Norwegian University of Life Sciences
Faculty of Chemistry, Biotechnology and Food Science

Philosophiae Doctor (PhD)
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Small intestinal microbiota – Regulation by reactive oxygen species and influence of diet

Tynntarmsmikrobiota – Regulering av reaktive oksygenforbindelser og påvirkning av diett

Chrysoula Kielland

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Summary

Over the last years multiple studies have analyzed the impact of intestinal microbiota on health. Certain changes in the composition of the microbial population in the intestine have been associated with several diseases including cancer, autoimmune diseases and conditions related to the metabolic syndrome. In many cases, the composition of the gut microbiota has been associated with certain components in the diet. However, most of these studies analyzed either the composition of bacteria in the large intestine or the one in feces. The small intestine (SI) is the organ where absorption of nutrients occurs and is the site where primarily the intestinal microbiota meets the macro- and micronutrients from the diet. Furthermore, the SI possess an enormous number of immune cells and it is known that both dietary compounds and alterations in gut microbial populations can elicit an inflammatory response.

Apart from being responsible for nutrient absorption, the SI, possesses another crucial role; it defends the host from a potential invasion by microorganisms. To achieve that, the small intestine employs several mechanisms including motility and secretion of antimicrobial substances. Reactive Oxygen Species (ROS) are part of the innate immune system and are known to be responsible for the respiratory burst in neutrophils, macrophages, and dendritic cells which reside inside the lamina propria. However, an antimicrobial role of ROS produced by the epithelial cells in the small intestine under steady state is less determined. This work is divided in two parts; first our aim was to elucidate to which extent the small intestinal microbiota (SIM) is inducing the production of ROS in an effort of the host to maintain the microbial homeostasis in the small intestine. In the second part we sought to analyze whether changes in diet would alter the composition of the small intestinal microbiota and consequently if this would impact the state of the immune system. Fat and coffee were the diet compounds that were tested.

Nitric oxide synthases (NOSes) are enzymes catalysing the reaction producing nitric oxide (NO). In this work we focused on the inducible NOS isoform (iNOS). The expression of iNOS is induced among others by bacterial products. NADPH 1 (NOX1) is expressed from the epithelial cells in the small and large intestine and is a catalyst in the production of

superoxide. Superoxide reacts with NO to produce peroxynitrite. *In vivo* imaging revealed very high levels of ROS in the ileum of normal healthy mice which was regulated by the number of intestinal bacteria at this site. In the ileum of iNOS- and NOX1-deficient mice, the bacterial load was higher, and the composition of the bacterial community resembled the one present in cecum. These data suggest a role of ROS produced in the terminal SI as a regulator of the amount and composition of the small intestinal microbiota, possibly contributing against reflux of microbes from the large intestine.

Introduction of a high fat diet (HFD) has been linked with onset of inflammation in the SI. Moreover, studies based on both human and animal models have shown that ingestion of a HFD leads to an unbalanced microbiota composition. Here, based on a mouse model we found that consumption of a HFD led to an unbalanced microbial profile in the SI. SIM in the HFD fed mice was characterized by an increase in the proinflammatory *γ-Proteobacteria* and *Peptoclostridium*. Additionally, we observed an increased inflammatory response in the SI characterised by higher production of pro-inflammatory cytokines and higher ratio of pro-inflammatory to anti-inflammatory macrophages.

Coffee is a widely consumed beverage rich in polyphenols that are known to be anti-inflammatory. A positive association of diets rich in polyphenols with the colonic gut microbiota has been previously described. In the current work, we aimed to identify whether the coffee would improve the changes in the intestinal microbiota and in certain inflammatory markers caused by HFD. The results showed that the microbiota in mice fed coffee together with HFD was altered and resembled the one in the mice fed a low-fat diet (LFD). In addition, the mice receiving coffee had a dampened inflammatory response in the SI.

Summing up, here we have shown that a very high ROS production is induced by the bacteria in the terminal SI. These ROS derive from the epithelial cell layer and act as a modulator of microbial homeostasis in the SI. Regarding the impact of high amount of fat in the diet we observed that fat negatively affected the community structure of SIM and the inflammatory status of the host, something that was improved after coffee addition.

Sammendrag

I de siste årene har flere studier analysert sammenhengen mellom tarmmikrobiota og helse. Mange av disse studiene har etablert at vise endringer i tarmens mikrobesammensetning er assosiert med flere sykdommer, deriblant kreft, auto-immune sykdommer og tilstander knyttet til metabolsk syndrom. I mange tilfeller har kostholdet og bestemte kostoldskomponenter hatt mye å si for å regulere denne sammensetningen. Imidlertid har de fleste studiene analysert sammensetningen av bakterier enten i tykktarmen eller fra avføring, men i liten grad er tynntarmens mikrobiota blitt studert. Tynntarmen er organet der absorpsjon av næringsstoffer skjer og er stedet der tarmmikrobiota i første rekke møter makro- og mikronæringsstoffer fra kostholdet. Videre har tynntarmen et enormt antall immunceller, og det er kjent at både ernæringsfaktorer og endringer i tarmmikrobielle populasjoner kan fremkalle en inflammatorisk respons.

Bortsett fra å være ansvarlig for næringsopptak, har tynntarmen en annen avgjørende rolle; den forsvarer verten mot en potensiell invasjon av mikroorganismer. For å oppnå det, bruker tynntarmen flere mekanismer, inkludert fysiske barrierer, motilitet og sekresjon av antimikrobielle peptider og immunoglobulin A. Reaktive oksygenforbindelser (ROS) er en del av det medfødte immunsystemet og er kjent for å være ansvarlig for respiratorisk «burst» (respiratorisk utbrudd) i nøytrofile granulocytter, makrofager og dendrittiske celler ved møte med bakterier under en infeksjon. Dette kan også finne sted i lamina propria. Imidlertid er det mulig at epitelcellene som også produserer og skiller ut ROS kan ha en antimikrobiell rolle i tynntarmen under stabile tilstander, men kunnskapen om dette er mindre etablert. Dette arbeidet er delt i to deler; først var målet vårt å belyse i hvilken grad ROS produsert i tynntarm blir påvirket av bakterier, dernest om produksjon av ROS av verten spiller en rolle i å opprettholde den mikrobielle homeostasen i tynntarmen. I den andre delen søkte vi å analysere om sentrale kostholdsfaktorer ville endre sammensetningen av tynntarmsmikrobiota og om dette ville påvirke immunsystemets tilstand. Fett og kaffe var diettforbindelsene som ble testet.

Nitrogenoksydsyntaser (NOSer) er enzymer som katalyserer reaksjonen som produserer nitrogenoksid (NO). I dette arbeidet fokuserte vi på den induserbare NOS-isoformen

(iNOS). Uttrykket av iNOS induseres blant annet av bakterielle produkter. NADPH 1 (NOX1) uttrykkes fra epitelcellene i tynntarmen og tykktarmen og er en katalysator i produksjonen av superoksid. Superoksid reagerer med NO for å produsere peroksynitritt. *In vivo* avbildning avslørte svært høye nivåer av ROS i distal del av tynntarm (ileum) hos normale friske mus, og ROS-nivåene var sammenfallende med både tilstedeværelse og mengde av bakterier på dette stedet. Videre ble både iNOS- og NOX1-knock-out mus benyttet for å undersøke om bakteriell belastning ble påvirket i ileum. Mengde bakterier ileum var høyere og sammensetningen av bakteriesamfunnet lignet den som var til stede i cecum ved fravær av iNOS og NOX1. Disse dataene antyder en rolle for ROS produsert i distal tynntarm som en regulator av mengden og sammensetningen av tynntarmsmikrobiota. Vi spekulerer at ROS kan ha rolle i å hindre bakterier fra tykktarm i å komme tilbake til tynntarm.

Innføring av en fettrik diett har vært knyttet til en lavgradsbetennelse i tynntarmen. Videre har studier i både mennesker og dyremodeller vist at inntak av en fettrik diett fører til en ubalansert mikrobiotasammensetning. Her, basert på en musemodell, fant vi at inntak av en fettrik diett førte til en ubalansert mikrobiell profil i tynntarmen. Tynntarmsmikrobiota i de fettrik-fôrede musene var preget av en økning i de proinflammatoriske *γ-Proteobakteriene* og *Peptoclostridium*. I tillegg observerte vi en økt inflammatorisk respons i tynntarmen preget av høyere produksjon av proinflammatoriske cytokiner og høyere forhold mellom proinflammatoriske og antiinflammatoriske makrofager i lamina propria.

Kaffe er en mye konsumert drikke rik på polyfenoler som er kjent for å være antiinflammatoriske. En positiv assosiasjon av dietter som er rike på polyfenoler med tykktarmsmikrobiota har blitt beskrevet tidligere. I det nåværende arbeidet hadde vi som mål å identifisere om kaffen ville forbedre endringene i tarmmikrobiota og i visse inflammatoriske markører forårsaket av en høyfettdiett. Resultatene viste at mikrobiota hos mus som ble tilført kaffe i fôret sammen med høyfettdietten ble endret og lignet den hos musene som fikk en lavfettdiett. I tillegg hadde musene som fikk kaffe en dempet inflammatorisk respons i tynntarmen.

Oppsummert, her har vi vist at en høy ROS-produksjon i den distale delen av tynntarm induseres av bakteriene. Disse ROSene produseres av epitelcellelaget og fungerer som en

modulator for mikrobiell homeostase i tynntarmen. Når det gjelder virkningen av høy mengde fett i dietten, observerte vi at fett påvirket samfunnsstrukturen til tynntarmsmikrobiota og vertens inflammatoriske status på en negativ måte, noe som ble forbedret etter tilsetning av kaffe.

List of papers

PAPER I

Matziouridou C*[1], Rocha SDC, Haabeth OA, Rudi K, Carlsen H, Kielland A (2018). iNOS- and NOX1-dependent ROS production maintains bacterial homeostasis in the ileum of mice.

Mucosal Immunol, 11(3), 774-784. doi:10.1038/mi.2017.106

PAPER II

Rocha SDC, Harvei S***, Kielland C***, Carlsen H, Kielland A. Small intestinal microbiota and immune status in high-fat dieting mice.

Manuscript

PAPER III

Kielland C*, Rocha SDC*, Harvei S, Carlsen H, Kielland A. Coffee consumption attenuates the unfavorable microbial composition and inflammation induced by high-fat feeding in mice.

Manuscript

*[1] birth name

* shared authorship

List of abbreviations

DC – Dendritic cells

DUOX – Dual Oxidase

GALT - gut-associated lymphoid tissues

GF – Germ free

GI – Gastrointestinal

HFD – High fat diet

IECs – Intestinal Epithelial Cells

KO – Knock out

LBP – Lipopolysaccharide binding protein

LFD – Low fat diet

LP – Lamina propria

LPS – Lipopolysaccharide

MLNs – Mesenteric lymph nodes

NOX - NADPH oxidase

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

NO – Nitric Oxide

SI – Small intestine

SIM – Small intestinal microbiota

Treg – Regulatory T cells

Introduction

The gastrointestinal tract (GI) is a long muscular tube stretching from the mouth to the anus. The intestine constitutes most of the GI tract and is divided in two parts; the small and the large intestine. Along the length of the intestine associated organs such as pancreas, liver and gall bladder are necessary for aiding in the digestion of food. The small and large intestine are physiologically different and each is responsible for the absorption of different compounds [1].

The GI tract is known to harbour a rich and diverse community of microorganisms which form a complex ecosystem. These microbes, known also as gut bacteria or gut microbiota, possess metabolic activities and interact with the host [2, 3]. Both the density as well as the community members differ between the small and the large intestine [4].

The present work examines aspects related to the function of the small intestine and the small intestinal microbiota. The main role of the small intestine (SI) is to digest and absorb nutrients and it is divided in three sections; the duodenum, jejunum and ileum. The SI consists of three main layers, namely the mucosa, submucosa and serosa. Each of these layers possesses a distinct function [1]. Of these, the mucosa is of particular interest for this work, due to dense immune system as well as due to its close proximity to the gut lumen and thereby the gut microbiota.

We focus on the community structure of small intestinal microbiota (SIM) and the regulation of the immune responses of the host. More specifically we explore the role of reactive oxygen species (ROS) production in the regulation of SIM and the impact of alterations in the diet in its community structure. This work is divided in two subparts, focusing on each of the two main objectives in question; namely whether SIM induces ROS production as well as how the secreted ROS contributes to the regulation of SIM (part I) and identify the impact of dietary changes (pictured by a diet rich in fat and a diet containing coffee – rich in phenolic compounds –) in the community structure of SIM and the small intestinal immune status of the host (part II).

Part I – Regulation of small intestinal microbiota by ROS production

Small intestine: A brief introduction to its anatomy

Beginning at the pyloric valve in the stomach and ending at the ileocecal valve in the terminal part of ileum, the SI is the entry point into the large intestine. It consists of three main segments, the duodenum which is described also as the proximal SI and is closest to the stomach, the jejunum, which is localized after the duodenum and the ileum, which is also referred to as the distal part of the SI. The SI consists of different layers with the mucosa being the one in contact with the gut lumen. The three constituents of the intestinal mucosa are the epithelium, the lamina propria (LP) and the muscularis mucosa [1]. The LP is a loosely packed connective tissue which contains many cells of the intestinal immune system and also provides the lymph drainage, blood and nervous supply to the mucosa **(Figure 1)** [5].

One of the main characteristics of the SI is the finger-like projections known as villi. The presence of villi creates a large surface area and thereby enhances digestion and uptake of nutrients [1, 5]. Multipotent stem cells differentiate into the different types of mature epithelial cells which are continuously renewed. The intestinal epithelial cells (IECs) layer consists mainly of nutrient absorptive enterocytes and goblet cells **(Figure 1)**. Additionally, Paneth cells and enteroendocrine cells are also part of the IECs, each having a different function. Paneth cells are known to secrete antimicrobial peptides. Enteroendocrine cells are responsible for the production of several digestion regulating hormones [5, 6].Apart from Paneth cells, the rest of the newly formed epithelial cells move along the crypts, from the bottom to the top. During this process, the epithelial cells undergo maturation and they only acquire all the essential properties for their absorptive functions when they reach the base of the villus [5].

Small intestinal immune system: An overview over its anatomical and functional features

The intestine is the site with the highest number of immune cells in the body, containing approximately 60% of all immune cells [5, 7]. The intestinal immune system consists of both inductive and effector sites. The main constituent of the inductive sites is the gut-associated lymphoid tissues (GALT) which comprises subepithelial lymphoid aggregates containing the microfold cells (M cells) **(Figure 1)**. Apart from being ports of entry of pathogens, the M cells specialize in uptake and transport of antigens from the lumen to the underlying dendritic cells (DC). Alternatively, antigens are transported through the epithelial cell layer and picked up by DC underneath the epithelium. DC next present the antigens to adaptive immune cells in GALT. GALT consists of Peyer's patches, which are mainly localized in ileum and consist of germinal centres and B cell lymphoid follicles which in turn consist of smaller T cell areas. Additionally, the GALT also consists of isolated lymphoid follicles (ILFs), which are prominent in colon. ILFs also have germinal centres and B cells, however they lack a T cell zone. Furthermore, the mesenteric lymph nodes (MLNs) which drain the intestine are also part of GALT [5].

The effector sites of the intestinal immune system are comprised by the LP and the epithelium. LP contains B cells, T cells; both $CD4^+$ and $CD8^+$, and various other innate immune cells, such as DC, macrophages, mast cells and eosinophils. The epithelium contains mainly T cells [5]. The B cells produce local polymeric immunoglobulin (Ig), mainly dimeric IgA [8]. Naïve T cells differentiate into T helper cells including Th1 and Th17 helper cells, regulatory T cells (Treg) and cytotoxic T cells. Th1 and Th17 cells are regulated by several mechanisms and participate in the production of inflammatory cytokines. Foxp3+ Treg cells on the other hand are central in creating tolerance towards commensals and dietary antigens by suppressing Th1 and Th17 cells. More specifically, the anti-inflammatory IL-10 and TGF-β originating from Treg cells, decreases the production of the Th1 cytokines; interferon (IFN)-γ and IL-12, and regulates intestinal myeloid cell activity [9]. Cytotoxic T cells upon activation by T helper cells are also able to produce cytokines and promote programmed apoptosis [10].

CD are the main antigen-presenting cells of the immune system. They constantly sample their environment for foreign and self-antigens. Intestinal CD acquire their antigen from the lumen using several mechanisms including the uptake of apoptotic or damaged cells, the extension of trans-epithelial dendrites into the lumen, via M cells, via goblet cell-associated antigen-passages, after transfer from tissue-resident macrophages or for soluble molecules, via paracellular and transcellular mechanisms. Once they have acquired an antigen, they migrate via the afferent lymphatics to the MLNs, where they present the processed antigen to the adaptive immune system cells. There, naïve T cells undergo differentiation and express a so-called, gut homing phenotype, draining the differentiated cells through the lymphatics and entering the bloodstream to return to the effector site. Intestinal DC express various cytokines as a response to inflammatory stimuli and microbial products [11].

The intestinal monocytes once they reach the mucosa, go through a differentiation process. The fully maturated macrophages, which in mice express high levels of CX3CR1 and MHCII, possess their properties progressively as they differentiate. These properties include the expression of receptors associated with the recognition and uptake of apoptotic cells, phagocytosis and bactericidal action and the production of mediators such as TNFα, IL-1β and IL-10 [11].

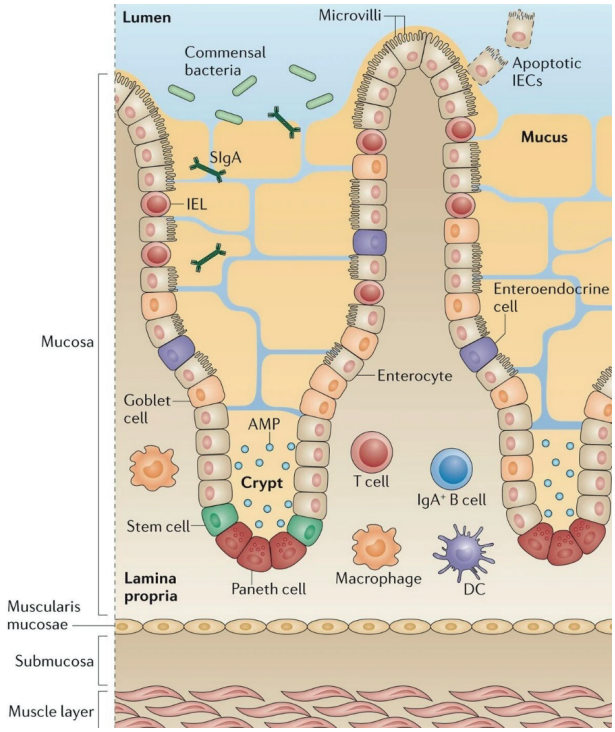


Figure 1: The small intestinal mucosa and lumen. Major anatomical features and immune cell populations. Adapted by Mowat et al., 2014 [5].

Small intestine: A dual role

The SI with its 6m^2 surface area is the organ in which nutrients from diet are absorbed. As mentioned above, the mucosa is the layer in the SI that is in touch with the gut lumen. The IECs is a single cell layer that separates the gut lumen from the interior of the host and interacts directly with microbes in the gut lumen [12]. It is therefore essential to maintain a well-regulated balance in these cells to fulfil both its absorptive role and protect the host from invasion of intestinal microbes. Therefore, maintaining the integrity of the intestinal barrier is an essential prerequisite for intestinal homeostasis. A damage to the barrier

would overexpose the immune cells to the intestinal microbes, which would initiate an inflammatory response [13].

Small intestinal mucosa: Sensing microorganisms and mechanisms preventing overgrowth

The epithelium in the GI has a turnover of approximately 4 – 5 days and is able to sense the quantity and type of the microbes in the gut lumen as well as their distance from the epithelial surface. Upon sensing of pathogens, the intestinal mucosa will initiate a signalling cascade aiming to trigger an antimicrobial action, whereas the response to commensal bacteria is characterized by immune tolerance signals [13].

An uncontrolled growth of bacteria in the SI would have detrimental effects to the host. Small Intestinal Bacterial Overgrowth (SIBO) is a condition in which there is an overgrowth of bacteria in the small intestine. SIBO has been associated with several complications including severe maldigestion of nutrients leading to high morbidity and mortality [14]. Therefore, several mechanisms are in place to prevent a microbial overgrowth in the small intestine, sxomething that would pose a risk to the barrier's integrity. These include, secretory molecules, such as mucins constituting the mucus layer, antimicrobial peptides such as defensins and RegIIIγ, bile, secretory IgA, as well as substances produced by the commensal microflora. Furthermore, to keep the bacterial numbers low, peristaltic movements in the SI will sweep bacteria distally towards the colon, by the migrating motor complex and the peristaltic rush [15, 16].

Additionally, the GI consists of several sphincters. These are muscle rings that retain the flow of intestinal contents in various sites across the GI tract. One such sphincter is the ileocecal valve which is located at the end of ileum and separates the small from the large intestine. The two major functions of the ileocecal valve are to 1) secure directional intestinal flow towards the large intestine and 2) to prevent colonic content, including bacteria from entering the SI [1, 17]. A defective ileocecal valve can result in severe reflux of bacteria from the large intestine to the SI, resulting in SIBO [18].

ROS: Characterization and function

Reactive Oxygen Species (ROS) are small molecules that include a variety of diverse chemicals derived from O_2, such as oxygen radicals [superoxide ($O_2\bullet^-$) and hydroxyl radical ($\bullet OH$)] and non-radicals such as hydrogen peroxide (H_2O_2), singlet oxygen($^1O_2$) and hypochlorous acid (HOCl). Some ROS can be very reactive, while others are more stable [19]. Superoxide and hydrogen peroxide can be detoxified by endogenous antioxidants and enzymes, are less reactive and therefore have less acute effect than those of hydroxyl radical and singlet oxygen, which can be very toxic and lethal [20]. Superoxide dismutates to hydrogen peroxide, catalysed by superoxide dismutase (SOD). Hydrogen peroxide which is more stable and diffusible can either be converted to hypochlorous acid (which is 100 times more reactive) or can be enzymatically decomposed to water and oxygen by catalase or glutathione peroxidase. Additionally, hydrogen peroxide can react non-enzymatically with superoxide anion under the presence of ferrous (Fe^{2+}) and create hydroxyl radicals, known as the Fenton reaction. Superoxide anion can also react with nitric oxide ($NO^{\cdot}$) forming peroxynitrite ($ONOO^-$). In its turn, peroxynitrite can form other NO-derived compounds, commonly referred to as reactive nitrogen species (RNS). NO is produced together with L-citrulline by the oxidation of the terminal nitrogen from L-arginine, a reaction that is catalysed by Nitric Oxide Synthases (NOS) [13, 20].

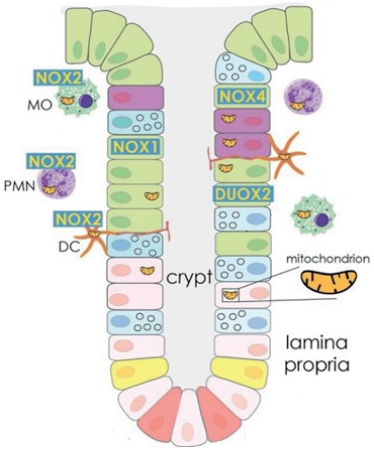
ROS are continuously produced endogenously both by enzymatic and non-enzymatic reactions. Mitochondria and phagocytic cells are some of the endogenous ROS sources [20]. Some of the physiological processes that ROS participate include mitogenic signalling, regulation of vascular tone, and host defence. ROS are a part of the innate immune system and they are responsible for the respiratory burst in phagocytic cells, namely neutrophils, macrophages, and dendritic cells. Macrophages and neutrophils produce superoxide in the phagosomes via NADPH oxidase 2 (NOX2). Nonimmune cells can also produce ROS, for example NADPH oxidase 1 (NOX1), Dual Oxidase 2 (DUOX2) and xanthine oxidase located in intestinal epithelial cells [19].

Due to their reactivity ROS can pose deleterious effects in cells and tissues. High concentrations of ROS can disturb cellular signalling, create DNA mutations, lead to protein oxidation and lipid peroxidation with a resultant irreversible damage to the function of

target molecules [13]. Prolonged production of high levels of ROS as for example during chronic inflammation can therefore induce severe oxidative stress and accumulation of oxidative damage in tissues and diseases such as diabetes, atherosclerosis, and certain types of cancers [19]. Although ROS is largely considered as harmful molecules, production of ROS to low or moderate levels promotes homeostasis by regulating intracellular signalling pathways and transcriptional regulation, such as reversible thiol oxidation of reactive cysteine residues within regulatory proteins and T cell tolerance [13, 19]. Additionally, apart from cell signalling, NO has been characterized as an anti-infective agent and as an antioxidant [20].

Intestinal ROS: Localization, function, and regulation

The main sources of ROS in the GI tract are NOXes, DUOXes, NOSes, RNS and the mitochondrial electron transport chain (mETC). In mammals the NOX complex consists of five isoforms, NOX1 to NOX5, whereas the DUOX complex consists of two, DUOX1 and DUOX2. Of these NOX5 is the only one not expressed in rodents. NOX1 and DUOX2 are highly expressed along the GI tract by epithelial cells, NOX2 is expressed by professional phagocytes and DC, while NOX4 can be found in epithelial cells, smooth muscle cells and fibroblasts [13], however NOX4 expression is very low in the GI tract **(Figure 2)** [21]. Expression of NOX1 is increasing along the length of the GI tract reaching a peak in ileum and colon. DUOX2 is mainly expressed in the central and terminal small intestine (jejunum and ileum) and to a lesser extent in colon. The NOX enzymes produce superoxide, which although does not possess a direct antimicrobial activity, it acts as a precursor of other ROS molecules with bactericidal activity such as hydrogen peroxide, hydroxyl radicals, hypochlorous acid, and peroxynitrite [13].



$2O_2 + NADPH \rightarrow 2O_2\bullet^- + NADP^+ + H^+$

$O_2 + NADPH \rightarrow 2O_2\bullet^- + NAD^+$

$O_2 \rightarrow 2O_2\bullet^-$

$NO\bullet + O_2\bullet^- \rightarrow ONOO^-$

$L\text{-arginine} + O_2 \rightarrow L\text{-citrulline} + NO\bullet$

Figure 2: Left: Localization of the intestinal NADPH oxidases. Right: Main reactions producing ROS and RONS. Adapted by Aviello et al., 2017 [13].

Intestinal ROS are known to play a role in intestinal homeostasis. They modulate cell signalling by oxidizing certain reactive cysteine residues within enzymes controlling cell signalling pathways. Therefore, such proteins can function as redox sensors and signalling transducers [22]. Both the expression and enzymatic activity of both NOX1 and DUOX2 are dependent on the presence of intestinal microbiota [21]. NOX1 remains inactive under physiological conditions, but it gets upregulated by certain bacterial products, such as LPS and certain pro-inflammatory cytokines, as for example IFN-γ [13, 23-25]. NOX1 needs to be activated to function optimally. In general, two events take place during oxidase activation: the exchange of GTP for GDP on the small G protein Rac and the phosphorylation of the p47phox subunit by protein kinase C triggering a change in conformation of the cytosolic complex [26, 27]. During bacterial infection caused by *Campylobacter jejuni* it has been shown that the expression of epithelial NOX1 and DUOX get upregulated disrupting bacterial signalling and thus acting as an early antibacterial defence system [24]. Furthermore, it has been shown that NOX1 plays an important role in colonic homeostasis by enhancing the proliferation of IECs in the colonic crypts [28].

Additionally, it has been suggested that NOX1-dependent ROS released in the colonic epithelium regulates the colonic homeostasis by interacting both with the host as well as with the bacteria that are present in the lumen. However, the mechanistic details and signalling pathways that are involved are not yet fully understood [22, 29]. Recently, it has been shown that the expression of NOX1 in the colonic epithelium is generated by proliferating stem cells and that NOX1 expression is regulated by TLR activation in response to the microbiota. The ROS produced by NOX1 in this site is enhancing the proliferation of stem cells by participating in the redox-dependent activation of the Epidermal Growth Factor Receptor (EGFR) signalling pathway [29].

Regarding DUOX, in *Drosophila* it has been shown that the barrier epithelial cells express DUOX2 which generates ROS to prevent the proliferation of gut microbes and protect the host against infections [30]. In *C. elegans* it has been shown that ROS deriving from DUOX although it does not exert a direct antimicrobial role, improves host survival after bacterial infection [31]. In mammals, DUOX2 has been shown to protect against *Helicobacter felis* infection by producing ROS with bactericidal action [32].

Out of the three NOS isoforms, (NOS1 or nNOS, NOS3 or eNOS and NOS2 or iNOS), the first two are constitutively expressed by either neuronal (nNOS) or endothelial (eNOS) cells, whereas the expression of the latter (iNOS) is induced by certain microbial products or cytokines. iNOS is expressed by immune (T cells, macrophages, mature dendritic cells), epithelial and neuronal cells and produces long-lasting, high levels of NO, whereas nNOS and eNOS give rise to Ca^{2+} dependent short-lasting, low levels of NO [13, 33]. NO can also be formed in the gut lumen and on the gut mucosa. This depends on mainly three conditions that are specific for the GI tract: the accumulation of nitrogen species (both endogenous as well as dietary-derived), the presence of commensal bacteria and the extreme redox milieu that is formed in this site [34].

The expression of iNOS is induced by LPS exposure [35], pathogen ingestion [36] and by proinflammatory cytokines [37, 38], and is largely regulated by the NF-κB family of transcription factors [39]. After LPS injection in rats the expression of iNOS in ileum, once induced, is limited to the epithelial cells and it shows an increasing gradient from crypt to villus [35]. Along the GI tract iNOS is under normal conditions mainly expressed in ileum

and to a lesser extent in jejunum and its expression is downregulated in germ – free rodents [8]. Low levels of NO contribute to the maintenance of mucosal capillaries and mucosal homeostasis as well as being scavengers of oxygen radicals. Furthermore, moderate levels of NO promote barrier integrity and gut motility and they also contribute to the control of gastric mucosal blood flow [13]. Additionally, it has been found that mice deficient in iNOS had an increased mortality after sepsis, suggesting that the function of iNOS provides a survival benefit [40]. However, overexpression of iNOS can lead to impaired gut barrier function and give rise to deleterious levels of RNS [13, 38]. NO is considered to have a limited direct antimicrobial action. Therefore, it is believed that NO exerts its antimicrobial effects through its downstream metabolites, such as ONOO−, S-nitrosothiols (RSNO), nitrogen dioxide (NO_2•), dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4), and dinitrosyl–iron complexes (DNIC) [20].

Production of ROS and RNS are key events in the progression of several gastrointestinal diseases including gastroesophageal reflux disease, gastritis, enteritis, inflammatory bowel disease, and associated cancers [41]. For example, it has been shown that iNOS drives colonic tumor angiogenesis [42] and that NOX1 is overexpressed in colon cancer patients with K-Ras mutations [13]. Production of very high levels of ROS because of the upregulation of NOXes or due to an altered mitochondrial function has been associated with ileitis and ulcerative colitis. It appears that keeping ROS levels under a strict balance is crucial. While high levels of ROS are considered to be damaging for the tissue with the level of ROS depicting the severity of the inflammation established in the colonic mucosa [41], absence of intestinal ROS caused by inactivation of genes encoding NOXes is also linked to diseases of the GI tract, as for example Crohn's disease and global intestinal inflammation (pancolitis) [13]. On the other hand, inducing ROS at physiological levels accelerates mucosal healing and restitution [43]. It is therefore important to maintain a balance in the levels of ROS to promote homeostasis in the gut.

Part II – The influence of dietary changes in the composition of SIM and the regulation of host's immune response

Gut microbiota: A brief overview

Gut microbiota refers to the collection of microorganisms, for example bacteria, eukaryotes, protozoa and archaea that colonises the GI tract. Gut microbes have co-evolved with the host over thousands of years. The number of microorganisms inhabiting the colon, which is the organ with the highest microbial density, has been estimated to be around 10^{13} and includes about 500-1000 species, predominantly anaerobic bacteria [44, 45]. The gut microbiota is acquired during birth and is largely composed of bacteria from the phyla of *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*. Intestinal microbes harbour several genes and gene products which add various functionalities to the host including a number of enzymes important for digestion of complex carbohydrates not encoded by the host [44, 46].

The microbiota has developed a mutualistic relationship with the host. The intestinal microbes exert a beneficial effect to the host, including harvesting energy, synthesizing vitamins, metabolizing bile acids, shaping the intestinal epithelium, protecting against pathogens, and regulating host immunity. However, when the composition of the gut microbiota gets dysregulated these mechanisms could be disrupted resulting in several health deteriorating conditions. Such dysregulation can be caused by multiple factors. Studies including twin individuals have shown that, although there is a heritable component to gut microbiota, environmental factors as age, dietary habits, illness, and antibiotic treatment determine to a larger degree the composition of gut microbes [47, 48].

A plethora of studies over the last years have brought gut microbiota into focus. Remarkably between 2013 and 2017, 12900 publications analyzed the gut microbiota [3]. Findings indicate a correlation between establishment of an unbalanced environment and health deterioration, including metabolic syndrome, cardiovascular and intestinal diseases, cancer, and central nervous system disorders [3, 49-52]. However, it should be noted that causality between specific microbiota composition is lacking for many of the conditions

reported [53]. The interaction of gut microbiota and diet as modulator of human metabolism has been previously described [54].

Influence of HF feeding in gut microbiota composition: what is known

A diet high in fat is demonstrated to cause fecal and colonic dysbiosis [49, 55], while on the other hand the gut microbiota has a major impact on host phenotype in humans and mice by regulating nutrient uptake and host metabolism [56-59]. Interestingly, gut microbiota of obese humans and mice is different compared with the one in lean subjects and is generally characterized by higher *Firmicutes* and lower *Bacteroidetes* in obesity [60-62]. Dysbiosis refers to a disruption in the balance of gut microbiota homeostasis. A new paradigm, namely the Anna Karenina principle, has been proposed to describe dysbiosis. Here, it is suggested that while under steady state the microbiota composition might show certain similarities, under stress factors the changes to microbiota are rather stochastic and therefore do not necessarily follow universally distinct patterns [63].

The presence of intestinal bacteria is of crucial importance for fat absorption. GF mice have lower body weight and excrete more fecal lipids under a HFD regimen [50, 64], while GF zebrafish absorb short and long chain fatty acids to a lesser extent than conventionalised animals [65]. Furthermore, it has been shown that obese twins have an altered microbiome characterised by an increased capacity to absorb fat in comparison to their lean siblings [66]. When gut microbes of twins discordant for obesity were transferred to GF mice, mice receiving the obese-deriving microbiota had a higher fat mass in comparison to GF mice receiving microbiota from the lean twin sibling. Of interest, cohousing the two mouse groups prevented the development of increased body mass and obesity-associated metabolic phenotypes indicating that the phenotype conferred by “lean” microbiota could be transferred to the mice that received the “obese” microbiota [67]. Additionally, antibiotics-treated rats fed a HFD showed a reduction in the lymphatic lipid transport and thus lipid absorption which was also associated with a reduction in the secretion of intestinal apolipoprotein B [68]. Another explanation for the resistance of GF mice to HFD-induced obesity may be related to the metabolism of fat in the gut and peripheral tissues as

for example in muscle and liver. For example, fecal transplantation in GF mice resulted in downregulation of jejunal genes involved in fatty acid oxidation, while genes involved in glycolysis were upregulated, suggesting that conventionalization shifted energy utilization [58, 59]. Furthermore, GF animals exhibit higher levels of phosphorylated AMP-activated protein kinase (AMPK) in liver and skeletal muscle, as well as of its downstream targets involved in fatty acid oxidation (acetylCoA carboxylase and carnitine-palmitoyltransferase) compared to their conventionalized counterparts [50]. All these would mean that under GF conditions increased oxidation of absorbed fats in the intestine would prevent their incorporation into chylomicrons and delivery to the periphery [59].

Small Intestinal Microbiota: under the shadow of its big brother

Despite the growing evidence on the interplay of gut microbiota and host, the majority of studies has so far limited their target group to fecal, cecal or colonic samples and neglected the composition of the small intestinal microbes. The SI is the part of the intestine where lipid digestion and absorption take place and where lipid levels are highest following consumption of dietary fats [69, 70]. Furthermore, the SI has been characterized as a site rich in microbe-microbe and microbe-host interactions [71, 72]. Since the SI is the first region where bacterial and nutrient interactions occur, it is important to understand the dynamics of the microbial community structure in this region. It is not unlikely that bacteria in the SI are involved in nutrient uptake under HF conditions as some bacteria that can be found in this site (for example *γ-Proteobacteria*) are able to transport and oxidize exogenous fatty acids [73-75]. The lack of evidence on the interaction between a HFD and the small intestinal microbiota becomes even more prominent considering that HFD has been linked with alterations in the innate and adaptive immune responses in the SI [76-78]. The SI has an enormous immune capacity and immune activation on this site has been linked with changes in the composition of gut microbiota [79]. However, very few studies so far have taken into consideration all these three factors simultaneously; namely HFD, small intestinal microbiota and immune response.

Small Intestinal Microbiota: Factors affecting its community structure

The community structure of the intestinal microbiota changes along the length of the gut and the composition in the SI is different than the one in colon or in feces [4, 70, 80-82]. The small intestinal microbiota is a complex ecosystem although it is less abundantly colonized (10^3-10^7 cells per gram versus 10^{12-13} in colon) and is less diverse than in the colon [83]. However, in the terminal ileum bacterial densities may reach levels found in colon [84, 85]. The differences in chemical and nutrient gradients as well as the difference in transit time and the degree of host immunity between the small and the large intestine affect which bacterial populations are able to reside in these two sites. The environment in the SI is more acidic, with higher presence of oxygen and higher levels of antimicrobial peptides than the one in colon. The microbiota is subject to these conditions and therefore SIM is mainly characterised by facultative anaerobes or aerobes that tolerate the combined effects of oxygen, bile acids and antimicrobial substances [84, 86]. Bile acids are secreted at the proximal SI and due to their surfactant properties can be bactericidal to certain species [84].

The existing findings on the predominant phyla in the SI in mice are somewhat contradictory. In some studies, the SI has been found to have a higher relative abundance of *Bacteroidetes* than *Firmicutes* [70, 80, 82]. *Bacteroidetes* are known to resist the bactericidal activity of bile acids [59]. However, other studies have suggested that *Firmicutes*, rather than *Bacteroidetes*, is the most abundant phylum in the SI [59, 87]. A possible explanation for this discrepancy may be that studies are done in different housing environments which affect the composition of intestinal microbes differently. Several studies have for instance shown that different animal facilities “carry” their own microbiota which can have a large impact on the interpretation of microbiota results [88, 89]. Additionally, sampling was not the same among these studies; some collected the luminal content, whereas others collected from the mucus layer, and some have scraped mucosa containing luminal content. Apart from *Firmicutes* and *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* are usually found [80, 87, 90]. At a family level, the microbiota composition in the ileum of mice has been described to be dominated by *Lactobacillaceae* and *Enterobacteriaceae* [82]. In humans the duodenal microbiota was found to be dominated by

Firmicutes and *Actinobacteria* [91], while the bacterial communities found in human ileostomy fluid samples reported the presence of members of the *Firmicutes*, especially *Streptococcus* and *Clostridium spp.*, and a member of the *Proteobacteria*, *Escherichia coli* {[92]Zoetendal, 2012 #19}. *Streptococcus* and *Clostridium spp.* dominated also human jejunal and ileal biopsy samples [93]. In general *Streptococcus*, *Lactobacilli*, and the *Bacteroides* group are common findings of human jejunal and ileal samples [86, 93, 94].

Small Intestinal Microbiota: community structure under HFD and its function in lipid uptake

Only a handful of studies has so far analyzed the impact of HF feeding on the community structure of the small intestinal microbiota **(Table 1)**. Martinez-Guryn and co-workers found that after consumption of a HFD for four weeks the microbial population in the SI of mice was characterized by increase in *Clostridiaceae* family in all three segments and especially in jejunum and ileum, while the abundance of the *Bifidobacteriaceae* and *Bacteroidaceae* families decreased. It was also suggested that when GF mice were conventionalized with jejunal microbiota collected from HFD-fed mice there was an increase in lipid absorption caused by a microbiome that was able to induce the expression of fat transport genes (*Fabp2* and *Cd36*) and impairment of cholecystokinin signalling [59]. Interestingly, in another study where a four-week intervention was applied, ileal *Clostridia* decreased, which is not what Martinez-Guryn *et al.*, observed. Apart from different animal facilities, differences in the experimental design including control diet type, duration and sampling strategies may explain this. Additionally, *Bacteroidetes (S24_7 family)* decreased, while *Erysipelotrichi* increased in the HFD group. These findings were similar to the ones found in cecal and fecal samples. However, unique changes in all three different segments were also observed. It was also shown that not only the composition changed, but the bacterial density increased in duodenum, jejunum, and ileum of the HFD fed mice. Additionally, the spatial distribution was altered characterized by colonization of the ileal intervillous space, which is usually bacteria-free. Despite the high microbial density in ileum, the expression of antimicrobial peptides as well as mucus production were reduced possibly due to the decreased production of chloride in this site. Finally, segmented

filamentous bacteria could be seen at the top of the villi in ileum in the standard diet fed mice, but not in the HFD group [90]. When the composition of ileal microbiota in rats was studied in relation with time, during a six week HFD administration, the results revealed that the community structure is dynamic, constantly adapting to the environment in the lumen and that this is characterised by three main patterns: 1) abundance is reduced or increased at week one on HFD and remaining at these levels at weeks three and six (*Prevotella* and *Bacteroides*); 2) abundance is increased or decreased after one week on HFD but restored to that of chow-fed rats at weeks three and six (*Lachnospiraceae* family and *Oscillospira* of the order *Clostridiales*); and 3) abundance is increased (*Bacteroidales order*) or decreased (*Blautia*) progressively with time on HFD. Additionally, the investigators observed increased paracellular permeability in the SI, which was accompanied by a significant correlation with genera in the *Clostridia class* [95]. In a 12-week study the SI of mice was not divided in the individual segments but was rather examined as an entity. No changes in microbiota composition at phylum level were observed [70]. In another 12-week intervention based on a rat-mouse model the relative abundance of *Bacteroidetes* and *Actinobacteria* decreased, while *Proteobacteria* increased in the small intestinal mucosa. At a genus level *Lactobacilli* and *Allobaculum* decreased, while *Acinetobacter* increased in the HFD fed mice. Furthermore, the composition of small intestinal microbiota was found to be different than the one in feces both under standard diet and under HFD. The researchers concluded that *Bacteroidetes* and *Allobaculum* in the SI negatively correlated with weight gain [87]. However, once again the SI was not divided into segments. Analysing each segment of the SI individually is important as it has been found that in humans the composition in jejunum is different than the one in ileum [93]. Summing up, the main outcome of the studies conducted so far on HF feeding and SIM is that: 1) alteration in the amount and type of fat in the diet has an impact on the community structure of SIM; 2) in most studies this was characterised by a decrease in members of the *Bacteroidales* order and in some by a concurrent increase in *Clostridiales* members [59, 87, 90, 95]; 3) although some similarities are observed in the HFD-affected taxa in the small intestine and colon or feces, still exist unique features in these intestinal regions [59, 87, 90]; 4) in ileum, compositional alterations are dynamic adapting to the changes in the gut lumen over increased time exposure to HF conditions [95] and are accompanied by spatial

changes [90]; and 5) a microbial genetic pool enhancing epithelial lipid transport and cholecystokinin signalling is a possible mechanism for the increased fat uptake in conventionalized mice **(Figure 3)** [59].

Table 1: Study design and main structural changes of the small intestinal microbiota under high fat diet.

Model	**Intervention time (weeks)**	**Small intestinal segment**	**Control diet**	**Main structural changes**
Mice	4	Duodenum, jejunum, ileum	Low fat	Increase: *Clostridiaceae* *Decrease:* *Bifidobacteriaceae* & *Bacteroidaceae* [59]
Mice	4	Ileum	Chow	Increase: *Erysipelotrichi* Decrease: *Clostridia* & *Bacteroidetes(S24_7 family)* [90]
Mice	12	Undivided small intestine	Matched in terms of carbs, fat and protein	No difference at phyla level [70]
Rats	1-6	Ileum	Chow	In relation with time. Increase: *Oscillospira* Decrease: *Clostridia* [95]
Rats	12	Undivided small intestine	Chow	Increase: *Proteobacteria* Decrease: *Bacteroidetes* & *Actinobacteria [87]*

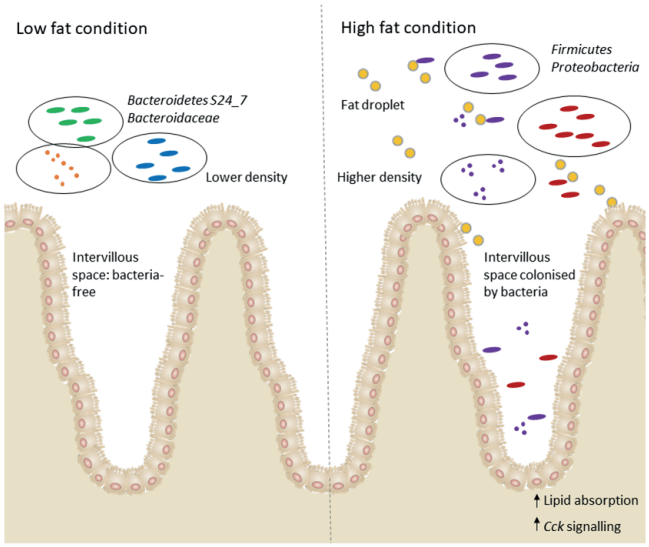


Figure 3: Under high fat conditions the small intestinal microbiota is characterized by increased members of the Firmicutes and Proteobacteria phyla and decreased members of the Bacteroidetes S24-and Bacteroidaceae families. Apart from taxonomic alterations, administration of a HFD leads to increased bacterial density in all segments of the small intestine, while the intervillous space in ileum becomes colonized by bacteria. This altered community structure enhances lipid absorption and the cholecystokinin pathway.

Small intestinal immune system: the impact of HFD

Numerous studies have shown an association between HF feeding and establishment of chronic inflammation in several organs and tissues, as for example adipose tissue, liver, hypothalamus, and muscles [76, 96-99]. In addition to these, recent evidence has shown that the immune system in the intestine is impaired by HF feeding leading to obesity and insulin resistance [76]. One of the suggested mechanisms is related to the alteration of the gut microbiota by HFD. It has been proposed that one of the main consequences of the unbalanced environment resulting from HF feeding is increased intestinal permeability. Under conditions of impaired gut barrier function increased leakage of bacteria or bacterial products has been observed leading to endotoxemia; a condition characterized by increased levels of plasma lipopolysaccharide (LPS) [76, 100, 101]. Increased permeability

of bacterial products is dependent on the microbial pattern recognition receptors NOD1 or CD14 and it results in activation of the innate immune system leading to chronic inflammation [76]. When low concentrations of LPS were infused in mice certain inflammation markers (TNF-α, IL-6, IL-1β) in several tissues increased resembling the effect of the HFD [100].

The innate intestinal immune system can be characterized as the first line of defence against infections. It possesses a very delicate function as it must maintain a balance between initiating inflammatory responses, while simultaneously providing immune tolerance to harmless antigens and commensal bacteria. Most studies analyzing the impact of HF feeding in the status of the intestinal immune system report that HFD leads to an inflammatory situation in the distal SI. More specifically, it has been shown that several pro-inflammatory cytokines and chemokines increase in the segments of the SI because of HFD consumption. Ding *et al*., showed that when conventional mice were fed HFD, TNF-α in ileum, as well as the expression of NF-κB along the intestine increased. This effect could not be observed in GF mice, pinpointing the role of intestinal bacteria in the detrimental effects caused by HF feeding [102]. In another mouse study, HFD consumption led to upregulation of intestinal genes expressing CCL5 and macrophage migration inhibitory factor (MIF) [103]. Furthermore, it has been shown that under HF feeding the expression of certain cytokines known to promote the barrier integrity (IL-10, IL-17A, IL-17F, IL-22) were downregulated in the ileum of mice and rats [95, 104].

The changes in the expression levels of cytokines are usually accompanied by a subsequent change in the intestinal cell population. It has been demonstrated in mice that after HF feeding for one week and before the onset of obesity, the intestinal eosinophils were depleted in the lamina propria coinciding with increased intestinal permeability [105]. In another mouse study, administration of HFD induced a pro-inflammatory shift in the immune cell populations illustrated by increased numbers of IFNγ-producing CD8+ T cells and Th1 CD4+ T cells and concurrently decreased number of CD4+ Foxp3+ Tregs [106]. A similar change in the Th1 cell population was also observed by another mouse study [104].

Coffee: Why is it relevant?

Coffee is the second most consumed beverage worldwide after water [107] and its daily annual consumption is estimated to be approximately 2,25 billion cups. It has been therefore suggested that coffee might have an impact on public health [108]. Coffee constitutes a mixture of numerous compounds, containing more than 800 volatile compounds [108]. The major components in coffee are caffeine, chlorogenic acids (CGAs), the diterpenes cafestol and kahweol and soluble dietary fiber, mainly galactomannans and arabinogalactan and minerals [109, 110]. Coffee is rich in dietary phenolic phytochemicals containing 200 - 550 mg per cup [111]. CGAs, which are esters formed between caffeic and quinic acids, belong to the plant polyphenols and have been shown to exhibit anti-inflammatory activities [112], while the other major compound in coffee, caffeine, exerts its effect through antagonism of adenosine receptors [108].

Several epidemiological studies have analyzed the impact of coffee consumption on health and the results appear to be conflicting at times. On the one hand, consumption of coffee has been postulated to exert health risks related to elevated levels of blood pressure and cardiovascular disease. However, recent well-controlled studies have shown that moderate consumption of coffee (3-4 cups/day) has either a neutral or a positive effect on blood pressure, cardiovascular disease, heart failure, and cardiac arrhythmias [108, 113]. Moreover, consumption of coffee was postulated to prevent against diabetes mellitus, complications of the metabolic syndrome, some types of cancer, as well as liver and neurological conditions [108, 114]. Overall, an observational study based on three large cohorts suggested that coffee consumption of one to five cups per day was associated with lower risk of mortality [115]. Interestingly, the positive correlations were stronger when the consumption of coffee was moderate rather than with low consumption (1 cup/day) [108].

Coffee, HFD, gut microbiota and inflammation: what is known

A couple of studies have examined how coffee affects various inflammatory markers in liver and blood. The results overall appeared to be contradictory [116-118]. Despite the increasing interest on the impact of coffee on metabolic disorders, few studies have so far

analyzed how consumption of coffee affects the composition of the gut microbiota. Coffee contains large amount of soluble dietary fiber and could therefore change the microbial community [109]. Additionally, coffee is a rich source of phenolic compounds, which are also known to alter the microbial composition [119-121]. In a study published in 2009 the fecal microbial composition from human samples was analyzed. The results showed that coffee consumption did not have an impact on dominant bacteria. Despite this, the probiotic *Bifidobacterium spp*. increased in the subjects consuming coffee [122]. A similar increase in the levels of fecal *Bifidobacteria* was also reported by another trial which analyzed the impact of colonic fermentation of the coffee oligosaccharides [123]. Last, in an obesity-prone mouse model coffee supplementation reduced liver inflammation, however, the microbial dysbiosis that was observed in the obese mice did not improve with coffee [110]. However, it should be highlighted here that all above mentioned studies focused on either fecal or colonic bacteria and did not evaluate the structural changes in SIM resulting from coffee consumption.

Few studies have also analyzed the impact of coffee when consumed in combination with HFD. A positive association of coffee consumption and modulation of the gut microbiota was found in a rat-based trial. More specifically, addition of caffeinated coffee attenuated the HFD-induced levels of the *Firmicutes* to *Bacteroidetes* ratio as well as the levels of *Clostridium Cluster XI* [124]. Finally, when mice were fed a HFD supplemented with 1.1% caffeine-containing instant coffee they had reduced metabolic markers as well as downregulated gene expression of IL-1β in liver and MCP-1 in white adipose tissue, suggesting an anti-inflammatory response caused by coffee consumption [125].

Objectives

The focus of this work is to investigate the homeostasis in the small intestine, including the impact of both ROS production and dietary changes in the structural community of the small intestinal microbiota and subsequently to the immune status of the host. To examine these, we used mouse models. More specifically, this work is divided in two subcategories:

1. the role of ROS produced by the epithelial cell layer in maintaining the homeostasis of SIM preventing bacterial overgrowth.

2. the impact of dietary changes in the composition of SIM and the regulation of the immune status of the host. Here, two different diet approaches were used:

i. HFD, containing 60 E% fat, using as a control diet a LFD containing 10 E% fat.
ii. HFD supplemented with two different coffee doses corresponding to 1 and 5 cups/day of human consumption.

The aim of this work was to:

- Explore whether high levels of ROS are produced in the epithelial cells in the terminal SI as a response to the bacterial load in this site and identify their role in maintaining the homeostasis of SIM (Paper I).
- Study the impact of HF feeding in the composition of SIM and examine how these would affect the immune status of the host (Paper II).
- Explore whether addition of coffee (rich in polyphenols) would attenuate the potential alterations in the composition of SIM and the activation of the immune system in the SI caused by the HFD (Paper III).

Main findings and discussion

Gut microbiota refers to the collection of microorganisms colonising the GI tract. It consists of various genes and exerts numerous health related effects to the host. Although the composition of gut microbiota shows some heritable traits, it can get shaped by several environmental factors, dietary habits being among them [47]. Despite the increasing number of studies analyzing the impact of diet on the gut microbiota composition, the vast majority of them uses samples from either the large intestine or feces.

The small intestine possesses two properties that could largely affect the microbial composition; 1. it possesses a very high number of immune cells and 2. it is the site where nutrient digestion occurs. It is known that in the small intestine several interactions of the host with the microbes occur [71, 72]. In this thesis we have taken small intestinal microbiota into focus. First, we have investigated the role of ROS in the homeostasis of SIM (Paper I). Next, we have evaluated the impact of high intake of fat (Paper II) and coffee -rich in phenolic compounds- (Paper III) in the composition of SIM.

To address these research questions, we have used different mouse models. Rodents present certain similarities in anatomy, physiology, and genetics with humans in addition to being small, easy to maintain and have a short life cycle. Thus, murine models have been widely used in biomedical research [126, 127]. In this work, we have additionally used genetically modified mouse models (i.e., the iNOS and NOX1 KO mice). The use of KO models has made an important turning point and is common in intestinal inflammation research [128]. In the field of gut microbiota, mouse models are also widely used to study the role and the association of gut bacteria with certain diseases. However, the composition of the core gut microbiota is different between the two species and although many genera are common the abundance levels differ [126, 129]. Thus, one should always keep in mind that results from animal studies are not always directly translatable to humans [126]. In our case though, given the fact that we focused on the small intestinal microbiota, obtaining samples from humans would be an invasive process and use of *in vitro* approaches would have big limitations.

PAPER I

We first assessed the *in vivo* ROS levels and we observed very high levels of ROS produced in the distal SI, the ileum. To measure the production of ROS we used *in vivo* imaging and the L-012 probe, a chemiluminescence imaging probe that is specific for peroxynitrite. This technique makes use of an IVIS camera and quantifies the chemiluminescent signal emerging upon the reaction of L-012 with ROS [130]. The very high production of ROS was accompanied by upregulated gene expression of iNOS and NOX1 in ileum. iNOS and NOX1 produce nitric oxide and superoxide respectively, which react together to form peroxynitrite. Some previous studies have studied the role of NOX1 in redox modifications of signalling pathways [22], however here our goal was to explore an antimicrobial role of NOX1.

Next, we used various strategies to inhibit iNOS and NOX1 or to scavenge their products. These included the NOS inhibitor L-NAME; the superoxide scavenger TEMPOL, the peroxynitrite scavenger MnTBAP, as well as iNOS and NOX1 knock out (KO) mice. We observed that in all cases the resulting ROS levels from ileum reduced significantly indicating that the intestinal ROS production is iNOS- and NOX1-dependent.

Although we have hypothesized that peroxynitrite might be the effector molecule, there exist also other candidates that could be responsible for the observed effects. Some examples include hydrogen peroxide and hypochlorous acid. Both of these are involved in the oxidative burst occurring inside the lamina propria [131]. However, hydrogen peroxide does not activate L-012 and is dependent on the presence of high concentrations of peroxidase, while hypochlorous acid requires myeloperoxidase, which normally is not expressed at such high levels in the ileum of normal healthy mice [132, 133].

Our next aim was to examine whether this production is induced by the bacterial load present in the SI. We have therefore administrated the mice with broad spectrum antibiotic concoctions. After one week the L-012 emission levels reduced in the antibiotic-treated mice and reduced even further after two weeks. To confirm that the amount of bacteria in ileum was reduced in the antibiotic-treated animals, we counted the amount of cultivable bacteria from the luminal content, represented as cfu/g and we observed a significant

reduction. In accordance with these findings, expression of both iNOS and NOX1 was downregulated in the mice receiving antibiotics. Previous studies have shown that in GF animals the expression of iNOS and NOX1 in the small intestine is reduced [21] and that ROS production is lower (assessed *ex vivo*) [134].

Last, we assessed both the microbial load and the community structure of SIM and the microbiota in cecum in the iNOS and NOX1 KO mice. Here, it should be pinpointed that these mice were bred in our animal facility and were cohoused for 3-4 weeks with wild type littermates after weaning to ensure same environmental exposure. The results showed that both KO mouse models had higher microbial load in SIM, but no difference in cecum. Additionally, the community structure in both KO models was similar and it resembled the composition of the microbiota in cecum indicating that under the absence of ROS in ileum a certain degree of reflux occurs from cecum.

Summing up, here we have shown that there is a high production of ROS levels, likely represented by peroxynitrite, from the epithelial cells in the ileum of normal healthy mice. This production is induced by the microbial load in the small intestine and acts as part of the defence of the innate immune system in order to control the homeostasis of SIM and prevent negative consequences to the host as for example bacterial overgrowth.

PAPER II

Since we have elucidated the role of ROS in ileum in the homeostasis of SIM in Paper I, here we sought to understand the influence of dietary components, and more specifically of fat in the community structure of SIM and the impact on the immune regulation of the host. We have therefore administrated mice with HFD diet for 18 weeks. As a control diet we chose to use a LFD, where the difference in fat percentage was replaced by corn starch.

We observed that the HFD fed mice had higher body weight. This was accompanied by higher energy intake, adipocyte size and impaired glucose regulation. Regarding the composition of SIM the α-diversity, measured by the Shannon Index, was not altered in the two groups. However, this was not surprising as it is known that SIM is not as phylogenetically diverse as the microbiota in the large intestine [72]. With regards to β-

diversity, we did observe differences between the two diet groups. More specifically, in jejunum and ileum we saw higher relative abundance of the *Firmicutes* to *Bacteroidetes* ratio and of the *Proteobacteria*, which was characterized by higher levels of the *γ-Proteobacteria* class whose several members are known to be pathogens [135]. Similar changes have been observed before as a result of HF feeding in studies analysing the structure of SIM [59, 136]. Furthermore, we observed higher levels of circulating LPS binding protein (LBP), which is considered to represent the levels of LPS. We hypothesized that this might be the result of the bloom in *Proteobacteria*, which carry LPS in their outer membrane. Last, the higher LPS levels could also be the result of its incorporation into chylomicrons together with fat and its subsequent release from the IECs [137].

Next, we measured the *in vivo* production of ROS, using the same technique as in Paper I. We saw that HF feeding led to higher levels of intestinal ROS. However, when we analysed the expression levels of iNOS and NOX1, we did not see an upregulation in the HFD group. Instead, we observed higher levels of NOX2, which is known to participate in the process of oxidative burst carried by macrophages and dendritic cells residing in the lamina propria. Indeed, after assessing the developmental stage of the monocyte-deriving macrophages, we saw that indeed there was a higher ratio of pro-inflammatory macrophages in the HFD group implying that HFD induces the recruitment of pro-inflammatory macrophages into the lamina propria.

In accordance with the findings described so far, we also observed an increase in the release of pro-inflammatory cytokines. This was most pronounced in jejunum followed by ileum. Additionally, the expression of certain pattern recognition receptors (PRRs) was as well upregulated. PRRs possess an important role in intestinal homeostasis and have been previously associated with increased permeability after HF feeding [101, 138]. However, when we measured the expression of the tight junction protein genes, zonula occludens and occluding, we saw that they were upregulated in the HFD group. Additionally, we observed an upregulation in the expression of RegIIIγ, an antimicrobial peptide. We hypothesize that these are the result of a compensatory action of the host to combat the detrimental effects induced by the changes in the microbial environment.

Taken together, in Paper II we have shown that administration of HF feeding associated with changes in the community structure of SIM, towards a more harmful composition, especially in ileum. This resulted in an adaptive response from the host as seen by an increased recruitment of proinflammatory macrophages and release of pro-inflammatory cytokines, as well as by a higher production of NOX2 dependent-ROS.

PAPER III

As a continuation of Paper II, here we sought to explore whether addition of coffee, a mixture rich in bioactive compounds, would attenuate the negative impact of HF feeding both with regards to the composition of SIM and the degree of intestinal immune activation from the host. To achieve this, we fed mice with different diets; LFD and HFD (as in Paper I), HFD supplemented with coffee representing the human consumption of 1 cup/day and HFD supplemented with coffee representative of 5 cups/day. Few previous studies have analysed the impact of coffee consumption on the composition of gut microbiota, however none has studied the composition of SIM [122, 123].

Our results showed that coffee supplementation on moderate doses (5 cups/day) was able to attenuate the negative effects of HFD on the composition of SIM and more specifically in jejunal and ileal microbiota. In more details, mice fed HFD had lower relative abundance of *Bacteroidetes* and higher abundance of *Firmicutes*, which was mainly represented by lower relative abundance of *Bacteroidales S24_7* family group and *Faecalibaculum* and higher abundance of *Peptoclostridium*. We observed that these altered levels were consistent for all segments of SI and even throughout the whole intestine, i.e., including the large intestine. *Firmicutes* and *Bacteroidetes* are the major phyla in the intestinal tract and an imbalance in their ratio induced by HFD has been referred to as dysbiosis [49, 51, 55, 87, 139, 140]. The diet group containing low dose of coffee showed no statistically significant differences regarding these taxa, while mice fed the diet containing 5 cups/day showed significant alterations in these taxa in a similar manner as the LFD group. Additionally, we observed that the coffee supplemented diets resulted in higher relative abundance in *Actinobacteria* which was attributed to higher *Coriobacteriaceae UCG-002*. Coffee is a

mixture rich in many bioactive compounds, and there exist several candidates that could explain the positive impact in the gut microbiota. For example, coffee contains high amounts of peptides as well as poly-and oligosaccharides that could act as nitrogen and carbon source and therefore provide a growth advantage to certain bacterial taxa present in SIM [141]. In addition, coffee contains phenolic compounds, especially chlorogenic acid which has been shown to positively affect the composition of the gut microbial population during HF feeding [142]. Last, caffeine has been also reported to positively affect the gut microbiota in the colonic mucosa [143].

Establishment of an unbalanced environment as a result of HF feeding has been associated with impairment in the gut barrier function resulting in bacterial leakage which is dependent on NOD1, a microbial PRR [76]. Indeed, in our study, despite the higher expression of tight junction protein genes in the HFD group, we observed higher expression levels of TLR4, NOD1 and circulating LBP indicating increased transcellular permeability. We then wanted to explore whether the improvement in the microbial profile after coffee consumption would restore the intestinal barrier. We saw that coffee addition to the HFD in moderate doses had lower levels of circulating LBP, and downregulated expression of intestinal NOD1. Furthermore, both coffee groups showed even higher expression levels of the tight junction genes.

As it has been previously shown that an increased inflammatory status follows HF feeding [76], here we sought to study whether coffee has an impact on intestinal inflammation. Coffee contains various substances, such as chlorogenic acid, caffeine and trigonelline, that could affect certain inflammatory markers. Previous studies have analysed the impact of coffee on inflammation in liver and blood with contradictory results [116-118]. We observed higher levels of intestinal ROS under HF feeding. The elevated ROS levels in the HFD group could potentially be the result of the unbalanced SIM. Upon coffee supplementation the elevated ROS levels caused by HFD were significantly lower. Regarding the changes in the developmental phase of the monocyte deriving macrophage population in lamina propria, we saw that that the ratio of the anti-inflammatory to pro-inflammatory macrophages was higher in the coffee groups (in a dose dependent manner) and that HFD alone had lower levels. Furthermore, addition of moderate doses of coffee in

the HFD downregulated the expression of TGF-β and TNF-α in the small intestine. Our data indicates that coffee consumption at moderate doses dampens, at least in parts, the expression of pro-inflammatory cytokines in the intestine.

Our overall findings showed that moderate coffee consumption ameliorated the negative effects that were induced by HF feeding in terms of small intestinal gut microbiota composition. This was accompanied by an enhancement of the barrier function and a dampened intestinal inflammatory status of the host.

Concluding remarks and future perspectives

In the present work we evaluated the interaction of the small intestinal microbiota with the immune system of the host and certain dietary compounds. Our overall results have revealed the regulation of SIM by epithelial ROS as well as the influence of fat and coffee on the community structure of SIM and the immune status in the intestine of the host.

In the first subpart of this work (represented by Paper I) we have shown that the presence of SIM is inducing very high levels of ROS produced from the epithelial cells in ileum and that this is a two-way interaction meaning that when the production of ROS is disturbed several changes in the population of SIM occur. Here, we have hypothesized that peroxynitrite might be the effector molecule. Overall, these findings provide the first step to further analyses following up closely the implications a disturbance in ROS production and a modulation of SIM would pose to the host. In addition, here we took advantage of two KO mouse models, the iNOS and NOX1 KO. It would be of interest to include other KO models of other intestinal ROS sources, as for example DUOX2.

Additionally, the higher microbial load in the SI suggested an antimicrobial role of ROS deriving from the epithelial cells. Considering that the production of ROS was particularly high in the terminal ileum which is also the site where the microbial load is highest in the SI, we believe that the production of ROS at this site plays a unique role in preventing uncontrolled bacterial growth which could potentially lead to pathogenic conditions as SIBO. Therefore, it would be interesting to use other genetically engineered models susceptible to bacterial overgrowth and modify the production or scavenge ROS.

In the second subpart (represented by Paper II and III) we have shown that changes in diet associate with changes in SIM. More specifically, under HF feeding we observed an unbalanced microbial composition in jejunum and ileum which further activated the intestinal immune system of the host which was depicted by a change in macrophage population in the lamina propria, production of higher levels of ROS in ileum and release of certain pro-inflammatory cytokines. When coffee, a mixture rich in several bioactive compounds, was supplemented in the diet together with HFD, it attenuated the negative effects of HF feeding to SIM and consequently to the above mentioned intestinal

inflammatory markers. Future perspectives to be followed up here, would be to explore the impact of coffee under non-HF feeding, for example in combination with the LFD or a chow diet. Furthermore, here we have speculated that the regulation of the immune system of the host is the result of the altered SIM profile, we see a benefit to clarify this by the use for example of animals under GF conditions. Last, as in this work we have shown an association of diet with SIM, we believe that a next important step is to study the underlying mechanisms. The use of GF models (and their colonization with microbiota from the different diets) would be a possible approach in this case.

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PAPER I

iNOS- and NOX1-dependent ROS production maintains bacterial homeostasis in the ileum of mice

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The intestinal epithelial cells constitute the first line of defense against gut microbes, which includes secretion of various antimicrobial substances. Reactive oxygen species (ROS) are well characterized as part of the innate phagocytic immunity; however, a role in controlling microorganisms in the gut lumen is less clear. Here, we show a role for nitric oxide synthase (iNOS)- and NOX1-produced ROS in maintaining homeostasis of the gut microbiota. *In vivo* imaging revealed distinctly high levels of ROS in the ileum of normal healthy mice, regulated in accordance with the amount of gut bacteria. The ROS level was dependent on the nitric oxide and superoxide producers iNOS and NOX1, respectively, suggesting peroxynitrite as the effector molecule. In the ileum of iNOS- and NOX1-deficient mice, the bacterial load is increased and the composition is more cecum like. Our data suggest a unique role of ileum in maintaining homeostasis of gut microbes through production of ROS with potential importance for preventing reflux from the large intestine, bacterial overgrowth, and translocation.

INTRODUCTION

The small intestine possesses a dual role: on the one hand, it is responsible for uptake of nutrients, which requires a large absorptive surface, whereas on the other hand it must prevent the gut lumen microbiota from entering this same surface. The gut lumen is separated from the host interior by only one cell layer, which is ideal for its absorptive role. However, this creates a challenge for its role as a barrier. As part of the barrier function, the epithelial cells interact with the microbes in the gut lumen. Several secretory molecules, such as mucins that make up the mucus layer, antimicrobial peptides, and secretory IgA participate in controlling the gut microbes. Reactive oxygen species (ROS) are universally accepted to be an essential component of the innate immune system through the respiratory burst in neutrophils, macrophages, and dendritic cells; however, an antimicrobial role of ROS in the gut is less determined. Here, we have explored a role of ROS production in maintaining bacterial homeostasis in the small intestine at the border with the large intestine.

The ROS producing enzymes, Dual oxidase (DUOX) and NADPH oxidase (NOX), are expressed in the intestinal epithelial cells in correlation with microbial content, indicating a role of these enzymes in ROS-based immune homeostasis of the gut.[1] In *Drosophila*, it is shown that DUOX produces ROS in the gut, which is directly bactericidal, and necessary to control gut bacteria and prevent infections. The DUOX in *Drosophila* contains two enzymatic domains: one oxidase domain that produces hydrogen peroxide and one peroxidase domain that converts the hydrogen peroxide to hypochlorous acid.[2] In *C. elegans*, DUOX-dependent ROS production in the gut was shown to improve survival during bacterial infections; however, no direct bactericidal effect was determined.[3] Hydrogen peroxide is suggested as the effector molecule, but this is a less bactericidal molecule than hypochlorous acid. In mammals, DUOX2 is reported to have an anti-bacterial role in cultured primary airway epithelial cells[4] and in defense against *Helicobacter felis* infection in gastric epithelium.[5] In these systems, it was suggested that DUOX2 produced hydrogen peroxide as a substrate for lactoperoxidase, with a subsequent formation of the bactericidal hypothiocyanous acid. In a DUOX2-deficient mouse, bacterial translocation was increased and expression of a ROS-inducible gene was downregulated in

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the mucosal-segmented filamentous bacteria.[6] NOX1 is highly expressed in epithelial cells, particularly, in colon and ileum. Most studies have focused on NOX1's role in redox modifications of signaling pathways involved in cell division and differentiation, particularly, with respect to wound healing in colon.[7] However, its role in controlling gut microbes is elusive. In one study of NOX1 knockout (KO) mice inoculated with *Salmonella typhimurium*, no effect on cecal concentration of the bacterium and changes in overall protection were observed.[8] In another study with *Citrobacter rodentium* infection, reduced cecal numbers and lower disease status were found in NOX1 KO mice.[9] They further provided evidence suggesting NOX1 to operate through regulation of DUOX2. The NOX enzymes generate superoxide, which does not kill microbes directly, but is precursor of other ROS with bactericidal activity such as hydrogen peroxide, hydroxyl radicals, hypochlorous acid, and peroxynitrite. Superoxide combines with nitric oxide (NO) to form peroxynitrite, which is regarded as one of the crucial bactericidal ROS in the respiratory burst of the innate immune system. NO is produced by inducible nitric oxide synthase (iNOS), which is expressed by epithelial cells of the small intestine in association with the amount of bacterial content.[1,10] Furthermore, NO is found in the gut lumen.[11] This raises the question whether peroxynitrite produced in the intestine can affect the luminal gut bacteria and thus participate in maintaining homeostasis of gut microbes.

Here, we have found evidence for a high NOX1- and iNOS-dependent ROS production in the ileum of normal healthy mice. We furthermore observed a strong impact of this ROS production on the microbial load and composition in the ileum. This suggests a unique role of ileum in maintaining homeostasis of gut microbes through production of peroxynitrite with potential importance for preventing reflux from the large intestine, bacterial overgrowth, and invasion.

RESULTS

iNOS- and NOX1-dependent production of ileal ROS

To determine whether ROS are present in the small intestine, we used the optical imaging probe L-012, which has high specificity for ROS, particularly, peroxynitrite.[12] *In vivo* imaging of L-012-dependent luminescence showed a strong signal coming from the abdominal region of the mice (**Figure 1a**). Dissection and imaging of individual organs *ex vivo* showed that the signal originated from the distal small intestine, the ileum (**Figure 1b**). This overlaps with the particularly high expression of iNOS in ileum in comparison to the rest of the intestine and the relatively high expression of NOX1 in ileum compared to more proximal parts (**Figure 1c**). As these enzymes catalyze the production of superoxide anion and NO, which rapidly react to form peroxynitrite we wanted to determine their involvement in the ROS production. We injected the NOS inhibitor L-NAME i.v. ($50\,mg\,kg^{-1}$) and imaged the L-012-mediated luminescence, which was reduced by 70% (**Figure 1d**). To elucidate the involvement of superoxide, we added the superoxide scavenger TEMPOL in the drinking water ($2.5\,g\,l^{-1}$) (**Figure 1e**). After 2 days, the L-012 signal was reduced by more than 60%. To assess the presence of peroxynitrite, we administrated the peroxynitrite scavenger MnTBAP chloride i.v ($20\,mg\,kg^{-1}$), and we here observed around 60% reduction in the L-012 signal (**Figure 1f**). Furthermore, we explored the ROS production in KO mice of iNOS and NOX1. We first imaged the L-012 signal in KO mice cohoused with wild type (WT) mice and then 4 weeks after being separated and housed as single genotypes (**Figure 1g**). The signal was reduced by around 60% in the iNOS KO mice and almost abolished in the NOX1 KO mice. We did not observe any significant difference between the mice during and after cohousing. Furthermore, as peroxynitrite is a strong oxidant, we gave the antioxidants vitamin C ($10\,g\,l^{-1}$) and tannic acid ($5\,g\,l^{-1}$) in the drinking water of WT mice. We measured the L-012 signal over 3 weeks (**Figure 1h**). The signal went gradually down reaching 70% reduction for both antioxidants. To determine whether the epithelial cells could be responsible for the peroxynitrite production, we measured the NO content in these cells with the probe DAF-FM using flow cytometry (**Figure 2a, b**). We observed in the cells staining positively for the epithelial cell marker CD326, a significantly larger fraction of cells with DAF-FM labeling in the distal small intestine compared with the proximal region. Furthermore, the distal cells showed higher mean fluorescent intensity indicating more NO production in the distal cells compared to the proximal. As innate immune cells in the lamina propria can mount a respiratory burst, we also determined the NO content in cells stained with CD11b, a marker for monocytes, dendritic cells, and macrophages. There was significantly higher signal from the epithelial cells in comparison to CD11b stained cells. Taken together, these observations support an iNOS- and NOX1-dependent ROS production in epithelial cells of ileum.

The amount of gut bacteria is associated with the ROS production

To elucidate whether gut bacteria can induce ROS production in ileum, we manipulated the amount of bacteria. To decrease the bacterial load, we first used an antibiotics treatment proven to almost fully deplete the gut microbiota ($1\,g\,l^{-1}$ neomycin, $0.5\,g\,l^{-1}$ vancomycin, $1\,g\,l^{-1}$ metronidazole, and $1\,g\,l^{-1}$ ampicillin, oral gavage daily for a week).[13] As this is a harsh treatment with potential unforeseen physiological effects and depletion of ileal microbiota is sufficient here, we also exposed the mice to a milder antibiotic treatment ($0.5\,g\,l^{-1}$ neomycin and $0.25\,g\,l^{-1}$ vancomycin through the drinking water over 4 weeks) (**Figure 3a, b**). In both cases, the L-012-mediated signal went down by $\sim$80%. As expected, the bacterial load went down in ileum (**Figure 3c**). Consistent with these observations, the mRNA expression of both iNOS and NOX1 was decreased in the ileum (**Figure 3d, e**). We also decreased the bacterial content in the intestine by 15 h fasting (**Figure 3f**). This caused a reduction in the L-012 signal from the mice by 70%. To study the ROS production during an increase in the bacterial load, we imaged mice before, during and after weaning, as this is a period where massive increase of intestinal bacteria occurs.[14] Here, we observed a large increase in

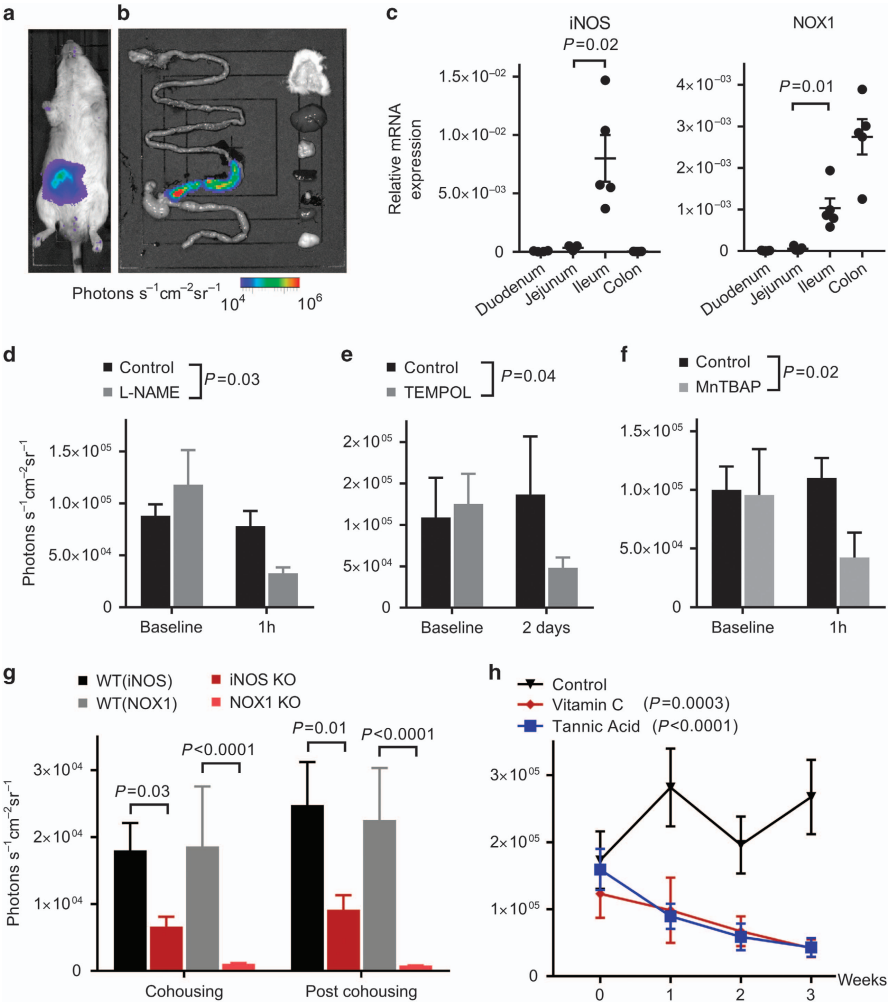


Figure 1 iNOS- and NOX1-dependent production of ileal ROS. (**a**, **b**) L-012-mediated luminescence signal (**a**) *in vivo* and (**b**) *ex vivo* indicating high ROS production in the terminal part of the small intestine, the ileum. Dissected organs displayed to the right in the panel illustrates low L-012 signal (from top to bottom: skin, liver, pancreas, spleen, inguinal lymph node, kidney and mesenteric fat). The pseudo colors represent photons s^{-1} cm^2 sr. (**c**) mRNA expression of iNOS and NOX1 in the small intestine and colon of WT mice. GADPH is used for normalization. Both of the genes are highly expressed in ileum, $n=5$. (**d**–**h**) The following conditions reduced the L-012-mediated signal: (**d**) L-NAME provided i.p., $n=6$; (**e**) TEMPOL in the drinking water, $n=8$–10; (**f**) MnTBAP provided i.v., $n=8$ (**g**) iNOS and NOX1 KO mice compared to WT that were imaged both during cohousing with WT mice and 4 weeks after separation to individual cages, $n=15$–30; (**h**) Vitamin C or tannic acid in the drinking water, $n=15$. Values are mean with s.e.m. (**c**) Student's *t*-test. (**d**–**f**) Student's *t*-test of the difference in reduction between control and treatment groups. (**g**) Kruskal–Wallis test with Dunn's correction for multiple comparisons. (**h**) One-way ANOVA. iNOS, nitric oxide synthase; ROS, reactive oxygen species; WT, wild type.

the L-012 signal the first week after separating the mice from their mother, which thereafter stabilized (**Figure 3g**). In conclusion, these finding suggest that the intestinal ROS production is dependent on the presence of bacteria in the gut.

iNOS and NOX1 KO mice had higher bacterial load in the distal small intestine

As we observed that iNOS and NOX1 are necessary for the ROS production, we sought to determine whether the bacterial content in the gut of iNOS and NOX1 KO mice is increased compared to WT mice. To balance the environmental influence, the KO and WT mice were cohoused for 3 to 4 weeks directly after weaning. The mice were then allocated to separate cages based on genotype for 4 weeks before sampling. We estimated the amount of bacteria in the luminal chyme by colony-forming units (CFU) counting (**Figure 4a**) and in the mucus by RT-qPCR against the 16S rRNA gene (**Figure 4b**).). In both KO

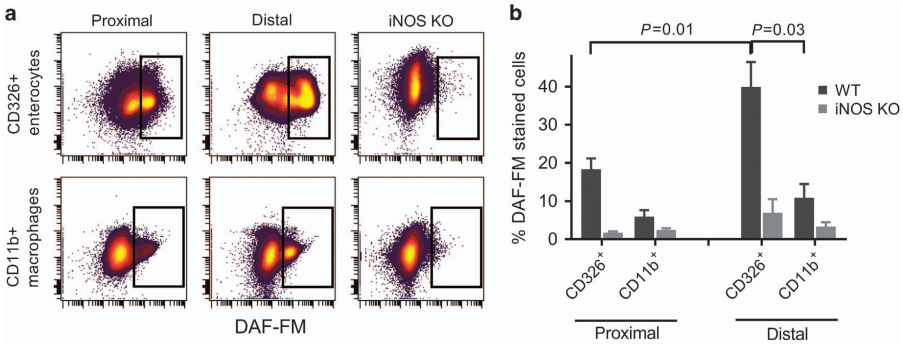


Figure 2 Flow cytometry analysis of NO content in cells of proximal and distal part of the small intestine. (**a**) Scatter plots of the NO probe DAF-FM staining combined with the epithelial cell marker CD326 and the phagocyte cell marker CD11b in cells isolated from the epithelial layer and lamina propria. Labeling of cells from iNOS KO is consistent with the absence of NO production (**b**) WT and iNOS KO mice show higher NO production in epithelial cells of distal intestine compared to both proximal intestine and macrophages of the lamina propria Mann–Whitney test. Furthermore, in all the corresponding cell populations of the iNOS KO and WT mice, the iNOS KOs showed significantly reduced NO production ($P < 0.03$ for all comparisons) Kruskal–Wallis test with Dunn's correction for multiple comparisons. Values are mean with s.e.m. iNOS, nitric oxide synthase; NO, nitric oxide; WT, wild type.

types, the amount of bacteria was higher in ileum and jejunum in comparison to the WT mice for the luminal samples. For the mucosal samples, we did observe higher bacterial load in the ileum of the two KOs, but it was only statistically significant for the NOX1. We also estimated the bacterial content in cecum samples by CFU counting (**Figure 4a**). Here, we found no significant difference between either of the KO with the WT mice. Interestingly, we observed that in the KOs the bacterial amount reached cecum-like numbers in ileum, consistent with potential bacterial overgrowth. In conclusion, the amount of bacteria in the distal small intestine is dependent on both iNOS and NOX1 suggesting involvement of a converging effect, possibly the formation of peroxynitrite.

The microbial composition in the small intestine is altered in a similar manner for the iNOS and NOX1 KO mice

To determine the impact of ROS production on potential alterations in the composition of the gut microbiota, we performed 16S rRNA gene sequencing of DNA extracted from mucosa and luminal chyme samples of the WTs and the iNOS and NOX1 KO mice. We observed that at phylum level, there is a similar shift in the microbial composition in jejunum and ileum for both KO mice (**Figure 5a**). Firmicutes increase and Bacteroidetes concurrently decrease. This can be seen for both mucosa and luminal chyme samples; however, it is more pronounced in the former. In cecum, the microbial composition appears to be unaltered between the KO and the WT mice. To investigate whether the gut microbiota is also altered at finer taxonomic levels, we performed a Principal component analysis (PCA) using the relative abundance of the taxa classified at family level (**Figure 5b–e**). Families that were present in at least five mice with relative abundance higher than 0.5% were included in the analysis. The PCA distinguished the ileal samples of the WT and KO, whereas the two KO types co-clustered. The results were similar for the mucus and chyme samples; however, the chyme samples show some degree of overlap with the WT. All cecum samples showed co-clustering. In addition, we sought to determine whether there is an overlap between the specifically differentiated taxa for each of the two KO mice. We performed a LEfSE analysis on the ileal mucosal samples to identify the taxa that were associated with the iNOS and the NOX1 KO mice. Five taxa were identified for the iNOS KO mice: four increasing (Actinobacteria, Actinomycetales, *Sporosarcina*, *Allobaculum*) and one decreasing (S24_7). For the NOX1 KO mice, nine taxa were identified: eight increasing (Actinobacteria, Bacillaceae, Coriobacteriaceae, *Dietzia*, *Turicibacter*, *Bifidobacterium*, *Sporosarcina*, *Allobaculum*) and one decreasing (*Lactobacillus*). We next compared the relative abundance of the enriched identified taxa in each of the KOs to the WT (**Figure 6**). Six out of the eight taxa associated with the NOX1 KO mice show a statistically significant increase in the iNOS KO mice, whereas three out of the four taxa associated with the iNOS KO mice increase in the NOX1 KO mice. However, the taxa that decreased in each of the KOs did not change significantly in the other KO type. Taken together, these findings suggest that iNOS- and NOX1-dependent production of ROS in the small intestine alters the gut microbial composition. The observed alterations appear to be similar for both KOs indicating a synergistic effect of NO and superoxide.

iNOS and NOX1 KO mice show a shift in ileal microbiota resembling more cecal composition

We observed a higher ratio of Firmicutes to Bacteroidetes for the two KOs (**Figure 7a**). Interestingly, this moves the microbiota composition in a direction resembling more the one in cecum. Furthermore, the increased Firmicutes/Bacteroidetes ratio is most pronounced in the ileum and becomes gradually less in more proximal parts of the small intestine. To elucidate whether this could be caused by

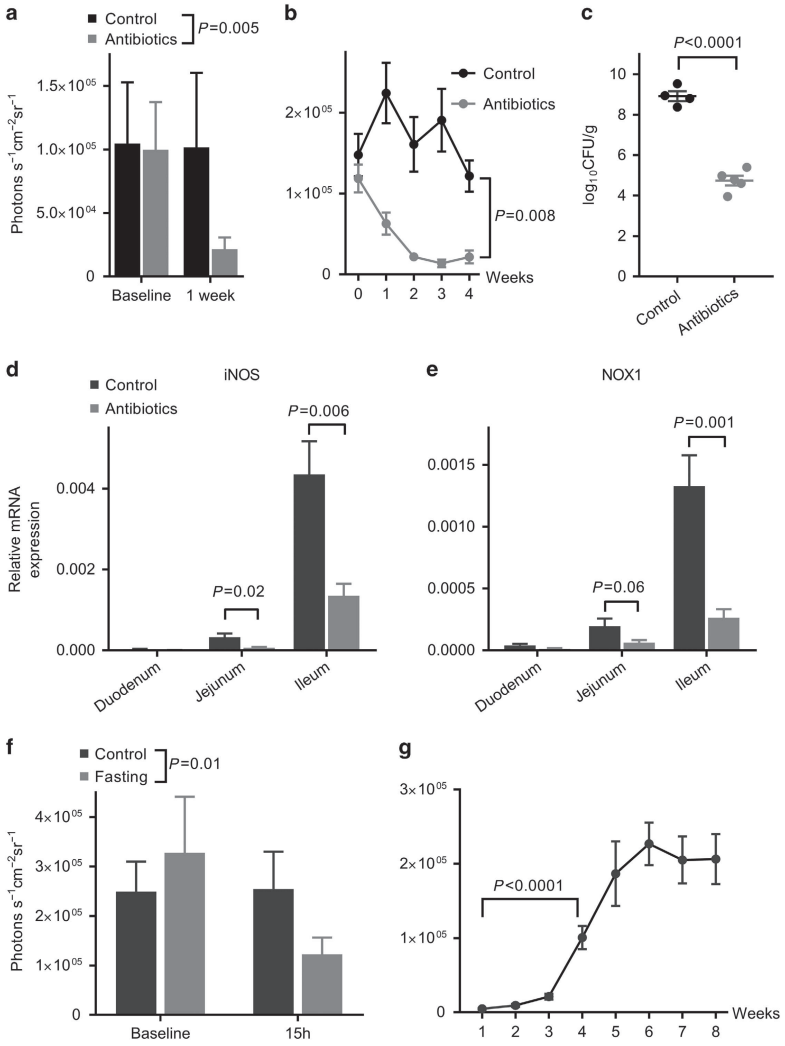


Figure 3 Effect of bacterial luminal load on ROS production. (**a**, **b**) The L-012-mediated signal is reduced in mice receiving antibiotics concoctions either by (**a**) daily oral gavage, $n = 5$–6 or (**b**) via the drinking water, $n = 10$. (**c**) The amount of cultivable luminal chyme bacteria in ileum is reduced in mice receiving antibiotics via gavage over 1 week, $n = 4$–5. (**d**, **e**) The mRNA expression of iNOS and NOX1 decreases in jejunum and ileum in mice receiving antibiotics in the drinking water over 4 weeks, $n = 10$. (**f**) The L-012-mediated signal goes down in mice after 15 h of fasting $n = 10$. (**g**) The L-012 signal is minimal in the 3 weeks prior to weaning and increases severely after separation from mother $n = 11$–16. Values are mean with s.e.m. (**a**, **f**) Student's *t*-test of the difference in reduction between control and treatment groups. (**c**) Student's *t*-test (**b**, **g**) one-way ANOVA. (**d**, **e**) One-way ANOVA with Sidak's correction for multiple comparisons. iNOS, nitric oxide synthase; ROS, reactive oxygen species.

migration of bacteria from cecum to ileum, we identified all genera that were significantly more abundant in cecum than in ileum of WT mice and determined if they were increased in the KO mice (**Figure 7b**). We observed the abundance in ileum of most of these genera to be higher in both of the two KO mouse types compared to WT. In conclusion, these results may imply that ROS production in ileum has a role in prevention of cecal microbiota reflux.

Bacterial DNA is higher in the liver of the KO mice

To evaluate potential implications of the intestinal ROS production on bacterial translocation, we determined the content of bacterial DNA in liver by RT-qPCR against the 16S rRNA gene. We found an increase in bacterial DNA of 56% ($n = 6$–10, $P = 0.02$, Kruskal–Wallis test with Dunn's correction for multiple comparisons) for iNOS KO mice and of 112% ($n = 10$–13, $P = 0.009$) for NOX1 KO mice compared to WT

mice, indicating a role for intestinal ROS production in defense against bacterial translocation.

DISCUSSION

In the present study, we have demonstrated that ROS, possibly peroxynitrite, are highly produced by iNOS and NOX1 in the ileum of normal healthy mice. Furthermore, the amount, composition and translocation of intestinal bacteria appear to be regulated by this ROS production.

To address temporal and spatial *in vivo* ROS production, we exploited the luminescent probe L-012 as an imaging marker for ROS.[15–18] We observed a strong L-012-mediated signal in ileum that we believe is the result of epithelial cell secretion of superoxide and NO, with a resultant peroxynitrite formation. This is supported by the following: firstly, L-012 is recognized to act extracellularly, and with a high specificity and sensitivity to peroxynitrite.[12] NOX1 together with iNOS are responsible for the signal and the major substrate of superoxide, in terms of reaction kinetics, is NO, which together form peroxynitrite.[19] Both L-NAME, a NOS inhibitor, and using iNOS KO mice reduced the signal, whereas the superoxide quencher TEMPOL and particularly knocking out NOX1 nearly abolished the L-012-generated signal. Secondly, NOX1 and iNOS are co-expressed in the small intestine, with a particularly high expression in the ileum. In addition, the L-012 signal is not present in the colon where NOX1 has high expression, but where iNOS is not detectable. Finally, we demonstrated that NO production is more abundant in ileal epithelial cells compared to more proximal intestinal regions and immune cells of the lamina propria. Others have reported that iNOS expression in the healthy gut is confined to the ileal villi.[10] We regard these data as convincing evidence of NO and superoxide production by epithelial cells of ileum, which may join to form peroxynitrite in the gut lumen.

Although peroxynitrite is likely detected in our experiments, there are other candidates such as hydrogen peroxide and hypochlorous acid, which both have prominent roles in respiratory burst.[9] However, hydrogen peroxide is in itself a poor activator of L-012 and requires the presence of high concentrations of peroxidase,[20] which to our knowledge is not expressed at high levels in the ileum of normal healthy mice. Hypochlorous acid can activate L-012, but its production is dependent on myeloperoxidase.[21] Hydrogen peroxide can also be converted to hydroxyl radicals in the presence of Fe^{2+}

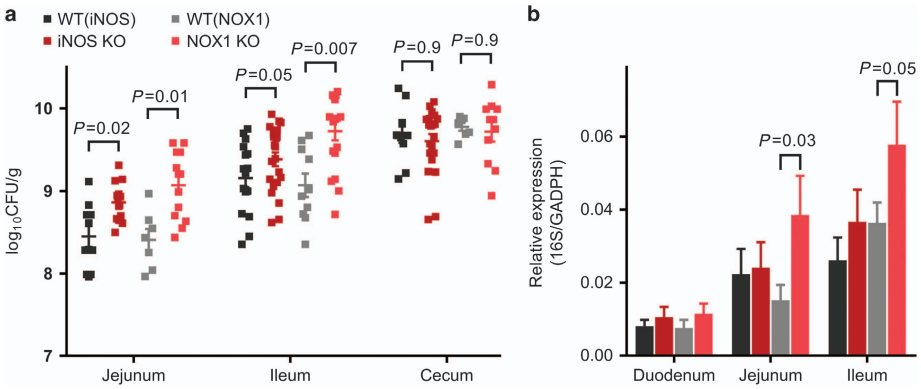
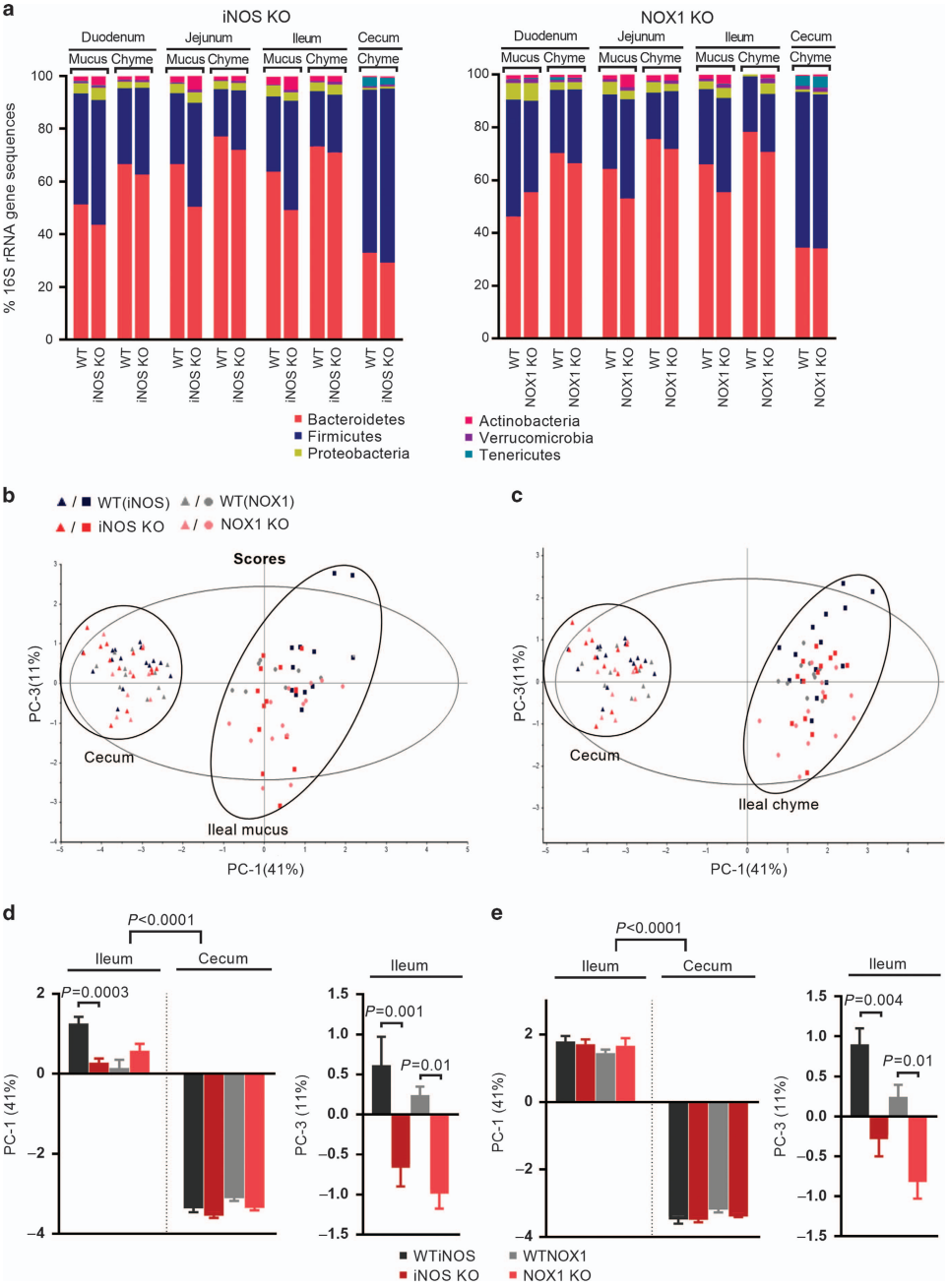


Figure 4 ROS production in the distal part of the small intestine affects the amount of mucosal and luminal bacteria. (**a**) Amount of cultivable bacteria in the luminal chyme of iNOS and NOX1 KO mice. In both jejunum and ileum, the amount of bacteria is increased in the KO mice in comparison to their, respectively, cohoused WT mice. In cecum, no such difference could be detected, $n = 10–16$. (**b**) Amount of bacteria in the mucus layer as determined by RT-qPCR of 16S rRNA. GADPH is used for normalization. The bacterial load is increased in the jejunum and ileum of the NOX1 KO mice, $n = 9–15$. Values are mean with s.e.m. (**a**) One-way ANOVA with Sidak's correction for multiple comparisons. (**b**) Kruskal–Wallis test with Dunn's correction for multiple comparisons. iNOS, nitric oxide synthase; ROS, reactive oxygen species; WT, wild type.

Figure 5 Gut microbial composition of the iNOS and NOX1 KO mice shows overlap. (**a**) Relative abundance of phyla in iNOS and NOX1 KO mice and their, respectively, cohoused WT mice. In the two KO mice, Firmicutes are increased in the mucus layer of both jejunum ($P < 0.0001$ for both KO) and ileum ($P < 0.0001$ for iNOS KO and $P = 0.01$ for NOX1 KO), whereas Bacteroidetes decrease (jejunum $P < 0.0001$ for both KO, ileum $P < 0.0001$ for iNOS KO and $P = 0.006$ for NOX1 KO). For the ileal chyme samples, Firmicutes are statistically increased only in NOX1 KO ($P = 0.004$), whereas Bacteroidetes increase only in jejunum in iNOS KO ($P = 0.02$) and in ileum in NOX1 KO ($P = 0.0002$). No differences could be detected in the cecum samples, $n = 10–16$. Values are mean with s.e.m. One-way ANOVA with Tukey's *post hoc*. (**b**, **c**) PCA plot of the taxa at family level of the ileum and cecum samples. After examining all possible principal components, we found that PC-1 and PC-3 could best explain the variation among the groups. All of the cecum samples were co-clustered regardless of mouse groups. (**b**) The ileal mucosa samples of the two KO mice cluster together and the two WT mice cluster together with minimal overlap between the KOs and the WTs, $n = 8–15$. For the ileal chyme samples (**c**) a similar pattern is observed; however, more overlap can be seen between the iNOS and the WT, $n = 10–15$. (**d**, **e**) Loading scores of cecum, ileal mucosa (**d**), and ileal chyme (**e**) samples presented in PCA plots in **b** and **c**. In accordance with the above findings, both KO mice for both of the ileal compartments differ from their respective WT on PC3, whereas the iNOS mucosal samples are also separated in PC1, $n = 8–15$. Values are mean with s.e.m. One-way ANOVA with Sidak's corrections for multiple comparisons. iNOS, nitric oxide synthase; PCA, principal component analysis; ROS, reactive oxygen species; WT, wild type.

through the Fenton reaction. Hydroxyl radicals can activate L-012, but the concentration of Fe^{2+} in the intestine is low. However, superoxide can through the Haber–Weiss reaction generate Fe^{2+} suggesting that a combination of NOX1 and DUOX2 could generate part of the intestinal ROS responsible for the L-012 signal. Indeed, when eliminating the iNOS activity



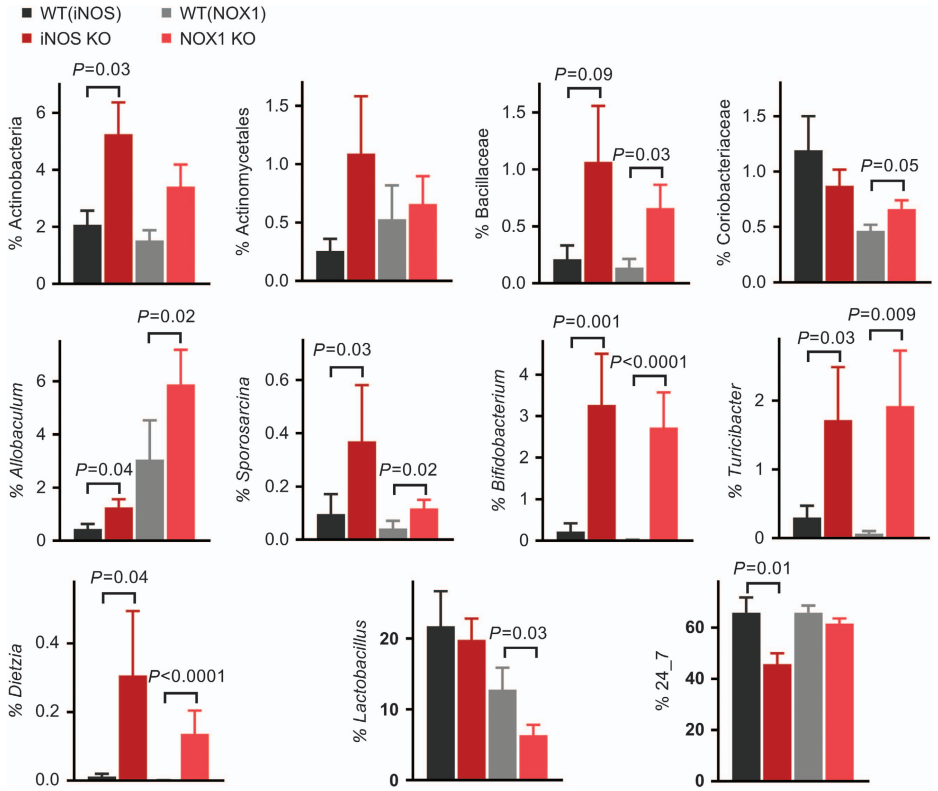


Figure 6 Overlap in identified taxa between iNOS and NOX1 KO mice. Relative abundance of the taxa that either increased or decreased in each of the KO mice as identified by LEfSE. The two KO types show a similar increase for most of these taxa, $n = 10–14$. Values are mean with s.e.m. For each taxon after logarithmic data transformation, one-way ANOVA with Sidak's correction for multiple comparisons. iNOS, nitric oxide synthase; LEfSE, linear discriminant analysis effect size.

not all the signal is abolished. In addition to ROS production related to immune defense, ROS are also produced by the electron transport chain inside the mitochondria and in connection with intracellular signaling, but this is a global phenomenon and comprise only low levels of ROS concentrations, and hence cannot be imaged *in vivo*.

The strength of the L-012-mediated signal is exceptionally high, which implies that the amount of ROS produced in the ileum is high. This is in accordance with previous studies where L-012 is used to image ROS during various conditions of acute inflammation.[15,17] Interestingly, the signal strength generated by L-012 in models of arthritis and skin inflammation is lower than the signal we observe in a healthy ileum. However, it resembles the amount of signal in models of colonic inflammation.[17,18] This raises the interesting question if a large amount of ROS production only is consistent with secretion in non-host tissue, such as the intestinal lumen. Certainly, constant exposure to an extensive oxidative environment can be detrimental to the host tissue.

We found that in the ileum of iNOS and NOX1 KO mice the bacterial load was increased, suggesting an antimicrobial role for the epithelium-deriving ROS. This could be seen overall in ileum, both in the luminal content and in the mucosa. However, the approach used for the assessment of the bacteria in the luminal content was based on CFU counting, which only assesses the cultivable bacteria, and thus gives an underestimate of the total bacteria that are present. In addition, the microbial composition of both KO mice was altered. The changes in bacterial composition were similar between NOX1 and iNOS KO mice and to some extent more evident in mucus compared to lumen samples, supporting a cooperative effect of superoxide and nitric oxide originating from the epithelium, as discussed above possibly through the secretion of peroxynitrite. The strong reduction of L-012-mediated signal following administration of broad-spectrum antibiotics, concurred with a downregulation of iNOS and NOX1. This observation fits well with comparable data showing that germ-free mice have four- and sevenfold lower expression of NOX1 and iNOS, respectively, than conventional mice.[1] Furthermore, intestinal ROS production is lower in germ-free mice, as detected by a broad range *ex vivo* ROS probe.[22] L-012 signals were also influenced by other conditions that change the bacterial load such as after

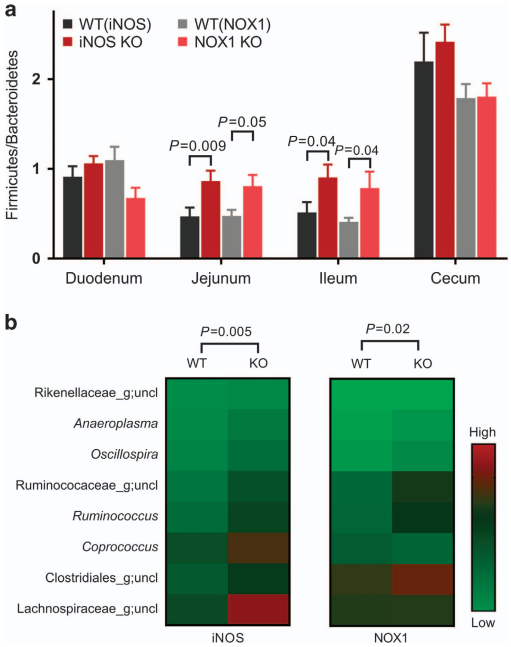


Figure 7 The gut microbial composition of the iNOS and NOX1 KO mice shifts in the direction of cecum. (**a**) Ratio of relative abundance of Firmicutes and Bacteroidetes in different mucosa gut segments. The ratio increases for both KO mice in jejunum and in ileum. No difference was observed for duodenum, $n = 11–16$. Values are mean with s.e.m. One-way ANOVA with Sidak's correction for multiple comparisons for jejunum and duodenum samples, Kruskal–Wallis test with Dunn's correction for multiple comparisons for ileum samples. (**b**) Shift in the relative abundance of the eight genera that were higher in cecum compared to the small intestine. The heat map shows the ratio of ileum to cecum. Columns represent the mean value of each group. In general, in both KOs the mice showed warmer colors compared to the WTs, indicating an ileal shift in these genera to a cecum-like profile, $n = 8–12$. One-way ANOVA. iNOS, nitric oxide synthase; WT, wild type.

introduction of solid food in weaned mice and during a fasting period.[14,23] It has been known for some time that ROS affect the luminal bacteria directly. It is reported that during an infectious state DUOX-dependent ROS exert antimicrobial properties in the gut of *Drosophila*.[2] In addition, hydrogen peroxide through the activity of DUOX2 is reported to directly influence the virulence of *Campylobacter jejuni* in an organ culture.[24] In a DUOX2 KO mouse, a ROS-inducible gene was lowered in mucosal associated bacteria suggesting a role of hydrogen peroxide against microbes close to the epithelial surface.[6] ROS through the action of NOX1 has foremost been associated with intracellular signaling related to tissue-wound repair in the colon.[7,25] Pircalabioru *et al.* [9] showed reduced DUOX2 expression in a NOX1 KO mouse during infection suggesting a regulatory role of NOX1 in relation to luminal bacteria. They further observed, in accordance with our results, that microbiota in cecum of the KO had the same distribution of the main phyla as in the WT mice. However, they found an increase in certain species of *Lactobacilli*. It is difficult to compare their data directly with ours as they did not assess the microbiota composition in ileum. Nevertheless, we found that *Lactobacilli* are reduced in the ileum of NOX1 KO mice. As *Lactobacilli* are known to harbor a strong defense against ROS,[26,27] we believe that the reduction of ROS rather removes a competitive advantage that can explain the decrease in *Lactobacilli*. Our data contribute to the knowledge about NOX1 by showing a direct role in controlling the gut microbiota.

The microbiota content in ileum is much higher than in the rest of the small intestine. This is likely caused by microbial reflux from the large load of microbes in cecum and colon. This suggests a unique role for ileum in preventing bacterial overgrowth and maintaining homeostasis of microbes in the small intestine. This is reflected by the higher density of lymphoid tissue in ileum compared to the rest of the gut. We believe that secretion of ROS, potentially peroxynitrite, from the ileal epithelial cells is an integral part of a defense system controlling the small intestinal microbiota and thus important for the nutrient uptake and barrier role of the gut. These insights may have implications for understanding the pathogenic mechanisms that underlie conditions or diseases often found in the ileum such as SIBO, Crohn's disease, and ileitis.

METHODS

Animals. Animal procedures were approved by the Norwegian Animal Research Authority (Mattilsynet). All experiments apart from the experiments with KO mice were performed on NMRI mice of both genders up to 3 months of age from Janvier Labs. In experiments where both genders were used, we could not observe any gender-specific differences. Breeding stocks of C57BL/6J, and the following KO mice: iNOS$^{-/-}$ and NOX1$^{-/-,\ y/-}$ on C57BL/6J background were purchased from The Jackson Laboratory. As the largest impact of environmental influence on gut microbiota composition occurs during weaning, we cohoused the KO mice for 3–4 weeks together with WT mice directly after separation from mother.[14,28] All mice used for the analysis of the gut microbiota composition were supplied by the same provider (The Jackson Laboratory, Bar Harbor, ME). The diet was RM1 diet (SDS Diet, Essex, UK) and mice were housed in humidity, temperature and by 12 h night/day light cycle controlled environment in individually ventilated cages (Innovive, San Diego, CA).

Imaging. Imaging was done with IVIS Lumina II (Perkin Elmer, Walthamn, MA). The luminescent probe L-012 (Wako Chemical, Neuss, Germany) was dissolved in saline and injected intraperitoneally (i.p.) at 10 mg kg^{-1}. During *in vivo* imaging the mice were immobilized using isoflurane (2.5–3.5%). *Ex vivo* imaging on dissected organs was performed within 5 min of L-012 injection. Data acquisition were done with the Living Image software (Perkin Elmer). Light emission from the region of interest was quantified as photons s^{-1} cm^{-2} steradian^{-1}.

Flow cytometry. Isolation of an epithelial cell fraction and a lamina propria cell fraction was done as previously described by Goodyear *et al.*[29] Briefly, after killing the mouse, the small intestine was dissected and divided into a proximal and a distal part to be analyzed separately. Peyers patches were removed, the intestine was opened longitudinally and cut into 5 mm long pieces and placed in ice-cold RPMI. The tissue successively went through a three-step process removing mucus, isolating epithelial cells, and isolating lamina propria cells. Mucus was removed by incubation in HBSS/5 mM DTT/2% FBS for 20 min at 37 °C with agitation. Epithelial cells were isolated by three incubation steps each in HBSS/5 mM EDTA/2% FBS for 15 min at 37 °C with agitation. Lamina propria cells were isolated by digestion in HBSS/

Liberase (Roche, Basel, Switzerland)/DNAse (Sigma Aldrich, St Louis, MO) for 30 min at 37 °C. The epithelial cell fraction and lamina propria cell fraction were further enriched for/or depleted-off epithelial cells using magnetic EpCam microbeads in accordance with the manufacturer's protocol (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were pre-blocked in 60% inactivated rat sera and 10 μg ml^{-1} of anti-CD16/32 (HB2.4) in PBS/2%FBS/2 mM EDTA for 10 min on ice. Cells where washed and stained in PBS/2%FBS/2 mM EDTA for 30 min on ice with 5 μl per test of the following antibodies: CD326 Pacific blue (Biolegend, San Diego, CA), CD11b PE-Cy7 (BD Bioscience, San Jose, CA), CD3-APC (BD Bioscience), and CD24-PE (Biolegend). NO was detected with the florescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) in accordance with the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA). The labeled cells were acquired on a LSR II flow cytometer (BD Bioscience). Flow data were analyzed in Cytobank.

Sampling. Biological samples were collected under sterile conditions after killing the mice by neck dislocation. The small intestine was divided in three: duodenum (most proximal 5 cm), jejunum (6 cm around the center), and ileum (most distal 6 cm). Cecum samples were taken directly opposite to the ileocecal valve to collect samples close to the valve that additionally are easy to anatomically identify to avoid sampling error. Luminal chyme for DNA extraction or microbe cultivation was squeezed out. The intestinal fragments were cut longitudinally and mucosal samples for RNA or DNA extraction were scraped off with a glass slide.

Counting of cultivable microbes. Luminal chyme samples were weighed, suspended in 1:10 sterile PBS (Sigma Aldrich) and homogenized by brief vortexing. The suspensions were serially diluted in the range of 10^{-1} to 10^{-8} and 100 μl of each dilution factor were plated on 7% freshly prepared horse blood agar plates (Oxoid, Basingstoke, UK). The procedure was performed in duplicates and the plates were incubated at 37 °C under anaerobic conditions for 48 h before counting CFU.

DNA extraction. Luminal chyme and mucosal samples were placed in S.T.A.R buffer (Roche) complimented with acid-washed glass beads (size < 106 μm, Sigma-Aldrich) directly after dissection. Cells were lysed by homogenization in a MagNaLyser (Roche) at 6500 rpm for 2 × 20 s with a cooling step in-between. Samples were centrifuged at 14,000 *g* for 5 min. The supernatants were transferred to 96-well plates and DNA was extracted using the Mag Mini LGC kit (LGC Genomics, Teddington, UK) according to the manufacturer's protocol in a KingFisher Flex DNA extraction robot (Thermo Fisher Scientific).

Real-time quantitative. Mucosal samples were placed in RNAlater (Sigma-Aldrich) directly after dissection. mRNA was isolated with NucleoSpin RNA/Protein Purification kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol and cDNA was synthesized with the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). The primers used for mRNA expression were as follows: Gapdh, forward 5′-CTTCAACAGCAACTCCCACTCTT-3′ and reverse 5′-GCCGTATTCATTGTCATACCAGG-3′ (T_m 60 °C); iNOS, forward 5′-GACATTACGACCCCTCCCAC-3′ and reverse 5′-ACTC TGAGGGCTGACACAAG-3′ (T_m 62 °C); NOX1, forward 5′-GTGATTACCAAGGTTGTCATGC-3′ and reverse 5′-AAGCCTC GCTTCCTCATCTG-3′ (T_m 64 °C). The primer used for genomic quantification were as follows: 16S rRNA gene (modified from ref. 30) forward 5′-TAGCTATTACCGCGGCTGCT-3′, and reverse 5′-AC TCCTACGGGAGGCAGCAGT-3′ (T_m 64 °C). Gapdh forward 5′-AATACGGCTACAGCAACAGG-3′ and reverse 5′-TCTCTTGC TCAGTGTCCTTG-3′ (T_m = 56 °C). The RT-qPCR was performed with FirePol EvaGreen qPCR Supermix (Solis BioDyne, Tartu, Estonia) in accordance with the manufacturer's protocol in a LightC ycler 480 Instrument II (Roche). The thermal cycle parameters were as follows: 12 min at 95 °C; 40 cycles of 15 s at 95 °C followed by 20 s at T_m (primer optimized); 20 s at 72 °C. LinReg Software was used to calculate Cq values and primer efficiency. The exact efficiency was used for the comparative C_q values analyzes.

16S rRNA gene sequencing. The analysis of the composition of the gut microbiota was performed on iNOS and NOX1 KO and their respective WT mice. The 16S rRNA gene sequence workflow analysis has previously been reported.[31] Briefly, after DNA extraction the 16S rRNA gene was PCR amplified for 25 cycles using prokaryotes-targeting primers developed by Yu *et al.*[32] The PCR product was purified with AMPure XP (Beckman-Coulter, Brea, CA) and 10 further PCR cycles were performed. The resulting amplicons were sequenced on Illumina MiSeq V3 platform (Illumina, San Diego, CA). Resulting 300 bp paired-end reads were further paired-end joined, quality-filtered using QIIME[33] and clustered with 97% identity level using closed-reference *usearch v7.0* algorithm[34,35] against Greengenes database v13.8.[36]

Statistical analyses. Statistical significance values were calculated in the GraphPad Prism software (La Jolla, CA). Averages are presented as mean and variances as standard error of the mean (s.e.m.). Statistical significance level was set as $\alpha = 0.05$. We chose 3,000 sequences per sample as a cut-off to normalize the sequencing data. Linear discriminant analysis effect size (LefSe) with LDA score > 2 was used to identify taxa associated with the KO groups.[37] PCA were performed in Unscrambler 14.1 (Camo software, Oslo, Norway).

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AUTHOR CONTRIBUTIONS

A.K. conceived and designed the study. C.M. and A.K. performed most of the experiments and analyzed data. S.D.C.R. and O.A.H. performed experiments. K.R. provided equipment and reagents. C.M., H.C., and A.K. wrote the paper. All authors reviewed and provided comments to the paper.

DISCLOSURE

The authors declare no conflict of interest.

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PAPER II

Small intestine microbiota and immune status in high-fat dieting mice

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Abstract

The small intestine's primarily role is to digest and absorb nutrient from the diet. However, the small intestine also has an important role in protecting the host against detrimental effects of the abundant microbes of the gut. The challenge of both absorbing nutrients and preventing microbes from harming the host along the same surface aspire for unique barrier and immune functionalities of the intestinal wall. Fat enriched diet is established to significantly change the composition of the microbiota in the large intestine. However, relatively few studies have explored the effect of diet rich in fat on the small intestinal microbiota and related responses in the gut wall. Here, we have fed mice high-fat diet (HFD) or low-fat control diet (LFD) to explore changes in microbiota composition in distinct segments of the small intestine and further assessed the intestinal barrier function and immune status. The HFD group showed an unbalanced gut microbiota composition characterized by a higher *Firmicutes* to *Bacteriodetes* ratio and lower abundance of *Proteobacteria*. At genus level we observed higher prevalence of *Peptoclostridium* and decrease in members of the *Bacteroidales S24_7* family and *Faecalibaculum*. Furthermore, the number of T-regulatory cells was reduced and the proportion of proinflammatory macrophages was higher. In line with this, the cytokines TNF-α, IL-1β, TGF-β and CCL5 were elevated and the production of antimicrobial cytotoxic reactive oxygen species was higher. When assessing the gut barrier integrity, we found upregulated expression of genes encoding the tight junction proteins and the antimicrobial peptide Reg3γ, while FITC dextran 4 influx was decreased, suggesting a strengthened barrier of the small intestinal wall. In conclusion, we observed an association between consumption of HFD and an unfavorable small intestinal microbiota profile and activation of defensive host responses possibly preventing putative detrimental systemic effects.

Introduction

The small intestinal epithelial layer is absorbing most of the food nutrients and has for that purpose a huge surface area in intimate contact with the luminal content of the small intestine (SI). However, the lumen is also the habitat of a vast number of microbes that ideally get prevented from crossing the intestinal wall. Thus, a well-controlled host-microbe interaction is required in the SI, which signify the importance of an elaborate intestinal barrier in order to maintain a healthy homeostatic environment in the SI. Here, we investigate how this homeostasis is influenced by a high-fat diet (HFD) with respect to microbial composition, barrier permeability and immune activity of the SI. This is of relevance for health beyond intestinal disorders as local intestinal conditions are linked to systemic metabolic diseases such as obesity and glucose dysregulation, cardiovascular disease and brain disorders [1-5].

Multiple pathways are suggested regarding the impact of HFD on systemic metabolic disorders, however, induction of intestinal chronic low-grade inflammation through a shift in the microbial composition of the gut is highly emphasized since diet-induced microbial and immunological changes in the intestine appear to affect immunity in other tissues relevant to metabolic disorders [6-14]. Compared to the large intestine, there are much less studies examining the influence of HFD on the microbiota in the SI [15]. In feeding interventions up to four weeks the *Bacteroidetes (S24-7)* and *Clostridiaceae* families were both decreased in the distal part of the SI [16] [17] [18]. Interestingly, the microbiota composition in jejunum shaped by the HFD feeding directly promoted lipid absorption [18]. In a 12 weeks intervention study *Bacteroidetes* and *Actinobacteria* decreased, while *Proteobacteria* increased [20]. However, in another study of equal length no changes were observed [19]. However, neither of these studies discriminated between different segments of the SI. In this study, we have divided the SI into the three segments duodenum, jejunum, and ileum. In general, it is unclear to what extent HFD changes the small intestinal microbiota, however, a switch to fewer *Bacteroidetes* (mainly characterized by the *S24-7* and *Bacteroidaceae* families) and more *Firmicutes* and *Proteobacteria* appears to be a relevant hypothesis. Such microbiota composition is regarded to be unbalanced with

respect to microbial homeostasis and it is suggested to have potential adverse effects on gut health [3].

Most studies of the immune response to HFD in the SI have characterized the cytokine profiles in mucosal tissue, and for the most, they suggest a proinflammatory profile by HFD [7]. This is particular prominent in the distal part after long interventions [15, 21] which is in accordance with observed increased NF-κB activity during HFD feeding [10]. Furthermore, the T-cell population after long-time HFD feeding shifts to a more inflammatory profile as expressed by reduced numbers of Foxp3/Treg cells and increased numbers of Th1, CD8 and Th17 cells [6, 22]. This is also more pronounced in the distal part of the SI. However, after one week of HFD eosinophils which normally accumulate during intestinal inflammation are depleted [23]. The effects of HFD on macrophages (MΦ) and dendritic cells (DC) are little studied, and only minor changes are observed [22, 23]. However, these studies did not distinguish between the different subtypes of MΦ and DC which have substantially different functions in regulating inflammation in the intestine.

We have here analyzed changes in MΦ subtype proportions in response to HFD. MΦ of the SI in the adult stage are entirely of hematopoietic origin and are constantly replenished. When blood monocytes extravasate into the mucosa, they are described to undergo a four-stage developmental process to mature resident MΦ [24]. The fully matured MΦ recognized by high expression of CX3CR1 and MHCII lack some of the classical inflammatory response patterns, including secretion of proinflammatory cytokines and oxidative burst, typical for most other MΦ [25, 26]. This MΦ still clear microbes and scavenge degraded cell material from the lamina propria, but without generating inflammatory responses of detrimental character to the surrounding tissue, making them well adapted to maintain homeostasis and tolerance in the constant immune challenged environment of the intestine. The fully mature MΦ are also involved in regulation of Treg cells and in the production of anti-inflammatory cytokine IL-10 [26]. However, in state of intestinal inflammation, the monocytes entering the mucosa do not reach full maturation but instead remain in an intermediate differential stage, recognized by intermediate expression of CX3CR1 and low to intermediate level of MHCII. These MΦ display classical proinflammatory behavior in response to exposure of microbial materials [25].

In previous studies of the effects of HFD in the SI, varying results and some contradictions are observed [7]. Of several potential explanations the dietary components substituting the fat in the control diet is of high relevance as this directly influences differences in the microbiota composition. In most studies, the control diet is a randomly chosen chow diet which substantially differences in nutrient composition compared to the HFD. Here, we have used a control diet that differs in only one component as the fat is substituted with corn starch.

In the study presented here, we fed mice either HFD or LFD for 18 weeks. We segmentally analyzed the SI both with respect to microbial composition and host barrier and immune functionality. Our data suggest an unbalanced bacterial community structure in the distal segments and more impermeable intestinal barrier and proinflammatory environment in HFD fed mice.

Results

HFD induced obesity and insulin resistance

18 weeks of HFD consumption resulted in higher body weight, larger adipocyte size in epididymal fat depots, and impaired glucose homeostasis compared to LFD control mice (**fig. 1**). HFD-fed mice gained 45% more weight than LFD-fed mice, and the difference in body weight became significant from the third week (**fig. 1a**). Energy intake in the HFD group was on average 20% higher than in the LFD-fed mice (**fig. 1b**), and the average transversal area of adipocytes was almost doubled (**fig. 1c-d**). Regarding glucose regulation, the HFD group had higher fasting blood glucose levels, reduced insulin sensitivity and significantly higher blood glucose concentrations in response to an oral glucose tolerance test (**fig. 1e-f**)

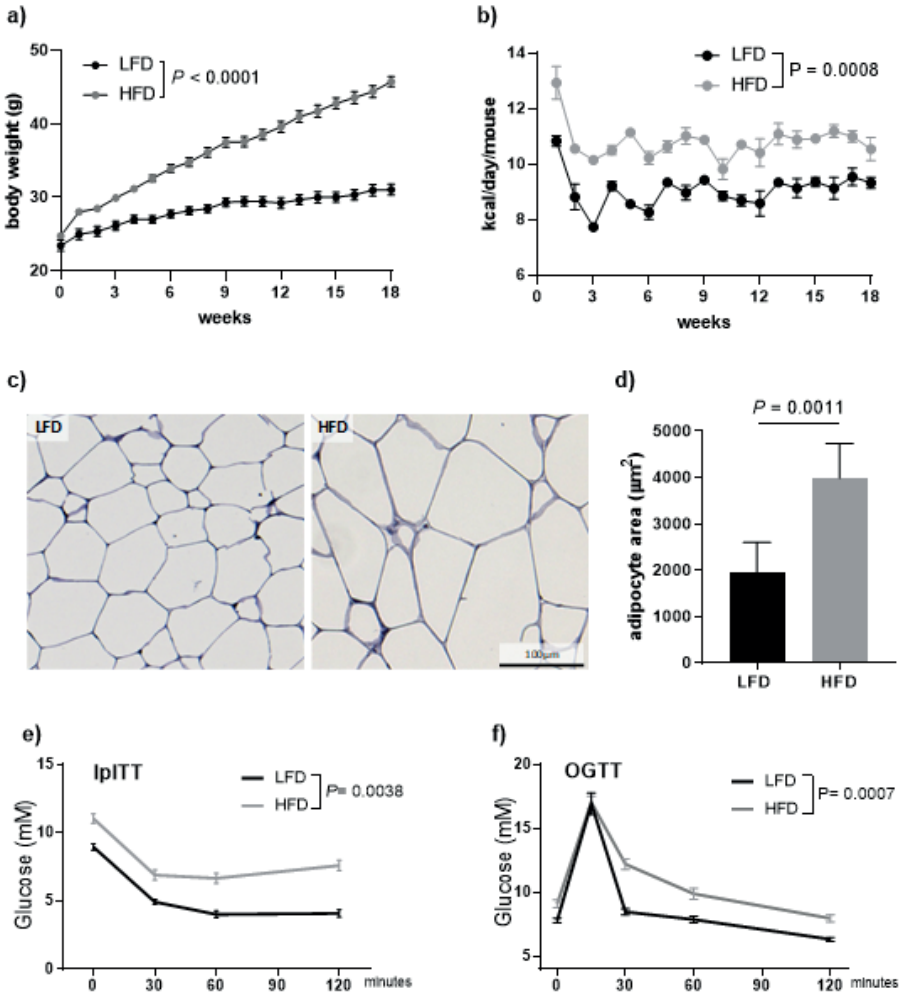

Figure 1. **Obesity and glucose regulation during 18 weeks of HFD feeding**.

(**a**) Body weight development; (**b**) Daily estimated energy intake; (**c**) Representative sections of epidydimal adipose tissue; (**d**) Average transversal area of adipocytes (n=6); (**e**) IpITT after 6 hours of fasting; (**f**) OgTT after 4 hours of fasting; (**a**, **b**, **e**, **f**) Mixed-effect model with Sidak's multiple comparisons test; (**d**). Unpaired t-test. All have n=11-12 besides the estimation of cell area. Values are presented as mean with s.e.m as error bar.

Intake of HFD promoted an unbalanced microbiota profile

To determine the impact of HFD on the composition of the gut microbiota, we evaluated the 16S rRNA gene profile of samples from duodenum, jejunum, ileum, and colon. At phylum level, we observed differences between the two diet groups throughout the SI (**fig. 2a**-**b**). In all SI segments, there was a higher *Firmicutes* to *Bacteroidetes* ratio in the HFD group (**fig**. **2c**). In addition, there was higher relative abundance of *Proteobacteria* in the ileal samples of the HFD group (**fig. 2b**). At genus level, the HFD group showed more *Peptoclostridium* in both jejunum and ileum, an uncultured genus of the *Lachnospiraceae* family in jejunum and *Lactobacillus* in ileum. Furthermore, we observed less *Bacteroidales S24_7* and *Faecalibac*ulum in both jejunum and ileum (**fig. 2d**). In colon, we found no significant group differences in samples from mucosa, while in the chyme the microbial composition resembled what we observed in ileum, namely more *Lactobacilli* and *Peptoclostridium* and less *Bacteroidales S24-7* in the HFD group (**fig. 2d**). PCoA plots reveal differences in microbial composition of ileum and colon in agreement with the results above (**fig. 2e-i**). Furthermore, we have evaluated the impact of the HFD on the α-diversity using the Shannon Index. We found no difference in the small intestinal segments, whereas in the colonic chyme samples the HFD group had a lower total number of different species in comparison to the control (**fig 2j**). In conclusion, our results indicate that HFD has an impact on the microbial composition of the whole intestine. Interestingly, the HFD appears to have particularly large effect in the ileal area.

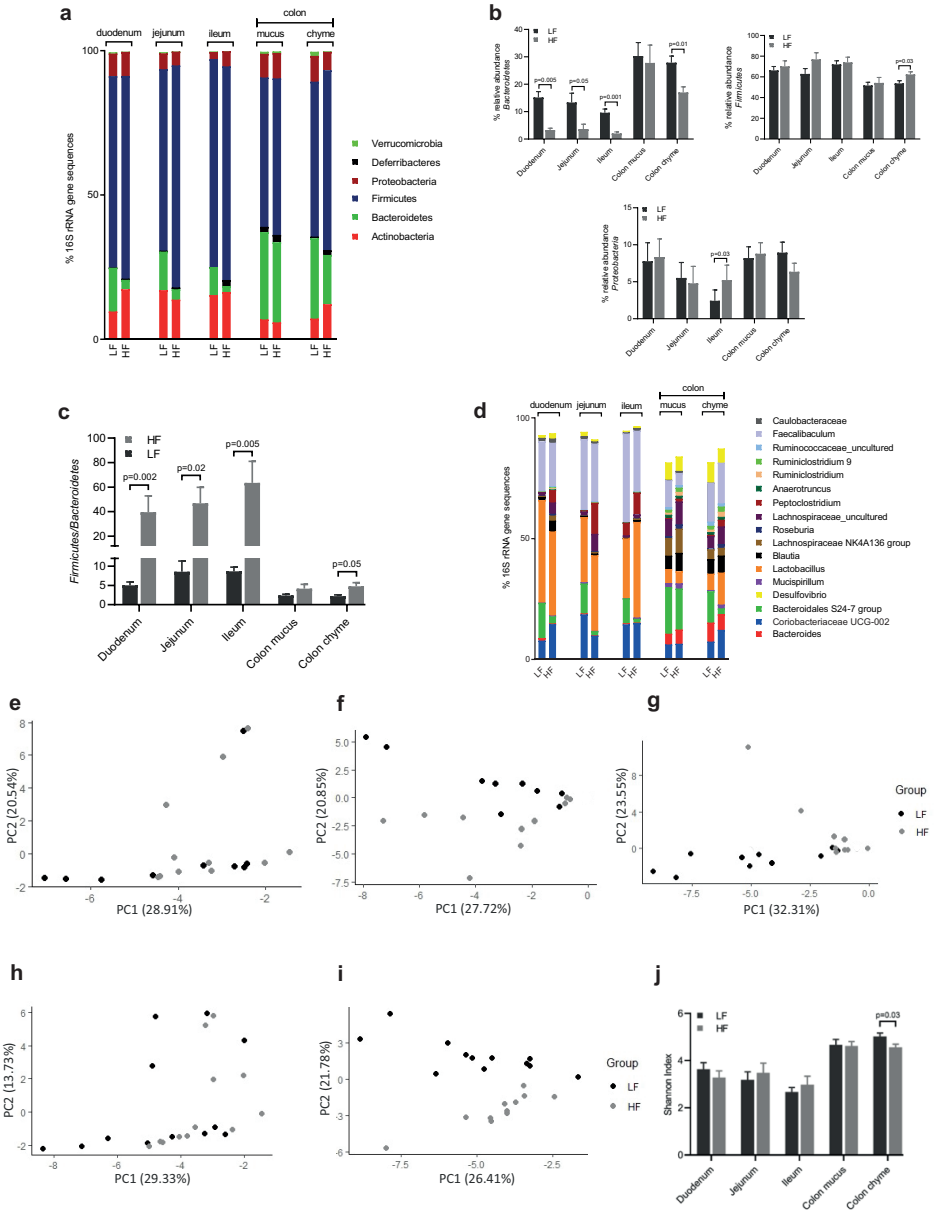

Figure 2 *(legend in the next page)*

←Figure 2. **Microbiota composition of the GI tract in mice fed LFD and HFD.** (**a, d**) Relative abundance of bacterial taxa at the phylum (a) and genus (d) levels. Only abundances > 1 % are shown (n=9-12); (**b**) Relative abundance of the phyla that significantly differ between the two groups (n=9-12). Values are presented as mean with s.e.m. Brown-Forsythe and Welch ANOVA tests with Tamhane's T2 multiple comparisons test; (**c**) The *Firmicutes:Bacteroidetes* ratio was higher in all intestinal segments in the mice fed the HFD. Values are presented as mean with s.e.m. Kruskal Wallis test with Dunn's correction for multiple comparisons (n=9-12); (**d**) HFD fed mice show more *Peptoclostridium* in jejunum and ileum, and more *Lachnospiraceae* (uncultured genus) in jejunum ($p<0.0001$, adj. $p=0.03$) and more *Lactobacillus* in ileum. The relative abundance of *Bacteroidales S24-7* and *Faecalibac*ulum was lower in HFD group in both jejunum and ileum segments. In colon content the HFD group had more *Lactobacillus, Coriobacteriaceae UCG-002, Lachnospiraceae* (uncultured genus) ($p<0.0001$ adj. $p=0.0008$) and *Peptoclostridium* ($p<0.0001$ adj.$p=0.01$). Bacteroidales S24-7, *Desulfovibrio* ($p<0.0001$ adj. $p=0.0006$) and *Clostridiales vadinBB60 group* ($p<0.0001$ adj. $p=0.02$) was lower in the colonic content of the HFD group. The observed significance level of the differences between groups was $p<0.0001$, adj. $p<0.0001$, unless otherwise stated. Multiple *t*-tests with Bonferonni corrections (n=10-12); (**e-i**) PCoA plots based on the Bray Curtis distance of the OTUs of samples taken from duodenum (**e**), jejunum (**f**), ileum (**g**), colon mucus (**h**) and colon luminal chyme (**i**) (n=8-12); (**j**) Species richness using Shannon index (n=10-12). Data are presented as mean with s.e.m. One-way ANOVA with Sidak's multiple comparisons.

HFD resulted in increased colonization of *Peptoclostridium* and multiple *Proteobacteria members* in the distal SI

To expand our understanding on the impact of HFD feeding on the gut microbiota we sought to identify the specifically differentiated taxa for the two diets in the gut segments using the Linear discriminant analysis (LDA) effect size algorithm (LEfSe) [28]. *Peptoclostridium* was identified for the HFD group in both jejunum and ileum (**fig. 3a** and **b**). Furthermore, in accordance with our findings related to the elevated relative abundance of *Proteobacteria* in ileum, we found that 11 out of the 14 identified taxa specifically identified in the HFD fed mice belonged to the *Proteobacteria* phylum. Most of them belonged to the α- and γ-*Proteobacteria* classes (**fig. 3b**). Regarding the colon luminal chyme samples, most of the HFD-identified taxa belonged to the class of *Clostridia* and to the *Actinobacteria* phylum (**fig. 3c**). *Peptoclostridium* and γ-*Proteobacteria* are described to be of proinflammatory nature in the gut suggesting more proinflammatory microbial environment in the distal SI of the HFD group [27].

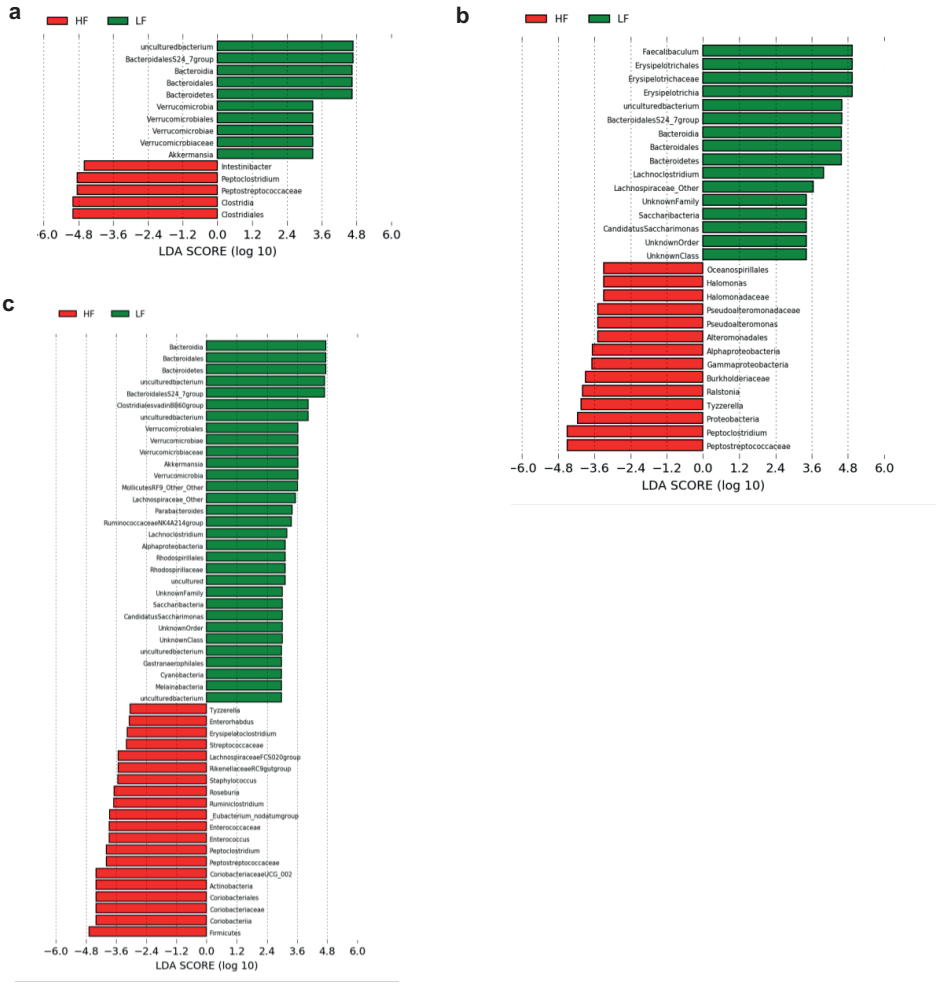

Figure 3. *(legend in the next page)*

←Figure 3. **LEfSe results of microbial taxa of HFD and LFD fed mice**. Histograms of the LDA scores computed for OTUs from the jejunal (**a**), ileal (**b**), and colon luminal chyme (**c**) samples. Red colored taxa were identified as specifically more abundant in HFD compared to LFD, while green colored taxa were higher in LFD fed mice.

HFD altered the integrity of the intestinal barrier

To determine whether the negative changes in the microbiota composition of the SI could cause microbes or microbial products to pass the intestinal lining we measured LPS binding protein (LBP) in plasma as a marker of LPS influx from the intestinal lumen. The LBP level was on average 27% higher in HFD mice compared to LFD (**fig. 4a**). Furthermore, we examined the expression of pattern recognition receptors (PRRs) in the lamina propria as they typically are upregulated by the presence of microbial components and are an important link to downstream host responses of microbial translocation [29]. TLR4 expression was consistently higher in all intestinal segments of the HFD group (**fig. 4b**). NOD1 also showed higher expression levels along the different intestinal segments, but only statistically significant in jejunum and colon (**fig. 4c**). NOD2 was only higher in jejunum (**fig. 4d**). To examine the permeability of the SI we measured the uptake of orally administered Fluorescein Isothiocyanate-dextran 4 (FD4). We observed 34% lower levels of FD4 in the plasma of mice fed HFD compared to the LFD group indicating reduction in the paracellular passage pathway (**fig. 4e**). Consistently, we observed a clear upregulation of the genes encoding the tight junction proteins occludin and zonula occludens in the HFD group (**fig. 4f-g**), where the expression of both genes was significantly higher in all segments from jejunum to colon. We also measured the antimicrobial peptide Reg3γ (**fig. 4h**) and observed an increase in the SI of the HFD group when evaluating the SI as a whole. Together, these data suggest that HFD caused an increased microbial challenge of the intestinal wall leading to the host strengthening the intestinal barrier.

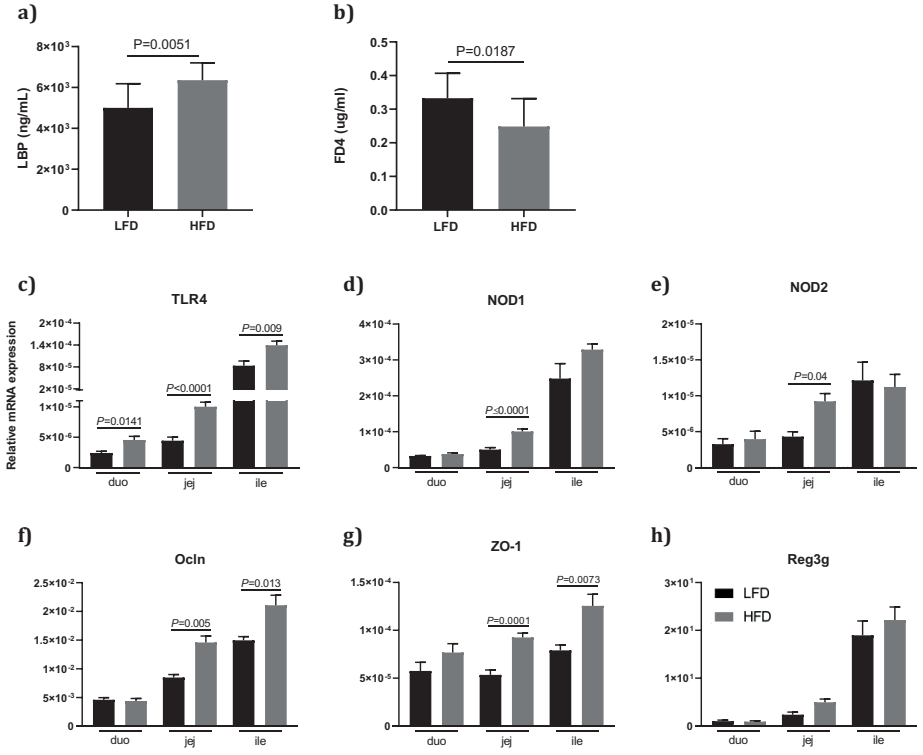

Figure 4. **Barrier function after long- term HFD consumption.** (**a**) LPS binding protein in plasma. (**b**) FD4 in plasma 1.5h after oral gavage. FD4 fixed amount was based on the average weight of the LFD group and normalized with body weight. (**a**-**b**) n=10-12. Values are presented as mean with s.e.m. Unpaired t-test. (**c**-**h**) mRNA expression of TLR4 (**c**), NOD1 (**d**), NOD2 (**e**), Ocln (**f**), ZO-1 (**g**) and Reg3γ (**h**) in SI and colon. When evaluated Reg3γ expression in the SI as a whole, we observed a significant increase (p=0.04). Gene expression normalized by GAPDH (n=10-12). Values are presented as mean with s.e.m. One-way ANOVA with Sidak's multiple comparisons test.

HFD promoted proinflammatory immune cells in the lamina propria

To examine the effect of HFD and the accompanied microbial alterations to the host immune response we investigated the amount of T regulatory (Tregs) cells and subtypes of MΦ in the small intestinal lamina propria by flow cytometry. We observed a significantly lower number of CD4+ Foxp3+ Tregs in the HFD group compared to control. To investigate the extent of inflammatory MΦ cells we compared the ratio between fully mature MΦ cells ($MHCII^{high}/CX3CR1^{high}$) with immature cells ($MHCII^{low\text{-}int}/CX3CR1^{int}$) (**fig. 5c**). The last subtype are known to be proinflammatory [25]. We found a significantly higher level of this subtype in the HFD fed mice (**fig. 5d**). Proinflammatory MΦ generally releases cytokines promoting an inflammatory environment [30, 31] and we did also observe more mRNA of TNF-α, IL-1β, as well as the MΦ-regulatory cytokines TGF-β and CCL5 [32, 33](**fig. 5e-h**), however, on a segmental level, only TNF-α and CCL5 in jejunum were statistical significant. Nevertheless, evaluating the SI as a whole showed a clear significant difference for all cytokines.

Proinflammatory MΦ contributes to antimicrobial activity by the production of cytotoxic reactive oxygen species (ROS) [34]. The production of such ROS is in MΦ NOX2-dependent. Upon measuring NOX2 expression we observed a significant increase in the distal part of the SI (**fig. 5i**). Additionally, we measured the abdominal ROS production *in vivo* through non-invasive imaging of the L-012 activity (**fig. 5j-k**). This method is shown to reflect distal SI ROS production [35]. At the three assessed timepoints of the dietary intervention, the signal was consistently higher in the HFD group compared to the control group. At the last timepoint of ten weeks the L-012 signal was 2.7 times higher in the HFD group (**fig. 5k**). Altogether, these suggest that HFD with the accompanied unbalanced microbiota composition promoted a proinflammatory immune environment of the SI.

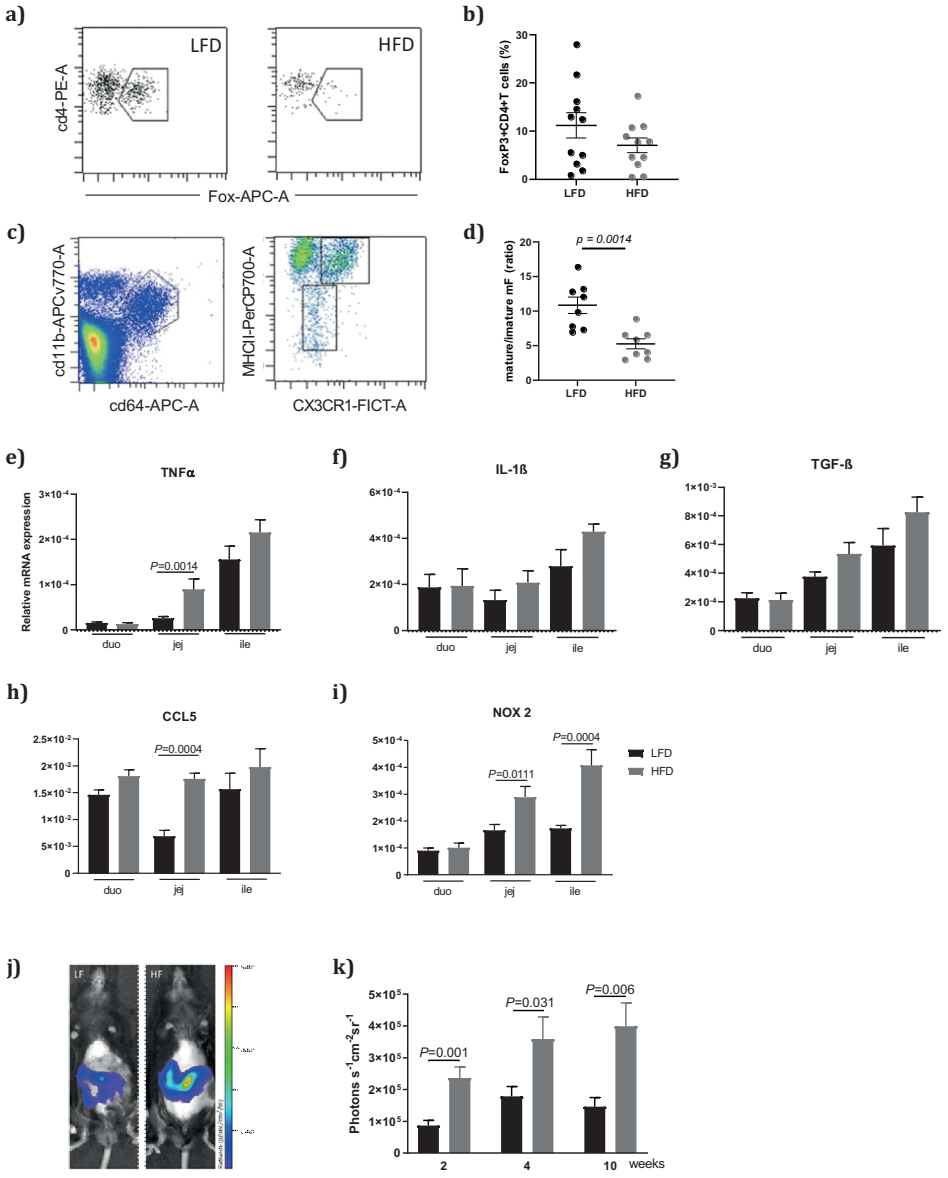

Figure 5 *(legend in the next page)*

←Figure 5. **Immune activity in SI mucosa.** (**a, c**) Gating scheme of the FOXP3+CD4+ Tregs (**a**) and CD64+ MHCII+ cells after exclusion of lymphocytes and NK cells (**c**). Polygons indicate gated cells subsets; (**b**) Proportion (%) of FOxP3+CD4+ T cells of total CD4+ gated cells (p=0.189) (n=11); (**d**) ratio between non-inflammatory MΦs ($MHCII^{high}/CX3CR1^{high}$) and inflammatory MΦs ($MHCII^{low\text{-}int}/CX3CR1^{int}$) (n=8); (**e-i**) mRNA expression of TNF-α (**e**), IL-1β (**f**), TGF-β (**g**), CCL5 (**h**) and NOX2 (**i**) in SI. When evaluating the SI as a whole TNF-α (p=0.03), IL-1β (p=0.04), TGF-β (p=0.04), CCL5 (p=0.01). Gene expression normalized by GAPDH (n=8-12). (**j**) L-012 luminescence of one representative mouse of each group after ten weeks of the dietary trial.; (**k**) Measurements of L-012 signal in LFD and HFD groups at three different timepoints (n=12); Values are presented as mean with s.e.m. (e-i, j) One-way ANOVA with Sidak's multiple comparisons test.

Discussion

In the present study, we investigated the effects of long term HFD feeding on microbiota, barrier function and immune status in the SI. We used LFD as control, where the fat was replaced with corn starch. Our main findings were that HFD caused an unbalanced profile of gut microbiota with concomitant changes primarily in distal SI-segments of innate immune markers including an increased proportion of inflammatory MΦ, decrease in Tregs and increased expression of proinflammatory cytokines, NOX2 and PRRs. Interestingly, we observed that the antibacterial peptide Reg3γ and TJ proteins occludin and ZO-1 were upregulated followed by reduced SI-permeability. These results indicate that host intestinal tissue elicited a defense response to attenuate the presumably negative impact of the altered microbial composition.

Numerous studies have investigated gut microbiota and its interaction with diet as a modulator of nutrient uptake and host metabolism in humans and mice [1, 3, 18, 36, 37]. Many studies have demonstrated an association of high fat diet with fecal and colonic dysbiosis [38, 39]. However, most of these studies have limited their analyses to colon and cecum and did not examine the impact of HFD on the small intestinal microbiota and its putative effect on the SI tissue. The SI is the primary site of nutrient absorption and due to the significant contact between food substrate and commensal bacteria, it is characterized as a site rich in microbe-microbe and host-microbe interactions [40]. We found that the microbial population showed more distinct differences between the two groups in the distal part of the SI (jejunum and ileum) than in duodenum. This is not so surprising considering that duodenum is characterized by low numbers of bacteria that are less diverse and that most of the fat uptake takes place in jejunum [18]. More specifically, at phylum level, we observed a shift of the microbiota in the animals fed HFD towards a higher *Firmicutes:Bacteroidetes* ratio along the GI tract. In addition, in the ileal samples, we observed a significant increase in the relative abundance of *Proteobacteria.* Overall, such alterations are known to be the result after HF feeding in cecal, colonic and fecal microbiota and have been previously reported by others to be associated with obesity and low-grade inflammation [38, 39, 41]. In the few studies analysing the impact of HFD on the small intestinal microbiota, a decrease in *Bacteroidetes* was a common finding [17, 18, 20], while

Meng and co-workers observed an increase in the small intestinal *Proteobacteria* in rats [20]. As regards alpha diversity, we found no significant differences between the two groups in any of the SI-segments. This was not surprising though, considering that the microbiota in this region is overall phylogenetically less diverse than in the large intestine [42]. At the genus level, we found that the abundance of *Peptoclostridium* is higher along all segments of the SI in the HFD fed mice, while the abundance of *Bacteroidales S24-7* and *Faecalibaculum* is lower. Lower levels of ileal *Bacteroidales S24-7* have been previously linked to HFD [17], while its presence in the feces of non-obese diabetic mice has been proposed to protect against diabetes [43]. Bacterial species belonging to the *Peptoclostridium* genus are thought to be pro-inflammatory. One of its members is *Peptoclostridium difficile aka Clostridium difficile* which is one of the main pathogens that have been associated with infectious diarrhea [44]. Furthermore, we found that in ileum most of the identified taxa that were associated with the HFD belonged to the *Proteobacteria* phylum. *Proteobacteria* consist of Gram-negative bacteria and are one of the most abundant phyla of the small intestinal microbiota consisting of several known pathogens. An increased presence of *Proteobacteria* has been characterized as a microbial signature of disease and a connection between *Proteobacteria* with low-grade inflammation and the metabolic syndrome has been previously described [27]. In this context LPS produced by Gram-negative bacteria is thought to induce endotoxemia that further leads to an inflammatory state in the gut [45]. In recent studies focusing on the identification of bacteria that might be involved in the genesis of endotoxemia, the prevalence of *Proteobacteria* has been found to be increased [27]. Together, our data clearly show that HFD leads to an unbalanced microbial environment in jejunum and ileum.

We observed an increase of LBP in the plasma of mice fed HFD, an indication of LPS leakage from the gut. LBP is an acute-phase protein primarily produced by the liver in response to LPS and then secreted into the blood. Its main role is to escort LPS to its target cells with subsequent binding to CD14 and TLR4 signaling induction. Indeed, Cani and coworkers have shown that feeding rats corn oil by oral gavage increases levels of LPS in plasma compared to water controls [46]. A possible explanation is that LPS can, together with fat, be incorporated into chylomicrons which subsequently facilitate LPS release from

intestinal epithelial cells [47]. As we see an upregulation of *Proteobacteria* in the HFD group, this can also explain the increased LPS flux in the intestinal lumen. However, we cannot rule out that LBP is also induced by proinflammatory cytokines originating from adipose tissue as a response to obesity with subsequent low-grade inflammation [48]. We also observed a lower intestinal permeability assessed by FD4 in mice fed HFD. We administered FD4 after 4h fasting and measured its concentration in plasma after 90 minutes, reflecting primarily an assessment of the SI barrier. Contrary to most other studies, we administered a fixed amount of FD4 based on the average weight of lean mice, as suggested by McGuinness *et al.* [49].

ROS are an integral part of the innate immune system, foremost by the induction of respiratory burst by phagocytic cells to efficiently kill microbes. In the present study, we observed increased levels of ROS using non-invasive imaging [35] after HFD consumption. Previous studies have shown that the highly reactive peroxynitrite made by the combination of nitric oxide and superoxide produced by iNOS and NOX1/NOX2, respectively is prominent in the SI [35]. NOX1 is expressed in epithelial cells [34] whereas NOX2 is primarily expressed in the lamina propria residing-MΦ. We have previously demonstrated that intestinal ROS production assessed *in vivo* in healthy animals is dependent on NOX1 expression [50]. However, here we observed reduced NOX1 expression and increased NOX2 expression suggesting that the increased ROS production in HFD fed mice is caused by the increased presence of NOX2 expressing MΦ. Indeed, we found an increased proportion of immature proinflammatory MΦ by assessing the expression of MHCII/CX3CR1^{int} as markers of monocyte derived immature MΦ. To our knowledge, this is not previously described, and the results imply that monocytes are recruited to the SI lamina propria as a consequence of the HFD. The increased proportion of these cells can also explain the reduced number of Treg cells, as mature intestinal MΦ are important for Treg formation [26]. A reduction in Treg cells by HFD has been observed by others, but only in the context where the low-fat control diet was a chow diet [6]. Our results thus confirm that reduction in Treg cells is indeed due to the increased fat consumption. We further observed a moderate increase in the expression of proinflammatory cytokines in the distal segments of the SI. The most robust changes in expression were found in jejunum.

Interestingly, we also found higher relative expressions of PRRs and barrier related genes in this area. As most of the fat is digested and absorbed in the jejunum, and very little fat remains in the ileum, it is fair to suggest that dietary fat is of relevance to these changes seen in jejunum [7, 51].

The increased expression of the PRRs TLR4, NOD1 and NOD2 by the HFD is in line with most other studies [51-54]. The PRRs are crucial for sensing microbiota and critical for eliciting downstream responses that maintain gut homeostasis [55]. However, their increased expression has been dominantly linked to increased permeability in HFD settings [15, 56]. We measured the expression of Reg3γ and the tight junction proteins occludin and ZO-1. Reg3γ is an antimicrobial peptide secreted by Paneth cells into the gut lumen, and dependent on TLRs via MyD88 signaling [55, 57, 58], whereas occludin and ZO-1's functions are to create a seal in the epithelium to avoid paracellular influx. In both cases, we found increased expression by HFD. These observations are contradictory to most other findings with respect to HFD, which find diminished expression of Reg3γ [59] and TJ encoding genes [7, 45]. For instance, increased NOD1 and TLR4 signaling are shown to decrease the expression of TJ proteins in HFD mice [56]. However, NOD2 on the other hand is crucial for maintaining normal TJ expression and maintaining barrier properties. Therefore, we believe that the induced expressions of Reg3γ and TJ genes are compensatory responses mediated by the host when challenged by a detrimental microbial environment imposed by HFD. As shown by Hamilton, the adaptation to new dietary regimes is a dynamic process which over time strengthens the intestinal barrier. In their study, rats fed HFD had diminished barrier function during the first week, but after 3 and 6 weeks, the barrier was essentially restored [16]. Importantly, many of the studies so far have compared HFD with a low-fat chow diet rich in fiber. In our study, we used a HFD matched low-fat diet that differed only with respect to carbohydrate content and fat. Indeed, Kless and coworkers demonstrated that HFD with matched LFD controls found little or no differences in intestinal barrier properties between HFD and LFD mice [60].

Overall, our results show that HFD compared with a matched LFD control, lead to an unfavorable microbial profile in the distal segments of the SI, particularly in ileum. These

conditions further elicit an adaptive host response resulting in reduced barrier permeability and shift to more inflammatory immune cells.

Material and Methods

Animal housing and diets

Twenty-four male mice, 9 weeks old (C57BL/6J; Envigo, The Netherlands), were housed in individually ventilated cages in a controlled environment (12 hours light-dark cycle; 24 ± 1 °C; 45-55% humidity). Initially, all 24 animals were fed with a LFD (D12540J, 10 E% fat, Research Diets) for 4 weeks to acclimatize. Then, mice were randomized divided into two groups and given a HFD (D12492, 60 E% fat, Research Diets) or LFD for 18 weeks. Mice had *ad libitum* access to food and water. Food and body weight were measured once a week to assess food intake and weight development. Activity wheels were removed at experiment start-up to exclude physical activity as a confounder.

Animals were euthanized by cervical dislocation under anesthesia, composed of Zolezepam (32 mg/kg), Tiletamin (32 mg/kg), Xylazine (4.5 mg/kg), and Fentanyl (26 ug/kg).

Ethical aspects

The animal experiment was performed with permission from The Norwegian Animal Research Authority (Mattilsynet, FOTS #8196), and conducted in compliance with the current guidelines of The Federation of European Laboratory Animal Science Associations (FELASA).

Glucose homeostasis and insulin sensitivity

Mice were subjected to intraperitoneal insulin tolerance test (IpITT) and oral glucose tolerance test (OGTT) to assess glucose homeostasis and insulin sensitivity. Blood was obtained from the tail. Blood glucose levels were measured by a glucometer (Accu-Chek, Roche Diagnostics). The IpITT was conducted at week 9 of the experiment. Human insulin (Sigma-Aldrich) was injected in a fixed-dose (0.75 U/kg) after 4 hours of fasting. The OGTT

was performed at week 10 of the experiment. A fixed-dose of D-glucose (Sigma-Aldrich) was administrated (2 g/Kg) after 6 hours of fasting.

Intestinal permeability by FITC-dextran

Intestinal permeability was determined at week 14 of the dietary trial using a protocol adapted from Johnson et al., PlosOne [23]. Briefly, mice were fasted for 4 hours prior to an oral administration of 650 mg/Kg of fluorescein isothiocyanate (FITC) dextran (FD4, Sigma-Aldrich). This dose was based on the average weight of the LF group. Blood was collected 1.5 hours after administration from vena saphena into EDTA-coated tubes to separate plasma through centrifugation. Plasma was diluted with one volume of saline solution and FITC dextran concentration was determined by fluorescence spectroscopy with excitation and emission wavelengths at 490 nm and 520 nm, respectively (Synergy H4 Hybrid microplate reader, Bio Tek instrument).

***In vivo* imaging of ROS using L-012**

Mice were shaved in the ventral side in the day prior to imaging. Immobilized using isoflurane (2.5-3.5%), 200 µl of chemiluminescent probe L-012 (Wako Chemical) was injected intraperitoneally, dissolved in saline (2.5 mg/mL). L-012 which reacts with peroxynitrite originated from the interaction between nitric oxide and superoxide anion [61]. Light emission from the ventral side was measured with IVIS Lumina II (Perkin Elmer) as photons per second per cm^2 per steradian using the Living Imaging software (Perkin Elmer) 5 minutes after injection and with 3 minutes exposure time.

Flow cytometry

Intestinal samples were harvested after animal euthanasia. Mesenteric fat, luminal content and Peyer's patches were removed. The intestine was opened longitudinally, cut into 5 mm long segments and placed in cold RPMI. Isolation of epithelial and lamina propria cells followed Goodyear *et al.* [62] description. Briefly, mucus was removed by incubation in DTT at 37 °C for 20 min with agitation. Epithelial cells were separated by three incubation steps in EDTA solution at 37 °C for 15 min with agitation. Loose connective tissue was removed

by adding a digestion step containing collagenases, Liberase and DNases (Sigma-Aldrich), for 15 min at 37 °C with agitation. Cells were stained after several washing steps with protein extraction buffer and then pre-blocked with FcR Block (Miltenyi Biotec). Cells were stained with the following intra- and extracellular antibodies: CD3-APCvio770, CD8-PEvio770, CD45-PerCPvio700, FoxP3-APC, CD11b-APCv770-A, cd64-AP-A, MHCII-PerCP700-A and CX3CR1-FITC-A (Miltenyi Biotec), following the manufacturer's instructions. For differentiation between live and dead cells, we used the LIVE/DEAD fixable Violet Stain kit (Thermo Fisher). We used MACQuant Analyzer 10 Flow cytometer and MACQuantify software (Miltenyi Biotec) for cell acquisition and analyses.

Small intestinal segmentation

For analysis performed in different segments of the SI, we consistently selected the same areas for sampling. Firstly, we identified the middle point of the SI. The most proximal 5 cm of SI was considered as the duodenum, 6 cm around the middle point was considered as jejunum and the most distal 6 cm was considered as the ileum.

Gene expression and 16S rRNA gene sequencing

The mucosa samples for RNA and DNA extraction were scraped off with a blunted microscope glass slide from the intestine after being open longitudinally. Colon content for DNA extraction was squeezed out with forceps. The segmentation of the SI was performed as described above and the colon mucosa was collected from the proximal half. Mucosal samples for RNA extraction were preserved in RNAlater (Sigma-ALdrich) after sampling. Mucosal samples and intestinal content for DNA extraction were placed in S.T.A.R buffer (Roche), together with <106 Mm acid-washed glass beads (Sigma-Aldrich), immediately after dissection.

Real-Time quantitative PCR (RT-qPCR)

RNA was isolated using the NucluoSpin RNA/Protein Purification kit (Macherye-Nagel). The kit iScript cDNA Synthesis (Bio Rad) was used to produce cDNA and FirePol EvaGreen qPCR Supermix (Solis BioDyne) for the RT-qPCR reaction. Used primers and optimized primer annealing temperature are listed in table 1.

Table1: primers used for RT-qPCR and their annealing temperature

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Tm °C
GAPDH	CTTCAACAGCAACTCCCACTCTT	GCCGTATTCATTGTCATACCAGG	60
CCL5	GCCCACGTCAAGGAGTATTT	CTTCGAGTGACAAACACGAC	59
IL-1β	GCAGCTGGAGAGTGTGGAT	AAACTCCACTTTGCTCTTGACTT	61
NOD1	TGACAGTAATCTGGCTGACC	GTCTGGTTCACTCTCAGCAT	59
NOD2	GCAGAAACTAGCTCTCTTCAAC	CGGCTGTGATGTGATTGTTC	61
NOX2	GGGAACTGGGCTGTGAATGA	CAGTGCTGACCCAAGGAGTT	61
Ocln	CTGTGAAAACCCGAAGAAAGATG	GCAGACACATTTTTAACCCACTC	57
Reg3γ	GTCAAGAGCCTCAGGATTTCT	ACCCATGATGTCAATTCTGTACTC	57
TGF-β	GAACCAAGGAGACGGAATACAG	CGTGGAGTTTGTTATCTTTGCTG	65
TLR4	GATCTGAGCTTCAACCCCTT	TGTTTCAATTTCACACCTGGA	61
TNF-α	CTGTCTACTGAACTTCGGGGTGAT	GGTCTGGGCCATAGAACTGATG	61
ZO-1	GAGAAAGGTGAAACTCTGCTG	ACGAGGAGTCGGATGATTTTAGA	59

RT-qPCR was performed in LightCycler 480 Instrument II (Roche) with the following parameters: 12 min at 95 ˚C; 40 cycles of 15 s at 95 ˚C followed by 20 s at optimized primer annealing temperature (listed above); 20 s at 72 ˚C. LinReg Software was used to calculate Cq values based on a common threshold and individual efficiencies.

16S rRNA gene sequencing

Samples were lysed in a MagNA Lyser Instrument (Roche) at 6500 rpm twice for 20 s with a cooling step in-between, followed by centrifugation for 5 min at 14000 *g.* The supernatant was collected, and DNA extracted using the Mag Mini LGC kit (LGC Genomics), according to the manufacturer's protocol in a KingFisher Flex DNA extraction robot (Thermo Scientific).

We followed the 16S rRNA amplicon sequence analysis workflow previously described by Avershina et al. [63]. Briefly, after DNA extraction we amplified the 16S rRNA gene for 25 cycles using prokaryote targeting primers developed by Yu et al. [64]. We used AMPure XP to purify the PCR product (Beckman-Coulter, Brea, CA) and we performed 10 further

PCR cycles. The resulting amplicons were sequenced on Illumina MiSeq V3 platform (Illumina, SanDiego, CA). The resulting 300 bp paired-end reads were further paired-end joined, quality-filtered using QIIME33 and clustered with 97% identity level using closed-reference *usearch v7.0* algorithm [65, 66] against Greengenes database v13.8 [67].

LBP in Plasma

LPS binding protein in plasma was used to measured, indirectly, the amount of LPS in samples through an enzyme immunoassay for determination of mouse LBP (Biometec, Greifswald). Briefly, plasma samples were diluted 1:800 and added to a pre-coated plate. Substrate was read with an absorbance at 450 nm and the amount of LBP was calculated based in a standard curve and dilution in a 4-parameter logistic curve fit.

Histology

After dissection, adipose tissue samples were surrounded with OCT, flash-frozen in liquid nitrogen and stored at -80^{o}C until fixation. Samples were fixed in ice-cold 10% formalin for 1 hour and stored overnight in fresh 10% formalin at 4 ^{o}C. Tissues were dehydrated in ethanol, assembled in paraffin blocks and cut in 5 μm thick sections.

Staining and image analysis.

Adipose tissue sections were stained with Hematoxilin and Eosin as described by Parlee et al. [68]. Adipocytes area was measured using Open Source Adiposoft Image Analysis in ImageJ software [69, 70]. The average transversal adipocyte section area of each sample was calculated from 150-250 cells per sample.

Statistical Analyses

Statistical analyses were done in the GraphPad Prism software (La Jolla, USA). Averages are presented as mean and variances as the standard error of the mean (s.e.m). The homogeneity of variances was tested with Bartlett's test. Normal distributed data from multiple groups were compared by one-way ANOVA with Sidak's correction for multiple comparisons. For non-normal distributed data the non-parametric Kruskal-Wallis one way-ANOVA was performed. When only two independent groups were compared, we used the

unpaired *t*-test or the non-parametric Mann-Whitney test. We chose 1.500 sequences per sample as a cut-off value to normalize the sequencing data. To identify taxa associated with either the LFD or the HFD group, we used linear discriminant analysis effect size (LEfSe) with LDA score > 2 [28]. PCoA was performed using R [71]. Only OTUs that were present with more than 10 reads in at least 5% of all samples were included for the PCoA.

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PAPER III

Coffee consumption attenuates the unfavorable microbial composition and inflammation induced by high-fat feeding in mice

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Abstract

An impaired balance in the composition of the gut microbes has been linked to deterioration of health. Consumption of a high-fat (HF) diet leads to unbalanced gut microbiota and inflammation in the small intestine. Coffee is widely consumed, and it contains several bioactive compounds. Although several studies have reported a positive correlation between health and coffee consumption, its impact on gut health remains rather unexplored. Here, we show that dietary supplementation of HF diet with coffee attenuated the HF diet-induced unbalanced microbial profile and dampened the immune response in the small intestine. The microbial profile of mice consuming coffee showed a shift into a more low-fat diet-like profile in a dose-dependent way characterized by lower *Firmicutes/Bacteroidetes* ratio, lower abundance of *Peptoclostridium* and higher abundance of members of the *Bacteroidales S24_7* family. In addition, the groups receiving coffee had lower number of proinflammatory macrophages expressing the CX3CR1 and MHCII surface markers. Last, coffee attenuated the levels of the circulating LPS binding protein and the expression levels of intestinal TLR4 and NOD1. Mice receiving coffee had lower intestinal expression of TNF-α, TGF-β and NOX2, and lower production of reactive oxygen species. Our data show that consumption of coffee can have a positive impact on gut health characterized by the presence of a symbiotic microbial environment, improved barrier function and dampened immune response.

Introduction

Coffee is the second most consumed beverage worldwide, after water, [1] and its daily consumption is estimated to be approximately 2,25 billion cups [2]. Coffee contains numerous bioactive compounds including phenolic polymers, chlorogenic acid, minerals and caffeine [3]. Due to its high consumption, coffee may have a substantial impact on public health [2]. Numerous studies have focused on the effect of coffee consumption in health, but sometimes the results are conflicting. However, in research over the past years, the benefits of coffee appear to outgrow the risks linked with its consumption. Daily coffee consumption is suggested to prevent several metabolic, liver and neurological conditions as well as certain cancer types. The strongest correlation was observed when the consumption was moderate, 3-4 cups per day, rather than when only 1 cup was consumed [2, 4]. Despite the increased interest in coffee and health, the relationship between coffee and the gut microbiota as well as whether downstream host-microbial interactions could be one of the mechanisms for the health claims of coffee, remain still rather unexplored.

The intestinal microbiota consists of approximately a hundred trillion microorganisms that form a complex ecological community. Although the gut microbiota composition is stable over time in healthy subjects, dietary modifications have a strong impact on its community structure [5]. More specifically, the consumption of HF diet has been associated with the establishment of an unbalanced profile that is characterized by higher *Firmicutes/Bacteroidetes* ratio as well as an increased prevalence of *Proteobacteria* [6]. Such a profile can be characterized as proinflammatory and a bloom of *Proteobacteria* has been linked with intestinal bowel disease and in general the presence of an inflammatory state in the intestine [7, 8].

Although coffee is the second most consumed beverage worldwide [1] and several studies have analyzed its impact on health focusing among others, on metabolic disorders [2, 4], there have not been many studies on the correlation of coffee with gut microbiota and whether this might possess a causal role in the observed health benefits of coffee. Jaquet *et al*. analyzed the microbial composition in human fecal samples after coffee consumption

and found that, although the dominant bacteria were not altered, coffee led to an increase of the probiotic *Bifidobacterium spp*. [9]. Nakayama *et al.* also reported an increase in fecal *Bifidobacteria* as a result of the colonic fermentation of the oligosaccharides present in coffee [10]. Nishitsuji *et al.* reported that although the liver lobular inflammation decreased after diet supplementation with coffee in a diabetes-prone mouse model, the microbial dysbiosis that has been established by a HF diet did not improve after coffee consumption [3]. However, only fecal or colonic bacteria were analyzed in these studies. It is well known that the community structure of microbes along the gastrointestinal tract is different [11, 12]. The composition of fecal samples differs not only in comparison to the one in the small intestine but also that in the large intestine [13-15]. Therefore, although convenient, using fecal samples can result in "false negatives" when screening for microbiota effects and results should be interpreted with caution when fecal samples are used as a proxy [11, 16].

Apart from changing the composition of the gut microbiota, consumption of HF diet has been linked with alterations in the innate and adaptive immune responses in the small intestine [17]. Coffee has been shown to affect the inflammatory status in various tissues, such as liver and blood, however with contradictory findings [18-21]. Furthermore, a couple of studies have analyzed the impact of polyphenols, chlorogenic acid and caffeine on intestinal inflammation revealing mainly a positive association [22-24].

The lack of evidence on the interplay among coffee, gut microbiota and intestinal inflammation becomes even more important, considering that the few reports on coffee and bacteria focus either on *in vitro* fermentation experiments based on fecal slurries [25] or on the composition of fecal bacteria after coffee consumption [3, 9, 26]. In addition, many of these studies base their analyses on real-time quantitative PCR and analyze only a selection of bacteria that are thought to be of relevance, neglecting possible differences of the whole microbial population in the intestine [10, 26].

The objective of this study was to examine the impact of coffee on gut health by examining whether coffee consumption would attenuate the negative effects caused by HF feeding. We have subjected mice to four different diets; low-fat (LF) diet, HF diet and HF diet together with two different amounts of coffee, representing the human consumption of 1 and 5 cups per day (HFC1 and HFC5 respectively). We found that coffee attenuated the HF diet-induced

changes in the microbiota in a dose-dependent manner, resulting in a very similar microbial composition as in the LF fed mice. Coffee additionally led to lower levels of circulating lipopolysaccharide-binding protein (LBP), higher expression of the intestinal tight junction protein occludin and zonula occludens (ZO-1), and lower expression of intestinal NOD1 and TLR4, suggesting an enhanced gut wall barrier. Furthermore, the macrophage (Mϕ) population of the lamina propria showed higher expression of CX3CR1 and MHCII in the coffee supplemented diets, which was accompanied by lower production of reactive oxygen species (ROS) and downregulation of certain proinflammatory cytokines (TNF-α, TGF-β and IL-1β), suggesting the presence of less proinflammatory and more homeostatic Mϕ [27]. In conclusion, our overall data support a positive impact of coffee on gut health.

Results

Coffee counteracted the weight gain caused by HF diet consumption

Mice fed the HF diet had higher body weight in comparison to the LF diet-fed mice. Mice fed the HFC1 diet had similar weight gain as the HF diet group. In fact, HFC1 was the group with the highest weight gain by the end of the experiment, gaining three times more weight than the LF diet group. On the other hand, mice fed the HFC5 diet showed a lower body weight gain in comparison to the HF diet group **(Fig. 1a)**. The HF diet group had higher energy intake per cage than the LF diet group. Mice fed the HFC1 diet had the highest energy intake, being 31.9% and 10.6% higher than the LF and HF diet groups, respectively. From the coffee groups, the HFC5 had a more similar energy intake when compared to LF diet **(Fig. 1b)**.

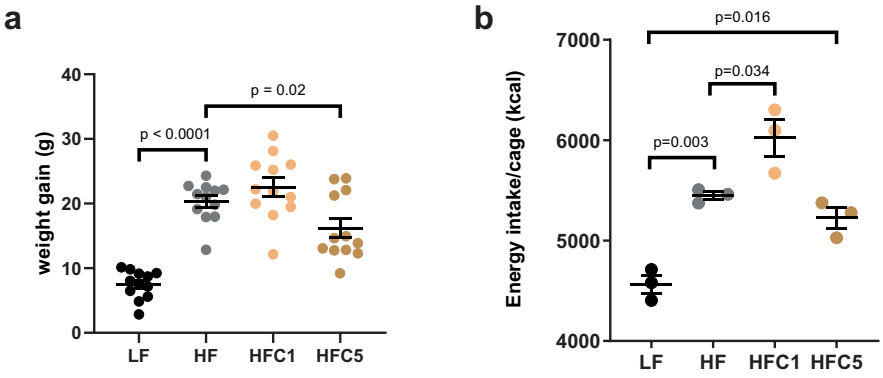

Figure 1. **Weight gain and energy intake**. **(a)** Weight gain after 18 weeks of dietary trial. n=12. **(b)** Energy intake (kcal) per cage (4 mice per cage) after 18 weeks. n=3. Values are presented as mean with s.e.m. One-way ANOVA with Sidak's correction for multiple comparisons.

Coffee supplementation attenuated the unfavorable microbial profile induced in the HF diet fed mice

Consumption of the HF diet led to an unbalanced microbial environment characterized by higher relative abundance of *Firmicutes* and lower abundance of *Bacteroidetes,* both in the small and in the large intestine. Diet supplementation with coffee resulted in a concentration-dependent shift to a profile resembling the one seen in the LF diet, namely, lower *Firmicutes* and higher *Bacteroidetes* levels **(Fig. 2a, 2c-d)**. In addition, there were higher levels of *Actinobacteria* observed only in the mice receiving diets supplemented with coffee **(Fig. 2e)**. At genus level, the coffee groups had again a concentration-dependent composition similar to the LF diet-induced profile **(Fig. 2b).** In the distal small intestine, the HF diet led to higher abundance of *Peptoclostridium* and *Lachnospiraceae (uncultured bacterium)* in jejunum ($p < 0.0001$, adj. $p = 0.03$), and *Lactobacillus* in ileum. Regarding the coffee supplemented groups, HFC1 had the least effect. In jejunum, HFC1 had lower *Peptoclostridium* and higher *Lachnospiraceae* levels, while *Lactobacillus* and *Coriobacteriaceae UCG-002* had a higher relative abundance in both ileum and jejunum. HFC5 led to lower levels of *Peptoclostridium* and *Lactobacillus* with concurrently higher levels of *Bacteroidales S24_7 family (uncultured bacterium)*, *Coriobacteriaceae UCG-002* and *Faecalibaculum* in both jejunum and ileum. In colon chyme *Coriobacteriaceae UCG-002, Lachnospiraceae, Peptoclostridium* and *Lactobacillus* were higher in the HF diet group, while *Bacteroidales S24_7 family, Desulfovibrio* and *Clostridiales vadin BB60* were lower. HFC1 led to higher *Bacteroidales S24_7 family, Coriobacteriaceae UCG-002* and *Bacteroides*. HFC5 had lower levels of *Lactobacillus, Blautia* ($p < 0.0001$, adj. $p < 0.001$), *Faecalibaculum* and *Peptoclostridium* ($p < 0.001$, adj. $p = 0.002$), while it led to higher abundance of *Coriobacteriaceae UCG-002, Bacteroidales S24-7 family, Bacteroides* and *Alistipes*. For all comparisons, unless stated otherwise, $p < 0.0001$, adj. $p < 0.0001$. Summing up, we observed a correlation between increasing doses of coffee and the relative abundance of *Bacteroidales S24-7 family, Peptoclostridium* in both the small and large instestine and *Faecalibaculum* in the small intestine **(Fig. 2f-k)**.

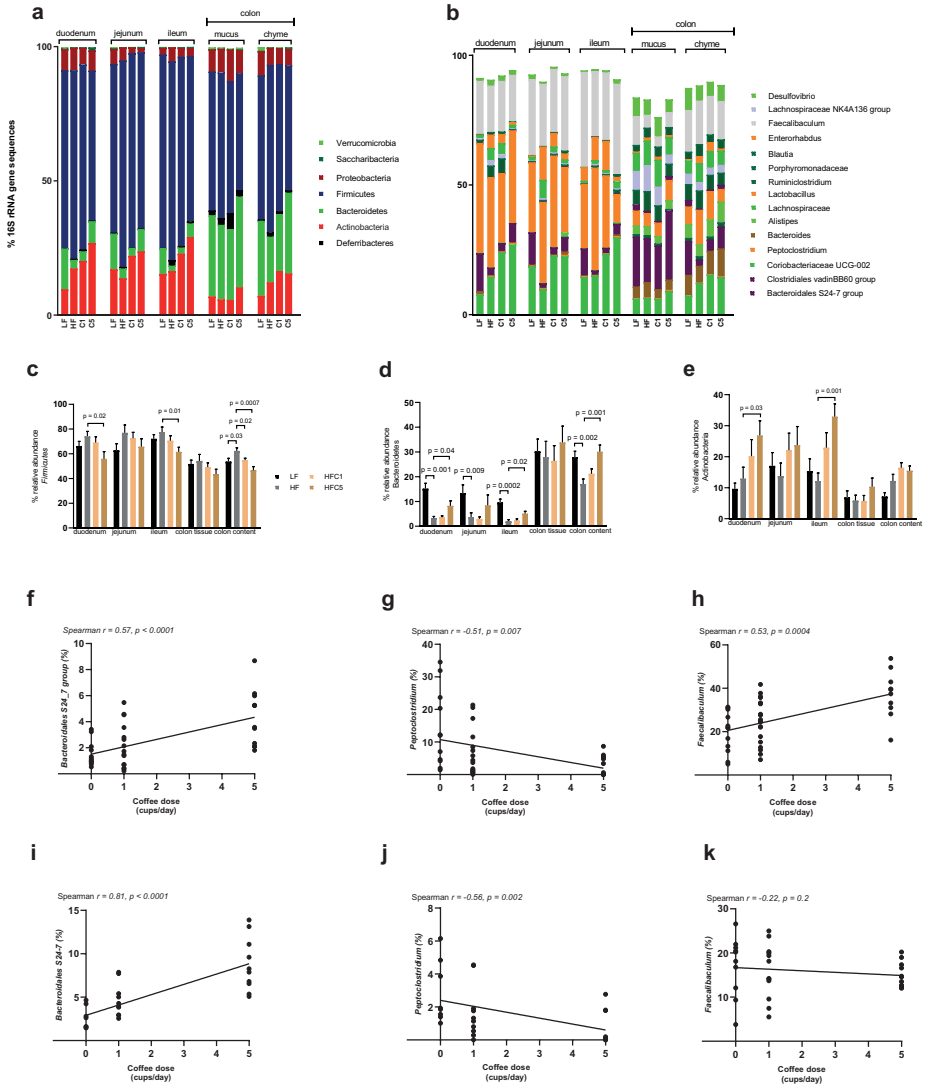

Figure 2 *(legend in the next page)*

Figure 2 – Gut microbiota composition of mice on coffee diets. **(a)** Relative abundance of bacterial taxa at phylum **(b)** and genus level based on the 16S rRNA amplicon sequences. Only abundances > 1 % are shown. **(c-e)** Relative abundance of *Firmicutes **(c)***, *Bacteroidetes* **(d)** and *Actinobacteria* **(e)**. These phyla are the ones that showed significant differences among the different diet groups. Values are presented as mean with s.e.m. One-way ANOVA with Sidak's multiple comparisons test. **(f-k)** Significant associations between coffee dosage and changes in genus abundance in the small intestine **(f-h)** of *Bacteroidales S24-7 family (uncultured bacterium)* **(f)**, *Peptoclostridium* **(g)**, and *Faecalibaculum* **(h)** and the colonic chyme **(i-k)** *Bacteroidales S24-7 family (uncultured bacterium)* **(i)**, *Peptoclostridium* **(j)**, and *Faecalibaculum* **(k)** as measured by the Spearman's correlation. n = 9-12.

The microbial population in coffee groups showed a higher level of overlap with the LF group

Regarding β-diversity, the principal coordinate analysis (PCoA) plots based on Bray Curtis distance **(Fig. 3a-e)** showed that in the small intestine **(Fig. 3a-c)** there was a more spread clustering of all groups than in colon chyme **(Fig. 3e).** However, we did see that in jejunum and ileum the HF and LF diet groups cluster separately **(Fig. 3b-c)**, indicating a high level of diversity in terms of bacterial composition, while the coffee groups, especially HFC5, clustered closer to the LF group. In colon chyme **(Fig. 3e)** we saw that HFC1 clustered in between the HF and LF groups, while HFC5 showed a higher level of overlap with the LF group indicating similar composition. For duodenum **(Fig. 3a)** and colon tissue **(Fig. 3d)** we observed a higher degree of co-clustering among all groups. It has been previously shown that consumption of a HF diet leads to decreased α-diversity in the distal gut (cecal and fecal samples) [28, 29]. We used Shannon Index as a measure to estimate the α-diversity and we saw, indeed, a reduction in the colon chymal content in the HF fed mice (**Fig. 3f**). Regarding the coffee groups, the HFC5 diet group showed higher levels of the Shannon Index in colon chyme. No significant changes were observed in the small intestine.

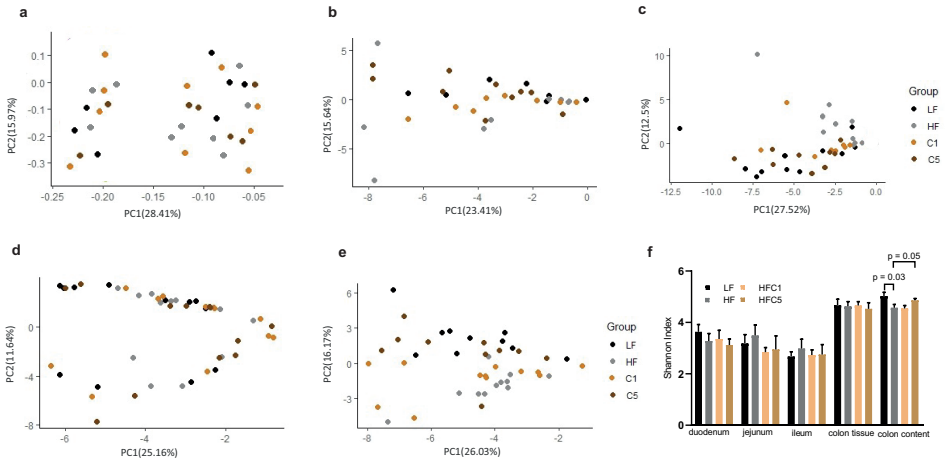

Figure 3 – **Beta and alpha diversity of the HF, LF and the HF supplemented with different doses of coffee. (a-e)** PCoA plots using Bray Curtis distances of the OTUs of samples taken from the duodenum **(a)**, jejunum **(b)**, ileum **(c)**, colon tissue **(d)**, and colon chyme **(e)**. **(f)** Alpha diversity shown by Shannon Index for the different diets, n = 8-12. Values are presented as mean with s.e.m. One-way ANOVA with Dunnett's multiple comparisons.

The LF diet-associated taxa are present in mice fed moderate coffee doses

As we observed similarities in the affected taxa both at phylum and genus level among the LF and the coffee groups, we next identified the genera that were significantly different between the LF diet and the HF diet groups in ileum and colon chyme (i.e., comparisons between these two groups only). The genera that were significantly lower in the LF group, we have referred them to as the LF-associated genera, while the ones that were higher in the HF group, have been called as the HF-associated genera. Further, we wished to examine how the presence of coffee influences these genera. We have clustered the different diet groups based on the relative abundance of the LF- and HF-associated genera. **Figure 4** consists of two heatmaps; one deriving from ileal samples **(Fig. 4a)** and one from colonic chyme samples **(Fig. 4b)** and it shows that most of the HFC5 fed mice cluster together with the LF group, while HFC1 cluster with HF fed mice. This indicates that supplementation of HF diet with coffee at moderate doses changed the relative abundance of the LF- and HF-associated genera to a LF-like profile.

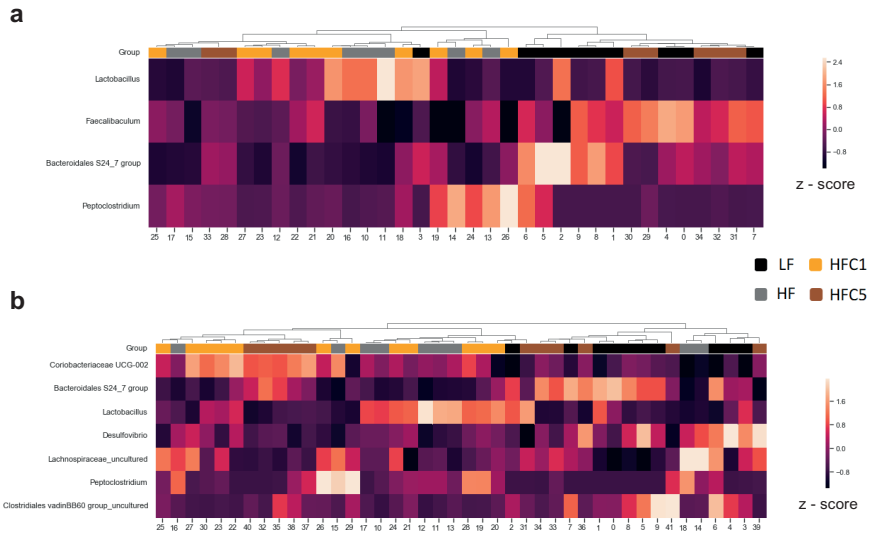

Figure 4. **Group clustering is based on the relative abundance of microbial genera associated with the LF and HF diets in ileum and colon chyme.** Clustering of mice according to relative abundance at genus level in the ileum **(a)** and colon chyme **(b)**. Heatmap visualization is based on the relative abundance of selected genera. Rows are clustered by Euclidean distance and ward linkage hierarchical clustering. Row-bar is colour-coded by the diet group. The colour scale represents the scaled abundance of each genus, denoted as Z-score, with red indicating high abundance and blue indicating low abundance. n = 9-12.

Coffee protected against HF diet-mediated endotoxemia

A change in the gut microbiota composition caused by HF diet is known to increase intestinal translocation of bacteria or bacterial products such as LPS [17]. Here, we observed that HF diet had higher levels of plasma LBP, while HFC5 was able to attenuate this. HFC1 had no impact on the plasma LBP levels **(Fig. 5a)**. We have additionally measured the levels of circulating FD4 and the expression of tight junction protein genes. We found no difference in plasma FD4 in any of the groups **(Fig. 5b)**. Regarding tight junctions, the expression of occludin and zonula occludens was higher in the HF diet fed mice in jejunum, ileum and colon and this effect was even more pronounced in the HFC5 group **(Fig. 5c-d)**.

In addition, we measured the expression of two pattern recognition receptors; TLR4 and NOD1. TLR4 is upregulated when LPS passes through the intestinal barrier [30], while it has been shown that bacterium-related leakage after consumption of HF diet is dependent on NOD1 [17]. We observed that the HF diet had higher levels of TLR4 and NOD1 throughout the whole intestine. Both receptors were lower in either of the coffee groups both in jejunum and in ileum. Regarding the large intestine, the HFC1 had lower expression levels of both receptors, while the HFC5 had only lowered NOD1 expression levels **(Fig. 5e-f)**. In summary, these data suggest that supplementation of HF diet with coffee could be able to enhance the barrier integrity and thus prevent bacteria and microbial products from passing through the gut wall.

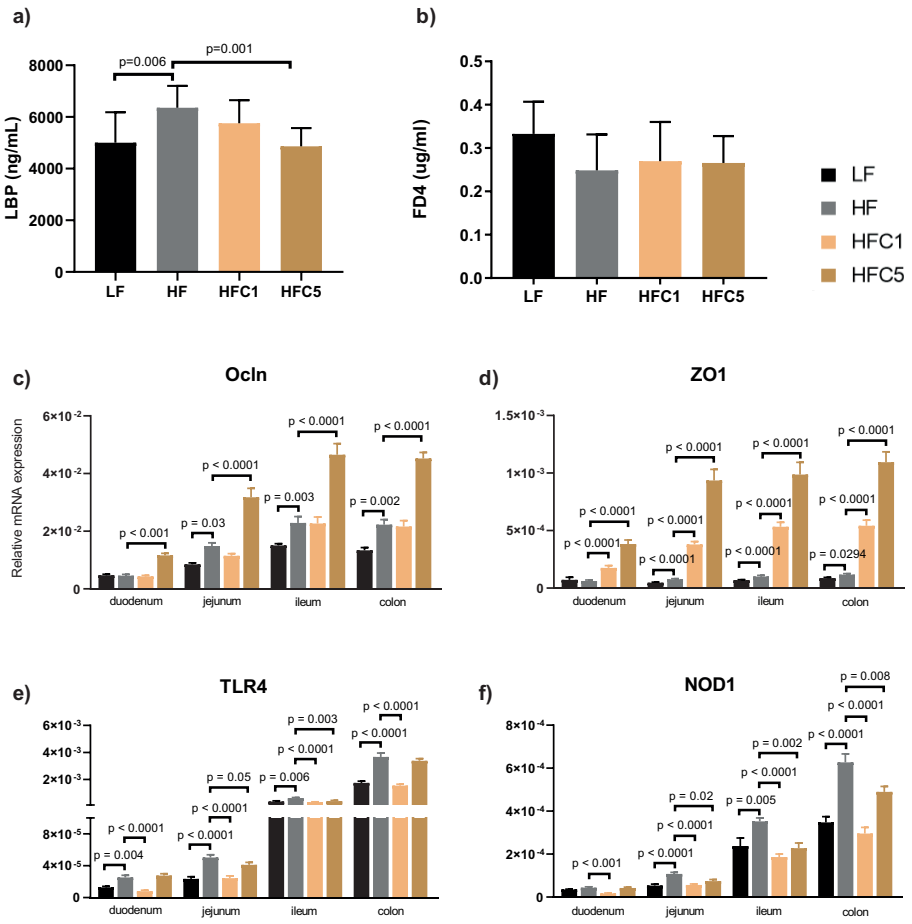

Figure 5 – **Intestinal barrier function in the LF, HF, and coffee supplemented HF diets. (a)** LBP protein in plasma. **(b)** FD4 in plasma 1.5h after oral gavage. **(c-f)** mRNA expression of Ocln **(c)**, ZO-1 **(d)**, TLR4 **(e)** and NOD1 **(f)** in the intestinal mucosa. n=10-12. Relative quantification by RT-qPCR normalized to GAPDH. Values are presented as mean with s.e.m. One-way ANOVA with Sidak's correction for multiple comparisons test.

Coffee consumption dampened the intestinal inflammation seen in the HF group

It has been previously shown that HF diet leads to a more inflammatory profile of the intestinal immune system [17]. Since mice fed coffee show a healthier microbial composition, we investigated whether supplementation of the HF diet with coffee would impact the Mφ cell population in the small intestinal lamina propria. We took advantage of the increased expression of the surface markers CX3CR1 and MHCII as the intestinal Mϕ mature in the lamina propria and the fact that the non-fully maturated Mϕ are proinflammatory [27]. Evaluating the ratio between cells expressing high CX3CR1 and MHCII (fully maturated) with cells expressing intermediate CX3CR1 and low to intermediate MHCII (immature and proinflammatory) showed a dose-dependent relationship between the amount of coffee in the diet and the relative amount of Mϕ with high CX3CR1 and MHCII expression **(Fig. 6a-b)**.

Furthermore, we investigated whether the changes in the Mϕ population are reflected in the release of typical proinflammatory cytokines. We saw that the HF diet had higher mRNA expression of TGF-β in ileum, and TNF-α and IL-1β in both jejunum and ileum **(Fig. 6c-e)**. HFC1 had lower TNF-α and IL-1β levels in ileum but no changes in the expression of TGF-β were observed. HFC5 had lower levels of TGF-β, TNF-α and IL-1β in ileum, while only higher TNF-a levels in jejunum **(Fig. 6c-e).** Proinflammatory Mϕ use cytotoxic ROS in their antimicrobial respiratory burst to combat microbes [31]. The production of these ROS in Mϕ are NOX2-dependent [32-35]. We observed that mice fed the HF diet showed higher expression of NOX2 in jejunum and ileum and both HFC1 and HFC5 had lower expression levels of these genes similar to the level observed in the LF diet group **(Fig. 6f)**. Furthermore, we recorded *in vivo* the ROS production by non-invasive imaging using the ROS probe L-012. We saw abdominal higher levels of ROS in the HF group after two weeks, which continued to be high until the final measuring timepoint at 10 weeks **(Fig. 6g)**. This L-012 dependent in vivo signal was previously shown to originate from the distal small intestine [32]. The coffee groups had significantly lower ROS production in the gut to a

similar level as in the LF diet group at all timepoints **(Fig. 6g)**. Taken together, our data indicate that coffee at moderate doses (HFC5) partly dampened the intestinal inflammatory response caused by the consumption of a HF diet.

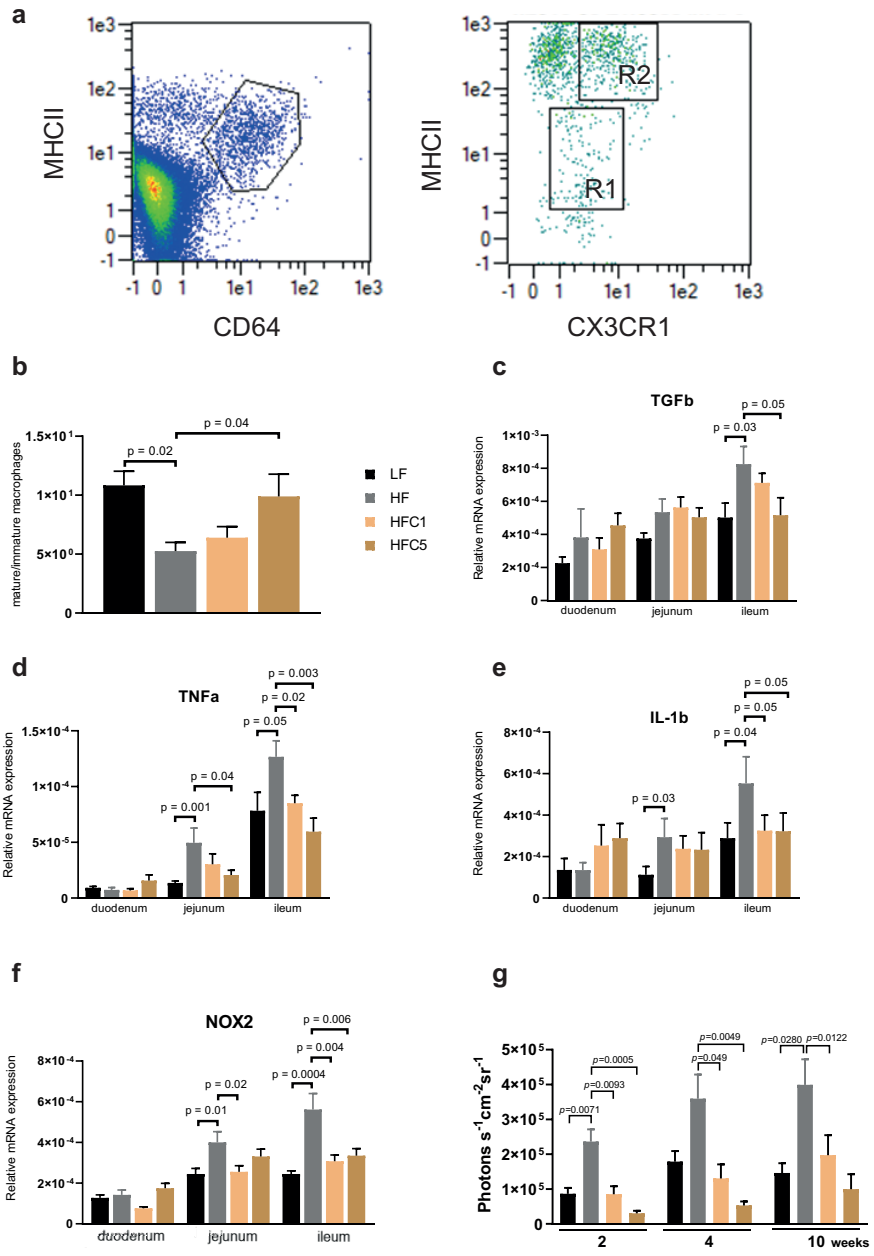

Figure 6 *(legend in the next page)*

←Figure 6 – **Intestinal immune response to HF diet supplemented with coffee.** **(a)** Following the exclusion of lymphocytes and NK-cells, Mφ was identified as CD64+/MHCII+ cells. Mφ was further subdivided into mature/homeostatic and immature/proinflammatory Mφ based on the expression level of CX3CR1 and MHCII. Proinflammatory was identified as intermediate CX3CR1 and low to intermediate MHCII (R1), while mature was identified as CX3CR1 high/MHCII high (R2). **(b)** The ratio of mature to proinflammatory Mφ showed a dose-dependent increase in response to coffee intake (linear regression, p=0.02). **(c-f)** mRNA expression of TGF-β **(c)**, TNF-α **(d)**, IL-1β **(e)** and NOX2 **(f)** mRNA expression in the intestinal mucosa, n = 8-12. Relative quantification by RT-qPCR normalized to GAPDH. **(g)** Measurement of L-012-mediated luminescence signal in all dietary groups at three different timepoints, n=12. Values are presented as mean with s.e.m. One-way ANOVA with Sidak's correction for multiple comparisons for all apart from **(g)** where Kruskal-Wallis with Dunn's correction for multiple comparisons was used instead.

Discussion

In the present study, we have investigated the impact of coffee on the microbial composition along the length of the intestine, including the segments of the small intestine, in mice fed a LF diet, a HF diet and HF diet supplemented with two different coffee doses. Our results indicate that consumption of coffee in moderate doses attenuated the unfavourable alterations in the microbial community structure along the intestine that were induced in the HF diet group. Interestingly, we saw that the microbiota composition in the groups receiving coffee was similar to the one in the LF fed mice. The changes in the microbiota were accompanied by an improved barrier function and a dampened immune response from the lamina propria in the small intestine, as seen by lower circulating LBP levels, upregulated expression of the genes encoding tight junction proteins, TLR4 and NOD1 as well as lower numbers of proinflammatory Mφ and lower production of cytotoxic ROS and release of proinflammatory cytokines.

Several previous rodent studies have shown that consumption of HF diet might unbalance the gut microbiota composition [6, 28, 29, 36-38]. However, the majority of these studies have investigated the microbial composition in feces and in the large intestine [39]. Here, we analyzed the microbial composition throughout the whole intestine including the three regions of the small intestine; duodenum, jejunum, and ileum. The small intestine is the primary site of nutrient absorption and the terminal small intestine, the ileum, possesses the most abundant immune tissue of the whole gastrointestinal tract. Additionally, the small intestine has been characterized as a site rich in microbe-microbe and host-microbe interactions that affect metabolic, immune and endocrine functions [40, 41]. Recently, some studies have shown that the small intestinal microbiota is subject to dietary changes and that it plays a role in nutrient digestion and transport [36, 42, 43]. Here, we chose to analyze the impact of the different diets in the composition of the small intestinal microbiota, divided into the three small intestinal segments, in addition to the gut microbiota in the large intestine.

The changes we observed in the gut microbial community of the HF diet-fed mice, namely higher levels of *Firmicutes* and lower levels of *Bacteroidetes*, mainly represented by higher relative abundance of *Peptoclostridium* and *Lachnospiraceae* and lower relative abundance of *Faecalibaculum* and members of the *Bacteroidales S24_7* family are in accordance with some of the previous findings on HF diet-induced microbiota alterations. *Firmicutes* and *Bacteroidetes* are the major phyla in the intestinal tract and an unbalance between them has been associated with HF diet-induced dysbiosis and disease [6, 28, 29, 38, 42, 44]. A decrease in the *S24_7* family has been previously correlated to HF diet, while *Faecalibaculum* (which had lower abundance levels in the HF diet group) has been proposed to promote body weight loss as a result of its ability to produce lactic acid as a metabolic end product [45, 46].

We saw that supplementation of coffee attenuated the unfavorable microbial environment observed in the HF diet group. In more details, the most pronounced changes of coffee in the gut microbiota were depicted by lower levels of *Firmicutes* and higher levels of *Bacteroidetes* and *Actinobacteria* (mainly *Coriobacteriaceae UCG-002*). Furthermore, we observed a positive correlation of coffee supplementation with *Faecalibaculum* and members of the *Bacteroidales S24_7* family, while we also saw a negative correlation with *Peptoclostridium*. Interestingly, we observed that when coffee was supplemented in the HF diet the microbial community structure both in ileum and in the large intestine changed towards a similar profile seen in the LF fed mice. With about 1500 chemical compounds present in coffee it is difficult to pinpoint the responsible molecule for the observed changes; it might also be a synergistic action of multiple compounds. Coffee contains several poly- and oligosaccharides, as well as peptides that could act as a carbon and nitrogen source and therefore alter the microbial composition [47], especially in the small intestine as this is the primary site of absorption. Coffee is also rich in phenolic compounds that could also alter the microbial composition [48-50], especially, chlorogenic acid, which has been found to modulate positively the gut microbiota in HF diet-fed mice [51]. Additionally, consumption of caffeine has been also shown to positively alter the gut microbiota [52]. Lastly, coffee contains high amounts of soluble dietary fiber, mainly

galactomannans and arabinogalactans, which act as energy sources for commensal bacteria [53].

The negative changes in the composition of the gut microbiota as a result of HF feeding have been associated with impairment in the gut barrier function, leading to leakage of bacteria or bacterial compounds dependent on the NOD1 microbial pattern recognition receptors. [17]. Here, we saw that the expression of NOD1 was upregulated in the HF fed mice and that coffee had lower levels. The same was the case for another pattern recognition receptor, TLR4. Pattern recognition receptors sense bacteria and bacterial products and thus possess a critical role in gut homeostasis by eliciting downstream responses [54]. Furthermore, we observed that coffee supplementation also eliminated the HF diet-elevated levels of LBP and that it led to an upregulation in the expression levels of the genes encoding the tight junction proteins.

In addition to an impairment in the gut barrier function, HF feeding has been also associated with increased inflammatory status in the small intestinal mucosa, possibly due to the posed alterations in the intestinal microbial milieu [17]. Here, we observed that supplementation of coffee in the HF diet attenuated the increased inflammatory response originating from the small intestine in mice fed the HF diet alone. This was characterized by lower levels of the immature proinflammatory Mφ in the lamina propria of the small intestine, followed by downregulation of NOX2 and lower expression levels of certain proinflammatory cytokines, i.e., TNF-α, IL-1β and TGF-β. Small intestinal Mφ under steady state are of hematopoietic origin and are constantly replenished. Blood monocytes undergo a four-stage maturation differentiation process [55]. The fully matured Mφ (expressing high CX3CR1 and MHCII) participate in maintaining homeostasis in the small intestine by clearing bacteria and bacterial products, but in a more “silent” mode, i.e. without generating an inflammatory response [27, 56]. However, this is not the case during intestinal inflammation, where the monocytes do not reach full maturation but remain in the intermediate differential stages (intermediate CX3CR1 and low to intermediate MHCII). These Mφ are known to display proinflammatory behavior in response to TLR ligands by inducing oxidative burst and producing proinflammatory mediators, such as TNF-α and IL-1β [27, 31]. ROS are part of the innate immune system participating in the respiratory burst

to kill microbes [32-34]. The production of ROS in the Mφ of lamina propria is originating from NOX2, which we observed to be lower in the coffee supplemented diet groups. This was also reflected in the levels of abdominal ROS assessed *in vivo*.

Previous studies have analyzed the impact of coffee on inflammation in liver and blood with contradictory results [18-20]. As regards the intestine, a recently published study using a co-culture cell model of the intestinal mucosa has shown that coffee exhibits anti-inflammatory properties via the inhibition of inflammatory mediator release and enhances the barrier function [57]. As with the case of gut microbiota, it is not clear whether one or many bioactive compounds present in coffee mediate the observed inflammatory response directly or whether it is a cascade effect. There are indications that caffeine, chlorogenic acid and diterpenes kahweol and cafestol can act directly at cell level and thus inhibit the activity of NF-κB and consequently reduce an inflammatory response and oxidative stress [58-60]. However, it has been previously shown that a change in the small intestinal microbial population can reduce inflammation in this site [61, 62]. Therefore, we hypothesize that the dampened inflammatory reaction observed in the small intestine in coffee fed mice could be the result of the beneficial modulation of microbiota.

In conclusion, our results show that coffee consumption ameliorated the negative effects that accompanied the high-fat diet in terms of gut health. This was depicted by a favorable microbial population, resembling the one seen in low-fat diet fed mice, dampened immune response in the small intestine and improved gut barrier integrity.

Material and Methods

Animal housing and diets

Nine-week-old (n = 12/group) male mice (C57BL/6J background, Envigo, The Netherlands) were housed in individually ventilated cages (4 mice per cage) in a controlled environment (12h-light-dark cycle; temperature, 24 ± 1 °C; humidity, 45-55%). Animals were acclimatized for 4 weeks with a LF diet (D12540J, 10 E% fat, Research Diets). Thereafter 12 mice/group were fed HF diet (D12492, 60 E% fat, Research Diets), HF diet supplemented with coffee equivalent to 1 cup/day (HFC1) or 5 cups/day (HFC5). Diets were obtained from Research Diets (New Brunswick, NJ, USA) by adding freeze-dried coffee to the D12492 diet. The conversion of estimated cups/day into the actual amount of coffee in feed was done by relating intake to body mass/body surface area (kg/m^2). Therefore, HFC1 and HFC5 had 5 and 25 g of coffee per kg of food. Mice had *ad libitum* access to food and water and weight measurements were performed weekly to assess food intake and weight development. To exclude physical activity as a confounder, we removed running wheels after acclimatization. Animals were euthanized by cervical dislocation under anesthesia (100 μL/10 g mouse), by Zolezepam (32 mg/Kg), Tiletamin (32 mg/Kg), Xylazine (4.5 mg/Kg) and Fentanyl (26 ug/Kg).

Ethical aspects

The animal experiment was performed with permission from The Norwegian Animal Research Authority and was conducted in compliance with the current guidelines of The Federation of European Laboratory Animal Science Associations (FELASA).

Intestinal permeability by FITC-dextran

Intestinal permeability was determined at week 14 using a protocol adopted from Johnson et al., [63]. Briefly, mice that have previously fasted for 4h received orally 650mg/Kg of fluorescein isothiocyanate (FITC) dextran (FD4, Sigma-Aldrich) and blood was collected after 1.5 hours. Concentration of FITC dextran in plasma was determined by fluorescence spectroscopy with excitation and emission wavelengths at 490 nm and 520 nm, respectively (Synergy H4 Hybrid microplate reader, Bio Tek instrument).

LBP measurement

LBP in plasma was measured through an enzyme immunoassay for the determination of mouse LBP (Biometec, Greifswald). Briefly, plasma samples were diluted 1:800 and loaded into a precoated plate. Subtracts were read with an absorbance at 450 nm and the amount of LBP was estimated based on a standard curve constructed in a 4-parameter logistic curve fit.

Flow cytometry

After isolating the small intestine, mesenteric fat, lumen content and Peyer's patches were removed. Then, it was opened longitudinally, cut into 5 mm long segments and placed immediately in cold RPMI. Isolation of epithelial and lamina propria cells followed the description by Goodyear and coworkers [64]. Briefly, mucus was removed after incubation in DTT at 37 °C/20min, epithelial cells were separated by three incubation steps in EDTA solution at 37 °C/15min and loose connective tissue was removed by adding a digestion step containing collagenases, liberase and DNases (Sigma-Aldrich), at 37 °C/15min. All incubation steps were performed together with agitation. Cells were washed with PEB buffer (Miltenyi Biotec) and pre-blocked with FcR Block reagent (Miltenyi Biotec). Cells were stained with the following intra- and extracellular antibodies: CD3-APCvio770, CD4-PE, CD8-PEvio770, CD45-PerCPvio700, FoxP3-APC, Mouse IgG1-APC, and CD11b-APCv770-A and MHCII-PerCP700-A (Miltenyi Biotec). Live cells were identified with the use of propidium iodide or LIVE/DEAD fixable Violet Stain kit (Thermo Fisher). Cell acquisition and analysis were performed with MACQuant Analyzer 10 Flow cytometer and MACQuantify software (Miltenyi Biotec) for cell acquisition and analyses.

Gene expression and 16S rRNA gene sequencing

Mucosal samples for RNA and DNA extraction were scraped off from longitudinally opened intestine with a glass slide. The small intestine was divided as: duodenum (most proximal 5 cm), jejunum (6 cm around the center) and ileum (most distal 6 cm). Mucosal samples for RNA extraction were preserved in RNAlater (Sigma-ALdrich) after sampling. RT-qPCR was performed in LightCycler 480 Instrument II (Roche). Used primers and optimized primer

annealing temperature are listed in **table 1**. LinReg Software was used to calculate Cq values based with a common threshold and individual efficiencies.

Mucosal samples and intestinal content for DNA extraction were placed in S.T.A.R buffer (Roche), together with <106 Mm acid-washed glass beads (Sigma-Aldrich), immediately after dissection.

Table1: primers used for RT-qPCR and their annealing temperature

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Tm °C
GAPDH	CTTCAACAGCAACTCCCACTCTT	GCCGTATTCATTGTCATACCAGG	60
IL-1β	GCAGCTGGAGAGTGTGGAT	AAACTCCACTTTGCTCTTGACTT	61
NOD1	TGACAGTAATCTGGCTGACC	GTCTGGTTCACTCTCAGCAT	59
NOX2	GGGAACTGGGCTGTGAATGA	CAGTGCTGACCCAAGGAGTT	61
Ocln	CTGTGAAAACCCGAAGAAAGATG	GCAGACACATTTTTAACCCACTC	57
TGF-β	GAACCAAGGAGACGGAATACAG	CGTGGAGTTTGTTATCTTTGCTG	65
TLR4	GATCTGAGCTTCAACCCCTT	TGTTTCAATTTCACACCTGGA	61
TNFα	CTGTCTACTGAACTTCGGGGTGAT	GGTCTGGGCCATAGAACTGATG	61
ZO-1	GAGAAAGGTGAAACTCTGCTG	ACGAGGAGTCGGATGATTTTAGA	59

16S rRNA based gut microbiota sequencing

The 16S rRNA amplicon sequence analysis protocol was previously described by Avershina et al. [65]. Briefly, after DNA extraction the 16S rRNA gene was amplified for 25 cycles using prokaryote targeting primers developed by Yu et al. [66]. AMPure XP was used for purification of the PCR product (Beckman-Coulter, Brea, CA) and 10 further PCR cycles followed. The resulting amplicons were sequenced on Illumina MiSeq V3 platform (Illumina, SanDiego, CA). The resulting 300 bp paired-end reads were further paired-end joined, quality-filtered using QIIME33 and clustered with 97% identity level using closed-reference *usearch v7.0* algorithm [67, 68] against Greengenes database v13.8 [69].

In vivo imaging ROS using L-012 probe

In vivo imaging was performed with IVIS Lumina II (Perkin Elmer). During the 2nd, 4th and 10th week of the experiment, 200µl of luminescence probe L-012 (Wako Chemical) was injected intraperitoneally in a concentration of 2.5mg/mL. Light emission from the ventral

side was measured as photons per second per cm^2 per steradian, 5 minutes after injection, using Living Imaging software (Perkin Elmer).

Statistical Analyses

Statistical analyses were done in the GraphPad Prism software (La Jolla, USA). Averages are presented as mean and variances as the standard error of the mean (s.e.m). The homogeneity of variances was tested with Bartlett's test. Normal distributed data from multiple groups were compared by one-way ANOVA with Sidak's correction for multiple comparisons. For non-normal distributed data, the non-parametric Kruskal-Wallis one way-ANOVA was performed. When only two independent groups were compared, we used the unpaired *t* test or the non-parametric Mann-Whitney test.

To identify the LF associated taxa students t-test adjusted for multiple comparisons with Bonferonni correction were performed between the LF and HF fed mice. Genera with adj. $p < 0.05$ were selected. The PCoA plots were performed in R by plotting the Bray-Curtis distance matrix using *vegan* and *ggplot*. Heatmaps and hierarchical clustering were created in Python using *seaborn* where rows were clustered by the Euclidean distance matrix and ward linkage hierarchical clustering.

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