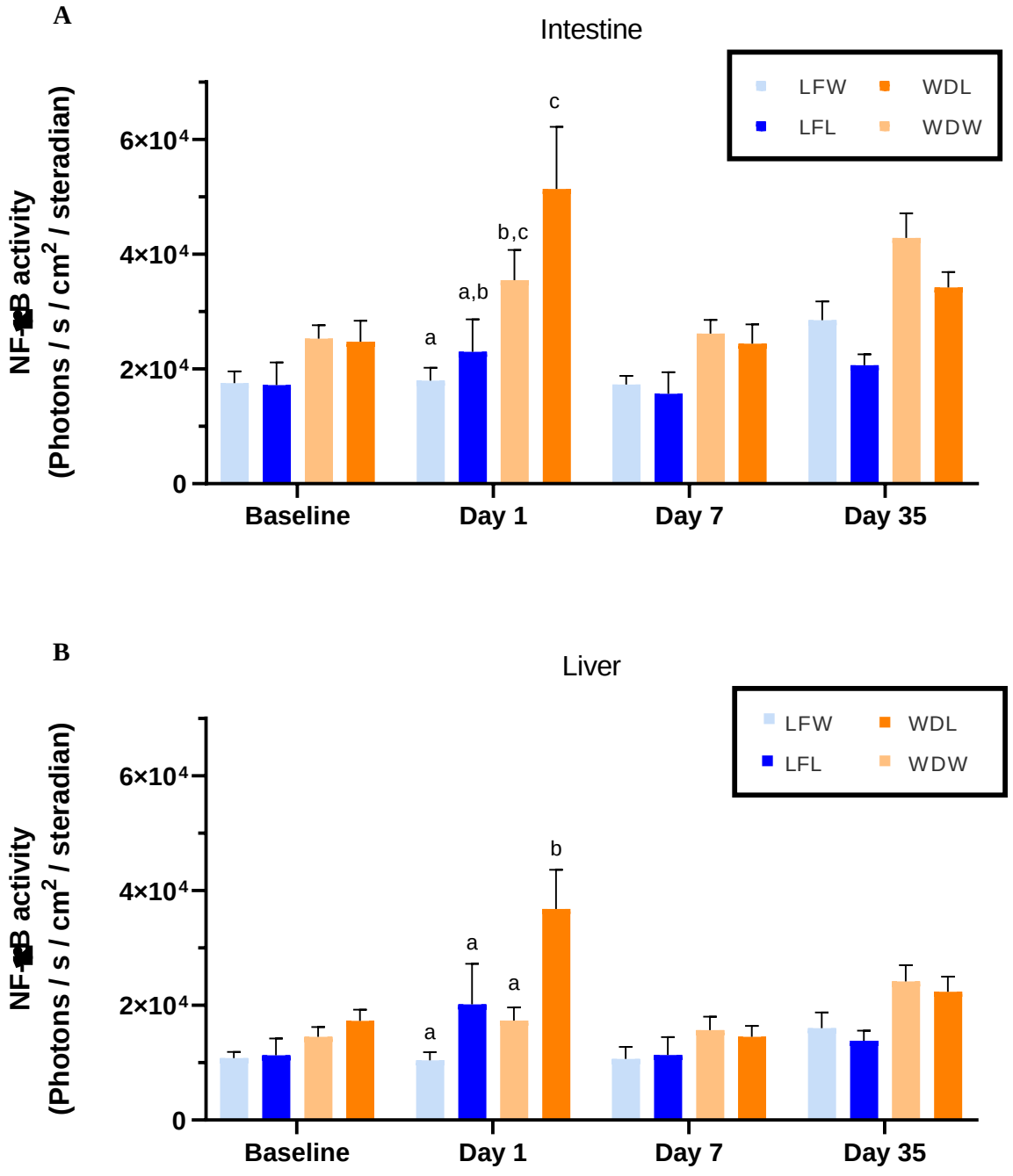

Figure 3.6: *In vivo* imaging analysis: Luciferase induced bioluminescence reflecting NF-κB activity (photons/s/cm^2/steradian) calculated from average radiance in the intestine (**A**) and in the liver (**B**) of mice at different time points. Baseline was measured 24hrs before LPS start up. n = 16-17. Data are means (± SEM). Analyzed by two-way ANOVA with Tukey's multiple comparisons test. Mean values with differing letters indicates a statistical difference ($P < 0.05$).

RESULTS

Endotoxin is elevated in the intestine of treated mice but LBP levels are unaffected

Following eight weeks of feeding, we hypothesized the LPS treated mice would have higher plasma LPS levels compared with mice receiving water. We also hypothezied that a Western diet receiving water would elevate LPS levels in plasma.

To our surprise LPS was not detectable in blood using the LPS detection assay in any of the experimental groups (data yielded negative concentration values and is not shown). To validate the HEK293 LPS assay for detection of LPS in blood, mice were intraperitoneally injected with LPS (50 µg per mouse in 100 µL) to serve as positive controls for comparison with negative controls receiving PBS. The LPS detection assay showed significantly higher LPS blood levels (ng/mL) in the positive controls compared to negative controls (P = 0.0045; Figure 3.7).

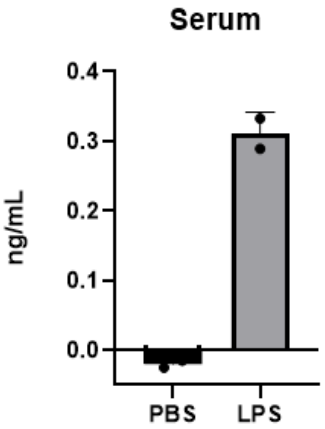

Figure 3.7: Serum endotoxin levels: Endotoxin units (ng/mL) in blood is elevated in mice intraperitoneally injected with LPS and not in mice injected with PBS. Blood was sampled by cardiac puncture within 5 minutes of LPS or PBS injection (n=2). Analyzed using Student's t test. Data are means (SD).

We then investigated whether levels of plasma LBP (ng/mL) was changed. LBP is considered a surrogate marker for the measurement of LPS, since its function is to detect LPS in blood and guide LPS to target cells. However, when measuring plasma LBP no major differences were found between any of the groups (Figure 3.8).

We conclude therefore that LPS given in drinking water at the given dose (330 µg/mL) is not detectable in blood and does not elevate plasma LBP levels.

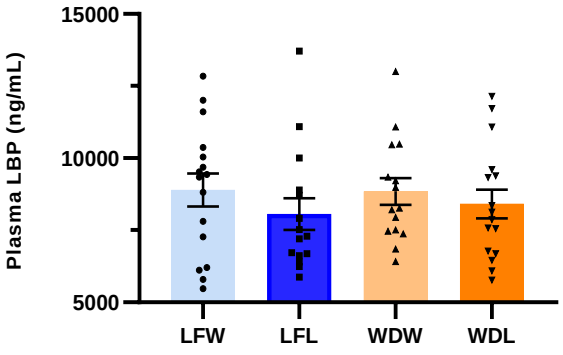

Figure 3.8: Plasma LBP levels: Oral administration of LPS did not alter plasma LBP levels (ng/mL). Sampled by terminal cardiac puncture. n = 16-17. An extreme outlier (23348 ng/mL) in the WDW group was removed using Rout's test (Q = 1%). Analyzed by one-way ANOVA. Data are means (± SEM).

RESULTS

To verify that LPS was in fact present in the intestine following ingestion, we measured luminal LPS levels in the duodenum and ileum of the small intestine and in the distal colon using the HEK293 cells.

As shown by figure 3.9, a difference in luminal endotoxin levels (ng/g) was only found between LPS-supplemented groups and the untreated groups ($P \leq 0.01$), wherein the LPS treated gorups had significantly elevated endotoxin levels, in both duodenum and ileum.
We therefore conclude that 330 µg/mL LPS given orally reaches the intestines in both Chow- and WD fed mice, without differing between these groups.

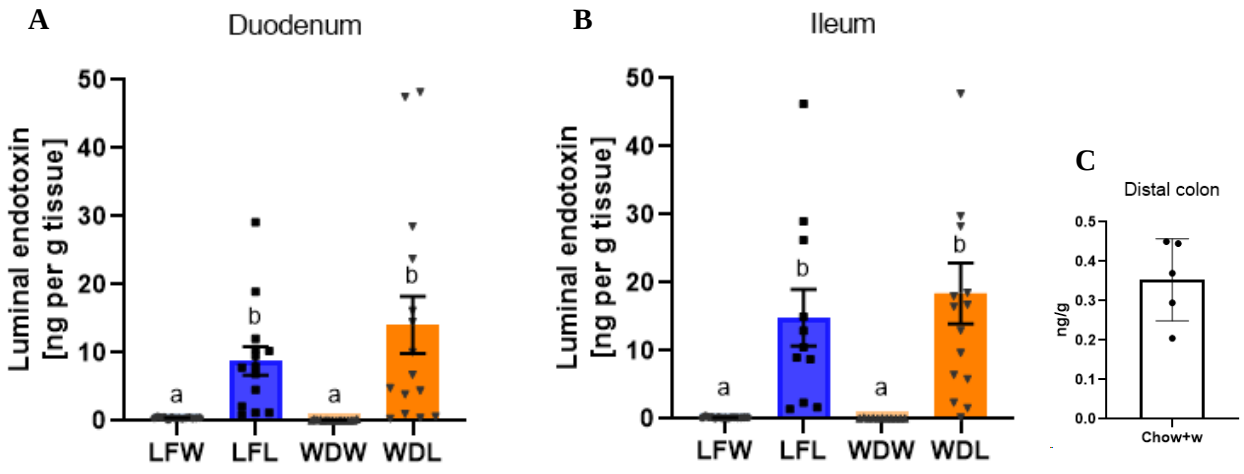

Figure 3.9: Endotoxin levels (ng/g) in the duodenum (**A**), ileum (**B**) (n=16-17) and (**C**) colon (n=5). Colon samples are from mice fed a low-fat Chow diet and water. Rout's test (Q = 1%) were used for identifying outliers. Data are means (± SEM). Analyzed by one-way ANOVA with Tukey's multiple comparisons test. Two groups (WDW, duodenum & WDL, ileum) did not pass D'agostino & Pearson normality test (P = 0.0088 and 0.0257). Negative concentration values were excluded. Mean values with differing letters indicates a statistical difference ($P < 0.05$).

RESULTS

IAP levels

Since levels of intact LPS can be modified along the small intestinal tract, we measured the levels of intestinal alkaline phosphatase (IAP), which is highly expressed in the proximal part of the small intestine. IAP expression and enzymatic activity is reported to be regulated amongst other by the presence of Gram-negative bacteria and bioactive LPS molecules. IAP can neutralize LPS molecules by removing its phosphate groups and thereby reduce the capacity of LPS to interact with TLR4. IAP levels was therefore measured to see if LPS given orally with or without a Western diet influenced IAP enzymatic activity in the duodenum.

Mice on a Western diet displayed slightly higher IAP levels (ng/mg) than Chow fed mice, but this was not statistically significant ($P \geq 0.051$; Figure 3.10).
A Western diet and 330 µg/mL LPS given orally does not elevate IAP levels.

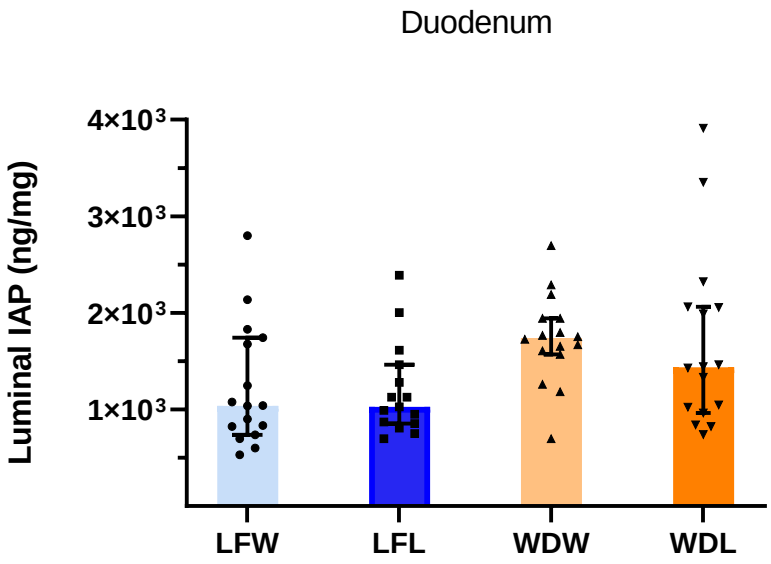

Figure 3.10: Oral administration of LPS did not alter luminal IAP levels (ng/mg) in the duodenum. n= 16-17 mice. Data are medians (with 95% CI). Analyzed by non-parametric one-way ANOVA with Dunn's multiple comparisons test.

RESULTS

3.3 A higher but physiologically relevant dose of LPS given orally is insufficient to detect LPS in plasma and for induction of systemic inflammation in mice

After finding no effect of oral supplementation of LPS (330 µg/mL), we investigated if higher doses (100 and 1000 µg/mL) of LPS given orally in the drinking water would yield detectable and elevated changes in systemic LPS levels and systemic inflammation reflected by NF-κB activity. This was investigated short term over 8 days. LPS levels in plasma from portal blood and vena saphena was assayed using TLR4 transfected HEK293 cells. NF-κB activity was measured by *in vivo* imaging. Still, the LPS detection assay was unable to detect LPS in blood in these doses (data yielded negative concentration values and is not shown).

As shown by figure 3.11: 100, and 1000 µg/mL of LPS given orally in mice fed a WD for eight days significantly elevates NF-κB activity 4 days post LPS administration in the intestine ($P = 0.036$) and liver ($P \leq 0.0009$). A small but statistical significant elevation in NF-κB activity is observed in the liver at day 8 ($P = 0.028$). An elevation in NF-κB activity is also seen at day 1 in both regions although without statistical significance.

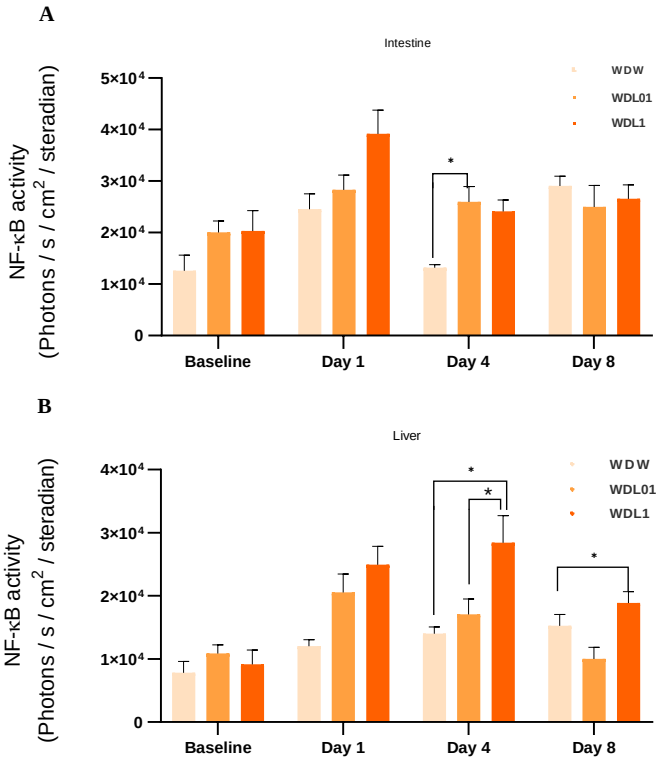

Figure 3.11: *In vivo* imaging analysis: Luciferase induced bioluminescence reflecting NF-κB activity (photons/s/cm^2/steradian) calculated from average radiance in the intestine (**A**) and in the liver (**B**) of mice at different time points. Baseline was measured prior to LPS start up. n = 5. Data are means (± SEM). Analyzed by two-way ANOVA with Tukey's multiple comparisons test. * indicates a statistical difference ($P < 0.05$). Data not shown for WDL001.

4. Discussion

Metabolic endotoxemia (ME) is suggested as a causal trigger of obesity and T2DM which are considered leading causes of death globally, part of an ever increasing epidemic. Reported inflammatory effects of LPS is often based on systemic delivery while effects of LPS during oral delivery remains to be determined. Still, the Western diet eating pattern with excess intake of dietary fat and SFAs is linked to low-grade inflammation, ME and chronic disease. As established by many studies, the TLR4 stimulatory effects of LPS is established, but contra to injection of LPS, oral ingestion of LPS tells another story. The aim of this study was to investigate the effects of a Western diet and oral intake of LPS on parameters of low-grade inflammation.

(Cani et al., 2007)[24] found that oral delivery of exogenous LPS in oil increased plasma LPS, before showing in subsequent experiments that ME-driven inflammation, obesity and T2DM is enabled by a fat rich diet, indicating a major role of LPS. Cani found that four weeks of high-fat feeding alone or continuous systemic infusion of LPS using a mini-osmotic pump (055:B5, 300 µg/kg/d) in mice on a low fat Chow diet induced ME, and elevated body weight, insulin resistance, and inflammation. We on the other hand found that oral intake of LPS did not affect weight gain, energy intake or glucose homeostasis beyond that of a Western diet. We did however demonstrate that LPS administered to WD fed mice elevated NF-κB activity transiently one day after start of LPS administration, but was later reduced to baseline values.

DISCUSSION

4.1 LPS given orally in the drinking water

4.1.2 Tolerance and endotoxin levels

Before conducting our experiments, we first needed to test whether low-fat Chow diet-fed mice tolerated oral ingestion of LPS for 8 days dissolved in drinking water. We concluded that mice tolerated oral ingestion of LPS primarily based on observations of maintained body weight as compared to controls. If mice had rejected the solution, it is probable that body weight would have been reduced as a result. Furthermore, we assessed LPS in the small intestine and found significantly higher luminal endotoxin levels in LPS treated mice compared to controls receiving tap water. We concluded from this study that the dose tested resulted in an increase in LPS concentrations in both duodenum and ileum. In our subsequent experiment we detected LPS in the small intestine of mice regardless of diet, but only in significantly elevated amounts in LPS treated mice.

Studies suggests that a Western diet amplifies LPS content in the intestine with a resulting increase in blood levels of LPS. This process is thought to be augmented by dietary fat and by the associated intestinal inflammatory environment due to the lipophilicity of LPS and the studies that have shown LPS uptake to increase in the presence of dietary fat (Pendyala et al., 2012)[78]; (Umoh et al., 2016)[96]; (Winter et al., 2013)[106]. In line with this, we would expect elevated luminal LPS levels, and in turn elevated plasma LPS and LBP levels in our WD-water fed mice due to the increased abundance of dietary fat. But this assumption is taken without firm confirmation of what role the respective uptake mechanisms and carriers in the portal or lymphatic circulation might have (or not have) on the bioactivity of LPS in escaping to the systemic circulation. Perhaps unexpectedly, the WD control group was not different from the LF Chow-fed group. We anticpitated that a WD would lead to elevated levels of proteobacteria in the small intestine due to dysbiosis reported by others. Bacterial load and endogenous LPS is substantial in the intestine and increases longitudinally (Eckburg et al., 2005)[37], but endogenous LPS may primarily act as antagonists with regards to TLR4 (Abreu et al., 2001)[1]. This is in contrast to LPS from processed food which is suggested to be highly agonistic (Erridge, 2011b)[45]. Importantly, LPS from food may greatly outnumber estimates of endogenous LPS both in availability and bioactivity, as LPS from food borne *E. coli*, has as of today the greatest capacity for stimulating TLR4 (Rietschel et al., 1994)[136]. Additionally,

DISCUSSION

endogenous LPS still attached to endogenous Gram-negative bacteria is less likely to be absorbed due to the physiochemical properties of a bacterium contra free LPS molecules (Faraj et al., 2019). Enodgenous bacteria may instead be in favor of the intestinal defence by being able to hinder exogenous LPS from being effectively distributed around absorptive surfaces in the small intestine, thus limiting uptake of exogenous LPS to some extent (Ravin et al., 1960). The information above taken together with our results introduces uncertainties on the ability of a WD alone in being able to increase agonistic luminal LPS content. In investigating potential effects of diet and LPS given orally on circulating LPS levels, we were in all experiments unable to detect LPS in blood with a cell based TLR4 assay. Similar limitations are reported elsewhere with regards to detection of circulating LPS when delivered orally (Tamai et al., 2000)[152]; (Harper et al., 2011)[67], whereas radiolabeling confirms ingested LPS to reach the liver first (Freudenberg et al., 1982)[55] (Faraj et al., 2019)[49]. In contrast, i.p injection of LPS showed significantly higher plasma LPS levels, thus we confirmed the validity of the method used for detecting LPS in blood. Detection of LPS with HEK293 cells was thus limited to luminal LPS regarding orally administered LPS. In addition, we found no difference in plasma LBP levels between any groups, which supports the notion that a Western diet and long-term oral administration of LPS does not induce ME in mice, as we hypothesized higher LBP levels in WD fed mice and LPS supplemented mice.

Continuous absorption of LPS from the intestine was thought to be part of a physiological process of unknown relevance (Ghoshal et al., 2009). This process is now connected to conditioning of the immune system (Vatanen et al., 2016); (Haller, 2018). For instance, IAP levels are regulated by Gram-negative bacteria, LPS and diet (Sefcikova et al., 2008)[145]. Expression of IAP is highest in the duodenum, while its enzymatic activity is highest in the ileum (Fawley & Gourlay, 2016). As such, altered duodenal IAP levels was expected in our LPS supplemented groups and WD fed mice, but no difference was found in IAP levels between any groups despite LPS treatment and higher body weight in WD fed mice. The evidence of the effect of diet and LPS on IAP expression is dual. Studies have shown that injected LPS increases IAP levels in animals (Poelstra et al., 1997)[127], but this is again bypassing potential effects conveyed by the gastrointestinal tract. A Western-like diet has on different occasions been shown to both stimulate and downregulate IAP expression in rodents (Kaliannan et al., 2013)[79]; (Serre et al., 2010)[146]; (Sefcikova et al., 2008)[145]. De la Serre and coworkers suggested that excessive fat intake alters the microbiota, ultimately downregulating

IAP. As such, the role of a Western diet and LPS in regulating IAP is still not clear-cut but based on this study IAP regulation is probably dependent on factors beyond 8 weeks of consuming a Western diet and oral ingestion of LPS.

4.2 Western diet and LPS on metabolism and low-grade inflammation

4.2.1 Effects on body weight, energy intake and glucose homeostasis

In investigating potential long-term effects of diet and LPS on parameters of MetS, we found generally no effect of LPS (330 µg/mL, O55:B5) given orally in mice when compared to control groups receiving water on either a Western diet or a low-fat Chow diet for 8 weeks:

Both LPS-supplemented groups had similar body weight developments as the control groups within the respective diets. There was a significant difference in body weight between diet groups wherein WD fed mice had the highest mean body weight for the duration of the experiment in accordance with energy intake.

If dietary fat is a limiting factor in LPS uptake: perhaps the difference in fat content between a HFD with > 70% of energy from fat, and a WD (40%) may account for why HFD fed mice and not our WD fed mice showed a substantial metabolic effect of LPS.

In line with our findings and contrasting that of Cani's low-fat Chow diet-fed mice with systemic infusion of LPS, a recent study by Faraj et al., (2019) found no effect after 10 weeks of supplementing the drinking water with 100 µg/mL LPS (*E. coli*, O111:B4) on weight gain nor glucose homeostasis (HOMA-IR) in Chow fed mice.

During the insulin tolerance test the mean fasting blood glucose values (mmol/L) of WD fed mice was significantly different than low-fat Chow diet-fed mice (Chow diet 7.27 vs WD 8.61 mmol/L). According to WHO criteria, subjects with fasting blood glucose levels ≥ 7.0 mmol/L are classified as diabetic, while fasting blood glucose levels of 5.6 – 6.9 mmol/L is considered prediabetic. However, considering that this test can be stressful for mice, the values might be slightly exaggerated, and it may be that only WD fed mice have a tendency in being above the 7.0 mmol/L

threshold which would be consistent with changes in body weight gain which is primarily due to increases in adipose tissue which correlates with insulin resistance and the fact that the low-fat Chow diet mice are lean compared to the WD mice. This would be in line what other studies have shown on with regards to effects of a Western diet on glucose homeostasis and more (Martinez et al., 2017)[100]. On the other hand, during the oral glucose tolerance test, all groups had fasting blood glucose levels over 8.0 mmol/L (LFW 8.11, LFL, 8.20 and WDW 8.86, WDL 8.60 mmol/L) without differing significantly. Both diet groups experienced a reduction in body weight during the week of OGTT but only the low-fat Chow diet mice experienced an increase in fasting blood glucose levels compared to the IpITT. This could be due to stress, but then an increase in fasting blood glucose level could be expected in the WD-fed mice seen together with the reduction in body weight. Although speculative, it might be that a certain weight treshold dictates the severity of possible changes in blood glucose levels. However, all responded well to injection of insulin, returning to baseline glucose levels after 2 hours and no difference was found in AUC values in either case. In any case we found that oral ingestion of LPS did not influence glucose homeostasis.

4.2.2 Effects on inflammation

To explore potential effects of diet and LPS on inflammation, we used NF-κB luciferase reporter mice as a model, and non-invasive *in vivo* imaging of NF-κB mediated luciferase activity as a method. Interestingly we found no overall long-term elevation in NF-κB activity between or within the diet groups, in either the liver or the intestine after 8 weeks of treatment. However, we observed an initial increase in NF-κB activity in both regions one day after start of LPS administration. In the liver, NF-κB activity differed significantly between the WD fed LPS-supplemented group (WDL) and the other groups, while only differing significantly in the intestine between WDL and low-fat Chow diet-fed mice. A similar scenario was realized in our short-term experiment after analyzing NF-κB activity wherein WD fed mice received either tap water or 100 (WDL01) or 1000 (WDL1) µg/mL LPS orally for 8 days. The analysis showed elevated NF-κB activity in the intestine of LPS-supplemented groups compared to the control group at day 4, although significance was restricted to WDL01. In the liver however, a signifcant elevation in NF-κB activity was found

between WDL1 and both the other groups, as well as a significant elevation between WDL1 and the control group at day 8.

Seen together, these findings might suggest that there is a regional specific effect of both the Western diet and LPS on NF-κB activity: wherein the intestine is less suceptive to the effect of LPS based on the significant elevation in NF-κB activity seen between but not within WD fed mice and low-fat Chow diet-fed mice. This contrasts with the liver, where a significant elevation in NF-κB activity is seen between WDL and all other groups, wherein the WDW group was on par with both low-fat Chow diet groups with regards to NF-κB activity. Again, this effect is in both experiments limited to an initial upregulation.

As mentioned above (ch. 4.2.1), a study by Faraj et al. (2019) showed similar effects of orally ingested LPS in line with our results on body weight and glucose homeostasis. Concordantly, they found no signs of inflammation in the gastro-intestinal tract, but rather an upregulation of acute phase proteins limited to liver tissue. LBP, lipoproteins and other transporters of LPS in the portal circulation could have influenced NF-κB activity in our experiments by sequestering the exogenous LPS that actually manages to enter the portal circulation (Rose et al., 2000)[137]; (Faraj et al., 2017)[48]; (Mathison et al., 1991)[101]. Components in blood (proteins, platelets, antibodies etc.) can also inhibit LPS but a reduction in LPS immune signalling is reported to be mostly related to lipoproteins (Flegel et al., 1989)[51]. Seen together, this may mask NF-κB signalling in our imaging measurements to some extent.

The initial elevation followed by receeding NF-κB activity observed in our experiments, could be related to the time it takes for the host to adapt to and then engage defenses to counter the LPS offensive. More specifically, this may be connected to the inherent molecular mimicry of molecules expressed by bacteria, enabling delayed recognition of and response to LPS by TLR4 (Duerr et al., 2009)[36]. With regards to NF-κB activity in the intestine, intestinal cells can retain and mask LPS in fat carriers (Ghoshal et al., 2009)[57]. Additionally, the intestine is tolerant to immune activation as it is continously exposed to agonistic and antagonistic stimulants of TLR4, including but not limited to LPS, which seems to rather induce an anti-inflammatory effect (Abreu et al., 2001)[1]. Endogenous LPS has in this regard been linked to elevated immune tolerance and maintenance of an anti-inflammatory intestinal environment (d'Hennezel et al., 2017)[32]. With regards to NF-κB activity in the liver, sequestering of LPS in the portal circulation and concomittant host immune

adaptation to LPS might together explain why the effect of orally ingested LPS on NF-κB activity in our experiments is not of a greater magnitude (Tsukomo, 2007); (Dudele, 2015); (Kitchens et al., 2001).

This also based on the absence of a long-term difference in NF-κB activity between WD and low-fat Chow diet mice. This further suggests that inflammatory effects of LPS might correlate with the composition and intactiveness of LPS and that a Western diet allows for LPS induced hepatic inflammation, albeit short term. With regards to hepatic responses to LPS, Ravin and co-workers detected and recovered intact LPS from the liver of healthy rabbits gavaged once with LPS (1500 µg/mL, *E. Coli* 0111B, exposure time < 10 hours). Less than 1% of the given dose was recovered. Interestingly, the recovered LPS was assayed for bioactivity but was found to be inactive (Ravin et al., 1960)[132], suggesting that LPS is absorbed but modified in the portal circulation.

In terms of inflammation, our experiments together support the notion of limited, but not inconsequential, cell responsiveness to LPS, primarily in the liver.

4.3 Methodological considerations

Dietary LPS can be present in uknown amounts in different animal laboratory diets. Adaptive responses by the body to LPS can affect measured variables in a time dependent manner. This can cause greater variations in results and may mitigate the outcome of a study when comparing results of intervention and control groups with respect to parameters such as, glucose homeostasis, and inflammation (Lindenberg et al., 2019)[92]. As such we may consider the possibility of such mitigations in other measured variables such as changes in body weight, LBP and IAP levels and, NF-κB activity, i.e. seeing less of a difference in results if for example control groups are affected by this.

Additionally, mice have been proven to have a higher resistance than humans towards the inflammatory effects of LPS as elaborated on in chapter 2.2. While a dose of 1 µg/kg can be lethal in humans, mice can tolerate 1000-fold higher doses or more (Warren et al., 2010)[170]. As such it

is hard to precisely decide what is relevant doses of LPS as well as physiological translatable doses with regards to not eliciting under- or overexaggarated metabolic responses translatable to humans. Depending on the concentration, LPS stored in solution can be stable for 1 month at 2-8°C. It is known that LPS adheres to glass and plastics although adherence is supposedly negligible if the concentration is > 1.0 mg/mL (Sigma Aldrich). In our long term experiment, LPS was stored at a concentration of 0.33 mg/mL for less than 4 weeks at 4°C. Although being stored for less than 1 month, the concentration may present issues with adherence of LPS, based on the information from Sigma Aldrich.

Mice as research animals

22 week old mice were chosen which represents the adult human. Smaller animals have higher metabolic rate and thus higher physiological processes. As such we can study effects in mice over a shorter time scale, but this also means that smaller animals require higher doses on a weight basis (Nair & Jacob, 2016). Furthermore, Toll-like receptor cascades are similar in mice and humans, as both species has over the centuries been exposed to TLR stimulants such as endotoxins, making mice good models for studying this aspect of the immune system (von Scheidt et al., 2017)[166]. However, differences in intestinal anatomy between mice and humans implies differences in the gastrointestinal micriobiota which may influence health in different ways (Perlman, 2016)[125].

***In vivo* imaging**

One limitation in measurements of NF-κB activity is that region of interests (ROIs) are manually configured, i.e. both inclusion area (size, location) is based on a subjective evaluation. This implies that inflammatory effects reported also reflects a subjective evaluation.

OGTT/IpITT

With regards to the choosing the design of OGTT/IpITT, the literature favors oral delivery of glucose for stimulating a natural physiological insulin response. This is related to release of

hormones in the GI tract that influence insulin release. A fixed dose related to the average weight of mice is favored as muscle mass will virtually remain the same in mice across a certain weight span, as weight gain is primarily due to increased fat mass. Muscle tissue, not fat tissue, is involved in glucose turnover (approx. ~5% due to fat tissue). As such, a higher glucose load can skew results leading to false inferrals. No anesthesia was used for the same reason, as this might influence insulin release causing more variability and less consistency in glucose levels across mice. However, the literature is not clear on the use of anesthesia which, given the right anesthetic agent, might help to reduce stress in animals, in turn yielding more consistent glucose levels. Furthermore, our animals had been handled regularly in the weeks prior to the OGTT and IpITT. By assuming that the animals were somewhat adapted to being handled, it would follow that any stress responses would be less frequent, which should reduce the influence of stress on blood glucose levels. A homeostatic modeling assessement (HOMA) score (insulin sensitivity index) could have been employed to determine if WD mice truly were diabetic, but the utility of this method is questioned and is under scrutiny in the literature. Moreover, effects of WD feeding on blood glucose levels is covered elsewhere in the literature (Haley et al., 2017)[63]; (Forbes et al., 2013)[53]. In any case we found that oral ingestion of LPS did not have an effect on glucose homeostasis.

HEK-Blue LPS detection assay

The sensitivity and ability of the assay to detect LPS in blood was confirmed using i.p injection of LPS in mice. Thus, the limitation is not with the assay per se, but rather with oral intake of LPS. HEK-blue TLR4 cells have previously been demonstrated to accurately reflect biological response to LPS, with a limitation of potential inhibition by LPS-binding components. As we cannot detect or measure free LPS in blood when LPS is ingested orally due to inhibition, we cannot be sure of how much of luminal LPS is absorbed. Cell based LPS detection assays are reported to be prone to errors and contamination (Mukundan, 2017)[112]. LBP, lipoproteins and components in blood could have influenced the ability of LPS in portal and systemic blood to bind to TLR4 (Faraj et al., 2017)[48]; (Mathison et al., 1991)[101].

This indicates an inhibitory LPS threshold level that must be surpassed in order for the LPS detection assay to be able to recognize LPS in blood.

4.4 Suggestions for further research

Additional samples were collected throughout this project, but further analysis was outside the scope of this thesis. Further elucidation of the effects of a Western diet and LPS on metabolic inflammation could be investigated in comparison to control groups: Liver- and mucus tissue samples could be used for analyzing gene expression of inflammation related genes (cytokines, chemokines, T-regs, interleukins etc.). Bacterial samples from different segments of the colon could be used for analyzing microbial profiles. Immunohistological analysis of visceral fat could be used to detect inflammatory molecules and monocyte infiltration in adipose tissue. Ileal IAP activity could be analyzed and compared to duodenal IAP activity. Furtermore, AOAH activity could be cross-checked with IAP activity.

5. Conclusion

Mice tolerate oral ingestion of LPS which is present in the small intestine regardless of diet, and to a larger extent in the ileum. A Western diet increases weight gain and is indicative of of reduced insulin sensitivity. LPS does not affect body weight gain nor glucose homeostasis. LPS and a WD has an initial regional dependent effect on NF-κB activity in the liver and in the intestine, without causing further long-term activation in either regions. LPS administered orally and a Western diet did not elevate LBP blood levels nor duodenal IAP levels. In conclusion, the gastrointestinal tract influences the fate and effects of orally ingested LPS which differs from effects of injected LPS reported by others.

However, the potential fates and effects of orally ingested LPS remains clouded by several factors which should be further investigated with regards to uptake mechanisms of LPS, inhibited detection of circulating LPS amongst other.

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Appendices

Kits

Product	Supplier
HEK-Blue™ LPS Detection Kit2	InvivoGen, San Diego, CA, USA
Mouse LBP Quantification ELISA kit	Biometec, Greifswald, Germany
SensoLyte® *p*NPP Alkaline Phosphatase Assay Kit	©AnaSpec, Inc., Fremont, CA, USA

Instruments and software

Product	Software	Supplier
Cell Countess II	Countess II/II FL	Life Technologies (Now Invitrogen), Thermo Fisher Sceintific , Inc., Waltham, MA, USA
IVIS Lumina II	Living Image® Software	Perkin Elmer, Walthamn, MA
Safe 2020 Class II Biological Safety Cabinets	--	Thermo Fisher Sceintific , Inc., Waltham, MA, USA
SpectraMax M2 Multimode Microplate Reader	SoftMax Pro	Molecular Devices, LLC., San Jose, CA, USA
Synergy H4 Hybrid Microplate Reader	Gen5™ Software	Bio Tek Instruments, Winooski, VT, USA

Equipment

Product	Supplier
Accu-Check Aviva glucometer	Roche Diagnostics, Mannheim, Germany
Millex® - HV 0.45µm Sterile Filter Unit	Merck Millipore, Ltd., Tullagreen, Ireland

APPENDICES

Chemicals

Product	**Supplier**
D-(+)-Glucose (G7021-5KG)	Sigma Aldrich Chemie GmbH, Schnelldorf, Germany
Human Insulin (12643-50 mg) 1M	Sigma Aldrich, St. Louis, MI, USA
IsoFlo® Vet 100% Isolfurane (Vnr 00 21 25)	Zoetis, New Jersey, USA
LPS (L2880-500MG)	Sigma Aldrich Chemie GmbH, Schnelldorf, Germany
LPS (L2880-25MG)	Sigma Aldrich Chemie GmbH, Schnelldorf, Germany

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway