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

Philosophiae Doctor (PhD)
Thesis 2022:58

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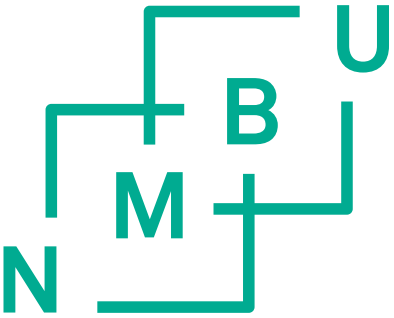

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Summary

English

Background: Various factors, including dietary components, the gut microbiota and host responses, are imperative for gut health. Optimal interactions between diet, gut microbiota and host are suggested to be important to avoid imbalanced gut microbial ecosystems and low-grade inflammation. Dietary fiber components, in particular, have been highlighted for their positive effects on the microbiota and gut inflammation status. Furthermore, although high-fat diets, mainly based on animal fat, have been associated with gut inflammation it is not clear to what extend different fat sources (i.e., animal type), and modes of fat intake affect the gut inflammatory process.

Objectives

The overall aim of the current thesis was to investigate the differential impact of fiber and protein fractions from faba beans and different fat types on the microbiota composition and gut health in mice with and without low-grade inflammation in the gut. Another aim was to assess the role of reactive oxygen species (ROS) induced by NADPH oxidase 1 (NOX1) for low-grade gut inflammation and microbial community in the colon.

Methods: After the establishment of a low-grade inflammation model with the chemical dextran sodium sulfate (DSS) feeding trials with C57BL/6J mice were performed. Besides the wild type (WT), C57BL/6J, mice deficient in the *NOX1* gene were used (NOX1-KO mice). The diets offered during the mouse experiments varied in macronutrient composition and energy intake. Briefly these diets were: a standard mouse diet (chow diet), a synthetic low-fat diet (LFD), a purified western diet (WD) and modified western diets (WDs), in which defined fractions from faba beans were incorporated. These fractions were protein fraction (PF) and fiber fraction (FF) that replaced partially casein and cellulose substances in WDs, respectively. Body weight, colon length, gene expression of inflammatory and reactive oxygen species related genes, biomarkers of inflammation in blood and fecal sample were parameters to be examined. Shifts of the colonic microbiota populations in phylum and genus level upon the termination of experiments were additionally evaluated using LEfSe (Linear discriminant analysis Effect Size).

Results: In Paper I, we established the low-grade inflammation mouse model and showed that NOX1 plays a significant role in ROS formation in the colon during pathogenesis towards the status of low-grade inflammation. We demonstrated that NOX1 modulates the colonic microbiota both in a steady-state (healthy) and during low grade inflammation. Also, while absence of NOX-1 did not influence the pathology scores, the inflammation-related genes and lipocalin 2 (LCN-2) expression tended to be higher in NOX1 deficient mice than in WT mice. From these results we conclude that NOX1-dependent ROS production is important for shaping microbiota composition and for protecting against insults to the colon.

In Paper II, we compared the impact of a low-fat diet (LFD) and different western diets (WD) with fat from various sources on colon health in low-grade inflammation. We falsified the hypothesis that WD fed mice would manifest more severe symptoms compared to LFD fed mice as we observed that LFD mice were more susceptible to DSS-induced inflammation and revealed a less diverse microbiota with increased relative abundance of the Proteobacteria phylum compared to WD fed mice. Both fat sources (milk and lard) tested as part of the WD induced similar immediate 'protective' effect as compared to the LFD. Because these results contradict many other studies, we suspect that the higher fat content protects against the establishment of the DSS-induced low-grade inflammation. Therefore, we raise the concern that the DSS model of mouse inflammation might be unfit for the purpose of studying the differential impact of dietary fats.

In Paper III, we investigated possible metabolic and colonic beneficial effects of faba bean protein and fiber fractions when ingested as part of a WD. Although the diets were isocaloric the mice that ingested diets with added faba bean protein content (WD+PF and WD+BF) had a higher increase in body weight compared to the pure WD or the WD+FF. However, no differences were revealed in glucose and insulin tolerance test between dietary groups. Also, neither the protein nor the fiber fraction protected against DSS induced low-grade gut inflammation as compared with mice fed a pure WD. Furthermore, marginal changes were found in the microbiota at the genus level.

Conclusions: Collectively, our results demonstrate a role for ROS in the healthy gut and in response to DSS induced inflammation which may be related to controlling and shaping the microbiota. Furthermore, unexpectedly, we found that a high fat content in the diet protected

against DSS induced inflammation as compared with the LFD group and speculate that the high fat content confounds the establishment of the DSS induced low-grade inflammation model. Also, a WD with a higher protein fraction from faba bean resulted in even higher gain of weight compared to standard WD. Only marginal changes were found for the microbiota composition when increasing the fiber fraction of the WD and it did not protect against weight gain.

Norwegian (Norsk sammendrag)

Bakgrunn: Ulike faktorer, slik som næringsstoffer, tarmmikrobiota og biologiske vertsresponser, er avgjørende for tarmhelsen. Optimal interaksjon mellom disse er viktig for å unngå ubalanserte mikrobielle tarmøkosystemer og lavgradig betennelse. Særlig har kostfiber blitt fremhevet som viktig og positiv for både mikrobiota og tarmbetennelsesstatus. I tillegg, selv om kosthold med høyt fettinnhold fra animalske kilder har vært assosiert med tarmbetennelse, er det ikke entydig klart i hvilken grad ulike fettkilder (dvs. dyretype), og inntak av fett som sådan påvirker betennelse i tarm.

Mål: Det overordnede målet med denne oppgaven var å undersøke i hvilken grad fiber- og proteinfraksjoner fra fababønner og ulike fetttyper påvirket mikrobiotasammensetningen og tarmhelsen hos mus med og uten lavgradig betennelse i tarmen. Et annet mål var å evaluere rollen til reaktive oksygenforbindelser (ROS) indusert av NADPH-oksidase 1 (NOX1) for lavgradig tarmbetennelse og mikrobielle samfunn i tykktarm.

Metoder: Etter etablering av en lavgradig inflammasjonsmodell med det kjemiske stoffet dekstran-natriumsulfat/dextran sodium sulfate (DSS) ble det utført fôringsforsøk med C57BL/6J-mus. I tillegg til å bruke villtypemus (WT), ble C57BL/6J-mus uten uttrykk av NOX1-genet benyttet (NOX1-KO-mus). De ulike fôrtypene som ble brukt under museforsøkene varierte i sammensetning av makronæringsstoffer og energiinnhold. Fôrtypene bestod (i korthet) av: standard musefôr også kalt chow, lav-fettdiett (LFD), en vestlig høy-fettdiett (WD) og modifiserte vestlige dietter, der fraksjoner fra faba bønner ble inkorporert. Disse fraksjonene var proteinfraksjon (PF) og fiberfraksjon (FF) som delvis erstattet henholdsvis kasein- og cellulose i WD-fôrtypene. Alle fôrtypene med unntak av chow var kontrollerte (kjent innhold av alle næringsstoffene og ingrediensene). Chow-fôret er mer udefinert fordi det er satt sammen av hele råvarer. Kroppsvekt, tykktarmslengde, genuttrykk av inflammatoriske og ROS-relaterte gener, biomarkører for betennelse i blod og avføringsprøver var parametere som skulle undersøkes. Endringer av tykktarmens mikroflora ved avslutning av eksperimenter ble i tillegg evaluert ved bruk av såkalt LEfSe (Linear discriminant analysis Effect size).

Resultater: I Artikkel I (Paper I) etablerte vi musemodell for lavgradig inflammasjon og viste at NOX1 spiller en betydelig rolle i ROS-produksjon i tykktarmen under lavgradig betennelse

indusert med DSS. Vi viste også at NOX1 modulerer tykktarmsmikrobiotaen både i en 'steady-state' (sunn) og under lavgradig betennelse. Videre så vi at, mens fravær av NOX-1 ikke påvirket patologi, hadde de betennelsesrelaterte genene og uttrykk av lipocalin 2 (LCN-2) en tendens til å være høyere i NOX1-knockout mus enn i WT-mus. Fra disse resultatene konkluderer vi med at NOX1-avhengig ROS-produksjon er viktig for å forme mikrobiotasammensetning og for å beskytte mot skader i tykktarmen.

I Artikkel II (Paper II) sammenlignet vi effekten av en lav-fettdiett (LFD) og ulike vestlige dietter (WD) med fett fra ulike kilder på tykktarmshelsen ved lavgradig betennelse. Vi forkastet derfor hypotesen om at WD-fôrede mus ville manifestere mer alvorlige symptomer sammenlignet med LFD-fôrede mus da vi observerte at LFD-mus var mer mottakelige for DSS-indusert betennelse og avslørte en mindre mangfoldig mikrobiota med økt relativ tilstedeværelse av Proteobakterier (fylum) sammenlignet med WD-fôrede mus. Begge fettkilder (melk og svinefett) testet som en del av WD førte i begge tilfeller til umiddelbar "beskyttende" effekt sammenlignet med LFD. Fordi disse resultatene motsier mange andre studier, mistenker vi at det høyere fettinnholdet beskytter mot etableringen av DSS-indusert lavgradig betennelse. Derfor reiser vi bekymringen for at DSS-modellen for musebetennelse kan være uegnet for det formål å studere den differensielle påvirkningen av fettrikt fôr med den hensikt å studere fysiologiske effekter av høyfett-dietter.

I Artikkel II (Paper III) undersøkte vi potensielt metabolske effekter av proteiner og fiber (begge var fraksjoner) isolert fra fababønner som ble blandet inn WD-fôret. Selv om diettene var isokaloriske, hadde musene som inntok fôret beriket med proteiner fra fababønner (WD+PF og WD+BF) fant vi at disse musene hadde en høyere økning i kroppsvekt sammenlignet med ren WD eller WD+FF (fiberfraksjon). Imidlertid ble det ikke avdekket forskjeller i glukose- og insulinregulering mellom de ulike diettgruppene. Heller ikke protein- eller fiberfraksjonen fra fababønner beskyttet mot DSS induserte lavgradig tarmbetennelse sammenlignet med mus som ble fôret med en ren WD. Videre ble det funnet marginale endringer i mikrobiotaen på slektsnivå.

Konklusjoner: Samlet viser resultatene våre en rolle for ROS i den sunne tarmen og som respons på DSS-indusert betennelse som kan være relatert til å kontrollere og forme mikrobiotaen. Videre, og noe uventet, fant vi at et høyt fettinnhold i kostholdet (WD)

beskyttet mot DSS-indusert betennelse sammenlignet med LFD-gruppen og spekulerer i at det høye fettinnholdet påvirker etableringen av den DSS-induserte lavgradige betennelsesmodellen, muligens direkte. Dessuten resulterte en WD med en høyere proteinfraksjon fra fababønne i enda høyere vektøkning sammenlignet med standard WD. Bare marginale endringer ble funnet for mikrobiotasammensetningen ved tilsetning av fiberfraksjonen til WD, og den beskyttet ikke mot vektøkning.

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Abbreviations

AOM: Azoxymethane

BF: Both protein and fiber fractions

CD: Crohn disease

DCs: Dendritic cells

DSS: Dextran sodium sulfate

F/B: Firmicutes/Bacteroidetes

FBDGs: food-based dietary guidelines

FF: Fiber fraction

GALT: Gut associated lymphoid tissue

GF: Germ free

GI: Gastrointestinal

HFD: High fat diet

IECs: Intestinal epithelial cells

IgA: Immunoglobulin A

IL: Interleukin

LCN-2: Lipocalin-2

LEfSe: Linear discriminant analysis Effect Size

LFD: Low fat diet

MAMPs: Microbial associated molecular patterns

NCDs: Non-communicable diseases

NF-κB: Nuclear factor-kappa B

NLRs: Nod like receptors

NNR: Nordic Nutrition Recommendations

NOX1-KO mice: NADPH oxidase 1 knockout mice

PAMPs: Pathogen associated molecular patterns

PF: Protein fraction

PRRs: Pattern recognition receptors

ROS: Reactive oxygen species

SCFAs: Short chain fatty acids

Th: T helper

TLR: Toll like receptor

Tregs: T regulatory cells

UC: Ulcerative colitis

WD: Western diet

WT: Wild type

List of papers

PAPER I

The ROS-generating enzyme NADPH oxidase 1 modulates the colonic microbiota but offers minor protection against dextran sulfate sodium-induced low-grade colon inflammation in mice.

Herfindal A.M., Rocha S.D.C., **Papoutsis D.**, Bøhn S.K., Carlsen H.

Published in the *Free Radical Biology & Medicine*, Volume 188, June 2022, Pages 298-311, https://doi.org/10.1016/j.freeradbiomed.2022.06.234

PAPER II

Western diet attenuates DSS effects in a low-grade inflammation mouse model compared with a low-fat diet.

Papoutsis D., Rocha S.D.C., Herfindal A.M., Bøhn S.K., Carlsen H.

Published in *The Journal of Nutrition*, Volume 152, Issue 3, December 2021, Pages 758–769, https://doi.org/10.1093/jn/nxab401

PAPER III

Intestinal effect of faba bean fractions in WD-fed mice treated with low dose of DSS.

Papoutsis D., Rocha S.D.C., Herfindal A.M., Bøhn S.K., Carlsen H.

Published in *PLOS ONE*, August 2022, https://doi.org/10.1371/journal.pone.0272288

1. **Introduction**

The large intestine, also known as colon, is an indispensable part of the digestive system contributing to a wide range of vital functions. It is responsible for the constant maintenance of fluid and electrolyte balance, the removal of waste metabolic products and is the region where the most dense and diverse microbial community, collectively referred as gut microbiota is harbored within the host. Along the gastrointestinal tract (GI-tract) numerous Bacteria, Archaea and Eukarya are found, with the former domain of life being the most dominant. Some of their primary functions are to recover energy from food through digestion of complex carbohydrates, to provide vitamins such as riboflavin (vitamin B2), biotin (vitamin B7), folate (vitamin B9) and vitamin K and to offer protection against pathogens [1].

The GI-tract receives daily a plethora of environmental factors which shape the gut microbiota assembly with beneficial or detrimental consequences in metabolic processes and immunity. Diet is probably the environmental determinant with the greatest influence on microbiota composition and function. Dietary fiber, fat and proteins can act as microbial modulators and cause metabolic implications and diseases [2]. Although a lot has been learned on how food nutrients are involved in microbiota profile, there are still a number of issues that are not clearly understood, like to what extent disease patterns influenced by diet are causally linked to the microbiota profile and importantly how host factors including the immune system affects microbiota. The use of animal models for research may provide useful insights and nutrition remains a compelling target to elaborate.

The current thesis focuses on whether diets varying quantitively and qualitative in macronutrients affect the colon state and the formation of colonic microbiota. The thesis also focuses on host factors important in shaping and regulating microbiota such as reactive oxygen species (ROS). Herein, the introductory chapter provides knowledge on diet, health and gut microbiota aspects. Apart from that, the structure and the immune system of the GI-tract are described together with the interplay between dietary choices, bacteria and host health. Lastly, commonly used mouse models for studying inflammatory bowel diseases and arguments for the utilization of rodents as experimental animals are displayed.

1.1 Diet and health

Searching in a dictionary for the word diet, which derives from the Greek word "*diaita*", we come across two main definitions. It can be either all the dietary choices that a person or an animal consumes habitually, or a list of food and drinks strictly followed by an individual person due to a medical problem or just with the intention to lose weight. Selection of food choices is depending on several individual and public determinants such as taste, income, socioeconomic status, availability of time, ethnicity, religion and culture[3, 4].

From the perspective of health, an optimal diet is often regarded as a balanced diet, which contains appropriate amounts of macronutrients and micronutrients with the aim to balance the energetic and physiological requirements of the body. The value of nutrition on health was stated by Hippocrates, around 400 BC, with the phrase "Let food be thy medicine". Proteins, fats, carbohydrates fibers and small proportions of minerals, vitamins are necessary for growth, metabolism and maintenance of life. Despite the constant evolving knowledge on dietary patterns through intervention trials, the endeavor to define a healthy diet and classify dietary regimens in energy balance is challenging [5].

In 2004, the World Health Assembly, a committee of health ministers from 194 member states, adopted the recommendations from World Health Organization (WHO). According to the report, named "WHO Global Strategy on Diet, Physical Activity and Health", the main objective was to prevent malnutrition and simultaneously reduce the prevalence of non-communicable diseases (NCDs) [6]. Nevertheless, as long as such aims remain unsolved, international policies require rapid actions for promoting appropriate dietary styles in order to deal with obesity, cardiovascular diseases, diabetes and cancer [7].

Globally, numerous studies strongly support that replacement of saturated and trans fats with unsaturated fats, limitations in salt and sugar, increased consumption of fruits, legumes and vegetables and restriction of the total energy intake have a major effect on reducing the risk for several diseases [8-10].

Recent advances in technological fields allow experts in nutrition to offer proper diet consultancy. In 2021, a study published in Nature Foods, assessed the impact of around

6.000 foods on disease burden. From the analysis it was suggested that individuals could gain up to 48 minutes added life span per day when 10% of caloric intake from beef and processed meats was replaced by fruits, vegetables and legumes in their daily meal [11].

In Europe, food-based dietary guidelines (FBDGs) set an example, where eating styles are adjusted to agreed principles by WHO and Food and Agriculture Organization (FAO) for improving public health [12]. Although these guidelines are primarily depending on dietary data, often other healthy related parameters, like physical activity and body weight, are included.

For Nordic countries, the national FBDGs came out between 2013 and 2015 (2014 for Norway) and their basis was the Nordic Nutrition Recommendations (NNR) 2012 [13]. Below (**Table 1**) is presented the most recent macronutrient intake indicated by NNR for the general population in Nordic regions and during 2022 the new recommendations would become available. For people with diseases or with other special needs, amendments might be required regarding the macronutrient uptake. Proteins, carbohydrates and fatty acids are presented as percentage of energy intake, whereas the estimation for dietary fiber is expressed as minimum grams per day or grams per Megajoule (MJ).

Macronutrients	**Percentage in energy intake (E%)**
Proteins	Adults & children (2>years) **10-20 E%**, elderly (≥65 years) **15 E%**
Carbohydrates	Total intake between **45-60 E%**
Fatty acids (triglycerides)	Monounsaturated fatty acids **10-20 E%** Polyunsaturated fatty acids **5-10 E%** Saturated fatty acids less than **10 E%**
Dietary fibers	Adults minimum **25-35g/d or 3g/MJ**, children (>2 years) **2-3g/MJ**

Table 1. Illustration of macronutrients intake for Nordic citizens by NNR.

1.1.1 The western diet

In western societies, where more than 80% of deaths are attributed to NCDs, western diet (WD) is regarded as a major reason for the onset of pathological conditions [14]. WD is a modern type of diet characterized by high quantities of saturated fats and omega-6 fatty acids, refined sugars, salt and reduced amounts of fibers and omega-3 fatty acids [15]. Processed and ultra-processed foods constitute a big part of the Western pattern diet and often diet recommendations suggest limiting or avoiding their consumption. Generally, they are agricultural commodities that go through a series of mechanical and chemical procedures such as heating, chopping, milling, canning, pasteurizing and addition of food additives [16]. On top of a western lifestyle, characterized by physical inactivity, air pollution, low exposure to sun and unhealthy habits (alcohol, smoking), WDs can trigger intestinal barrier dysfunction, increase gut permeability and lead to leakage of bacteria and harmful bacterial metabolites into the blood circulation, a condition known as endotoxemia [17]. These pathophysiology's are responsible for the development of low-grade systemic inflammation and are associated with metabolic syndrome, cancer and autoimmune diseases [18, 19]. IBDs, multiple sclerosis rheumatoid arthritis and psoriasis compose a heterogeneous group of autoimmune diseases that present multifactorial etiologies. Diet and specifically WD appears to be a major risk factor for the occurrence of the above-mentioned diseases and prominently for IBDs [20]. Based on recent data for the worldwide prevalence of IBDs, countries in Europe and North America report the highest values [21]. Indicatively, the incidence of ulcerative colitis in Norway is 505 per 100.000 persons whereas the estimation for Crohn's disease in Germany is 322 per 100.000 persons [22]. Although WD is typically considered as a diet without macronutrient and micronutrient deficiencies, it may alter the immune system function and hence influence immune-mediated diseases [23]. Consumption of WD or other diets high in fat content can lead to systemic inflammation due to immune system activation via proliferation of immune cells and cytokine production [24]. Food additives and substances, added in order to enhance the texture, the stability and the flavor of the products, are often considered as factors that affect intestinal health. For instance, it has been shown that two dietary emulsifiers, carboxymethylcellulose (CMC) and polysorbate 80 (P80), can induce alterations in human and mouse gut microbiota in a

manner that subsequently promotes chronic intestinal inflammation and colon carcinogenesis [25-27].

The impact of dietary components on gastrointestinal health is revealed through *in vitro* studies, animal models and human studies. It has been proposed that elevated intake of animal proteins, gives rise to active metabolites like ammonium, branched-chain fatty acids, hydrogen sulfide upon degradation. Ultimately, those metabolites may affect gut barrier function and increase predisposal to Crohn's disease [28]. Interestingly, the macronutrient metabolism has the capacity to modulate intestinal microbiota. According to interventions studies, proteins, fats, carbohydrates can promote the presence of commensal bacteria or induce a dysbiotic environment [29]. The source and the quantity of macronutrients are significant factors in terms of the gut response in colitis and other inflammatory conditions.

1.1.2 Legumes and pulses

Legume is any plant or seed of a plant that belongs to the Fabaceae or Leguminosae family [30], counting more than 800 genera and 20.000 species [31]. Legumes, which include dry and oil seeds, constitute the second most edible crops after cereals [32].

Dry seeds or dry grains, is categorized as pulses by the Food and Agriculture Organization of the United Nations (FAO) to discriminate them from fresh green crops like green beans and green peas, which are categorized as vegetables. Dry beans, dry peas, lentils and chickpeas are characteristic examples of the 11 primary pulses recognized by FAO and possess a significant position in the nutritional and economic value chain worldwide [33]. India, Canada, China, USA, Russia, Brazil are the leading countries in pulse production with a diversity of cultivars and numerous varieties depending on the size, the color and the shape of that yield [34].

Interestingly, consumers in Europe over the last years have shown an increased interest in legumes for health and environmental reasons. Grain legumes can be discriminated to the warm season legumes, which include soybeans (*Glycine max L.*) and common beans (*Phaseolus vulgaris L.*), and the cold season legumes. Faba beans (*Vicia faba L.*) and field peas (*Pisum sativum*) set an example of the latter group. These protein crops are highly preferred

for cultivation in Northern Europe due to their tolerance of low temperatures. Yield differences in legumes even between Nordic countries can be influenced by high latitude conditions[35]. Therefore, pulses like the faba beans can be exploited by Norwegian farmers and ultimately contribute to the effort of a more sustainable environment characterized by protein self-sufficiency and reduced of soybeans and other imported plant-based sources.

Pulses are acknowledged in human diet as an important food mainly because they are a dish of high nutritional value due to their macronutrient and micronutrient composition. In particular, pulses are among the edible plants with the highest protein content with an approximate number of 7.7g/0.5cup serving size and 17-30% of dry weight [36, 37].

Additionally, the high amounts of carbohydrates (55-65% of their total dry weight), minerals (e.g. iron, magnesium, potassium), insoluble and soluble fibers (approximately 7g/0.5cup serving) [38], results in recognizing pulses as a valuable nutritional dish for humanity. It should be clarified that the nutrient composition of pulses varies among the species, variety and seeding process used. Although pulses are protein rich, they are somewhat low in some essential amino acids (methionine, tryptophan, cysteine), but when combined with other animal or plant protein sources, they complement each other. Thus, for vegetarians, intake of a combination of different pulses is recommended to cover intake of all essential amino acids in the diet [39].

Legumes also comprise a pool of non-nutritional compounds which includes antinutrients and toxic factors. These compounds may interfere with digestion and uptake of other nutrients, cause micronutrient malnutrition and mineral deficiencies and thus influence metabolism [40]. Prominent antinutrients in legumes are phytic acid, saponins, a-galactosides, protease inhibitors, amylase inhibitors and lectins. The presence of these antinutrients can be drastically reduced by physical and biochemical methods such as dehulling, soaking, milling, cooking, fermentation and germination. Furthermore, legumes contain other phytochemicals such as flavonoids, other polyphenols, and phytosterols which can contribute beneficially to human health. Flavonoids have been shown to increase insulin secretion and reduce insulin resistance [41, 42], suggesting that these phytochemicals have positive outcomes against obesity and diabetes [43]. Polyphenols, can affect bodyweight by diminishing appetite and enhancing lipid metabolism [44] and also interact with

gastrointestinal microbiota in a bi-directional manner [45]. Through bacterial metabolism polyphenols are converted to more bioactive compounds that can affect gut ecology [46, 47]. Observational studies also highlight anticancer, antimicrobial and antioxidant properties of legumes [48-51].

Findings from systematic reviews and meta-analyses of prospective cohort studies provide a series of indications for legumes and diseases. In 2009 Aune et al., found an inverse association between legume intake and cancer risk. Particularly, populations consuming at least 100g of legumes weekly, demonstrated lower risk in manifesting colorectal, stomach and kidney cancer [52].

Later, Afshin et al., showed that legume consumption was related to reduced ischemic heart disease, but no significant associations were revealed for stroke and diabetes [53]. Similar results were obtained in 2017 and 2019 from Marventano [54] and Viguiliouk [55], respectively. Both evaluated the role of dietary patterns rich in legume and pulse proportions at coronary heart disease incidence and other cardiometabolic outcomes. Interesting are the results from two very recent randomized controlled trials. In 2021, Hafiz after examining the impact of pulses on long-term glycemic control in adults with and without type 2 diabetes, concluded that pulse consumption can improve glycemic control parameters [56], while data from Ferreira et al., suggested that intake of 150g pulses daily can benefit against cardiovascular risk factors, including blood lipid profile, glycemic control and inflammatory status [57].

1.2 The gastrointestinal system

The digestive system is composed of the gastrointestinal tract (GI-tract), a hollow tube starting from the mouth and ending at the anus with a length of 7-11 meters, and accessory organs which includes the pancreas, gall bladder and liver. Thus, teeth, tongue, pharynx, esophagus, stomach, small and large intestine, with the assistance of secreting enzymes and bile from salivary glands, pancreas, gall bladder and liver, contribute to the digestion and absorption of nutrients and water into the body. The human GI-tract, which is the biggest interface with our environment, spans around 32 m^2 surface area, of which 2 m^2 are ascribed to the large intestine [58]. Besides that, the removal of dead microorganisms, waste and undigested food through feces and the involvement on host's defense, as part of the immune system, are fundamental functions of the GI-tract.

1.2.1 Anatomy and physiology of the gut

The regional specialization along the GI-tract is attributed to differences in the tissue structure of intestines. The small intestine is separated into three parts; the duodenum in the proximal part, followed by jejunum and the distal region, ileum. Characteristic for the small intestine is the presence of villi and microvilli folds. These finger-like lumen formations increase the surface area and assist the uptake of nutrients together with several enzymes known as brush border enzymes. Ileum participates also in nutrient absorption but also with specific roles in reuptake of bile acids and uptake of vitamin B12.

The large intestine, separated from the small intestine by the ileocecal valve is the last part of GI-tract and consists of the appendix, cecum, colon, rectum and anus. With an average length of 1.5 meters, it is involved in water absorption, formation of stools removed by defecation and fermentation of indigestible food material by commensal bacteria. In contrast to the small intestine, colon is lacking villi and microvilli protrusions and contain only crypts [59].

Mucosa, submucosa, muscularis externa or propria and serosa are the four tissue layers found similarly in the gastrointestinal wall structure (**Figure 1**). From the luminal side, mucosa, the innermost tissue layer of the GI-tract, is a mucous membrane that comprises

epithelium and lamina propria. The former is a continuous sheet of different specialized epithelial cells whereas the latter is a thin layer of connective tissue mainly responsible for protection against microorganisms. Mucus secretion into the stomach and intestines is important because it acts as a shield against digestive enzymes, bacteria and also assists food transfer through lubrication. Submucosa is composed of connective tissue, a complex nerve plexus blood vessels and lymphatics. It supports mucosa by joining it to the third layer called muscularis externa which contain circular and longitudinal smooth muscle. Together with enteric neurons this layer is instrumental in regulating movements such as peristalsis and segmentation. The last layer is serosa, which is a smooth membrane coated by fluid from body cavities to avoiding mechanical damage between adjacent organs or surfaces [60].

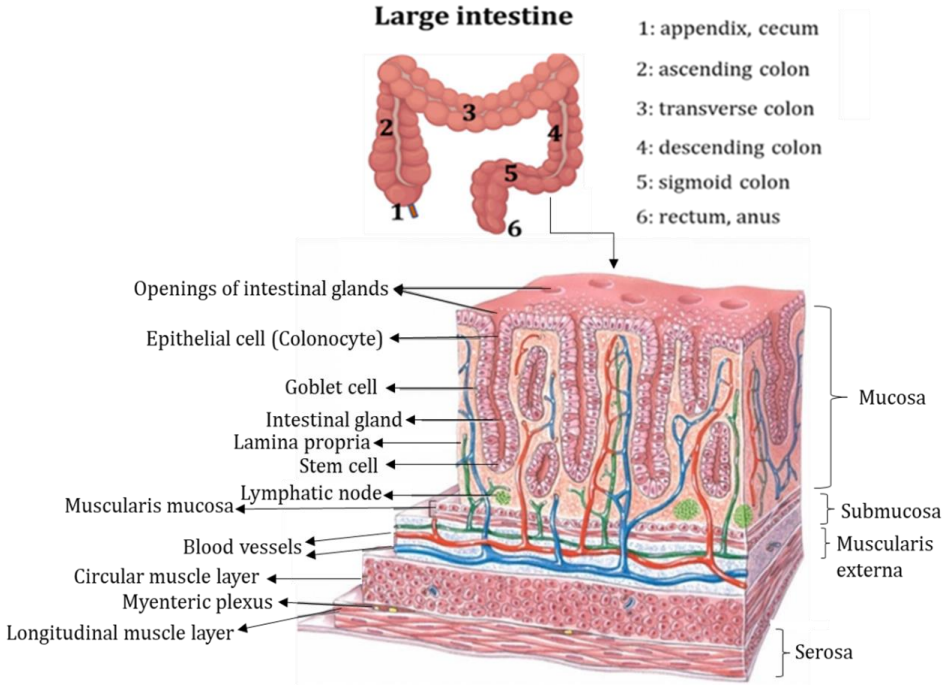

Figure 1: Three-dimensional view of the anatomy and structure at the large intestine. Picture adopted and edited from https://healthjade.com/.

Cells in the epithelium arise from stem cells situated in the crypts and apart from the absorptive-type intestinal epithelial cells (enterocytes in small intestine, colonocytes in colon), they give rise to secretory type cells including goblet, Paneth, enteroendocrine and tuft cells.

Goblet cells secrete mucus (a dense structure of glycoproteins known as mucins) for the formation of a mucus layer, which separates the epithelium content from the lumen and acts as a protective barrier for host's defense against pathogens and avoid commensal bacteria reaching the epithelium [61, 62]. These cells are present in higher numbers in colonic crypts than in small intestine and justifies why mucus thickness is enhanced at the colon with two distinct layers. Particularly in mice, the inner dense stratified mucus layer, which lines next to the epithelial cells is approximately 50 μm whereas the loose outer layer is approximately 100μm.

Paneth cells reside in the bottom of small intestinal crypts but are missing from a healthy colon. They are important for stem cell maintenance and production of anti-microbial peptides such as lysozyme, defensins, C-type lectin regenerating islet-derived protein IIIγ and defensins that contribute to the protection of epithelial barrier and maintenance of a balance with intestinal microbiota [63-65].

Enteroendocrine cells have a major role in the regulation of appetite, glucose levels by sensing the presence of nutrients in the small intestine and controlling the secretion of peptide hormones. So far, more than 10 subsets of enteroendocrine cells are known to exist between stomach and large intestine with different functions. The absorbed food nutrients affect the hormones secreted by those cells in small intestine, whereas the response of enteroendocrine cells in the large intestine is depended on microbial metabolites and products [66].

Tuft cells or brush cells along the GI-tract are a type of chemosensory cells with primary role to induce type 2 immune responses to parasites. Specifically, when parasites like intestinal helminths or protozoa are recognized in the intestine, tuft cells induce the secretion of a cytokine called interleukin 25, which causes the stimulation of lymphoid cells. From their

side, they produce another cytokine (interleukin 13) and as a result those unwelcomed microorganisms are faced effectively [67, 68].

M cells (microfold cells) are a special cell-type located within the epithelium overlying lymphoid tissues of the small intestine such as Peyer's patches and the large intestine. Their role is to take up particulate antigens including viruses and bacteria and deliver them to the underlying lymphoid follicles containing dendritic cells to initiate adaptive immune responses [69].

1.2.2 The gut immune system

The GI-tract constitutes an external mucosal surface that separates the outer environment from the inner body, made up of a single layer of epithelial cells body and is the biggest compartment for the immune system homeostasis. Since it is exposed to numerous and diverse microorganisms and environmental factors, it requires prompt protective mechanisms. Mechanical, chemical and microbiological barriers in the skin and mucosa areas are defensive mechanisms constantly ready to prevent the passage of microbes from the luminal content, which after crossing the epithelium can colonize tissues and cause infections. Thus, the constant effort of the host on a routine basis to eliminate harmful microorganisms, control the growth of commensal microorganisms and develop tolerance on foreign antigens like nutrients is quite challenging.

Starting from the stomach, the low pH attributed to parietal cell secreting HCl, is an effective chemical barrier to decrease the number of microorganisms entering the oral route into the intestine. Besides the mucus layer, tight junctions formed between adjacent intestinal epithelial cells restricts passage of pathogens into the lamina propria. The latter barrier can be weakened in case of inflammation or other disorders [70]. The mucosal immune system consists of regions with different functional properties known as inductive and effector sites. The cellular procedure for gut immune response is achieved by migration of immune cells from the inductive sites to the effector sites through the lymphatic system. Effector T cells and B cells achieve this by expressing surface proteins acting as address tags in order to "home" back to the mucosal effector sites [71].

The gut-associated lymphoid tissue (GALT), which is composed of lymphoid tissues and immune cells, has a crucial role for maintaining an adaptive immune response and protecting against pathogens and infections. The major inductive sites in the GALT are Peyer's patches and isolated lymphoid follicles. Peyer's patches are distinctive secondary lymphoid organs present in the small intestine (mainly in the ileum) that comprise T cells, B cells, dendritic cells (DCs), whereas isolated lymphoid follicles are dominant in the large intestine and consist B cells [72]. The effector sites are found in the lamina propria, the underlying connective tissue, in which macrophages, T cells, B cells and DCs migrate. Mesenteric lymph nodes are additional inductive sites of the intestine and is the place where food and microbial antigens are presented to naïve T cells. Their dual function is based upon their ability to tolerate food nutrients and to maintain homeostasis by preventing spread of microorganisms. Almost 70% of the total immune cells in human body are present in the GALT [73].

The first line of immune defense is the innate system that includes macrophages, eosinophils, neutrophils, stromal, innate lymphoid and DCs (**Figure 2**). A common feature of innate immune cells and intestinal epithelial cells is the expression of pattern recognition receptors (PRRs), which have the ability to recognize different molecular structures of the invaders known as pathogen associated molecular patterns (PAMPs) or microbial associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) on their apical or basolateral surfaces [74]. DNA, RNA and various compounds of the cell wall like the lipopolysaccharide of specific bacteria (Gram-negative) are examples of PAMPS/MAMPs, while Toll-like receptors (TLRs) and Nod-like receptors (NLRs) are the most well-studied PRRs and demonstrate structural and ligand similarities [75, 76].

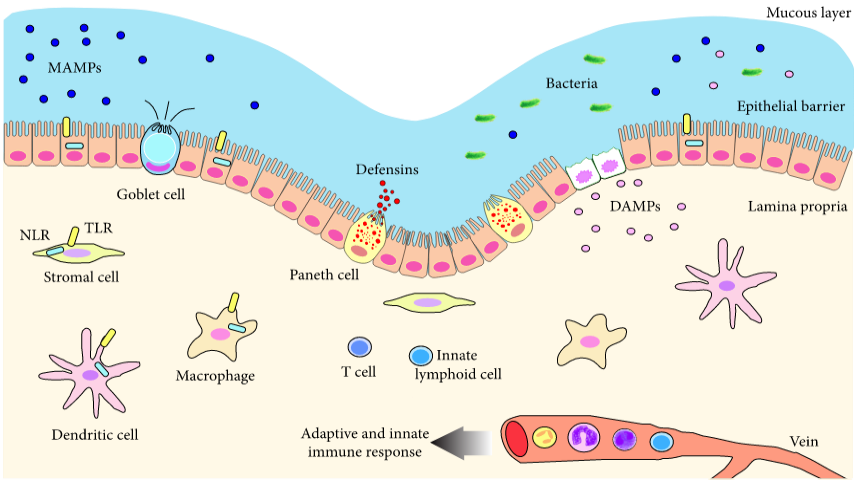

Figure 2: Adaptive and innate immune mechanisms in the intestine. Picture adopted from Elia et al, 2014 [74].

TLRs comprises 10 transmembrane proteins that are expressed on the surface or the inner side of immune and non-immune cells. Signaling of TLRs results in the activation of the transcription factor nuclear factor-kappa B (NF-κB), interferon regulatory factors or mitogen-activated protein kinases. Subsequently, those transcription factors regulate cytokines' expression (interleukins, chemokines, and interferons) and determine the result of innate immune responses [77]. Cytokines can act either in a synergistic or antagonistic manner and depending their role to trigger inflammatory response or repress the immune system are classified as pro-inflammatory (e.g., TNF-alpha, IL-1 beta) and anti-inflammatory (e.g., IL-10, IL-11, IL-13) cytokines respectively [78]. NLRs are a group of intracellular proteins (22 known members in humans, 34 in mice) that detect by-products due to tissue damage or other intracellular pathogens and regulate inflammation [79].

In contrast to the unspecific and rapid responses of the innate immune system, the activation of the adaptive immunity, is mediated by lymphocytes. The naïve forms of B and T cells depend on antigen presenting cells for proliferation and differentiation, in which DCs are most prominent. These cells present antigens to T helper cells (Th cells) at the inductive site and depending on whether the antigen poses a threat or is harmless, different subsets of Th cells are induced including Th1, Th2, Th17 or T regulatory cells (Tregs). Th cells are mainly involved in activating the immune system to evoke an immune response, whereas Tregs

suppress and control the immune response [80]. In mucosal tissues, immune tolerance against food antigens and commensals is the default state to prevent an overt immune reaction every time an antigen is presented to the immune system. DCs are important in this regard because they produce several metabolites including retinoic acid that can induce T cells to become Tregs. The contribution of Treg cells is pivotal, and largely dependent on the interaction with dendritic cells because they suppress immune response to self-antigens, harmless food antigens and commensals. This is a critical feature that ensures a tolerant state when no danger is present (immune tolerance) [81]. B cells are especially important for immune exclusion, by secreting large amounts of IgA. IgA's main role is to act in the gut lumen through binding to microbes and restrict access to the host (immune exclusion). In this process, IgA produced by B cells in the lamina propria is transported through the epithelia cell layer by transcytosis [82, 83]. In contrast to IgG and IgM, IgA does not activate the complement system, which plays a key role in defense against pathogens and in host homeostasis, and that is important for keeping immune responses in low rhythm or low activity and is considered anti-inflammatory.

1.2.3 Reactive oxygen species

Reactive oxygen species (ROS) are unstable active oxygen-derived compounds with short lifetime. They constitute essential effectors of the innate immune system, but in excess, ROS can cause damage to cells and are implicated in disease risk. Under normal physiological conditions, their endogenous presence in low or moderate amounts is important since they regulate signaling pathways. Mitochondria are the cellular organelles where most of the ROS are produced during cell respiration, but self-generated ROS is also prevalent in many cells that contain specific enzymes with the aim of producing ROS. Although generation and elimination rhythm is largely controlled by the host, an abnormal rise from various sources (**Figure 3**) can cause oxidative stress (inability to remove excess ROS), often involved in the pathogenesis of intestinal, cardiovascular and other diseases [84].

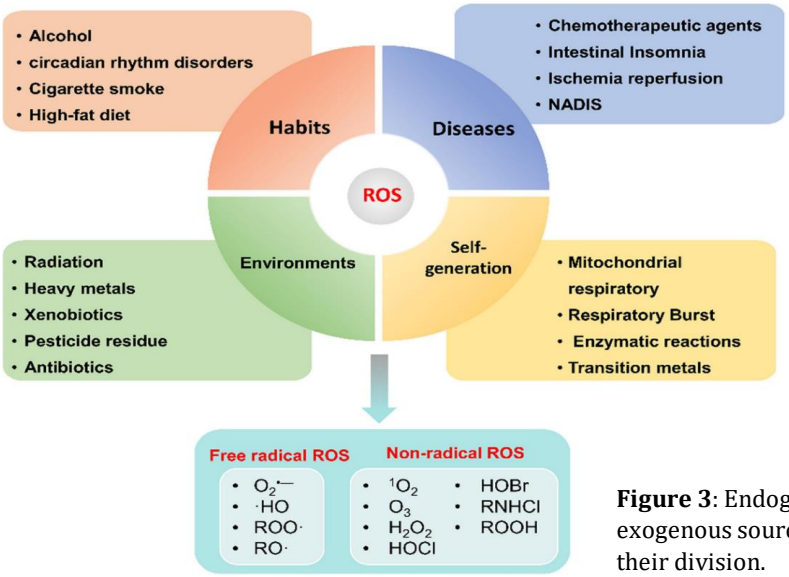

Figure 3: Endogenous & exogenous sources of ROS and their division.

Many of the ROS are free radicals such as superoxide ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) whereas hydrogen peroxide(H_2O_2) and hypochlorous acid (HOCl) are non-radical ROS. Similar to ROS is another type of highly active molecules named reactive nitrogen species (RNS) observed also in oxidative stress state. Nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($^{\bullet}NO_2$) and

peroxynitrite ($ONOO^-$) are members of that group. Particularly for $ONOO^-$ it is formed after the reaction of $NO^\bullet$ with $O_2^{\bullet-}$ occurs [85].

Apart from the mitochondrial electron transport chain, ROS/RNS production is achieved by phagocytic cells during respiratory burst and non-immune cells. Through NAPDH oxidase enzymes (NOX isoforms, DUOX isoforms) and NO synthases (NOS), macrophages, neutrophils and dendritic cells give rise to ROS/RNS. That process is an additional defensive mechanism of the immune system, which promotes homeostasis. However, abnormal levels of ROS/RNS are harmful since they are leading to impairments in basic components of cells and tissues. Mutations in the DNA, protein oxidation and lipid peroxidation are conditions attributed to high concentrations of those molecules [86].

In mammals five NOX (NOX1-5) and two DUOX isoforms are expressed in the GI tract but their abundance varies within the gut. NOX1 is expressed in intestinal epithelial cells, with highest expression in the ileum and colon, NOX2 in phagocytes in the lamina propria upon immune cell infiltration or in phagocytic cells residing in this area, whereas DUOX2 is found in all regions of intestine [87]. The composition of intestinal microbiota is a factor that affects expression and activity of NOX and DUOX isoforms [88]. In terms of NOS, it is expressed in the epithelial cells of the small intestine and is responsible for the production of $NO^\bullet$. Specifically, that enzyme converts L-arginine into $NO^\bullet$ and citrulline. Endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) are the three isoforms known. Under inflammation, microbes and their products or proinflammatory cytokines upregulate the *iNOS* gene and stimulate iNOS synthesis [89]. Generally, overexpression of ROS in the gut damages intestinal epithelial cells, affects the barrier integrity and is linked with the progression of gastrointestinal diseases [90]. Nevertheless, while IBDs and colon cancer are often outcomes due to intense ROS production, complete removal of them may also cause gastrointestinal problems [86]. Thus, maintaining a balance on ROS molecules is crucial for gut welfare.

1.3 The gut microbiota

The human GI-tract is estimated to host around $3.9x10^{13}$ bacteria [91]. This diverse community, which comprises mainly bacteria but also fungi, archaea, protozoa and viruses, is collectively referred as human gut microbiota or human microflora. While the number of

bacterial species found in the human gut exceeds 1000 different species, approximately only 160 species are found in fecal sample per individual [92]. The distribution of microbiota varies enormously between duodenum at the small intestine (10^3 bacteria/ml) and the large intestine (10^{12} bacteria/ml). Antimicrobial peptides, oxygen concentration, pH, thickness of the mucus layer have the ability to condition bacterial amount along the GI-tract [93].

Intestinal microbiota is actively involved in innate immunity by helping the immune cells to distinguish on what compounds present in the gut are unnecessary/harmful and which are needed. Synthesis of certain vitamins (vitamin B and K), production of short chain fatty acids (SCFAs) metabolites after anaerobic fermentation of non-digestible dietary fibers and metabolism of primary bile acids into secondary bile acids before their return to the liver are processes where bacteria are importantly involved. Moreover, bacterial metabolites have the potential to regulate appetite and enhance indirectly gut protection since their presence stimulates mucus secretion from the goblet cells. Apart from beneficial relationships with the host, known as symbiosis, some microorganisms, defined as pathogens, can induce negative consequences for the overall health. Dysbiosis is a frequently used term to describe rise of pathogens, loss of commensal bacteria with beneficial properties and reduced microbiota diversity in the gut [94-96].

Colonization of gut microbiota begins from the early stages of life by the transfer of microbes from the mother during birth and the local environment and is more or less stable after 2-3 years of age [97]. Microbiota shifts will nevertheless change throughout life and are contingent on various factors (**Figure 4**). Maternal microbiota, mother's lifestyle, and the way of delivery, vaginal or C-section, play a pivotal role to microbiota establishment. Breastfeeding is another important process for shaping microbiota in the infant gut. Breast milk is rich in probiotics (facultative and strict anaerobes bacteria) and prebiotics (e.g., human milk oligosaccharides). Disruptions in microbial colonization at the early stages of life may affect immune and metabolic pathways which can increase risk for diseases later in life [98]. Inflammation, stress, infections, antibiotic treatment and habits like alcohol, smoking and physical activity are additional factors that influence to a higher or a lower extent microbiota development [92, 99]. Last but not least, geographical location and ethnicity reveal divergences in the microbiota profile [100].

During the last two decades the main method for identification, quantification and classification of gut microbiota is by DNA based assays. Among them, 16S ribosomal RNA sequencing is the most common procedure because the 16S rRNA gene contains both conserved and variable regions, which allow the designing of suitable primers and the recognition between the bacteria taxa. The taxonomic system for bacteria has the following descending order: Phylum, Class, Order, Family, Genus, Species. The five most dominant phyla in the intestine are Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Verrucomicrobia, with Firmicutes and Bacteroidetes comprising 80% of all gut bacteria [101]. In addition to classification of taxa, evaluating microbial diversity through alpha and beta diversity is also important to better distinguish bacterial structure and profiles. Alpha diversity refers to the variation within one sample whereas beta diversity refers to the variation between samples [95, 102].

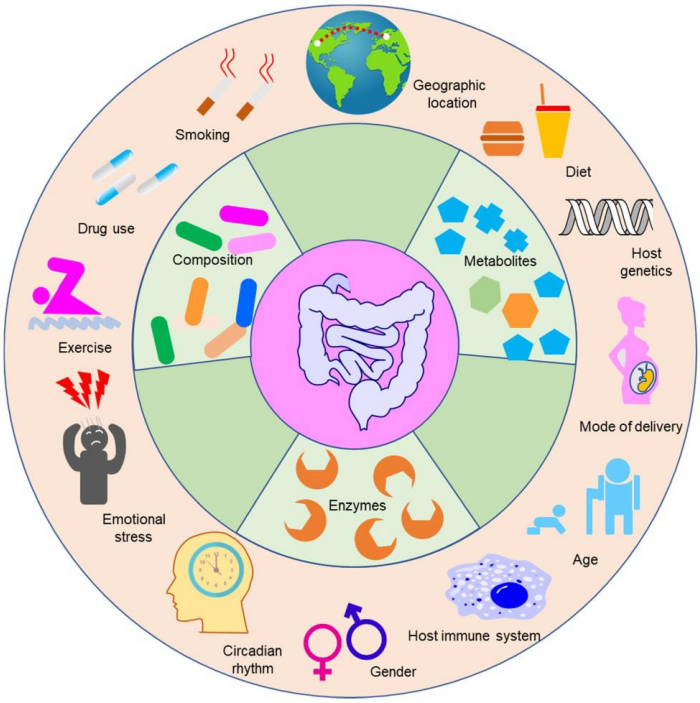

Figure 4: Environmental and genetical factors that affect gut microbiota composition. Adopted from Feng et al., 2020 [103].

1.3.1 Gut microbiota, health and diets

Over the last decades, researchers have demonstrated a growing interest in microbial diversity because of the consensus that gut microbiota has a key role for host's welfare. Microbiota studies with animals provide advantages and therefore more comprehensive knowledge in that field compared to outcomes from human studies, due to limitations. The ability to utilize not only stools but also tissues samples, animals without bacterial flora (germ-free animals), the human interference with a specific strain or even combinations of bacteria and the controlled experimental conditions assist researchers to understand better different aspects of microbiota on intestinal health.

Despite the flexibility given by animal studies and the potential interesting findings obtained, conclusions must be drawn carefully when translating animal data for human's benefit. Regardless the gut microbiome similarities between humans and experimental animals, that dynamic community can easily alter by external factors. Focusing on the mouse intestinal microbiota, in 2018 Ericsson and his group proved that laboratory equipment and materials such as cages and beddings, often not considered by many researchers, are able to influence microbiota [104]. Earlier, another relevant study from Jakobsson et al., showed that mice housed in the same facility had different microbiota which affected barrier structures such as the mucus phenotype [105].

As mentioned above, diet constitutes a major determinant of microbiota, since dietary changes promote the overgrowth of some species over others and consequently influence host's metabolism and immune system. It has therefore been suggested that nutrition can affect gut microbiota composition up to 57% whereas host genes account for less than 12% [106].

So far, many reviews highlight the causative effect of the dietary pattern on gut microbes [99, 107]. Human studies reveal the ability of animal and plant-based diets to modify the gut microbiota composition within 24 hours [108, 109]. Lawrence and his colleagues demonstrated that short-term consumption of an animal diet was responsible for the rise in bile-tolerant bacteria (*Alistipes*, *Bacteroides*) and simultaneously the decline of Firmicutes-belonging bacteria that metabolize plant polysaccharides (*Roseburia*, *Eubacterium rectale*)

[109]. Also, another study revealed the dominance of *Bacteroides* and *Prevotella* genera in humans consuming animal proteins and plant diets respectively [110]. Moreover, populations with frequent consumption of starchy carbohydrates are associated with high levels of *Bifidobacterium* genus [111], while preference for WDs boost *Bacteroides* and *Bilophila* [112].

Insights from animal studies, where HFDs were investigated extensively, suggest that mice fed with abundant amounts of saturated fats are characterized by an elevated Firmicutes/ Bacteroidetes ratio (F/B ratio) [113-115] and a significant drop of the Actinobacteria phylum, which is actively involved in obesity maintenance [116]. Findings from Cani and Delzenne revealed that diets rich in fat caused a marked reduction of Bifidobacteria species [117] and notable is the fact that SCFAs production is decreasing under a HFD. Furthermore, animals following diets with increased fat content presented high relative abundance of Proteobacteria [118, 119]. Several studies demonstrated the significant rise of species included in that phylum (*Desulfovibrio*, *Escherichia*, *Shigella*), which produce endotoxins, release proinflammatory cytokines and act as opportunistic pathogenic bacteria. Normally a healthy human gut encompasses only minor amounts of Proteobacteria. Therefore, in cases where increased proportions are observed, they are proposed as a microbial marker of dysbiosis and linked to human diseases [120, 121]. Lastly from Verrucomicrobia phylum, *Akkermansia muciniphila*, a mucin-degrading bacterium with a relative abundance of around 4% in the gut of healthy subjects, has gained attention [122, 123]. That genus was decreased in HFD-fed mice [124] and both in mice [124] and humans [125] there is an inverse correlation with body weight.

Many studies have demonstrated that animals fed a HFD with lard lead to an increased F/B ratio, whereas the seemingly opposite outcome is observed in humans consuming animal-based diets rich in fat and proteins (low F/B ratio) [126]. Intriguingly this can be attributed to differences in fat source between animal and human studies. This was demonstrated by Devkota et al., who conducted a mouse experiment with a HFD (lard), a HFD (milk). While, HFD (lard) revealed an increased F/B ratio, mice fed HFD with milk had a reduction in Firmicutes and an increase of Bacteroidetes compared to LFD-fed mice [127].

Mediterranean and middle east diets, which are typically high in fibers from fruits, vegetables, legumes and grains, can rapidly shift microbiota in a positive manner. Indicatively, a study from 2016 revealed that Mediterranean diet was associated with a higher abundance of Bacteroidetes, Prevotellaceae and *Prevotella*, while the concentration of Firmicutes and Lachnospiraceae was lower [128]. However, another study conducted by De Filippo in children from Africa showed an increase of Bacteroidetes and a decline of Proteobacteria and Firmicutes compared to children from Europe [129]. The outcome was attributed to their rural diet, which is abundant in fibers, starch and other carbohydrates, while poor in animal protein and fats. Thus, it becomes evident the complex relationship between diet and microbiota.

In a plethora of mouse studies with legumes and pulses, researchers point out the increased microbial diversity and the elevated abundance of SCFA-producing genera (*Roseburia*, *Prevotella*, *Dorea*), Bifidobacteria strains (able to reduce production of proinflammatory cytokines) and *Ruminococcus flavefaciens* (usually reduced relative abundance in overweight subjects). Collectively, these plant-based diets promote the dominance of the above-mentioned bacteria, which from their side benefit intestinal epithelial cells in order to thrive, maintain barrier integrity and ultimately protect the host from pathological conditions [130-133].

1.3.2 Relationships between gut microbiota and inflammation

In broad terms, inflammation is body's response triggered by a noxious stimulus, responsible for causing tissue injury, with ultimate aim to repair the damage and prevent the spread of an infection. As part of the defensive system, that dynamic procedure, which restores tissue's integrity and function, is important for maintaining homeostasis. Various factors can affect the advent of inflammation including infective pathogens (viruses, bacteria), non-infectious conditions (chemicals, damaged cells, radiation, burn, physical injury) and autoimmune diseases (inflammatory bowel disease, celiac disease, type-1 diabetes, etc.). In the latter case, the immune system is mistakenly activated against host's tissues or cells, because they are recognized as foreign or abnormal. The etiology for an inflammatory response is multifactorial but the symptoms that usually accompany an inflamed tissue are redness, swelling, warmth and pain.

A major discrimination that should be clarified is systemic inflammation in nonmucosal tissues versus inflammation in mucosal tissues. Briefly the main features of the former inflammation in the affected tissue are activation of macrophages that secrete inflammatory cytokines, recruitment of innate immune cells from the blood to that tissue, migration of dendritic cells to secondary lymphoid tissues to initiate adaptive immune responses. From these responses, effector T cells and antibodies are transferred the infected tissue until infection is terminated. On the contrary, in nonmucosal tissues that interact sporadically with commensal microorganisms, the strategy applied differs. In the gut any disruption of the epithelial layer can lead to influx of bacteria from the lumen and thus infection. In that case a rapid adaptive response occurs in order recruit memory and effector immune cells. A feature of the mucosal immune system is the effort to prevent inflammation because it causes tissue damage and gut inflammation, which is often linked with chronic diseases, can worsen infection instead of solving the problem.

Gut microbiota is acknowledged as a key driver for inflammation since it interacts with pathways and performs different actions depending on the individual's profile. NF-κB pathway is a characteristic example that has an active role in the expression of many inflammation-related genes [134]. During inflammation immune cells secrete plenty signaling molecules with various functions called cytokines. The main classification for those molecules based on their properties is pro-inflammatory and anti-inflammatory [135]. In the context of that thesis TNFa, IL-1b and IL-6 were the pro-inflammatory cytokines studied. Increased levels of TNFa are linked to disorders of the metabolic syndrome [136] and often attributed to microbiota. In 2007 a study revealed that humans with higher relative abundance of a *Bifidobacterium* strain (probiotic genus) had lower levels of TNFa [137]. IL-1b is another molecule released from intestinal myeloid cells (macrophages, monocytes) and a versatile mediator for inflammation. Although its function is important for host-responses against infections and injuries, elevated levels are apparent in chronic diseases [138]. Although limited are the insights about the role of IL-1a and IL-1b in colitis-models, recently Menghini et al., demonstrated that neutralization of IL-1a reduces intestinal symptoms by causing modifications of gut microbiota [139]. IL-6, which is secreted from macrophages and is associated with inflammation and type-2 diabetes [140], possess a role in intestinal

microbial formation. *Faecalibacterium* is inversely related with IL-6 production and that could be explained by its ability to produce butyrate, a SCFA capable of inhibiting NF-κB pathway [141]. Lastly, a human study in obese and overweight adults showed a correlation of IL-6 with *Lactobacillus* strains [142].

Lipopolysaccharide (LPS) is a component of the cell wall of gram-negative bacteria. When the intestinal barrier is damaged, due to diet, pathogens, chemicals etc., that endotoxin can pass from the intestine layers to the blood circulation leading to what is commonly referred as leaky gut. High concentration of LPS in the serum or plasma is observed in subjects suffering from metabolic diseases [143]. Also, LPS can bind to a particular toll-like receptor (TLR4) expressed on cells of the immune system and induce a cascade of inflammatory reactions in the intestine and other areas. Rodent studies with *Lactobacillus*, also belonging to probiotics, reduced the levels and LPS effects, therefore proposing an association [144].

1.4 Types of inflammation, IBDs and detection methods

Different parameters, like the duration, the degree and the region (local or systemic) of inflammation, define the inflammatory response type and influence the immune reactions at molecular and cellular level. The most common classification for inflammation is acute or chronic. Normally during acute inflammation, harmful compounds are faced rapidly and efficiently in a controlled manner by the immune system, without creating a risk for impending events (injuries, infections). That type of inflammation is beneficial because it resolves the problem and promotes tissue recovery.

On the other side, the body's inability to eliminate a threat, frequently gives rise to a prolonged inflammatory state, known as chronic inflammation. However, the constant evoke of immune cells from the bloodstream can damage and destroy healthy tissues. In addition to microbes, the modern way of life with a variety of features such as diet, physical inactivity, smoking, stress and age may turn an uncontrolled acute inflammation into a chronic inflammation, usually linked with inflammatory bowel diseases, type-2 diabetes mellitus, obesity, fatty liver disease and cancer.

Regardless the severity and the location of inflammation, the mechanism characterizing an inflammatory response briefly contains the following steps: i) recognition of the stimulus from innate immune sensors, ii) activation of inflammatory pathways, iii) release of inflammatory markers, iv) recruitment of immune and non-specific immune cells.

When inflammation rises in the intestine, two forms of IBDs, Crohn's disease (CD) and ulcerative colitis (UC) can occur. The general feature for IBDs is that the intestine is experiencing relapsing inflammation due to uncontrolled activation of the mucosal immune system[145]. The main difference is that CD has a patchy appearance affecting both the small intestine (most often in the ileum) and colon, while ulcerative colitis is a continuous inflammation of the colon. Common symptoms for both diseases are diarrhea, rectal bleeding, abdominal pain, fatigue and weight loss. The molecular mechanisms remain elusive, however in the pathogenesis of IBDs genetic and environmental factors can affect host's susceptibility [146]. For example, mutations in the *NOD2* gene, which result in defective responses of the immune system, are observed in patients suffering from CD [147]. Diet and lifestyle are considered factors for the development of intestinal pathologies and

together with their cross talk with commensal bacteria flora can modify both risk and severity of IBDs [148-150]. The role of microbiota is highly important since decreased microbial diversity, termed often as dysbiosis, is apparent in individuals with IBDs [151, 152]. Importantly, the presence of microbes in the gut is necessary for developing IBD as germ-free mice are resistant towards this disease, due to the absence of antigenic stimuli for activating the mucosal immune system [153].

Endoscopy is the gold standard method to monitor the disease activity of IBDs, but one of the major focuses in medical fields is the identification of non-invasive, sensitive, simple, cheap and effective biomarkers [154]. In the literature several potential biological markers for studying intestinal inflammation are available either from fecal material or blood samples, which are easy to obtain and process for the diagnosis. Although the degree of reliability varies among them, it is likely that fecal biomarkers (e.g., neutrophil gelatinase B-associated lipocalin, calprotectin, neutrophil myeloperoxidase) may reflect better local inflammation in the gut compared to serum markers (like C-reactive protein, α1-acid glycoprotein, β2-microglobulin and sialic acid). Potential explanation is the fact that feces have direct contact with mucosal areas and signals detected in blood may be higher attributed to systemic inflammatory response[155].

Lipocalin-2 (LCN2) or neutrophil gelatinase-associated lipocalin (NGAL), the second name stands also for the protein human analog, is a promising and widely used stool biomarker for IBDs due to its high sensitivity. Elevated concentration of that innate immune protein has been identified in feces during infections and other intestinal pathologies [156]. LCN2 is produced from intestinal epithelial cells and immune cells such as neutrophils and macrophages. It deactivates macrophages and induce apoptosis of immune cells, in order to terminate systemic hyper-inflammation [157]. Apart from these functions, LCN2 is participating in iron homeostasis. Specifically, it inhibits iron uptake from gut bacteria and thus it prevents their excessive development [158].

Intestinal inflammation and impairments in the gut integrity are often linked with bacterial translocation. Due to increased permeability toxins like LPS and peptidoglycan trigger the rise of proinflammatory signals, which may lead to chronic diseases. A suitable biomarker for measuring indirectly LPS is the lipopolysaccharide binding protein (LBP)[159]. That

protein is mainly secreted by hepatocytes and its concentration is depending on the circulatory LPS levels. Studies have shown that people suffering from type 2 diabetes mellitus and obesity manifest elevated levels of LBP [160].

1.4.1 Mouse models of intestinal inflammation

For exploring mechanisms involved on intestinal inflammation and thus the pathogenesis of IBDs, different mouse models are utilized depending on the research interest (**Table 2**). In the context of the current thesis, DSS was the option for inducing acute colonic inflammation.

Models for IBDs	Achieved by	Region	Type of Response
Chemically Induced	DSS	Colon	Epithelial damage
	TNBS		Epithelial damage Immune-mediated
	Oxazolone		Epithelial damage Immune-mediated
Spontaneous Mutation	C3H/HeJBir		Immune-mediated
Adoptive T Cell Transfer	$CD4^{+}CD45RB^{hi}$		Immune-mediated
Genetically Engineered	IL-10 knockout mice		Immune-mediated
Microbiome Induced	Germ-free IL-10 knockout mice with/without microbial transfer		Immune-mediated

Figure 4: Common experimental mouse models for investigating intestinal inflammation.

The DSS model

It was 1990 when Okayasu et al., administered a derivative from dextran, known as dextran sodium sulfate (DSS), in mice through drinking water and tried to understand the role of immune cells in an acute and chronic ulcerative colitis state. DSS is a synthetic sulfated branched polysaccharide, negatively charged and water soluble. It has the ability to disrupt the epithelial barrier, allowing the passage of microorganisms into the mucosa and mimics enterocolitis disease [161]. Additionally, the activation of immune cells has a crucial role in mucosal inflammation. Macrophages, which are apparent in DSS colitis, give rise to proinflammatory cytokines and other cytokines that have a role in barrier function [162]. In addition, tissue damage can be aggravated by the presence of neutrophils [163]. Among the advantages it offers as a chemical of preference compared to others, is that experiments are carried out in a simple, quick, easily reproducible and controllable manner.

The induction of epithelial damage and thus inflammation is attributed to its ability to form nano-lipocomplexes with medium-chain-length fatty acids (MCFAs) in the colon [164]. However, while MCFAs and long chain fatty acids long chain fatty acids promote inflammation, the opposite happens with SCFAs such as butyrate, propionate and acetate.

These fatty acids have the property to attenuate inflammation by influencing immune cell differentiation and gut microbiota profile [165]. The doses and the treatment cycles of DSS applied in each experimental model allows the study of different forms of intestinal inflammation.

The TNBS and oxazolone model

2,4,6-trinitro benzene sulfonic acid (TNBS) is a chemical used to investigate intestinal inflammation and provides many similarities with Crohn's disease. Intrarectal administration of that chemical to mice causes colitis recognized by immune cell infiltration in the lamina propria together with diarrhea and weight loss. On the other hand, oxazolone is a chemical that elicits inflammation in the colon but in a different manner compared to TNBS and shares many properties not with Crohn's diseases but with ulcerative colitis. In ulcerative colitis increased production of IL-13 and IL-9 is noticed and lamina propria contains high amounts of natural killer T-cells. Both those chemicals are considered to behave as haptens because they bind to endogenous proteins in the colonic mucosa and induce an immune response through macrophage and T cell activation [163].

IL-10 knockout mice

Interleukin-10 (IL-10) is an important anti-inflammatory cytokine in shaping mucosal immune responses in the intestine. This cytokine is produced by regulatory T cells, epithelial cells, macrophage and dendritic cells and is characterized by significant properties, such as stimulation of B-cell differentiation, immunoglobulin secretion and suppression of macrophage activation to inhibit inflammatory cytokines production. IL-10 is a good therapeutic candidate against IBD due to its immunosuppressive activity and central role in downregulating inflammatory cascades. Genetically engineered mice lacking expression of IL-10 gene are widely used due to the fact they develop spontaneous inflammation in the colon and they increased cell infiltration there [163]. Experimentally, Leoni et al., and Treton et al., used IL-10-KO mice to study ROS production by NOX1 during colitis induced by DSS or TNBS because they supported that only NOX1 deficiency was not able to produce a phenotype that represents colitis [166, 167]. After crossing IL-10-KO with NOX1-KO mice,

development of spontaneous colitis was visible to mice and that mimicked patients suffering from UC and CD.

1.5 Rodents as animal models for human gut diseases

In biomedical studies, animal utilization is an indispensable tool for studying systemically several pathological conditions. Nematodes, *Drosophila*, Zebrafish, rodents, rabbits, pigs are some of the available options with advantages and limitations.

Murine models provide a variety of advantageous features, including their small size, the low cost of maintenance and the high reproductive rate compared to other animals. Another rational reason for why researchers show a preference on mice is the available knowledge over genetics. Two decades have passed since the whole mouse-genome sequencing completed (December 2002), allowing the application of techniques, such as gene editing or silencing. Gene interference and the creation of transgenic and knockout mice, assist scientists to study human diseases and disorders in a more comprehensive way. Modifications of genetic lines can lead to new phenotypes, which potentially provide answers on how the immune system regulation affects various aspects of gut inflammation. CB7BL/6 mouse belongs to the common mouse species (*Mus musculus*) and is the most widely used strain in the lab. The fact that those mice are inbred, thus characterized by the same genotypic background, make them a good experimental option for investigating the role of genetics in the disease occurrence [168]. Currently over 400 inbred strains of mice are available for research purposes [169].

Focusing on gut research, the overall anatomy of the GI-tract, the composition of sectional tissue in the small and the large intestine, the presence of similar cells along the intestine are important parameters closely shared between humans and mice. Nevertheless, despite the many common similarities, prominent differences are also found between the species, like the cecum size, the distribution of Paneth and Goblet cells and appendix presence.

Several are the advantages of using mouse models in gut health and its microbiota. Firstly, the small size of mice together with. The fact that it allows scientists to conduct interventions that would not be easy or manageable in humans with the aim to explore effect of diet or microbiota in a healthy or unhealthy state. As already mentioned, the extensive knowledge

of mouse genetics and the plethora of mouse models available that allow either gene editing or gene silencing are benefits provided by them [168].

On the other side, despite the common features shared in terms of genes, physiology, immunity and intestinal function, the use of mouse models comprises limitations and potential drawbacks in translating the knowledge acquired from mice to humans. A notable example is the lesions found in patients suffering from IBDs that are not the same with the lesions observed in mice after DSS administration or any other chemical that induces intestinal inflammation [170]. Besides that, in contrast to humans, mice are coprophagic animals meaning that this behavioral pattern may affect intestinal health through changes in nutrient balance and shifts in intestinal microbiota population [171]. Particularly for intestinal microbiota, studies have revealed significant differences at genera level. The abundance of *Akkermansia, Alistipes and Lactobacillus* varied among mouse strains such as C57BL/6 and BALB/c [172, 173]. As a result, the interpretations may be inaccurate and provide wrong conclusions.

2. Research objectives

The overall objectives of this thesis were to understand the impact of NOX1 induced ROS for colon health state in mice and study the interaction effect between diets and bacteria during chemically induced low-grade intestinal inflammation.

Therefore, the main aims were:

I To establish a mouse model for low-grade inflammation using a low dose of DSS and evaluate the role of NOX1 induced ROS in the colon, both in the steady state (without inflammation) and during low-grade inflammation, measuring biomarkers of intestinal inflammation and effects on the microbiota (**Paper I**).

II To compare the impact of high fat; WD, versus low fat intake; LFD, on colon and its microbiota prior and during low-grade inflammation induced by 1% DSS and investigate the impact of different fat sources (**Paper II**)

III To evaluate a potential beneficial role of adding protein and fiber fractions from faba beans as part of a WD, by examining the effects of the different macronutrient compositions on the inflammatory responses and changes in the microbiota profile (**Paper III**)

3. Summary of the papers

Paper I

The goal of paper I was to establish a colonic low-grade inflammation mouse model to study the role of the *Nox1* gene for ROS production and inflammation. First, different doses of DSS (0.5%, 1%, 2%, 4%) were given in drinking water to find the optimal dose, where mice revealed none or minor visible signs of disease, while having a moderate up-regulation of inflammation-related genes in the colon. After concluding that 1% of DSS for six days was an adequate concentration to induce colonic low-grade inflammation, we assessed the role of *NOX1* gene to wild type (WT) and NOX1-KO mice. A disease activity index (DAI) score was used to grade colonic inflammation after DSS exposure, based on improper body weight gain, stool quality, activity level and hunched posture. *Ex vivo* imaging showed that NOX1-KO mice, both DSS treated and untreated, manifested lower levels of peroxynitrite than WT, confirming that *NOX1* is important for ROS production. Expression of inflammatory genes (*TNF-a*, *IL-1b*, *IL-6*, *Ptgs2*, *Lcn-2*) were up-regulated in both genotypes during DSS treatment. *TNF-a* and *IL-6* expression were higher in NOX1-KO mice than WT mice. Also, analysis of LCN-2 protein in fecal samples revealed an increase in NOX1-KO mice after DSS exposure but no histological differences in colon were observed between NOX1 KO mice and WT mice treated with DSS. The effect of NOX1 in colonic microbiota composition was visible by assessing alpha diversity and beta diversity between WT and NOX1-KO mice. Generally, prior to DSS, there was a decrease in the alpha diversity of NOX1-KO mice compared to WT mice whereas the beta diversity of fecal samples analyses demonstrated that all groups were statistically different from each other. Further analysis at phylum level, indicated an increased *Firmicutes*/*Bacteroidetes* ratio in NOX1-KO untreated mice, whereas at genus level, the absence of *Nox1* gene led to an increase of genera related to inflammation.

To sum up, the role of NOX1 in the colon in NOX1-deficient and wild type mice, under mild and subclinical low-grade inflammation was investigated and presence of the *Nox1* gene was found to be important for keeping the gut healthy. However, NOX1 only marginally protected against the severity of inflammation which indicate the involvement of compensatory mechanisms.

Paper II

Effects of two matched purified diets, a LFD and a WD were compared, in a chemically induced low-grade inflammation model established earlier (**PAPER I**). The main finding was that WD fed mice were less prone to DSS as manifested by decreased degree of inflammation and minor microbiota changes compared to the LFD fed mice treated with DSS. Genes related to proinflammatory cytokines (*TNF-a, IL-1b, IL-6, Ptgs2*), ROS (*iNOS, Nox1, Nox2*) and barrier function (*ZO-1*, *occludin*) were up-regulated significantly in LFD fed mice with DSS in their drinking water. In addition, DAI score and biomarkers assessed for intestinal inflammation (LCN-2, LBP) were significantly increased in LFD fed mice after DSS treatment. Furthermore, 16S rRNA sequencing revealed less fecal microbial diversity and less pathogenic-related bacteria belonging to Proteobacteria phylum in the LFD fed mice compared with WD fed mice leading us to hypothesize that the milk-fat content in the WD could protect against DSS-induced colitis. That impelled us to carry out two short additional experiments to compare the ability of different fat sources to protect against DSS-induced colitis. Initially, we compared WD with milk fat to that of lard, as well as LFD. Both fat sources protected equally well against DSS-induced intestinal damage based on DAI score and proinflammatory genes. LFD were again more severely affected. In order to reveal whether the fat in the WD protects against the damaging effect of DSS, we carried out a second follow-up study, where the diet in the LFD fed mice was switched one day before 1% DSS was provided. The exposure to WD immediately prior to DSS treatment protected the mice against the adverse effects of DSS.

In overall, the high fat proportion in the WD, both milk fat and lard (41% of energy content) protected against chemically (1% DSS) induced low-grade inflammation as compared to LFD fed mice. However, the microbiota was less diverse in the WD fed mice compared to LFD fed mice, from phyla level to genus level. Future trials should investigate whether the fat intake induces protective mechanisms against DSS disease development or inhibits the establishment of the DSS-induced colitis model.

Paper III

The goal of paper III was to investigate whether adding the protein fraction (PF) and/or fiber fraction (FF) of faba beans to the mice diets would protect against the adverse effects of a WD. First, mice were fed a WD for seven weeks with the intention to cause symptoms of the metabolic syndrome (obesity, glucose intolerance/insulin resistance), before dividing the mice into four diet groups: 1) WD, 2) WD+PF, 3) WD+FF, 4) WD+BF (both fractions). To ensure that modified diets were equal in energy content, 30% of the casein was substituted by PF and 7% of the cellulose was replaced by FF. The mice with added faba bean protein content (WD+PF and WD+BF) had a higher increase in body weight compared to the pure WD or the WD+FF. There was no difference observed between the diets for glucose regulation. The L-012 signal from peroxynitrite production in the intestine, an indicator of ROS production (unpublished data), was very low in mice consuming protein, fiber or both faba bea fractions. After inducing low-grade inflammation using 1% DSS for six days, no differences were revealed between the diet groups with regards to gene expression, biomarkers of gut leakage and inflammation (LCN-2, LBP). On the contrary, in both the WD+PF or WD+FF fed mice we found a slight but not significant increase of *TNF-a*, *Ptgs2* and *NOX2*. In terms of microbiota analysis among the DSS treated groups, alpha diversity showed a significant difference only between WD and WD+PF fed mice. Futhermore, the DSS treated mice following a pure WD had a tendency for a higher relative abundance of Actinobacteria and Proteobacteria.

In summary, enrichment of WDs with protein and fiber faba bean fractions respectively, led to no significant differences on colonic health, indicators of metabolic syndrome or inflammation but marginally modulated the intestinal microbiota.

4. Discussion

The presence of ROS is imperative for the proper function of the immune system and the maintenance of a steady state for the host. Aging, cancer and cardiovascular problems are pathologies associated with irregular levels of ROS production [174]. Focusing on gut health, ROS have been given considerable attention because apart from constituting harmful byproducts, they are crucial regulators for gut homeostasis. In paper I we studied the way a highly expressed in colonocytes ROS-generating enzyme, NOX1, was involved in modulating colonic microbiota and how it affected colon in a normal and in a low-grade inflammation state induced by 1% DSS. Earlier studies from our group in mice have shown that peroxynitrite, a highly reactive nitrogen intermediary formed when nitric oxide (from *iNOS*) and superoxide (from *Nox1, Nox2*) react, is regulating microbiota in the small intestine [175]. The reason for applying 1% DSS in our model was that it represents a condition many individuals are experiencing through lifespan without manifesting serious symptoms. In other words, it causes a mild irritation in the colon due to various genetical and environmental factors. However, even if the outcomes from an animal model tries to mimic human's intestinal disturbance, interpretation should be done critically and carefully. It is frequently discussed the dissimilar outcomes obtained among the use of different chemicals and mouse strains [161, 176], raising the issue of experimental reproducibility.

Herein, *ex vivo* imaging revealed that NOX1-KO mice with and without DSS, had lower level of ROS/RNS signal than in WT mice, confirming the *NOX1* dependency for peroxynitrite production. Regardless the genotype, expression of *iNOS* significantly increased in DSS treated mice compared to untreated mice. The ROS production in NOX1-KO mice treated with 1% DSS may be attribute to other ROS sources forming peroxynitrite such as NOX3 [177] and myeloperoxidase enzymes [178]. In terms of inflammation, although prior DSS treatment no significant differences were revealed between NOX1-KO and WT mice, higher expression of proinflammatory genes was observed in the knockout mice when DSS was administrated. Additionally, analysis of a sensitive inflammatory biomarker, LCN-2 protein, in fecal samples showed a modest rise in the NOX1-KO mice given DSS but not in the WT with DSS. That result contradicts Makhezer's findings, where *Lcn-2* expression in colonocytes is partly dependent on *Nox1* gene [179]. However, one explanation of our outcome can be the

fact that bacteria affect LCN-2 production [158] and DSS increases bacterial exposure due to colon tissue damage [180]. Thus, the increased permeability of the mucus layer allows the passage of bacterial populations. Another explanation could be the state of a study that different LCN-2 levels are associated with the initial microbiota present before DSS treatment [181].

Indeed, microbiota comparisons of both fecal and colon tissue samples demonstrated a decrease in alpha diversity of NOX1-KO mice. For beta diversity fecal samples were statistically different in all groups. These data point out the influence of NOX gene in microbiota population. At the phylum level, the ratio of the Firmicutes/Bacteroidetes was increased in NOX1-KO mice, as it happens with chronic low-grade inflammation induced by obesity [182]. Also, Verrucomicrobia phylum, with *Akkermansia* as representative in our results, had an elevated abundance in NOX1 KO mice than WT prior to DSS, whereas after treatment the difference was not visible because there was a bloom in WT mice. The significance of that genus remains a topic of discussion although references provide a positive impact on intestinal homeostasis [122, 183, 184]. Considering that NOX1-KO mice have an increased number of goblet cells and as a result more mucus secretion [185], the high relative abundance in untreated NOX1-KO mice could be an argument. The harmful effects of DSS on the mucus layer [180] may justify the reason for rising *Akkermansia* in WT mice. Nevertheless, it remains vague why *Akkermansia* in NOX1-KO mice is unaffected by DSS. Another interesting observation was that NOX1 absence induced shifts in microbiota. Reduction of bacteria related to barrier function stability and anti-inflammatory response[186, 187] in NOX1-KO mice and simultaneously increase of genera linked to inflammation and diseases [188, 189] were observations worth to be mentioned. Finally, it could be said that the bacterial profile of NOX1-KO mice had a closer resemblance to mice treated with 1% DSS.

Generally, despite the microbiota shifts in NOX1-KO mice towards a more dysbiotic profile, no pathological changes were manifested. Observations from different assays, support an enhanced inflammatory response and suggest a meaningful function for *NOX1* gene in intestinal welfare.

In paper II we observed that mice following a WD (41% fat in energy content) were less affected by DSS compared with mice fed a LFD (10% fat). Quite surprisingly, LFD-fed mice displayed a significantly higher degree of inflammation and a more dysbiotic microbiota profile in response to the DSS treatment compared to the WD fed mice. In paper I we also used 1%DSS in mice fed a standard unrefined low-fat diet (chow), however these mice did not experience severe symptoms of colonic inflammation compared to mice fed the synthetic low-fat diet (LFD) demonstrated in paper II. The main difference between these two low-fat diets was the richness of fibers in the chow diet, whereas the LFD is a purified diet with only cellulose as a fiber source. As diet specialists claim, researchers are often using improper control diets when they want to study metabolic syndrome and other diseases in animal models. Therefore, these results demonstrate the importance of including a proper control diet where factors such as fiber remain the same to avoid potential confounding effects leading to misinterpretation of data [190].

Another macronutrient that proportionally varied in our food pellets was the fat. For many years, the consensus for dietary advice was a diet with not excessive amounts of fat in an attempt to limit chronic diseases. However, over the last years there are indications that ketogenic diets, characterized by high fat levels and low carbohydrates, may have pleiotropic effects on health [191]. In animal studies, the vast majority of experiments with HFDs indicated that fat negatively modulates intestinal microbiota, exacerbates colonic inflammation under DSS exposure and is a risk factor for colon cancer [192-194]. In contrast to those outcomes, according to Enos and his colleagues, high fat diets offered a protection against azoxymethane/dextran sodium sulfate-induced colon cancer (AOM/DSS model) [195], which is line with what we observed in DSS-treated mice. Also, Wolters in a systemic review pointed out that in most cases fat amount and fat sources matter on gut microbiota and metabolic health but data are not always consistent [196]. In the initial feeding experiment we used a WD containing milk fat. Milk fat contains short chain fatty acids (SCFAs) and is particularly abundant in the butyrate. We therefore suspected that milk fat rich in butyrate was partially responsible for the moderate inflammatory response in mice following DSS treatment. Since no data about oral administration of butyrate and colon health during an inflammatory state are available in the literature, we tested in a short follow

up experiment whether lard fat would provide the same protection as milk fat in the WD-fed mice during DSS treatment. Both DAI score and expression of *TNF-a, IL-1b and Ptgs2* genes revealed no differences between milk and lard fat. While fat type had no significance, its presence during DSS treatment proved to contribute since LFD-fed mice when switched to WD reduced their disease phenotype.

Regarding microbiota composition, we observed an increased Firmicutes/Bacteroidetes ratio in the WD fed mice, which agreed with other studies [197, 198]. Furthermore, we found a strikingly higher abundance of *Bifidobacteria* (phylum Actinobacteria) in the LFD fed mice prior to DSS treatment, which has been reported by others [199]. After DSS treatment the abundance of *Bifidobacteria* dramatically decreased. Although controversial views exist, *Bifidobacteria* are generally considered a beneficial commensal and exploited for probiotic purposes [200, 201]. A recent report found that supplementing mice with Bifidobacteria could protect against DSS-induced colitis, which argues against an unbeneficial effect of high pre-DSS levels in the LFD-fed mice [202]. Proteobacteria (including *Escherichia, Shigella* and *Parasutterella* genera) increased in LFD-fed mice treated with DSS. That phylum is proposed as an indicator of an inflammatory phenotype with a potential disease [120]. Earlier studies showed that different doses of DSS (1%, 2%, 3 %) increased the abundance of the family *Enterobacteriaceae* (which includes *Escherichia*, *Shigella*) and depleted *Bifidobacteria* [203].

Overall, conclusions in terms of the direct or indirect impact of fat are difficult to be drawn when many parameters are involved in an animal study. The animal model, the strain of animal, the initial microbiota profile, the environment the experiment is taking place, the chemicals used are determinants, which can manipulate the outcomes.

In paper III we wanted to investigate the effects of faba bean fractions in the colon when incorporated into the WD used in Paper II. So far, it is supported the contribution of legumes and pulses at reducing the risk of 10 chronic diseases [204]. Reduction of the risk for colon cancer [205], blood cholesterol levels [206] and cardiovascular disease [54] are some of the health benefits. The presence of soluble fibers, vitamins, lignans, minerals, phenolic acids, phytoestrogens, flavonoids and isoflavones are key players for the protective action. Much interest has given to fiber fractions from legumes and pulses because their mechanisms to prevent intestinal inflammation and types of cancer are attributed to mediation in intestinal

microbiota [207]. That is why dietary fiber is proposed to have a major role in shaping microbiota and influences the occurrence of IBDs [208]. For humans is often recommended a minimum fiber uptake of 38 g and 25 g per day for adult men and women respectively [209]. One of our hypothesis was that under 1% DSS-induced inflammation, a WD supplemented with soluble fibers (pectins, fructans, hemicelluloses, gums, etc.) from faba bean hulls [210] could improve colonic health compared to the pure WD, which contained only the insoluble fiber cellulose.

Apart from fibers and phytochemicals with antioxidant and anti-inflammatory activity, recently the plant protein content is gaining attention [211]. Bioactive compounds in the protein part of legumes, such as Bowman and Birk protease inhibitors (BBIs), trypsin-chymotrypsin inhibitors, lectins, are defined non-nutrients or antinutrients. Soaking, cooking, germination are processes applied in order to remove or reduce substantially their presence [206, 212]. On the other hand, statements reveal beneficial properties of bioactive compounds, which can be applicable in bioscience and biomedicine [213-215]. For our experiment protein and fiber fractions were cooked before making the modified WDs because we wanted to limit the negative factors mentioned above and also follow the way humans consume pulses.

Prior to DSS, *in vivo* imaging for ROS, glucose tolerance, insulin resistance and body weight were measured. In ROS production assay, the L-012 signal was drastically reduced in all three groups fed faba bean fractions. One explanation could be the macronutrient content of our fractions. Analysis showed that protein fraction contained small but not negligible proportions of fibers, starch and other polysaccharides whereas fiber fraction had proteins. Therefore, the use of concentrates and not isolate fractions might have an influence.

Legumes are often mentioned as a food dish that increases satiety [216]. In our case, mice fed WD+PF and WD+BF gained significantly more weight than pure WD and WD+FF probably because the food was more appealing to them. After five weeks in WDs with faba bean fractions did not reveal a better metabolic profile neither to body weight nor to glucose tolerance compared to those following a pure WD. Our outcome was in accordance with a study in 2017, where Lamming and his group stated that short term consumption of a plant

protein diet (three weeks) from C57BL/6J mice did not show improvement in glucose homeostasis [217].

Studies for investigating the role of legumes in the large intestine under an inflammatory condition are not new. First of all, Monk and her colleagues have conducted a series of experiments trying to elucidate the role of different pulses in DSS-induced colitis. Kidney beans, cranberry beans, navy beans, chickpeas and lentils were some of the dietary substances used in mice feeding trials [131, 218-221]. Their results highlight the ability of pulses to improve colon health prior to DSS treatment and reduce the disease severity manifested. Expression of antimicrobial and epithelial barrier integrity genes (*TLR4, Relmb, MUC1-3*), serum levels of proinflammatory cytokines (TNF-a, IL-1b, IL-6) and colonic histological damage (cell proliferation, crypt height, mucus content) were basic assays performed. In our case no significant differences were obtained when expression of proinflammatory (*TNF-a, IL-6, IL-1b*) and ROS (*iNOS, NOX1, NOX2*) genes were measured. On the contrary the *NOX2* gene, which is up regulated during colonic inflammation, was higher expressed in mice following the faba bean supplemented WDs than those kept on the standard WD.

Compared to our experimental design Monk's group decided to stop feeding mice with pulses during the DSS exposure. They argued that this would avoid potential confounding effects and wanted to mimic humans' eating habits while experiencing gastrointestinal disturbances. Another difference was that they preferred to use pulse flours in a basal purified diet (AIN 93G) whereas we opted protein and fiber concentrate fractions in WD. One explanation for their significant results after DSS exposure could be the presence of macronutrients and micronutrients in those flours, whereas in our case although the fractions were purer in composition, maybe they were lacking some important compounds. However, it should be mentioned that the amounts of the two faba bean fractions utilized in our experiment was close to Monk's diets. From their side they applied the highest intake level of pulse consumers in Canada that is approximately 295 g of pulses per day [222].

Searching the literature, we came across studies describing the positive outcomes in the gut from pea proteins [223, 224]. Characteristically, Utrilla and her group showed that pea albumin extracts manifested anti-inflammatory activities in mice treated with 3.5% DSS.

That conclusion was drawn by assessing the mRNA expression of proinflammatory genes, toll-like receptors, inducible enzymes in addition to proteins involved in barrier function. The ameliorated damage in colon was partially attributed to active BBIs present in the pea protein fraction [224].

Finally, Monk's group examined how diets supplemented with pulses altered the colonic microbiota composition and mitigated colitis severity, but they admitted that further research must be done [225]. Some of their findings indicated that pulse consumption in mice reduced the relative abundance of Clostridiaceae family, which contains harmful bacteria often seen in CD patients. Also, genera from Prevotella, a genus related to SCFAs production [129], showed a rise in mice following the chickpea diet.

From our sequencing data in fecal samples, alpha diversity was elevated in WD fed mice with fractions compared to WD fed mice but no changes appeared in beta diversity. There was a decrease of Proteobacteria and an increase of several members belonging to Bacteroidetes, in mice fed modified WDs. Those phyla, which are related to pathogenic conditions [120, 121] and necessary functions [226] respectively provide indications on how that outcomes may be manipulated for human's benefit. In that experiment, from LEfSe analysis, the relative abundance of Prevotellaceae family was significantly higher in WD+FF, WD+BF groups, while the genus *Bifidobacterium* was elevated in WD+PF. On the other side, *Tyzzerella*, a genus which is overrepresented in CD patients [227] showed an increase in WD mice.

5. Conclusions

In the current work, we successfully established a low-grade inflammation mouse model to investigate the importance of NOX1 protein for colon health and its interaction with gut microbiota. Moreover, the role of different diets in composition, with primary emphasis on WDs, were explored regarding their contribution to the inflamed colon and microbiota formation. Although overexpression of ROS is linked with gut damage and progression of gastrointestinal diseases, our results indicate that a certain amount of ROS (i.e., peroxynitrite produced by NOX1) is needed to keep the microbial content in check and ensure a healthy colon. Mice without the *NOX1* gene had compromised gut health. However, NOX1 only marginally protected against the severity of the DSS induced inflammation which indicate the involvement of compensatory protective mechanisms.

In line with previous findings, we demonstrated that WD induced unbeneficial effects in mice in terms of metabolic profile, inflammation and weight gain under steady-state conditions. However, unexpectedly, we found that the high fat proportion in the WD, both milk fat and lard (41% of energy content) protected against chemically (1% DSS) induced low-grade inflammation when compared to LFD fed mice. The microbiota was less diverse in the WD-fed mice compared to LFD-fed mice, from phyla level to genus level which is in line with previous studies. We speculated that the fat content in the WD inhibits the establishment of the DSS induced colitis model. A less likely explanation is that the fat could induce protective mechanisms but more research is needed to clarify these findings.

Finally, we investigated if the enrichment of WDs with protein and fiber faba bean fractions would protect against the unbeneficial impacts of a WD. We found no significant effects of the faba-bean fractions on colonic health, indicators of metabolic syndrome or inflammation and only marginally modulation of the intestinal microbiota. It is possible that such shifts in the microbiota by the enrichment of the diet with faba bean fractions could offer health beneficial effects but clinical trials of substantial sizes are required to investigate the importance for human health. Nevertheless, the faba bean fractions were well tolerated and from a sustainable point of view and due to the nutritional content faba beans can be recommended to be included as part of a healthy diet.

6. Future perspectives

The findings on the research conducted for this thesis have increased the knowledge about the importance of genes, diets, microbiota and their interactions on intestinal health. Although the mechanisms involved in maintaining intestinal homeostasis are not entirely clear and depend on a range of genetic and environmental factors, further studied could be performed for answering some of the queries. The use of mice as experimental animals for studying aspects of human nutrition and gut health provides promising results for the impact of diet but have to be verified in human studies in order to gain more attention.

While the current work showed the importance of ROS production for colonic microbiota (Paper I), we only focused on one gene (*NOX1*) generating ROS. There are many other genes involved in the pathway of generating ROS. Also, the ROS group includes a large variety of molecules with a wide spectrum of effects and interactions, impacting not only in the gut but in other host's tissues and cells. Thus, silencing and knocking other ROS related genes in mice could be useful to elucidate the importance of different types of ROS and the upstream pathways that are involved in the induction of the responses. More advanced techniques for identifying different ROS may be applied for more accurate and reliable measurements. Considering that a standard chow diet was used, it would be interesting an experiment containing both a chow and a purified diet.

The results of a beneficial contribution of fat presence in a WD against the DSS effects in the colon were unexpected. These outcomes, which in our case were not attributed to fat type, should be studied more thoroughly in order to explain why higher proportion of fat in diet can have a positive function in terms of protecting against DSS-induced damage. Whether the fat directly protects against DSS damage in the gut or induce beneficial effects directly in the colonocytes or indirectly by modulating the microbiota are proposed hypotheses that could be investigated.

In the end, the fact that we found no benefits of adding faba bean fractions to a WD during DSS-induced low-grade inflammation, raises questions whether the type and the amounts of faba bean fractions used in WD were sufficient for the mice. Importantly, the DSS model may not be the best model to study moderate effects of food components as the effects of DSS will

be too big in comparison. Last but not least, faba beans represent only one member of pulses among the wide variety that exists. For that reason, peas, lentils, chickpeas and even other types of beans can be utilized in future nutritional studies. Besides the short or long feeding trials with fractions of different legumes in animal models, the performance of clinical trials in humans may provide more versatile clinical evidence on the attempt to prevent or treat a spectrum of human diseases.

References

1. Malesza, I.J., et al., *High-Fat, Western-Style Diet, Systemic Inflammation, and Gut Microbiota: A Narrative Review*. Cells, 2021. **10**(11).
2. Zmora, N., J. Suez, and E. Elinav, *You are what you eat: diet, health and the gut microbiota.* Nature Reviews Gastroenterology & Hepatology, 2019. **16**(1): p. 35-56.
3. Dimitri, C. and S. Rogus, *FOOD CHOICES, FOOD SECURITY, AND FOOD POLICY.* Journal of International Affairs, 2014. **67**(2): p. 19-31.
4. Larson, N. and M. Story, *A review of environmental influences on food choices.* Annals of behavioral medicine : a publication of the Society of Behavioral Medicine, 2009. **38 Suppl 1**: p. S56-73.
5. Bo, S., et al., *A Critical Review on the Role of Food and Nutrition in the Energy Balance.* Nutrients, 2020. **12**(4).
6. Organization, W.H., *Global strategy on diet, physical activity and health.* 2004: Geneva.
7. Organization, W.H., *Global Action Plan for the Prevention and Control of NCDs 2013-2020*. 2013: Geneva.
8. Nothlings, U., et al., *Intake of vegetables, legumes, and fruit, and risk for all-cause, cardiovascular, and cancer mortality in a European diabetic population.* J Nutr, 2008. **138**(4): p. 775-81.
9. Ascherio, A., *Epidemiologic studies on dietary fats and coronary heart disease.* Am J Med, 2002. **113 Suppl 9B**: p. 9s-12s.
10. de Souza, R.J., et al., *Intake of saturated and trans unsaturated fatty acids and risk of all cause mortality, cardiovascular disease, and type 2 diabetes: systematic review and meta-analysis of observational studies.* BMJ : British Medical Journal, 2015. **351**: p. h3978.
11. Stylianou, K.S., V.L. Fulgoni, and O. Jolliet, *Small targeted dietary changes can yield substantial gains for human health and the environment.* Nature Food, 2021. **2**(8): p. 616-627.
12. Bechthold, A., et al., *Perspective: Food-Based Dietary Guidelines in Europe-Scientific Concepts, Current Status, and Perspectives.* Adv Nutr, 2018. **9**(5): p. 544-560.
13. *Nordic Council of Ministers. Nordic Nutrition Recommendations 2012: Integrating nutrition and physical activity*. Copenhagen (Denmark); 2014.
14. Christ, A., et al., *Western Diet Triggers NLRP3-Dependent Innate Immune Reprogramming.* Cell, 2018. **172**(1-2): p. 162-175 e14.
15. Cordain, L., et al., *Origins and evolution of the Western diet: health implications for the 21st century.* Am J Clin Nutr, 2005. **81**(2): p. 341-54.
16. Hall, K.D., et al., *Ultra-Processed Diets Cause Excess Calorie Intake and Weight Gain: An Inpatient Randomized Controlled Trial of Ad Libitum Food Intake.* Cell Metab, 2019. **30**(1): p. 67-77 e3.
17. Fuke, N., et al., *Regulation of Gut Microbiota and Metabolic Endotoxemia with Dietary Factors.* Nutrients, 2019. **11**(10).
18. Cani, P.D., et al., *Metabolic endotoxemia initiates obesity and insulin resistance.* Diabetes, 2007. **56**(7): p. 1761-72.
19. Mohammad, S. and C. Thiemermann, *Role of Metabolic Endotoxemia in Systemic Inflammation and Potential Interventions.* Front Immunol, 2020. **11**: p. 594150.
20. Manzel, A., et al., *Role of "Western diet" in inflammatory autoimmune diseases.* Curr Allergy Asthma Rep, 2014. **14**(1): p. 404.
21. Ananthakrishnan, A.N., *Epidemiology and risk factors for IBD.* Nat Rev Gastroenterol Hepatol, 2015. **12**(4): p. 205-17.

22. Ng, S.C., et al., *Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies.* Lancet, 2017. **390**(10114): p. 2769-2778.
23. Myles, I.A., *Fast food fever: reviewing the impacts of the Western diet on immunity.* Nutr J, 2014. **13**: p. 61.
24. Duan, Y., et al., *Inflammatory Links Between High Fat Diets and Diseases.* Front Immunol, 2018. **9**: p. 2649.
25. Naimi, S., et al., *Direct impact of commonly used dietary emulsifiers on human gut microbiota.* Microbiome, 2021. **9**.
26. Chassaing, B., et al., *Dietary emulsifiers directly alter human microbiota composition and gene expression ex vivo potentiating intestinal inflammation.* Gut, 2017. **66**(8): p. 1414-1427.
27. Viennois, E., et al., *Dietary Emulsifier-Induced Low-Grade Inflammation Promotes Colon Carcinogenesis.* Cancer Res, 2017. **77**(1): p. 27-40.
28. Kostovcikova, K., et al., *Diet Rich in Animal Protein Promotes Pro-inflammatory Macrophage Response and Exacerbates Colitis in Mice.* Front Immunol, 2019. **10**: p. 919.
29. Statovci, D., et al., *The Impact of Western Diet and Nutrients on the Microbiota and Immune Response at Mucosal Interfaces.* Front Immunol, 2017. **8**: p. 838.
30. Stagnari, F., et al., *Multiple benefits of legumes for agriculture sustainability: an overview.* Chemical and Biological Technologies in Agriculture, 2017. **4**(1): p. 2.
31. Lewis G, S.B., Mackinder B, Lock M., *Legumes of the World*. 2005: Kew: Royal Botanic Gardens.
32. Rebello, C.J., F.L. Greenway, and J.W. Finley, *Whole grains and pulses: a comparison of the nutritional and health benefits.* J Agric Food Chem, 2014. **62**(29): p. 7029-49.
33. Nations, F.a.A.O.o.t.U., *Definition and classification of commodities: pulses and derived products*. 1994: Rome.
34. George Amponsah Annor, Z.M.a.J.I.B., *Crops-Legumes*, in *Food Processing: Principles and Appliations* S.J.a.B.L. Stephanie Clark, Editor. 2014, John Wiley & Sons, Ltd.
35. Peltonen-Sainio, P., L. Jauhiainen, and H. Lehtonen, *Land Use, Yield and Quality Changes of Minor Field Crops: Is There Superseded Potential to Be Reinvented in Northern Europe?* PLoS One, 2016. **11**(11): p. e0166403.
36. McCrory, M.A., et al., *Pulse consumption, satiety, and weight management.* Adv Nutr, 2010. **1**(1): p. 17-30.
37. Boye, J., F. Zare, and A. Pletch, *Pulse proteins: Processing, characterization, functional properties and applications in food and feed.* Food Research International, 2010. **43**(2): p. 414-431.
38. Tosh, S. and S. Yada, *Dietary fibres in pulse seeds and fractions: Characterization, functional attributes, and applications.* Food Research International, 2010. **43**: p. 450-460.
39. Mariotti, F. and C.D. Gardner, *Dietary Protein and Amino Acids in Vegetarian Diets-A Review.* Nutrients, 2019. **11**(11).
40. Samtiya, M., R.E. Aluko, and T. Dhewa, *Plant food anti-nutritional factors and their reduction strategies: an overview.* Food Production, Processing and Nutrition, 2020. **2**(1): p. 6.
41. Al-Ishaq, R.K., et al., *Flavonoids and Their Anti-Diabetic Effects: Cellular Mechanisms and Effects to Improve Blood Sugar Levels.* Biomolecules, 2019. **9**(9).
42. Soares, J.M.D., et al., *Influence of Flavonoids on Mechanism of Modulation of Insulin Secretion.* Pharmacogn Mag, 2017. **13**(52): p. 639-646.
43. Kawser Hossain, M., et al., *Molecular Mechanisms of the Anti-Obesity and Anti-Diabetic Properties of Flavonoids.* Int J Mol Sci, 2016. **17**(4): p. 569.
44. Jiao, X., et al., *Blueberry polyphenols extract as a potential prebiotic with anti-obesity effects on C57BL/6 J mice by modulating the gut microbiota.* J Nutr Biochem, 2019. **64**: p. 88-100.

45. Makarewicz, M., et al., *The Interactions between Polyphenols and Microorganisms, Especially Gut Microbiota.* Antioxidants, 2021. **10**(2): p. 188.
46. Morais, C.A., et al., *Anthocyanins as inflammatory modulators and the role of the gut microbiota.* The Journal of Nutritional Biochemistry, 2016. **33**: p. 1-7.
47. Mithul Aravind, S., et al., *Role of dietary polyphenols on gut microbiota, their metabolites and health benefits.* Food Research International, 2021. **142**: p. 110189.
48. Middleton, E., Jr., C. Kandaswami, and T.C. Theoharides, *The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer.* Pharmacol Rev, 2000. **52**(4): p. 673-751.
49. Wang, Y., et al., *Legume consumption and colorectal adenoma risk: a meta-analysis of observational studies.* PLoS One, 2013. **8**(6): p. e67335.
50. Hartman, T.J., et al., *Consumption of a legume-enriched, low-glycemic index diet is associated with biomarkers of insulin resistance and inflammation among men at risk for colorectal cancer.* J Nutr, 2010. **140**(1): p. 60-7.
51. Kanatt, S.R., A. K, and A. Sharma, *Antioxidant and antimicrobial activity of legume hulls.* Food Research International, 2011. **44**(10): p. 3182-3187.
52. Aune, D., et al., *Legume intake and the risk of cancer: a multisite case-control study in Uruguay.* Cancer Causes Control, 2009. **20**(9): p. 1605-15.
53. Afshin, A., et al., *Consumption of nuts and legumes and risk of incident ischemic heart disease, stroke, and diabetes: a systematic review and meta-analysis.* Am J Clin Nutr, 2014. **100**(1): p. 278-88.
54. Marventano, S., et al., *Legume consumption and CVD risk: a systematic review and meta-analysis.* Public Health Nutr, 2017. **20**(2): p. 245-254.
55. Viguiliouk, E., et al., *Associations between Dietary Pulses Alone or with Other Legumes and Cardiometabolic Disease Outcomes: An Umbrella Review and Updated Systematic Review and Meta-analysis of Prospective Cohort Studies.* Adv Nutr, 2019. **10**(Suppl_4): p. S308-S319.
56. Hafiz, M.S., et al., *Pulse consumption improves indices of glycemic control in adults with and without type 2 diabetes: a systematic review and meta-analysis of acute and long-term randomized controlled trials.* Eur J Nutr, 2022. **61**(2): p. 809-824.
57. Ferreira, H., et al., *Benefits of pulse consumption on metabolism and health: A systematic review of randomized controlled trials.* Critical Reviews in Food Science and Nutrition, 2021. **61**(1): p. 85-96.
58. Helander, H.F. and L. Fandriks, *Surface area of the digestive tract - revisited.* Scand J Gastroenterol, 2014. **49**(6): p. 681-9.
59. Barrett, K.E., *Gastrointestinal physiology*. 2nd ed, ed. L.P. Series. 2014, New York: Lange Medical Books/McGraw-Hill Education.
60. Mowat, A.M. and W.W. Agace, *Regional specialization within the intestinal immune system.* Nat Rev Immunol, 2014. **14**(10): p. 667-85.
61. Salzman, N.H., et al., *Enteric defensins are essential regulators of intestinal microbial ecology.* Nat Immunol, 2010. **11**(1): p. 76-83.
62. Peterson, L.W. and D. Artis, *Intestinal epithelial cells: regulators of barrier function and immune homeostasis.* Nat Rev Immunol, 2014. **14**(3): p. 141-53.
63. Sato, T., et al., *Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts.* Nature, 2011. **469**(7330): p. 415-8.
64. Johansson, M.E.V., J.M.H. Larsson, and G.C. Hansson, *The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host–microbial interactions.* Proceedings of the National Academy of Sciences, 2011. **108**(supplement_1): p. 4659-4665.

65. Ali, A., H. Tan, and G.E. Kaiko, *Role of the Intestinal Epithelium and Its Interaction With the Microbiota in Food Allergy.* Frontiers in Immunology, 2020. **11**.
66. Gribble, F.M. and F. Reimann, *Enteroendocrine Cells: Chemosensors in the Intestinal Epithelium.* Annu Rev Physiol, 2016. **78**: p. 277-99.
67. Gerbe, F. and P. Jay, *Intestinal tuft cells: epithelial sentinels linking luminal cues to the immune system.* Mucosal Immunol, 2016. **9**(6): p. 1353-1359.
68. Howitt, M.R., et al., *Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut.* Science, 2016. **351**(6279): p. 1329-33.
69. Kobayashi, N., et al., *The Roles of Peyer's Patches and Microfold Cells in the Gut Immune System: Relevance to Autoimmune Diseases.* Front Immunol, 2019. **10**: p. 2345.
70. Lee, B., K.M. Moon, and C.Y. Kim, *Tight Junction in the Intestinal Epithelium: Its Association with Diseases and Regulation by Phytochemicals.* J Immunol Res, 2018. **2018**: p. 2645465.
71. Mowat, A.M., *To respond or not to respond - a personal perspective of intestinal tolerance.* Nat Rev Immunol, 2018. **18**(6): p. 405-415.
72. Parham, P., *The Immune System* 5th ed. ed. 2021: W. W. Norton & Company.
73. Vighi, G., et al., *Allergy and the gastrointestinal system.* Clin Exp Immunol, 2008. **153 Suppl 1**: p. 3-6.
74. Elia, P.P., et al., *The Role of Innate Immunity Receptors in the Pathogenesis of Inflammatory Bowel Disease.* Mediators of Inflammation, 2015. **2015**: p. 936193.
75. Abul Abbas, A.L., Shiv Pillai, *Cellular and Molecular Immunology*. 8th ed. 2014: Saunders.
76. Langefeld, T., et al., *Toll-like receptors and NOD-like receptors: domain architecture and cellular signalling.* Adv Exp Med Biol, 2009. **653**: p. 48-57.
77. Kawasaki, T. and T. Kawai, *Toll-like receptor signaling pathways.* Front Immunol, 2014. **5**: p. 461.
78. Zhang, J.M. and J. An, *Cytokines, inflammation, and pain.* Int Anesthesiol Clin, 2007. **45**(2): p. 27-37.
79. Motta, V., et al., *NOD-like receptors: versatile cytosolic sentinels.* Physiol Rev, 2015. **95**(1): p. 149-78.
80. Macdonald, T.T. and G. Monteleone, *Immunity, inflammation, and allergy in the gut.* Science, 2005. **307**(5717): p. 1920-5.
81. Rocamora-Reverte, L., et al., *The Complex Role of Regulatory T Cells in Immunity and Aging.* Front Immunol, 2020. **11**: p. 616949.
82. Brandtzaeg, P. and H. Prydz, *Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins.* Nature, 1984. **311**(5981): p. 71-3.
83. Johansen, F.E., et al., *Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice.* J Exp Med, 1999. **190**(7): p. 915-22.
84. Wang, Y., et al., *New insights in intestinal oxidative stress damage and the health intervention effects of nutrients: A review.* Journal of Functional Foods, 2020. **75**: p. 104248.
85. McCafferty, D.M., *Peroxynitrite and inflammatory bowel disease.* Gut, 2000. **46**(3): p. 436-9.
86. Aviello, G. and U.G. Knaus, *ROS in gastrointestinal inflammation: Rescue Or Sabotage?* Br J Pharmacol, 2017. **174**(12): p. 1704-1718.
87. Bedard, K. and K.H. Krause, *The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology.* Physiol Rev, 2007. **87**(1): p. 245-313.
88. Larsson, E., et al., *Analysis of gut microbial regulation of host gene expression along the length of the gut and regulation of gut microbial ecology through MyD88.* Gut, 2012. **61**(8): p. 1124-31.
89. Shaked, H., et al., *Chronic epithelial NF-kappaB activation accelerates APC loss and intestinal tumor initiation through iNOS up-regulation.* Proc Natl Acad Sci U S A, 2012. **109**(35): p. 14007-12.

90. Aw, T.Y., *Intestinal glutathione: determinant of mucosal peroxide transport, metabolism, and oxidative susceptibility.* Toxicology and Applied Pharmacology, 2005. **204**(3): p. 320-328.
91. Sender, R., S. Fuchs, and R. Milo, *Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans.* Cell, 2016. **164**(3): p. 337-340.
92. Rodriguez, J.M., et al., *The composition of the gut microbiota throughout life, with an emphasis on early life.* Microb Ecol Health Dis, 2015. **26**: p. 26050.
93. Donaldson, G.P., S.M. Lee, and S.K. Mazmanian, *Gut biogeography of the bacterial microbiota.* Nat Rev Microbiol, 2016. **14**(1): p. 20-32.
94. Flint, H.J., et al., *The role of the gut microbiota in nutrition and health.* Nat Rev Gastroenterol Hepatol, 2012. **9**(10): p. 577-89.
95. Al Bander, Z., et al., *The Gut Microbiota and Inflammation: An Overview.* Int J Environ Res Public Health, 2020. **17**(20).
96. Brandl, K., V. Kumar, and L. Eckmann, *Gut-liver axis at the frontier of host-microbial interactions.* Am J Physiol Gastrointest Liver Physiol, 2017. **312**(5): p. G413-G419.
97. Voreades, N., A. Kozil, and T.L. Weir, *Diet and the development of the human intestinal microbiome.* Frontiers in Microbiology, 2014. **5**.
98. Laforest-Lapointe, I. and M.C. Arrieta, *Patterns of Early-Life Gut Microbial Colonization during Human Immune Development: An Ecological Perspective.* Front Immunol, 2017. **8**: p. 788.
99. Graf, D., et al., *Contribution of diet to the composition of the human gut microbiota.* Microb Ecol Health Dis, 2015. **26**: p. 26164.
100. Gupta, V.K., S. Paul, and C. Dutta, *Geography, Ethnicity or Subsistence-Specific Variations in Human Microbiome Composition and Diversity.* Frontiers in Microbiology, 2017. **8**.
101. Ley, R.E., et al., *Evolution of mammals and their gut microbes.* Science, 2008. **320**(5883): p. 1647-51.
102. Valdes, A.M., et al., *Role of the gut microbiota in nutrition and health.* BMJ, 2018. **361**: p. k2179.
103. Feng, W., et al., *Targeting gut microbiota for precision medicine: Focusing on the efficacy and toxicity of drugs.* Theranostics, 2020. **10**(24): p. 11278-11301.
104. Ericsson, A.C., et al., *The influence of caging, bedding, and diet on the composition of the microbiota in different regions of the mouse gut.* Sci Rep, 2018. **8**(1): p. 4065.
105. Jakobsson, H.E., et al., *The composition of the gut microbiota shapes the colon mucus barrier.* EMBO Rep, 2015. **16**(2): p. 164-77.
106. Zhang, C., et al., *Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice.* Isme j, 2010. **4**(2): p. 232-41.
107. Flint, H.J., S.H. Duncan, and P. Louis, *The impact of nutrition on intestinal bacterial communities.* Curr Opin Microbiol, 2017. **38**: p. 59-65.
108. Wu, G.D., et al., *Linking long-term dietary patterns with gut microbial enterotypes.* Science, 2011. **334**(6052): p. 105-8.
109. David, L.A., et al., *Diet rapidly and reproducibly alters the human gut microbiome.* Nature, 2014. **505**(7484): p. 559-563.
110. Hartstra, A.V., et al., *Insights into the role of the microbiome in obesity and type 2 diabetes.* Diabetes Care, 2015. **38**(1): p. 159-65.
111. Singh, R.K., et al., *Influence of diet on the gut microbiome and implications for human health.* J Transl Med, 2017. **15**(1): p. 73.
112. Zinöcker, M.K. and I.A. Lindseth, *The Western Diet-Microbiome-Host Interaction and Its Role in Metabolic Disease.* Nutrients, 2018. **10**(3): p. 365.
113. Daniel, H., et al., *High-fat diet alters gut microbiota physiology in mice.* ISME J, 2014. **8**(2): p. 295-308.

114. Christ, A., M. Lauterbach, and E. Latz, *Western Diet and the Immune System: An Inflammatory Connection.* Immunity, 2019. **51**(5): p. 794-811.
115. Candido, F.G., et al., *Impact of dietary fat on gut microbiota and low-grade systemic inflammation: mechanisms and clinical implications on obesity.* Int J Food Sci Nutr, 2018. **69**(2): p. 125-143.
116. Turnbaugh, P.J. and J.I. Gordon, *The core gut microbiome, energy balance and obesity.* J Physiol, 2009. **587**(Pt 17): p. 4153-8.
117. Cani, P.D. and N.M. Delzenne, *The gut microbiome as therapeutic target.* Pharmacol Ther, 2011. **130**(2): p. 202-12.
118. Tomas, J., et al., *High-fat diet modifies the PPAR-gamma pathway leading to disruption of microbial and physiological ecosystem in murine small intestine.* Proc Natl Acad Sci U S A, 2016. **113**(40): p. E5934-E5943.
119. de La Serre, C.B., et al., *Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation.* Am J Physiol Gastrointest Liver Physiol, 2010. **299**(2): p. G440-8.
120. Shin, N.R., T.W. Whon, and J.W. Bae, *Proteobacteria: microbial signature of dysbiosis in gut microbiota.* Trends in Biotechnology, 2015. **33**(9): p. 496-503.
121. Rizzatti, G., et al., *Proteobacteria: A Common Factor in Human Diseases.* Biomed Res Int, 2017. **2017**: p. 9351507.
122. Derrien, M., et al., *The Mucin degrader Akkermansia muciniphila is an abundant resident of the human intestinal tract.* Appl Environ Microbiol, 2008. **74**(5): p. 1646-8.
123. Belzer, C. and W.M. de Vos, *Microbes inside--from diversity to function: the case of Akkermansia.* ISME J, 2012. **6**(8): p. 1449-58.
124. Everard, A., et al., *Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity.* Proc Natl Acad Sci U S A, 2013. **110**(22): p. 9066-71.
125. Karlsson, C.L., et al., *The microbiota of the gut in preschool children with normal and excessive body weight.* Obesity (Silver Spring), 2012. **20**(11): p. 2257-61.
126. Arora, T. and F. Backhed, *The gut microbiota and metabolic disease: current understanding and future perspectives.* J Intern Med, 2016. **280**(4): p. 339-49.
127. Devkota, S., et al., *Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice.* Nature, 2012. **487**(7405): p. 104-8.
128. Gutierrez-Diaz, I., et al., *Mediterranean diet and faecal microbiota: a transversal study.* Food Funct, 2016. **7**(5): p. 2347-56.
129. De Filippo, C., et al., *Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa.* Proceedings of the National Academy of Sciences, 2010. **107**(33): p. 14691-14696.
130. Graf, D., et al., *Red lentil supplementation reduces the severity of dextran sodium sulfate-induced colitis in C57BL/6 male mice.* Journal of Functional Foods, 2020. **64**: p. 103625.
131. Monk, J.M., et al., *Chickpea-supplemented diet alters the gut microbiome and enhances gut barrier integrity in C57Bl/6 male mice.* Journal of Functional Foods, 2017. **38**: p. 663-674.
132. Tamanai-Shacoori, Z., et al., *Roseburia spp.: a marker of health?* Future Microbiol, 2017. **12**: p. 157-170.
133. Schwiertz, A., et al., *Microbiota and SCFA in Lean and Overweight Healthy Subjects.* Obesity, 2010. **18**(1): p. 190-195.
134. Lawrence, T., *The nuclear factor NF-kappaB pathway in inflammation.* Cold Spring Harb Perspect Biol, 2009. **1**(6): p. a001651.
135. Cavaillon, J.M., *Pro- versus anti-inflammatory cytokines: myth or reality.* Cell Mol Biol (Noisy-le-grand), 2001. **47**(4): p. 695-702.

136. Srikanthan, K., et al., *Systematic Review of Metabolic Syndrome Biomarkers: A Panel for Early Detection, Management, and Risk Stratification in the West Virginian Population.* Int J Med Sci, 2016. **13**(1): p. 25-38.
137. Schirmer, M., et al., *Linking the Human Gut Microbiome to Inflammatory Cytokine Production Capacity.* Cell, 2016. **167**(7): p. 1897.
138. Lopez-Castejon, G. and D. Brough, *Understanding the mechanism of IL-1beta secretion.* Cytokine Growth Factor Rev, 2011. **22**(4): p. 189-95.
139. Menghini, P., et al., *Neutralization of IL-1alpha ameliorates Crohn's disease-like ileitis by functional alterations of the gut microbiome.* Proc Natl Acad Sci U S A, 2019.
140. Pradhan, A.D., et al., *C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus.* JAMA, 2001. **286**(3): p. 327-34.
141. Di Sabatino, A., et al., *Efficacy of butyrate in the treatment of mild to moderate Crohn's disease.* Digestive and Liver Disease Supplements, 2007. **1**(1): p. 31-35.
142. Cooper, D., et al., *Relationship between Human Gut Microbiota and Interleukin 6 Levels in Overweight and Obese Adults.* The FASEB Journal, 2016. **30**(S1): p. 146.4-146.4.
143. Ghosh, S.S., et al., *Intestinal Barrier Dysfunction, LPS Translocation, and Disease Development.* Journal of the Endocrine Society, 2020. **4**(2).
144. Tian, P., et al., *Antidiabetic (type 2) effects of Lactobacillus G15 and Q14 in rats through regulation of intestinal permeability and microbiota.* Food Funct, 2016. **7**(9): p. 3789-3797.
145. Hyun, J.G. and L. Mayer, *Mechanisms underlying inflammatory bowel disease.* Drug Discovery Today: Disease Mechanisms, 2006. **3**(4): p. 457-462.
146. Ramos, G.P. and K.A. Papadakis, *Mechanisms of Disease: Inflammatory Bowel Diseases.* Mayo Clin Proc, 2019. **94**(1): p. 155-165.
147. Hugot, J.P., et al., *Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease.* Nature, 2001. **411**(6837): p. 599-603.
148. Albenberg, L.G. and G.D. Wu, *Diet and the intestinal microbiome: associations, functions, and implications for health and disease.* Gastroenterology, 2014. **146**(6): p. 1564-72.
149. de Castro, M.M., et al., *Role of diet and nutrition in inflammatory bowel disease.* World J Exp Med, 2021. **11**(1): p. 1-16.
150. Rozich, J.J., A. Holmer, and S. Singh, *Effect of Lifestyle Factors on Outcomes in Patients With Inflammatory Bowel Diseases.* Am J Gastroenterol, 2020. **115**(6): p. 832-840.
151. Sartor, R.B. and G.D. Wu, *Roles for Intestinal Bacteria, Viruses, and Fungi in Pathogenesis of Inflammatory Bowel Diseases and Therapeutic Approaches.* Gastroenterology, 2017. **152**(2): p. 327-339 e4.
152. Frank, D.N., et al., *Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases.* Proc Natl Acad Sci U S A, 2007. **104**(34): p. 13780-5.
153. Bouma, G. and W. Strober, *The immunological and genetic basis of inflammatory bowel disease.* Nat Rev Immunol, 2003. **3**(7): p. 521-33.
154. Wagatsuma, K., Y. Yokoyama, and H. Nakase, *Role of Biomarkers in the Diagnosis and Treatment of Inflammatory Bowel Disease.* Life (Basel), 2021. **11**(12).
155. Dragoni, G., T. Innocenti, and A. Galli, *Biomarkers of Inflammation in Inflammatory Bowel Disease: How Long before Abandoning Single-Marker Approaches?* Digestive Diseases, 2021. **39**(3): p. 190-203.
156. Moschen, A.R., et al., *Lipocalin-2: A Master Mediator of Intestinal and Metabolic Inflammation.* Trends Endocrinol Metab, 2017. **28**(5): p. 388-397.
157. Lu, F., et al., *Functions and regulation of lipocalin-2 in gut-origin sepsis: a narrative review.* Critical Care, 2019. **23**(1): p. 269.

158. Singh, V., et al., *Microbiota-inducible Innate Immune, Siderophore Binding Protein Lipocalin 2 is Critical for Intestinal Homeostasis.* Cell Mol Gastroenterol Hepatol, 2016. **2**(4): p. 482-498 e6.
159. Hoshiko, H., et al., *Identification of leaky gut-related markers as indicators of metabolic health in Dutch adults: The Nutrition Questionnaires plus (NQplus) study.* PLoS One, 2021. **16**(6): p. e0252936.
160. Moreno-Navarrete, J.M., et al., *Circulating lipopolysaccharide-binding protein (LBP) as a marker of obesity-related insulin resistance.* Int J Obes (Lond), 2012. **36**(11): p. 1442-9.
161. Chassaing, B., et al., *Dextran sulfate sodium (DSS)-induced colitis in mice.* Curr Protoc Immunol, 2014. **104**: p. Unit 15 25.
162. Dieleman, L.A., et al., *Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines.* Clin Exp Immunol, 1998. **114**(3): p. 385-91.
163. Kiesler, P., I.J. Fuss, and W. Strober, *Experimental Models of Inflammatory Bowel Diseases.* Cell Mol Gastroenterol Hepatol, 2015. **1**(2): p. 154-170.
164. Laroui, H., et al., *Dextran sodium sulfate (DSS) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon.* PLoS One, 2012. **7**(3): p. e32084.
165. Lee, J.G., et al., *Impact of short-chain fatty acid supplementation on gut inflammation and microbiota composition in a murine colitis model.* J Nutr Biochem, 2022. **101**: p. 108926.
166. Treton, X., et al., *Combined NADPH oxidase 1 and interleukin 10 deficiency induces chronic endoplasmic reticulum stress and causes ulcerative colitis-like disease in mice.* PLoS One, 2014. **9**(7): p. e101669.
167. Leoni, G., et al., *Annexin A1, formyl peptide receptor, and NOX1 orchestrate epithelial repair.* J Clin Invest, 2013. **123**(1): p. 443-54.
168. Hugenholtz, F. and W.M. de Vos, *Mouse models for human intestinal microbiota research: a critical evaluation.* Cell Mol Life Sci, 2018. **75**(1): p. 149-160.
169. Beck, J.A., et al., *Genealogies of mouse inbred strains.* Nat Genet, 2000. **24**(1): p. 23-5.
170. Low, D., D.D. Nguyen, and E. Mizoguchi, *Animal models of ulcerative colitis and their application in drug research.* Drug Des Devel Ther, 2013. **7**: p. 1341-57.
171. Bogatyrev, S.R., J.C. Rolando, and R.F. Ismagilov, *Self-reinoculation with fecal flora changes microbiota density and composition leading to an altered bile-acid profile in the mouse small intestine.* Microbiome, 2020. **8**(1): p. 19.
172. Xiao, L., et al., *A catalog of the mouse gut metagenome.* Nat Biotechnol, 2015. **33**(10): p. 1103-8.
173. Nguyen, T.L., et al., *How informative is the mouse for human gut microbiota research?* Dis Model Mech, 2015. **8**(1): p. 1-16.
174. Alfadda, A.A. and R.M. Sallam, *Reactive oxygen species in health and disease.* J Biomed Biotechnol, 2012. **2012**: p. 936486.
175. Matziouridou, C., et al., *iNOS- and NOX1-dependent ROS production maintains bacterial homeostasis in the ileum of mice.* Mucosal Immunology, 2017. **11**: p. 774.
176. Suzuki, R., et al., *Strain differences in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice.* Carcinogenesis, 2006. **27**(1): p. 162-9.
177. Aviello, G., et al., *Colitis susceptibility in mice with reactive oxygen species deficiency is mediated by mucus barrier and immune defense defects.* Mucosal Immunol, 2019. **12**(6): p. 1316-1326.
178. Chassaing, B., et al., *Fecal lipocalin 2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation.* PLoS One, 2012. **7**(9): p. e44328.
179. Makhezer, N., et al., *NOX1-derived ROS drive the expression of Lipocalin-2 in colonic epithelial cells in inflammatory conditions.* Mucosal Immunol, 2019. **12**(1): p. 117-131.
180. Johansson, M.E., et al., *Bacteria penetrate the inner mucus layer before inflammation in the dextran sulfate colitis model.* PLoS One, 2010. **5**(8): p. e12238.

181. Li, M., et al., *Initial gut microbiota structure affects sensitivity to DSS-induced colitis in a mouse model.* Sci China Life Sci, 2018. **61**(7): p. 762-769.
182. Ley, R.E., et al., *Obesity alters gut microbial ecology.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11070-5.
183. Derrien, M., C. Belzer, and W.M. de Vos, *Akkermansia muciniphila and its role in regulating host functions.* Microb Pathog, 2017. **106**: p. 171-181.
184. Derrien, M., et al., *Modulation of Mucosal Immune Response, Tolerance, and Proliferation in Mice Colonized by the Mucin-Degrader Akkermansia muciniphila.* Front Microbiol, 2011. **2**: p. 166.
185. Coant, N., et al., *NADPH oxidase 1 modulates WNT and NOTCH1 signaling to control the fate of proliferative progenitor cells in the colon.* Mol Cell Biol, 2010. **30**(11): p. 2636-50.
186. Liang, Y.N., et al., *Indigo Naturalis Ameliorates Dextran Sulfate Sodium-Induced Colitis in Mice by Modulating the Intestinal Microbiota Community.* Molecules, 2019. **24**(22).
187. Sokol, H., et al., *Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients.* Proc Natl Acad Sci U S A, 2008. **105**(43): p. 16731-6.
188. Zhao, H., X. Jiang, and W. Chu, *Shifts in the gut microbiota of mice in response to dexamethasone administration.* Int Microbiol, 2020. **23**(4): p. 565-573.
189. Parker, B.J., et al., *The Genus Alistipes: Gut Bacteria With Emerging Implications to Inflammation, Cancer, and Mental Health.* 2020. **11**(906).
190. Pellizzon, M.A. and M.R. Ricci, *The common use of improper control diets in diet-induced metabolic disease research confounds data interpretation: the fiber factor.* Nutr Metab (Lond), 2018. **15**: p. 3.
191. Ludwig, D.S., et al., *Dietary fat: From foe to friend?* Science, 2018. **362**(6416): p. 764-770.
192. Teixeira, L.G., et al., *The combination of high-fat diet-induced obesity and chronic ulcerative colitis reciprocally exacerbates adipose tissue and colon inflammation.* Lipids Health Dis, 2011. **10**: p. 204.
193. van der Logt, E.M., et al., *Westernized high-fat diet accelerates weight loss in dextran sulfate sodium-induced colitis in mice, which is further aggravated by supplementation of heme.* J Nutr Biochem, 2013. **24**(6): p. 1159-65.
194. Day, S.D., et al., *Linking inflammation to tumorigenesis in a mouse model of high-fat-diet-enhanced colon cancer.* Cytokine, 2013. **64**(1): p. 454-62.
195. Enos, R.T., et al., *High-fat diets rich in saturated fat protect against azoxymethane/dextran sulfate sodium-induced colon cancer.* Am J Physiol Gastrointest Liver Physiol, 2016. **310**(11): p. G906-19.
196. Wolters, M., et al., *Dietary fat, the gut microbiota, and metabolic health - A systematic review conducted within the MyNewGut project.* Clin Nutr, 2019. **38**(6): p. 2504-2520.
197. Clarke, S.F., et al., *The gut microbiota and its relationship to diet and obesity: new insights.* Gut Microbes, 2012. **3**(3): p. 186-202.
198. Kim, S.J., et al., *Dietary fat intake and age modulate the composition of the gut microbiota and colonic inflammation in C57BL/6J mice.* BMC Microbiol, 2019. **19**(1): p. 193.
199. Ang, Q.Y., et al., *Ketogenic Diets Alter the Gut Microbiome Resulting in Decreased Intestinal Th17 Cells.* Cell, 2020. **181**(6): p. 1263-1275 e16.
200. Picard, C., et al., *Review article: bifidobacteria as probiotic agents -- physiological effects and clinical benefits.* Aliment Pharmacol Ther, 2005. **22**(6): p. 495-512.
201. Di Giacinto, C., et al., *Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells.* J Immunol, 2005. **174**(6): p. 3237-46.

202. Singh, S., et al., *Anti-inflammatory Bifidobacterium strains prevent dextran sodium sulfate induced colitis and associated gut microbial dysbiosis in mice.* Sci Rep, 2020. **10**(1): p. 18597.
203. Park, H., et al., *Longitudinal Microbiome Analysis in a Dextran Sulfate Sodium-Induced Colitis Mouse Model.* Microorganisms, 2021. **9**(2).
204. Kromhout, D., et al., *The 2015 Dutch food-based dietary guidelines.* European Journal of Clinical Nutrition, 2016. **70**(8): p. 869-878.
205. Aune, D., et al., *Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies.* BMJ, 2011. **343**: p. d6617.
206. Clemente, A. and R. Olias, *Beneficial effects of legumes in gut health.* Current Opinion in Food Science, 2017. **14**: p. 32-36.
207. Aranda-Olmedo, I. and L.A. Rubio, *Dietary legumes, intestinal microbiota, inflammation and colorectal cancer.* Journal of Functional Foods, 2020. **64**: p. 103707.
208. Ananthakrishnan, A.N., et al., *A Prospective Study of Long-term Intake of Dietary Fiber and Risk of Crohn's Disease and Ulcerative Colitis.* Gastroenterology, 2013. **145**(5): p. 970-977.
209. Dahl, W.J. and M.L. Stewart, *Position of the Academy of Nutrition and Dietetics: Health Implications of Dietary Fiber.* Journal of the Academy of Nutrition and Dietetics, 2015. **115**(11): p. 1861-1870.
210. Tiwari, U. and E. Cummins, *Chapter 7 - Legume fiber characterization, functionality, and process effects*, in *Pulse Foods (Second Edition)*, B.K. Tiwari, A. Gowen, and B. McKenna, Editors. 2021, Academic Press. p. 147-175.
211. Duranti, M., *Grain legume proteins and nutraceutical properties.* Fitoterapia, 2006. **77**(2): p. 67-82.
212. Lannoo, N. and E.J.M. Van Damme, *Lectin domains at the frontiers of plant defense.* Frontiers in Plant Science, 2014. **5**(AUG).
213. Clemente, A. and C. Arques Mdel, *Bowman-Birk inhibitors from legumes as colorectal chemopreventive agents.* World J Gastroenterol, 2014. **20**(30): p. 10305-15.
214. Pusztai, A., S. Bardocz, and S.W.B. Ewen, *Uses of plant lectins in bioscience and biomedicine.* Frontiers in Bioscience-Landmark, 2008. **13**(3): p. 1130-1140.
215. Lopez-Barrios, L., J.A. Gutierrez-Uribe, and S.O. Serna-Saldivar, *Bioactive peptides and hydrolysates from pulses and their potential use as functional ingredients.* J Food Sci, 2014. **79**(3): p. R273-83.
216. Kristensen, M.D., et al., *Meals based on vegetable protein sources (beans and peas) are more satiating than meals based on animal protein sources (veal and pork) - a randomized cross-over meal test study.* Food Nutr Res, 2016. **60**: p. 32634.
217. Lamming, D.W., et al., *Short-term consumption of a plant protein diet does not improve glucose homeostasis of young C57BL/6J mice.* Nutr Healthy Aging, 2017. **4**(3): p. 239-245.
218. Monk, J.M., et al., *White and dark kidney beans reduce colonic mucosal damage and inflammation in response to dextran sodium sulfate.* J Nutr Biochem, 2015. **26**(7): p. 752-60.
219. Monk, J.M., et al., *Navy and black bean supplementation primes the colonic mucosal microenvironment to improve gut health.* J Nutr Biochem, 2017. **49**: p. 89-100.
220. Monk, J.M., et al., *Chickpea supplementation prior to colitis onset reduces inflammation in dextran sodium sulfate-treated C57Bl/6 male mice.* Appl Physiol Nutr Metab, 2018. **43**(9): p. 893-901.
221. Zhang, C., et al., *Cooked navy and black bean diets improve biomarkers of colon health and reduce inflammation during colitis.* Br J Nutr, 2014. **111**(9): p. 1549-63.
222. Mudryj, A.N., et al., *Pulse consumption in Canadian adults influences nutrient intakes.* British Journal of Nutrition, 2012. **108**(S1): p. S27-S36.

223. Rubio, L.A., et al., *Characterization of pea (Pisum sativum) seed protein fractions.* J Sci Food Agric, 2014. **94**(2): p. 280-7.
224. Utrilla, M.P., et al., *Pea (Pisum sativum L.) seed albumin extracts show anti-inflammatory effect in the DSS model of mouse colitis.* Mol Nutr Food Res, 2015. **59**(4): p. 807-19.
225. Monk, J.M., et al., *Diets enriched with cranberry beans alter the microbiota and mitigate colitis severity and associated inflammation.* J Nutr Biochem, 2016. **28**: p. 129-39.
226. Seong, E., et al., *Positive influence of gut microbiota on the effects of Korean red ginseng in metabolic syndrome: a randomized, double-blind, placebo-controlled clinical trial.* EPMA Journal, 2021. **12**(2): p. 177-197.
227. Olaisen, M., et al., *Bacterial Mucosa-associated Microbiome in Inflamed and Proximal Noninflamed Ileum of Patients With Crohn's Disease.* Inflamm Bowel Dis, 2021. **27**(1): p. 12-24.

Papers I-III

PAPER I

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The ROS-generating enzyme NADPH oxidase 1 modulates the colonic microbiota but offers minor protection against dextran sulfate sodium-induced low-grade colon inflammation in mice

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ABSTRACT

The enzyme NADPH oxidase 1 (NOX1) is a major producer of superoxide which together with other reactive oxygen and nitrogen species (ROS/RNS) are implicated in maintaining a healthy epithelial barrier in the gut. While previous studies have indicated NOX1's involvement in microbial modulation in the small intestine, less is known about the effects of NOX1-dependent ROS/RNS formation in the colon. We investigated the role of NOX1 in the colon of NOX1 knockout (KO) and wild type (WT) mice, under mild and subclinical low-grade colon inflammation induced by 1% dextran sulfate sodium (DSS). *Ex vivo* imaging of ROS/RNS in the colon revealed that absence of NOX1 strongly decreased ROS/RNS production, particularly during DSS treatment. Furthermore, while absence of NOX1 did not affect disease activity, some markers of inflammation (mRNA: *Tnfa*, *Il6*, *Ptgs2*; protein: lipocalin 2) in the colonic mucosa tended to be higher in NOX1 KO than in WT mice following DSS treatment. Lack of NOX1 also extensively modulated the bacterial community in the colon (16S rRNA gene sequencing), where NOX1 KO mice were characterized mainly by lower α-diversity (richness and evenness), higher abundance of Firmicutes, *Akkermansia*, and *Oscillibacter*, and lower abundance of Bacteroidetes and *Alistipes*. Together, our data suggest that NOX1 is pivotal for colonic ROS/RNS production in mice both during steady-state (i.e., no DSS treatment) and during 1% DSS-induced low-grade inflammation and for modulation of the colonic microbiota, with potential beneficial consequences for intestinal health.

1. Introduction

Reactive oxygen and nitrogen species (ROS/RNS) are reactive molecules produced by cells during normal cellular metabolism and their production can be triggered in cells of the innate immune system to kill bacteria [1–3]. They include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($^{\bullet}NO$), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl). Although ROS/RNS can lead to cellular damage and diseases their essential roles in cellular signaling are widely recognized [1–3]. Several enzymes are responsible for ROS/RNS production, with expression patterns and functions that vary depending on cell type [4].

During normal steady-state conditions in the intestine, NADPH oxidase (NOX) 1 (NOX1) and dual oxidase 2 (DUOX2) in epithelial cells are the primary sources of ROS, producing superoxide and hydrogen peroxide, respectively [4–7]. The role of NOX1 could be particularly important in the colon where the expression is highest [8–13], with an increasing expression gradient from proximal to distal end [9,10,13,14]. To convert oxygen to superoxide, NOX1 is dependent on a stabilizing protein ($p22^{phox}$), an activator (NOXA1), an organizer ($NOXO1/p47^{phox}$), and a Rac GTPase [4]. Colonic NOX1 is suggested to produce a basal level of ROS in the colon [4,15], which could be further enhanced during specific circumstances like inflammation [16] and bacterial invasion [15]. Another relevant RNS-producing enzyme in the intestine, not belonging to the NOX family, is inducible nitric oxide synthase (iNOS). iNOS produces nitric oxide which can spontaneously create peroxynitrite when reacting with superoxide [17]. While the expression of iNOS is high in the small intestine during normal conditions [18,19],

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colonic iNOS is mainly expressed during inflammation or infection [20]. During inflammation, NOX2 in phagocytic cells will also contribute significantly to ROS/RNS generation [4].

The role of NOX1 in the colon is not fully understood. Still, studies of NOX1 have indicated its involvement in several processes, including endoplasmic reticulum stress in goblet cells [21], mucosal wound repair [16,22,23], and epithelial cell proliferation and differentiation [24]. NOX1 has also been proposed to be an important participant in crosstalk between the gut bacteria and the host [25,26]. Induced NOX1 mRNA expression in the colon of specific pathogen-free compared to germ-free mice [13] further indicates a relationship between colonic NOX1 and gut bacteria, although this result was not observed by others [19]. Together, these findings suggest important roles of NOX1 in colonic homeostasis. We have previously shown that NOX1 impacts bacterial levels and composition in the ileum [8], possibly through mechanisms involving NOX1- and iNOS-dependent formation of peroxynitrite, as assessed by *in vivo* and *ex vivo* imaging using the chemiluminescent molecule L-012 [8,27]. Similar mechanisms could be present in the colon during inflammation when both NOX1 and iNOS are active. Previous studies of NOX1's role during colon inflammation have mostly used pathological concentrations of dextran sulfate sodium (DSS; 2–4% in drinking water) [16,21,22,28], a widely used inducer of colitis. In those studies, NOX1-deficient mice exhibited overall small differences in colitis pathology compared to wild type (WT) mice. However, WT mice recover faster after the withdrawal of DSS [16] in line with NOX1's beneficial role in wound healing [22,23].

Severe colitis is an extreme situation with a massive release of inflammatory mediators and high production of ROS/RNS from many sources, which may mask the effects of the absence or presence of NOX1 in the DSS models using high concentrations. Thus, a more relevant condition for studying effects of NOX1 is low-grade colon inflammation, characterized by mild signs of inflammation with few or no signs of pathology [29], which can lead to impaired gut barrier integrity and disturbances in the bacterial composition of the colon. Low-grade intestinal inflammation is a state frequently encountered in patients with irritable bowel syndrome, in patients with inflammatory bowel disease in remission, and in people with a poor diet [29–33]. Low-grade inflammation can be induced in mice by applying low doses of DSS typically in the range of 0.5–1% [34]. Under these conditions, elevated levels of nitrotyrosine have been observed in the epithelial layers of the colon, indicative of enhanced peroxynitrite production [35], most likely facilitated by NOX1- and iNOS-dependent ROS/RNS formation. Since NOX1 is highly expressed in the colonic epithelial cells in healthy mice, it is plausible that NOX1 together with iNOS are the primary sources of peroxynitrite during low-grade inflammation induced by 1% DSS, and we speculate that in this condition peroxynitrite will have an impact on colon inflammation and bacterial composition.

The aim of this study was therefore to investigate the role of NOX1-dependent ROS/RNS production in the colon both during steady-state and during a mild and subclinical low-grade inflammation. Specifically, we wanted to investigate (1) whether ROS/RNS production in the colon is dependent on NOX1, (2) whether NOX1 has a protective role during low-grade colon inflammation, and (3) whether and how NOX1 impacts the bacterial composition in the colon.

2. Material and methods

2.1. Mice

All mice had C57BL/6J genetic background (The Jackson Laboratory, Bar Harbor, ME). NOX1 knockout (KO) mice (Jax stock #018787) [85] were bred by homozygous breeding ($Nox1^{-/-} \times Nox1^{-/Y}$) and the genotype was confirmed by standard PCR with specific primer pairs as recommended by The Jackson Laboratory (Table S1). WT and NOX1 KO mice were bred and housed in separate individually ventilated cages (Innovative, San Diego, CA) in the same controlled environment (rack) (12h-light-dark cycle, 24 ± 1°C, 45–55% humidity), with standard rodent chow (RM1, SDS Diet, Essex, UK) and water *ad libitum*. A low number of mice per cage (2–3) was maintained to reduce potential cage effects. Prior to the experiments, both genotypes were bred for several generations in the same rack with identical feeding and handling routines, thereby giving them the same external bacterial exposures.

2.2. Mouse experiments

Two mouse experiments were performed (Fig. 1). In Experiment 1, mice were treated with different doses of DSS in drinking water to find the optimal dose and length of DSS treatment to induce low-grade inflammation in the colon. In the present study, colonic low-grade inflammation refers to the presence of few or no visible signs of disease (i.e., weight loss and change in stool quality) together with a moderate up-regulation of inflammation-related genes. For this purpose, 24 WT male mice (10–14 weeks old) were divided into four groups (n=6) which were given 0, 0.5, 1 or 2% (w/w) DSS (Dextran Sulfate Sodium Salt Colitis Grade, 36,000–50,000 MW, MP Biomedicals, Santa Ana, CA) in water for eight days. In Experiment 2, WT and NOX1 KO mice were used to investigate the effect of NOX1-deficiency on ROS/RNS production, gut microbiota, and colonic low-grade inflammation. Nineteen WT and 20 NOX1 KO mice (14–17 weeks old) were used for this purpose. Mice of each genotype were divided into two groups (n=9–10, two males per group), each of which were given 0 or 1% (w/w) DSS in water for six days. For both experiments, fresh DSS solutions in new bottles were prepared every second day as recommended [36]. Water for the control mice was changed accordingly. Animal experiments were performed with permission from The Norwegian Food Safety Authority (FOTS #14805), and they were conducted in compliance with the current guidelines of The Federation of European Laboratory Animal Science Associations.

2.3. Sampling

Mice were weighed and stool quality was inspected every second day of the experiments. On the last day of the experiments, prior to termination, feces was collected for enzyme-linked immunosorbent assay (ELISA). Mice were then anesthetized by intraperitoneal injection with ZRF cocktail (10 μL/g mouse, Table S2). The feed was removed ~4 h before the ZRF injection. Blood for ELISA was collected through cardiac

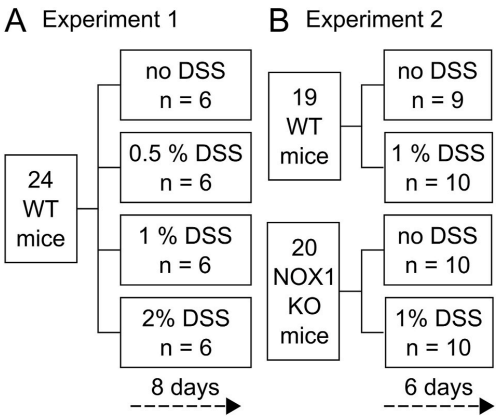

Fig. 1. Experimental group design for (A) Experiment 1 and (B) Experiment 2. In Experiment 1, WT mice were treated with 0 (no DSS), 0.5, 1, or 2% DSS for eight days (n=6 per group). In Experiment 2, WT and NOX1 KO mice were treated with 0 (no DSS) or 1% DSS for six days (n=9–10 per group). WT, wild type; DSS, dextran sulfate sodium; NOX1, NADPH oxidase 1; KO, knockout.

puncture with syringes coated with disodium EDTA as anticoagulant (0.05 M, final concentration 2–5 mM). After termination by cervical dislocation, the intestine was isolated and *ex vivo* imaging was performed (Experiment 2 only). After measuring the colon length, the colon was opened longitudinally, 2 cm from both sides. From these sections, 10–30 mg of lamina propria was scraped off with a glass slide for RNA extraction. The remaining middle segment was fixed for histological observations. In addition, 1 cm of the colon (referred to as "colon tissue") and one fecal pellet were collected from the proximal-middle section for DNA extraction (Experiment 2 only).

2.4. Ex vivo imaging of ROS/RNS using L-012 luminescence probe

Ex vivo imaging of the intestine in Experiment 2 was performed with IVIS Lumina II (PerkinElmer, Waltham, MA). On the termination day, L-012 luminescence probe (Wako Chemical, Neuss, Germany) was injected intraperitoneally (10 mg/kg mouse) after the ZRF injection. Light emission from intestine was measured as photons/second/cm^2/steradian 3 min after the L-012 injection with 5 min exposure time using the Living Imaging software (PerkinElmer). While presented images show both the small intestine and the colon, only the light emission from the colon was of interest and used for statistical analyses.

2.5. Quantification of LBP in plasma and LCN2 in feces using ELISA

ELISA was performed on plasma and feces from Experiment 2. Blood samples with disodium EDTA were placed on ice immediately after sampling. After centrifugation (10 min, 6000×*g*), plasma was collected and stored at −20°C until further processing. Plasma levels of lipopolysaccharide (LPS) binding protein (LBP) as a surrogate marker for LPS [37] were measured with Mouse LBP Quantification ELISA kit (Biometric, Greifswald, Germany) after diluting the plasma samples 800 times. After sampling, feces was stored at −80°C until further processing. Levels of lipocalin 2 protein (LCN2) in feces were used as an indicator of DSS-induced colon inflammation. Mouse Lipocalin-2/NGAL DuoSet ELISA and DuoSet ELISA Ancillary Reagent Kit 2 (R&D Systems, Minneapolis, MN) were used following the manufacturer's protocol. Feces was processed as described by Chassaing et al. [34] and supernatants were diluted between 20 and 20,000 times prior to the assay procedure. For both assays, levels of target protein were estimated using standard curves created using 4-parameter logistic curve fit and samples were analyzed in duplicates.

2.6. Histology

Histological analyses were performed on colon tissue from three mice per group from Experiment 2. Colon segments were fixed as a *swiss-roll* [38]. Briefly, the colon lumen was washed out with modified Bouin's fixative (50% ethanol, 5% acetic acid in dH_2O) and opened longitudinally. Segments were rolled into *swiss-roll* arrangement, with the luminal side facing inwards. Samples were kept in 10% buffered formalin overnight at room temperature and then stored in 70% ethanol at 4°C until standard ethanol dehydration procedures and paraffin embedding. Samples were cut with 7 μm thickness and stained with hematoxylin and eosin to be analyzed under an optical light microscope. The histological evaluation was based on the infiltration of immune cells in the lamina propria, space between epithelial cells bases and muscularis mucosa, and crypt structure, parameters commonly observed during DSS-induced colon inflammation [16,36].

2.7. RNA isolation and mRNA expression analyses of lamina propria

Lamina propria samples were placed in RNAlater (Sigma-Aldrich, St. Louis, MO) directly after sampling, stored at 4°C for 24 h, and then stored at −20°C until further processing. For RNA extraction, NucleoSpin RNA/Protein Purification kit (Macherey-Nagel, Düren, Germany) was used. DSS is known to inhibit both reverse transcriptase and PCR reactions [39,40]. Extracted RNA was therefore cleaned following the lithium chloride method as recommended [40]. Reverse transcriptase conversion of cleaned RNA to cDNA was performed using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). For the qPCR reactions, we used HOT FirePol EvaGreen qPCR Supermix (Solis BioDyne, Tartu, Estonia) and measured fluorescence in LightCycler 480 Instrument II (Roche, Basel, Switzerland). Samples were analyzed in duplicates. LinRegPCR Software (version 2018.0) [41] was used to calculate quantification cycle (Cq) values based on a common threshold for all primers and individual primer efficiencies. The following target genes (*symbol*) were assessed: Tumor necrosis factor alpha (*Tnfa*), interleukin 6 (*Il6*), prostaglandin-endoperoxide synthase 2 (*Ptgs2*), interleukin 1 beta (*Il1b*), lipocalin 2 (*Lcn2*), cytochrome *b*-245 (*Cybb*, also known as *Nox2*), dual oxidase 2 (*Duox2*), inducible nitric oxide synthase 2 (*Nos2*, also known as *iNOS*), and NADPH oxidase 1 (*Nox1*, Experiment 1 only). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as reference, and relative mRNA expression for each target gene in each sample was calculated using the formula $R = (E_{target}{}^{(-Cq_{target})})/(E_{reference}{}^{(-Cq_{reference})})$ where E denotes the primer efficiency. See Tables S3–S7 for reagents, primers, and temperature cycles for cDNA synthesis and qPCR. Results are only presented for proximal colon.

2.8. DNA extraction from feces and colon tissue

Fecal pellets (~0.05 g) and colon tissue from Experiment 2 were placed in S.T.A.R buffer (300 μL, Roche) with acid-washed glass beads (app. 0.2 g, <106 m, Sigma-Aldrich) directly after sampling and stored at −80°C until further processing. Later, the fecal samples were added an additional 300 μL S.T.A.R buffer to obtain the same volumes (600 μL) as used during method testing. All samples were processed twice on FastPrep 96 (1800 rpm, 40 s, 5 min cooling step in-between, MP Biomedicals, Irvine, CA) to obtain cell lysis. Processed samples were centrifuged (~13,226×*g*, 10 min) and 50 μL supernatants were transferred to 96-well plates for protease treatment followed by DNA extraction using Mag Midi LGC kit (LGC Genomics, Teddington, UK) according to the manufacturer's protocol on a KingFisher Flex DNA extraction robot (Thermo Fisher Scientific, Waltham, MA).

2.9. Library preparation and gene sequencing of 16S rRNA

A similar workflow of 16S rRNA gene sequencing has been reported by others [42]. After DNA extraction, 16S rRNA genes were amplified by PCR using prokaryote-targeting primers (target region V3–V4, Table S8&S9). As DSS in feces has inhibitory effects on PCR (identified through dilution series on qPCR), we diluted the extracted DNA from fecal samples 1:4 prior to amplicon PCR (total dilution of 1:100 in PCR reaction). PCR products (~466 bp) were purified with AMPure XP (Beckman-Coulter, Indianapolis, IN) and 10 further PCR cycles with index primers modified with Illumina adapters were performed (Tables S10–S12), resulting in PCR products of ~594 bp. All PCR products were qualitatively confirmed by gel electrophoresis. Quantification, normalization, and pooling of individual libraries were followed by purification by AMPure XP and quantification of the pooled library. The pooled library was diluted to 6 pM and sequenced with the MiSeq Reagent Kit V3 (cat. nr. MS-102-3003, Illumina, San Diego, CA) on the Illumina MiSeq following Illumina's protocol (16S Metagenomic Sequencing Library Preparation Part# 15044223 Rev. B), except we used nuclease free-water instead of Tris for PhiX library dilution. 20% PhiX served as an internal control.

2.10. Processing of 16S rRNA gene sequencing data

The resulting 300 bp paired-end reads from gene sequencing of 16S

rRNA were paired-end joined and split into their respective samples, quality-filtered using QIIME [43], and clustered into taxonomically assigned operational taxonomic units (OTUs) with ≥97% identity using closed-reference *usearch* algorithm (version 8) [44,45] against the SILVA database (version 128) [46]. The resulting dataset included 2,392,173 high-quality and chimera-checked sequences from the 78 samples (8644 to 61,079 sequences/sample). The OTU counts for each sample were normalized by even subsampling (rarefaction) in QIIME with 6500 sequences per sample as normalization cut-off. In the normalized dataset, 538 OTUs were identified in total (rarefaction curves in Fig. S1). The OTUs were taxonomically binned into phylum- and genus-level abundance tables. Abundances of bacterial taxa are presented as relative abundances (%) where the lowest detectable abundance was 0.015%.

2.11. Alpha- and beta-diversity

Measures of bacterial diversity within (α-diversity; number of observed species, Shannon-Wiener index, and evenness) and between (β-diversity; Bray-Curtis and weighted UniFrac distances [47]) samples were calculated based on the normalized OTU table.

The number of observed species in one sample was calculated as the number of OTUs with sequence count >0. The Shannon-Wiener index for one sample was calculated as $H = -\sum_{i=1}^{s}(p_i ln(p_i))$, where s denotes the number of OTUs with sequence count >0 and p_i the proportion of the community represented by OTU number i. Equitability (evenness) for one sample was calculated as $E = H/H_{max}$ where $H_{max} = \ln(\text{number of OTUs with sequence count} > 0)$.

β-diversities (Bray-Curtis and weighted UniFrac distances) between samples were calculated using QIIME default scripts (core_diversity_analyses.py). Analyses of β-diversities were conducted in R [48] (version 4.1.2). Ordination of β-diversities by non-metric multidimensional scaling (NMDS) was performed using the metaMDS() function from the *vegan* package [49] (version 2.5–7), with autotransform=FALSE and try=100. Ordination of β-diversities by principal coordinate analyses (PCoA) was performed using the cmdscale () function from the *stats* package [48] (version 4.1.2) with k=2. Global PERMANOVA of β-diversities was performed using the adonis() function from the *vegan* package with permutations=999, p.method="BH", and nperm=999 (four groups: WT, WT + 1% DSS, NOX1 KO, and NOX1 KO + 1% DSS). Dispersion homogeneity between groups was assessed using the function betadisper() from the *vegan* package. Significant global PERMANOVA was followed by pairwise PERMANOVA, performed by applying the pairwise.perm.manova() function from the *RVAideMemoire* package (version 0.9–79) [50] with nperm=999 and p.method="BH". The p-values reported from global and pairwise PERMANOVA are the mean p-values from 100 PERMANOVA runs. PERMANOVA was performed separately for feces and colon tissue.

2.12. Statistics

All statistical analyses, except PERMANOVA (section 2.11) and linear discriminant analysis (LDA) effect size (LEfSe), were performed using GraphPad Prism (version 9.3.0 for Windows, GraphPad Software, San Diego, California USA). All reported p-values are two-tailed where $p<0.05$ was considered significant.

Colon length and mRNA expression data from Experiment 1 (0, 0.5, 1 and 2% DSS in WT mice) were analyzed using one-way ANOVA while body weight change was analyzed using repeated measures 2-way ANOVA. Significant effect of DSS in one-way ANOVA was followed by pairwise comparison of the four different DSS doses using Tukey's test for multiple comparisons. Significant effect of day and DSS dose in the repeated measures 2-way ANOVA was followed by pairwise comparison of DSS doses within each day using Tukey's test for multiple comparisons. mRNA expression data were log10-transformed to obtain normally distributed model residuals with equal residual variance between groups.

Colon length, L-012 signal, mRNA expression, protein biomarkers, α-diversity, and phylum abundance data from Experiment 2 (effects of 1% DSS treatment and genotype) were analyzed using 2-way ANOVA when appropriate while body weight change was analyzed using repeated measures 3-way ANOVA. Significant interactions between treatment and genotype in 2-way ANOVA were followed by the assessment of simple main effects (effect of treatment within each genotype and effect of genotype within each treatment) with Bonferroni correction for multiple comparisons. mRNA expression, protein biomarkers, and some phylum abundance data (specified in figure captions) were log10-transformed to obtain normally distributed model residuals with equal residual variance between groups. In cases where residuals from 2-way ANOVA were not reasonably normally distributed or when equality of residual variance between groups could not be reasonably assumed (even after data transformation), comparisons of genotypes (WT versus NOX1 KO) were performed within each treatment (0 and 1% DSS) using a model suitable for each case (*t*-test, Welch's *t*-test, Mann-Whitney test, or Fisher's exact test, specified in figure captions).

Normality of model residuals was evaluated using Q-Q plot and Shapiro-Wilk test and homoscedasticity of model residuals was evaluated using residual plots and Brown-Forsythe test. Data are presented as individual values (with some exceptions) with group means ± standard error of the mean (SEM) or geometric group mean ×/÷ geometric SD factor in cases where statistical analyses were performed on log10-transformed data.

To identify differentiated bacterial genera between groups, we used LEfSe [51] available at https://huttenhower.sph.harvard.edu/galaxy/, with $p<0.05$ and the LDA effect size 2.0 to explain differences between groups.

3. Results

3.1. Establishment of the low-grade colon inflammation model

The main aim of the study was to investigate the role of NOX1-dependent ROS/RNS production in the colon during steady-state and DSS-induced low-grade colon inflammation. The criteria set for a relevant inflammation model were few or no visible signs of disease together with a moderate up-regulation of inflammation-related genes in the colonic mucosa. To find the optimal time-dose for inducing low-grade colon inflammation, we treated WT mice with 0, 0.5, 1, or 2% DSS for eight days (Experiment 1).

Mice treated with the highest dose of 2% DSS had significant weight loss after six days (Fig. S2A). Mice treated with 0.5 or 1% DSS showed only marginal or no weight loss and did not differ significantly from untreated mice. From day four, 2% DSS-treated mice presented poorer stool quality i.e., loose consistency and traces of blood, while 1 and 0.5% DSS-treated mice only presented minor changes by day six and eight, respectively. At termination (day eight), 2% DSS-treated mice had significantly shorter colons than all other groups, whereas the colon length of 0.5 and 1% DSS-treated mice were not different from untreated mice (Fig. S2B).

Compared to untreated mice, DSS-treated groups had a dose-dependent higher mRNA expression of the inflammation-related genes *Tnfa*, *Ptgs2*, *Il6*, *Il1b*, and *Lcn2*, and of the ROS/RNS-related genes *Nox2*, *Duox2*, and *iNOS* in the colonic mucosa at termination (Figs. S3A–H). The lowest dose of 0.5% DSS was not associated with higher expression of the inflammation- or ROS/RNS-related genes, except for *Tnfa*. Expression of *Nox1* was unaffected by all DSS doses tested (Fig. S3I).

Based on the results from Experiment 1, we concluded that treatment with 1% DSS for six days would be a suitable dose and duration to induce low-grade colon inflammation due to lack of marked clinical signs while high enough to significantly up-regulate inflammation- and relevant ROS/RNS-related genes.

3.2. NOX1 is important for ROS/RNS production in the colon

For the main experiment, where WT and NOX1 KO mice were treated with 0 or 1% DSS for six days (Experiment 2), we first sought to investigate whether the colonic ROS/RNS production was dependent on NOX1, especially during DSS-induced inflammation. We hypothesized that the ROS/RNS levels in NOX1 KO mice would be significantly lower than in WT mice, mainly due to lack of NOX1-dependent peroxynitrite formation.

L-012 luminescence from *ex vivo* imaging of the colons at termination (day six) was used as a sensor of ROS/RNS (Fig. 2A&B). Regardless of genotype, the L-012 mediated ROS/RNS signal was higher in DSS-treated mice than in untreated mice. Furthermore, in agreement with our hypothesis, the ROS/RNS signal was significantly lower in NOX1 KO than in WT mice, both with and without DSS treatment. In untreated and DSS-treated mice, the average (median) ROS/RNS signal in WT mice was ~2 and ~5 times higher, respectively, than in NOX1 KO mice.

Next, we investigated the importance of the ROS/RNS-related genes *iNOS*, *Duox2*, and *Nox2* for the ROS/RNS production by assessing the mRNA expression in the colonic mucosa at termination. All these genes encode enzymes which can contribute to the L-012 signal (i.e., ROS/RNS production) through different reaction pathways. Expression of *iNOS* was higher in DSS-treated than in untreated mice, but no difference was found between WT and NOX1 KO mice (Fig. 2C). *Duox2* expression was only moderately elevated in DSS-treated mice with no differences between WT and NOX1 KO mice (Fig. 2D) while expression of *Nox2* was unaffected by both DSS treatment and genotype (Fig. 2E). The median expression of *iNOS* and *Duox2* was about 40 and 2 times higher, respectively, in DSS-treated compared to untreated mice.

Taken together, these data show that NOX1-deficiency abolished ROS/RNS-dependent L-012 luminescence despite DSS-induced high expression of both *iNOS* and *Duox2*. Additionally, we show that expression of *iNOS* and *Duox2* was not dependent on NOX1.

3.3. NOX1 has minor impact on the susceptibility for DSS-induced low-grade colon inflammation

Since *ex vivo* imaging with L-012 indicated reduced ROS/RNS production in the NOX1 KO mice, we next asked whether the lack of NOX1-dependent ROS/RNS would affect the severity of the 1% DSS-induced low-grade colon inflammation. We hypothesized that NOX1 would have a protective role where NOX1 KO mice would be more susceptible to inflammation.

Six days of 1% DSS treatment did not result in significant changes in body weight for WT or NOX1 KO mice (Fig. S4A). Most of the DSS-treated mice displayed changes in stool quality from day four and they had reduced colon length at termination compared to untreated mice, but these changes were not different between WT and NOX1 KO mice (Fig. S4B).

To further evaluate the severity of the inflammation, we measured the mRNA expression of the inflammation-related genes *Tnfa*, *Il6*, *Ptgs2*, *Il1b*, and *Lcn2* in the colonic mucosa at termination. All genes were significantly higher expressed in DSS-treated than in untreated mice (Fig. 3A–E). Further, for *Tnfa*, there was a significant interaction

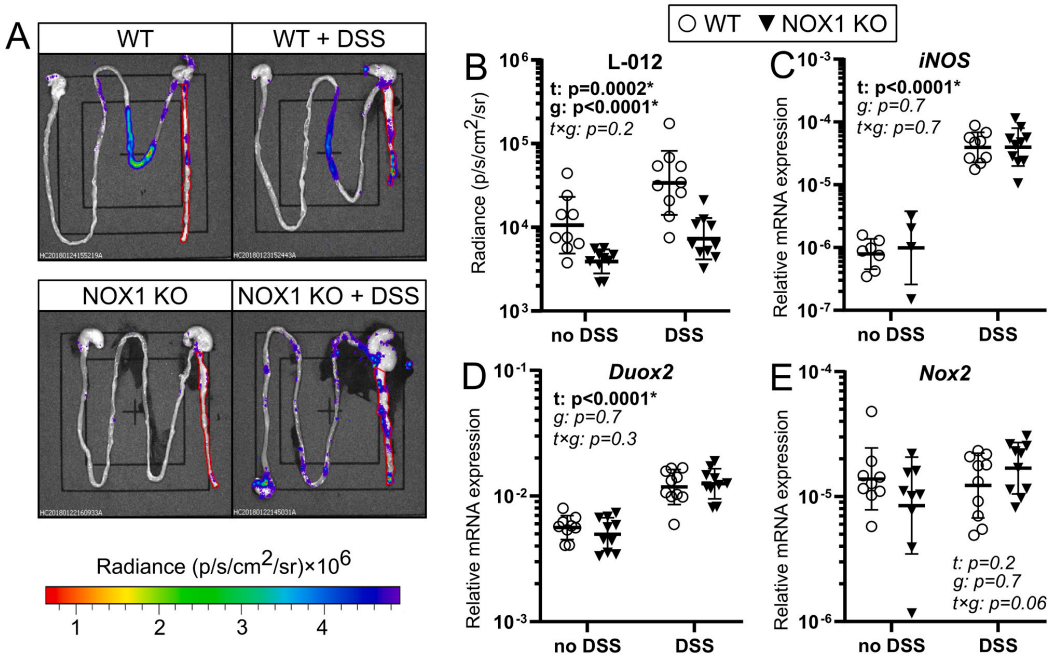

Fig. 2. ROS/RNS production in the colon of WT and NOX1 KO mice with or without 1% DSS treatment for six days. (A) Representative *ex vivo* images of one animal from each of the four groups after injection with L-012. Pseudo colors represent light intensity expressed as photons/sec/cm^2/steradian (p/s/cm^2/sr). Red markings show the region of interest (ROI) in the colon. (B) L-012-induced chemiluminescence from the colon ROI expressed as average radiance (p/s/cm^2/sr). n=9–10 per group. (C–E) Relative mRNA expression of (C) inducible nitric oxide synthase 2 (*iNOS*) (D), dual oxidase 2 (*Duox2*), and (E) cytochrome *b*-245 (*Nox2*) in mucosa from the proximal colon. n=4–10 per group. P-values from 2-way ANOVA (main effect of treatment (t; no DSS versus 1% DSS) and genotype (g; WT versus NOX1 KO), and interaction effect (t×g)) on log10-transformed data. Horizontal lines and whiskers are geometric group mean ×/÷ geometric SD factor. ROS, reactive oxygen species; RNS, reactive nitrogen species, WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium. *p<0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

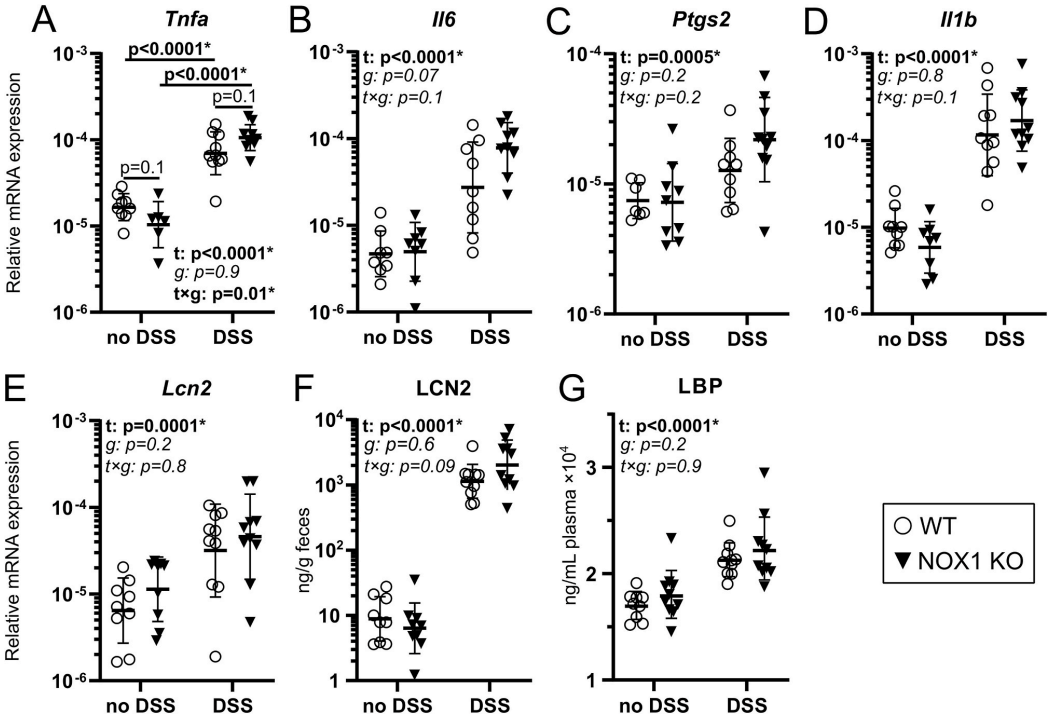

Fig. 3. Mucosal inflammation and intestinal barrier integrity of WT and NOX1 KO mice with or without 1% DSS treatment (6 days). (A–E) Relative mRNA expression of (A) tumor necrosis factor alpha (*Tnfa*), (B) interleukin 6 (*Il6*), (C) prostaglandin-endoperoxide synthase 2 (*Ptgs2*), (D) interleukin 1 beta (*Il1b*), and (E) lipocalin 2 (*Lcn2*) in mucosa from the proximal colon. n=6–10 per group. (F) Concentration of fecal lipocalin 2 protein (LCN2). n=9–10 per group. (G) Concentration of lipopolysaccharide binding protein (LBP) in plasma. n=9–10 per group. P-values from 2-way ANOVA (main effect of treatment (t; no DSS versus 1% DSS) and genotype (g; WT versus NOX1 KO), and interaction effect (t×g)) on log10-transformed data. In cases with significant interaction, p-values for simple main effects from post-hoc tests with Bonferroni correction for multiple comparisons are presented. Horizontal lines and whiskers are geometric group mean ×/÷ geometric SD factor. WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium. *p<0.05.

between treatment and genotype, indicating a stronger DSS-induced up-regulation of *Tnfa* in NOX1 KO than in WT mice (Fig. 3A). Similar trends were observed for *Il6* (Fig. 3B) and *Ptgs2* (Fig. 3C).

In addition to mRNA expression, we also investigated if the protein levels of LCN2 in feces were different between DSS-treated WT and NOX1 mice, as fecal LCN2 has been shown to be a sensitive marker of colon inflammation [34]. As for *Lcn2* mRNA expression, the LCN2 protein levels were higher in DSS-treated than in untreated mice. Furthermore, DSS-treated NOX1 KO mice tended to have higher LCN2 levels than DSS-treated WT mice, although not significantly (Fig. 3F). As a measure of intestinal barrier breach, commonly associated with colon inflammation, we also measured the protein levels of LBP in plasma. LBP levels were higher in DSS-treated than in untreated mice, but no differences were found between WT and NOX1 KO mice (Fig. 3G).

Finally, we evaluated the structural impacts of the DSS treatment on colon tissue in a selection of WT and NOX1 KO (n=3 per group). Both WT and NOX1 KO mice displayed similar mild signs of colon inflammation in response to DSS, with infiltration of immune cells in lamina propria and increased space between epithelial cell bases and muscularis mucosa (Fig. 4). In mice not treated with DSS, no signs of colon inflammation were observed in neither of the genotypes.

3.4. NOX1 affects the community structure and diversity of the colonic microbiota

To assess the role of NOX1 on the colonic microbiota both during steady-state and inflammatory conditions, we performed 16S rRNA gene sequencing of both feces and colon tissue samples, representing the luminal- and mucosa-associated microbiota, respectively.

First, two different measures of between-sample diversity (β-diversity) were used to assess the overall differences in bacterial community structures between the four groups of mice: 1) Bray-Curtis distances (based on OTU abundances) and 2) weighted UniFrac distances (abundance-weighted phylogenetic distances). As illustrated in NMDS ordination plots in Fig. 5A&C, feces samples clustered significantly according to both DSS treatment and genotype for both Bray-Curtis and weighted Unifrac distances, where all four groups were significantly different from each other. For colon tissue, we also found significant clustering according to both DSS treatment and genotype when applying Bray-Curtis distances (Fig. 5B) while for weighted Unifrac, samples clustered only according to treatment with no difference between WT and NOX1 KO mice (Fig. 5D). The same clustering patterns were obtained when applying PCoA as ordination method (Fig. S5).

We next examined the effect of NOX1 and DSS treatment on fecal bacterial diversity (richness and evenness) by applying three selected indices of within-sample diversity (α-diversity): 1) The Shannon-Wiener index (richness and evenness combined; Fig. 6A), 2) number of observed species (richness; Figs. 6B), and 3) evenness (Fig. 6C). Untreated NOX1 KO mice had lower diversity than untreated WT mice for all the three indices. Further, regardless of genotype, DSS-treated mice had higher Shannon-Wiener index and evenness compared to untreated mice, while number of observed species was unaffected by DSS treatment.

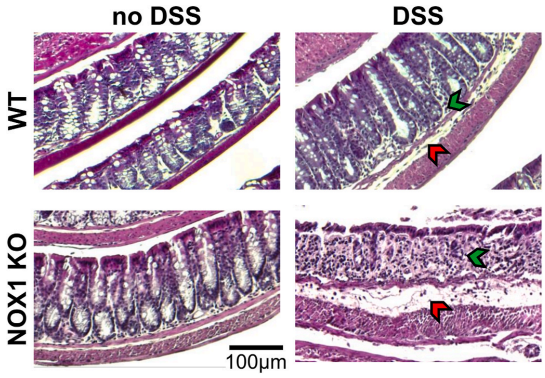

Fig. 4. Colon tissue of WT and NOX1 KO mice with or without 1% DSS treatment (6 days). Representative images (n=3 per group) except from DSS-treated NOX1 KO mouse. In this case we present the most severe observed case. The other DSS-treated NOX1 KO mice could not be distinguished from the DSS-treated WT mice. Green arrows indicate the infiltration of immune cells into the lamina propria. Red arrows indicate increased space between epithelial cell bases and muscularis mucosa. Colon sections (7 μm) were stained with hematoxylin and eosin. WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Interestingly, with DSS treatment, the difference in evenness between WT and NOX1 KO mice observed in untreated mice was no longer present. Similar patterns were found for colon tissue, although not as prominent (Fig. 6D–F).

In conclusion, the results on β- and α-diversity illustrate 1) that both NOX1 and DSS treatment highly affects the overall structures of the bacterial communities, 2) that NOX1 KO mice generally display a lower bacterial diversity than WT mice, and 3) that the differences between WT and NOX1 KO mice are more prominent for the luminal-associated microbiota.

3.5. NOX1 affects colonic bacterial composition at the phylum level

Based on the assessment of overall bacterial community structure and diversity, it was clear that the NOX1 KO and WT mice had considerable differences in the colonic microbiota, both during steady-state and inflammatory conditions. To obtain a more detailed understanding of these differences, we first assessed the effects of NOX1 and DSS treatment on the relative bacterial abundances at the phylum level (Fig. 7A). Only phyla with average relative abundance above 1% in at least one group were included in the analyses (individual plots for all analyzed phyla in Fig. S6).

In both feces and colon tissue, representing the luminal- and mucosa-associated microbiota, respectively, the main effects of DSS treatment were higher abundance of Firmicutes and Tenericutes, while lower abundance of Bacteroidetes and Proteobacteria. No main effect of genotype was found for any of the above-mentioned phyla. However, in feces, we found significant interaction between treatment and genotype for Bacteroidetes (Fig. 7B, Fig. S6B) and Firmicutes (Fig. 7C, Fig. S6A). Untreated NOX1 KO mice had lower abundance of Bacteroidetes than untreated WT mice, while there was no difference between the genotypes when treated with DSS, mainly due to reduced abundance in the WT mice. For Firmicutes, no significant differences were found between NOX1 KO and WT mice, but a tendency towards higher abundance in untreated NOX1 KO than in untreated WT mice. In addition to the higher Firmicutes/Bacteroidetes ratio in NOX1 KO mice, the phylum Verrucomicrobia was significantly more abundant in feces from NOX1 KO than from WT mice, both with and without DSS treatment (Fig. 7D, Fig. S6I). Furthermore, WT mice treated with DSS had higher abundance of Verrucomicrobia than untreated WT mice, but the levels were still lower than in NOX1 KO mice. The Verrucomicrobia abundance showed similar patterns in colon tissue.

3.6. NOX1 affects colonic bacterial composition at the genus level

To assess the effect of NOX1 and DSS treatment on the relative abundances at the genus level, we performed LEfSe separately for feces and colon tissue for the four relevant comparisons: comparison of genotypes (WT and NOX1 KO) within each treatment condition (0 and 1% DSS), and comparison of treatments within each genotype. From the LEfSe results (Figs. S7–S10), 21 genera in total were identified as different between NOX1 KO and WT mice in feces and/or colon tissue. Twelve LEfSe-identified genera were considered abundant (average abundance above 0.1% in feces and/or colon tissue, Fig. 8) while nine were low abundant (Fig. S11).

Of the abundant LEfSe-identified genera, *Lachnospiraceae FCS020 group* was the only one to be unaffected by DSS treatment itself and the abundance was generally lower in NOX1 KO than in WT mice (Fig. 8A). The genera *Faecalibaculum*, *Lachnospiraceae UCG-001*, and *unknown Bacteroidales S24-7 group* (identical to family *Bacteroidales S24-7 group*) had, with some exceptions, lower abundance in NOX1 KO than in WT mice and were decreased by DSS treatment in both genotypes (Fig. 8B–D). *Alistipes* (identical to family *Rikenellaceae*) was overall decreased by DSS, but more strongly in NOX1 KO mice, resulting in lower abundance of this genus in NOX1 KO mice compared to WT when treated with DSS (Fig. 8E). The abundance of *Akkermansia* (identical to phylum Verrucomicrobia, see section 3.5) was much higher in NOX1 KO mice compared to WT both in untreated and DSS-treated mice, and DSS led to enrichment of *Akkermansia* only in the WT mice (Fig. 8F). In untreated mice, *Oscillibacter* and *Ruminococcaceae UCG*-005 had similar abundances between WT and NOX1 KO mice, while DSS led to enrichment only in the NOX1 KO mice (Fig. 8G&H). Three genera *Ruminococcus1*, *unknown Peptococcaceae* and *unknown MollicutesRF9* (identical to order MollicutesRF9) showed an abundance pattern where DSS treatment led to enrichment, but only the WT mice (Fig. 8I–K). Finally, NOX1 KO mice had lower abundance of *Blautia* compared to WT when not treated with DSS, and while DSS treatment led to decreased levels in WT mice, *Blautia* was enriched in DSS-treated NOX1 KO mice (Fig. 8L).

Of the low abundant genera identified by LEfSe, two genera showed particularly distinct differences between NOX1 KO and WT mice. *Ruminiclostridium1* was only detected in NOX1 KO mice (Fig. S11A), while *Peptococcus* was almost exclusively found in WT mice (Fig. S11B).

4. Discussion

As a primary producer of ROS/RNS in colonic epithelial cells, NOX1 contributes to intestinal homeostasis [16,21–24] and its role could be particularly relevant when combined with other ROS/RNS-generating enzymes such as iNOS [8]. In this study, we hypothesized that NOX1-dependent ROS/RNS production influences the severity of low-grade inflammation and bacterial composition in the colon. To explore this hypothesis, we investigated the effects of NOX1-deficiency on ROS/RNS production, markers of inflammation, and bacterial composition in the colon both during steady-state and in 1% DSS-induced subclinical low-grade colon inflammation. While reduced levels of ROS/RNS in the colon of NOX1 KO mice only marginally aggravated the severity of inflammation, the colonic microbiota was highly affected by the lack of NOX1-depedent ROS/RNS.

As a marker of extracellular ROS/RNS in the colon, we used L-012, a chemiluminescent molecule that emits light when it reacts with various ROS/RNS, most prominently peroxynitrite, hypochlorous acid and hydroxyl radical [52–57]. While DSS treatment increased colonic ROS/RNS levels, the levels were substantially lower in NOX1 KO mice than in WT. Reduced L-012 signal in the colon of NOX1-deficient mice in

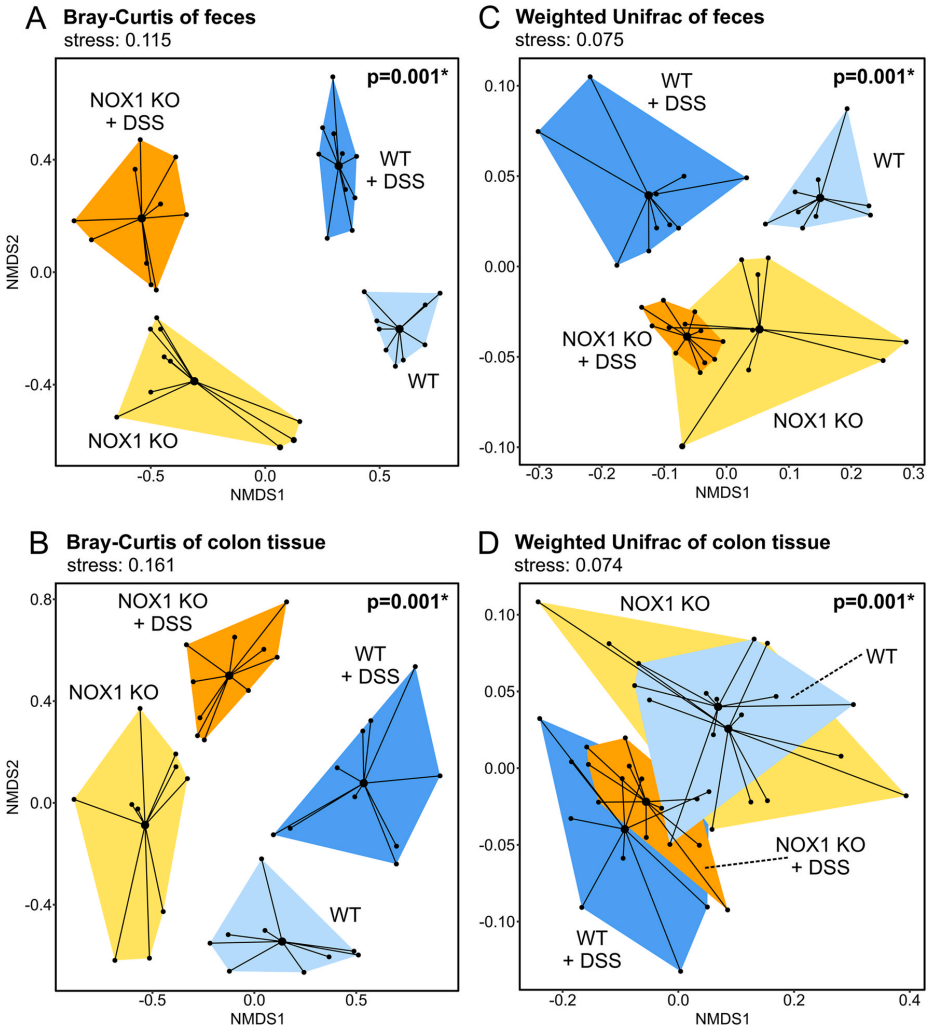

Fig. 5. Bacterial β-diversity between WT and NOX1 KO mice with or without 1% DSS treatment (6 days). NMDS ordination plots of (A,B) Bray-Curtis and (C,D) weighted UniFrac distances between (A,C) feces and (B,D) colon tissue samples. Colors indicate which group individual samples belong to. Stress values indicate the NMDS goodness-of-fit. n=9–10 per group. P-values in graph from global PERMANOVA. For data presented in A, B, and C, pairwise PERMANOVA showed a significant difference between all four groups (all $p \leq 0.007$). For data presented in D, there was no difference between genotypes in neither treatment condition (no DSS: all $p > 0.2$; DSS: all $p > 0.7$), only across treatment independent of genotype (all p's< 0.03). WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium; NMDS, non-metric multidimensional scaling. *$p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

steady-state conditions has been reported by others [11,58], but we show here that NOX1 is also important for colonic ROS/RNS production during DSS-induced inflammation. Together with the marked DSS-induced up-regulation of *iNOS* mRNA, but not of *Nox1*, *Nox2*, or *Duox2*, our results suggest that the DSS-increased L-012 signal in WT mice originated primarily from peroxynitrite, formed by NOX1-and iNOS-dependent production of superoxide and nitric oxide, respectively. Still, although *Nox1* expression was not affected by DSS treatment, we cannot claim that iNOS alone was responsible for the increased L-012 signal, as colitis can cause increased protein expression [16] and enzyme activity [59] of NOX1. Further, since DSS also increased the L-012 signal in the absence of NOX1, although modestly, it appears that peroxynitrite was not the sole source of the L-012 signal. One possible explanation for this observation is that *Duox2,* which can give rise to hydroxyl radical and hypochlorous acid via hydrogen peroxide, was modestly up-regulated by DSS. The formation of hypochlorous acid from hydrogen peroxide is catalyzed by myeloperoxidase, which can be induced by 1% DSS [34]. Lastly, deletion of NOX1 may cause up-regulation of other NOXs such as NOX3 [28] as a compensatory mechanism that can also contribute to the ROS/RNS mediated L-012 signal.

Even though the ROS/RNS production was clearly reduced in NOX1 KO mice, these mice did not have higher susceptibility towards low-grade inflammation than WT with regard to crude inflammatory

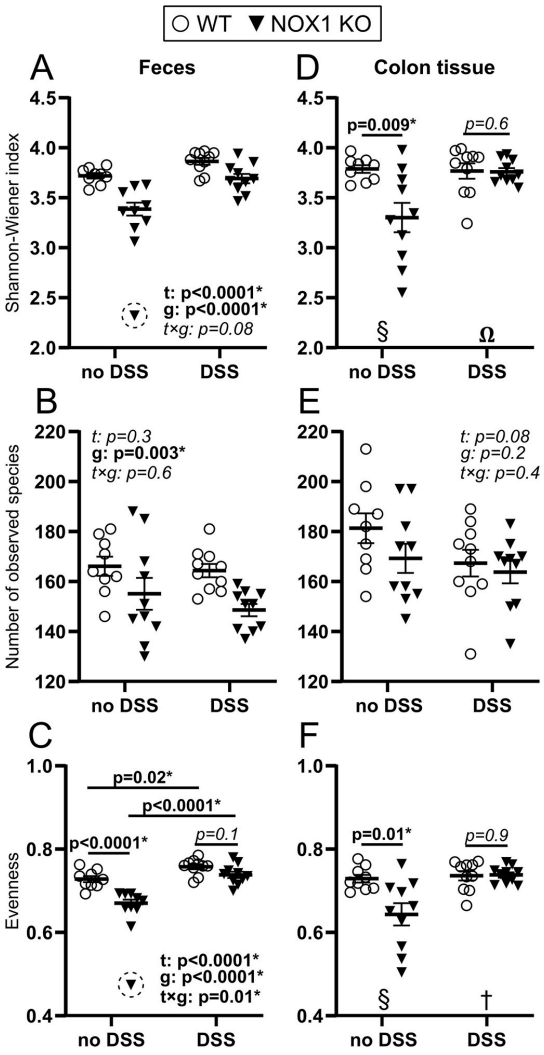

Fig. 6. Bacterial α-diversity in the colon of WT and NOX1 KO mice with or without 1% DSS treatment (6 days). (A,D) Shannon-Wiener index, (B,E) number of observed species (OTUs), and (C,F) evenness (equitability) of bacterial communities in (A–C) feces and (D–F) colon tissue. P-values from 2-way ANOVA (main effect of treatment (t; no DSS versus 1% DSS) and genotype (g; WT versus NOX1 KO), and interaction effect (t×g)). In cases with significant interaction, p-values for simple main effects from post-hoc tests with Bonferroni correction for multiple comparisons are presented. In two cases (A and C), extreme value was excluded from statistical analysis (dotted circles). When included, the significance of the interaction increased for the Shannon-Wiener index (p=0.06) and decreased for evenness (p=0.02). In cases where 2-way ANOVA could not be performed due to heteroskedasticity and/or violation of normality assumption alternative tests were used within the two treatment groups: (§) Welch's *t*-test, (Ω) Mann-Whitney test, or (†) *t*-test. n=9–10 per group. Horizontal lines and whiskers are group mean ± SEM. WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium; OTU, operational taxonomic unit. *p<0.05.

disease markers evaluated by body weight, colon length, stool quality, and histology. These observations are in line with other studies of NOX1-deficient mice using higher DSS concentrations [21,28]. However, the lack of more pronounced inflammation in NOX1 KO mice could be attributed to a NOX3 compensatory mechanism [28], as alluded to above. Mice deficient of the enzyme NOXO1, the organizing subunit of NOX1 and NOX3 [4], display a stronger pathological response than WT mice during DSS-induced colitis [11]. Thus, in the absence of NOX1-generated superoxide, up-regulation of NOX3 may to some extent compensate for NOX1-loss.

Despite no differences between NOX1 and WT mice regarding crude signs of inflammation after DSS treatment, mRNA levels of genes encoding the pro-inflammatory mediators TNF-α, IL-6, and PTGS2 (COX2) tended to be higher in the colonic mucosa of NOX1 KO than of WT mice. In line with our results, Kato *et al.* demonstrated that NOX1 KO mice had higher COX2 expression than WT mice following DSS colitis during the restitution phase [16]. The concentration of fecal LCN2, a highly sensitive marker of colon inflammation [34] was also higher in NOX1 KO than in WT mice. Collectively, these findings indicate a slightly elevated susceptibility to DSS-induced low-grade inflammation in NOX1-deficient mice. Since LCN2 expression is dependent on bacterial exposure [60], we speculate that the absence of NOX1 weakens the epithelial barrier, resulting in closer contact between the colonic bacteria and epithelial cells which again can result in compromised epithelial layer and more LCN2 in the lumen. NOX1-generated ROS has been implicated in cytoprotection in epithelial cells, mediated through the redox-sensitive transcription factor Nrf2 [61]. Impaired barrier function in NOX1-deficient mice could therefore be related to the inability to withstand the stress imposed by DSS treatment. That Nrf2 is important for protection against DSS-induced colitis has been demonstrated previously [62], possibly by inhibiting NF-κB activation [63]. Additionally, the absence of NOX1-generated superoxide could lead to altered bacterial composition in the colon, analogous to observations of the ileum [8] with implications for LCN2 expression. Indeed, Li and coworkers showed that DSS-induced LCN2 levels were dependent on the initial gut microbiota profile [64].

Regarding the colonic microbiota, our initial hypothesis was that NOX1-dependent extracellular ROS/RNS, particularly peroxynitrite, could act as bactericidal molecules affecting the colonic microbiota, and that this effect would be most prominent during DSS-induced inflammation due to marked up-regulation of iNOS. However, the results from 16S sequencing show that the WT and NOX1 KO mice had large differences in bacterial composition both with and without DSS treatment, indicating that peroxynitrite is not decisive for NOX1-dependent changes in the microbiota. NOX1-deficiency may affect the microbiota during steady-state through more indirect effects including NOX1-dependent signaling in epithelial cells that could affect goblet cell abundance and the mucus layer [16,24].

Specifically, we found that the colonic microbiota of NOX1-deficient mice was characterized by lower fecal α-diversity and changes in the relative abundance of specific bacterial taxa. Analogously, others have demonstrated that pharmacological removal of luminal ROS/RNS in the colon can lead to decreased bacterial diversity [65], indicative of a less stable community more vulnerable to perturbations [66]. When focusing on the relative abundance of individual bacterial taxa, a general pattern was that DSS-untreated NOX1 KO mice had abundance shifts resembling those induced by DSS treatment in WT mice, characterized mainly by a higher abundance of Firmicutes and Verrucomicrobia (genus *Akkermansia*), and lower abundance of Bacteroidetes (mainly family *Bacteroidales S24-7 group*). When treated with DSS, the most striking differences between the genotypes were the higher and lower abundances of *Oscillibacter* and *Alistipes*, respectively, in NOX1 KO mice. Low abundance of both S24-7 and *Alistipes* has been found to correlate with higher levels of pro-inflammatory cytokines [67], and *Alistipes* has been suggested to have a protective role in DSS-induced colitis [67,68].

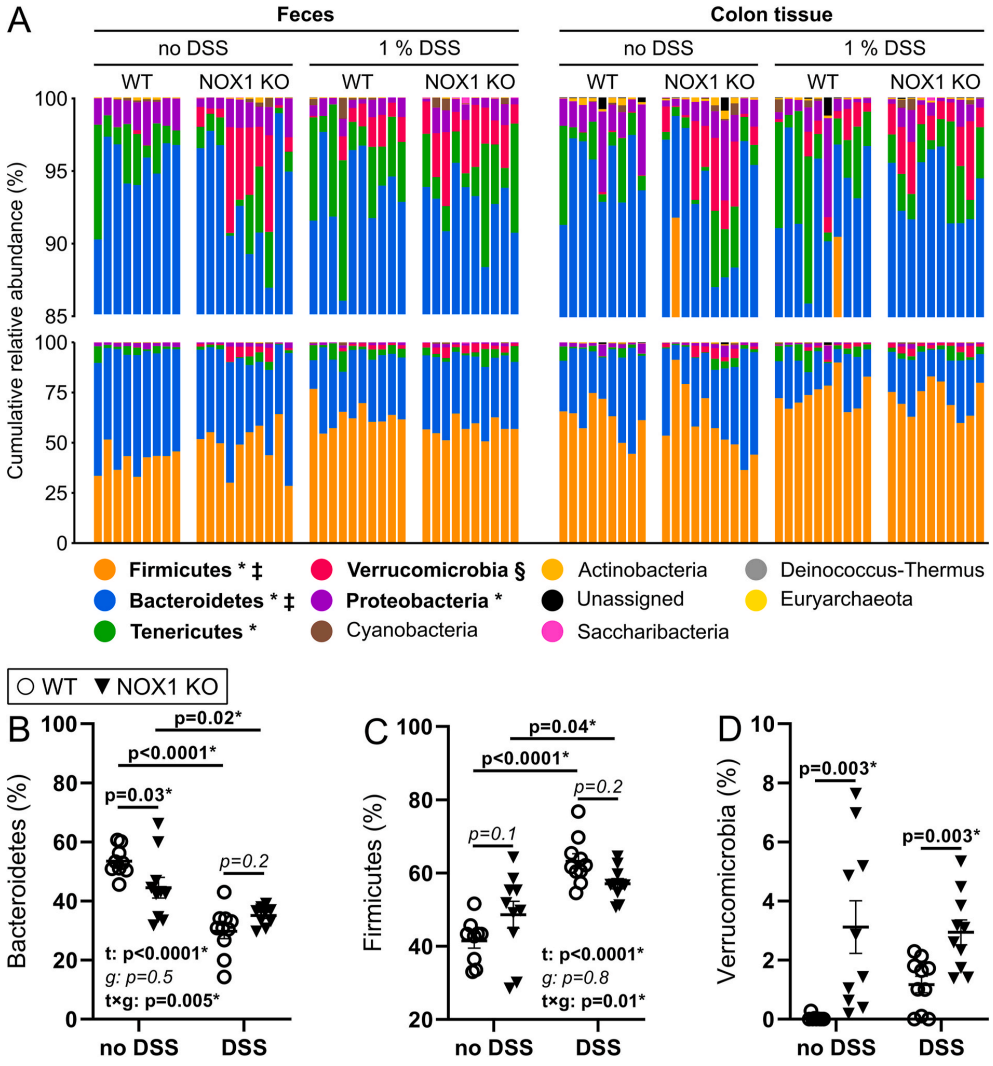

Fig. 7. Bacterial phyla characteristics of WT and NOX1 KO mice with or without 1% DSS treatment (6 days). (A) Cumulative relative abundance (%) of all detected phyla in individual feces and colon tissue samples. Phyla are sorted according to average abundance across all groups. Phyla in bold had average abundance above 1% in at least one group. *Indicates significant effect of DSS treatment for both feces and colon tissue, ‡ indicates significant interaction between genotype and treatment in feces, and § indicates higher abundance in NOX1 KO mice compared to WT mice in both feces and colon tissue. (B–D) Fecal relative abundance (%) of (B) Bacteroidetes, (C) Firmicutes, and (D) Verrucomicrobia. (B,C) P-values from 2-way ANOVA (main effect of treatment (t; no DSS versus 1% DSS) and genotype (g; WT versus NOX1 KO), and interaction effect (t×g)). Since significant interaction, p-values for simple main effects from post-hoc tests with Bonferroni correction for multiple comparisons are presented. *$p<0.05$. (D) Since 2-way ANOVA could not be performed due to heteroskedasticity and/or violation of normality assumption alternative tests were used within the two treatment groups: Fisher's exact test (no DSS) and *t*-test (DSS). Horizontal lines and whiskers are group mean ± SEM. n=9–10 per group. WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium.

The abundance of the phylum Verrucomicrobia, completely dominated by genus *Akkermansia*, was much higher in NOX1 KO than in WT mice. However, 1% DSS treatment reduced the difference between the genotypes due to a bloom of *Akkermansia* in WT mice, in line with previous observations [69–73]. *Akkermansia* spp. are anaerobic mucus-associated commensal bacteria that feeds on mucus, reported to have beneficial roles in intestinal homeostasis [74,75] and wound repair [23]. However, their role in aggravation of intestinal inflammation is debated with somewhat conflicting results [76]. While some studies have shown that *Akkermansia* supplementation reduces inflammation induced by a high-fat diet [77,78], others have suggested that *Akkermansia* contributes to enhancing colitis [64,71]. Since DSS is known to cause structural changes of the inner mucus layer of the colon making it more available for bacterial penetration [79], this could explain the increase of *Akkermansia* after DSS treatment in WT mice. Coant *et al.* observed that NOX1 KO mice had an increased number of goblet cells, and consequently more mucus protein [24], which could explain the increase in *Akkermansia* abundance in NOX1 KO mice. However, the

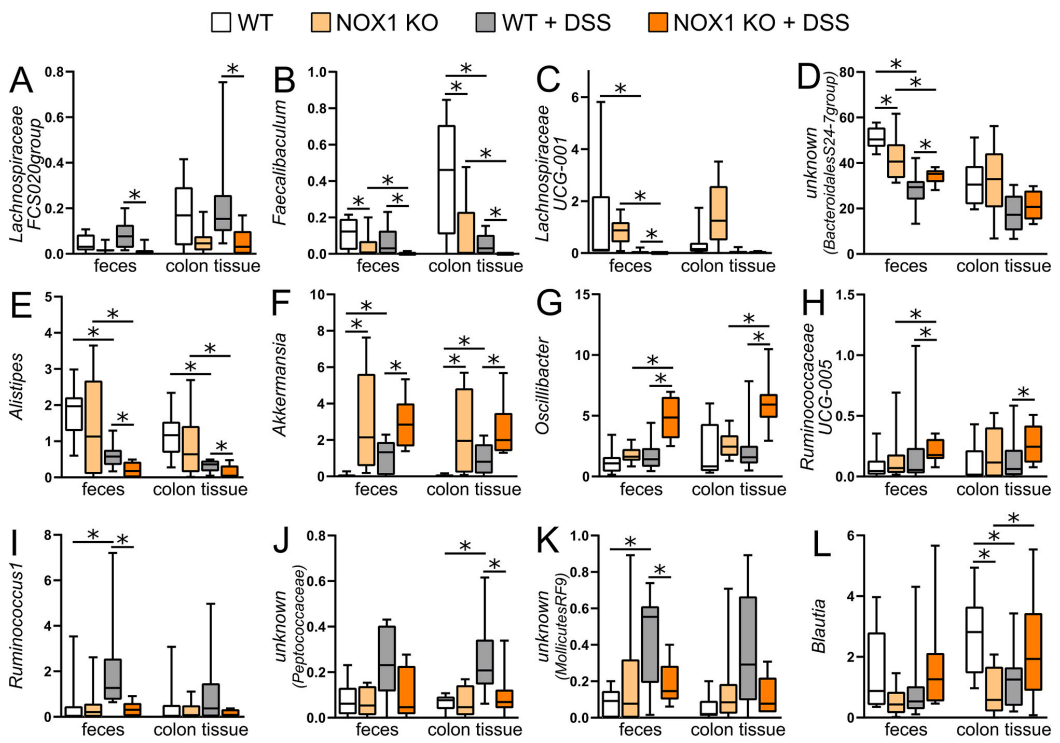

Fig. 8. Relative abundance (%) of abundant bacterial genera in feces and colon tissue from WT and NOX1 KO mice with or without 1% DSS treatment for (6 days). (A) *Lachnospiraceae FCS020 group*, (B) *Faecalibaculum*, (C) *Lachnospiraceae UCG-001*, (D) *unknown Bacteroidales S24-7group* (identical to family *Bacteroidales S24-7group*), (E) *Alistipes* (identical to family *Rikenellaceae*), (F) *Akkermansia* (identical to phylum Verrucomicrobia), (G) *Oscillibacter*, (H) *Ruminococcaceae UCG-005*, (I) *Ruminococcus1*, (J) *unknown Peptococcaceae*, (K) *unknown MollicutesRF9*, and (L) *Blautia*. n=9-10 per group. The presented genera are the ones that were found to have different abundance between WT and NOX1 KO mice in feces and/or colon tissue through LEfSe analysis (comparison of genotypes (WT, NOX1 KO) within each treatment (no DSS, 1% DSS), and between treatments within each genotype), and that had average relative abundance above 0.1% in feces and/or colon tissue. WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium; LDA, linear discriminant analysis; LEfSe, LDA effect size. *Indicates the LEfSe-identified significant comparisons.

mucus seems to be unaffected by NOX1-deficiency in more recent studies [28,80]. Why *Akkermansia* in NOX1 KO mice seems unaffected by DSS remains unclear but is perhaps related to the less extensive increase in colonic ROS/RNS during inflammation in the NOX1-deficient mice.

The genus *Oscillibacter* (order Clostridiales in phylum Firmicutes) was highly increased in DSS-treated NOX1 KO mice, but not in WT mice. The fact that *Oscillibacter* abundance increases after antibiotic treatment [81] supports the notion that *Oscillibacter* spp. are opportunistic bacteria that thrive when the normal balance is disturbed. The implication of *Oscillibacter* abundance in health and disease seems unresolved, but studies of colon inflammation suggest that increased *Oscillibacter* abundance could be related to impaired barrier function [82], increased markers of colon inflammation [67,83], and increased colitis susceptibility [64]. Thus, high abundance of *Oscillibacter* in NOX1 KO mice after DSS treatment could be interpreted as a sign of increased disease.

The findings that both *Akkermansia* and *Oscillibacter* have higher abundances in the NOX1 KO mice may be attributed to changes in the redox environment caused by the NOX1-deficiency. Intriguingly, both *Akkermansia* and *Oscillibacter* increase in abundance following a polyphenol-rich diet [84]. As polyphenols are poorly taken up in the small intestine and therefore transported to the colon, it is plausible that they act as antioxidants there and thereby create a more reducing environment. To our knowledge, redox changes in NOX1 KO compared to WT mice have not been measured, but it is pertinent to speculate that such changes occur and lack of NOX1 will therefore favor the growth of *Akkermansia* and *Oscillibacter* in a reducing environment. The fact that *Oscillibacter* is only blooming in DSS-treated NOX1 KO mice and not in those not treated with DSS is not straightforward to explain. However, as mentioned above, *Oscillibacter* is probably opportunistic and will thrive when homeostasis is disturbed [81]. Thus, when a low dose of DSS is introduced, this stimulus is not sufficient to create a niche for *Oscillibacter* unless deficiency in NOX1 creates this unbalance.

As already discussed, in the experimental setup where we compare NOX1 KO and WT mice, it is not possible to assess the direct effects of NOX1-dependent ROS/RNS-deficiency on low-grade colon inflammation due to initial differences in the colonic microbiota between the genotypes prior to DSS treatment. Treating WT mice with ROS/RNS inhibitors could therefore be an alternative approach to investigate the impact of NOX1-dependent ROS/RNS formation during inflammation, avoiding the impact of genetic background. As peroxynitrite most likely is one of the major ROS/RNS-contributors during colon inflammation, formed by NOX-dependent superoxide and iNOS-dependent nitric oxide, the NOS inhibitor L-NAME could be a suitable candidate [8].

In conclusion, this study demonstrates that the superoxide-producing enzyme NOX1 is important for the formation of colonic extracellular ROS/RNS and modulates the colonic microbiota both during steady-state and in 1% DSS-induced colonic low-grade inflammation. Further,

while NOX1 offered no protection against pathological changes induced by low-grade inflammation, analyses of inflammation-associated genes indicated a trend for enhanced inflammation in NOX1-deficient mice which was further supported by the increment of LCN2. We therefore propose that NOX1-dependent ROS/RNS have a role in shaping the colonic microbiota, with potential beneficial consequences for intestinal health.

Author contributions

AMH, SDCR and HC: designed the study; AMH, SDCR, DP and HC: performed the experiments; AMH: conducted the statistical analyses; AMH, SDCR, DP, SKB and HC: interpreted the results; AMH, SDCR, SKB and HC: wrote the manuscript; AMH: prepared the figures for the manuscript; AMH, SDCR and HC: supervised the study; AMH, SDCR and HC: prepared the animal protocol; AMH, SDCR, SKB, DP, and HC: discussed the results and edited manuscript drafts; and all authors: read and approved the final manuscript.

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Declaration of competing interest

None.

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Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2022.06.234.

References

[1] T. Finkel, Oxygen radicals and signaling, Curr. Opin. Cell Biol. 10 (2) (1998) 248–253, https://doi.org/10.1016/s0955-0674(98)80147-6.
[2] M.J. Jackson, S. Papa, J. Bolanos, R. Bruckdorfer, H. Carlsen, R.M. Elliott, J. Flier, H.R. Griffiths, S. Heales, B. Holst, M. Lorusso, E. Lund, J. Oivind Moskaug, U. Moser, M. Di Paola, M.C. Polidori, A. Signorile, W. Stahl, J. Vina-Ribes, S. B. Astley, Antioxidants, reactive oxygen and nitrogen species, gene induction and mitochondrial function, Mol. Aspect. Med. 23 (1–3) (2002) 209–285, https://doi.org/10.1016/s0098-2997(02)00018-3.
[3] S.G. Rhee, Redox signaling: hydrogen peroxide as intracellular messenger, Exp. Mol. Med. 31 (2) (1999) 53–59, https://doi.org/10.1038/emm.1999.9.
[4] K. Bedard, K.H. Krause, The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology, Physiol. Rev. 87 (1) (2007) 245–313, https://doi.org/10.1152/physrev.00044.2005.
[5] C.C. Faria, R.S. Fortunato, The role of dual oxidases in physiology and cancer, Genet. Mol. Biol. 43 (1 suppl. 1) (2020), e20190096, https://doi.org/10.1590/1678-4685/GMB-2019-0096.
[6] T.L. Leto, M. Geiszt, Role of Nox family NADPH oxidases in host defense, Antioxidants Redox Signal. 8 (9–10) (2006) 1549–1561, https://doi.org/10.1089/ars.2006.8.1549.
[7] B. Rada, T.L. Leto, Oxidative innate immune defenses by Nox/Duox family NADPH oxidases, Contrib. Microbiol. 15 (2008) 164–187, https://doi.org/10.1159/000136357.
[8] C. Matziouridou, S.D.C. Rocha, O.A. Haabeth, K. Rudi, H. Carlsen, A. Kielland, iNOS- and NOX1-dependent ROS production maintains bacterial homeostasis in the ileum of mice, Mucosal Immunol. 11 (3) (2018) 774–784, https://doi.org/10.1038/mi.2017.106.
[9] T. Schwerd, R.V. Bryant, S. Pandey, M. Capitani, L. Meran, J.B. Cazier, J. Jung, K. Mondal, M. Parkes, C.G. Mathew, K. Fiedler, D.J. McCarthy, WGS500 Consortium, Oxford IBD cohort study investigators, COLORS in IBD group investigators, UK IBD Genetics Consortium, P.B. Sullivan, A. Rodrigues, S.P. L. Travis, C. Moore, J. Sambrook, W.H. Ouwehand, D.J. Roberts, J. Danesh, INTERVAL Study, R.K. Russell, D.C. Wilson, J.R. Kelsen, R. Cornall, L.A. Denson, S. Kugathasan, U.G. Knaus, E.G. Serra, C.A. Anderson, R.H. Duerr, D.P. McGovern, J. Cho, F. Powrie, V.S. Li, A.M. Muise, H.H. Uhlig, NOX1 loss-of-function genetic variants in patients with inflammatory bowel disease, Mucosal Immunol. 11 (2) (2018) 562–574, https://doi.org/10.1038/mi.2017.74.
[10] I. Szanto, L. Rubbia-Brandt, P. Kiss, K. Steger, B. Banfi, E. Kovari, F. Herrmann, A. Hadengue, K.H. Krause, Expression of NOX1, a superoxide-generating NADPH oxidase, in colon cancer and inflammatory bowel disease, J. Pathol. 207 (2) (2005) 164–176, https://doi.org/10.1002/path.1824.
[11] F. Moll, M. Walter, F. Rezende, V. Helfinger, E. Vasconez, T. De Oliveira, F. R. Greten, C. Olesch, A. Weigert, H.H. Radeke, K. Schroder, NoxO1 controls proliferation of colon epithelial cells, Front. Immunol. 9 (2018) 973, https://doi.org/10.3389/fimmu.2018.00973.
[12] M. Geiszt, K. Lekstrom, S. Brenner, S.M. Hewitt, R. Dana, H.L. Malech, T.L. Leto, NAD(P)H oxidase 1, a product of differentiated colon epithelial cells, can partially replace glycoprotein 91 phox in the regulated production of superoxide by phagocytes, J. Immunol. 171 (1) (2003) 299–306, https://doi.org/10.4049/jimmunol.171.1.299.
[13] H. Grasberger, J. Gao, H. Nagao-Kitamoto, S. Kitamoto, M. Zhang, N. Kamada, K. A. Eaton, M. El-Zaatari, A.B. Shreiner, J.L. Merchant, C. Owyang, J.Y. Kao, Increased expression of DUOX2 is an epithelial response to mucosal dysbiosis required for immune homeostasis in mouse intestine, Gastroenterology 149 (7) (2015) 1849–1859, https://doi.org/10.1053/j.gastro.2015.07.062.
[14] M. Kamizato, K. Nishida, K. Masuda, K. Takeo, Y. Yamamoto, T. Kawai, S. Teshima-Kondo, T. Tanahashi, K. Rokutan, Interleukin 10 inhibits interferon gamma- and tumor necrosis factor alpha-stimulated activation of NADPH oxidase 1 in human colonic epithelial cells and the mouse colon, J. Gastroenterol. 44 (12) (2009) 1172–1184, https://doi.org/10.1007/s00535-009-0119-6.
[15] G. Aviello, U.G. Knaus, NADPH oxidases and ROS signaling in the gastrointestinal tract, Mucosal Immunol. 11 (4) (2018) 1011–1023, https://doi.org/10.1038/s41385-018-0021-8.
[16] M. Kato, M. Marumo, J. Nakayama, M. Matsumoto, C. Yabe-Nishimura, T. Kamata, The ROS-generating oxidase Nox1 is required for epithelial restitution following colitis, Exp. Anim. 65 (3) (2016) 197–205, https://doi.org/10.1538/expanim.15-0127.
[17] R. Radi, Peroxynitrite, a stealthy biological oxidant, J. Biol. Chem. 288 (37) (2013) 26464–26472, https://doi.org/10.1074/jbc.R113.472936.
[18] H. Shaked, L.J. Hofseth, A. Chumanevich, A.A. Chumanevich, J. Wang, Y. Wang, K. Taniguchi, M. Guma, S. Shenouda, H. Clevers, C.C. Harris, M. Karin, Chronic epithelial NF-kappaB activation accelerates APC loss and intestinal tumor initiation through iNOS up-regulation, Proc. Natl. Acad. Sci. U. S. A. 109 (35) (2012) 14007–14012, https://doi.org/10.1073/pnas.1211509109.
[19] E. Larsson, V. Tremaroli, Y.S. Lee, O. Koren, I. Nookaew, A. Fricker, J. Nielsen, R. E. Ley, F. Backhed, Analysis of gut microbial regulation of host gene expression along the length of the gut and regulation of gut microbial ecology through MyD88, Gut 61 (8) (2012) 1124–1131, https://doi.org/10.1136/gutjnl-2011-301104.
[20] J.O. Lundberg, E. Weitzberg, Biology of nitrogen oxides in the gastrointestinal tract, Gut 62 (4) (2013) 616–629, https://doi.org/10.1136/gutjnl-2011-301649.
[21] X. Treton, E. Pedruzzi, C. Guichard, Y. Ladeiro, S. Sedghi, M. Vallee, N. Fernandez, E. Bruyere, P.L. Woerther, R. Ducroc, N. Montcuquet, J.N. Freund, I. Van Seuningen, F. Barreau, A. Marah, J.P. Hugot, D. Cazals-Hatem, Y. Bouhnik, F. Daniel, E. Ogier-Denis, Combined NADPH oxidase 1 and interleukin 10 deficiency induces chronic endoplasmic reticulum stress and causes ulcerative colitis-like disease in mice, PLoS One 9 (7) (2014), e101669, https://doi.org/10.1371/journal.pone.0101669.
[22] G. Leoni, A. Alam, P.A. Neumann, J.D. Lambeth, G. Cheng, J. McCoy, R.S. Hilgarth, K. Kundu, N. Murthy, D. Kusters, C. Reutelingsperger, M. Perretti, C.A. Parkos, A. S. Neish, A. Nusrat, Annexin A1, formyl peptide receptor, and NOX1 orchestrate epithelial repair, J. Clin. Invest. 123 (1) (2013) 443–454, https://doi.org/10.1172/JCI65831.
[23] A. Alam, G. Leoni, M. Quiros, H. Wu, C. Desai, H. Nishio, R.M. Jones, A. Nusrat, A. S. Neish, The microenvironment of injured murine gut elicits a local pro-restitutive microbiota, Nat Microbiol 1 (2016) 15021, https://doi.org/10.1038/nmicrobiol.2015.21.
[24] N. Coant, S. Ben Mkaddem, E. Pedruzzi, C. Guichard, X. Treton, R. Ducroc, J. N. Freund, D. Cazals-Hatem, Y. Bouhnik, P.L. Woerther, D. Skurnik, A. Grodet, M. Fay, D. Biard, T. Lesuffleur, C. Deffert, R. Moreau, A. Groyer, K.H. Krause, F. Daniel, E. Ogier-Denis, NADPH oxidase 1 modulates WNT and NOTCH1 signaling to control the fate of proliferative progenitor cells in the colon, Mol. Cell Biol. 30 (11) (2010) 2636–2650, https://doi.org/10.1128/MCB.01194-09.
[25] R.M. Jones, L. Luo, C.S. Ardita, A.N. Richardson, Y.M. Kwon, J.W. Mercante, A. Alam, C.L. Gates, H. Wu, P.A. Swanson, J.D. Lambeth, P.W. Denning, A.S. Neish, Symbiotic lactobacilli stimulate gut epithelial proliferation via Nox-mediated generation of reactive oxygen species, EMBO J. 32 (23) (2013) 3017–3028, https://doi.org/10.1038/emboj.2013.224.
[26] R.M. Jones, A.S. Neish, Redox signaling mediated by the gut microbiota, Free Radic.Biol & med. (2016), https://doi.org/10.1016/j.freeradbiomed.2016.10.495.
[27] A. Kielland, T. Blom, K.S. Nandakumar, R. Holmdahl, R. Blomhoff, H. Carlsen, In vivo imaging of reactive oxygen and nitrogen species in inflammation using the luminescent probe L-012, Free Radic.Biol & med. 47 (6) (2009) 760–766, https://doi.org/10.1016/j.freeradbiomed.2009.06.013.
[28] G. Aviello, A.K. Singh, S. O'Neill, E. Conroy, W. Gallagher, G. D'Agostino, A. W. Walker, B. Bourke, D. Scholz, U.G. Knaus, Colitis susceptibility in mice with reactive oxygen species deficiency is mediated by mucus barrier and immune

defense defects, Mucosal Immunol. 12 (6) (2019) 1316–1326, https://doi.org/10.1038/s41385-019-0205-x.
[29] B. Chassaing, A.T. Gewirtz, Gut microbiota, low-grade inflammation, and metabolic syndrome, Toxicol. Pathol. 42 (1) (2014) 49–53, https://doi.org/10.1177/0192623313508481.
[30] A. Agus, J. Denizot, J. Thevenot, M. Martinez-Medina, S. Massier, P. Sauvanet, A. Bernalier-Donadille, S. Denis, P. Hofman, R. Bonnet, E. Billard, N. Barnich, Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive *E. coli* infection and intestinal inflammation, Sci. Rep. 6 (2016), 19032, https://doi.org/10.1038/srep19032.
[31] G. Barbara, C. Cremon, V. Stanghellini, Inflammatory bowel disease and irritable bowel syndrome: similarities and differences, Curr. Opin. Gastroenterol. 30 (4) (2014) 352–358, https://doi.org/10.1097/MOG.0000000000000070.
[32] B. Chassaing, O. Koren, J.K. Goodrich, A.C. Poole, S. Srinivasan, R.E. Ley, A. T. Gewirtz, Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome, Nature 519 (7541) (2015) 92–96, https://doi.org/10.1038/nature14232.
[33] A.C. Moss, The meaning of low-grade inflammation in clinically quiescent inflammatory bowel disease, Curr. Opin. Gastroenterol. 30 (4) (2014) 365–369, https://doi.org/10.1097/MOG.0000000000000082.
[34] B. Chassaing, G. Srinivasan, M.A. Delgado, A.N. Young, A.T. Gewirtz, M. Vijay-Kumar, Fecal lipocalin 2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation, PLoS One 7 (9) (2012), e44328, https://doi.org/10.1371/journal.pone.0044328.
[35] R. Suzuki, H. Kohno, S. Sugie, H. Nakagama, T. Tanaka, Strain differences in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice, Carcinogenesis 27 (1) (2006) 162–169, https://doi.org/10.1093/carcin/bgi205.
[36] B. Chassaing, J.D. Aitken, M. Malleshappa, M. Vijay-Kumar, Dextran sulfate sodium (DSS)-induced colitis in mice, Curr. Protoc. Im. 104 (2014) 15.25.1–15.25.14, https://doi.org/10.1002/0471142735.im1525s104.
[37] F.I. Umoh, I. Kato, J. Ren, P.L. Wachowiak, M.T.t. Ruffin, D.K. Turgeon, A. Sen, D. E. Brenner, Z. Djuric, Markers of systemic exposures to products of intestinal bacteria in a dietary intervention study, Eur. J. Nutr. 55 (2) (2016) 793–798, https://doi.org/10.1007/s00394-015-0900-7.
[38] A.B. Bialkowska, A.M. Ghaleb, M.O. Nandan, V.W. Yang, Improved Swiss-rolling technique for intestinal tissue preparation for immunohistochemical and immunofluorescent analyses, J Vis Exp 113 (2016), e54161, https://doi.org/10.3791/54161.
[39] T.A. Kerr, M.A. Ciorba, H. Matsumoto, V.R. Davis, J. Luo, S. Kennedy, Y. Xie, A. Shaker, B.K. Dieckgraefe, N.O. Davidson, Dextran sodium sulfate inhibition of real-time polymerase chain reaction amplification: a poly-A purification solution, Inflamm. Bowel Dis. 18 (2) (2012) 344–348, https://doi.org/10.1002/ibd.21763.
[40] E. Viennois, F. Chen, H. Laroui, M.T. Baker, D. Merlin, Dextran sodium sulfate inhibits the activities of both polymerase and reverse transcriptase: lithium chloride purification, a rapid and efficient technique to purify RNA, BMC Res. Notes 6 (2013) 360, https://doi.org/10.1186/1756-0500-6-360.
[41] J.M. Ruijter, C. Ramakers, W.M. Hoogaars, Y. Karlen, O. Bakker, M.J. van den Hoff, A.F. Moorman, Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data, Nucleic Acids Res. 37 (6) (2009) e45, https://doi.org/10.1093/nar/gkp045.
[42] E. Avershina, K. Lundgard, M. Sekelja, C. Dotterud, O. Storro, T. Oien, R. Johnsen, K. Rudi, Transition from infant- to adult-like gut microbiota, Environ. Microbiol. 18 (7) (2016) 2226–2236, https://doi.org/10.1111/1462-2920.13248.
[43] J.G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E. K. Costello, N. Fierer, A.G. Pena, J.K. Goodrich, J.I. Gordon, G.A. Huttley, S. T. Kelley, D. Knights, J.E. Koenig, R.E. Ley, C.A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J.R. Sevinsky, P.J. Turnbaugh, W.A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, R. Knight, QIIME allows analysis of high-throughput community sequencing data, Nat. Methods 7 (5) (2010) 335–336, https://doi.org/10.1038/nmeth.f.303.
[44] R.C. Edgar, Search and clustering orders of magnitude faster than BLAST, Bioinformatics 26 (19) (2010) 2460–2461, https://doi.org/10.1093/bioinformatics/btq461.
[45] R.C. Edgar, UPARSE: highly accurate OTU sequences from microbial amplicon reads, Nat. Methods 10 (10) (2013) 996–998, https://doi.org/10.1038/nmeth.2604.
[46] E. Pruesse, C. Quast, K. Knittel, B.M. Fuchs, W. Ludwig, J. Peplies, F.O. Glockner, SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB, Nucleic Acids Res. 35 (21) (2007) 7188–7196, https://doi.org/10.1093/nar/gkm864.
[47] C.A. Lozupone, M. Hamady, S.T. Kelley, R. Knight, Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities, Appl. Environ. Microbiol. 73 (5) (2007) 1576–1585, https://doi.org/10.1128/AEM.01996-06.
[48] R Core Team, R, A Language and Environment for Statistical Computing (Version 4.1.2), R Foundation for Statistical Computing, Vienna, Austria, 2021. https://www.R-project.org/.
[49] J. Oksanen, F.G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R. Minchin, R.B. O'Hara, G.L. Simpson, P. Solymos, M.H.H. Stevens, E. Szoecs, H. Wagner, Vegan: Community Ecology Package (Version 2.5-7), 2020. https://CRAN.R-project.org/package=vegan.
[50] M. Hervé, RVAideMemoire: Testing and Plotting Procedures for Biostatistics (Version 0.9-79), 2021. https://CRAN.R-project.org/package=RVAideMemoire.
[51] N. Segata, J. Izard, L. Waldron, D. Gevers, L. Miropolsky, W.S. Garrett, C. Huttenhower, Metagenomic biomarker discovery and explanation, Genome Biol. 12 (6) (2011) R60, https://doi.org/10.1186/gb-2011-12-6-r60.
[52] S. Gross, S.T. Gammon, B.L. Moss, D. Rauch, J. Harding, J.W. Heinecke, L. Ratner, D. Piwnica-Worms, Bioluminescence imaging of myeloperoxidase activity in vivo, Nat. Med. 15 (4) (2009) 455–461, https://doi.org/10.1038/nm.1886.
[53] A. Daiber, M. Oelze, S. Steven, S. Kroller-Schon, T. Munzel, Taking up the cudgels for the traditional reactive oxygen and nitrogen species detection assays and their use in the cardiovascular system, Redox Biol. 12 (2017) 35–49, https://doi.org/10.1016/j.redox.2017.02.001.
[54] K. Van Dyke, E. Ghareeb, M. Van Dyke, D.H. Van Thiel, Ultrasensitive peroxynitrite-based luminescence with L-012 as a screening system for antioxidative/antinitrating substances, e.g. Tylenol (acetaminophen), 4-OH tempol, quercetin and carboxy-PTIO, Luminescence 22 (4) (2007) 267–274, https://doi.org/10.1002/bio.959.
[55] R.J. Goiffon, S.C. Martinez, D. Piwnica-Worms, A rapid bioluminescence assay for measuring myeloperoxidase activity in human plasma, Nat. Commun. 6 (2015) 6271, https://doi.org/10.1038/ncomms7271.
[56] J. Zielonka, J.D. Lambeth, B. Kalyanaraman, On the use of L-012, a luminol-based chemiluminescent probe, for detecting superoxide and identifying inhibitors of NADPH oxidase: a reevaluation, Free Radic.Biol & med. 65 (2013) 1310–1314, https://doi.org/10.1016/j.freeradbiomed.2013.09.017.
[57] A. Daiber, M. August, S. Baldus, M. Wendt, M. Oelze, K. Sydow, A.L. Kleschyov, T. Munzel, Measurement of NAD(P)H oxidase-derived superoxide with the luminol analogue L-012, Free Radic.Biol & med. 36 (1) (2004) 101–111, https://doi.org/10.1016/j.freeradbiomed.2003.10.012.
[58] H. Yokota, A. Tsuzuki, Y. Shimada, A. Imai, D. Utsumi, T. Tsukahara, M. Matsumoto, K. Amagase, K. Iwata, A. Nakamura, C. Yabe-Nishimura, S. Kato, NOX1/NADPH oxidase expressed in colonic macrophages contributes to the pathogenesis of colonic inflammation in trinitrobenzene sulfonic acid-induced murine colitis, J Pharmacol Exp Ther 360 (1) (2017) 192–200, https://doi.org/10.1124/jpet.116.235580.
[59] D. Liu, J.C. Marie, A.L. Pelletier, Z. Song, M. Ben-Khemis, K. Boudiaf, C. Pintard, T. Leger, S. Terrier, G. Chevreux, J. El-Benna, P.M. Dang, Protein kinase CK2 acts as a molecular brake to control NADPH oxidase 1 activation and colon inflammation, Cell Mol Gastroenterol Hepatol 13 (4) (2022) 1073–1093, https://doi.org/10.1016/j.jcmgh.2022.01.003.
[60] V. Singh, B.S. Yeoh, B. Chassaing, B. Zhang, P. Saha, X. Xiao, D. Awasthi, R. Shashidharamurthy, M. Dikshit, A. Gewirtz, M. Vijay-Kumar, Microbiota-inducible innate immune, siderophore binding protein lipocalin 2 is critical for intestinal homeostasis, Cell Mol Gastroenterol Hepatol 2 (4) (2016) 482–498, https://doi.org/10.1016/j.jcmgh.2016.03.007, e6.
[61] R.M. Jones, C. Desai, T.M. Darby, L. Luo, A.A. Wolfarth, C.D. Scharer, C.S. Ardita, A.R. Reedy, E.S. Keebaugh, A.S. Neish, Lactobacilli modulate epithelial cytoprotection through the Nrf2 pathway, Cell Rep. 12 (8) (2015) 1217–1225, https://doi.org/10.1016/j.celrep.2015.07.042.
[62] T.O. Khor, M.T. Huang, K.H. Kwon, J.Y. Chan, B.S. Reddy, A.N. Kong, Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis, Cancer Res. 66 (24) (2006) 11580–11584, https://doi.org/10.1158/0008-5472.CAN-06-3562.
[63] W. Gao, L. Guo, Y. Yang, Y. Wang, S. Xia, H. Gong, B.K. Zhang, M. Yan, Dissecting the crosstalk between Nrf2 and NF-κB response pathways in drug-induced toxicity, Front. Cell Dev. Biol. 9 (2021), 809952, https://doi.org/10.3389/fcell.2021.809952.
[64] M. Li, Y. Wu, Y. Hu, L. Zhao, C. Zhang, Initial gut microbiota structure affects sensitivity to DSS-induced colitis in a mouse model, Sci. China Life Sci. 61 (7) (2018) 762–769, https://doi.org/10.1007/s11427-017-9097-0.
[65] C.B. Van Buiten, G. Wu, Y.Y. Lam, L. Zhao, I. Raskin, Elemental iron modifies the redox environment of the gastrointestinal tract: a novel therapeutic target and test for metabolic syndrome, Free Radic.Biol & med. 168 (2021) 203–213, https://doi.org/10.1016/j.freeradbiomed.2021.03.032.
[66] A. Karkman, J. Lehtimaki, L. Ruokolainen, The ecology of human microbiota: dynamics and diversity in health and disease, Ann. N. Y. Acad. Sci. 1399 (1) (2017) 78–92, https://doi.org/10.1111/nyas.13326.
[67] M. Wu, P. Li, Y. An, J. Ren, D. Yan, J. Cui, D. Li, M. Li, M. Wang, G. Zhong, Phloretin ameliorates dextran sulfate sodium-induced ulcerative colitis in mice by regulating the gut microbiota, Pharmacol. Res. 150 (2019), 104489, https://doi.org/10.1016/j.phrs.2019.104489.
[68] R. Dziarski, S.Y. Park, D.R. Kashyap, S.E. Dowd, D. Gupta, Pglyrp-regulated gut microflora prevotella falsenii, parabacteroides distasonis and Bacteroides eggerthii enhance and Alistipes finegoldii attenuates colitis in mice, PLoS One 11 (1) (2016), e0146162, https://doi.org/10.1371/journal.pone.0146162.
[69] C. Schwab, D. Berry, I. Rauch, I. Rennisch, J. Ramesmayer, E. Hainzl, S. Heider, T. Decker, L. Kenner, M. Muller, B. Strobl, M. Wagner, C. Schleper, A. Loy, T. Urich, Longitudinal study of murine microbiota activity and interactions with the host during acute inflammation and recovery, ISME J. 8 (5) (2014) 1101–1114, https://doi.org/10.1038/ismej.2013.223.
[70] D. Berry, C. Schwab, G. Milinovich, J. Reichert, K. Ben Mahfoudh, T. Decker, M. Engel, B. Hai, E. Hainzl, S. Heider, L. Kenner, M. Muller, I. Rauch, B. Strobl, M. Wagner, C. Schleper, T. Urich, A. Loy, Phylotype-level 16S rRNA analysis reveals new bacterial indicators of health state in acute murine colitis, ISME J. 6 (11) (2012) 2091–2106, https://doi.org/10.1038/ismej.2012.39.
[71] Q. Zhang, Y. Wu, J. Wang, G. Wu, W. Long, Z. Xue, L. Wang, X. Zhang, X. Pang, Y. Zhao, L. Zhao, C. Zhang, Accelerated dysbiosis of gut microbiota during aggravation of DSS-induced colitis by a butyrate-producing bacterium, Sci. Rep. 6 (2016) 27572, https://doi.org/10.1038/srep27572.

[72] G. Lo Sasso, B.W. Phillips, A. Sewer, J.N.D. Battey, A. Kondylis, M. Talikka, B. Titz, E. Guedj, D. Peric, D. Bornand, R. Dulize, C. Merg, M. Corciulo, S. Ouadi, R. Yanuar, C.K. Tung, N.V. Ivanov, M.C. Peitsch, J. Hoeng, The reduction of DSS-induced colitis severity in mice exposed to cigarette smoke is linked to immune modulation and microbial shifts, Sci. Rep. 10 (1) (2020) 3829, https://doi.org/10.1038/s41598-020-60175-3.

[73] M.A. Borton, A. Sabag-Daigle, J. Wu, L.M. Solden, B.S. O'Banion, R.A. Daly, R. A. Wolfe, J.F. Gonzalez, V.H. Wysocki, B.M.M. Ahmer, K.C. Wrighton, Chemical and pathogen-induced inflammation disrupt the murine intestinal microbiome, Microbiome 5 (1) (2017) 47, https://doi.org/10.1186/s40168-017-0264-8.

[74] M. Derrien, P. Van Baarlen, G. Hooiveld, E. Norin, M. Muller, W.M. de Vos, Modulation of mucosal immune response, tolerance, and proliferation in mice colonized by the mucin-degrader Akkermansia muciniphila, Front. Microbiol. 2 (2011) 166, https://doi.org/10.3389/fmicb.2011.00166.

[75] M. Derrien, M.C. Collado, K. Ben-Amor, S. Salminen, W.M. de Vos, The Mucin degrader Akkermansia muciniphila is an abundant resident of the human intestinal tract, Appl. Environ. Microbiol. 74 (5) (2008) 1646–1648, https://doi.org/10.1128/AEM.01226-07.

[76] T. Zhang, X. Ji, G. Lu, F. Zhang, The potential of Akkermansia muciniphila in inflammatory bowel disease, Appl. Microbiol. Biotechnol. 105 (14–15) (2021) 5785–5794, https://doi.org/10.1007/s00253-021-11453-1.

[77] A. Everard, C. Belzer, L. Geurts, J.P. Ouwerkerk, C. Druart, L.B. Bindels, Y. Guiot, M. Derrien, G.G. Muccioli, N.M. Delzenne, W.M. de Vos, P.D. Cani, Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity, Proc. Natl. Acad. Sci. U. S. A. 110 (22) (2013) 9066–9071, https://doi.org/10.1073/pnas.1219451110.

[78] N.R. Shin, J.C. Lee, H.Y. Lee, M.S. Kim, T.W. Whon, M.S. Lee, J.W. Bae, An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice, Gut 63 (5) (2014) 727–735, https://doi.org/10.1136/gutjnl-2012-303839.

[79] M.E. Johansson, J.K. Gustafsson, K.E. Sjoberg, J. Petersson, L. Holm, H. Sjovall, G. C. Hansson, Bacteria penetrate the inner mucus layer before inflammation in the dextran sulfate colitis model, PLoS One 5 (8) (2010), e12238, https://doi.org/10.1371/journal.pone.0012238.

[80] G. Pircalabioru, G. Aviello, M. Kubica, A. Zhdanov, M.H. Paclet, L. Brennan, R. Hertzberger, D. Papkovsky, B. Bourke, U.G. Knaus, Defensive mutualism rescues NADPH oxidase inactivation in gut infection, Cell Host Microbe 19 (5) (2016) 651–663, https://doi.org/10.1016/j.chom.2016.04.007.

[81] H. Yan, B. Yu, J. Degroote, T. Spranghers, N. Van Noten, M. Majdeddin, M. Van Poucke, L. Peelman, J. De Vrieze, N. Boon, I. Gielen, S. Smet, D. Chen, J. Michiels, Antibiotic affects the gut microbiota composition and expression of genes related to lipid metabolism and myofiber types in skeletal muscle of piglets, BMC Vet. Res. 16 (1) (2020) 392, https://doi.org/10.1186/s12917-020-02592-0.

[82] Y.Y. Lam, C.W. Ha, C.R. Campbell, A.J. Mitchell, A. Dinudom, J. Oscarsson, D. I. Cook, N.H. Hunt, I.D. Caterson, A.J. Holmes, L.H. Storlien, Increased gut permeability and microbiota change associate with mesenteric fat inflammation and metabolic dysfunction in diet-induced obese mice, PLoS One 7 (3) (2012), e34233, https://doi.org/10.1371/journal.pone.0034233.

[83] Y. Peng, Y. Yan, P. Wan, D. Chen, Y. Ding, L. Ran, J. Mi, L. Lu, Z. Zhang, X. Li, X. Zeng, Y. Cao, Gut microbiota modulation and anti-inflammatory properties of anthocyanins from the fruits of Lycium ruthenicum Murray in dextran sodium sulfate-induced colitis in mice, Free Radic.Biol & med. 136 (2019) 96–108, https://doi.org/10.1016/j.freeradbiomed.2019.04.005.

[84] F.F. Anhe, T.V. Varin, M. Le Barz, G. Pilon, S. Dudonne, J. Trottier, P. St-Pierre, C. S. Harris, M. Lucas, M. Lemire, E. Dewailly, O. Barbier, Y. Desjardins, D. Roy, A. Marette, Arctic berry extracts target the gut-liver axis to alleviate metabolic endotoxaemia, insulin resistance and hepatic steatosis in diet-induced obese mice, Diabetologia 61 (4) (2018) 919–931, https://doi.org/10.1007/s00125-017-4520-z.

[85] G. Gavazzi, B. Banfi, C. Deffert, L. Fiette, M. Schappi, F. Herrmann, K.H. Krause, Decreased blood pressure in NOX1-deficient mice, FEBS Lett 580 (2) (2006) 497–504, https://doi.org/10.1016/j.febslet.2005.12.049.

Online Supplementary Material for: "The ROS-generating enzyme NADPH oxidase 1 modulates the colonic microbiota but offers minor protection against dextran sulfate sodium-induced low-grade colon inflammation in mice"

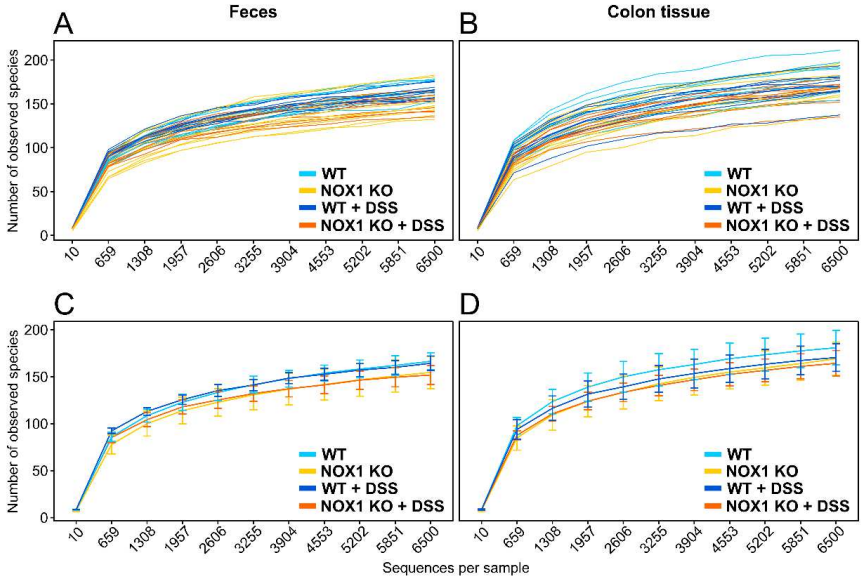

Fig. S1. Rarefaction curves for number of observed species (OTUs) in (A,C) feces and (B,D) colon tissue from WT and NOX1 KO mice with or without 1 % DSS treatment (6 days). (A,B) Shows individual samples while (C,D) shows group mean ± SD for each experimental group for each sequencing depth (10, 659, 1308, 1957, 2606, 3255, 3904, 4553, 5202, 5851 and 6500 sequences per sample). n=9-10 per group. The number of observed species for each sequencing depth is the mean of ten rarefaction iterations. OTU, operational taxonomic unit; WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium.

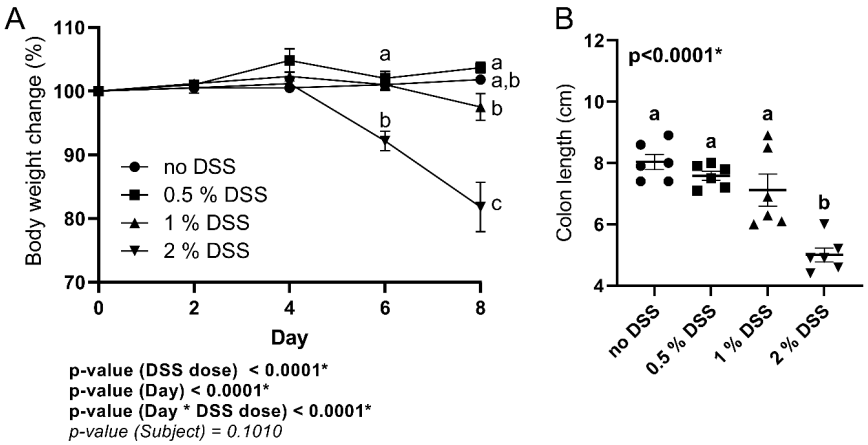

Fig. S2. Disease evaluation of WT mice treated with 0 (no DSS), 0.5, 1, or 2 % DSS for eight days**.** (A) Change in body weight (%) for day 2, 4, 6, and 8. P-values from repeated measures 2-way ANOVA (main effect of DSS dose, day, the interaction effects between day and DSS dose, and the subject effect). Characters ("a", "b", "c") indicate significant differences between DSS doses within each day with Tukey correction for multiple comparisons. (B) Colon length (cm) at termination (day 8). P-value from 1-way ANOVA. Characters ("a", "b") indicate significant differences between DSS doses, with Tukey correction for multiple comparisons. (A,B) Horizontal lines and whiskers are group mean ± SEM. n=6 per group. WT, wild type. DSS, dextran sulfate sodium. *p<0.05.

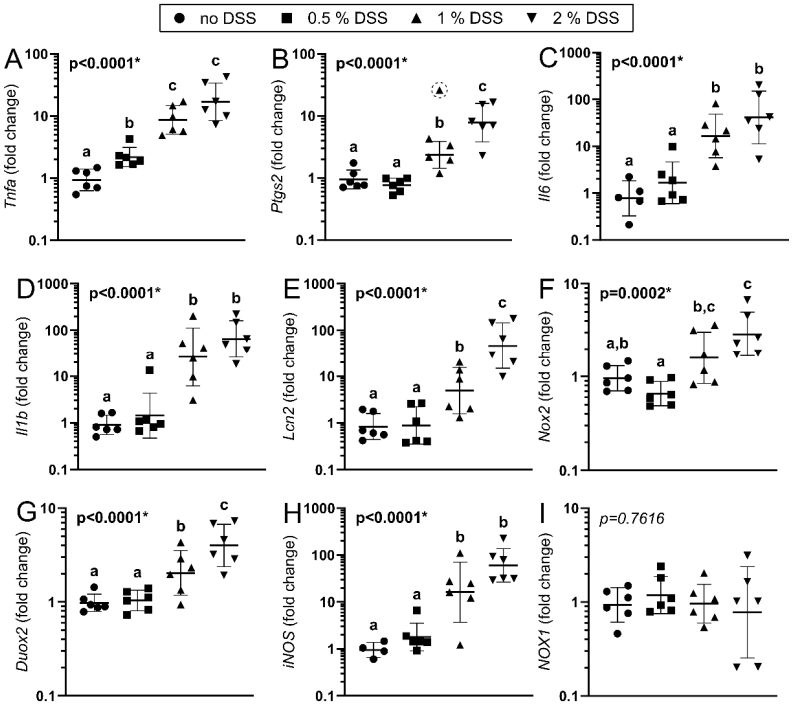

Fig. S3. mRNA expression of inflammation- and ROS-related genes in WT mice treated with 0 (no DSS), 0.5, 1, or 2 % DSS for eight days. Fold change in mucosa from the proximal colon. (A) Tumor necrosis factor alpha (*Tnfa*), (B) prostaglandin-endoperoxide synthase 2 (*Ptgs2*), (C) interleukin 6 (*Il6*), (D) interleukin 1 beta (*Il1b*), (E) lipocalin 2 (*Lcn2*), (F) cytochrome b-245 (*Nox2*), (G) dual oxidase 2 (*Duox2*), (H) inducible nitric oxide synthase 2 (*iNOS*), and (I) NADPH oxidase 1 (*Nox1*). Data from each mouse was normalized by dividing by the mean expression of the group with no DSS treatment. P-values from 1-way ANOVA on log10-transformed data. Characters ("a", "b", "c") indicate significant differences between DSS doses, with Tukey correction for multiple comparisons. Horizontal lines and whiskers are geometric group mean ×/÷ geometric SD factor. n=4-6 per group. (B) One extreme value was excluded from statistical analysis (dotted circles). When included, the DSS effect was significant (P<0.0001) but letters "a, a, b, c" would be exchanged for "a, a, b, b". For this model however, the assumption of normality was violated. WT, wild type. DSS, dextran sulfate sodium. *p<0.05.

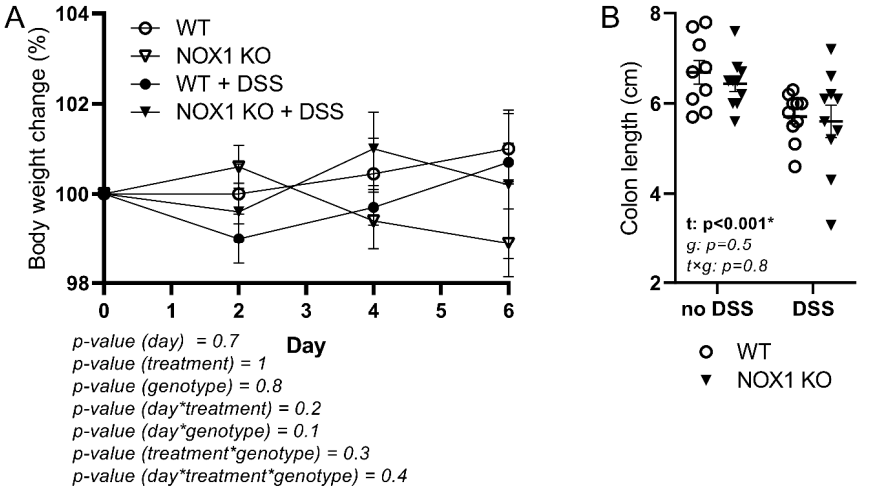

Fig. S4. Disease evaluation of WT and NOX1 KO mice with or without 1% DSS treatment (6 days). (A) Change in body weight (%) measured on day 0, 2, 4 and 6. P-values from repeated measures 3-way ANOVA (main effect of day, treatment and genotype, the interaction effects). Symbols and whiskers are group mean ± SEM. n=9-10 per group. (B) Colon length (cm) at termination (day 6). P-values from 2-way ANOVA (main effect of treatment (t; no DSS versus 1 % DSS) and genotype (g; WT versus NOX1 KO), and interaction effect (t×g)). Horizontal lines and whiskers are group mean ± SEM. n=9-10 per group. WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium. *p<0.05.

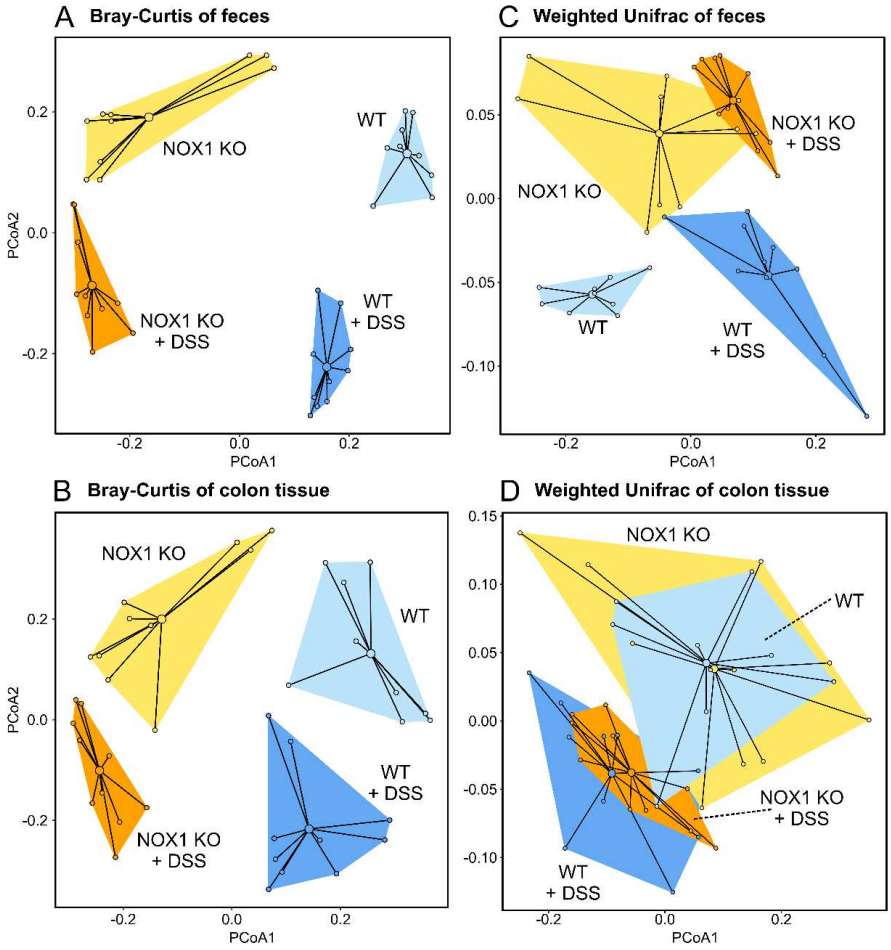

Fig. S5. Bacterial β-diversity between WT and NOX1 KO mice with or without 1% DSS treatment (6 days). PCoA ordination plots of (A,B) Bray-Curtis and (C,D) weighted UniFrac distances between (A,C) feces and (B,D) colon tissue samples. Colors indicate which group individual samples belong to. n=9-10 per group. WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium; PCoA, principal coordinate analysis.

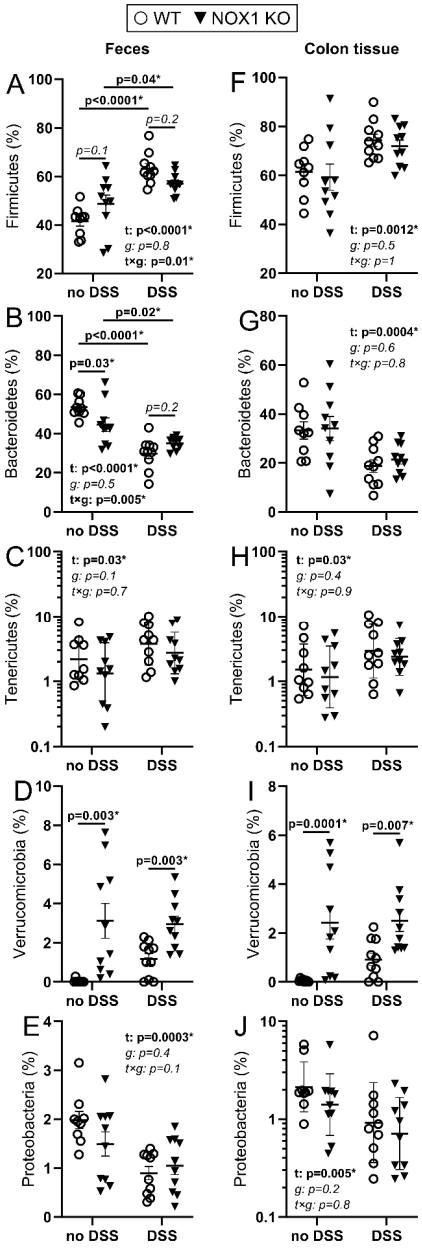

Fig. S6. Relative abundance (%) of bacterial phyla in (A-E) feces and (F-J) and colon tissue from WT and NOX1 KO mice with or without 1 % DSS treatment (6 days). Only phyla with average relative abundance above 1 % in at least one group is presented. n=9-10 per group. (A,F) Firmicutes, (B,G) Bacteroidetes, (C,H) Tenericutes, (D,I) Verrucomicrobia, and (E,J) Proteobacteria. (A,B,C,E,F,G,H,J) P-values from 2-way ANOVA (main effect of treatment (t; no DSS versus 1 % DSS) and genotype (g; WT versus NOX1 KO), and interaction effect (t×g)). In cases of significant interaction, p-values for simple main effects from post-hoc tests with Bonferroni correction for multiple comparisons are presented. In cases where 2-way ANOVA could not be performed due to heteroskedasticity and/or violation of normality assumption alternative tests were used within the two treatment groups: (D, no DSS) Fisher's exact test, (D, DSS) t-test, and (I) Mann-Whitney U test. (C,H,J) Statistical analyses were performed on log10-transformed data. Horizontal lines and whiskers are geometric group mean ×/÷ geometric SD factor. (A,B,D,E,F,G,I) Horizontal lines and whiskers are group mean ± SEM. WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium. *p<0.05.

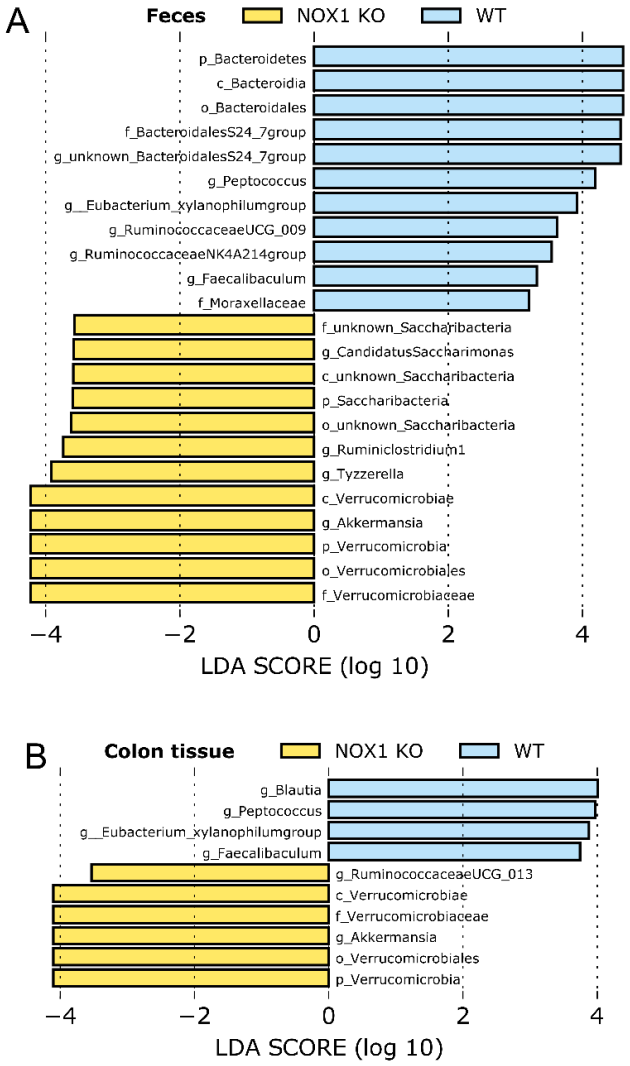

Fig. S7. LEfSe results from comparison of the relative abundance of bacterial taxa in (A) feces and (B) colon tissue between WT and NOX1 KO mice without 1 % DSS treatment. Higher LDA score (longer horizontal bar) indicate more significantly differentiated abundance between the two groups. Bacterial taxa next to blue bars had higher abundance in WT than in NOX1 KO mice, while bacterial taxa next to yellow bars had higher abundance in NOX1 KO than in WT mice. n=9-10 per group. LDA, linear discriminant analysis; LEfSe, LDA effect size; WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium.

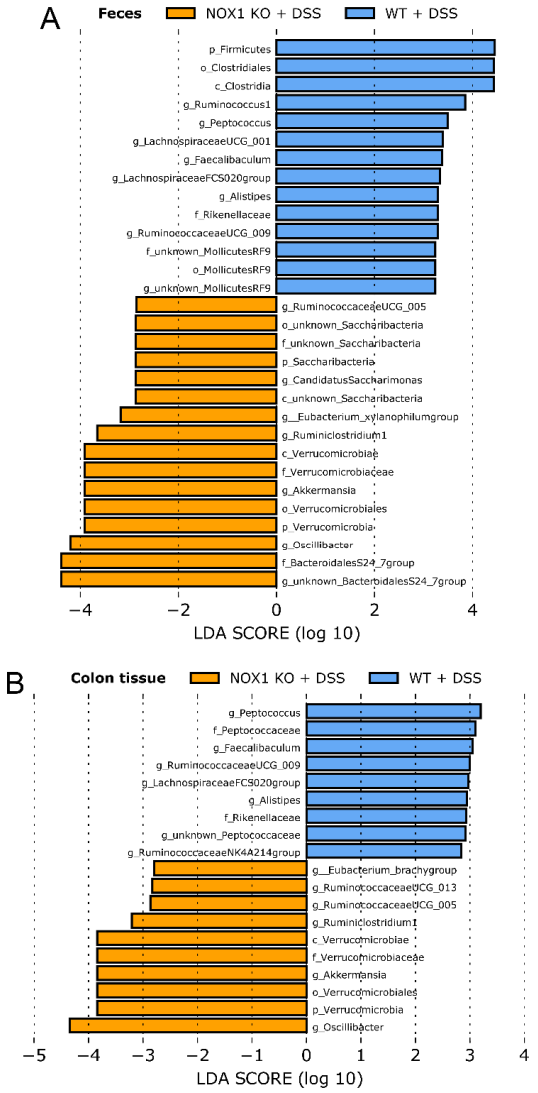

Fig. S8. LEfSe results from comparison of the relative abundance of bacterial taxa in (A) feces and (B) colon tissue between WT and NOX1 KO mice with 1 % DSS treatment (6 days). Higher LDA score (longer horizontal bar) indicate more significantly differentiated abundance between the two groups. Bacterial taxa next to blue bars had higher abundance in WT than in NOX1 KO mice, while bacterial taxa next to orange bars had higher abundance in NOX1 KO than in WT mice. n=10 per group. LDA, linear discriminant analysis; LEfSe, LDA effect size; WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium.

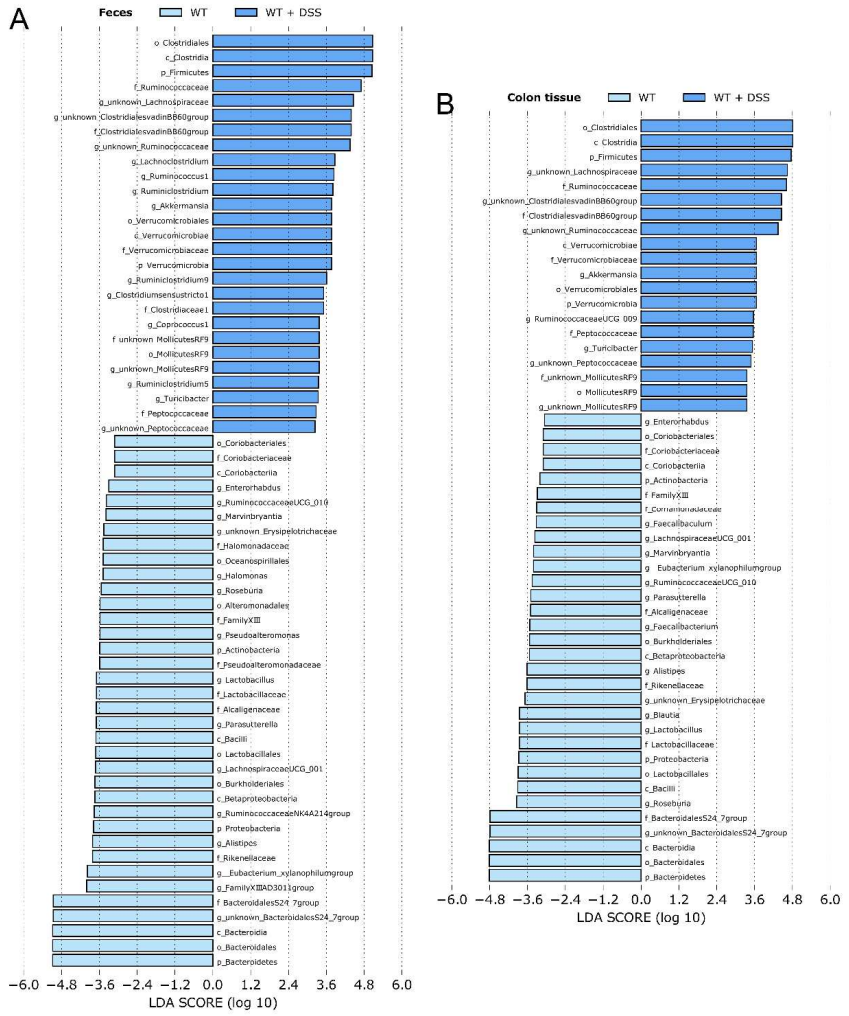

Fig. S9. LEfSe results from comparison of the relative abundance of bacterial taxa in (A) feces and (B) colon tissue between WT mice with or without 1 % DSS treatment (6 days). Higher LDA score (longer horizontal bar) indicate more significantly differentiated abundance between the two groups. Bacterial taxa next to light blue bars had higher abundance untreated than in DSS-treated mice, while bacterial taxa next to dark blue bars had higher abundance in DSS-treated than in untreated mice. n=9-10 per group. LDA, linear discriminant analysis; LEfSe, LDA effect size; WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium.

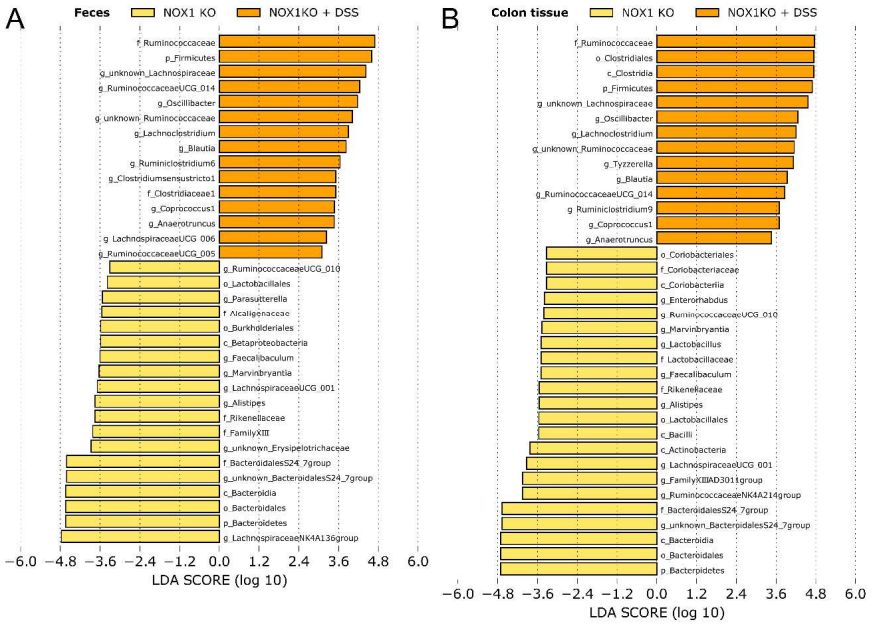

Fig. S10. LEfSe results from comparison of the relative abundance of bacterial taxa in (A) feces and (B) colon tissue between NOX1 KO mice with or without 1 % DSS treatment (6 days). Higher LDA score (longer horizontal bar) indicate more significantly differentiated abundance between the two groups. Bacterial taxa next to yellow bars had higher abundance untreated than in DSS-treated mice, while bacterial taxa next to orange bars had higher abundance in DSS-treated than in untreated mice. n=9-10 per group. LDA, linear discriminant analysis; LEfSe, LDA effect size; WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium.

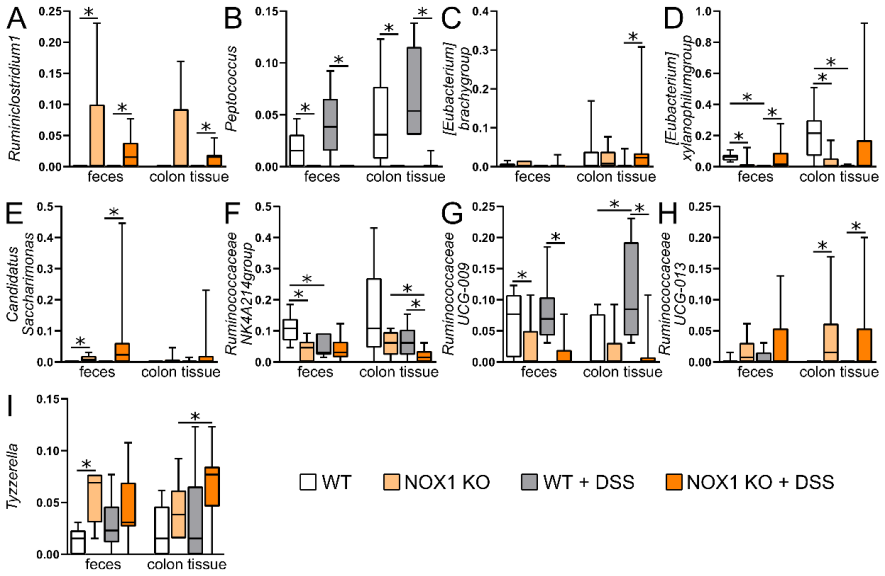

Fig. S11. Relative abundance (%) of low abundant bacterial genera in feces and colon tissue from WT and NOX1 KO mice with or without 1 % DSS treatment (6 days). (A) *Ruminiclostridium1*, (B) *Peptococcus*, (C) *[Eubacterium]brachygroup*, (D) *[Eubacterium]xylanophilumgroup*, (E) *Candidatus Saccharimonas*, (F) *RuminococcaceaeNK4A214group*, (G) *RuminococcaceaeUCG-009*, (H) *RuminococcaceaeUCG-013*, and (I) *Tyzzerella.* The presented genera are the ones that were found to have differentiated abundance between WT and NOX1 KO mice in feces and/or colon tissue through LEfSe analysis (comparison of genotypes (WT, NOX1 KO) within each treatment (no DSS, 1 % DSS), and between treatments within each genotype), and that had average relative abundance below 0.1 % in both feces and colon tissue. n=9-10 per group. WT, wild type; NOX1, NADPH oxidase 1; KO, knockout; DSS, dextran sulfate sodium; LDA, linear discriminant analysis; LEfSe, LDA effect size. *Indicates the LEfSe-identified significant comparisons.

Table S1. Primers used for genotyping of NOX1 knockout mice. NOX1, NADPH oxidase 1.

Primer name	**Sequence 5' --> 3'**
Mutant Reverse	CGAGCGCTCTGAAGTTCCT
Common	TAGCTGCCATGGAACTGAG
Wild type Reverse	TTGCAGTTGTTGGGTGATCT

Table S2. ZRF cocktail used to anesthetize mice by intraperitoneal injection.

Product	**Active substance**	**Dosage of active substance**
Zoletil forte vet (Virbac)	Zolezepam	32 mg substance/kg mouse
	Tiletamin	32 mg substance/kg mouse
Rompun vet (Bayer Animal Health GmbH)	Xylazine	4.5 mg substance/kg mouse
Fentadon vet (Eurovet)	Fentanyl	26 µg substance/kg mouse

Table S3. Reaction mixture for cDNA synthesis using the iScript cDNA Synthesis kit (Bio-Rad).

Component	**Per reaction**
5x iScript reaction mix	4 µL
iScript reverse transcriptase	1 µL
Nuclease-free water	11 µL
RNA template (200ng/µL)	4 µL

Table S4. Temperature program used for cDNA synthesis.

Operation	**Temperature (°C)**	**Duration**
Primer annealing	25	5 min
cDNA synthesis	42	30 min
cDNA synthesis termination	85	5 min
-	4	∞

Table S5. Reaction mixture for qPCR.

Component	**Per reaction**
HOT FirePol EvaGreen qPCR Supermix (Solis BioDyne)	2 µL
Forward primer (10 pmol/µL)*	0.2 µL
Reverse primer(10 pmol/µL)*	0.2 µL
Nuclease-free water	4.6 µL
cDNA template (8ng/µL)	3 µL

* See Supplemental Table S7.

Table S6. Temperature program used for qPCR.

Operation	**Temperature (°C)**	**Duration**	**Cycles**
Initial activation	95	12 min	1
Denaturation	95	15 sec	40
Annealing	*	20 sec	
Elongation	72	20 sec	
Final elongation	72	7 min	1
Melting curve	95	1 min	1
	45	1 min	
	60-90(+0.02/sec)	25 min	

* See Supplemental Table S7.

Table S7. Primers (Solis BioDyne, Tartu, Estonia) used for quantitative real-time PCR and their annealing temperature (Tm).

Gene	Forward Primer	Reverse Primer	Tm (°C)
Gapdh	CTTCAACAGCAACTCCCACTCTT	GCCGTATTCATTGTCATACCAGG	60
Doux2	TGTGAATGACGGGTCCAAGT	GGAGGCGAAGACGTACATGA	59
Il1b	GCAGCTGGAGAGTGTGGAT	AAACTCCACTTTGCTCTTGACTT	61
Il6	CGTGGAAATGAGAAAAGAGTTGT	AGTGCATCATCGTTGTTCATACA	61
iNOS	GACATTACGACCCCTCCCAC	ACTCTGAGGGCTGACACAAG	62
Lcn2	CACCACGGACTACAACCAG	TGGTTCTTCCATACAGGGTAAT	59
Nox2	GGGAACTGGGCTGTGAATGA	CAGTGCTGACCCAAGGAGTT	61
Ptgs2	AATATCAGGTCATTGGTGGAGA	TCTACCTGAGTGTCTTTGACTG	61
Tnfa	CTGTCTACTGAACTTCGGGGTGAT	GGTCTGGGCCATAGAACTGATG	61
Nox1	GTGATTACCAAGGTTGTCATGC	AAGCCTCGCTTCCTCATCTG	64

Table S8. Reaction mixture for amplicon PCR during library preparation for gene sequencing of 16S rRNA.

Component	Per reaction
5x HOT FIREPol® Blend Master Mix Ready to Load (Solis BioDyne)	5 µL
Forward primer, PRK341F (1 µM)*	0.5 µL
Reverse primer, PRK806R (1 µM)*	0.5 µL
Nuclease-free water	18 µL
Template DNA (0.07-57 ng/µL*)	1 µL

* Forward 5'- CCTACGGGRBGCASCAG-3', reverse 5'- GGACTACYVGGGTATCTAAT-3'[1].
** Measured by Qubit.

Table S9. Temperature cycles used for amplicon PCR during library preparation for gene sequencing of 16S rRNA.

Operation	Temperature (°C)	Duration	Cycles
Initial activation	95	15 min	1
Denaturation	95	30 sec	25/30*
Annealing	55	30 sec	
Elongation	72	45 sec	
Final elongation	72	7 min	1
-	4	∞	-

*25 for DNA samples from feces and 30 for DNA samples from colon tissue.

Table S10. Reaction mixture for index PCR during library preparation for gene sequencing of 16S rRNA.

Component	Per reaction
5x FIREPol® Master Mix Ready to Load (Solis BioDyne)	5 µL
Forward primer (1 µM)*	5 µL
Reverse primer (1 µM)*	5 µL
Nuclease-free water	8 µL
Template DNA	2 µL

* See Table S12.

Table S11. Temperature cycles used for index PCR during library preparation for gene sequencing of 16S rRNA.

Operation	Temperature (°C)	Duration	Cycles
Initial activation	95	5 min	1
Denaturation	95	30 sec	10
Annealing	55	1 min	
Elongation	72	45 sec	
Final elongation	72	7 min	1
-	4	∞	-

Table S12. Primers modified with Illumina adapters used for index PCR during library preparation for gene sequencing of 16S rRNA [2]. Unique combination of forward and reverse primer was used for each sample.

Primer name	Sequence, 5' -> 3'	Target region/gene	Direction
F1	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttcc gatctagtcaaCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F2	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttcc gatctagttccCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F3	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttcc gatctatgtcaCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F4	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttcc gatctccgtccCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F5	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttcc gatctgtagagCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F6	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttcc gatctgtccgcCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F7	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttcc gatctgtgaaaCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F8	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttcc gatctgtggccCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
R1	caagcagaagacggcatacgagatCGTGATgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R2	caagcagaagacggcatacgagatACATCGgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R3	caagcagaagacggcatacgagatGCCTAAgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R4	caagcagaagacggcatacgagatTGGTCAgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R5	caagcagaagacggcatacgagatCACTCTgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R6	caagcagaagacggcatacgagatATTGGCgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R7	caagcagaagacggcatacgagatGATCTGgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R8	caagcagaagacggcatacgagatTCAAGTgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R9	caagcagaagacggcatacgagatCTGATCgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R10	caagcagaagacggcatacgagatAAGCTAgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R11	caagcagaagacggcatacgagatGTAGCCgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R12	caagcagaagacggcatacgagatTACAAGgtgactggagttcagac gtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse

References

[1] Y. Yu, C. Lee, J. Kim, S. Hwang, Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction, Biotechnol Bioeng 89(6) (2005) 670-9. https://doi.org/10.1002/bit.20347.

[2] E. Avershina, K. Lundgard, M. Sekelja, C. Dotterud, O. Storro, T. Oien, R. Johnsen, K. Rudi, Transition from infant- to adult-like gut microbiota, Environ Microbiol 18(7) (2016) 2226-36. https://doi.org/10.1111/1462-2920.13248.

PAPER II

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A High-Fat Western Diet Attenuates Intestinal Changes in Mice with DSS-Induced Low-Grade Inflammation

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ABSTRACT

Background: A Western diet (WD) is associated with increased inflammation in the large intestine, which is often ascribed to the high dietary fat content. Intestinal inflammation in rodents can be induced by oral administration of dextran sodium sulfate (DSS). However, most studies investigating effects of WD and DSS have not used appropriate low-fat diets (LFDs) as control.

Objectives: To compare the effects of a WD with those of an LFD on colon health in a DSS-induced low-grade colonic inflammation mouse model.

Methods: Six-week-old male C57BL/6JRj mice were fed an LFD (fat = 10.3% energy, $n = 24$) or a WD (fat = 41.2% energy, $n = 24$) for 15 wk [Experiment 1 (Exp.1)]. Half the mice on each diet ($n = 12$) then received 1% DSS in water for 6 d with the remainder ($n = 12$ in each diet) administered water. Disease activity, proinflammatory genes, inflammatory biomarkers, and fecal microbiota (16S rRNA) were assessed (Exp.1). Follow-up experiments (Exp.2 and Exp.3) were performed to investigate whether fat source (milk or lard; Exp.2) affected outcomes and whether a shift from LFD to WD 1 d prior to 1% DSS exposure caused an immediate effect on DSS-induced inflammation (Exp.3).

Results: In Exp.1, 1% DSS treatment significantly increased disease score in the LFD group compared with the WD group (2.7 compared with 0.8; $P < 0.001$). Higher concentrations of fecal lipocalin (11-fold; $P < 0.001$), proinflammatory gene expression (≤82-fold), and *Proteobacteria* were observed in LFD-fed mice compared with the WD group. The 2 fat sources in WDs (Exp.2) revealed the same low inflammation in WD+DSS mice compared with LFD+DSS mice. Finally, the switch from LFD to WD just before DSS exposure resulted in reduced colonic inflammation (Exp.3).

Conclusions: Herein, WDs (with milk or lard) protected mice against DSS-induced colonic inflammation compared with LFD-fed mice. Whether fat intake induces protective mechanisms against DSS-mediated inflammation or inhibits establishment of the DSS-induced colitis model is unclear. *J Nutr* 2022;00:1–12.

Keywords: dextran sodium sulfate, gut inflammation, intestinal microbiota, low-fat diet, Western diet

Introduction

Western-type diets are characterized by a high content of saturated fat, cholesterol, and refined sugars and are low in dietary fiber. They are associated with inflammation, both systemically and in the gastrointestinal tract (1–3). Although inflammation generally constitutes a central process of the host's innate immune system, chronic inflammation can initiate pathological conditions. Even a modest increase in inflammatory status (low-grade inflammation) experienced over time, can drive the development of many diseases such as metabolic syndrome, obesity, nonalcoholic fatty liver disease, cardiovascular disease, and cancer (4, 5).

In the gastrointestinal tract, a low-grade inflammation is frequently present and defined as a state of higher inflammatory tonus in mucosal tissue of both small intestine and colon, even though not necessarily manifesting clear pathology (6). Low-grade intestinal inflammation can lead to impaired gut barrier integrity. This can result in leakage of bacterial endotoxins, such as LPSs, as well as other metabolites, and can induce both local and systemic responses (7). It has been shown in animal studies that high-fat Western diets (WDs) can induce or exacerbate intestinal inflammation (8). Specifically, ingestion of fat-rich diets can increase the presence of LPSs systemically (9) and diminish expression of genes related to tight junction proteins in epithelial cells, thereby increasing intestinal permeability (10). The gut microbiota is also affected by high-fat diets (HFDs) and changes can promote an inflammatory status in the host (11). Hallmarks of the effect of a WD on microbiota composition in both humans and mice are decreased bacterial richness (12), increased *Firmicutes*/*Bacteroidetes* ratio (13, 14), and higher

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abundance of Gram-negative bacteria (15), mainly belonging to the *Proteobacteria* phylum. The low content of dietary fiber in WD has been suggested to be a main driver of microbiota changes with adverse effects on colon health (16, 17). However, a high fat content per se is also suggested as being crucial for the negative effects of a WD (18, 19).

Dextran sodium sulfate (DSS), a synthetic sulfated polysaccharide, is widely used for inducing colitis in rodent models because the induced pathogenesis resembles features of inflammatory bowel disease found in humans (20). DSS-induced inflammation primarily affects the colon through a poorly defined mechanism. DSS concentrations ranging from 2.5% to 5%, either in drinking water or in food, are sufficient to cause an inflamed gut in mouse models (21). Most studies have demonstrated that HFDs, particularly those rich in saturated fats, worsen the colonic effects of DSS, both in DSS-induced colitis mouse models and in cancer models where DSS is combined with the carcinogen azoxymethane (AOM) (22–26). In a study by Lee and coworkers (27) HFD-fed mice manifested aggravated experimental colitis compared with mice following a standard fiber-rich, unpurified rodent diet after DSS exposure. This was shown by more severe histological changes in the colon, decrease of goblet cells, disruption of gut barrier, and alterations of intestinal microbiota. Benninghoff et al. (28) showed that AOM/DSS-induced colorectal cancer was exacerbated with a diet that mimicked an extreme version of a WD (reduced amounts of micronutrients in addition to high concentrations of fat and refined sugar). However, when the same diet was used, but with micronutrients matched to the control diet, they observed no differences in tumorigenesis or inflammation when compared with a low-fat control diet. Therefore, the effect of an HFD on induced colonic inflammation is not fully clear.

Previous studies reporting effects of WD or HFD on inflammation in mice have used high doses of DSS (2–5%) to induce inflammation (24, 29, 30). However, others have demonstrated that a lower concentration of DSS (1% DSS) results in a subclinical inflammatory state with few or no visible signs of intestinal damage and with a moderate induction of proinflammatory genes (31, 32). This is relevant for a number of clinical conditions including inflammatory bowel disease when in remission (33) and irritable bowel syndrome (34). In addition to using high concentrations of DSS, most other studies have also used low-fat control diets, which were poorly matched with regard to fiber content (23, 35, 36). In standard rodent maintenance diet (unpurified diet), commonly used as control, fiber content is higher and more diverse than synthetic experimental rodent diets high in fat. In our experiments, we used a low-fat control diet with fiber content equal to the WD (7% cellulose).

Previous studies investigating the relative effects on colonic inflammation of HFDs compared with low-fat diets (LFDs) have provided inconsistent results. The aim in this study was therefore to conduct a series of experiments to elucidate whether a high-fat WD impacted more adversely colonic inflammation compared with a properly controlled LFD. We hypothesized initially that a WD would exacerbate colonic inflammation more than an LFD. We further hypothesized that both fat source and timing of the high-fat feeding in relation to DSS treatment would influence the outcome.

Methods

Animals and diets

Experiment 1.

Six-week-old male C57BL/6JRj mice ($n = 48$) were purchased from JANVIER LABS and housed in ventilated cages (4 mice per cage) under controlled conditions (12-h light-dark cycle; 25 ± 2°C; 45–55% humidity). After 2 wk of acclimatization with a regular mouse maintenance diet (7.4% fat, 75.1% carbohydrate, 17.5% protein; RM1; Special Diets Services), mice were randomly allocated to 4 experimental groups in a 2 × 2 factorial design ($n = 12$ for each group): *1*) LFD, *2*) LFD+DSS, *3*) WD, and *4*) WD+DSS. The experimental diets were purchased from Research Diets: an LFD (D1404270, 10.3% energy from milk fat) and a WD (D12079B, 41.2% of total energy from milk fat). The diets were matched in terms of protein (casein 15.2% of energy), fiber (7% cellulose), and micronutrients. The difference apart from fat content was that the carbohydrate content (74.5% of energy) in the LFD was primarily maltodextrin and corn starch. Corn starch was partially replaced by sucrose as the main carbohydrate source in the WD. Also, 1.5 g/kg cholesterol was added to the WD but not in the LFD. Combined with naturally occurring cholesterol in milk fat, the WD contained ~2 g/kg (0.2%) cholesterol. Detailed description of the diets is found in **Supplemental Table 1**. After 15 wk on a WD or LFD, 24 mice (groups 2 and 4) received 1% DSS in their drinking water for 6 d whereas the rest received water.

Experiment 2.

To test the effects of 2 different types of fat in WD (milk and lard), 32 mice were allocated to the following groups ($n = 8$); *1*) LFD, *2*) LFD+DSS, *3*) $WD_{milk\ fat}$ +DSS and *4*) $WD_{lard\ fat}$ +DSS. Housing and acclimatization conditions for both Experiment 2 (Exp.2) and Exp.3 were the same as in Exp.1 mentioned above.The feeding trial lasted for 6 wk and then 1% DSS was introduced in the drinking water of groups 2–4 for 6 d. The first 2 groups were used as controls to determine whether the results from Exp.1 could be reproduced. Both WDs (milk- or lard-based) were purchased from Research Diets (Cat no: D12079B) and had the same energy content in all macronutrients including milk fat and lard fat (41.2%). The fatty acid profiles in the 2 types of fat are presented in **Supplemental Table 2**.

Experiment 3.

To investigate whether the effect of DSS on intestinal health was directly affected by a WD, 18 mice were allocated to 3 groups ($n = 6$)—2 LFD groups and 1 WD group—for 4 wk of feeding. One day before 1% DSS treatment, 1 of the LFD groups was switched to the WD.

All DSS groups were supplied with freshly made 1% DSS in water every second day for 6 d. Animal welfare was evaluated every second day and scored for disease activity according to a score sheet (**Supplemental Table 3**). Food and water were supplied ad libitum. Body weights and food consumption were recorded once per week.

Experimental procedures were approved by the Norwegian Animal Research Authority (Mattilsynet, FOTS ID 14805) in accordance with the guidelines and recommendations of the Federation of European Laboratory Animal Science Associations.

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Supplemental Tables 1–11 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
Address correspondence to HC (e-mail: harald.carlsen@nmbu.no).
Abbreviations used: AOM, azoxymethane; DAI, disease activity index; DSS, dextran sodium sulfate; Exp., Experiment; FD4, FITC (fluorescein isothiocyanate) dextran 4 kDa; HFD, high-fat diet; *Il1b*, interleukin-1 beta; *Il6*, interleukin 6; LBP, lipopolysaccharide binding protein; LCN, lipocalin; LEfSe, linear discriminant analysis effect size; LFD, low-fat diet; Muc2, mucin 2; NGAL, neutrophil gelatinase-associated lipocalin; *Nod*, nucleotide-binding oligomerization domain; *Nos2*, nitric oxide synthase 2; *Nox2*, NADPH oxidase 2; PERMANOVA, permutational multivariate analysis of variance; *Ptgs2*, prostaglandin-endoperoxide synthase 2; QIIME, Quantitative Insights Into Microbial Ecology; *Tlr4*, toll-like receptor 4; *Tnf-a*, tumor necrosis factor alpha; WD, Western diet; *Zo1*, zonula occludens-1.

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Sampling

Samples were collected on day 6 of DSS exposure. Initially, whole blood was collected by cardiac puncture following anesthesia by a cocktail of Zoletil Forte (Virbac), Rompun (Bayer), and Fentadon (Eurovet Animal Health) (ZRF; intraperitoneally 0.1 mL ZRF/10 g body weight), with the following active ingredients: zolezepam (32 mg/kg), tiletamine (32 mg/kg), xylazine (4.5 mg/kg), and fentanyl (26 μg/kg). Blood (0.5–1 mL) was drawn into tubes containing ~50 μL NaEDTA (50 mM) as anticoagulant and mice were then killed by cervical dislocation. Blood was centrifuged (6000 × *g*, 10 min, 4°C) to obtain plasma. Colon mucosa was collected by opening the colon longitudinally and kept in RNAlater (Sigma-Aldrich). Fecal pellets were collected from the colon. All samples were stored at −80°C. Due to failure of collecting and processing some of the samples, the number of data points differed occasionally between groups.

Epithelial barrier permeability

Barrier permeability was measured by using fluorescein isothiocyanate dextran [FITC dextran, 4 kDa (FD4); Sigma-Aldrich), according to Johnson et al. (37). In brief, mice on termination day (Exp.1) were fasted for 4 h before 600 mg/kg FD4 was orally administered. Whole blood was collected by cardiac puncture ~3 h post FD4 administration. Plasma was obtained as described above and diluted 1:5 in PBS. FITC dextran was determined by fluorescence-spectroscopy (Synergy H4 Hybrid microplate reader, BioTek instruments; 490 nm $_{Ex}$/520 nm $_{Em}$). FITC dextran concentration was calculated using a standard curve based on 5 points of serial dilutions of FITC dextran in control plasma.

RNA extraction and qRT-PCR

RNA from colonic mucosa samples was extracted with the NucleoSpin RNA/Protein Purification kit (Macherye-Nagel). Because DSS reduces efficiency of both reverse transcriptase and PCR reactions (38, 39), all colon RNA samples were purified using lithium chloride according to Viennois et al. (39).

cDNA synthesis from RNA was performed (**Supplemental Tables 4** and **5**) using iScript cDNA Synthesis Kit (1708891, Bio-Rad), whereas FirePol EvaGreen qPCR Supermix (08-36-00001, Solis BioDyne) was used for the qRT-PCR reaction in a Light Cycler 480 Instrument II (Roche). The parameter settings were: 12 min at 95°C; 40 cycles of 15 s at 95°C followed by 20 s at optimized primer annealing temperature; 20 s at 72°C. LinRegPCR Software (2017.1.0.0) was used to calculate quantification cycle values and primer efficiency (40). Primers used for mRNA expression (Thermo Fisher Scientific) are presented in **Supplemental Table 6**.

Lipocalin-2 measurement

Mouse Lipocalin-2/NGAL DuoSet ELISA (R&D systems) was used for measuring lipocalin-2 protein (LCN2) from fecal samples collected on day 6 of DSS exposure based on a protocol described earlier (32). Briefly, fecal suspensions were made by vortexing fecal samples (20 min) in PBS containing 0.1% Tween 20 (100 mg feces in 1 mL buffer). Suspensions were centrifuged (13,500 × *g*, 10 min, 4°C) and supernatants were collected and subjected to analysis. Samples were diluted 20 times (untreated mice) and 20,000 times (DSS-treated). Optical density at 450 nm was determined with a spectrophotometer (SpectraMax M2; Molecular Devices). LCN2 concentration was estimated from a standard curve using 4-parameter logistic curve fit.

Lipopolysaccharide binding protein measurement

Lipopolysaccharide binding protein (LBP) in plasma was measured with an ELISA assay according to the manufacturer (Biometec). Plasma was obtained at day 6 (termination day). Plasma samples from control mice were diluted 800 times, whereas samples from 1% DSS-treated mice were diluted ~1500 times. The concentration was measured by optical density as described for Lipocalin-2/NGAL measurements above.

16S rRNA gene sequencing

The workflow has been described previously (41). Briefly fecal pellets were placed in 400 μL S.T.A.R buffer (Roche) containing glass beads (Sigma-Aldrich). Samples were processed by FastPrep 96 (1800 rpm, 40 s, 5 min cooling step in between; MP BioMedicals) to lyse cells and centrifuged (15,900 × *g*, 10 min, 21°C). Supernatants were treated with protease using the Mag Mini LGC kit (LGC Genomics), and KingFisher Flex DNA extraction robot (Thermo Fisher Scientific) for DNA extraction. Because DSS has an inhibitory effect on PCR (39), extracted DNA from fecal samples was diluted 1:4 prior to amplicon PCR (total dilution of 1:100 in the PCR reaction).

After DNA extraction, the 16S rRNA gene was amplified by PCR ("amplicon PCR") using prokaryote-targeting primers specific for the variable region of V3-V4 of the 16S rRNA gene (25 cycles) (42). Primer sequences and PCR conditions are listed in **Supplemental Tables 7** and **8**. PCR product was purified with AMPure XP (Beckman-Coulter) and 10 further PCR cycles ("index PCR") were performed (**Supplemental Tables 9** and **10**) resulting in PCR product of ~594 bp. The sequences of primers in index PCR are shown in **Supplemental Table 11**. All PCR products were qualitatively confirmed by electrophoresis on a 1.5% agarose gel. Quantification of DNA concentrations of index PCR products, and normalization and pooling of these index PCR products were followed by purification of the pooled library with Sera Mag Beads by following the AMPure XP protocol. The pooled library was diluted to 6 pM and sequenced with the MiSeq Reagent Kit V3 (cat. no. MS-102-3003) on the Illumina MiSeq following Illumina's protocol, generating 300-bp paired-end reads that were further paired-end joined and split into their respective samples, quality-filtered using QIIME (Quantitative Insights Into Microbial Ecology) (43), and clustered with 97% identity and higher using the closed-reference usearch algorithm (version 8) (44, 45) against the SILVA database (version 128) (46). To normalize (rarefy) the sequencing data, 6500 sequences per sample were chosen as a cut-off.

Statistics

Statistical analyses were performed using GraphPad Prism (version 8.3.1 for Windows; GraphPad Software). Data are presented as individual values with group means ± SEM. When necessary, data were $\log_{10}$-transformed to achieve stabilized variance and normality, and geometric group mean with geometric SD was applied as the best way to express the center of distribution. Normal distribution was tested using the Shapiro–Wilk normality test. Using the Brown–Forsythe test, following normality testing and possible transformation, it was investigated whether the variation (SD) within the groups (homogeneity of variance) was significantly different. Based on whether normal distribution was achieved or not, parametric and nonparametric models were used respectively. P values < 0.05 were considered significant.

In Exp.1, prior to DSS treatment, body weight change and food intake were analyzed by the mixed effects model. In the case of significant interaction (time × diet), data were analyzed for simple main effect of diet within each time point with Bonferroni correction for multiple comparisons. During DSS exposure, body weight changes (Exp.1, Exp.2, and Exp.3) and disease activity index (DAI) (Exp.1) were analyzed using repeated measures 2-factor ANOVA with Geisser–Greenhouse correction. In case of significant interactions (time × diet) we assessed simple main effect of diet for each time point using Tukey or Bonferroni correction for multiple comparisons. Comparisons of untreated and DSS-treated groups were analyzed using 2-factor ANOVA (effects of diet and treatment). When interactions (treatment × diet) were significant we compared all groups with Bonferroni correction for multiple comparisons. If assumptions for ANOVA were not met, comparisons were performed using another suitable approach as specified in figure legends (unpaired t test with Welch correction or Mann–Whitney test). Also, in Exp.1 outliers identified by the Rout method, Q = 1% were excluded. In Exp.2 and Exp.3, 1-factor ANOVA was used for the DSS groups followed by Tukey post hoc analysis for the expression of inflammatory genes. Untreated LFD-fed mice in Exp.2 were not included in the statistical analysis.

Analysis of β diversity was conducted in R (version 4.0.0). Weighted UniFrac distances were calculated using QIIME default scripts (core_diversity_analyses.py) and are based on the normalized (rarefied) OUT table. Nonmetric multidimensional scaling of weighted UniFrac distances was performed using the metaMDS function from the vegan

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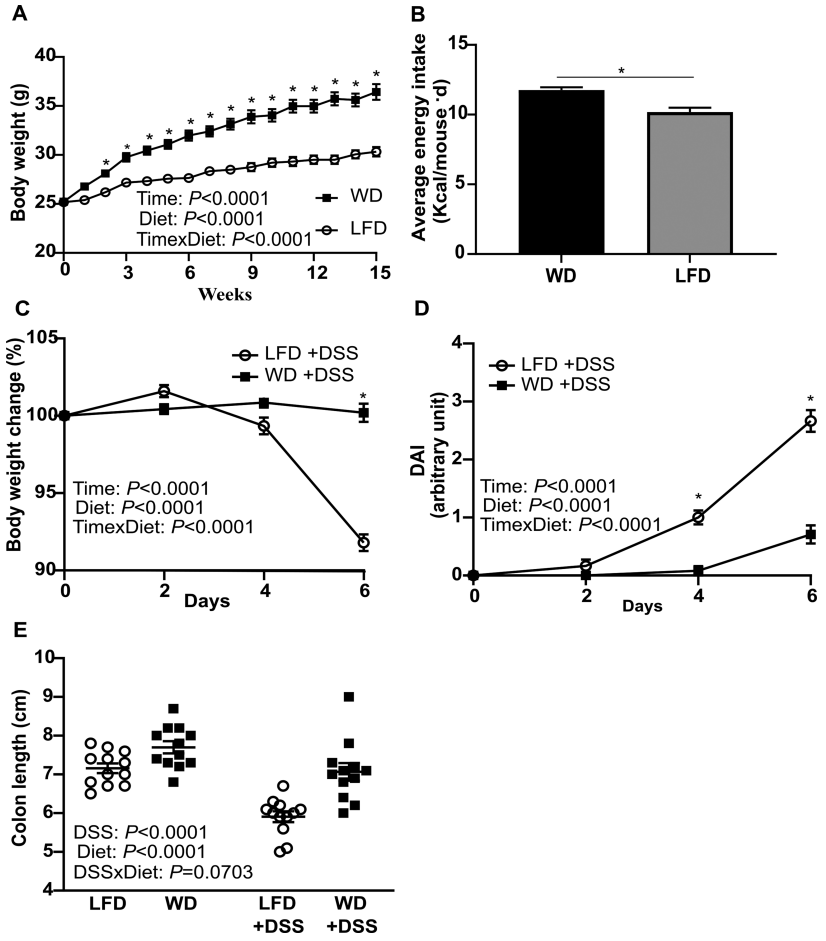

FIGURE 1 Body weight development and food intake during 15 wk prior to 1% DSS exposure (A, B). Change in body weight (%) measured on days 0, 2, 4, and 6 following start of 1% DSS exposure (C). DAI score for mouse welfare during 1% DSS treatment (D). Colon length from LFD-fed and WD-fed mice with or without 1% DSS (E). Values are means ± SEM ($n = 12$). For panels A, C, and D: *significantly different from LFD at that time, $P < 0.05$. DAI, disease activity index; DSS, dextran sodium sulfate; LFD, low-fat diet; WD, Western diet.

package (47) with autotransform = FALSE and try = 100. Global permutational multivariate analysis of variance (PERMANOVA) on weighted UniFrac distances was performed using the adonis function from the vegan package with 999 permutations. Pairwise PERMANOVA was performed by applying the pairwise.perm.manova function from the RVAideMemoire package (48).

For linear discriminant analysis effect size (LEfSe), relative abundances of taxa were used. Software is available at https://huttenhower.sph.harvard.edu/galaxy, with linear discriminant analysis (LDA) score set at 2.0 and $P \leq 0.05$.

Results

Exp.1

1% DSS induced a disease phenotype in LFD-fed mice.

After a feeding period of 15 wk and before administering 1% DSS, weight gain in WD-fed mice was significantly higher compared with LFD-fed mice (**Figure 1**A). Weight gain corroborated with an increased energy intake in the WD group compared with LFD-fed mice (11.7 compared with 10.2 kcal/mouse/d; $P < 0.01$) (Figure 1B).

After 6 d of DSS treatment LFD mice experienced an average 8% weight loss whereas WD-fed mice showed no change in body weight (Figure 1C). In addition, LFD mice had a significantly higher DAI score than WD mice from day 4 after DSS exposure (Figure 1D). With regard to colon length, LFD+DSS caused shorter colons compared with WD+DSS treatment. Overall there was a significantly shorter colon length due to both diet ($P < 0.0001$) and DSS ($P < 0.0001$) (Figure 1E).

Levels of proinflammatory cytokines and LCN2 were increased in LFD-fed mice.

The expression of the inflammatory genes, tumor necrosis factor alpha (*Tnf*-a), interleukin 1 beta (*Il1b*), interleukin 6 (*Il6*), and prostaglandin-endoperoxide synthase 2 (*Ptgs2*), in the distal colon was compared between the diet groups with and without DSS treatment (**Figure 2**A–D). For all genes except *Ptgs2* an interaction effect was found between diet and treatment ($P <$

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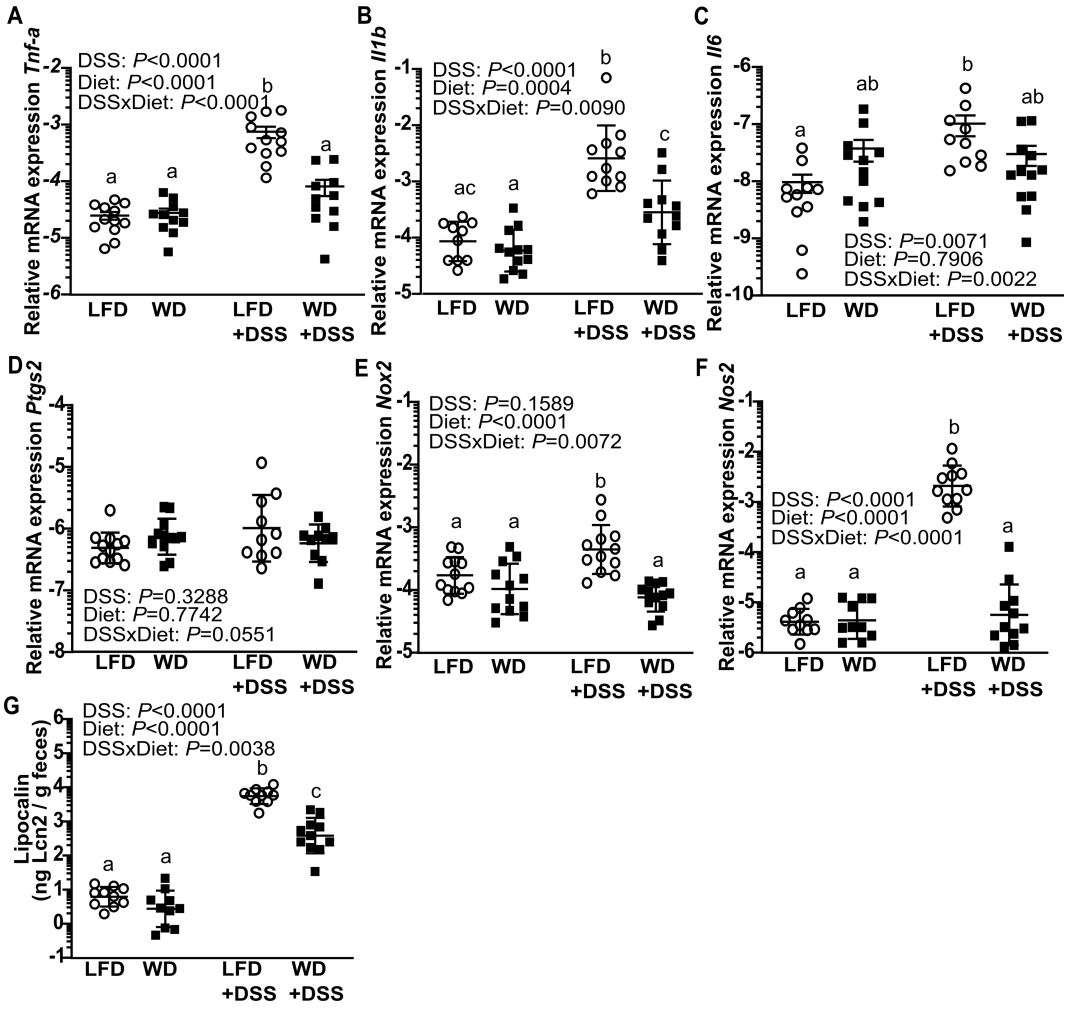

FIGURE 2 Relative mRNA expression of tumor necrosis factor α (*Tnf-a*) (A), interleukin-1 beta (*Il1b*) (B), interleukin 6 (*Il6*) (C), prostaglandin-endoperoxide synthase 2 (*Ptgs2*) (D), NADPH oxidase 2 (*Nox2*) (E), and nitric oxide synthase 2 (*Nos2*) (F) in mucosa from the proximal colon of LFD-fed and WD-fed mice with or without 1% DSS. Concentration of LCN2 in feces (G). Values are means ± SEM ($n = 9$–12). Labeled means without a common letter differ, $P < 0.05$. DSS, dextran sodium sulfate; LCN, lipocalin; LFD, low-fat diet; WD, Western diet.

0.01). In untreated mice, no differences were found between WD and LFD groups whereas DSS treatment led to a significant upregulation of these genes in the LFD+DSS mice compared with WD+DSS mice ($P < 0.05$). In WD-fed mice the expression levels of the above mentioned genes were not affected by DSS.

The same pattern of treatment × diet interaction was also observed for nitric oxide synthase 2 (*Nos2*) (also known as *iNos*) and NADPH oxidase 2 (*Nox2*), which was significantly higher in LFD+DSS mice ($P < 0.001$) compared with WD+DSS mice (Figure 2E,F). Finally we assessed the concentrations of LCN2 in feces, a sensitive marker of colonic inflammation. In both diet groups DSS treatment led to an increase of fecal LCN2, but the effect was significantly higher ($P < 0.001$) in LFD+DSS mice compared with WD+DSS mice (Figure 2G), also suggesting an interaction between treatment and diet for LCN2.

Gene expression for gut permeability was affected in LFD-fed mice.

In addition to inflammatory related genes, expression of genes related to gut barrier and pattern recognition receptors was examined. DSS treatment led to a higher expression of toll-like receptor 4 (*Tlr4*), zonula occludens-1 (*Zo1*), and nucleotide-binding oligomerization domain 2 (*Nod2*) in the LFD mice when compared with WD-fed animals ($P < 0.001$).

To investigate potential breach in the gut barrier, we assessed concentrations of lipopolysaccharide-binding protein (LBP) in plasma, which is an indicator of LPS leakage from the gut. A significantly higher concentration of LBP was found in

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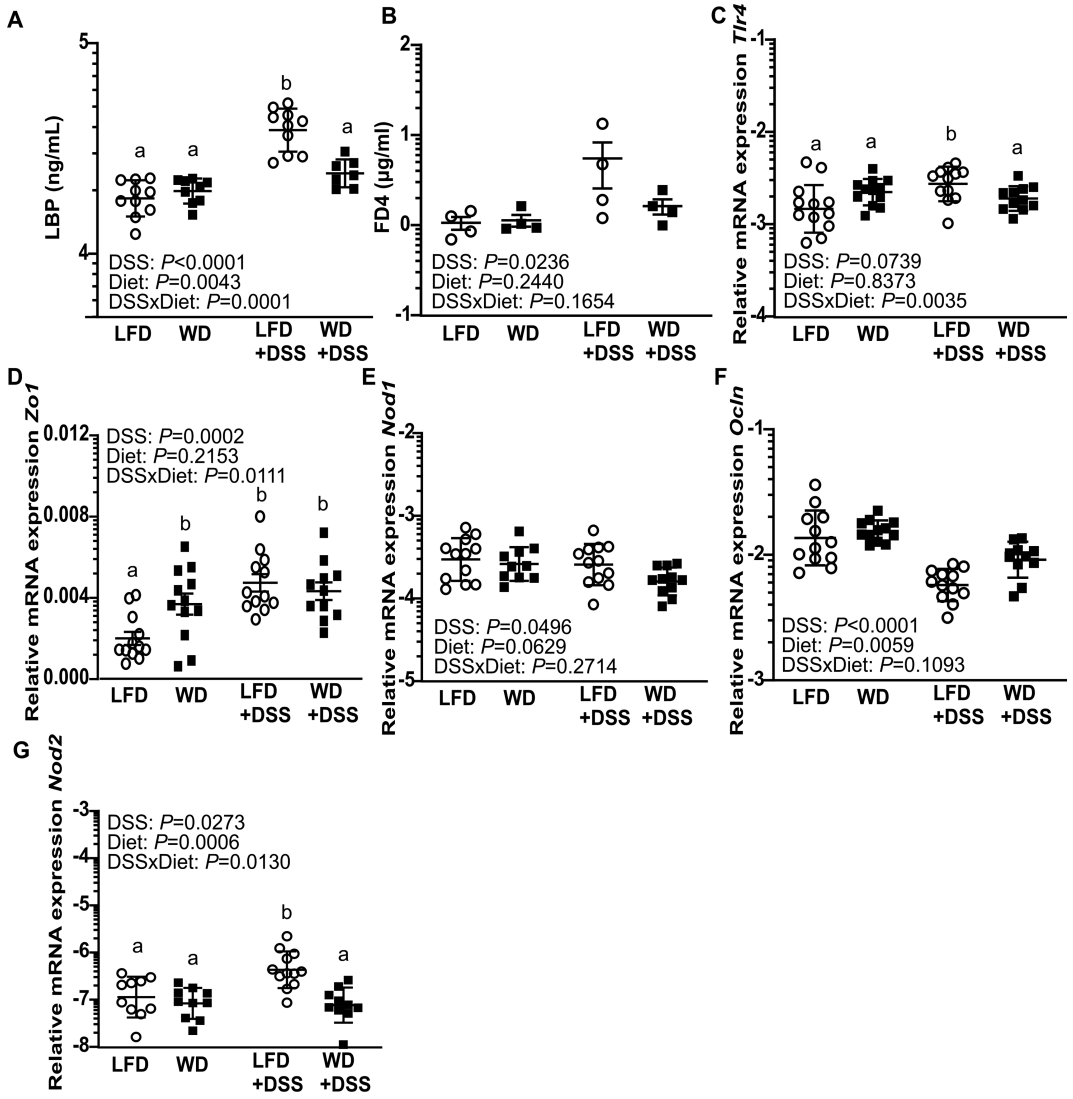

FIGURE 3 Concentration of LBP in plasma from LFD-fed and WD-fed mice with or without 1% DSS (A). FD4 in plasma 2 h after oral gavage (B). Relative mRNA expression of toll-like receptor 4 (*Tlr4*) (C), zonula occludens-1 (*Zo1*) (D), nucleotide-binding oligomerization domain 1 (*Nod1*) (E), occludin (*Ocln*) (F), and nucleotide-binding oligomerization domain 1 (*Nod2*) (G) in mucosa from the proximal colon of LFD-fed and WD-fed mice with or without 1% DSS. Values are means ± SEM ($n = 7$–12 apart from FD4 assay where $n = 4$). Labeled means without a common letter differ, $P < 0.05$. DSS, dextran sodium sulfate; FD4, FITC (fluorescein isothiocyanate) dextran 4 kDa; LBP, lipopolysaccharide binding protein; LFD, low-fat diet; WD, Western diet.

LFD mice with DSS compared with WD mice with DSS (Figure 3A). The permeability of the gut influenced by diet and DSS, was further examined by assessment of plasma FD4 in 4 randomly selected mice per diet. We observed that DSS significantly increased plasma concentrations of FD4 ($P = 0.024$), but found no difference between the diet groups (Figure 3B).

When comparing untreated LFD and WD mice for *Tlr4* and *Zo1* mRNA abundance we observed that WD caused a higher expression of both these genes compared with LFD mice ($P < 0.05$) (Figure 3C,D). However, DSS treatment increased abundance of *Tlr4* and *Zo1* only in LFD mice ($P < 0.001$) and not in WD mice. Expression levels of *Nod1* and occludin (*Ocln*) genes were marginally downregulated by DSS treatment ($P < 0.05$), but no differences were noted between the 2 diet groups (Figure 3E,F).

DSS treatment caused a marked change in microbiota composition of LFD-fed mice.

16S rRNA sequencing was performed on fecal pellets to elucidate differential effects of diets and DSS treatment on

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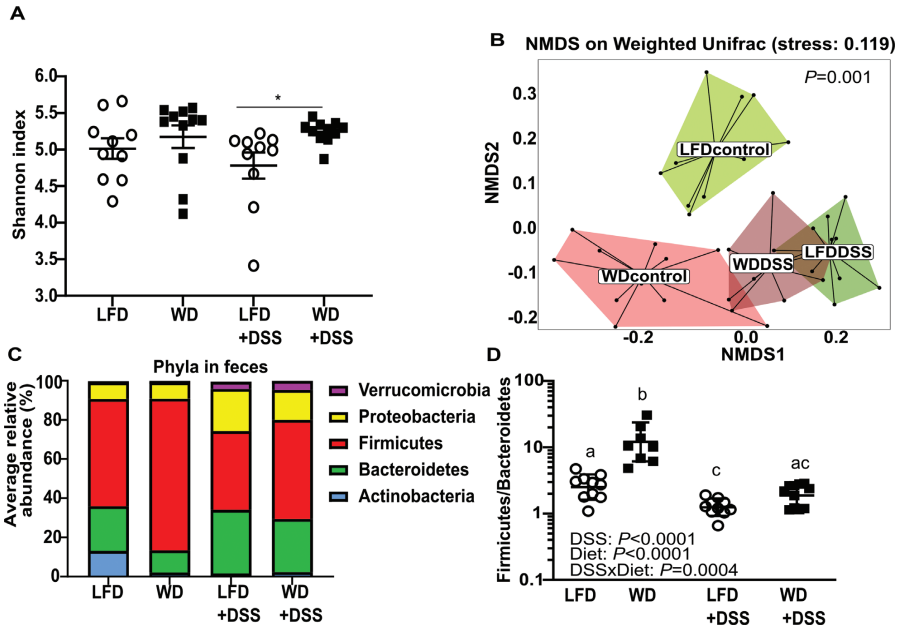

FIGURE 4 Microbiota analyses in feces from LFD-fed and WD-fed mice with or without 1% DSS. α Diversity with Shannon index (A), and β diversity with nonmetric multidimensional scaling (NMDS) of weighted UniFrac distances between groups (B). Colors indicate which group individual samples belong to (LFD control, WD control, LFD+DSS, WD+DSS). $P = 0.001$ in the 2-dimensional representation plot is from global PERMANOVA. Average relative abundance for all detected phyla for each group in fecal samples (C). *Firmicutes/Bacteroidetes* ratio in feces (D). Values are expressed as means ± SEM ($n = 9$–11). *Statistically significant difference, $P < 0.05$. Labeled means without a common letter differ, $P < 0.05$. DSS, dextran sodium sulfate; LFD, low-fat diet; PERMANOVA, permutational multivariate analysis of variance; WD, Western diet.

microbiota. LFD+DSS mice had a lower α diversity (within-sample diversity) compared with WD+DSS mice ($P = 0.0006$), whereas in untreated mice, no significant difference was found between the diet groups (**Figure 4**A).

β Diversity (between-sample diversity) showed significant differences between groups (Figure 4B). The LFD control group (untreated mice) was more diverse than the other groups, whereas the LFD+DSS mice were more similar to the WD control (untreated) and WD+DSS mice. The 5 most abundant phyla (relative average abundance >0.5%) were compared between all groups (Figure 4C). As illustrated by the *Firmicutes/Bacteroidetes* ratio (Figure 4D), untreated LFD-fed mice showed a higher abundance of the phylum *Bacteroidetes* and lower abundance of *Firmicutes* than untreated WD-fed mice. Notably untreated LFD-fed mice had high abundance of *Actinobacteria*, which was hardly detected in WD-fed mice. Abundance of *Proteobacteria* was similar in LFD- and WD-fed mice. Following 1% DSS administration, the abundance of *Proteobacteria* increased in both groups compared with untreated mice and a slight increase in *Bacteroidetes* and *Verrucomicrobia* was observed. *Firmicutes*, however decreased in abundance after DSS administration but with slightly higher levels in WD mice. *Actinobacteria* phylum was almost eliminated in LFD+DSS mice.

LEfSe analyses (49) for non–DSS-treated and DSS-treated animals (**Figure 5**A,B) showed that genera belonging to the *Proteobacteria* phylum, such as *Parasutterella* and *Escherichia-Shigella*, increased significantly ($P < 0.05$) (Figure 5C,D) and there was a striking reduction of the genus *Bifidobacterium* (Figure 5E) in LFD mice treated with 1% DSS.

Exp.2

WD reduced DSS inflammation regardless of fat source.

To investigate whether fat source was important for reducing DSS-mediated inflammation we compared LFD+DSS mice with WD+DSS mice where either milk fat or lard was used as the fat source in the WD (Exp.2). The results revealed similar protection against 1% DSS in both WD groups compared with LFD+DSS, regardless of fat source. As in Exp.1, the LFD+DSS mice showed the same pattern of weight loss (**Figure 6**A) and strong upregulation of *Tnf-a* and *Il1b* gene expression compared with WD+DSS containing either milk fat or lard as the fat source (Figure 6B,C).

Exp.3

WD rapidly attenuated DSS-mediated inflammation.

To test whether a WD offered an immediate "rescue effect" independent of long-term WD feeding, a third experiment was conducted (Exp.3). We here switched the diet from LFD to WD 1 d before applying DSS (LFD-WD+DSS) and compared this group with 2 other groups that were kept on the same diet from the start to the end of the experiment (WD-WD+DSS and LFD-LFD+DSS). WD introduced to LFD mice just prior to DSS treatment partially attenuated the DSS-mediated effects, both with regard to change in body weight (Figure 6D) and expression of *Tnf-a* and *Il1b* (Figure 6E,F). In line with the outcomes from Exp.1 and Exp.2, LFD-LFD+DSS mice experienced more weight loss and greater increase

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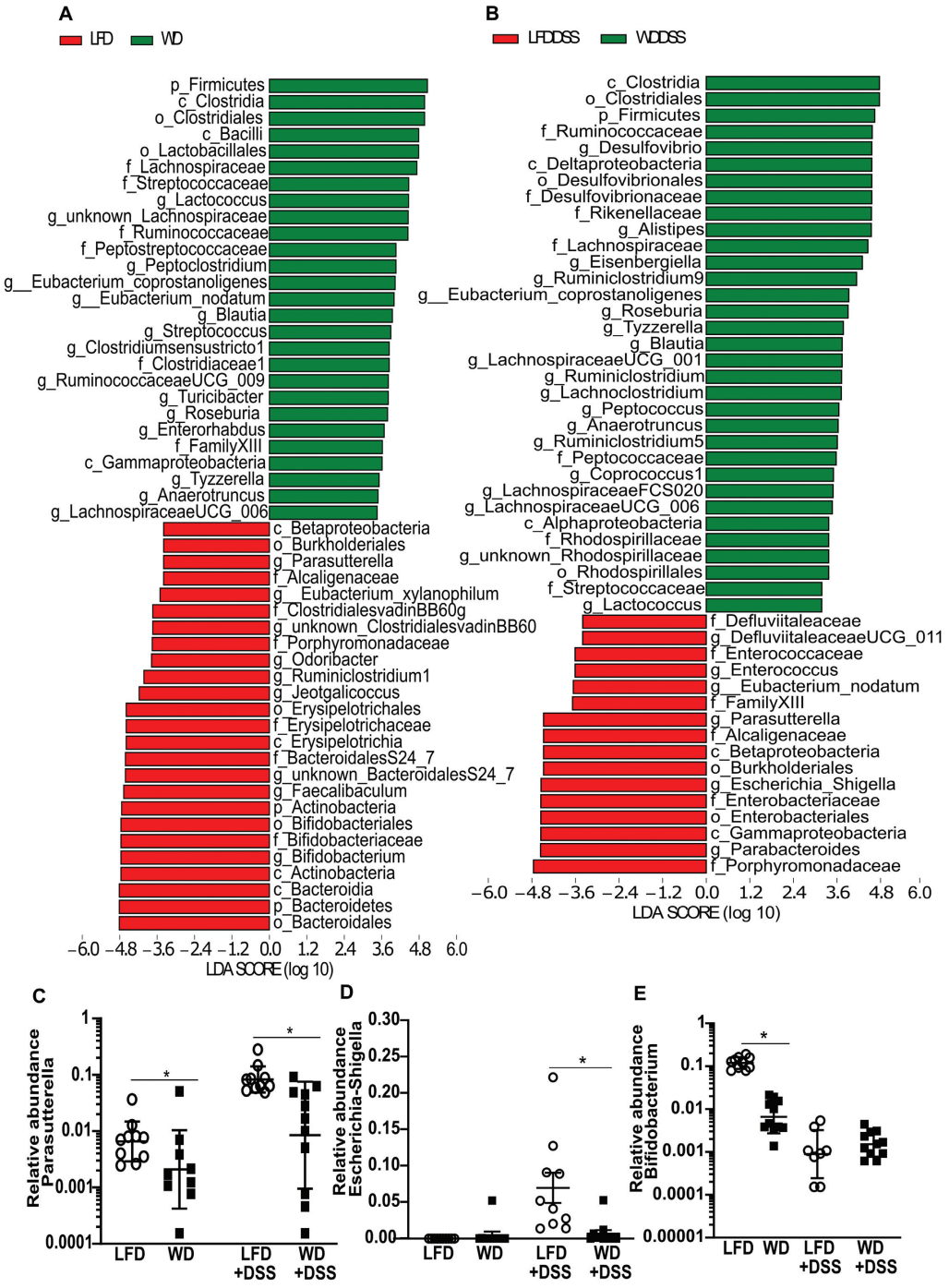

FIGURE 5 Comparison of the operational taxonomic units using linear discriminant effect size analysis and genera presence from *Actinobacteria* and *Proteobacteria* between the LFD-fed and WD-fed mice with or without 1% DSS. The histograms (A, B) present the taxa that explain the greatest differences between the LFD-fed and WD-fed mice untreated and treated with 1% DSS. Relative abundance of *Parasutterella* (C), *Escherichia-Shigella* (D), and *Bifidobacterium* (E) ($n = 8–11$). In panels C–E, *statistically significant difference, $P < 0.05$. c, class; DSS, dextran sodium sulfate; f, family; g, genus; LFD, low-fat diet; o, order; p, phylum; WD, Western diet.

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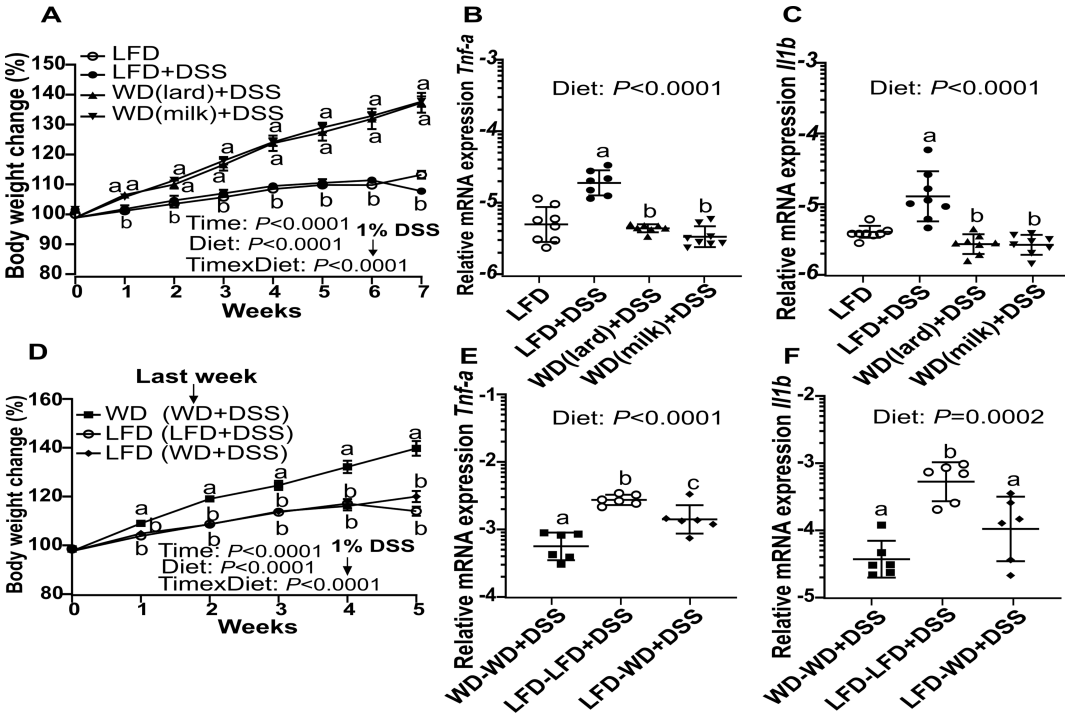

FIGURE 6 Body weight development (A) comparing the effects on 1% DSS treatment between the groups receiving $WD_{milkfat,}$ WD_{lard}, or LFD (Exp.2). Relative mRNA expression of tumor necrosis factor α (*Tnf-a*) (B) and interleukin-1 beta (*Il1b*) (C) in mucosa from the proximal colon was compared between the groups (Exp.2). Values are means ± SEM ($n = 8$). Body weight development (D) comparing the group that changed from LFD into WD in the last week before DSS treatment and the groups that continued on LFD or WD (Exp.3). Relative mRNA expression of *Tnf-a* (E) and *Il1b* (F) in mucosa from the proximal colon were compared between the groups (Exp.3). Values are means ± SEM ($n = 6$). Labeled means without a common letter differ, $P < 0.05$. DSS, dextran sodium sulfate; Exp., Experiment; LFD, low-fat diet; WD, Western diet; WD_{lard}, Western diet with lard fat; $WD_{milkfat}$, Western diet with milk fat.

in proinflammatory genes compared with both WD groups ($P < 0.05$).

Discussion

In the present study we investigated the effects of a WD on colon health and microbiota composition with and without a low-grade inflammation induced by 1% DSS. The main aim was to compare the effects of a WD with an LFD in mice. The 2 diets differed primarily in fat content and cholesterol. The main findings were that WD-fed mice were markedly less affected by DSS treatment compared with LFD-fed mice, which displayed a significantly increased degree of inflammation and had a microbiota composition deviating from that of non-DSS LFD mice.

Based on numerous reports on the detrimental effects of HFDs on intestinal health we initially hypothesized that WD would intensify colonic inflammation induced by DSS when compared with mice fed a LFD. It was therefore unexpected that the WD-fed group was only weakly affected by the DSS treatment whereas LFD mice were severely affected. This was shown not only when assessed clinically but also by using various inflammatory markers including proinflammatory gene expression, biomarker in feces (LCN2), and barrier integrity.

Our results hence contrast with most studies that have investigated the impact of fat-rich diets on DSS-induced colitis, which overall demonstrate that HFDs exacerbate DSS-induced colitis (22, 24, 26). However, in most of these experiments higher DSS concentrations were used and the results might therefore not be directly comparable.

Moreover, most of the studies finding adverse effects of WDs or HFDs, have used standard LFDs rich in dietary fiber as low-fat controls (unpurified diets). Such diets are poorly matched with the commonly used purified HFDs or WDs, which use the metabolically inert cellulose as the fiber source. The high diversity of fiber in unpurified diets, therefore, represents a confounder when interpreting results regarding effects of WDs or HFDs. Thus, it is possible that the adverse effect of WDs or other HFDs seen in many studies could be the result of a diet devoid of dietary fiber, which creates both a less diverse bacterial composition and blooming of bacteria that weakens the intestinal barrier (50) and not the high-fat content per se. In a study by Miles et al. (51), mice fed an unpurified diet were significantly more protected against DSS than mice fed a synthetic LFD.

Although our results contradict most studies investigating the impact of a WD, Enos and coworkers (52) demonstrated

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that mice fed a WD had significantly less tumor burden and inflammation in an AOM/DSS model of colorectal cancer compared with an LFD. The authors suggested that the protection against inflammation in that model could be ascribed to a higher content of mucin 2 (Muc2), which is the dominant protein in the protective mucus layer, and thereby strengthens the intestinal barrier . However, we did not find any difference in *Muc2* mRNA expression between LFD or WD mice (not shown).

Despite a more severe clinical impact of DSS in LFD-fed mice compared with WD, we found no differences in colonic inflammation between the 2 groups that were not exposed to DSS. However, WD-fed mice not exposed to DSS manifested increased expressions of *Zo1* and *Tlr4*, changes that could potentially strengthen the gut barrier and integrity. Tlr4 is a pathogen-recognition receptor and is important for eliciting downstream responses that maintain gut homeostasis (53, 54). Although permeability, as measured by FD4 leakage from gut to the blood, was unchanged in the LFD compared with the WD group, we cannot rule out that upregulation of *Zo1* and *Tlr4* are beneficial responses induced by WD for creating a more robust intestinal wall.

In terms of microbiota composition, we observed no difference in α diversity between LFD and WD in non–DSS-treated mice, but did notice a substantial difference in community structure (β diversity). In agreement with other studies (55, 56), we observed an increased *Firmicutes*/*Bacteriodetes* ratio in WD-fed mice. Interestingly, we also found a strikingly higher abundance of *Bifidobacteria* (phylum *Actinobacteria*) in LFD-fed mice prior to DSS treatment, which has also been reported by others (57). After DSS treatment, the abundance of *Bifidobacteria* dramatically decreased. Both the initial high level of *Bifidobacteria* and the sudden shift in abundance during DSS treatment could be possible drivers of the inflammatory process in the current experiment.

Bifidobacteria are generally considered beneficial commensals and are exploited for probiotic purposes (58, 59). Interestingly, a recent report found that supplementing mice with *Bifidobacteria* could protect against DSS-induced colitis, which argues against an unbeneficial effect of high pre-DSS levels in the LFD mice (60). However, certain strains of *Bifidobacteria* can promote intestinal inflammation through T helper 17 cells in the lamina propria (61). In this study, we did not detect any increase in other proinflammatory markers in LFD compared with WD in non–DSS-treated mice. Therefore, our results do not suggest that the higher *Bifidobacteria* abundance in the LFD before DSS treatment negatively influenced colitis development. Rather, we argue that the sudden shift in the abundance of *Bifidobacteria* during DSS treatment in the current study is a more likely explanation for the colitis development. Considering that *Bifidobacteria* are strict anaerobes, this genus is vulnerable to increased oxygen content in the gut following DSS treatment (62). In line with this argument, we found that expression of genes involved in production of reactive oxygen species, *Nox2* and *Nos2,* was upregulated in LFD+DSS mice.

Proteobacteria phylum increased in abundance in LFD+DSS mice compared with the WD+DSS mice. This is in agreement with other studies showing that *Proteobacteria* can be an indicator of an inflammatory phenotype with disease potential (63). In the LFD+DSS mice we also noticed a significant rise of genera belonging to the *Proteobacteria* such as *Escherichia*, *Shigella*, and *Parasutterella*. These observations agree with a recent study, where different doses of DSS (1%, 2%, 3%) increased the abundance of the family *Enterobacteriaceae* (which includes *Escherichia* and *Shigella*) and depleted *Bifidobacteria* (64).

The mechanism of how DSS induces colitis is not entirely known but it appears that DSS molecules disrupt the epithelial layer resulting in increased colonic epithelial permeability (21). Because DSS is a water-soluble, negatively charged sulfated polysaccharide we speculate that a WD with its high-fat content could create a hydrophobic layer on the intestinal surfaces that interferes with DSS and thereby inhibits the inflammatory action of DSS. To test whether WD had a direct effect on DSS, we performed a follow-up experiment in LFD mice switching the diet to WD just prior to DSS treatment. The WD given concomitantly with the DSS treatment protected against the DSS-induced colitis but the mice that were fed WD throughout the whole experiment were more protected. To the best of our knowledge we cannot find studies supporting that ingested fat can interfere with or neutralize induction of inflammation due to DSS. On the contrary, a study has shown that medium-chain fatty acids can chemically interact with DSS but lead to aggravated effects instead of a reduced colitis (65). There could also be other factors that interfere with establishing the DSS colitis. As suggested by Nell et al. (66) the induction of DSS-induced colitis depends on different factors, such as mouse strain, age, gender, body weight, lot number, molecular weight, concentration, and duration of exposure. To test whether the LFD mice had higher intake of DSS we also assessed water consumption, but found no difference between WD and LFD mice.

An alternative explanation for the observed effect of WDs is the influence of cholesterol (0.2%), which was added to the WD but not the LFD. Dietary cholesterol influences cholesterol homeostasis and leads to increased secretion of both free cholesterol and bile acids in the feces (67). Although we did not measure bile acids in this study, we can assume that concentrations of secondary bile acids in the colon were increased, with a potential impact on both microbiota composition and colonic health. Indeed concentrations of secondary bile acids in feces correlated with exacerbated DSS-induced colitis in mice (68), whereas in another study, secondary bile acids protected against DSS-induced colitis (69). Hence, based on the latter study we cannot rule out that cholesterol can in fact mediate some of the anti-inflammatory effects we observed.

In conclusion, our data demonstrate that a WD reduced DSS-induced colonic outcomes compared with an LFD regardless of whether the fat source was milk or lard. Although these data are somewhat conflicting with the general consensus that a WD adversely affects intestinal health, most previously reported experiments on this subject have rarely used LFD controls that match fiber content in the diet. Whether the protection against DSS is caused by a potential positive contribution of fat in WD or by other nutrients such as cholesterol should be further investigated. It is also possible that the DSS colitis mouse model, despite its popularity due to its rapidity, simplicity, and controllability, is not optimal to investigate the effects of HFDs on the development of colitis.

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The authors' responsibilities were as follows—DP and HC: designed the study; DP, SDCdR, and HC: performed the experiments; DP, SDCdR, and AMH: conducted the statistical analysis; DP, HC, and SKB: interpreted the results; DP and HC: wrote the manuscript; DP, SDCdR, and AMH: prepared the figures for the manuscript; DP, HC, and SKB: supervised the study; DP and HC: prepared the animal protocol; DP, HC, SKB, SDCdR, and AMH: discussed the results and edited manuscript drafts; DP and AMH: analyzed the 16S ribosomal RNA gene profiling data; and all authors: read and approved the final manuscript.

References

1. Poti JM, Duffey KJ, Popkin BM. The association of fast food consumption with poor dietary outcomes and obesity among children: is it the fast food or the remainder of the diet? Am J Clin Nutr 2014;99(1):162–71.
2. Medina-Remon A, Kirwan R, Lamuela-Raventos RM, Estruch R. Dietary patterns and the risk of obesity, type 2 diabetes mellitus, cardiovascular diseases, asthma, and neurodegenerative diseases. Crit Rev Food Sci Nutr 2018;58(2):262–96.
3. Liu TC, Kern JT, Jain U, Sonnek NM, Xiong S, Simpson KF, VanDussen KL, Winkler ES, Haritunians T, Malique A, et al. Western diet induces Paneth cell defects through microbiome alterations and farnesoid X receptor and type I interferon activation. Cell Host Microbe 2021;29(6):988–1001.e6.
4. Minihane AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, Teeling JL, Blaak EE, Fenech M, Vauzour D, et al. Low-grade inflammation, diet composition and health: current research evidence and its translation. Br J Nutr 2015;114(7):999–1012.
5. Christ A, Lauterbach M, Latz E. Western diet and the immune system: an inflammatory connection. Immunity 2019;51(5):794–811.
6. Chassaing B, Gewirtz AT. Gut microbiota, low-grade inflammation, and metabolic syndrome. Toxicol Pathol 2014;42(1):49–53.
7. Andre P, Laugerette F, Feart C. Metabolic endotoxemia: a potential underlying mechanism of the relationship between dietary fat intake and risk for cognitive impairments in humans? Nutrients 2019;11(8):1887.
8. Pendyala S, Walker JM, Holt PR. A high-fat diet is associated with endotoxemia that originates from the gut. Gastroenterology 2012;142(5):1100–1.e2.
9. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56(7):1761–72.
10. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 2008;57(6):1470–81.
11. Tagliabue A, Elli M. The role of gut microbiota in human obesity: recent findings and future perspectives. Nutr Metab Cardiovasc Dis 2013;23(3):160–8.
12. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto JM, Kennedy S, et al. Richness of human gut microbiome correlates with metabolic markers. Nature 2013;500(7464):541–6.
13. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006;444(7122):1022–3.
14. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A 2005;102(31):11070–5.
15. Rizzatti G, Lopetuso LR, Gibiino G, Binda C, Gasbarrini A. Proteobacteria: a common factor in human diseases. Biomed Res Int 2017;2017:9351507.
16. Kuo SM. The interplay between fiber and the intestinal microbiome in the inflammatory response. Adv Nutr 2013;4(1):16–28.
17. Sonnenburg ED, Sonnenburg JL. Starving our microbial self: the deleterious consequences of a diet deficient in microbiota-accessible carbohydrates. Cell Metab 2014;20(5):779–86.
18. Kennedy A, Martinez K, Chuang CC, LaPoint K, McIntosh M. Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: mechanisms of action and implications. J Nutr 2009;139(1):1–4.
19. Lam YY, Ha CW, Hoffmann JM, Oscarsson J, Dinudom A, Mather TJ, Cook DI, Hunt NH, Caterson ID, Holmes AJ, et al. Effects of dietary fat profile on gut permeability and microbiota and their relationships with metabolic changes in mice. Obesity 2015;23(7):1429–39.
20. Kiesler P, Fuss IJ, Strober W. Experimental models of inflammatory bowel diseases. Cell Mol Gastroenterol Hepatol 2015;1(2):154–70.
21. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. Curr Protoc Immunol 2014;104(1):15.25.1–15.25.14.
22. Ma X, Torbenson M, Hamad AR, Soloski MJ, Li Z. High-fat diet modulates non-CD1d-restricted natural killer T cells and regulatory T cells in mouse colon and exacerbates experimental colitis. Clin Exp Immunol 2008;151(1):130–8.
23. van der Logt EM, Blokzijl T, van der Meer R, Faber KN, Dijkstra G. Westernized high-fat diet accelerates weight loss in dextran sulfate sodium-induced colitis in mice, which is further aggravated by supplementation of heme. J Nutr Biochem 2013;24(6):1159–65.
24. Cheng L, Jin H, Qiang Y, Wu S, Yan C, Han M, Xiao T, Yan N, An H, Zhou X, et al. High fat diet exacerbates dextran sulfate sodium induced colitis through disturbing mucosal dendritic cell homeostasis. Int Immunopharmacol 2016;40:1–10.
25. Wunderlich CM, Ackermann PJ, Ostermann AL, Adams-Quack P, Vogt MC, Tran ML, Nikolajev A, Waisman A, Garbers C, Theurich S, et al. Obesity exacerbates colitis-associated cancer via IL-6-regulated macrophage polarisation and CCL-20/CCR-6-mediated lymphocyte recruitment. Nat. Commun 2018;9(1):1646.
26. Teixeira LG, Leonel AJ, Aguilar EC, Batista NV, Alves AC, Coimbra CC, Ferreira AV, de Faria AM, Cara DC, Alvarez Leite JI. The combination of high-fat diet-induced obesity and chronic ulcerative colitis reciprocally exacerbates adipose tissue and colon inflammation. Lipids Health Dis 2011;10(1):204.
27. Lee JC, Lee HY, Kim TK, Kim MS, Park YM, Kim J, Park K, Kweon MN, Kim SH, Bae JW, et al. Obesogenic diet-induced gut barrier dysfunction and pathobiont expansion aggravate experimental colitis. PLoS One 2017;12(11):e0187515.
28. Benninghoff AD, Hintze KJ, Monsanto SP, Rodriguez DM, Hunter AH, Phatak S, Pestka JJ, Wettere AJV, Ward RE. Consumption of the total Western diet promotes colitis and inflammation-associated colorectal cancer in mice. Nutrients 2020;12(2):544.
29. Zhao D, Cai C, Chen Q, Jin S, Yang B, Li N. High-fat diet promotes DSS-induced ulcerative colitis by downregulated FXR expression through the TGFB pathway. Biomed Res Int 2020;2020:3516128.
30. Li X, Wei X, Sun Y, Du J, Li X, Xun Z, Li YC. High-fat diet promotes experimental colitis by inducing oxidative stress in the colon. Am J Physiol Gastrointest Liver Physiol 2019;317(4):G453–G62.
31. Suzuki R, Kohno H, Sugie S, Nakagama H, Tanaka T. Strain differences in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice. Carcinogenesis 2005;27(1):162–9.
32. Chassaing B, Srinivasan G, Delgado MA, Young AN, Gewirtz AT, Vijay-Kumar M. Fecal lipocalin 2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation. PLoS One 2012;7(9):e44328.
33. Moss AC. The meaning of low-grade inflammation in clinically quiescent inflammatory bowel disease. Curr Opin Gastroenterol 2014;30(4):365–9.
34. Barbara G, Cremon C, Stanghellini V. Inflammatory bowel disease and irritable bowel syndrome: similarities and differences. Curr Opin Gastroenterol 2014;30(4):352–8.
35. Hasegawa Y, Chen SY, Sheng L, Jena PK, Kalanetra KM, Mills DA, Wan YY, Slupsky CM. Long-term effects of western diet consumption in male and female mice. Sci Rep 2020;10(1):14686.
36. Agus A, Denizot J, Thevenot J, Martinez-Medina M, Massier S, Sauvanet P, Bernalier-Donadille A, Denis S, Hofman P, Bonnet R, et al. Western diet induces a shift in microbiota composition enhancing susceptibility to adherent-invasive E. coli infection and intestinal inflammation. Sci Rep 2016;6(1):19032.
37. Johnson AM, Costanzo A, Gareau MG, Armando AM, Quehenberger O, Jameson JM, Olefsky JM. High fat diet causes depletion of intestinal eosinophils associated with intestinal permeability. PLoS One 2015;10(4):e0122195.

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38. Kerr TA, Ciorba MA, Matsumoto H, Davis VR, Luo J, Kennedy S, Xie Y, Shaker A, Dieckgraefe BK, Davidson NO. Dextran sodium sulfate inhibition of real-time polymerase chain reaction amplification: a poly-A purification solution. Inflamm Bowel Dis 2012;18(2):344–8.
39. Viennois E, Chen F, Laroui H, Baker MT, Merlin D. Dextran sodium sulfate inhibits the activities of both polymerase and reverse transcriptase: lithium chloride purification, a rapid and efficient technique to purify RNA. BMC Res Notes 2013;6(1):360.
40. Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, Moorman AF. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 2009;37(6):e45.
41. Avershina E, Lundgård K, Sekelja M, Dotterud C, Storrø O, Øien T, Johnsen R, Rudi K. Transition from infant- to adult-like gut microbiota. Environ Microbiol 2016;18(7):2226–36.
42. Yu Y, Lee C, Kim J, Hwang S. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol Bioeng 2005;89(6):670–9.
43. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7(5):335–6.
44. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;26(19):2460–1.
45. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 2013;10(10):996–8.
46. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glockner FO. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 2007;35(21):7188–96.
47. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, et al. vegan: community ecology package. R-package version 2.5-6. The R Foundation; 2019.
48. Hervé M. RVAideMemoire: testing and plotting procedures for biostatistics. R package version 0.9-78. The R Foundation; 2020.
49. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. Genome Biol 2011;12(6):R60.
50. Desai MS, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, Pudlo NA, Kitamoto S, Terrapon N, Muller A, et al. A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. Cell 2016;167(5):1339–53.
51. Miles JP, Zou J, Kumar MV, Pellizzon M, Ulman E, Ricci M, Gewirtz AT, Chassaing B. Supplementation of low- and high-fat diets with fermentable fiber exacerbates severity of DSS-induced acute colitis. Inflamm Bowel Dis 2017;23(7):1133–43.
52. Enos RT, Velazquez KT, McClellan JL, Cranford TL, Nagarkatti M, Nagarkatti PS, Davis JM, Murphy EA. High-fat diets rich in saturated fat protect against azoxymethane/dextran sulfate sodium-induced colon cancer. Am J Physiol Gastrointest Liver Physiol 2016;310(11):G906–G19.
53. Kramer B, Franca LM, Zhang Y, Paes AMA, Gerdes AM, Carrillo-Sepulveda MA. Western diet triggers Toll-like receptor 4 signaling-induced endothelial dysfunction in female Wistar rats. Am J Physiol Heart Circ Physiol 2018;315(6):H1735–H47.
54. Rivera CA, Gaskin L, Singer G, Houghton J, Allman M. Western diet enhances hepatic inflammation in mice exposed to cecal ligation and puncture. BMC Physiol 2010;10(1):20.
55. Clarke SF, Murphy EF, Nilaweera K, Ross PR, Shanahan F, O'Toole PW, Cotter PD. The gut microbiota and its relationship to diet and obesity: new insights. Gut Microbes 2012;3(3):186–202.
56. Kim SJ, Kim SE, Kim AR, Kang S, Park MY, Sung MK. Dietary fat intake and age modulate the composition of the gut microbiota and colonic inflammation in C57BL/6J mice. BMC Microbiol 2019;19(1):193.
57. Ang QY, Alexander M, Newman JC, Tian Y, Cai J, Upadhyay V, Turnbaugh JA, Verdin E, Hall KD, Leibel RL, et al. Ketogenic diets alter the gut microbiome resulting in decreased intestinal Th17 cells. Cell 2020;181(6):1263–75.
58. Picard C, Fioramonti J, Francois A, Robinson T, Neant F, Matuchansky C. Review article: bifidobacteria as probiotic agents—physiological effects and clinical benefits. Aliment Pharmacol Ther 2005;22(6):495–512.
59. Di Giacinto C, Marinaro M, Sanchez M, Strober W, Boirivant M. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. J Immunol 2005;174(6):3237–46.
60. Singh S, Bhatia R, Khare P, Sharma S, Rajarammohan S, Bishnoi M, Bhadada SK, Sharma SS, Kaur J, Kondepudi KK. Anti-inflammatory Bifidobacterium strains prevent dextran sodium sulfate induced colitis and associated gut microbial dysbiosis in mice. Sci Rep 2020;10(1):18597.
61. Derrien M, van Passel MW, van de Bovenkamp JH, Schipper RG, de Vos WM, Dekker J. Mucin-bacterial interactions in the human oral cavity and digestive tract. Gut Microbes 2010;1(4):254–68.
62. Ruiz L, Ruas-Madiedo P, Gueimonde M, de Los Reyes-Gavilan CG, Margolles A, Sanchez B. How do bifidobacteria counteract environmental challenges? Mechanisms involved and physiological consequences. Genes Nutr 2011;6(3):307–18.
63. Shin NR, Whon TW, Bae JW. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol 2015;33(9):496–503.
64. Park H, Yeo S, Kang S, Huh CS. Longitudinal microbiome analysis in a dextran sulfate sodium-induced colitis mouse model. Microorganisms 2021;9(2):370.
65. Laroui H, Ingersoll SA, Liu HC, Baker MT, Ayyadurai S, Charania MA, Laroui F, Yan Y, Sitaraman SV, Merlin D. Dextran sodium sulfate (DSS) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon. PLoS One 2012;7(3):e32084.
66. Nell S, Suerbaum S, Josenhans C. The impact of the microbiota on the pathogenesis of IBD: lessons from mouse infection models. Nat Rev Microbiol 2010;8(8):564–77.
67. Escolà-Gil JC, Llaverias G, Julve J, Jauhiainen M, Méndez-González J, Blanco-Vaca F. The cholesterol content of Western diets plays a major role in the paradoxical increase in high-density lipoprotein cholesterol and upregulates the macrophage reverse cholesterol transport pathway. Arterioscler Thromb Vasc Biol 2011;31(11):2493–99.
68. Stenman LK, Holma R, Forsgård R, Gylling H, Korpela R. Higher fecal bile acid hydrophobicity is associated with exacerbation of dextran sodium sulfate colitis in mice. J Nutr 2013;143(11):1691–7.
69. Sinha SR, Haileselassie Y, Nguyen LP, Tropini C, Wang M, Becker LS, Sim D, Jarr K, Spear ET, Singh G, et al. Dysbiosis-induced secondary bile acid deficiency promotes intestinal inflammation. Cell Host Microbe 2020;27(4):659–70.e5.

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Online Supplementary Material for: "A high-fat Western diet attenuates intestinal changes in mice with DSS-induced low-grade inflammation"

SUPPLEMENTARY TABLE 1
Compositions of experimental diets

Diets	**LFD**		**WD**	
Ingredients	**g**	**kcal**	**g**	**kcal**
Casein	195	780	195	780
DL-methionine	3	12	3	12
Corn starch	695.38	2780	50	200
Maltodextrin 10	150	600	100	400
Sucrose	0	0	341	1364
Milk fat, cow, anhydrous	42.5	320	200	1800
Corn oil	10	90	10	90
Cellulose, BW200	70	0	70	0
Ethoxyquin	0.04	0	0.04	0
Mineral mix S10001	35	0	35	0
Calcium carbonate	4	0	4	0
Vitamin mix V10001	10	40	10	40
Choline bitartrate	2	0	2	0
Cholesterol	0	0	1.5	0
Total	1216.9	4592.4	1021.5	4592.4
Protein, %	14.3	15.2	17.1	15.2
Carbohydrate, %	70.2	74.5	49	43.6
Fat, %	4.3	10.3	20.1	41.2
kcal/g	3.8		4.5	

LFD, low fat diet (D14042701, Research Diets); WD, Western diet (D12079B, Research Diets).

SUPPLEMENTARY TABLE 2
Fatty acid profile of fat sources.

Fat source	**Milk**	**Lard**
Ingredients	g	g
Butter, Anhydrous	200	0
Corn Oil	10	10
Lard		200
Total	210	210
Fatty Acid	g	g
C4, Butyric	6.4	0.0
C6, Caproic	3.8	0.0
C8, Caprylic	2.2	0.0
C10, Capric	5.0	0.1
C12, Lauric	5.6	0.2
C14, Myristic	20.0	2.3
C14:1, Myristoleic	3.0	0.0
C15, Pentadecanoic	0.0	0.2
C16, Palmitic	53.5	39.7
C16:1, Palmitoleic	4.6	2.7
C17, Heptadecanoic	0.0	0.7
C18, Stearic	24.4	21.4
C18:1, Oleic	52.7	68.2
C18:2, Linoleic	10.6	54.8
C18:3, Linolenic	2.9	2.8
C20, Arachidic	1.9	0.3
C20:1, Eicosenoic	0.0	1.2
C20:2, Eicosadienoic	0.0	1.6
C20:3, Dihomo-gamma-linolenic	0.0	0.3
C20:4, Arachidonic	0.0	0.6
C22:5, Docosapentaenoic	0.0	0.2
Total	196.6	197.3
Saturated	122.8	64.9
Monounsaturated	60.3	72.2
Polyunsaturated	13.6	60.2
	% (wt: wt)	% (wt: wt)
Saturated	62.4	32.9
Monounsaturated	30.7	36.6
Polyunsaturated	6.9	30.5

Wt, weight.

SUPPLEMANTARY TABLE 3

Criteria for DAI and scoring way for assessing during exposure to 1% DSS.

	day 0	day 2		day 4		Termination (day 6)	
	Initial weight	weight	% change from start	weight	% change from start	weight	% change from start
Animal 1							
Animal 2							
Animal 3							
Animal 4							

Score given regarding body weight loss:

For weight
0=<5% weight loss
1= 5-15% weight loss
5= 15-20% weight loss
10=> 20% weight loss

	Activity level		Hunched posture		Stool quality	
	DAY 2	DAY 4	DAY 2	DAY 4	DAY 2	DAY 4
Animal 1						
Animal 2						
Animal 3						
Animal 4						

Score given regarding mouse welfare:

For activity, hunched posture, stool quality
0: normal symptoms
1: mild symptoms
3: severe symptoms

SUPPLEMENTARY TABLE 4
Reaction mixture for cDNA synthesis using the iScript cDNA Synthesis kit (Bio Rad).

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

SUPPLEMENTARY TABLE 5
Temperature program used for cDNA synthesis.

Operation	Temperature (°C)	Duration
Primer annealing	25	5 min
cDNA synthesis	42	30 min
cDNA synthesis termination	85	5 min
-	4	∞

SUPPLEMENTARY TABLE 6
Primer sequences for RT-qPCR and melting temperatures

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Tm °C
Gapdh	CTTCAACAGCAACTCCCACTCTT	GCCGTATTCATTGTCATACCAGG	60
Il1b	GCAGCTGGAGAGTGTGGAT	AAACTCCACTTTGCTCTTGACTT	61
Il6	CGTGGAAATGAGAAAAGAGTTGT	GCATCATCGTTGTTCATACA	61
Nod1	TGACAGTAATCTGGCTGACC	GTCTGGTTCACTCTCAGCAT	59
Nod2	GCAGAAACTAGCTCTCTTCAAC	CGGCTGTGATGTGATTGTTC	61
Nos2	GACATTACGACCCCTCCCAC	ACTCTGAGGGCTGACACAAG	62
Nox2	GGGAACTGGGCTGTGAATGA	CAGTGCTGACCCAAGGAGTT	61
Ocln	CTGTGAAAACCCGAAGAAAGATG	GCAGACACATTTTTAACCCACTC	57
Ptgs2	AATATCAGGTCATTGGTGGAGA	TCTACCTGAGTGTCTTTGACTG	61
Tlr4	GATCTGAGCTTCAACCCCTT	TGTTTCAATTTCACACCTGGA	61
Tnfa	CTGTCTACTGAACTTCGGGGTGAT	GGTCTGGGCCATAGAACTGATG	61
Zo1	GAGAAAGGTGAAACTCTGCTG	ACGAGGAGTCGGATGATTTTAGA	59

Abbreviations: *Gapdh***,** glyceraldehyde 3-phosphate dehydrogenase; *Il1b*, interleukin-1 beta; *Il6*, interleukin 6; *Nod1*, nucleotide-binding oligomerization domain 1; Nod2, nucleotide-binding oligomerization domain 2; *Nos2*, nitric oxide synthase 2; *Nox2*, NADPH oxidase 2; *Ocln*, Occludin; *Ptgs2*, prostaglandin endoperoxide synthase 2; *Tlr4*, toll like receptor 4; *Tnf*a, tumor necrosis factor alpha; *Zo1*, Zonula occludens 1.

SUPPLEMENTARY TABLE 7
Reaction mixture for amplicon PCR during library preparation for gene sequencing of 16S rRNA.

Component	Per reaction
5x HOT FIREPol® Blend Master Mix Ready to Load (Solis BioDyne)	5 µL
Forward primer, PRK341F (1 µM)*	0.5 µL
Reverse primer, PRK806R (1 µM)*	0.5 µL
Nuclease-free water	18 µL
Template DNA (0.003-2 ng/µL**)	1 µL

* Forward 5'- CCTACGGGRBGCASCAG-3', reverse 5'- GGACTACYVGGGTATCTAAT-3'

** Measured by Qubit.

SUPPLEMENTARY TABLE 8
Temperature cycles for amplicon PCR during library preparation for gene sequencing of 16S rRNA.

Operation	Temperature (°C)	Duration	Cycles
Initial activation	95	15 min	1
Denaturation	95	30 sec	
Annealing	55	30 sec	25
Elongation	72	45 sec	
Final elongation	72	7 min	1
-	4	∞	-

SUPPLEMENTARY TABLE 9
Reaction mixture for index PCR during library preparation for gene sequencing of 16S rRNA.

Component	Per reaction
5x FIREPol® Master Mix Ready to Load (Solis BioDyne)	5 µL
Forward primer (1 µM)*	5 µL
Reverse primer (1 µM)*	5 µL
Nuclease-free water	8 µL
Template DNA	2 µL

* See Supplementary table 11

SUPPLEMENTARY TABLE 10

Temperature cycles for index PCR during library preparation for gene sequencing of 16S rRNA.

Operation	Temperature (°C)	Duration	Cycles
Initial activation	95	5 min	1
Denaturation	95	30 sec	
Annealing	55	1 min	10
Elongation	72	45 sec	
Final elongation	72	7 min	1
-	4	∞	-

SUPPLEMENTARY TABLE 11

Primers modified with Illumina adapters used for index PCR during library preparation for gene sequencing of 16S rRNA. Unique combination of forward and reverse primer was used for each sample.

Primer name	Sequence, 5' -> 3'	Target region	Direction
F9	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtttcgCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F10	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctcgtacgCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F11	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgagtggCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F12	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctggtagcCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F13	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctactgatCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F14	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctatgagcCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F15	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctattcctCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F16	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctcaaaagCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
R25	caagcagaagacggcatacgagatATCAGTgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R26	caagcagaagacggcatacgagatGCTCATgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R27	caagcagaagacggcatacgagatAGGAATgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R28	caagcagaagacggcatacgagatCTTTTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R29	caagcagaagacggcatacgagatTAGTTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R30	caagcagaagacggcatacgagatCCGGTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R31	caagcagaagacggcatacgagatATCGTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R32	caagcagaagacggcatacgagatTGAGTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R33	caagcagaagacggcatacgagatCGCCTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R34	caagcagaagacggcatacgagatGCCATGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R35	caagcagaagacggcatacgagatAAAATGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse

PAPER III

PLOS ONE

RESEARCH ARTICLE

Intestinal effect of faba bean fractions in WD-fed mice treated with low dose of DSS

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Abstract

Rodent studies have shown that legumes can reduce chemical induced colonic inflammation, but the role of faba bean fractions for colon health has not been described. We have investigated the role of protein and fiber fractions of faba beans for colonic health and microbiota composition in a low-grade inflammation mice-model when incorporated in a Western diet (WD). The diet of sixty C57BL/6JRj male mice was standardized to a WD (41% fat, 43% carbohydrates) before were randomly assigned to four groups (n = 12) receiving either 1) WD with 30% of the protein replaced with faba-bean proteins, 2) WD with 7% of the fiber replaced with faba-bean fibers, 3) WD with protein and fiber fractions or 4) plain WD (n = 24). Low-grade inflammation was induced by 1% dextran sodium sulfate (DSS) given to mice for the last six days of the trial. Half (n = 12) in group 4) were given only water (controls). Prior to DSS, body weight, energy intake, glucose and insulin tolerance assays were performed. Inflammatory status in the colon was assessed by biomarkers of inflammation and qRT-PCR analyses of inflammatory related genes. Fecal microbiota composition was assessed by 16S rRNA gene sequencing. 1% DSS treatment increased levels in fecal lipocalin-2 and induced disease activity index score, but the presence of faba bean fractions in WD did not influence these indicators nor the expression level of inflammatory associated genes. However, the mice that had faba-bean proteins had a lower amount of Proteobacteria compared the group on plain WD. The Actinobacteria abundance was also lower in the group that had fiber fraction from faba-beans. Overall, outcomes indicated that in a low-grade inflammation model, replacement of protein and or fiber in a WD with faba bean fractions had marginal effects on inflammatory parameters and colonic microbiota.

Introduction

Legumes and pulses (the dry seed of the legume), which belong to the Fabaceae family, constitute an inexpensive food source with high nutritional value, often attributed to their richness in proteins (20–35%), dietary fibers and various phytochemicals such as polyphenols. The World Health Organization has actively encouraged to include more legumes in the daily meal due to their high nutritional value [1–3].

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Data from epidemiological studies have shown that legume consumption is positively associated with improved blood cholesterol profile [4], reduced risk of cardiovascular diseases [5, 6], type-2 diabetes [6, 7], metabolic syndrome [8–10] and colorectal cancer [11, 12]. In addition, feeding trials in rodents with different types of legumes have demonstrated a positive impact against colonic inflammation, chemically induced by dextran sodium sulfate (DSS) [13–15].

The beneficial health effects of legumes are probably multicausal and it is unlikely that one single nutrient is responsible. Dietary fibers, which increase the bulk of stool, maintain regular bowel movements and are sources of short chain fatty acids from bacterial fermentation, can prevent the advent of inflammation and several chronic diseases [16, 17]. Furthermore, plant proteins are proposed to contribute more on overall health state than animal proteins [18]. According to some studies, high intake of animal proteins, particularly from red meat, is linked to inflammatory bowel diseases (IBDs) [19], whereas the risk is lower with increased consumption of plant proteins [20]. These positive indications may be related to differences in amino acid profiles [21], or the presence of bioactive peptides arising during the digestion of plant proteins [22, 23]. A mouse study with pea albumin extracts in the diet demonstrated reduced inflammation and differences in microbiota composition after DSS treatment. The authors suggested that these effects were attributed to certain bioactive protein components called Bowman Birk inhibitors, present in peas and other legumes [24].

The presence of antinutrients in the legume protein package, such as lectins, saponins, and enzyme inhibitors, may be responsible for adverse health effects and often constitutes a major concern. However, through processing (soaking, cooking or other thermal treatments) the concentration of antinutritional factors can be significantly reduced [25]. Moreover, the presence of antinutrients in moderate amounts help to reduce blood glucose, plasma cholesterol, triglycerides an even reduce cancer risk [26–28].

Faba bean (*Vicia faba L*), which is also referred as broad bean, field bean and horse bean [29], represents a popular dish mainly in Middle East and Mediterranean region [30]. Faba beans is the fourth most cultivated legume after peas, chickpeas and lentils (FAOSTAT, 2019). Faba beans can tolerate lower temperature [31], allowing growth in different climate zones, including the Nordic countries [32]. Notably, not only levels of nutrients and antinutrients among various cultivars of faba beans can vary significantly [33] but also the amount of soluble fiber in different forms of faba bean protein ingredients (protein-rich flour, protein isolate) [34].

So far very few studies on potential health effects of faba beans have been carried out. We therefore performed a feeding trial with high fat Western-like diet (WD), where we incorporated fractions from faba beans (protein, fiber or both fractions together). The aim was to investigate colon health status under a WD and low-grade inflammation induced by a low concentration of DSS (1%) and we hypothesized that adding cooked fractions of faba beans in a WD would manifest a lower degree of intestinal inflammation (after 1% DSS treatment).

Prior to DSS treatment, the WD with protein fraction significantly increased body weight gain of mice ($P<0.05$) but not glucose and insulin tolerances compared to those fed the WD without faba bean fractions. During 1% DSS treatment in drinking water, evaluation of colon through inflammatory markers (LCN2, LBP) showed no significant effects in mice following WDs with faba bean fractions compared to WD-fed mice. Evaluation of microbiota composition between the different dietary groups demonstrated minor shifts from phyla level to genera level.

Materials and methods

Animals and diets

Six-week-old male C57BL/6JRj mice (n = 60) were purchased from JANVIER LABS (Le Genest-Saint-Isle, France) and housed in individually ventilated cages (Innorack, Innovive, San

Diego, CA; n = 4 per cage) under controlled conditions (12 hours light-dark cycle; 23±2˚C, 45–55% humidity). Upon arrival, mice were acclimatized for two weeks and fed standard rodent chow diet (RM1, SDS Diet, Essex, UK). After acclimatization, all mice switched to a Western Diet (WD) (D12079B, 43.6% CHO—41.2% fat) for eight weeks before they were allocated to four dietary groups (for another eight weeks), in which fiber, protein or both fractions from faba beans (type Vertigo, Denmark) were included. Those groups were i) a WD with no supplement (WD, n = 24) ii) a WD supplemented with 30% faba bean protein fraction (WD +PF, n = 12), iii) a WD supplemented with 7% of faba bean fiber fraction (WD+FF, n = 12) and iv) a WD supplemented with both faba bean protein (30%) and fiber fraction (7%) (WD +BF, n = 12). The faba bean strain, Vertigo was grown and harvested in Denmark and shipped to Norway for further processing. The protein fraction was obtained by dry milling and air-classification (Skjelfoss Korn AS, Hobøl, Norway) of faba beans after the hull removal (hull fraction). The fiber fraction was acquired from the hull fraction. The hull fraction was milled using a Retsch ZM 100 mill (Retsch Gmbh, Haan, Germany) comprising a 0.5mm sieve before further use in the mice feeds.

Faba bean fractions were cooked (10 min) and freeze-dried before being shipped to Research Diets (Madison, WI) for final preparation of the mouse diets. The reason for cooking them was to reduce the concentration of toxic antinutrients such as vicine and convicine, which often may cause in humans and animals a hemolytic disease called favism [35, 36]. Composition of the diets is presented in S1A and S1B Table.

Following the six-week feeding with the WD +/- faba bean fractions, 1% DSS was introduced in the drinking water of mice (n = 12/group) for six days to induce low-grade colon inflammation. To score disease activity from the 1% DSS treatment, mice were evaluated every two days to determine a disease activity (DAI) index score, which considered body weight, activity level, posture and stool quality. Half of the WD-fed mice (n = 12) were given water only (without DSS) and used as negative controls. The experimental timeline is presented in Fig 1.

Water and food were provided *ad libitum*. Body weights and food intake were recorded once per week. Experimental procedures were approved by the Norwegian Animal Research Authority (Mattilsynet, FOTS ID 14805) in accordance with the Federation of European Laboratory Animal Science Associations (FELASA).

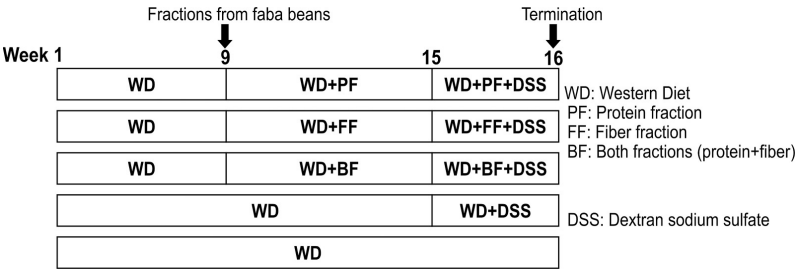

Fig 1. Timeline of feeding trial prior and during 1% DSS treatment. For eight weeks, all C57BL/6JRj mice (n = 60) were fed a WD. From week 9 mice (n = 12) were divided into four dietary groups, in which faba bean fractions were introduced in the WD: 1) WD incorporated with 30% faba-bean protein fraction (WD+PF), 2) WD with 7% fiber fraction (WD+FF), 3) WD with both protein and fiber fraction (WD+BF) or 4) WD without faba bean content (n = 24). The mice were followed for another eight weeks. The last six days before experiment termination, mice were exposed to 1% DSS in the drinking water expect half of the WD only group (n = 12), serving as negative controls for the DSS effect.

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Glucose tolerance test (OGTT) and insulin tolerance test (ITT)

In the third and fourth week of the feeding trial with the different diets (WD, WD+PF, WD +FF, WD+BF), assays for glucose homeostasis and insulin sensitivity were carried out, respectively. For the oral glucose tolerance test (OGTT), animals were fasted for 6 hours and a fixed dose of D-glucose (Sigma-Aldrich, 2 g/kg, meaning 300 μl of glucose solution 20% per mouse) was administered *per os* and blood (~3–5 μL) was collected from tail at different time points. Blood glucose levels were measured by a glucometer (Accu-Check ®, Roche Diagnostics) at baseline and 15, 30, 60 and 120 minutes after glucose administration. For the insulin tolerance test, mice fasted for 4 hours before human insulin (I2643, Sigma-Aldrich) was injected i.p. (0.25 U/kg). Blood glucose was measured at the baseline and 30, 60, 120 minutes after insulin injection.

Sampling and histology

Samples were collected on day six of 1% DSS treatment (termination of experiment). Mice were anesthetized with a cocktail containing Zoletil Forte (Virbac, Carros, France), Rompun (Bayer, Oslo, Norway), and Fentadon (Eurovet Animal Health, Bladel, The Netherlands) (ZRF; i.p. 0.1 mL ZRF/10 g body weight), with the following active ingredients: Zolezepam (32 mg/kg), Tiletamine (32 mg/kg), Xylazine (4.5 mg/kg), and Fentanyl (26 mg/kg). Prior to mice euthanasia by cervical dislocation, blood (0.5–1 mL) was collected by cardiac puncture into syringes with ~50 μL NaEDTA (50 mM) as anticoagulant. To obtain plasma blood was centrifuged at 6,000xg for 10 min at 4˚C. After opening the colon longitudinally, mucosa was collected by gentle scraping with a sharp glass slide. Colonic mucosal samples were kept in RNAlater (Sigma-Aldrich) to preserve RNA. Fecal pellets were collected from the colon. All samples were stored in -80˚C.

Sections from the distal colon were fixed following the protocol already described [37]. Generally, the colon lumen was washed with modified Bouin's fixative (50% ethanol, 5% acetic acid in dH_2O), opened longitudinally to expose the luminal side and then wrapped around a toothpick with the luminal side facing inwards. Samples were then kept in 10% buffered formalin overnight at room temperature and transferred to 70% ethanol and stored at 4˚C until samples were embedded in paraffin according to protocol provided by the university imaging core facility. The paraffin embedded samples were cut in 7μm thick sections and stained with hematoxylin and eosin before they were judged for structural damage under a light microscope (DM6B, Leica, Germany).

RNA extraction and qRT-PCR

RNA from colonic mucosa samples collected on day six of the DSS treatment (termination ay) was extracted with the NucleoSpin RNA/Protein Purification kit (Macherye-Nagel, Düren, Germany). Because DSS reduces efficiency of both reverse transcriptase and PCR reactions, all RNA samples were purified using lithium chloride (LiCl) described by Viennois *et al*. [38].

cDNA synthesis from RNA was performed (S3 Table) using iScript cDNA Synthesis Kit (1708891, Bio Rad), whereas FirePol EvaGreen qPCR Supermix (08-36-00001, Solis BioDyne) was used for the qRT-PCR reaction in a Light Cycler 480 Instrument II (Roche). The parameter settings were: 12 min at 95˚C; 40 cycles of 15 sec at 95˚C followed by 20 sec at optimized primer annealing temperature; 20 sec at 72˚C (S4 Table). LinRegPCR Software (2017.1.0.0, Amsterdam UMC) was used to calculate Cq values of the colon samples and each primer efficiency. Primers used for mRNA expression (Thermo Fisher Scientific) and are presented in S5 Table.

LBP measurement

ELISA assay for determination of lipopolysaccharide binding protein (LBP) was performed in plasma samples collected on day six of the DSS treatment (termination day) according to the manufacturer (Biometec, Greifswald-Rostock, Germany). Plasma samples from control mice were diluted 800 times (as recommended in the guidelines), whereas those from 1% DSS-treated mice were diluted from 1,200 to 1,600 times. Optical density of each sample at 450nm was measured with a spectrophotometer (SpectraMax M2, Molecular Devices) and concentration of the protein estimated based on standard curve using 4-parameter logistic curve fit.

Lipocalin-2 measurement

Mouse Lipocalin-2/NGAL DuoSet ELISA from Research and Development systems (R&D systems, USA) was used for measuring lipocalin-2 protein in extracts from fecal samples collected on day six (termination day) of DSS treatment. Fecal extracts were made from feces samples (weighed and stored at -80C) reconstituted in PBS containing 0,1% Tween 20 (1ml buffer per 100mg feces). The samples were vortexed for 20min to obtain a homogenous fecal suspension. The supernatant was collected from each sample after centrifuging the samples for 10min at 12.000 rpm (4˚C), as described by Chassaing *et al.* [39]. Prior to the assay, fecal samples from control mice and 1% DSS was diluted 20 and 20.000 times respectively. The concentration was measured by optical density as described for LBP measurements.

Microbiota analysis

DNA extraction from feces and library preparation. Right after dissection, fecal samples were collected from the colon and placed in 400 μL S.T.A.R buffer (Roche, USA) with acid-washed glass lysing beads (approximately 0.2g <106 μm, 0.2g of 425–600 μm and 2–4 beads of 2mm Sigma-Aldrich) and stored at -80˚C for further processing. All samples were homogenized twice on FastPrep 96 (1,800 rpm, 40 sec, 5 min cooling step in-between, MP BioMedicals). Processed samples were then centrifuged (13,000 rpm, 10 min) and 50 μL supernatant was transferred to 96-well plates for protease treatment and DNA extraction using Mag Mini LGC kit (LGC Genomics, UK) on KingFisher Flex DNA extraction robot (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Gene sequencing of 16S Rrna. After DNA extraction, 16S rRNA gene was amplified by PCR using prokaryote-targeting primers against the variable region V3-V4 with an amplicon length of 466bp. PCR primers for amplification and conditions for library preparation are presented in the S6 and S7 Tables. As DSS in fecal samples has an inhibitory effect on the PCR reaction (identified through dilution series on qPCR), we diluted the extracted DNA from feces 1:4 prior to amplicon PCR (total dilution of 1:100 in the PCR reaction). PCR product (466 bp) was purified with AMPure XP (Beckman-Coulter) and 10 further PCR cycles with index primers were performed (S8 and S9 Tables) resulting in PCR product of approximately 594 bp. The sequences of primers in index PCR are shown in S10 Table. All PCR products were qualitatively confirmed by electrophoresis on 1.5% agarose gel. Quantification of DNA concentrations of index PCR products, normalization and pooling of these index PCR products were followed by purification of the pooled library with Sera Mag Beads by following the AMPure XP protocol. The pooled library was diluted to 6 pM and sequenced with the MiSeq Reagent Kit V3 (cat. nr. MS-102-3003) on the Illumina MiSeq following Illumina's protocol (16S Metagenomic Sequencing Library Preparation Part# 15044223 Rev. B), except we used nuclease free-water instead of Tris for PhiX library dilution. 20% PhiX served as an internal control.

Assigning taxonomy. Resulting 300 bp paired-end reads from MiSeq platform were filtered using QIIME and OTU (Operationally Taxonomic Unit) clustered based on 97% identity using closed-reference OTU *usearch* algorithm (version 8) [40, 41] against SILVA database (version 128) [42]. 6,500 sequences per sample were chosen as a cut-off to normalize (rarefy) the sequencing data.

Statistical analysis. Statistical analyses were performed using GraphPad Prism (version 8.3.1 for Windows, GraphPad Software, San Diego, CA). Data are presented as individual values with group means ± standard error of the mean (SEM). Normal distribution was tested using the Shapiro-Wilk normality test. Based on whether normal distribution was achieved or not, parametric and non-parametric models were used respectively. *P*-values smaller than 0.05 were considered significant.

Prior to DSS treatment, body weight, energy intake and insulin tolerance were analyzed by the mixed effect model whereas glucose tolerance by repeated measures 2-way ANOVA. The latter statistical method was used for analyzing the body weight change (%) of the four diet groups during the six days of DSS treatment whereas one-way ANOVA followed by Tukey's post-hoc analysis and the non-parametric test Kruskal-Wallis with Dunn's multiple comparisons for colon length and DAI on day six of 1% DSS treatment respectively. For the statistical analysis of proinflammatory cytokines and biomarkers of inflammation, the control mice group of mice was excluded and either one-way ANOVA followed by Tukey's post-hoc analysis or Kruskal-Wallis with Dunn's multiple comparisons was applied. Analysis of beta-diversity was conducted in R (version 4.0.0). Weighted UniFrac distances were calculated using QIIME default scripts (core_diversity_analyses.py) and are based on the normalized (rarefied) OUT table. Non-metric multidimensional scaling (NMDS) of weighted UniFrac distances was performed using the metaMDS function from the *vegan* package [43] with autotransform = FALSE and try = 100. Global PERMANOVA on weighted UniFrac distances was performed using the adonis function from the *vegan* package with 999 permutations. Pairwise PERMANOVA was performed by applying the pairwise.perm.manova function from the *RVAideMemoire* package [44].

For linear discriminant analysis effect size (LEfSe), relative abundances of taxa were used [45]. To identify statistical differences, factorial Kruskal-Wallis sum test, followed by pairwise Wilcoxon test, both with an alpha value of 0.05, were used. The threshold on the linear discriminant analysis (LDA) score was set at 2.0 with a multi-class analysis against all.

Results

Body weight, energy intake and glucose regulation before DSS treatment

Prior to DSS treatment the eight-week impact of the different diets was examined. Mice fed a WD+PF gained significantly more weight ($P<0.0001$) compared to mice given WD or WD +FF. No difference in weight gain was observed between WD and WD+FF fed mice (Fig 2A). The energy intake *per* mouse was significantly higher in mice fed WD+PF compared to WD +FF fed mice ($P = 0.0148$) (Fig 2B), but no difference was found between any of the other groups. In terms of glucose regulation, no significant differences were observed between the dietary groups judged by the glucose tolerance test ($P = 0.8037$) or the insulin tolerance test ($P = 0.1269$) (Fig 2C and 2D).

Impact of faba-bean fractions on body weight and colon during 1% DSS treatment

Before evaluating the impact of the faba-bean fractions initial investigations were performed to assess the low-inflammation model induced by 1% DSS treatment. During the first four

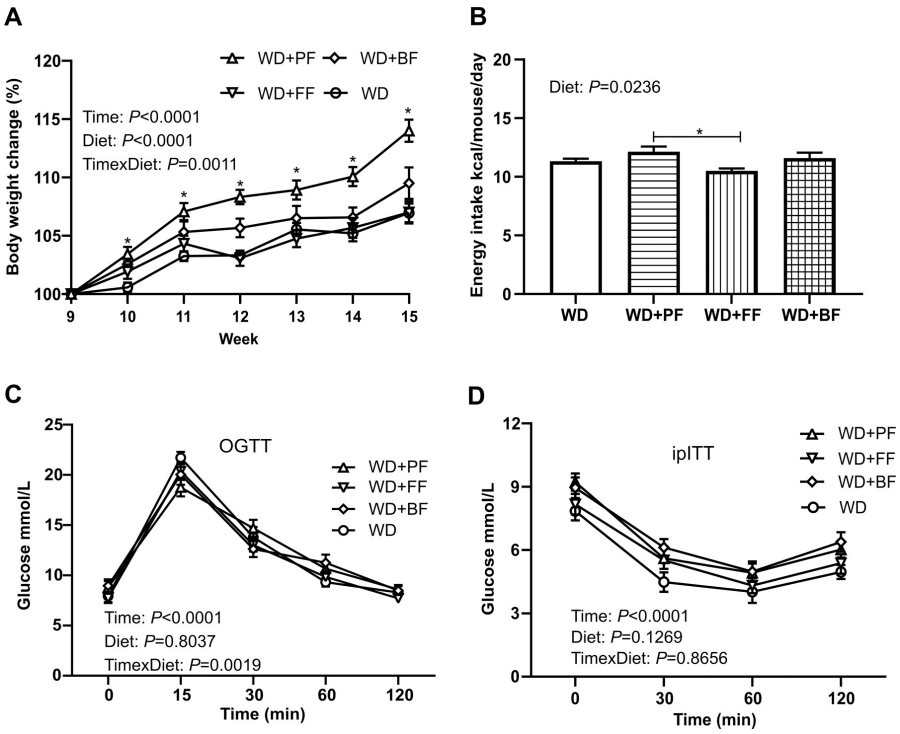

Fig 2. Bodyweight development, energy intake and glucose regulation prior to DSS treatment in WD fed C57BL/6JRj mice. Bodyweight development **(2A)** during the feeding trial with modified WDs (n = 12 except WD-fed mice, n = 24). Estimated energy intake **(2B)** per mouse per day from week 9 to week 15. OGTT **(2C)** after 4 hours of fasting at week 3 (n = 12). IpITT **(2D)** after 6 hours of fasting at week 4 (n = 12). * means significantly different ($P<0.05$). Values are means ± SEM. For panels A, B, D mixed-effect model whereas for panel C 2-way ANOVA with repeated measures was applied.

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days of 1% DSS administration, no visible signs of disease were observed in any of the mice i.e., the mice continued to increase in weight due to WD (Fig 3A). At day six the weight increase had ceased in all diet groups and even mildly decreased from week 4. Also, mild signs of disease were observed in the majority of the mice judged by the animal researcher, visually inspecting stool quality, body posture and activity levels summarizing the DAI score. At termination day, colon length of all mice treated with 1% DSS was shorter than WD control mice, but no significant differences were found in terms of colon length between diet groups ($P = 0.1556$) (Fig 3B). Furthermore, histological staining of colon tissue for 1% DSS treated mice revealed no structural damage in colonic mucosal tissue (S1 Fig). Also, neither of the faba bean fractions impacted the overall DAI scores ($P = 0.6768$) on day six (termination day) (Fig 3C).

Effect of diet on gene expression and biomarkers of inflammation with 1% DSS

We next assessed mRNA expression levels of the inflammatory related genes; *Tnf*-a, *Il-1b*, *Il-6*, *Nox2*, *Nos2* and *Ptgs2* in colonic mucosa. While mild signs of disease were observed as

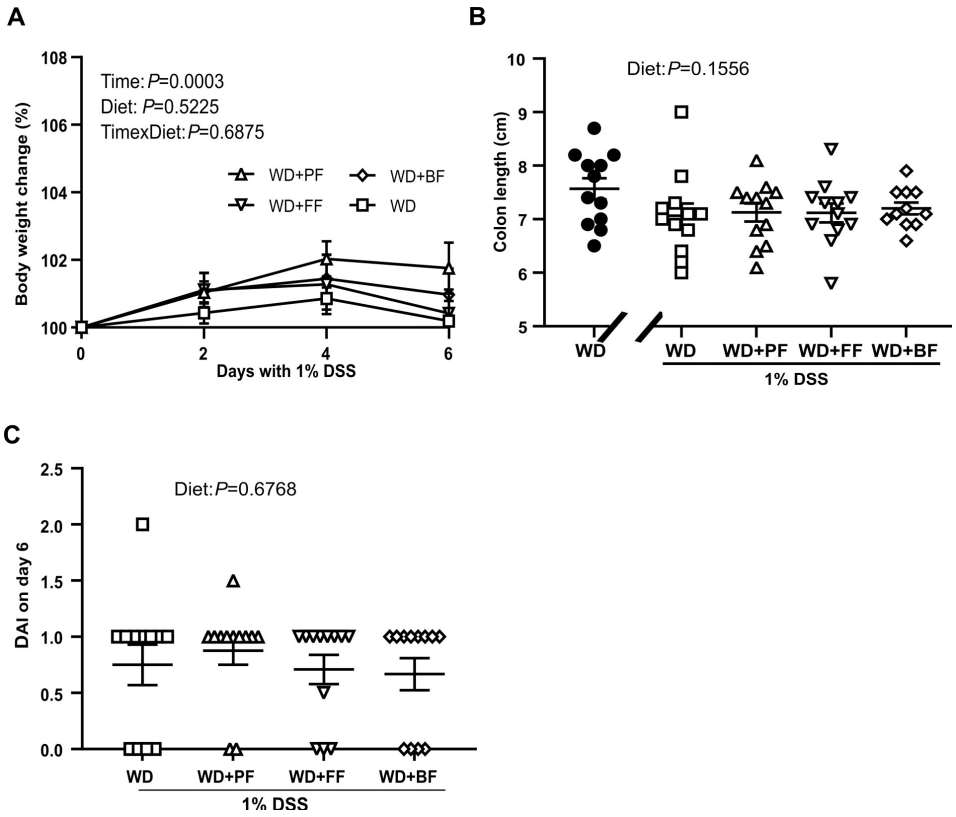

Fig 3. Bodyweight (%) development during 1% DSS in WD fed C57BL/6JRj mice during 6-days treatment. Change in bodyweight (%) measured on day 0, 2, 4 and 6 **(3A)**. Colon length in cm **(3B)**. Control mice indicated by black circles in the figure. For panel A 2-way ANOVA was used whereas for panels B and C one-way ANOVA and Kruskal Wallis test, respectively. Values are mean ± SEM. DAI score on day six of 1% DSS treatment **(3C)**.

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described in the previous section the 1% DSS treatment did not lead to induced expression of these genes when compared to the negative controls that were not exposed to DSS. Furthermore, no impact of the faba bean fractions was found on the gene expression (Fig 4A–4F). In contrast, the fecal levels of LCN2 were robustly upregulated by 1% DSS ($P = 0.0431$), but apart from WD+PF vs WD+BF ($P = 0.0286$) no differences in LCN2 levels were observed between the faba bean fraction groups (Fig 4G). Also, even as the plasma levels of LBP were overall higher in 1% DSS exposed mice compared to the negative WD-control mice, no difference could be observed between the faba-bean fraction groups ($P = 0.2217$) (Fig 4H).

The presence of faba bean fractions in WD and during 1% DSS treatment brought shifts in microbiota profile

Furthermore, we investigated if replacement of protein and fiber content in the WD with different faba bean fractions could induce microbial shifts in colonic content. Alpha-diversity in

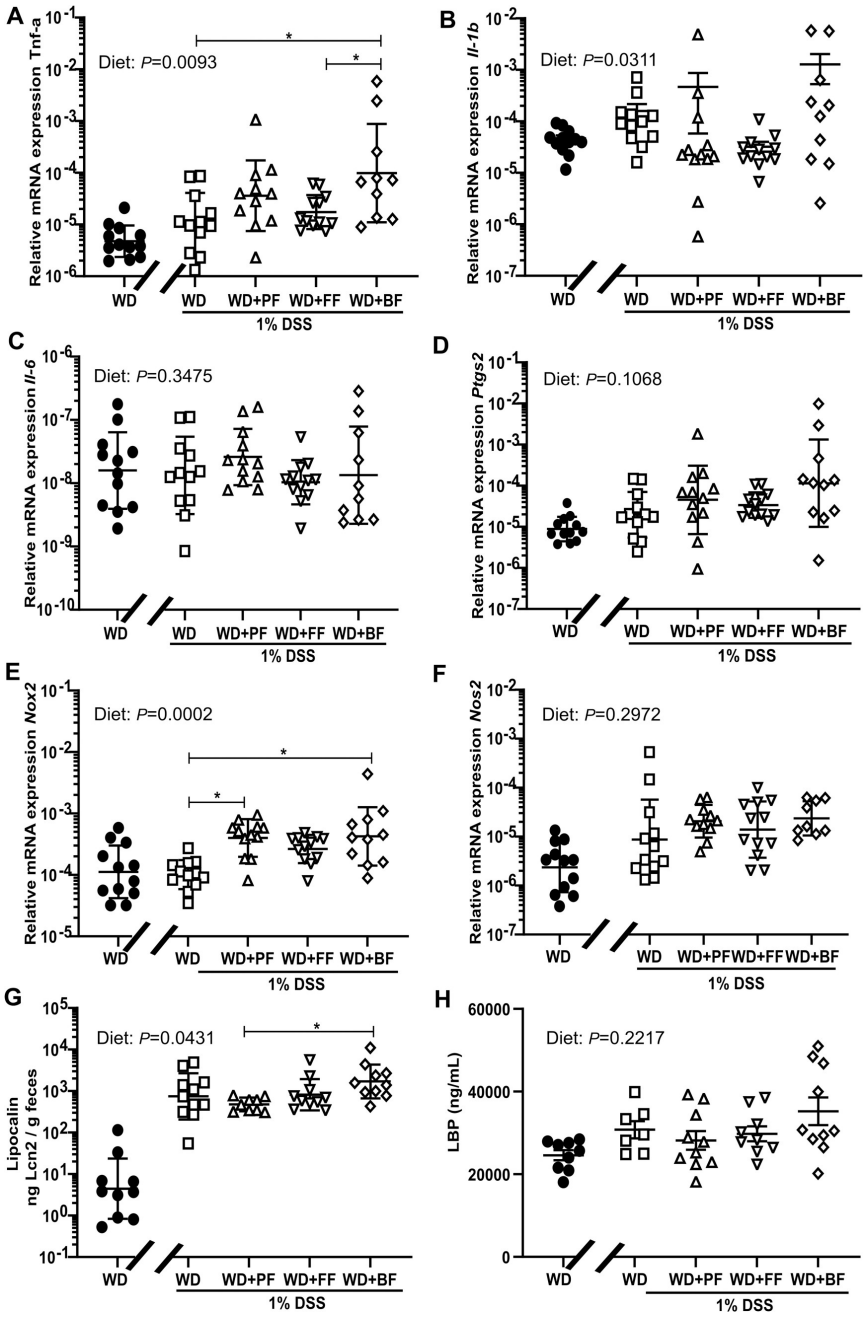
A
Relative mRNA expression Tnf-a
Diet: P=0.0093
B
Relative mRNA expression Il-1b
Diet: P=0.0311
C
Relative mRNA expression Il-6
Diet: P=0.3475
D
Relative mRNA expression Ptgs2
Diet: P=0.1068
E
Relative mRNA expression Nox2
Diet: P=0.0002
F
Relative mRNA expression Nos2
Diet: P=0.2972
G
Lipocalin
ng Lcn2 / g feces
Diet: P=0.0431
H
LBP (ng/mL)
Diet: P=0.2217
WD
WD
WD+PF
WD+FF
WD+BF
1% DSS

Fig 4. Gene expression and inflammatory markers. Impact of faba bean fractions on the expression of proinflammatory (*Tnf-a* (**4A**), *Il-1b* (**4B**), *Il-6* (**4C**), Ptgs2 (**4D**)), and ROS associated genes (*Nox2* (**4E**), *Nos2* (**4F**)) in mucosa from the proximal colon (n = 10–12). Lipocalin-3 concentration in feces (**4G**) and LBP levels (n = 9–11) (**4H**) in plasma of WD fed C57BL/6JRj mice after 1% DSS treatment. WD+PF: WD incorporated with 30% faba-bean protein fraction; WD+FF; WD with 7% fiber fraction, WD +BF; WD with both protein- and fiber fraction, WD without faba bean content (n = 24). Values are mean ± SEM. One-way ANOVA with Tukey's multiple comparison for analysis in panels A-H, except panels B and E, where Kruskal-Wallis with Dunn's multiple comparisons test was used. Control mice indicated by black circles in the figures.

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fecal samples was assessed by using the Shannon index on 16S rRNA sequencing data. No impact of DSS was found on the alpha-diversity when comparing the DSS exposed mice to the negative controls. However, among the DSS treated groups a significant difference was found between the WD and the WD+PF ($P = 0.0104$) while no significance was observed between the remaining groups (Fig 5A). Moreover, beta-diversity by using non-metric multidimensional scaling (NMDS) of weighted UniFrac distances, was examined giving an overall $P = 0.001$ between dietary groups. The DSS treatment was the main factor for group dispersion with an overlap between the clusters of groups exposed to 1% DSS indicating no impact of the faba-bean fractions on the beta-diversity (Fig 5B).

When examining differences in taxonomy at the phyla level, differences in the relative abundances were found between the groups (Fig 5C). Firstly, the negative control had a significantly higher level of Firmicutes and lower level of Bacteroidetes as visualized in Fig 5D showing the Firmicutes/Bacteroidetes ratio (Fig 5D). When comparing the diet groups that were exposed to DSS (one-way ANOVA), the relative abundance of Actinobacteria was significantly higher in WD ($P = 0.0130$) and WD+PF mice ($P = 0.0255$) compared to the WD+FF mice (Fig 5E). In addition, all DSS treated WD mice had higher relative abundance levels of Proteobacteria and Verrucomicrobia compared to the WD control mice (Fig 5F and 5G). Interestingly, DSS and fed with faba bean fractions had lower relative abundance levels of those two phyla compared to DSS-treated mice fed solely a WD. Although statistical significance was only found in the WD+PF fed mice ($P = 0.04$), the tendency for changes in the microbiota profile at phylum level was evident when faba bean fractions were present in WD.

To get a deeper understanding on the taxonomic levels, we applied the linear discriminant analysis effect size (LEfSe), which can identify differences in microbial communities based on relative abundances and hence allow us to statistically test differences among the experimental groups. When comparing the WD group and the diet groups that had replaced protein and or fiber content with faba-bean fractions several bacterial clades were detected to be different (Fig 6A). Genera of *Bifodobacterium (*Fig 6B), *Alloprevotella* (Fig 6C) together with *Prevotellaceae UCG-001* (Fig 6F) and *Enterorhabdus* (Fig 6G) were indicated in WD+PF, WD+FF, WD+BF respectively (Fig 6B). In WD+DSS fed-mice *Tyzzerella* (Fig 6D) and *Ruminococcaceae UCG-005* (Fig 6E) showed significantly high relative abundance ($P<0.05$) compared to the faba-bean fraction groups.

It is worth mentioning that *Bifidobacteria* in mice consuming WD+PF was similar to WD-fed mice without DSS treatment (as illustrated earlier in Fig 5A), while *Alloprevotella* and *Prevotellaceae UCG-001* manifested higher relative abundance in mice eating a WD supplemented with faba bean fractions (mainly fiber fraction) than mice following a WD with or without 1% DSS treatment.

Discussion

In the present study we examined the effect of a WD supplemented with two different fractions from faba beans in mice treated with 1% DSS for six days. Contrary to commonly used 2–5% DSS concentration for inducing colitis, we used 1% DSS to cause an irritation of the colon

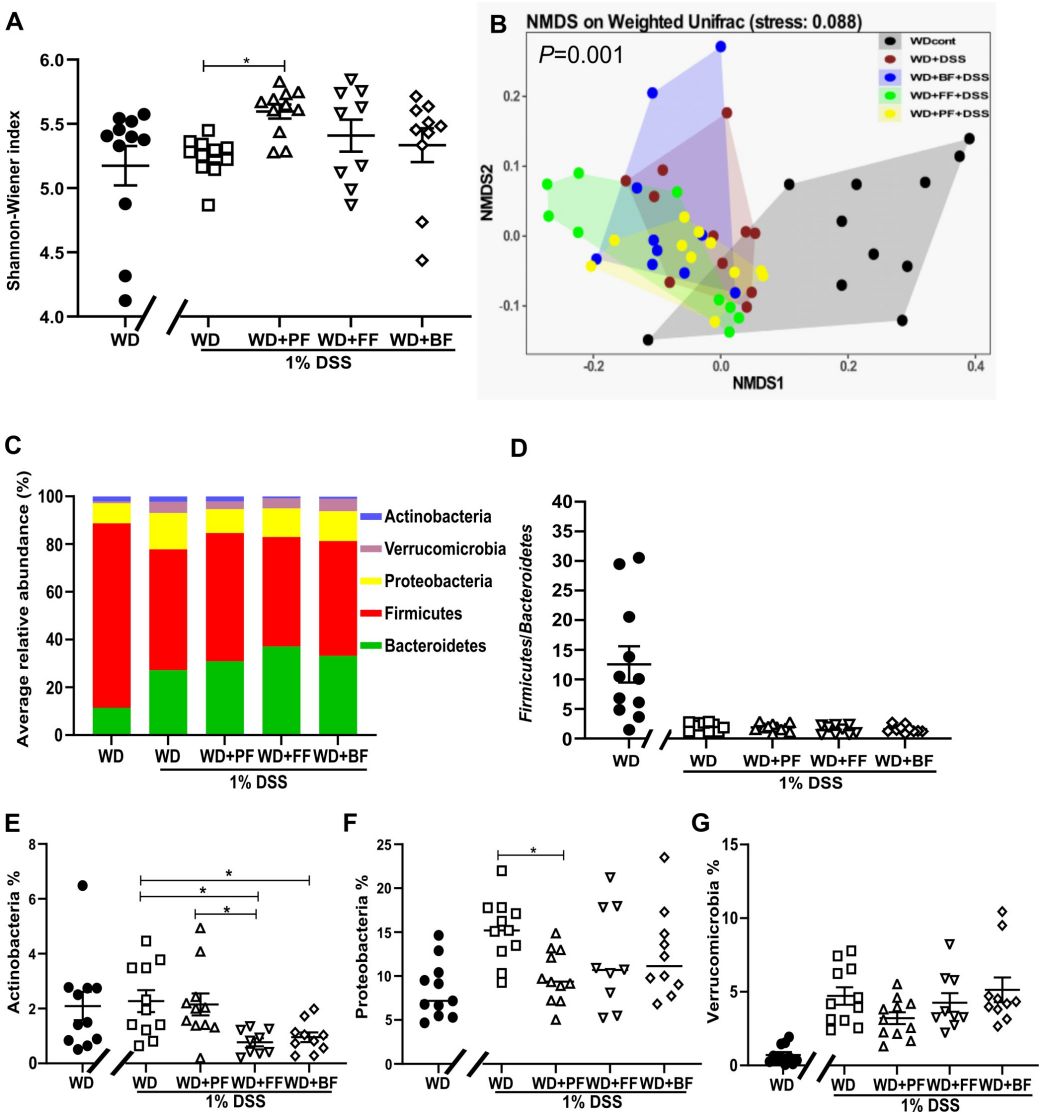

Fig 5. Impact of various faba-bean fractions with WD (WD-PF, WD-FF and WD-BF) on microbiota measures in C57BL/6JRj exposed to 1% DSS. WD without DSS treatment served as negative control. Alpha-diversity (Shannon index) of colonic bacterial communities in feces from WD mice represented as mean with SEM n = 8–11 in each dietary group (**5A**). Groups were compared using Kruskal-Wallis test since one-way ANOVA could not be performed due to violation of normality assumption. Impact of faba bean fractions on beta-diversity was explored using non-metric multidimensional scaling (NMDS) of weighted UniFrac distances between fecal samples (**5B**). Colors indicate dietary group. *P*-value in the plot from global PERMANOVA. Average relative abundance for all detected phyla for each group in fecal samples (**5C**). Firmicutes/Bacteroidetes ratio in feces (**5D**), relative abundance of Actinobacteria (**5E**), Proteobacteria (**5F**) and Verrucomicrobia (**5G**) phyla. For panel D Kruskal-Wallis test with Dunn's multiple comparisons whereas for panel E-G one-way ANOVA with Tukey's multiple comparison was conducted. * means significantly different ($P<0.05$). Values are expressed as means ± SEM, n = 9–11. Control mice indicated by black circles in the figures.

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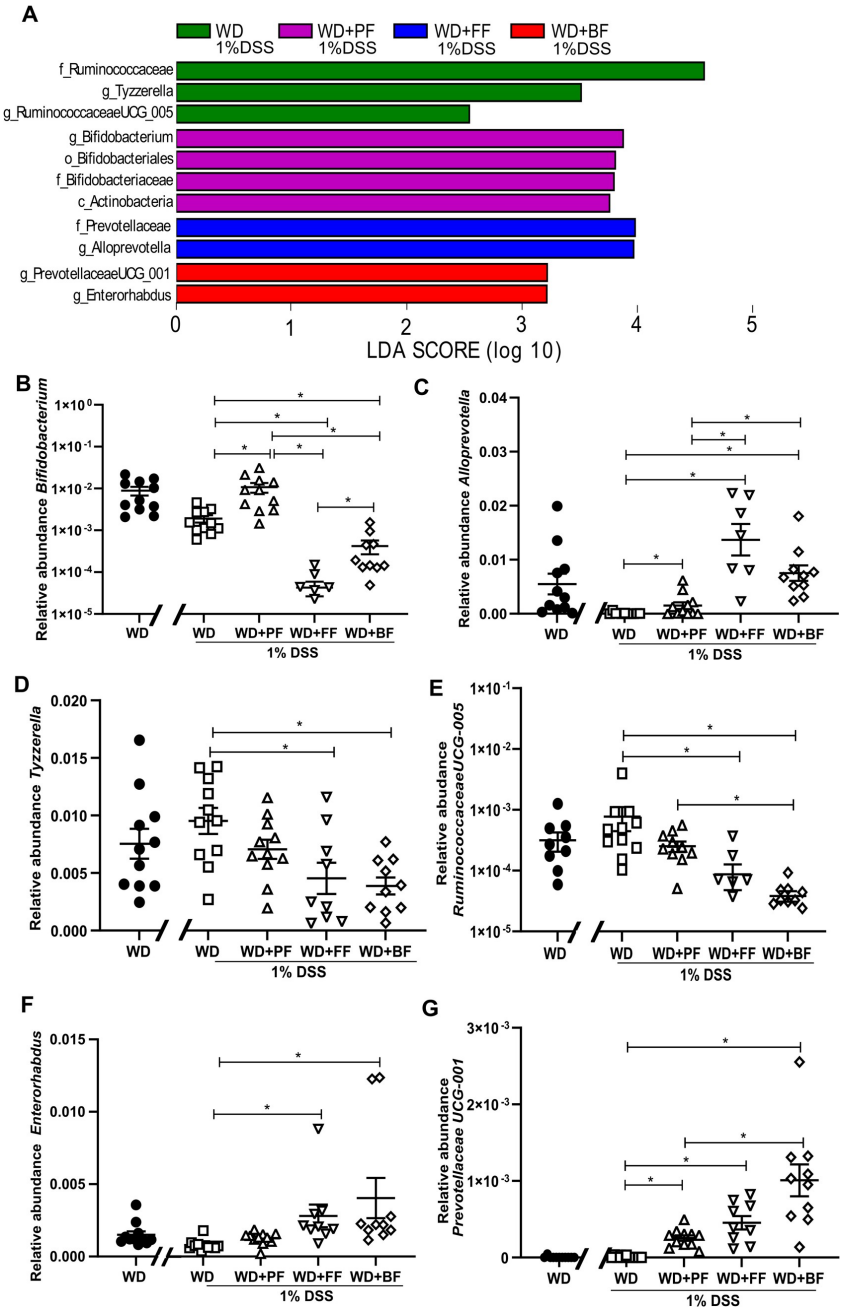
A
WD 1%DSS
WD+PF 1%DSS
WD+FF 1%DSS
WD+BF 1%DSS
f_Ruminococcaceae
g_Tyzzerella
g_RuminococcaceaeUCG_005
g_Bifidobacterium
o_Bifidobacteriales
f_Bifidobacteriaceae
c_Actinobacteria
f_Prevotellaceae
g_Alloprevotella
g_PrevotellaceaeUCG_001
g_Enterorhabdus
0
1
2
3
4
5
LDA SCORE (log 10)
B
Relative abundance Bifidobacterium
C
Relative abundance Alloprevotella
D
Relative abundance Tyzzerella
E
Relative abudance RuminococcaceaeUCG-005
F
Relative abundance Enterorhabdus
G
Relative abundance Prevotellaceae UCG-001
WD
WD+PF
WD+FF
WD+BF
1% DSS

Fig 6. Linear discriminant analysis (LDA) effect size (LEfSe) analysis of fecal microbiota changes following consumption of a high-fat diet (WD) with various faba-bean fractions (PF, FF, BF) during dextran sodium sulfate (DSS) treatment. Histogram (**6A**) shows the LDA scores of the taxa (genera -g, and family -f) with the greatest differences between the groups. The relative abundance of the genera from histogram are presented in figures (**6B**-**6G**). Values are expressed as means ± SEM, n = 9–11. For panels B, C, D, E, and G, one-way ANOVA with Tukey's multiple comparison was applied, whereas for panel F, the nonparametric test Kruskal-Wallis test with Dunn's multiple comparisons test. * means significantly different ($P<0.05$). Control mice indicated by black circles in the figures.

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ideally mimicking a mild colitis phenotype consistent with low-grade colonic inflammation [46–48]. DSS treatments were combined with a commonly assumed unhealthy WD rich in fat and sucrose and low in dietary fibers, which may create an additional stress to the intestine [49]. We hypothesized that protein and/or fiber fraction(s) from faba beans would alleviate the effects of an induced low-grade colonic inflammation imposed by the 1% DSS treatment in WD fed mice.

Prior to inducing low grade inflammation with 1% DSS we observed that mice in all groups fed WD were healthy and tolerated the faba bean fractions well. Intriguingly mice consuming the WD+PF diet gained more weight and had higher food and energy intake compared to all the other diet groups ($P<0.05$). This could simply be a result of a more palatable diet provided by the proteins in the faba beans, of unknown reasons. It may also be explained by a reduced amount in casein in the WD+PF diet independent of palatability. In the WD+PF diet we exchanged 30% of casein with proteins from faba beans, which could have relevance for weight gain. Indeed, a mouse study from 2016 showed that casein, compared to many other protein sources had a significant weight reducing effect in mice fed a high fat diet [50]. When assessing glucose regulation, we did not observe differences between the groups despite weight differences. This is line with Lamming and coworkers who noticed that consumption of plant proteins for a short term period did not affect glucose homeostasis in C57BL/6J mice [51].

When mice were challenged with 1% DSS, we observed that the effect on inflammatory markers in the colon were overall low, and we speculate that the dose used was borderline to induce a robust inflammation in the colon judged from the variable and low induction of proinflammatory gene expression. Nevertheless, we found that 1% DSS led to a significant shortening of colon lenght in all the groups, a robustly higher level of fecal LCN2 and a modest elevation of LBP in plasma, all indicative of a low grade inflammatory state in the colon following the 1% DSS treatment. When assessing the effects of the different faba bean fractions in the DSS exposed enviroment, our results do not support that they were able to mitigate any of the clinical markers of low grade inflammation.

Although we found variable expression of proinflammatory genes, we in fact observed that some of the genes were more highly expressed in the WD+faba bean fractions than mice fed the pure WD following DSS treatment. This was particularly apparent for *Nox2*, a gene expressed in activated macrophages and associated to the respiratory burst during inflammation. We therefore speculate that, in the current conditions, faba beans could potentially increase the DSS effects and impact colon health adversely. This assumption is partially in agreement with an earlier mouse study, in which bean diets exerted both beneficial and adverse effects in colons exposed to 2% DSS when mice were fed bean diets together with DSS [52]. In other relevant studies, legume containing diets were instead swithed to a common basal diet just before DSS exposure and kept on this diet during DSS challenge [13–15, 52–56]. A rationale for such approach was to mimic IBD patients consumption pattern when they experience intestinal problems (thus abstain from legumes and other fermentable sources). The impact of the faba bean diets employed in our study could therefore be impacted by the order of feeding and DSS. Although our results contrast other findings with respect to

legumes' impact on inflammation, a study by Bibi and colleagues showed that high fat fed mice supplemented with peas displayed no difference in colitis symptoms between HFD and HFD+peas during DSS challenge in mice exposed to DSS [57]. Nevertheless, they found that the recovery phase became shorter in the HFD+pea mice. In our case we terminated the experiment at day six of DSS treatment, thus we do not have results from any recovery phase and therefore we are not able to refer to potential contribution [57].

Regarding microbiota in fecal samples of 1% DSS treated mice, we observed a trend of increased alpha-diversity in all faba bean fraction fed mice compared to the WD+DSS mice, whereas the beta-diversity was only clearly different between control mice and those exposed to DSS. These results, which are in line with previous observations using higher doses of DSS (2–5%) [58, 59], indicate that even a low dose of DSS is capable of shifting the bacterial community bacteria structure but not the bacterial species richness. According to Singh *et al.*, plant-protein diets in humans are linked with higher microbiota richness and diversity than animal-protein diets [60]. One potential reason is that dietary fiber [61] and phenolic compounds [62] present in legumes, which are metabolised by intestinal microbiota causing shifts in gut bacterial populations. At phylum level, the relative abundance of Proteobacteria and Verucomicrobia was higher in all mice exposed to 1% DSS, which is in agreement with other DSS administered studies [63]. In mice fed solely a WD, these two phyla had a tendency to be more abundant compared to mice fed a WD supplemented with faba bea fractions. Increased abundance of Proteobacteria is common in IBD and is regarded as indicator of an inflammatory phenotype [64]. The phylum Verrucomicrobia, which has only one representative in the human and mouse gut (*Akkermansia muciniphila*), is characterized by mucin degrading properties. The role of genus *Akkermansia* is conflicting since some studies support its beneficial contribution to intestinal homeostasis [65], whereas other studies indicate that they exacerbate intestinal inflammation [66]. Furthermore, *Akkermansia muciniphila* in mice is linked with both a positive effect in mice fed a high fat diet [67] and harmful effect such as enhanced colitis [68].

Moreover, WD and WD+PF after DSS treatment had significantly higher relative abundance of the phylum Actinobacteria when compared to WD+FF. In addition, we noticed that the F/B ratio was high in WD-fed mice not treated with 1% DSS, whereas all DSS treated mice regardless of diet, revealed a low F/B ratio. The former condition is commonly associated to obesity [69, 70] and the latter with IBDs [71, 72]. Further Lefse analysis, provided detailed information about lower taxonomic groups. Characteristically, the genus Alloprevotella, a carbohydrate fermenting bacteria had high relative abundance in the mice following a WD+FF whereas the genus *Bifidobacterium*, which belongs to probiotic bacteria was highly present in the WD+PF-fed mice. Initially, Hayakawa showed that purifiried raffinose oligosaacharides family promotes Bifidobacteria growth [73]. Our protein fraction diet contains higher amounts of starch and non starch-digestible carbohydrates (raffinose, stachyose, verbascose), whereas the fiber fraction diet is mainly rich in cellulose and other indigestible fibers. Finally, it is important to note that we cannot rule out that differences between the faba-bean fraction groups could have happened already before the DSS treatment. The protein fraction, in particular, led to an increase in weight prior to DSS due to increased energy intake. Optimally, future studies should include non-DSS groups receiving similar diets. Alternatively, the microbiota composition should also be characterized at start of DSS treatment to account for differential impact of the diets prior to DSS.

In conclusion, we assessed whether a high fat Western diet supplemented with faba bean fractions reduced vulnerability towards colonic inflammation induced by a low DSS dose. Herein, our results suggest that although Faba bean fractions could modulate microbiota, they were not able to influence colonic inflammation induced by DSS.

Supporting information

S1 Fig. Histology images. Representative images of colon tissue from mice exposed to 1% DSS. Colon sections (7μm) were stained with hematoxylin and eosin. BF, both fractions; DSS, dextran sodium sulfate; FF, fiber fraction; PF, protein fraction; WD, western diet.
(PDF)

S1 Table. a. Composition of food pellets. b. Content of cooked faba bean fractions.
(PDF)

S2 Table. Criteria for DAI and scoring way for assessing during exposure to 1% DSS.
(PDF)

S3 Table. Reaction mixture for cDNA synthesis using the iScript cDNA Synthesis Kit.
(PDF)

S4 Table. Temperature program used for cDNA synthesis.
(PDF)

S5 Table. Primer sequences for RT-qPCR and melting temperatures.
(PDF)

S6 Table. Reaction mixture for amplicon PCR during library preparation for gene sequencing of 16S rRNA.
(PDF)

S7 Table. Temperature cycles for amplicon PCR during library preparation for gene sequencing of 16S rRNA.
(PDF)

S8 Table. Reaction mixture for index PCR during library preparation for gene sequencing of 16S rRNA.
(PDF)

S9 Table. Temperature cycles for index PCR during library preparation for gene sequencing of 16S rRNA.
(PDF)

S10 Table. Primers modified with Illumina adapters used for index PCR during library preparation for gene sequencing of 16S rRNA.
(PDF)

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References

1. Maphosa Y, Jideani V. The Role of Legumes in Human Nutrition. 2017.
2. George Amponsah Annor ZMaJIB. Crops-Legumes. In: Stephanie Clark SJaBL, editor. Food Processing: Principles and Appliations Second ed: John Wiley & Sons, Ltd; 2014.
3. Organization WH. Global Action Plan for the Prevention and Control of NCDs 2013–2020. Geneva; 2013.
4. Bazzano LA, Thompson AM, Tees MT, Nguyen CH, Winham DM. Non-soy legume consumption lowers cholesterol levels: a meta-analysis of randomized controlled trials. Nutr Metab Cardiovasc Dis. 2011; 21 (2):94–103. https://doi.org/10.1016/j.numecd.2009.08.012 PMID: 19939654
5. Afshin A, Micha R, Khatibzadeh S, Mozaffarian D. Consumption of nuts and legumes and risk of incident ischemic heart disease, stroke, and diabetes: a systematic review and meta-analysis. Am J Clin Nutr. 2014; 100(1):278–88. https://doi.org/10.3945/ajcn.113.076901 PMID: 24898241
6. Nothlings U, Schulze MB, Weikert C, Boeing H, van der Schouw YT, Bamia C, et al. Intake of vegetables, legumes, and fruit, and risk for all-cause, cardiovascular, and cancer mortality in a European diabetic population. J Nutr. 2008; 138(4):775–81. https://doi.org/10.1093/jn/138.4.775 PMID: 18356334
7. Grundy SM. Pre-diabetes, metabolic syndrome, and cardiovascular risk. J Am Coll Cardiol. 2012; 59 (7):635–43. https://doi.org/10.1016/j.jacc.2011.08.080 PMID: 22322078
8. Mollard RC, Luhovyy BL, Panahi S, Nunez M, Hanley A, Anderson GH. Regular consumption of pulses for 8 weeks reduces metabolic syndrome risk factors in overweight and obese adults. Br J Nutr. 2012; 108 Suppl 1:S111–22. https://doi.org/10.1017/S0007114512000712 PMID: 22916807
9. Sajjadi F, Gharipour M, Mohammadifard N, Nouri F, Maghroun M, Alikhasi H. Relationship between legumes consumption and metabolic syndrome: Findings of the Isfahan Healthy Heart Program. ARYA atherosclerosis. 2014; 10:18–24. PMID: 24963309
10. Rebello CJ, Greenway FL, Finley JW. A review of the nutritional value of legumes and their effects on obesity and its related co-morbidities. Obes Rev. 2014; 15(5):392–407. https://doi.org/10.1111/obr.12144 PMID: 24433379
11. Lanza E, Hartman TJ, Albert PS, Shields R, Slattery M, Caan B, et al. High dry bean intake and reduced risk of advanced colorectal adenoma recurrence among participants in the polyp prevention trial. J Nutr. 2006; 136(7):1896–903. https://doi.org/10.1093/jn/136.7.1896 PMID: 16772456
12. Perera T, Takata Y, Bobe G. Grain Legume Consumption Inhibits Colorectal Tumorigenesis: A Meta-Analysis of Human and Animal Studies. 2016.
13. Graf D, Monk JM, Wu W, Wellings HR, Robinson LE, Power KA. Red lentil supplementation reduces the severity of dextran sodium sulfate-induced colitis in C57BL/6 male mice. Journal of Functional Foods. 2020; 64:103625.
14. Monk JM, Lepp D, Wu W, Graf D, McGillis LH, Hussain A, et al. Chickpea-supplemented diet alters the gut microbiome and enhances gut barrier integrity in C57Bl/6 male mice. Journal of Functional Foods. 2017; 38:663–74.
15. Monk JM, Zhang CP, Wu W, Zarepoor L, Lu JT, Liu R, et al. White and dark kidney beans reduce colonic mucosal damage and inflammation in response to dextran sodium sulfate. J Nutr Biochem. 2015; 26(7):752–60. https://doi.org/10.1016/j.jnutbio.2015.02.003 PMID: 25841250
16. Dhingra D, Michael M, Rajput H, Patil RT. Dietary fibre in foods: a review. J Food Sci Technol. 2012; 49 (3):255–66. https://doi.org/10.1007/s13197-011-0365-5 PMID: 23729846

17. Trinidad TP, Mallillin AC, Loyola AS, Sagum RS, Encabo RR. The potential health benefits of legumes as a good source of dietary fibre. Br J Nutr. 2010; 103(4):569–74. https://doi.org/10.1017/S0007114509992157 PMID: 19825218

18. Levine ME, Suarez JA, Brandhorst S, Balasubramanian P, Cheng CW, Madia F, et al. Low protein intake is associated with a major reduction in IGF-1, cancer, and overall mortality in the 65 and younger but not older population. Cell Metab. 2014; 19(3):407–17. https://doi.org/10.1016/j.cmet.2014.02.006 PMID: 24606898

19. Jantchou P, Morois S, Clavel-Chapelon F, Boutron-Ruault MC, Carbonnel F. Animal protein intake and risk of inflammatory bowel disease: The E3N prospective study. Am J Gastroenterol. 2010; 105 (10):2195–201. https://doi.org/10.1038/ajg.2010.192 PMID: 20461067

20. Hou JK, Abraham B, El-Serag H. Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. Am J Gastroenterol. 2011; 106(4):563–73. https://doi.org/10.1038/ajg.2011.44 PMID: 21468064

21. Stolt E, Olsen T, Elshorbagy A, Kožich V, van Greevenbroek M, Øvrebø B, et al. Sulfur amino acid restriction, energy metabolism and obesity: a study protocol of an 8-week randomized controlled dietary intervention with whole foods and amino acid supplements. Journal of Translational Medicine. 2021; 19 (1):153. https://doi.org/10.1186/s12967-021-02824-3 PMID: 33858441

22. Hertzler SR, Lieblein-Boff JC, Weiler M, Allgeier C. Plant Proteins: Assessing Their Nutritional Quality and Effects on Health and Physical Function. Nutrients. 2020; 12(12). https://doi.org/10.3390/nu12123704 PMID: 33266120

23. Lopez-Barrios L, Gutierrez-Uribe JA, Serna-Saldivar SO. Bioactive peptides and hydrolysates from pulses and their potential use as functional ingredients. J Food Sci. 2014; 79(3):R273–83. https://doi.org/10.1111/1750-3841.12365 PMID: 24547749

24. Utrilla MP, Peinado MJ, Ruiz R, Rodriguez-Nogales A, Algieri F, Rodriguez-Cabezas ME, et al. Pea (Pisum sativum L.) seed albumin extracts show anti-inflammatory effect in the DSS model of mouse colitis. Mol Nutr Food Res. 2015; 59(4):807–19. https://doi.org/10.1002/mnfr.201400630 PMID: 25626675

25. Margier M, George S, Hafnaoui N, Remond D, Nowicki M, Du Chaffaut L, et al. Nutritional Composition and Bioactive Content of Legumes: Characterization of Pulses Frequently Consumed in France and Effect of the Cooking Method. Nutrients. 2018; 10(11). https://doi.org/10.3390/nu10111668 PMID: 30400385

26. Popova A, Mihaylova D. Antinutrients in Plant-based Foods: A Review. The Open Biotechnology Journal. 2019; 13:68–76.

27. Clemente A, Olias R. Beneficial effects of legumes in gut health. Current Opinion in Food Science. 2017; 14:32–6.

28. Clemente A, Arques Mdel C. Bowman-Birk inhibitors from legumes as colorectal chemopreventive agents. World J Gastroenterol. 2014; 20(30):10305–15. https://doi.org/10.3748/wjg.v20.i30.10305 PMID: 25132747

29. Basheer-Salimia R, Camilli B, Scacchi S, Noli E, Awad M. Genetic Diversity among Palestinian Faba Bean (Vicia faba L.) Ecotypes based on Single Nucleotide Polymorphisms. European Journal of Horticultural Science. 2014; 79:1611–4426.

30. Alghamdi SS, Migdadi HM, Ammar MH, Paull JG, Siddique KHM. Faba bean genomics: current status and future prospects. Euphytica. 2012; 186(3):609–24.

31. Maalouf F, Hu J, O'Sullivan DM, Zong X, Hamwieh A, Kumar S, et al. Breeding and genomics status in faba bean (Vicia faba). Plant Breeding. 2019; 138(4):465–73.

32. Singh A, Bharati R, Manibhushan NC, Pedpati A, editors. An assessment of faba bean (Vicia faba L.) current status and future prospect2013.

33. Mayer Labba IC, Frokiaer H, Sandberg AS. Nutritional and antinutritional composition of fava bean (Vicia faba L., var. minor) cultivars. Food Res Int. 2021; 140:110038. https://doi.org/10.1016/j.foodres.2020.110038 PMID: 33648264

34. Vogelsang-O'Dwyer M, Petersen IL, Joehnke MS, Sorensen JC, Bez J, Detzel A, et al. Comparison of Faba Bean Protein Ingredients Produced Using Dry Fractionation and Isoelectric Precipitation: Techno-Functional, Nutritional and Environmental Performance. Foods. 2020; 9(3). https://doi.org/10.3390/foods9030322 PMID: 32168773

35. Rizzello CG, Losito I, Facchini L, Katina K, Palmisano F, Gobbetti M, et al. Degradation of vicine, convicine and their aglycones during fermentation of faba bean flour. Scientific Reports. 2016; 6(1):32452. https://doi.org/10.1038/srep32452 PMID: 27578427

36. Cardador-Martínez A, Maya-Ocaña K, Ortiz-Moreno A, Herrera-Cabrera BE, Dávila-Ortiz G, Múzquiz M, et al. Effect of Roasting and Boiling on the Content of Vicine, Convicine and L-3,4-dihydroxyphenylalanine in Vicia faba L. Journal of Food Quality. 2012; 35(6):419–28.

37. Bialkowska AB, Ghaleb AM, Nandan MO, Yang VW. Improved Swiss-rolling Technique for Intestinal Tissue Preparation for Immunohistochemical and Immunofluorescent Analyses. J Vis Exp. 2016(113). https://doi.org/10.3791/54161 PMID: 27501188

38. Viennois E, Chen F, Laroui H, Baker MT, Merlin D. Dextran sodium sulfate inhibits the activities of both polymerase and reverse transcriptase: lithium chloride purification, a rapid and efficient technique to purify RNA. BMC Res Notes. 2013; 6:360. https://doi.org/10.1186/1756-0500-6-360 PMID: 24010775

39. Chassaing B, Srinivasan G, Delgado MA, Young AN, Gewirtz AT, Vijay-Kumar M. Fecal lipocalin 2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation. PLoS One. 2012; 7 (9):e44328. https://doi.org/10.1371/journal.pone.0044328 PMID: 22957064

40. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013; 10(10):996–8. https://doi.org/10.1038/nmeth.2604 PMID: 23955772

41. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010; 26 (19):2460–1. https://doi.org/10.1093/bioinformatics/btq461 PMID: 20709691

42. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 2007; 35(21):7188–96. https://doi.org/10.1093/nar/gkm864 PMID: 17947321

43. Oksanen J F.G. Blanchet, et al., vegan: Community Ecology Package. R package version 2.5–6. 2019.

44. Hervé M. RVAideMemoire: Testing and Plotting Procedures for Biostatistics. R package version 0.9–78. 2020.

45. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011; 12(6):R60. https://doi.org/10.1186/gb-2011-12-6-r60 PMID: 21702898

46. Papoutsis D, da Rocha SDC, Herfindal AM, Bohn SK, Carlsen H. A high-fat Western Diet Attenuates Intestinal Changes in Mice with DSS-induced Low-grade Inflammation. J Nutr. 2021.

47. Suzuki R, Kohno H, Sugie S, Nakagama H, Tanaka T. Strain differences in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice. Carcinogenesis. 2006; 27 (1):162–9. https://doi.org/10.1093/carcin/bgi205 PMID: 16081511

48. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. Curr Protoc Immunol. 2014; 104:Unit 15 25. https://doi.org/10.1002/0471142735.im1525s104 PMID: 24510619

49. Christ A, Lauterbach M, Latz E. Western Diet and the Immune System: An Inflammatory Connection. Immunity. 2019; 51(5):794–811. https://doi.org/10.1016/j.immuni.2019.09.020 PMID: 31747581

50. Liisberg U, Myrmel LS, Fjaere E, Ronnevik AK, Bjelland S, Fauske KR, et al. The protein source determines the potential of high protein diets to attenuate obesity development in C57BL/6J mice. Adipocyte. 2016; 5(2):196–211. https://doi.org/10.1080/21623945.2015.1122855 PMID: 27386160

51. Lamming DW, Baar EL, Arriola Apelo SI, Tosti V, Fontana L. Short-term consumption of a plant protein diet does not improve glucose homeostasis of young C57BL/6J mice. Nutr Healthy Aging. 2017; 4 (3):239–45. https://doi.org/10.3233/NHA-170025 PMID: 29276793

52. Zhang C, Monk JM, Lu JT, Zarepoor L, Wu W, Liu R, et al. Cooked navy and black bean diets improve biomarkers of colon health and reduce inflammation during colitis. Br J Nutr. 2014; 111(9):1549–63. https://doi.org/10.1017/S0007114513004352 PMID: 24521520

53. Graf D, Monk JM, Lepp D, Wu W, McGillis L, Roberton K, et al. Cooked Red Lentils Dose-Dependently Modulate the Colonic Microenvironment in Healthy C57Bl/6 Male Mice. Nutrients. 2019; 11(8). https://doi.org/10.3390/nu11081853 PMID: 31405019

54. Monk JM, Lepp D, Wu W, Pauls KP, Robinson LE, Power KA. Navy and black bean supplementation primes the colonic mucosal microenvironment to improve gut health. J Nutr Biochem. 2017; 49:89–100. https://doi.org/10.1016/j.jnutbio.2017.08.002 PMID: 28915390

55. Monk JM, Lepp D, Zhang CP, Wu W, Zarepoor L, Lu JT, et al. Diets enriched with cranberry beans alter the microbiota and mitigate colitis severity and associated inflammation. J Nutr Biochem. 2016; 28:129–39. https://doi.org/10.1016/j.jnutbio.2015.10.014 PMID: 26878790

56. Monk JM, Wu W, McGillis LH, Wellings HR, Hutchinson AL, Liddle DM, et al. Chickpea supplementation prior to colitis onset reduces inflammation in dextran sodium sulfate-treated C57Bl/6 male mice. Appl Physiol Nutr Metab. 2018; 43(9):893–901. https://doi.org/10.1139/apnm-2017-0689 PMID: 29522694

57. Bibi S, de Sousa Moraes LF, Lebow N, Zhu MJ. Dietary Green Pea Protects against DSS-Induced Colitis in Mice Challenged with High-Fat Diet. Nutrients. 2017; 9(5). https://doi.org/10.3390/nu9050509 PMID: 28524086

58. Munyaka PM, Rabbi MF, Khafipour E, Ghia JE. Acute dextran sulfate sodium (DSS)-induced colitis promotes gut microbial dysbiosis in mice. J Basic Microbiol. 2016; 56(9):986–98. https://doi.org/10.1002/jobm.201500726 PMID: 27112251

59. Berry D, Schwab C, Milinovich G, Reichert J, Ben Mahfoudh K, Decker T, et al. Phylotype-level 16S rRNA analysis reveals new bacterial indicators of health state in acute murine colitis. The ISME Journal. 2012; 6(11):2091–106. https://doi.org/10.1038/ismej.2012.39 PMID: 22572638

60. Singh RK, Chang HW, Yan D, Lee KM, Ucmak D, Wong K, et al. Influence of diet on the gut microbiome and implications for human health. J Transl Med. 2017; 15(1):73. https://doi.org/10.1186/s12967-017-1175-y PMID: 28388917

61. Abreu y Abreu AT, Milke-García MP, Argüello-Arévalo GA, Calderón-de la Barca AM, Carmona-Sánchez RI, Consuelo-Sánchez A, et al. Dietary fiber and the microbiota: A narrative review by a group of experts from the Asociación Mexicana de Gastroenterología. Revista de Gastroenterología de México (English Edition). 2021; 86(3):287–304. https://doi.org/10.1016/j.rgmxen.2021.02.002 PMID: 34144942

62. Cardona F, Andrés-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuño MI. Benefits of polyphenols on gut microbiota and implications in human health. The Journal of Nutritional Biochemistry. 2013; 24(8):1415–22. https://doi.org/10.1016/j.jnutbio.2013.05.001 PMID: 23849454

63. Chang CS, Liao YC, Huang CT, Lin CM, Cheung CHY, Ruan JW, et al. Identification of a gut microbiota member that ameliorates DSS-induced colitis in intestinal barrier enhanced Dusp6-deficient mice. Cell Rep. 2021; 37(8):110016. https://doi.org/10.1016/j.celrep.2021.110016 PMID: 34818535

64. Shin NR, Whon TW, Bae JW. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 2015; 33(9):496–503. https://doi.org/10.1016/j.tibtech.2015.06.011 PMID: 26210164

65. Derrien M, Van Baarlen P, Hooiveld G, Norin E, Muller M, de Vos WM. Modulation of Mucosal Immune Response, Tolerance, and Proliferation in Mice Colonized by the Mucin-Degrader Akkermansia muciniphila. Front Microbiol. 2011; 2:166. https://doi.org/10.3389/fmicb.2011.00166 PMID: 21904534

66. Zhang T, Ji X, Lu G, Zhang F. The potential of Akkermansia muciniphila in inflammatory bowel disease. Appl Microbiol Biotechnol. 2021; 105(14–15):5785–94. https://doi.org/10.1007/s00253-021-11453-1 PMID: 34312713

67. Shin NR, Lee JC, Lee HY, Kim MS, Whon TW, Lee MS, et al. An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut. 2014; 63(5):727–35. https://doi.org/10.1136/gutjnl-2012-303839 PMID: 23804561

68. Zhang Q, Wu Y, Wang J, Wu G, Long W, Xue Z, et al. Accelerated dysbiosis of gut microbiota during aggravation of DSS-induced colitis by a butyrate-producing bacterium. Sci Rep. 2016; 6:27572. https://doi.org/10.1038/srep27572 PMID: 27264309

69. Huazano-Garcia A, Shin H, Lopez MG. Modulation of Gut Microbiota of Overweight Mice by Agavins and Their Association with Body Weight Loss. Nutrients. 2017; 9(9). https://doi.org/10.3390/nu9090821 PMID: 28832493

70. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A. 2005; 102(31):11070–5. https://doi.org/10.1073/pnas.0504978102 PMID: 16033867

71. Kho ZY, Lal SK. The Human Gut Microbiome—A Potential Controller of Wellness and Disease. Front Microbiol. 2018; 9:1835. https://doi.org/10.3389/fmicb.2018.01835 PMID: 30154767

72. Zhou Y, Zhi F. Lower Level of Bacteroides in the Gut Microbiota Is Associated with Inflammatory Bowel Disease: A Meta-Analysis. Biomed Res Int. 2016; 2016:5828959. https://doi.org/10.1155/2016/5828959 PMID: 27999802

73. Hayakawa K, Mizutani J, Wada K, Masai T, Yoshihara I, Mitsuoka T. Effects of Soybean Oligosaccharides on Human Faecal Flora. Microbial Ecology in Health and Disease. 1990; 3(6):293–303.

Supplementary Material for: "Intestinal effect of faba bean fractions in WD-fed mice treated with low dose of DSS"

S1 Table
a. Composition of food pellets

Diets	**WD**		**WD+PF**		**WD+FF**		**WD+BF**	
Ingredients	**g**	**kcal**	**g**	**kcal**	**g**	**kcal**	**g**	**kcal**
Casein	195	780	128.52	514	186.04	744.16	126.88	507.52
DL-methionine	3	12	3	12	3	12	3	12
Corn starch	50	200	38.91	155.64	45.46	181.84	36.07	144.28
Maltodextrin 10	100	400	100	400	100	400	100	400
Sucrose	341	1364	341	1364	341	1364	341	1364
Milk fat, cow, anhydrous	200	1800	196.95	1772	200	1800	197.22	1774
Corn oil	10	90	10	90	10	90	10	90
Cellulose, BW200	70	0	63.91	0	0	0	70	5.6
Ethoxyquin	0.04	0	0.04	0	0.04	0	0.04	0
Mineral mix S10001	35	0	35	0	35	0	35	0
Calcium carbonate	4	0	4	0	4	0	4	0
Vitamin mix V10001	10	40	10	40	10	40	10	40
Choline bitartrate	2	0	2	0	2	0	2	0
Cholesterol	1.5	0	1.5	0	1.5	0	1.5	0
Protein fraction	0	0	89.97	358.65	0	0	82	518.13
Fiber fraction	0	0	0	0	122.5	238.24	103	
Total	1021.5	4592.4	1024.8	4592.4	1060.5	4592.4	1057.3	4592.4
Protein, %	17.1	15.2	17.1	15.2	17.1	15.2	17.1	15.2
Carbohydrate, %	49	43.6	49	43.6	49	43.6	49	43.6
Fat, %	20.1	41.2	20.1	41.2	20.1	41.2	20.1	41.2
kcal/g	4.5		4.5		4.3		4.3	

BF, both fractions; FF, fiber fraction; PF, protein fraction; WD, Western diet (D12079B, Research Diets).

b. Content of cooked faba bean fractions

Sample	**Starch+Glucose+Fructose+Sucrose** Total available CHO (g/100g)	**NSP+NDO CHO** based Total Dietary Fiber (g/100g)	**NDF** (g/100g)	**Protein** (g/100g)	**Crude fat** (g/100g)
Fiber fraction (from hull)	3,70	57,14	62,68	6,44	0,27
Protein fraction	12,34	6,76	10,09	65,02	3,39

NSP: Non-starch polysaccharides, NDO: Non-digestible oligosaccharides, NDF: Neutral detergent fiber.

S2 Table
Criteria for DAI and scoring way for assessing during exposure to 1% DSS.

	Day 0	Day 2		Day 4		Day 6 (termination)	
	Initial weight	Weight	% change from initial	Weight	% change from initial	Weight	% change from initial
Mouse 1							
Mouse 2							
Mouse 3							
Mouse 4							

	Activity		Hunched posture		Stool quality	
	Day 2	Day 4	Day 2	Day 4	Day 2	Day 4
Mouse 1						
Mouse 2						
Mouse 3						
Mouse 4						

Scores given	**For weight**	**For activity, hunched posture, stool quality**
	0=<5% weight loss	0: normal symptoms
	1= 5-15% weight loss	1: mild symptoms
	5= 15-20% weight loss	3: severe symptoms
	10=> 20% weight loss	

S3 Table
Reaction mixture for cDNA synthesis using the iScript cDNA Synthesis kit (Bio Rad).

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

S4 Table

Temperature program used for cDNA synthesis.

Operation	Temperature (°C)	Duration
Primer annealing	25	5 min
cDNA synthesis	42	30 min
cDNA synthesis termination	85	5 min
-	4	∞

S5 Table

Primer sequences for RT-qPCR and melting temperatures.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Tm °C
Gapdh	CTTCAACAGCAACTCCCACTCTT	GCCGTATTCATTGTCATACCAGG	60
Il-1b	GCAGCTGGAGAGTGTGGAT	AAACTCCACTTTGCTCTTGACTT	61
Il-6	CGTGGAAATGAGAAAAGAGTTGT	GCATCATCGTTGTTCATACA	61
Nos2	GACATTACGACCCCTCCCAC	ACTCTGAGGGCTGACACAAG	62
Nox2	GGGAACTGGGCTGTGAATGA	CAGTGCTGACCCAAGGAGTT	61
Ptgs2	AATATCAGGTCATTGGTGGAGA	TCTACCTGAGTGTCTTTGACTG	61
Tnf-a	CTGTCTACTGAACTTCGGGGTGAT	GGTCTGGGCCATAGAACTGATG	61

Abbreviations: *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Il-1b*, interleukin-1 beta; *Il-6*, interleukin 6; *Nos2*, nitric oxide synthase 2; *Nox2*, NADPH oxidase 2; *Ptgs2*, prostaglandin endoperoxide synthase 2; *Tnf*-a, tumor necrosis factor alpha.

S6 Table

Reaction mixture for amplicon PCR during library preparation.

Component	Per reaction
5x HOT FIREPol® Blend Master Mix Ready to Load (Solis BioDyne)	5 µL
Forward primer, PRK341F (1 µM)*	0.5 µL
Reverse primer, PRK806R (1 µM)*	0.5 µL
Nuclease-free water	18 µL
Template DNA (0.003-2 ng/µL**)	1 µL

* Forward 5'- CCTACGGGRBGCASCAG-3', reverse 5'- GGACTACYVGGGTATCTAAT-3'

** Measured by Qubit.

S7 Table

Temperature cycles for amplicon PCR during library preparation.

Operation	Temperature (°C)	Duration	Cycles
Initial activation	95	15 min	1
Denaturation	95	30 sec	
Annealing	55	30 sec	25
Elongation	72	45 sec	
Final elongation	72	7 min	1
-	4	∞	-

S8 Table

Reaction mixture for index PCR during library preparation

Component	Per reaction
5x FIREPol® Master Mix Ready to Load (Solis BioDyne)	5 µL
Forward primer (1 µM)*	5 µL
Reverse primer (1 µM)*	5 µL
Nuclease-free water	8 µL
Template DNA	2 µL

* See Supplementary table 10

S9 Table

Temperature cycles for index PCR during library preparation for gene sequencing of 16S rRNA.

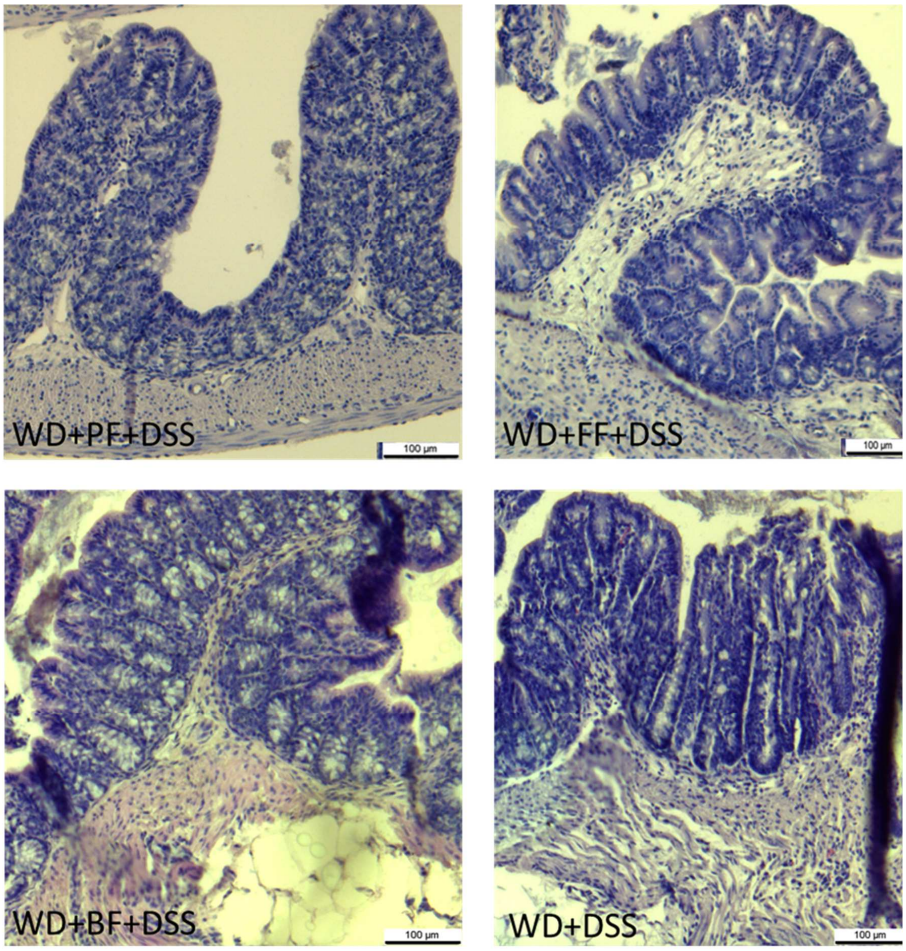
Operation	Temperature (°C)	Duration	Cycles
Initial activation	95	5 min	1
Denaturation	95	30 sec	
Annealing	55	1 min	10
Elongation	72	45 sec	
Final elongation	72	7 min	1
-	4	∞	-

S10 Table

Primers modified with Illumina adapters used for index PCR during library preparation for gene sequencing of 16S rRNA. Unique combination of forward and reverse primer was used for each sample.

Primer name	Sequence, 5' -> 3'	Target region	Direction
F9	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtttcgCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F10	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctcgtacgCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F11	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgagtggCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F12	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctggtagcCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F13	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctactgatCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F14	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctatgagcCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F15	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctattcctCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
F16	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctcaaaagCCTACGGGRBGCASCAG	16S rRNA (V3-V4)	Forward
R25	caagcagaagacggcatacgagatATCAGTgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R26	caagcagaagacggcatacgagatGCTCATgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R27	caagcagaagacggcatacgagatAGGAATgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R28	caagcagaagacggcatacgagatCTTTTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R29	caagcagaagacggcatacgagatTAGTTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R30	caagcagaagacggcatacgagatCCGGTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R31	caagcagaagacggcatacgagatATCGTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R32	caagcagaagacggcatacgagatTGAGTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R33	caagcagaagacggcatacgagatCGCCTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R34	caagcagaagacggcatacgagatGCCATGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse
R35	caagcagaagacggcatacgagatAAAATGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT	16S rRNA (V3-V4)	Reverse

S1 Fig.
Histology images



Supplementary figure 1. Representative images of colon tissue from mice exposed to 1% DSS. Colon sections (7µm) were stained with hematoxylin and eosin. BF, both fractions; DSS, dextran sodium sulfate; FF, fiber fraction; PF, protein fraction; WD, western diet.

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