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Dietary fat source and nutritional ketosis in intestinal permeability and inflammation

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ACADEMIC DISSERTATION

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'Aut inveniam viam aut faciam.'

'I shall either find a way or make one.'

- Hannibal Barca

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Abstract

Intestinal permeability and inflammation are interrelated processes in which increased paracellular permeability can evoke a pro-inflammatory response in the intestine, and inflammation further impairs barrier function. Indeed, defects in intestinal barrier function have been linked to inflammatory bowel diseases. Low-carbohydrate, high-fat ketogenic diets (KDs) have anti-inflammatory properties but previous observations of their effects on intestinal inflammation have been contradictory. Since the type of dietary fat can influence the physiological effects of a diet, it is possible that the effects of a KD on the intestine depend on its fat source.

This thesis set out to characterize the effects of a KD and its fat source on intestinal permeability and inflammation. Studies were conducted in healthy mice and mice with dextran sodium sulfate-induced experimental colitis. Intestinal permeability was determined *in vivo*, and other parameters of barrier function and intestinal inflammation were analyzed from tissue samples. First, the mice were fed KDs high either in saturated fatty acids or polyunsaturated linoleic acid to compare their effects in animals with or without induced intestinal inflammation. As the KDs were beneficial in alleviating colitis, the effect of the exogenous administration of the ketone body β-hydroxybutyrate was investigated as a possible mediator of the anti-inflammatory effects. While β-hydroxybutyrate provided no benefit, the expression of intestinal renin-angiotensin-aldosterone system components, involved in inflammation, was studied as a possible link between the KDs and the improved colitis outcomes.

The KDs conferred protection against experimental colitis, manifested as a reduction in macroscopic changes, histological damage, and the expression of inflammatory markers in the colon. The beneficial effect was pronounced with a diet rich in linoleic acid compared to saturated fatty acids. While β-hydroxybutyrate

was hypothesized to contribute to these effects, supplementation with this compound alone did not alleviate colitis. Neither of the KDs affected intestinal permeability in healthy or inflamed intestine but the diets upregulated the expression of specific tight junction proteins in the healthy intestine and maintained their levels in the presence of inflammation. The KDs had little influence on the expression of intestinal renin-angiotensin-aldosterone system components in the absence of induced inflammation but the diets mitigated inflammation-related changes in the system upon exposure to dextran sodium sulfate.

These results imply that KDs protect from intestinal inflammation without affecting paracellular permeability, and with the level of the protective effect being dependent on the dietary fat source. The exogenous administration of β-hydroxybutyrate does not lead to the same outcome, indicating that other features in addition to the increased supply of ketone bodies are responsible for the diet's impact. Finally, KDs can prevent inflammation-related changes in the intestinal renin-angiotensin-aldosterone system. Taken together, KDs may be of benefit in inflammatory conditions of the intestine.

Tiivistelmä

Suoliston läpäisevyys ja tulehdus ovat toisiinsa liittyviä prosesseja, joissa lisääntynyt läpäisevyys voi johtaa tulehdusvasteeseen, joka taas entisestään heikentää suoliston seinämän toimintaa. Lisääntynyt suoliston läpäisevyys onkin linkitetty tulehduksellisiin suolistosairauksiin. Vähähiilihydraattinen ja runsasrasvainen, ketogeeninen ruokavalio voi vähentää systeemistä tulehdusta, mutta aiemmat havainnot sen vaikutuksista suolitulehdukseen ovat ristiriitaisia. Ruokavalion rasvakoostumus voi määrittää osan sen fysiologisista vaikutuksista, joten on mahdollista, että myös ketogeenisen ruokavalion vaikutus riippuu sen rasvan lähteestä.

Tämä väitöstutkimus selvitti ketogeenisen ruokavalion ja sen rasvan lähteen vaikutusta suoliston läpäisevyyteen ja tulehdukseen. Osatöissä käytettiin terveitä hiiriä sekä dekstraaninatriumsulfaatilla indusoitua paksusuolitulehduksen hiirimallia. Suoliston läpäisevyys määritettiin *in vivo* ja muut suolen seinämän toimintaa sekä suoliston läpäisevyyttä kuvaavat parametrit analysoitiin kudosnäytteistä. Eläimiä ruokittiin ensin joko runsaasti tyydyttyneitä rasvahappoja tai monityydyttymätöntä linolihappoa sisältävillä ketogeenisillä ruokavalioilla ja verrattiin näiden vaikutuksia suolen läpäisevyyteen ja tulehdukseen sekä terveellä että suolitulehduksen mallilla. Koska ruokavaliot suojasivat kokeelliselta paksusuolitulehdukselta, selvitettiin, onko ketoaine β-hydroksibutyraatti vastuussa vaikutuksista antamalla eläimille sitä tavallisen ruoan mukana. β-hydroksibutyraatti ei saanut aikaan suojavastetta, joten suoliston tulehdukseen liittyvän, paikallisen reniini-angiotensiini-aldosteronijärjestelmän komponenttien ilmentymistä tutkittiin toisena mahdollisena linkkinä ketogeenisen ruokavalion ja lievemmän suolistotulehduksen välillä.

Ketogeeninen ruokavalio suojasi tulehdukselta, mikä ilmeni lievempinä makroskooppisina muutoksina, histologisina vaurioina ja tulehduksen merkkiaineiden ilmentymisenä paksusuolessa. Suojavaikutus oli voimakkaampi linolihappopitoisella ruokavaliolla verrattuna tyydyttyneitä rasvahappoja sisältävään ruokavalioon. Vaikka hypoteesin mukaan β-hydroksibutyraatti voisi olla tekijä suojavaikutuksen taustalla, sen lisääminen tavalliseen ruokavalioon ei yksinään suojannut tulehdukselta. Kumpikaan ketogeenisistä ruokavalioista ei muuttanut läpäisevyyttä terveessä tai tulehtuneessa suolessa, mutta lisäsi tiettyjen tiivisliitosproteiinien ilmentymistä terveessä suolessa ja lievensi tulehduksen aiheuttamia muutoksia niiden ilmentymisessä. Ruokavaliot eivät juurikaan vaikuttaneet terveen suolen reniini-angiotensiini-aldosteronijärjestelmän komponenttien ilmentymiseen, mutta lievittivät dekstraaninatriumsulfaatilla indusoituun tulehdukseen liittyviä muutoksia järjestelmässä.

Tulosten perusteella ketogeeniset ruokavaliot voivat suojata paksusuolitulehdukselta ilman vaikutusta suoliston läpäisevyyteen ja suojavasteen voimakkuus on riippuvainen ruokavalion rasvan lähteestä. Ruokinta β-hydroksibutyraatilla ei suojaa suolitulehdukselta, mikä viittaa lisääntyneen ketoaineiden saatavuuden ohella muiden ketogeenisen ruokavalion ominaisuuksien selittävän suojavaikutuksen. Ketogeeniset ruokavaliot voivat estää tulehdukseen liittyviä muutoksia suolen reniini-angiotensiini-aldosteronijärjestelmän komponenttien ilmentymisessä. Yhteenvetona, ketogeeniset ruokavaliot ovat mahdollinen apukeino suoliston tulehduksen lievittämisessä.

List of abbreviations

^{51}Cr-EDTA = radioactive chromium complexed with ethylene diamine tetraacetic acid
ACE = angiotensin-converting enzyme
ACE2 = angiotensin-converting enzyme 2
AT1R = angiotensin II receptor type 1
AT2R = angiotensin II receptor type 2
BHB = β-hydroxybutyrate
DSS = dextran sodium sulfate
DHA = docosahexaenoic acid
ELISA = enzyme-linked immunosorbent assay
EPA = eicosapentaenoic acid
E% = percentage of energy
FITC-dextran = fluorescein isothiocyanate dextran
HFD = high-fat diet
IBD = inflammatory bowel disease
IL = interleukin
I-FABP = intestinal fatty acid binding protein
KD = ketogenic diet
LA = linoleic acid
LPS = lipopolysaccharide
MasR = Mas receptor
MCT = medium-chain triglyceride
MLCK = myosin light-chain kinase
PEG = polyethylene glycol
RAAS = renin-angiotensin-aldosterone system
SFA = saturated fatty acid
sCD14 = soluble CD14

TEER = transepithelial electric resistance

TJ = tight junction

TNBS = trinitrobenzene sulfonic acid

List of original publications

This thesis is based on the following publications:

I **Toivio, L.**, Launonen, H., Lindén, J., Lehto, M., Vapaatalo, H., Salmenkari, H., & Korpela, R. (2023). Ketogenic diet high in saturated fat promotes colonic claudin expression without changes in intestinal permeability to iohexol in healthy mice. *Nutrients*, *16*(1), 18.

II **Toivio, L.**, Lindén, J., Lehto, M., Salmenkari, H., & Korpela, R. (2024). Ketogenic diet protects from experimental colitis in a mouse model regardless of dietary fat source. *Nutrients*, *16*(9), 1348.

III **Toivio, L.**, Toivio, J., Lindén, J., Lee, K., Lehto, M., Salmenkari, H., & Korpela, R. (2025). Free acid β-hydroxybutyrate supplementation does not ameliorate dextran sodium sulfate-induced colitis similar to ketogenic diet in male mice. *PharmaNutrition, 31,* 10043.

IV Launonen, H., **Toivio, L.**, Lindén, J., Salmenkari, H., & Korpela, R. (2025). Ketogenic diet counteracts the proinflammatory alterations in the renin-angiotensin-aldosterone system in murine experimental colitis. Submitted to *J Nutr Biochem*.

The publications are referred to in the text by their Roman numerals. The original publications are reprinted under the Creative Commons license.

1 Introduction

The intestine has the crucial functions of absorbing dietary nutrients and preventing harmful compounds and microbes from entering the body. If the intestinal barrier is dysfunctional, paracellular permeability to luminal contents of the intestine increases. This results in local and systemic inflammation, which further promotes intestinal permeability, which may create a vicious cycle (Fasano, 2020). Increased intestinal permeability has been linked to chronic diseases, like inflammatory bowel diseases (IBDs) which are relapsing conditions of inflammation of the intestine (Vanuytsel et al., 2021).

Dietary factors, such as fat quantity and source, can influence intestinal health. Regular high-fat diets (HFDs), high also in carbohydrate but low in fiber, may increase intestinal permeability and worsen inflammation through diverse mechanisms such as influencing the expression and morphology of tight junction (TJ) proteins and modulating the gut microbiota (Rohr et al., 2020). Importantly, the effect of the diet can be dependent on its fat source with different fats either predisposing the intestine to or protecting it from insults (Bartoszek et al., 2020; Haskey et al., 2022)

Despite the negative effects of regular HFDs, the ketogenic diet (KD), a low-carbohydrate, high-fat dietary approach that results in the elevated production and utilization of ketone bodies as an energy source, may promote the health of the intestine through several mechanisms. KDs fundamentally differ from typical HFDs in their physiological effects that are mostly anti-inflammatory (Ji et al., 2024). The postulated benefits for intestinal health may be mediated through mechanisms such as an increase in the circulating levels of the ketone body β-hydroxybutyrate (BHB), which has anti-inflammatory cellular and mitochondrial effects (Graff et al., 2016), and modulation of the gut microbiota (Ang et al., 2020).

KDs modulate the expression of the components of the local renin-angiotensin-aldosterone system (RAAS) in tissues such as lung and adipose tissue (Da Eira et al., 2021; Da Eira et al., 2023). In addition to the systemic RAAS, which regulates blood pressure, most tissues express RAAS components locally. These tissues include the intestine, where the system is involved in inflammation (Fändriks, 2011). As KDs promote the expression of anti-inflammatory RAAS components in other tissues (Da Eira et al., 2021; Da Eira et al., 2023), it is possible that the diet has the same effect in the intestine.

The focus of this thesis was on the impact of dietary fat source and nutritional ketosis on paracellular permeability and inflammation of the intestine. First, the effect of KDs with different fatty acid compositions on markers of intestinal permeability and inflammation in healthy animals were tested. Then, the impact of the diets and exogenously administered BHB on these parameters was investigated in experimental colitis. Further, the influence of the KDs on the expression of intestinal RAAS components in experimental colitis was studied.

2 Review of the literature

2.1 The intestinal barrier – a dynamic interface between the host and the environment

2.1.1 Overview of the intestine

The intestine forms a semi-permeable barrier, the main function of which is to regulate the passage of compounds to, and to some extent, from, the body. The lining of the intestine is made up of four different layers: mucosa, submucosa, muscular layer, and serosal layer (Figure 1). The first layer from the luminal side, mucosa, can further be divided into epithelium, *lamina propria*, and *muscularis mucosae*. The main cell types that comprise the epithelial layer (Figure 2) are enterocytes or colonocytes, enteroendocrine cells, goblet cells, Paneth cells, and microfold cells, all of which originate from intestinal stem cells, located in the intestinal crypts (Choi & Augenlicht, 2024). The turnover of epithelial cells is rapid: the human enterocyte population is renewed every three to five days (Darwich et al., 2014). Epithelial cells carry out most of the digestive, absorptive, and secretory functions of the intestine with enterocytes in the small intestine and colonocytes in the colon being the most abundant cell types. Especially enterocytes are responsible for the absorptive functions of the intestine, but they also participate in immune functions through sensing antigens and delivering them to other antigen-sensing cells (Wosen et al., 2018). The less abundant enteroendocrine cells respond to luminal nutrients and release peptide hormones that control the secretion of enzymes, influence motility, and regulate appetite. However, enteroendocrine cells

are also recognized to modulate the function of the intestinal immune system via mechanisms such as peptide and cytokine signaling (Worthington et al., 2018).

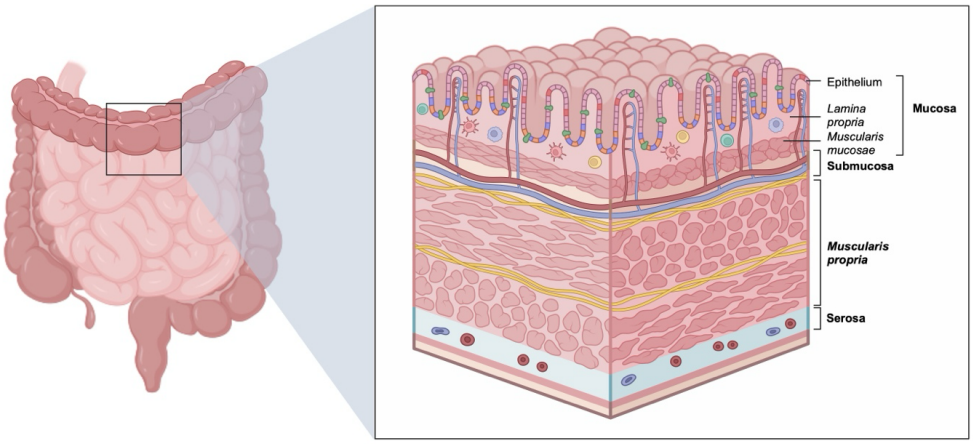

Figure 1. Structure of the intestinal wall. The intestinal wall comprises of four layers: mucosa, submucosa, *muscularis propria*, and serosa. The mucosa is further divided into epithelium, *lamina propria*, and *muscularis mucosae*. Created with BioRender.com.

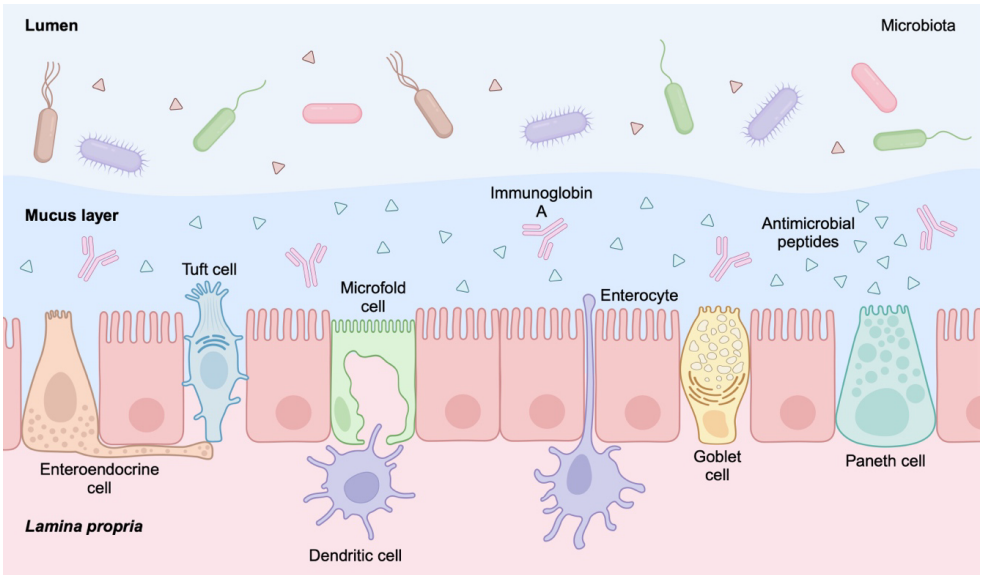

Figure 2. The intestinal barrier. The epithelial layer is comprised of different cell types, the most abundant of which are enterocytes. Goblet cells produce protective mucus that separates the epithelium from the intestinal microbiota and Paneth cells secrete antimicrobial peptides that further protect the epithelium. Created with BioRender.com.

The epithelium is a single-cell layer covered by protective mucus formed of glycosylated mucin proteins, most importantly mucin-2, that are secreted by epithelial mucosal cells, mainly goblet cells (Johansson et al., 2011). In the small intestine, the mucus is comprised of a single layer only, whereas in the colon there is a tight inner mucus layer covered by a looser outer layer (Atuma et al., 2001). While microbes can reside within the outer layer, the inner layer is normally impermeable to bacteria (Johansson et al., 2008). In addition to being covered by the mucus layer, the epithelium is protected by antimicrobial peptides, such as lysozyme and defensins, secreted by Paneth cells. In normal conditions, these cells are present in the small intestine but not in the colon (Wallaeys et al., 2023), which is logical as the abundance of commensal microbes is orders of magnitude higher in the latter.

Importantly, the epithelium is the primary regulator of paracellular permeability, also known as intestinal permeability, which refers to the passage of compounds between epithelial cells and through their cell-to-cell junctions (Vancamelbeke & Vermeire, 2017). The cell-to-cell junctions include anchoring junctions, gap junctions, and TJs which are multi-protein complexes that connect epithelial cells to each other. Of these, the anchoring junctions and gap junctions are responsible for the mechanical linkage between epithelial cells, whereas TJs control the paracellular passage. In addition to the junctional complexes, other components such as keratin filaments link epithelial cells to each other and assist in maintaining barrier integrity (Polari et al., 2020).

The intestine is also the largest site of the immune system in the body. The gut-associated lymphoid tissues are located within the mucosal layer and are responsible for maintaining tolerance to the commensal gut microbes while protecting the body from pathogenic organisms (Mörbe et al., 2021). These tissues comprise of Peyer's patches in the small intestine and isolated lymphoid follicles throughout the intestinal tract. Microfold cells residing in intestinal lymphoid tissues take up luminal antigens and present them to dendritic cells that are located in the submucosa (Ohno, 2016). The dendritic cells then further present the antigens to B and T cells, and thus, guide their activation and differentiation.

Dendritic cells with dendrites extending into the lumen via intraepithelial spaces can also participate in luminal antigen sampling. T cells are found in the epithelium as the major intraepithelial lymphocytes but also in *lamina propria* whereas B cells mainly reside in the latter (Mowat & Agace, 2014). Differentiated B cells, *i.e.* plasma cells, secrete immunoglobulin A into the gut lumen. This compound prevents the binding of pathogenic organisms to the epithelium (Mantis et al., 2011). Macrophages are the most abundant innate immune cells in the intestine and in addition to bacterial scavenging, they participate in antigen presentation, clearance of apoptotic cells, and immune modulation (Hegarty et al., 2023; Mowat & Agace, 2014).

Finally, the intestine hosts the microbiota, the majority of which resides in the colon and is estimated to contain 10^{13} to 10^{14} microbes (Gill et al., 2006). Bacteria are the most dominant microorganisms of the gut microbiota and comprise of six major phyla: *Firmicutes*, *Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria,* and *Verrucomicrobia*, of which the first two are the most abundant (Hou et al., 2022). Alongside bacteria, the gut microbiota contains commensal fungi, archaea, and viruses. The specific composition of the microbiota is individual to each host. Microbial colonization takes place already at birth with the delivery mode affecting the diversity and colonization pattern (Rutayisire et al., 2016). Many physiological factors, such as the host's genetics, immune system, and age further influence the composition of the microbiota (Hou et al., 2022). However, the microbiota can rapidly change based on environmental factors such as diet (David et al., 2014) and medications, especially antibiotics (Fishbein et al., 2023). The commensal microbiota forms a symbiotic relationship with the host by metabolizing non-digestible substances, such as short-chain fatty acids that colonocytes use for energy (Boets et al., 2017), and synthesizing vitamins, including group B vitamins and vitamin K (Tarracchini et al., 2024). Furthermore, these microbes are important in training the developing immune system (Donald & Finlay, 2023). They also contribute to immune defense by secreting antimicrobial compounds that suppress the growth of pathogens (Garcia-Gutierrez et al., 2019).

2.1.2 Intestinal permeability – pathways, regulation, and assessment

Pathways of intestinal permeability

The translocation of molecules through the gut epithelium can happen via two different routes: the transcellular or the paracellular pathway. The main function of the former pathway is the carrier-mediated absorption of dietary molecules and electrolytes via enterocytes. In addition, many drugs are absorbed transcellularly, and as previously described, the epithelial microfold cells take up microbial antigens to maintain tolerance for commensal microbes and protect the organism from pathogens. In contrast to the transcellular pathway, passage through the paracellular route is passive – the movement of solutes is driven by electrochemical and concentration gradients (Horowitz et al., 2023). The paracellular pathway is involved in both the influx and efflux of nutrients and in health, works in concert with transcellular transport. An important example of this is the efflux of sodium ions to the gut lumen through the paracellular route which ensures that the luminal sodium concentration is high enough to facilitate sodium-coupled transcellular nutrient transport (Tamura et al., 2011). There are two regulated paracellular routes across the intestinal epithelia: the pore and the leak pathway (France & Turner, 2017; Horowitz et al., 2023) (Figure 3). The first one is a high-capacity route which selectively allows the passage of molecules up to 0.6 nm in diameter based on their charge. The leak pathway has a lower capacity but is non-selective in terms of charge and permits the flux of solutes up to ~ 12.5 nm in size. Both routes are dependent on the expression and function of TJ proteins but are mostly regulated independently from each other. The tips of the intestinal villi are less permeable and mainly allow passage through the pore pathway, whereas the leak pathway is more dominant in the crypts (Shen et al., 2011). This restricts the permeability to larger molecules to areas further from the luminal contents without compromising the passage of ions and small molecules at the villus tips. If the epithelium is damaged, even larger particles, such as microbes, can pass the intestinal barrier

through an unrestricted pathway, which is unregulated and independent of TJ function (Horowitz et al., 2023) (Figure 3).

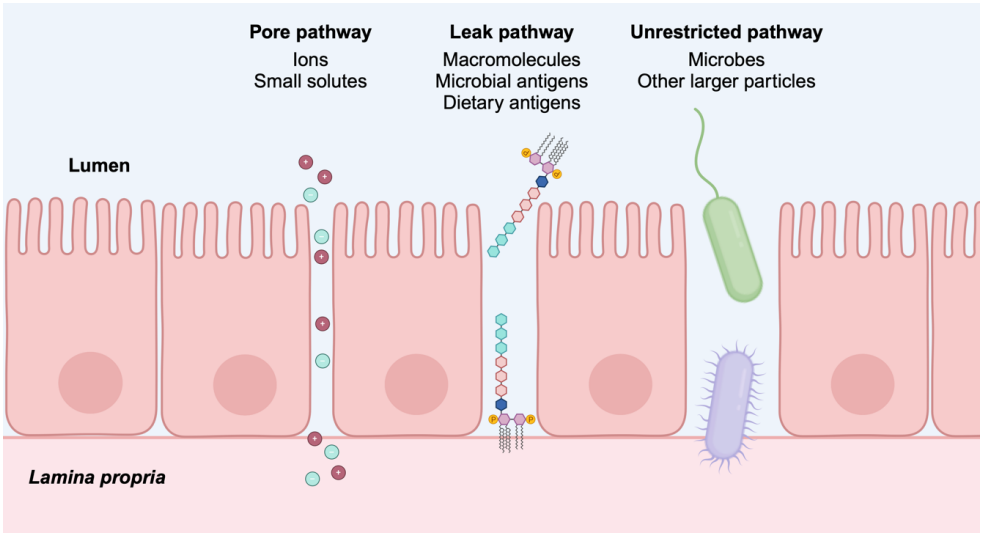

Figure 3. Pathways of paracellular permeability in the intestine. Paracellular flux can occur through three different pathways. The regulated pathways are the high-capacity, charge-selective pore pathway which allows the passage of small molecules; and the lower-capacity, non-selective leak pathway, which permits larger molecules to pass. An epithelial injury can result in flux through the unrestricted pathway. Created with BioRender.com.

Tight junctions as regulators of paracellular passage in the intestine

TJs are dynamic protein complexes that form cell-to-cell connections and link them to the cytoskeleton not only in the intestine but throughout the body, such as in the epidermis (O'Neill & Garrod, 2011) and the endothelium (Cong & Kong, 2020). They are the most apical junctions of the intestinal epithelium and are the main regulators of paracellular passage. TJs comprise of over 150 recognized intra- and extracellular proteins (Fasano, 2020). The most widely studied proteins making up the transmembrane part of the junction can be divided into three classes: claudins, TJ-associated MARVEL protein family members, and immunoglobulin superfamily proteins. These proteins are then connected to the actin cytoskeleton via scaffolding proteins, like those of the *zonula occludens* family.

The claudin family proteins are core components of these junctions. This family consists of 27 members, some of which, like claudins 1 and 4, have barrier-sealing properties, *i.e.*, they decrease the flux of ions and molecules through the intestinal wall (Tsukita et al., 2019). Many of these barrier-forming claudins are vital – for example, the knockout of claudin 1 or claudin 7 is lethal since it causes severe barrier defects (Furuse et al., 2002; Xu et al., 2019). The claudin family also contains pore-forming claudins, such as claudin-2. These claudins form charge- and size-selective paracellular channels that have a key function in the pore pathway. In the intestine, only cation-selective claudins are found. While knocking out either claudin-2 or claudin-15, another pore-forming claudin, is not lethal (Tamura et al., 2011), mice that lack both of these proteins do not survive (Wada et al., 2013), illustrating that there is some functional redundancy between different claudins. The expression of pore-forming claudins is more prominent in the proximal parts of the intestine whereas the expression of barrier-sealing claudins is higher in the distal intestine (Lameris et al., 2013).

The TJ-associated MARVEL family proteins include occludin, tricellulin, and marvelD3 (Raleigh et al., 2010). In contrast to the claudins, especially occludin is more involved in the regulation of the leak pathway. While in mice and Caco-2 cells, transepithelial electric resistance (TEER), a measure of electrical resistance across a cellular layer, does not change as a response to the knockout of occludin, permeability to a 10 kDa probe does increase (Al-Sadi et al., 2011; Buschmann et al., 2013; Saitou et al., 2000). This suggests that occludin regulates the leak but not the pore pathway. Tricellulin is enriched at tricellular TJs, *i.e.* the contact sites between three cells, but it is also found in bicellular junctions. In epithelial cell lines, a tricellulin knockout results in an increased flux of fluorescein isothiocyanate dextran (FITC-dextran) (Ikenouchi et al., 2005), a probe commonly used in permeability measurements. However, the absence of tricellulin also decreases TEER, indicating that it regulates both the pore and the leak pathway. There are limited data on the effects of marvelD3 on intestinal permeability, but its knockout decreases TEER in Caco-2 cells and thus, it may be involved in the regulation of the pore pathway (Raleigh et al., 2010).

Specific proteins of the immunoglobulin superfamily are expressed in TJs. These are transmembrane proteins that include junctional adhesion molecules 1, 3, and 4, coxsackie and adenovirus receptor, coxsackie and adenovirus receptor-like membrane protein, endothelial cell-selective adhesion molecule, and angulins. In mice, the knockout of junctional adhesion molecule 1 leads to increased intestinal permeability to FITC-dextran and decreased TEER, indicating that it is involved in the function of both the pore and the leak pathway (Laukoetter et al., 2007). The expression of other junctional adhesion molecules in intestinal TJs is low and they do not appear to have a central role in regulating epithelial permeability (Hartmann et al., 2020). While coxsackie and adenovirus receptor is more abundant in intestinal TJs, it is not an essential component as its deletion does not alter intestinal permeability to FITC-dextran nor change the structure of TJs in mice (Pazirandeh et al., 2011). This receptor likely functions similar to junctional adhesion molecules, and these can hypothetically compensate for its deficiency in a knockout model (Hartmann et al., 2020). Endothelial cell-selective adhesion molecule is more critical in the endothelium, but increasing its abundance via propionate administration leads to lower permeability to FITC-dextran in Caco-2 cells, suggesting that it may also regulate intestinal leak pathway permeability (Isayama et al., 2023). Little research has been conducted on intestinal angulins 1, 2, and 3, but they are present in tricellular TJs and likely have redundant functions as colonic knockout of angulin-2 leads to an increase in angulin-1 expression without changes in paracellular permeability (Hempstock et al., 2020). Overall, the specific roles of most immunoglobin superfamily proteins in the regulation of intestinal paracellular permeability are still unclear.

Mechanisms of regulation of paracellular permeability via tight junctions

The regulation of paracellular permeability is crucial for the prevention of unwanted compounds, *e.g.*, microbe- or food-derived antigens, from entering the body but also to permit the passage of water and some nutrients, such as monosaccharides (Pappenheimer, 1993) as well as the efflux of substances, most importantly sodium (Tamura et al., 2011). Permeability through the pore pathway

is mainly determined by the expression and function of claudins (Horowitz et al., 2023). As the integration of claudins in the TJ complex is relatively stable compared to the more dynamic TJ proteins, such as *zonula occludens* 1 and occludin, the rapid integration or dissociation of claudins is not a major regulatory mechanism of permeability. Thus, the balance in the amount and activity of barrier- and pore-forming claudins determines the level of permeability through the pore pathway. While the exact mechanisms that regulate pore pathway activity are incompletely characterized, interleukins 1, 6, 13, and 22, as well as tumor necrosis factor α (TNF-α) promote permeability through this pathway (Horowitz et al., 2023). Acute infection-related intestinal inflammation appears to increase the flux through the pore pathway via interleukin signaling and, specifically, by promoting claudin-2 expression (Tsai et al., 2017). This may be a protective mechanism since increased efflux of sodium and water can aid in the clearance of luminal pathogens. Claudin-2 overexpression is also protective in DSS-induced colitis (Ahmad et al., 2014), and its knockout detrimental (Nishida et al., 2013). Thus, in the face of luminal insults, inflammatory signaling may increase pore pathway activity as a protective mechanism.

The most important regulator of the leak pathway permeability is the enzyme myosin light-chain kinase (MLCK) (He et al., 2020). The key mechanisms through which it controls permeability were discovered already over twenty years ago. MLCK phosphorylates myosin light-chain, leading to the remodeling of the perijunctional actomyosin ring (Turner et al., 1997), an intracellular component of TJs, and the dissociation of *zonula occludens* 1 from the junctional complex, resulting in increased paracellular permeability (Madara et al., 1993). This pathway appears to serve a function in normal physiology, and is triggered, *e.g.*, by the activation of sodium-coupled transcellular nutrient transport (Berglund et al., 2001). In this context, the paracellular permeability is increased only to small molecules, such as nutrient monomers. The MLCK-driven permeability functions in concert with the passage through the pore-forming claudins to allow the transepithelial nutrient uptake to exceed the capacity of transcellular transport (He et al., 2020). This highlights the importance of the MLCK-mediated paracellular permeability in normal intestinal physiology.

MLCK is also an essential mediator of the inflammation-related increases in the leak pathway permeability. Proinflammatory cytokines like TNF-α and interferon-γ upregulate the transcription of MLCK, and its inhibition prevents the cytokine-induced flux through the leak pathway (Clayburgh et al., 2005). Upon induced inflammation, MLCK activity remodels the perijunctional actomyosin ring which results in *zonula occludens* 1 dissociation similar to that occurring in nutrient transport. Furthermore, inflammation-induced MLCK activation promotes the endocytosis of occludin (Clayburgh et al., 2005). In Caco-2 monolayers, treatment with TNF-α increases permeability only in cells that express occludin (Buschmann et al., 2013). In occludin knockout cells, permeability is similar as in TNF-α treated occludin-expressing cells, and does not increase further upon stimulation with TNF-α. In addition, when occludin is overexpressed or its endocytosis is prevented in mice, the animals are protected from TNF-α-induced increase in permeability (Marchiando et al., 2010). Thus, the MLCK-governed occludin regulation appears critical for inflammation-induced increases in paracellular permeability.

In addition to MLCK, another potential regulator of TJs is zonulin (Sturgeon & Fasano, 2016). Zonulin is an endogenously produced protein which increases leak pathway permeability *ex vivo* (Wang et al., 2000). Zonulin-overexpressing mice also exhibit increased permeability and higher susceptibility to DSS-induced colitis (Sturgeon et al., 2017). Zonulin binds to the proteinase activated receptor 2 and downstream, *zonula occludens* 1 and myosin 1C are phosphorylated (Sturgeon & Fasano, 2016). This leads to the redistribution of *zonula occludens* 1, occludin, and F-actin, resulting in increased permeability. However, zonulin has been less actively researched lately as the commercial detection methods have been shown to lack accuracy (Ajamian et al., 2019).

Assessment of intestinal permeability

Intestinal permeability can be assayed with several methods (Vancamelbeke & Vermeire, 2017). In the Ussing chamber method, pieces of intestinal tissue are mounted between two chambers, and the permeability to a probe molecule, such as

FITC-dextran, and TEER are measured. *In vivo*, orally ingested probes that are known to only use the paracellular route can be used to measure permeability by calculating the ratio of the amount of the ingested probe and the amount recovered in urine or plasma. *In vitro*, Caco-2 cell monolayers are used to model the intestinal barrier, and TEER and the flux of a selected probe molecule through the cell layer can be determined. The methods and common probes for permeability assessments are listed in Table 1.

In humans, sugar tests are the most common methods used for assessing intestinal permeability. The tests utilize two or more different saccharides that have different absorption ratios and, in some cases, sites (van Wijck et al., 2013). Monosaccharides such as mannitol and rhamnose are more effectively absorbed than disaccharides like lactulose and sucralose. Whereas the former use the transcellular pathway, the latter only permeate the intestinal barrier via the paracellular route, and thus, the disaccharide to monosaccharide ratio, *e.g.* lactulose to mannitol ratio, is considered to reflect the level of paracellular permeability. However, due to the similar size of these molecules, increased paracellular permeability may not only lead to elevated levels of lactulose but also mannitol, which may weaken the accuracy of the test (Camilleri, 2019). Most of these sugars can also undergo microbial metabolism and thus, the method may be prone to more error due to the degradation of the sugar molecules by the intestinal microbiota (Vancamelbeke & Vermeire, 2017). Another challenge with these molecules is their potential 'background consumption' in the regular diet which has led to the increased use of radiolabeled saccharides (Camilleri, 2019). In addition, sugar molecules are relatively small (164-398 Da) and thus, their levels do not reveal the permeability to larger molecules.

Iohexol (820 Da) is used as a contrast medium in X-ray imaging and is not as widely recognized as a probe molecule for intestinal permeability assessments. Nevertheless, it reflects epithelial permeability when administered orally and collected from urine (Halme et al., 2000). Determining permeability in different parts of the intestine is possible by collecting urine at set timepoints after the administration of iohexol (Ortin-Piqueras et al., 2021). The molecule is also highly

stable. However, the assessment of iohexol from plasma has not been validated as a marker of permeability which has limited its use, *e.g.* in animal models that develop diarrhea, due to possible fecal contamination of the urine. At the moment, the use of fluorescent probes such as FITC-dextrans of several sizes ranging from 4 to 150 kDa, are more common in animal research, with 4 kDa being the most popular molecular weight. As opposed to saccharides and iohexol, FITC-dextrans are relatively large and thus, it is impossible to investigate permeability to smaller compounds with these markers. They also need to be detected from plasma, making the method error-prone due to blood sample hemolysis which may interfere with the fluorometric detection. Other markers such as polyethylene glycols (PEGs) of various sizes are in use and have an equal performance to lactulose-mannitol test in humans (van Wijck et al., 2012). The advantage of PEGs is that different sizes can be combined to determine the approximate size of molecules being allowed to permeate the intestinal barrier. However, there can be some background use of PEGs trough diet or medical applications. Radioactive chromium complexed with ethylene diamine tetraacetic acid (^{51}Cr-EDTA, 340 Da) can be used both in animals and humans. Even though ^{51}Cr-EDTA is easily detectable and there is no risk of background exposure of the compound, it exposes subjects to low-level radiation which is why it is not generally recommended, especially for human studies. When compared to sugars and PEGs, the rest of the described methods are less able to account for individually determined factors since ratios of different molecules cannot be calculated (van Wijck et al., 2012). Finally, no method for intestinal permeability measurements has a standardized protocol and both within and between studies, a high inter-individual variability in permeability is observed. This complicates both comparing studies and applying the tests for clinical use.

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Table 1. The methods and most used probes for assessing intestinal permeability. Modified from Vancamelbeke & Vermeire (2017). ^{51}Cr-EDTA = radioactive chromium complexed with ethylene diamine tetraacetic acid, FITC-dextran = fluorescein isothiocyanate dextran, PEG = polyethylene glycol, TEER = transepithelial electric resistance.

	Marker	Description	Advantages	Limitations
In vivo	Sugar molecules	Used in combination in multi-sugar tests to determine the permeability of different parts of the intestine	Region-specific	Background consumption Degradation of some sugars by intestinal bacteria
	Sucrose *Lactulose* *Mannitol* *Rhamnose* *Sucralose*	Specific for stomach Specific for small intestine Specific for small intestine Specific for colon		Laborious detection Less accurate
	^{51}Cr-EDTA	Radioactive molecule, determines the permeability of the whole intestine	Not naturally present	Radioactivity Single probe Not region-specific
	PEGs	Polymers of different sizes, determine the permeability of the whole intestine	Probes of different sizes	Background consumption Laborious detection Not region-specific
	FITC-dextrans	Fluorescent molecules of different sizes, determine the permeability of the whole intestine		Interference from hemolysis Not region-specific
	Iohexol	Contrast medium, determines the permeability of the whole intestine	Not naturally present Easy detection	Single probe Not region-specific
Ex vivo	Ussing chambers	TEER and permeability to various probes measured from fresh tissue	Region-specific Various probes	Need for fresh tissue Limited viability Laborious
In vitro	Cell monolayers	Models the intestinal barrier, TEER and permeability to various probes measured	Various probes Long follow-up times Different test conditions	Less representative of permeability *in vivo*

a marker of permeability which has limited its use, *e.g.*, in animal models that develop diarrhea, due to the possible fecal contamination of the urine. At the moment, the use of fluorescent probes such as FITC-dextrans of several sizes ranging from 4 to 150 kDa, are more common in animal research, with 4 kDa being the most popular molecular weight. As opposed to saccharides and iohexol, FITC-dextrans are relatively large and thus, it is impossible to investigate permeability to smaller compounds with these markers. They also need to be detected from plasma, making the method error-prone due to blood sample hemolysis which may interfere with the fluorometric detection. Other markers such as polyethylene glycols (PEGs) of various sizes are in use and have an equal performance to lactulose-mannitol test in humans (van Wijck et al., 2012). The advantage of PEGs is that different sizes can be combined to determine the approximate size of molecules being allowed to permeate the intestinal barrier. However, there can be some background intake of PEGs trough diet or medical applications. Radioactive chromium complexed with ethylene diamine tetraacetic acid (^{51}Cr-EDTA, 340 Da) can be used both in animals and humans. Even though ^{51}Cr-EDTA is easily detectable and there is no risk of background exposure of the compound, it exposes subjects to low-level radiation which is why it is not generally recommended, especially for human studies. When compared to sugars and PEGs, the rest of the described methods are less able to account for individually determined factors since ratios of different molecules cannot be calculated (van Wijck et al., 2012). Finally, no method for intestinal permeability measurements has a standardized protocol and both within and between studies, a high inter-individual variability in permeability is observed. This complicates both comparing studies and applying the tests for clinical use.

There are other markers that do not necessarily reflect paracellular permeability but are widely used, nonetheless. While these biomarkers have value in providing information on other barrier properties, they are often mistakenly presented as direct indicators of permeability (Stevens et al., 2018). Of these, the morphology and expression of TJ proteins is occasionally utilized as a proxy marker for permeability even though a correlation between the two parameters is often lacking. For example, the protein expression of both barrier-sealing and pore-forming claudins, specifically claudins 1, 2, and 4, is elevated in active IBDs (Weber et al.,

2008), diseases characterized by increased permeability (Halme et al., 2000; Weber et al., 2008). While formerly considered to be a marker of paracellular permeability, lipopolysaccharide (LPS), a bacterial endotoxin, is, in fact, mainly translocated through enterocytes via lipid rafts (Ghoshal et al., 2009) and thus, is more indicative of the transcellular endotoxin transport. The same applies to LPS-binding protein, and soluble CD14 (sCD14) which are used as markers reflective of LPS levels. In addition, plasma LPS and LPS-binding protein levels do not necessarily correlate (Opal et al., 1999). Intestinal fatty acid binding protein (I-FABP) is used as a marker of enterocyte shedding and therefore, it is indicative of damage to the epithelium (Schellekens et al., 2014) but does not reflect permeability. Additionally, intestinal I-FABP expression increases, *e.g.*, as a response to a diet higher in fat which may make the marker unsuitable for dietary intervention studies (Lau et al., 2016; Mahmood et al., 2023). Finally, plasma zonulin has been popular as a biomarker of intestinal integrity due to the recognized role of this signaling molecule in the regulation of TJ function. However, its use is discouraged due to the lack of an accurate commercial detection method (Ajamian et al., 2019). While zonulin is problematic due to this reason, the other markers described here can provide important information, *e.g.*, on tight junction dynamics or endotoxin translocation. However, a clear distinction between them and true permeability should be made.

In summary, the intestine has the vital function of regulating the passage of compounds to the body. Alongside transport through epithelial cells, molecules can passively cross the epithelial layer through the paracellular pathway, which is regulated by tight junctions. In addition to epithelial damage, the dynamics of these complex protein structures determine the level of intestinal permeability which can be directly assessed with different probe molecules.

2.2 Intestinal inflammation – mechanisms and manifestations

2.2.1 Overview of intestinal inflammation

Acute inflammation is a vital response of the immune system triggered by pathogens, toxins, damaged cells, and tissue injury. The immune cells sense pathogen-associated molecular patterns, conserved structural components of pathogenic organisms (Li & Wu, 2021), or danger-associated molecular patterns, which are endogenously expressed upon stress (Ma et al., 2024). These molecular patterns are recognized by pattern-recognition receptors, such as Toll-like receptors and nucleotide oligomerization domain-like receptors, that are mainly expressed in the cells of the innate immune system (Li & Wu, 2021). This recognition initiates the acute immune response at the site of the infection or damage where tissue resident macrophages and mast cells release proinflammatory mediators, such as chemokines, cytokines, vasoactive amines, and polyunsaturated fatty acid-derived pro-inflammatory eicosanoids, in particular prostanoids, that promote vasodilation and endothelial permeability that allow leukocytes to enter the tissue, as well as blood coagulation (Medzhitov, 2008). In the case of an infection, neutrophils release effectors such as lysozyme, matrix metalloproteinases, and myeloperoxidase in an attempt to destroy the foreign organism. Lymphocytes are the next line of defense. These cells secrete cytokines and produce antibodies and immune complexes. When successful, the acute inflammation eliminates the inflammatory stimulus and is then followed by a macrophage-mediated repair of the host tissue and a resolution characterized by a switch in the lipid mediator profile from the pro-inflammatory prostanoids to anti-inflammatory, specialized pro-resolving mediators such as lipoxins, resolvins, protectins, and maresins, that promote the resolution of inflammation by limiting the neutrophil infiltration of the tissue and stimulating the clearance of neutrophils, cellular debris, and pathogens (Panigrahy et al., 2021).

A failure to initiate the resolution phase, which can be due to an unsuccessful clearance of the inflammatory agent or a dysregulated immune system, leads to a chronically persisting inflammation which is more often linked to the danger-associated molecular pattern-signaling as opposed to pathogenic organisms (Furman et al., 2019; Panigrahy et al., 2021). Nutritional factors such as an excess of glucose can also contribute to chronic inflammation which is logical since immune and metabolic systems are closely integrated (Hotamisligil & Erbay, 2008). This may also explain why nutrition is linked to the development of chronic diseases. In chronic inflammation, the immune cell profile changes from that of acute inflammation with neutrophils being replaced by pro-inflammatory macrophages and lymphocytes (Medzhitov, 2008).

Intestinal inflammation is characterized by a dysregulation in the dynamic crosstalk between intestinal epithelium, microbiota, and local immune cells (Maloy & Powrie, 2011). In a homeostatic state, these interactions allow for the recognition of and protection from pathogenic organisms while simultaneously maintaining tolerance to commensal microbes. Toll-like receptors and nucleotide oligomerization domain-like receptors may be especially important for immune homeostasis since mice deficient in their specific subtypes are more prone to DSS-induced colitis (Burgueño & Abreu, 2020). However, excessive activation of these receptors may also contribute to intestinal inflammation as their expression is elevated in patients with IBD (Lu et al., 2018). The inflammatory response in the intestine can be triggered by a disruption of the epithelial barrier function and defective secretion of antimicrobial peptides by Paneth cells (Maloy & Powrie, 2011). A weakened barrier function and antimicrobial defense allows the translocation of microbial antigens, leading to chemokine and cytokine secretion by intestinal epithelial cells and antigen-presenting cells which further results in the recruitment of immune cells that attempt to destroy the pathogens. The inflammatory response can also increase intestinal permeability and upon inappropriate regulation, may trigger a vicious cycle that is manifests as diseases such as IBD (Michielan & D'Inca, 2015).

2.2.2 Inflammatory bowel diseases – pathogenesis, clinical features and dietary therapies

IBDs are chronic, immune-mediated disorders that are characterized by relapsing and remittent inflammation of the gastrointestinal tract (Khor et al., 2011). There are two main types of IBD – Crohn's disease and ulcerative colitis. Whereas ulcerative colitis is localized in colon and rectum, Crohn's disease can affect any part of the intestine although it most often manifests in ileum. The inflammation and lesions in Crohn's disease can be transmural, while in ulcerative colitis, they are limited to the epithelial lining. The symptoms of both diseases include abdominal pain, diarrhea, rectal bleeding, severe internal cramps in the pelvic region, and weight loss. The most common extraintestinal manifestation of IBDs is anemia, as a result of intestinal bleeding as well as the dysfunctional absorption of nutrients (Gordon et al., 2024). When active, IBDs result in an elevation in fecal calprotectin which is used as a biomarker of the disease. The diagnosis of IBDs is confirmed with endoscopy and tissue biopsies.

The etiology of IBDs is likely an interplay between a genetic predisposition, the host's immune system, the intestinal microbiota, and environmental factors (Maloy & Powrie, 2011). Polymorphisms in genes involved in barrier function, epithelial restitution, microbial defense, immune system regulation, reactive oxygen species generation, autophagy, endoplasmic reticulum stress, and metabolic pathways involved in cellular homeostasis are associated with an increased risk of IBD (Khor et al., 2011) Many of these are also genetic risk factors for autoimmune diseases (Harroud & Hafler, 2023). However, genetic factors account for only 20 % of the risk of developing IBD. This is corroborated by the increasing prevalence of IBDs as such rapid changes cannot be explained by genes and highlights the importance of non-genetic factors in the development of the disease.

A dysfunction of the intestinal barrier may drive the development of IBDs as increased permeability allows the inappropriate passage of compounds, such as bacterial and dietary antigens, through the epithelial lining, causing an

inflammatory response (Michielan & D'Inca, 2015). Increased permeability is a hallmark of IBDs, and permeability measured as the lactulose-mannitol ratio has been reported to precede and predict Crohn's disease development (Turpin et al., 2020). These findings strengthen the hypothesis that barrier dysfunction has a causative role in IBDs. In addition to TJ permeability, the intestinal mucus layer has an important, protective role as a loss of mucin-producing goblet cells is observed in IBD and the levels of major structural components of mucus, including mucin-2, are reduced (van der Post et al., 2019). As mucus defects are detected also in non-inflamed tissue of these patients, it is possible that a weakened mucus barrier may contribute to the activation of the disease. In line with this, mice deficient in mucin-2 ($Muc2^{-/-}$) spontaneously develop colitis (Van der Sluis et al., 2006). IBDs are also linked to dysbiosis of the gut microbiota with patients exhibiting both elevated levels of pathogenic organisms like *Escherichia coli* (Zheng et al., 2024), and global changes in the composition of the microbiota such as reduced diversity, decreased *Firmicutes*, and increased *Proteobacteria* (Lee & Chang, 2021; Ni et al., 2017). In addition, IBDs can respond to antibiotic treatments (Wang et al., 2012) which further supports the hypothesis of the microbiota being a contributing factor in the development of these diseases. However, while intestinal permeability, the mucus layer, and microbiota are likely all involved in the development of IBDs, causative relationships have not yet been established, and it is also possible that the increased permeability, mucus abnormalities, and microbial dysbiosis are consequences, not causes of IBDs (Michielan & D'Inca, 2015; Ni et al., 2017). It is likely that while all three phenomena may be predisposing factors, over time the inflammation itself further contributes to barrier defects and dysbiosis.

Conventionally, IBDs have been treated with pharmacological agents including aminosalicylates, corticosteroids, immunomodulators such as thiopurines and methotrexate, and antibiotics (Cai et al., 2021). Newer options include biologic drugs, such as TNF-α inhibitors. However, many of the conventional therapies, such as corticosteroids, are associated with serious adverse effects. Also, the effectiveness of many of these treatments decreases over time and a substantial number of patients fails to respond to them (Alsoud et al., 2021). While the need for surgical

treatments has decreased with the development of biologic drugs, removal of the inflamed intestine is still necessary in the most severe cases (Cai et al., 2021).

Dietary interventions for inflammatory bowel diseases

Dietary factors may influence the development and progression of IBDs and there is growing interest in nutritional therapies for these conditions (Fitzpatrick et al., 2022). For patients without contraindications, a Mediterranean diet is generally recommended (Hashash et al., 2024) as it is sustainable and some studies suggest that it may decrease the severity of IBD (Chicco et al., 2021; Lewis et al., 2021). The most extensively researched dietary therapy for IBDs, specifically Crohn's disease, is exclusive enteral nutrition which can induce a remission in pediatric patients and has some efficacy in adults (Narula et al., 2018). However, the diet is challenging to maintain long-term, and cessation often results in the recurrence of the symptoms. A Crohn's disease exclusion diet is a more sustainable dietary strategy that has shown promise in both pediatric patients (Levine et al., 2019) and adults (Yanai et al., 2022). The diet is a standardized whole-foods dietary approach with three different stages (Sigall Boneh et al., 2024), focusing on removing ingredients, such as artificial sweeteners and emulsifiers, that are scientifically established to negatively impact barrier integrity and microbiota, while adding dietary components that are considered to support intestinal health, like starchy vegetables. Other diets such as the specific carbohydrate diet and the low-FODMAP diet have shown some efficacy in alleviating the symptoms (Fitzpatrick et al., 2022). While the overall evidence on the efficacy of dietary strategies for IBDs is limited, the existing data suggest there is a potential for dietary therapies to alleviate the symptoms of these diseases.

Many of the dietary therapies focus on eliminating or adding specific dietary components as they may impact the trajectory of IBD. However, the human data on individual foods or nutrients are often observational and thus, causative relationships cannot be established. A meta-analysis of clinical trials found no effect on relapse from decreased consumption of red meat or refined carbohydrates nor did eliminating gluten or milk proteins from the diet confer benefit (Limketkai et

al., 2023). Supplementation with polyunsaturated fatty acids also had little or no effect on the prevention or treatment of IBDs (Ajabnoor et al., 2021) . These data suggest that the diet as a whole may be more important than individual dietary components.

2.2.3 Experimental models of intestinal inflammation

Dextran sodium sulfate-induced model of colitis

DSS-induced colitis is one of the most extensively used models of experimental intestinal inflammation. The model, established over thirty years ago (Okayasu et al., 1990), leads to a progressive weight loss, bloody diarrhea, ulcer formation, epithelial cell loss, and infiltration of the intestinal layer with neutrophils. It is widely used due to its simplicity and reproducibility which enable the adjustment of doses and treatment cycles to allow the modeling of acute, relapsing, and chronic forms of colitis. In the model, colonic inflammation is induced by administering DSS to the animals in their drinking water as 1-5 % (w/v) solution for a few days or longer, with the administration regime determining the severity of the colitis (Wirtz et al., 2017).

DSS is a negatively charged sulfated polysaccharide comprised of a (1 → 6)-linked dextran chain which has two to three sulfate moieties at the second, third, and/or fourth carbon of the chain. The molecular weight of DSS is a critical determinant of the level of the damage and the location of the effects in the intestine, with molecules of ~ 40 kDa in size resulting in the most severe colitis with the distal colon being the most affected (Kitajima et al., 2000). Strains of mice differ in their susceptibility to colitis, *e.g.*, C57BL/6 mice tend to develop a more severe colitis than other strains with the effects of DSS being permanent, whereas Balb/c mice are more resistant and recover after the administration has ended (Melgar et al., 2008). Male mice are more susceptible to the effects of DSS than females (Mähler et al., 1998), which may be due to the protective role of estradiol (Bábíčková et al.,

2015). Age also influences the severity of colitis with older mice being more affected (Liu et al., 2020).

While the mechanisms by which DSS exerts its effects are not fully understood, DSS is recognized to be toxic to the epithelial cells of the intestine. It forms complexes with colonic medium-chain fatty acids which then fuse with the colonocyte membrane and further move to the cytoplasm where they can interfere with cell metabolism (Laroui et al., 2012). DSS compromises the function of the intestinal barrier by increasing epithelial permeability and promoting colonocyte apoptosis. Thus, mucosal, and submucosal immune cells are exposed to luminal antigens which trigger an inflammatory response, characterized by the secretion of inflammatory cytokines such as TNF-α, interleukin 1β (IL-1β), IL-6, IL-10, and interferon-γ (Yan et al., 2009).

The effects of DSS on the intestine also depend on the microbiota. Germ-free mice exhibit marked differences compared to conventionally raised mice in their response to DSS. Despite a more drastic systemic deterioration, blood loss, and even increased death rate in germ-free mice, the DSS-induced colonic inflammation and the overall immune response is abated in these animals (Hernandez-Chirlaque et al., 2016; Kitajima et al., 2001). The absence of the microbiota appears to elicit a dual response: lowered inflammation combined with exacerbated epithelial damage. This is likely a result of a defective development of the immune system in a germ-free setting since antibiotic-treated mice are protected from blood loss, epithelial damage, and mortality when compared to the germ-free mice (Hernandez-Chirlaque et al., 2016). While the mice given antibiotics develop normal DSS-colitis, the treatment attenuates the degree of the inflammation. The composition of the microbiota is a major factor influencing the response to DSS and drives the variation among genetically identical animals. When a pre-DSS microbiota from animals that respond severely is transplanted to germ-free mice, the response of the latter to DSS is indistinguishable from the donors, emphasizing the significance of the composition of the microbiota. Specifically, the presence of two microbes, *Duncaniella muricolitica* and *Alistipes okayasuensis*, appears to worsen colitis (Forster et al., 2022).

Other models of experimental colitis

In addition to DSS, several other models are used to study intestinal inflammation in experimental settings. The most common models are briefly discussed here and listed in Table 2. Other chemically induced models of intestinal inflammation include the oxalozone model and trinitrobenzene sulfonic acid (TNBS) model. Both substances are haptenating, *i.e.*, they attach to native proteins making them immunogenic. They are administered intrarectally and cause a model-specific T-cell mediated immune response by attaching to colonic or microbiota-derived proteins (Wirtz et al., 2017). Oxalozone administration results in colonic ulceration and inflammatory infiltration of *lamina propria*. TNBS causes an acute inflammation which is usually more severe than that induced with DSS. The symptoms are weight loss, diarrhea, and immune cell infiltration that is often transmural and leads to chronic fibrosis. However, similar to the DSS model, the severity of the inflammation can be controlled via the administration regime (Antoniou et al., 2016). TNBS was found to cause colitis over thirty years ago (Morris et al., 1989) and many important discoveries were made already then. For example, similar to DSS, TNBS does not induce typical colitis in the absence of the commensal microflora (García-Lafuente et al., 1999). In line with this, treatment with broad-spectrum antibiotics alleviates inflammation in this model (Videla et al., 1994).

Table 2. Examples of commonly used experimental colitis models.

	Model	Main mechanism
Chemical	DSS	Epithelial barrier defects
	TNBS	Aberrant adaptive T cell responses
	Oxalozone	Aberrant adaptive T cell responses
Genetic	IL-10$^{-/-}$	Regulatory and effector T cell imbalance
	Muc2$^{-/-}$	Epithelial barrier defects
Other	Adoptive transfer	Regulatory and effector T cell imbalance

Several genetic models for colitis are in use. The most popular one of these is the IL-10 knockout model (IL-10$^{-/-}$) which was established nearly thirty years ago (Berg et al., 1996). In this model, colitis develops spontaneously due to inadequate immunosuppression by IL-10-producing T regulatory cells. The model is

characterized by elevated colonic cytokine production and immune cell infiltration. As the disease progresses, ulcers and transmural lesions develop. Despite its genetic origin, colitis only develops in IL-10$^{-/-}$ mice in the presence of intestinal microbes, highlighting the significance of the microbiota in the development of intestinal inflammation (Sellon et al., 1998). Muc2$^{-/-}$ mice is another common genetic model. In this model, intestinal mucin production is compromised which leads to a progressively worsening colitis characterized by weight loss, bloody diarrhea, crypt morphology alterations, *lamina propria* abnormalities, increased immune cell infiltration, and elevated expression of inflammatory cytokines such as TNF-α (Van der Sluis et al., 2006).

In the adoptive transfer model of colitis, the function of T-regulatory cells is suppressed and colitis develops due to unrestrained aberrant T cell activation in the intestine (Yang & Cong, 2023). This model was also developed already thirty years ago when it was discovered that transferring naïve T helper cells to mice that are deficient in T and B lymphocytes causes intestinal inflammation (Powrie et al., 1993). While some variations of this model have been developed, the original method is still the most commonly used (Yang & Cong, 2023). Typical features of adoptive transfer colitis include transmural immune cell infiltration, epithelial hyperplasia, loss of mucin-secreting cells, ulceration, and increased levels of cytokines such as interferon-γ. Again, the development of colitis in this model is microbiota-dependent, since germ-free mice are resistant to adoptive transfer-induced intestinal inflammation (Niess et al., 2008).

The etiology of IBDs is complex and multifactorial and thus, no animal model can fully replicate the pathology of the human disease. However, the DSS model shares key characteristics of human IBDs, especially ulcerative colitis, including immune dysfunction, intestinal permeability, and histological lesions (Yang & Merlin, 2024). The model is simple and reproducible and exhibits similar responses to treatments as human IBDs. Therefore, it was chosen as the model of colitis in this thesis.

2.2.4 The intestinal renin-angiotensin-aldosterone system and its role in inflammation

RAAS is a multi-organ hormonal system, the main functions of which are the regulation of blood pressure and fluid balance (Fändriks, 2011). The system is also involved in inflammation (Cantero-Navarro et al., 2021) and fibrosis (Bernasconi & Nyström, 2018). RAAS is divided into two arms, the classical and the alternative pathways and the functions of these arms are the opposite (Fändriks, 2011). Angiotensinogen, produced mainly by the liver, is the precursor of the peptide signaling molecules of RAAS. When blood flow to kidneys is reduced, prorenin is activated to renin which converts angiotensinogen into angiotensin I. Angiotensin I is further cleaved into angiotensin II by angiotensin-converting enzyme 1 (ACE). Angiotensin II acts mainly via angiotensin II receptor type 1 (AT1R) and to some extent via angiotensin II receptor type 2 (AT2R). In the classic pathway, systemic AT1R activity leads to aldosterone secretion, fluid and sodium retention, and vasoconstriction, all of which increase blood pressure. Aldosterone, a key RAAS hormone produced mainly in the cortex of the adrenal glands, upregulates sodium and fluid reabsorption and potassium excretion in the kidney. AT1R also promotes nuclear factor-κB signaling which activates a proinflammatory cascade resulting in cytokine production, leukocyte recruitment, apoptosis, cell proliferation, and fibrosis (Cantero-Navarro et al., 2021). AT2R, on the other hand, decreases aldosterone secretion, promotes fluid and renal sodium excretion, increases vasodilation, and reduces the proliferation of cells. The alternative pathway is activated when angiotensin II is converted to angiotensin 1-7 by angiotensin-converting enzyme 2 (ACE2). Ang 1-7 binds to the Mas receptor (MasR) which has effects resembling those of AT2R. A simplified illustration of RAAS components and function is presented in Figure 4.

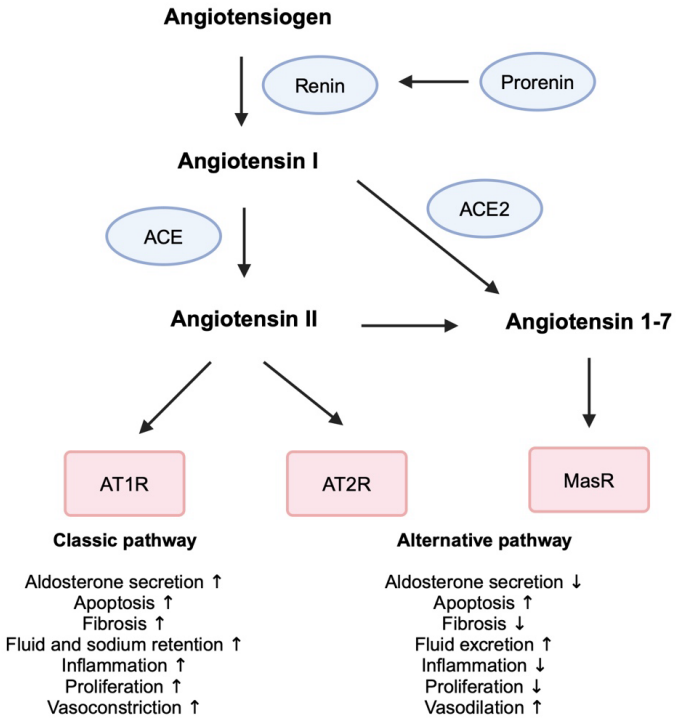


Figure 4. The main components of RAAS and functions of the classical and alternative pathways. Angiotensinogen is the precursor of the main effector peptides of the system. The effects are mainly exerted via three receptors, AT1R, AT2R, and MasR, upon the binding of angiotensin II and angiotensin 1-7. The downstream effects AT1R signaling are mostly opposite to those of AT2R and MasR. Modified from Salmenkari et al. (2021). Created with BioRender.com.

In addition to the systemic RAAS, the components of this system are also expressed in other tissues, such as the intestine, where they are involved in the absorption of fluid, electrolytes, and glucose, motility, and, importantly, inflammation (Fändriks, 2011). Both ACE and ACE2 are expressed in both the small intestine and the colon. The intestinal epithelium expresses AT1R, AT2R, and MasR, of which the first is highly expressed and the two others to a lesser extent. In addition, aldosterone synthase CYP11B2 is expressed in the intestine and aldosterone production has been detected locally (Launonen et al., 2021; Pang et al., 2022; Varmavuori et al., 2020). The effects of angiotensin II on intestinal fluid and electrolyte absorption

appear to be dependent on its concentration. Low levels of angiotensin II increase the absorption via AT1R whereas high levels have inhibitory properties.

In the intestine, the local RAAS is involved in the inflammatory processes (Fändriks, 2011). Intestinal angiotensin II levels are increased in inflammation, with its proinflammatory effects being mediated through AT1R signaling that leads to nuclear factor-κB activation. Indeed, angiotensin II administration leads to an exacerbation of TNBS-induced colitis (Shi et al., 2016). In line with this, in various models of colitis, the inhibition of ACE or blockade of AT1R with pharmacological agents leads to improved outcomes (Salmenkari et al., 2021). AT1R knockout mice also develop a less severe DSS-induced colitis (Katada et al., 2008; Mizushima et al., 2010). On the other hand, a MasR knockout worsens colitis whereas administration of angiotensin 1-7 alleviates the symptoms (Shi et al., 2016), indicating that the activity of the alternative RAAS pathway may be protective against colonic inflammation. Interestingly, intestinal CYP11B2 and aldosterone levels remained unchanged in DSS-colitis and the pharmacological inhibition of adrenal CYP11B2 and the subsequent decrease in aldosterone production did not improve colitis outcomes (Launonen et al., 2023). Thus, the effect of aldosterone on intestinal inflammation is still unclear. Overall, the activation of the classical pathway generally promotes intestinal inflammation while the alternative pathway alleviates it.

The intestine is an important site of immune system. However, a disruption of the dynamic interplay between intestinal epithelium, microbiota, and immune cells can lead to chronic inflammation and manifest as diseases such as IBDs.

2.3 Dietary fat and ketogenic diets – metabolism and health effects

2.3.1 Overview of dietary fats and fatty acid metabolism

Classification and sources of dietary fatty acids

Fatty acids are categorized based on the length of the hydrocarbon chain and the number and position of the double bonds within the chain (Saini & Keum, 2018). Saturated fatty acids (SFAs) have no double bonds, monounsaturated fatty acids contain one, and polyunsaturated fatty acids have two or more. Unsaturated fatty acids can take either the cis- or trans-orientation although most naturally occurring fatty acids exist in the former. Most dietary fatty acids are bound to a glycerol backbone in the form of triglycerides. Examples of different fatty acid types are shown in Figure 5.

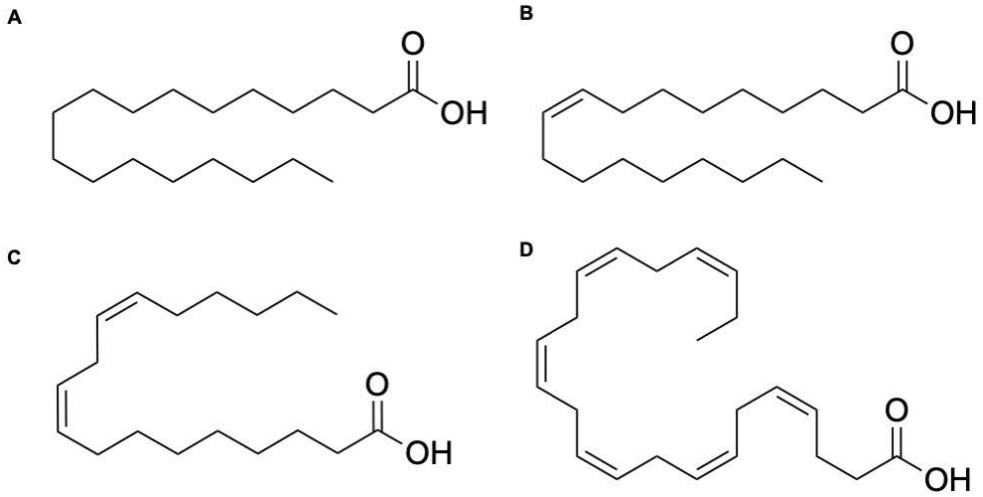


Figure 5. Examples of different fatty acid types. A. Stearic acid, a saturated fatty acid; B. oleic acid, a monounsaturated fatty acid; C. linoleic acid, an omega-6 polyunsaturated fatty acid; and D. docosahexaenoic acid, an omega-3 polyunsaturated fatty acid. Created with ChemDraw.

The most important dietary sources of SFAs are full-fat dairy products, especially butter, red meat, and poultry (O'Sullivan et al., 2013). They are also present in high amounts in tropical oils such as coconut and palm oil. The SFAs in the diet are mostly long-chain fatty acids such as stearic acid and palmitic acid. Medium-chain fatty acids, such as caprylic acid are found in low amounts in foods such as full-fat dairy products and coconut oil. Medium-chain triglycerides (MCTs) do not require bile for digestion and are transported directly to the liver through the portal vein after absorption and are readily converted into ketone bodies (Augustin et al., 2018). Short-chain fatty acids, such as butyrate and propionate, are a special class of SFAs. Some foods such as full-fat dairy products and fermented foods can contain small amounts of these fatty acids, but their main source is the intestinal microbiota which synthesizes them from indigestible dietary components such as fiber. They are mainly used for energy by colonocytes (Boets et al., 2017).

Oleic acid is the most abundant dietary unsaturated fatty acid (Schwingshackl & Hoffmann, 2014). The major sources of this monounsaturated fatty acid include olive oil, rapeseed oil, eggs, chicken, pork, and beef. Oleic acid can be synthesized in the body from long-chain SFAs and thus, it is not an essential fatty acid. Based on the location of the first double bond, polyunsaturated fatty acids are divided into two classes, omega-3s and omega-6s. Humans do not produce the desaturase enzyme required for the endogenous synthesis of these fatty acids (Malcicka et al., 2018). Therefore, alpha- linolenic acid and linoleic acid (LA), which are precursors for longer-chain polyunsaturated fatty acids, are considered to be essential. However, the conversion of these fatty acids to their more bioactive metabolites eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid is poor in humans, with only an estimated average 0.2 % of alpha-linolenic acid being converted to EPA and 0.05 % to DHA (Burdge & Calder, 2005). Food sources of alpha-linolenic acid include specific vegetable oils such as flax, hemp, rapeseed, and soy oil (Saini & Keum, 2018). Some nuts and seeds are also rich in this fatty acid. Plant foods do not contain the more bioactive polyunsaturated fatty acids EPA and DHA. These are found mainly in seafood and, to a lesser extent, red and other meat products. Similarly to alpha-linolenic, LA is present in at the highest levels in vegetable oils, especially in safflower, sunflower, hemp, corn, and soy oil. The most

important sources of arachidonic acid are pork, chicken, eggs, fish, and cheese (Kawashima, 2019).

Energy production from fatty acids and their other functions

The body obtains energy mainly from glucose and fatty acids, and to some extent, from amino acids. These substrates are either metabolized to produce ATP or, when consumed in excess, stored (Liu et al., 2025). Glucose is catabolized via glycolysis and fatty acids via β-oxidation. Of the three macronutrients, fats yield the most energy per gram (~ 9 kcal/gram), and thus, triglycerides are the most common energy storage form in the body. Some glucose is stored in liver and muscle tissue as glycogen, and liver stores are broken down as a response to a reduction in blood glucose levels. In addition, glucose can be produced from amino acids and the glycerol backbone of fatty acids via gluconeogenesis.

In energy production, fatty acid chains are removed from the glycerol backbone by lipases. A simplified illustration of fatty acid catabolism is presented in Figure 6. Fatty acids enter cells via specific transport proteins, such as fatty acid transport proteins, fatty acid translocase, caveolins, and plasma membrane fatty acid binding proteins (Longo et al., 2016) . Through intermediate steps catalyzed by acyl-CoA synthases, fatty acids are metabolized into fatty acyl-CoA. As the mitochondrial membrane is impermeable to acyl-CoA, it is conjugated into carnitine and transported to the mitochondrial matrix in a process called the carnitine shuttle. In the matrix, the fatty acyl-CoA is metabolized via β-oxidation where the fatty acid chain is broken down into two-carbon units through four steps: dehydrogenation, hydration, another dehydrogenation, and thiolytic cleavage. The end product of β-oxidation is acetyl CoA (Longo et al., 2016). Upon entrance into the Krebs cycle, it combines with oxaloacetate to form citrate before eventually being transformed into ATP during oxidative phosphorylation.

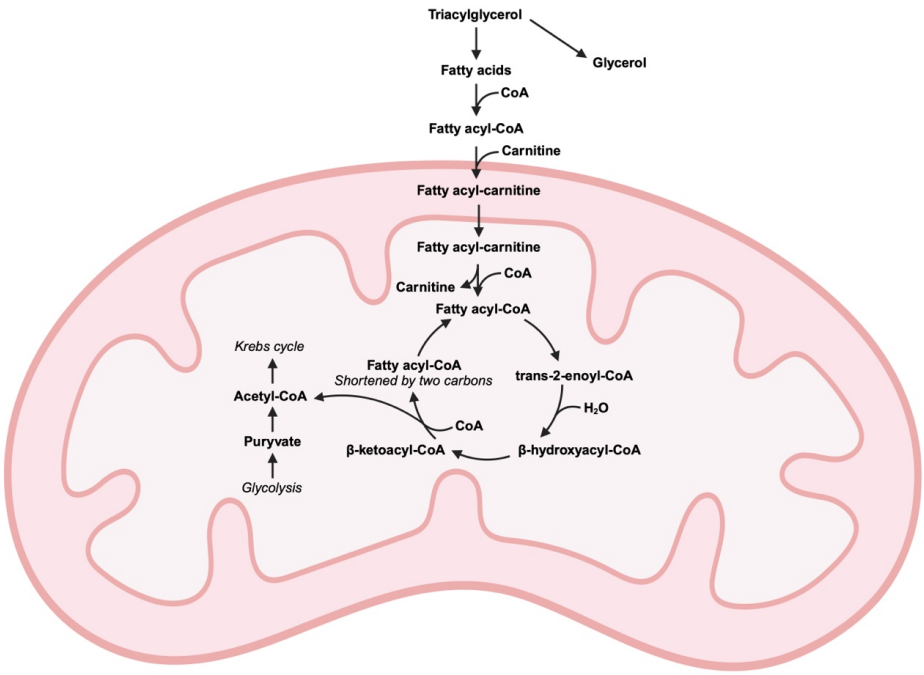


Figure 6. A simplified illustration of fatty acid catabolism. Triacylglycerols are broken down into individual fatty acids and further into fatty acyl-CoA molecules which the carnitine shuttle transports to the mitochondrial matrix. In β-oxidation, fatty acids are metabolized into two-carbon units which form acetyl-CoA. Created with BioRender.com.

Fatty acids are also integral components of the cell membrane phospholipids. The fatty acids in membrane phospholipids can be cleaved by phospholipase C and serve as intracellular signaling molecules (Sunshine & Iruela-Arispe, 2017). Polyunsaturated fatty acids can also be metabolized into oxylipins, namely eicosanoids and docosanoids, which are oxidized fatty acids with diverse signaling functions. Eicosanoids and docosanoids are produced by cyclooxygenases, lipoxygenases, and cytochrome P450 enzymes, but can also form spontaneously via free radical mechanisms (Dennis & Norris, 2015). Of these, prostanoids, such as prostaglandins and thromboxanes, as well as leukotrienes, are arachidonic acid- or EPA-derived molecules, most of which serve crucial roles in the pro-inflammatory response, but some also have anti-inflammatory properties. Another class of oxylipins, specialized pro-resolving mediators, namely lipoxins, resolvins,

protectins, and maresins are essential for the resolution of inflammation (Basil & Levy, 2016). Thus, fatty acids are not only important as an energy source, but they are also essential components of cell structures and have roles in physiological signaling.

Metabolic pathway of ketogenesis

When availability of glucose is limited, the acetyl CoA from fatty acid β-oxidation accumulates in the liver and exceeds the amount of oxaloacetate required for its metabolism in the Krebs cycle. Unlike most cells that can only metabolize acetyl CoA through this cycle, in liver cells, the mitochondria are able to use it for ketogenesis when oxaloacetate is required for gluconeogenesis (Liu et al., 2025; Zhu et al., 2022). Figure 7 illustrates the pathway of ketone body production where two acetyl CoA molecules are combined and, through intermediate steps, metabolized into acetoacetate. Acetoacetate is then converted into BHB, the main ketone body in the circulation, or spontaneously carboxylated into acetone. These ketone bodies, acetoacetate, BHB, and acetone, are amphipathic three- to four-carbon molecules (Figure 8) that can cross the blood-brain barrier. BHB is a reduced form of acetoacetate, and most tissues can readily convert both of these compounds back to acetyl CoA for energy production, whereas acetone is only metabolized in the liver (Zhu et al., 2022). Ketogenesis becomes crucial for the brain in low-glucose states, due to the organ's inability to use most fatty acids for ATP production. Besides the brain, other tissues, such as the intestine (Windmueller & Spaeth, 1978), readily use ketone bodies for energy. Interestingly, intestinal stem cells can also produce ketone bodies (Cheng et al., 2019).

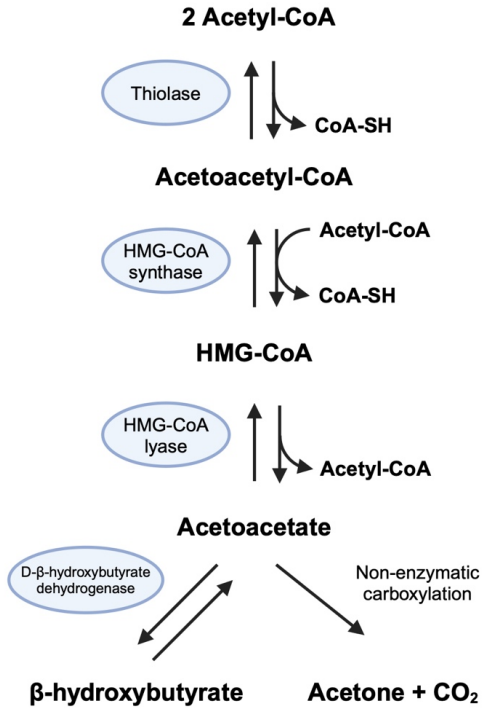


Figure 7. The biochemical pathway of ketogenesis. Acetyl-CoA molecules, mainly derived from β-oxidation of fatty acids, are metabolized into acetoacetate via intermediate steps. Acetoacetate is further converted into β-hydroxybutyrate, the major ketone body present in circulation, and acetone. HMG-CoA = hydroxymethylglutaryl-CoA, CoA-SH = coenzyme A. Created with BioRender.com.

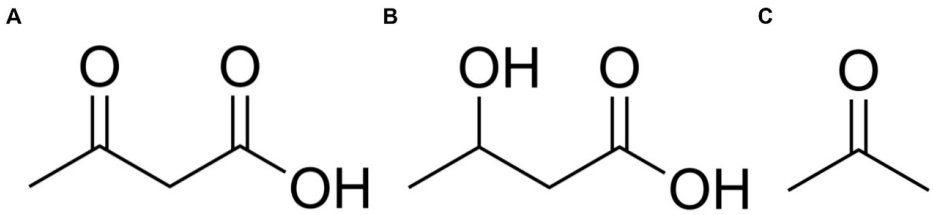


Figure 8. Ketone bodies produced in the body. Acetate forms from acetoacetate through spontaneous carboxylation, whereas β-hydroxybutyrate is formed by enzymatic conversion of the ketone group to a hydroxyl group. A. Acetoacetate, B. β-hydroxybutyrate, and C. acetone. Created with ChemDraw.

2.3.2 High-fat diets – different types and their implications for health

A high-fat diet (HFD) is a collective name for diets with over 30 percentage of energy (E%) is derived from fat. In animal models, HFDs usually contain 40-60 E% from fat (Buettner et al., 2007). In addition to fat, these diets most often contain up to 50 E% from carbohydrates, mainly sugar and/or starch, and are moderate to low in protein, thus resembling the so-called Western diet. This combination of fat and carbohydrate is hyperpalatable (Fazzino, 2022) and results in overeating which leads to diet-induced obesity and metabolic disturbances such as insulin resistance, high blood glucose and triglycerides, and fat accumulation in the liver (Asgharpour et al., 2016; Buettner et al., 2007). Hence, the term obesogenic diet is also often used. Most evidence on the health effects of high fat intake originates from studies conducted with these regular HFDs.

Low-carbohydrate diets are distinct from the regular HFDs. While they are generally high in fats, they should be differentiated from other HFDs due to their macronutrient ratio which makes the physiological effects different. Low-carbohydrate diets are defined as having 10-25 E% from carbohydrates, whereas in a very low-carbohydrate diet, less than 10 E% comes from carbohydrates (Kirkpatrick et al., 2019). Especially the latter, which are most often ketogenic, are distinguishably different in their metabolic and satiety effects when compared to the carbohydrate-rich HFDs. In general, they promote metabolic health by reducing weight, improving glycemic control, and lowering plasma triglyceride levels (Choi et al., 2020). They also improve satiety (Gibson et al., 2015). Thus, the physiological effects of low-carbohydrate diets are mostly different from those of the regular HFDs with higher carbohydrate content.

2.3.3 Ketogenic diets – physiological effects and therapeutic possibilities

Ketogenic diets (KDs) are a class of high-fat, very-low-carbohydrate diets in which the production of ketone bodies by the liver and their availability in plasma as an energy substrate are elevated due to the restricted supply of dietary glucose. This metabolic state is called ketosis which is generally defined as a condition where plasma BHB levels are increased to 0.5 mM or above (Gross et al., 2019). In addition to a carbohydrate-restricted diet, physiological ketosis can be achieved through fasting or ketone body supplementation. Ketosis induced via these three means is referred to as nutritional ketosis. Unlike most other animals, the human body begins to rely readily on ketone body metabolism upon low-carbohydrate diets even without protein restriction, with plasma levels of BHB ranging from 0.5 to 3.0 mM (Mohammadifard et al., 2022). For human consumption, 10 E% or less from carbohydrate and 70 E% or more from fat is the generally recommended KD formula. In comparison, a diet with essentially no carbohydrate and limited protein is used in rodents. Interestingly, the amount of protein in a KD appears to be a factor determining many of its effects. In rodents, a KD with severe protein restriction leads to decreased body weight and a substantial elevation in plasma BHB, likely due to suppressed gluconeogenesis, when compared to a KD with adequate protein intake (Stemmer et al., 2015). However, muscle wasting is also observed. Despite the recognized anticatabolic effect of ketone bodies (Koutnik et al., 2019), skeletal muscle atrophy is expected when the dietary supply of protein is inadequate due to its significance in muscle preservation (Paddon-Jones & Rasmussen, 2009). When ~ 10 % or more of energy is derived from protein on a KD, the weight development in rodents is comparable to controls (Stemmer et al., 2015) or can even exceed that (Newman et al., 2017). Therefore, the amount of protein in the KD is an important factor influencing the effects of the diet.

Physiological ketosis induced via modifications in nutrition is different from ketoacidosis which is a pathological state resulting from uncontrolled ketogenesis. It leads to medical acidosis, in which plasma BHB levels can rise above 10 mM

(Laffel, 1999). In most cases, ketoacidosis is caused by insulin deficiency in type I diabetes or late-stage type II diabetes. Even though blood glucose levels are high, with impaired insulin signaling, glucose is not taken up to the cells and fatty acids are uncontrollably released from adipose tissue and used for β-oxidation and the formation of acetyl CoA. Oxaloacetate is diverted to gluconeogenesis, resulting in acetyl CoA being used for ketogenesis. Until insulin is administered in sufficient amounts, the body continues ketone production, leading to pathophysiological plasma levels and acidosis. With normal insulin signaling, the production of ketone bodies is tightly regulated, and the rate is negligible when there is a supply of glucose from the diet. Prolonged and heavy alcohol intake in the setting of poor nutrition (McGuire et al., 2006), starvation during pregnancy (Frise et al., 2013), and certain medications and toxins (Cartwright et al., 2012) can lead to ketoacidosis. Thus, the origin and manifestations of ketoacidosis are very different from those of physiological ketosis and the two conditions should be clearly distinguished from each other.

Therapeutic uses of ketogenic diets

Before the invention of pharmaceutical therapies for epilepsy, a KD was used as a treatment for this condition and are still utilized for drug-resistant cases (Ruan et al., 2022). In addition to epilepsy, patients with inborn errors of glucose metabolism, such as glucose transporter type 1 or pyruvate dehydrogenase complex deficiency, need to follow a KD to prevent the manifestations of these genetic mutations (Kim et al., 2023; Sofou et al., 2017). During the past two decades, KDs have also been used therapeutically for some other conditions. They are effective for type II diabetes (Choi et al., 2020) and metabolic dysfunction associated steatotic liver disease (Watanabe et al., 2020). KDs and ketone bodies have neuroprotective effects (Gough et al., 2021) and outside epilepsy, there are preliminary human reports on the efficacy of these diets for neurodegenerative diseases such as Alzheimer's disease (Phillips et al., 2021), Parkinson's disease (Phillips et al., 2018), and multiple sclerosis (Brenton et al., 2022).

Dietary fat quality and quantity are generally considered to influence the risk for cardiovascular disease with diets high in fat thought to be detrimental. As KDs are mainly comprised of fat, concerns about their effects on cardiovascular health have been raised. Despite this, in high-risk subjects, such as people with obesity (Bueno et al., 2013) or type II diabetics (Choi et al., 2020), KDs result in improvements in cardiovascular disease risk markers including weight, glycemic control, triglycerides, HDL-C, and blood pressure. The effects on LDL-C are less consistent and likely dependent on the metabolic status and body composition of the individual upon the start of the diet (Patikorn et al., 2023). However, most trials that have characterized the influence of KDs on these parameters have been relatively short in duration and there are few long-term studies examining the effect of KDs on cardiovascular disease risk. In a five-year prospective study on glucose transporter type 1 deficient children on a KD, the subjects did not exhibit changes in blood lipids associated with cardiovascular disease risk (De Amicis et al., 2023). The same was observed after a ten-year follow-up in a case series of similar subjects who experienced no changes in carotid intima-media thickness, an indicator of subclinical atherosclerosis (Heussinger et al., 2018). While there are no prospective studies assessing cardiovascular mortality on a KD thus far, KDs do not result in a similar risk profile as carbohydrate-rich HFDs.

Mechanisms of action of ketogenic diets

KDs induce a pronounced change in energy metabolism which influences most cells of the body. Many of the beneficial effects of KDs are thought to be mediated by ketone bodies, especially BHB. Even if glucose utilization is impaired, such as in insulin resistance, cells are still able to use ketone bodies for energy. This may partly explain the benefits in neurological diseases; damaged neurons cannot use glucose efficiently for fuel but are still able to obtain energy from ketone bodies (Gough et al., 2021). While these compounds are mainly considered as fuel sources and many of their physiological effects can result from differences in energy metabolism, they also act as potent signaling molecules. BHB functions as a histone deacetylase inhibitor and therefore, regulates gene expression (Shimazu et al., 2013). For

example, its protective effect against experimental colorectal cancer is dependent on the upregulation of the expression of homeodomain-only protein Hopx, a tumor-suppressant (Dmitrieva-Posocco et al., 2022). In cell lines and mice, BHB decreases inflammation through inhibiting the NLRP3 inflammasome (Youm et al., 2015) and protects from oxidative stress (Shimazu et al., 2013), both of which are important components in the development of chronic diseases such as type II diabetes, cardiovascular disease, and neurodegenerative diseases. The mechanisms behind the BHB-mediated blockade of NLPR3 inflammasome are incompletely understood but have been linked to a prevention of cellular potassium efflux, required for NLPR3 activation (Swanson et al., 2019), and interference with the assembly of the inflammasome (Youm et al., 2015). The effects of BHB on oxidative stress take place via histone deacetylation that leads to an elevated expression of genes related to oxidative stress resistance (Shimazu et al., 2013). KDs also cause a pronounced shift in the gut microbiota (Santangelo et al., 2023) which may be mediating some of its effects as will be discussed below. Overall, KDs produce a distinct physiological state which influences metabolism and signaling in the body and this way, the diet may interfere with pathological processes.

In conclusion, fatty acids are important as a dietary energy source and come in various forms. When glucose availability is limited, fatty acids can take alternative metabolic pathways, resulting in the production of ketone bodies. This is achieved with KDs, high-fat, very low-carbohydrate diets, which differ from regular HFDs in their physiological effects.

2.4 Dietary fat and intestinal health – effects on permeability, inflammation, and microbiota

2.4.1 High-fat diets and dietary fatty acids in intestinal health

Effects of high-fat diets and dietary fat source on the intestinal barrier

Regular HFDs are generally considered to have a negative effect on intestinal barrier function (Rohr et al., 2020). While feeding an obesogenic HFD has been reported to increase intestinal permeability to various probes (Cani et al., 2008; Stenman et al., 2012), its effect may be dependent on the baseline microbiota. When mice in conventional housing conditions were compared to those in more hygienic, specific-pathogen-free conditions, increased permeability and endotoxemia in response to a HFD were observed only in the conventionally housed mice and the diet did not have an impact on permeability in mice living in the specific-pathogen-free facility (Muller et al., 2016). These microbiota-dependent effects may be mediated through bile acid metabolism (Muller et al., 2016; Stenman et al., 2013a). Thus, the effects of HFDs on intestinal barrier are not only dependent on the diet's macronutrient composition but can also be modulated by microbiota-modifying environmental factors outside the diet.

The effects of a HFD on the intestinal barrier may depend on the fat source of the diet. Animal studies comparing the effects of dietary fat sources on paracellular permeability are listed in Table 3. In mice, a liquid HFD with 40 E% from fat derived from high-LA corn oil decreased the mRNA expression of genes coding for TJ proteins claudin 1, occludin, and *zonula occludens* 1 as well as TJ adaptor proteins when compared to a combination of beef tallow and MCT oil (Kirpich et al., 2012). When these animals were also given ethanol, a substance recognized to damage the intestinal barrie, corn oil exacerbated the barrier dysfunction in ileum and promoted endotoxemia whereas the other diet did not, suggesting that an LA-rich HFD may worsen barrier function upon insult. The same was observed in Muc2$^{-/-}$

Table 3. Effects of dietary interventions comparing different fat sources on intestinal permeability in animal models. The direction of permeability is compared to healthy controls unless otherwise stated. DHA = docosahexanoic acid, EPA = eicosapentanoic acid, E% = energy percentage, FITC-dextran = fluorescein isothiocyanate dextran, HFD = high-fat diet, LA = linoleic acid, LFD = low-fat diet, LPS = lipopolysaccharide, MCT = medium chain triglyceride, TJ = tight junction.

Model	Dietary intervention	Main methods	Main results	Permeability	Ref.
Healthy Sprague Dawley male rats	Diet with 18.4 E% from butter, olive oil, safflower oil, or fish oil for 30 days Diets compared to LFD with 5.2 E% from fat	Jejunal *ex vivo* permeability to mannitol and diazepam	Permeability ↔ with all fat sources	Butter ↔ Olive oil ↔ Safflower oil ↔ Fish oil ↔	Vine et al., 2002
LPS-treated Sprague-Dawley male rats	Intragastric corn oil or MCT oil (5 g/kg/day) for 1 week Fat sources compared against each other	Small intestinal *ex vivo* permeability to horseradish peroxidase	Permeability with or without LPS administration ↑ with corn oil Mortality upon LPS exposure ↑ with corn oil	Corn oil ↑ Corn oil + LPS ↑ MCT oil ↔ MCT oil + LPS ↔	Kono et al., 2003
Ethanol-induced barrier dysfunction in C57BL/6N male mice	Liquid HFD with 40 E% from corn oil or MCT oil + beef tallow for 8 weeks Diets compared against each other	Ileal *ex vivo* permeability to FITC-dextran (4 kDa) Ileal expression of TJ protein-coding genes Plasma activity of LPS	Permeability and LPS activity upon ethanol exposure ↑ with corn oil TJ protein-coding gene expression with or without ethanol exposure ↓ with corn oil	Corn oil ↔ Corn oil + ethanol ↑ MCT oil + beef tallow ↔ MCT oil + beef tallow + ethanol ↔	Kirpich et al., 2012
Healthy C57BL/6J female mice	HFD with 60 E% from lard, sunflower oil, or fish oil for 8 weeks Diets compared to LFD with 10 E% from fat	Colonic *ex vivo* TEER Bacterial translocation to mesenteric fat	TEER and bacterial translocation ↑ with lard	Lard ↑ Sunflower oil ↔ Fish oil ↔	Lam et al., 2015
Heatstroke-induced barrier dysfunction in Wistar male rats	Intragastric corn oil, EPA, or DHA (1g/kg/day) for 21 days Fat sources compared to saline	Ileal *ex vivo* permeability to horseradish peroxidase TJ ultrastructure TJ protein levels and distribution Plasma activity of LPS	Permeability upon heat exposure ↑ with corn oil LPS activity upon heat exposure ↑ with corn oil and DHA	Corn oil + heat ↑ EPA + heat ↔ DHA + heat ↔	Xiao et al., 2015

mice that spontaneously develop colitis: compared to other fat sources, permeability in these mice was elevated upon corn oil feeding (Haskey et al., 2022). On the other hand, in healthy mice, a HFD with 60 E% from fat derived from either

Table 3. Continues.

Model	Dietary intervention	Methods	Main results	Permeability	Ref.
DSS-treated C57BL/6 male mice	Diet with 7 E% from sunflower oil, walnut oil, or soybean oil Healthy controls received only soybean oil diet Diets compared against each other	Colonic *ex vivo* permeability to FITC-dextran (4 kDa) Colonic expression of TJ protein-coding genes	Permeability upon DSS exposure ↑ with sunflower oil	Sunflower oil + DSS ↑ Walnut oil + DSS ↔ Soybean oil + DSS ↑	Bartoszek et al., 2020
Healthy Wistar female rats and their pups	Diet with 21 E% from fat of which 2 (low) or 12 % LA (high) for duration of pregnancy and lactation for dams and 3 or 6 months post-weaning for pups Diets compared against each other	Cecal *ex vivo* permeability to FITC-dextran (4 kDa) and horseradish peroxidase	Permeability to FITC-dextran at 3 mo ↑ and at 6 mo ↓ in all pups exposed to high LA	LA 3 mo ↑ LA 6 mo ↓	Marchix et al., 2020
Muc2$^{-/-}$ female and male mice	HFD with 40.8 E% from corn oil, olive oil, milkfat or Mediterranean blend (corn oil + olive oil + milkfat + fish oil) for 84 days No healthy controls Diets compared against each other	*In vivo* intestinal permeability to FITC-dextran (4 kDa)	Permeability ↑ with corn oil	Milkfat + Muc2$^{-/-}$ ↔ Corn oil + Muc2$^{-/-}$ ↑ Olive oil + Muc2$^{-/-}$ ↔ Mediterranean blend + Muc2$^{-/-}$ ↔	Haskey et al., 2022

lard, sunflower oil, or fish oil, decreased colonic *ex vivo* TEER only in the lard group (Lam et al., 2015). Even though this was attributed to the effects of saturated fat, a closer analysis of the dietary fatty acid compositions revealed that the diets differed more regarding other, unspecified fatty acids than SFAs. No negative effects from

high-LA sunflower oil on barrier function were reported. Overall, while the data on the impacts of specific fats are inconclusive, these studies support the concept that the diet's effects on intestinal barrier are dependent on the dietary fat source.

In the context of a diet lower in fat, the effects of different fatty acids on the intestine can also differ. In healthy rats, supplementation with corn oil increased *ex vivo* permeability to horseradish peroxidase when compared to MCT oil (Kono et al., 2003). Administration of LPS further increased the permeability and caused intestinal necrosis and hemorrhagic changes in the corn oil group whereas MCTs blunted these responses. Furthermore, all the corn oil-fed rats died after LPS administration, while MCT oil feeding promoted survival. Thus, these results also suggest that LA might be detrimental upon exposure to harmful substances. On the other hand, in healthy rats, a diet with 18.4 E% from fat sourced from either butter, olive oil, safflower oil, or fish oil did not change paracellular permeability, indicating that the fat source of the diet might not influence permeability in healthy adult animals (Vine et al., 2002). However, especially LA may impact permeability during development; when female rats were fed a diet with 21 E% from fat, of which 2 or 12 E% was derived from LA, during gestation and lactation, the diet influenced the offspring's intestinal permeability (Marchix et al., 2020). The offspring were allocated to either the same or the different diet upon weaning, and at three months of age, cecal permeability to FITC-dextran was higher in all groups that had been exposed to the high-LA diet at some stage. However, at six months of age, the impact of the maternal diet on permeability had disappeared and, interestingly, those fed 12 E% LA at weaning exhibited reduced fluxes of FITC-dextran, suggesting that LA may have a negative effect on intestinal barrier early but not later in life. In addition, while a pretreatment with corn oil did not worsen ileal barrier function in rats subjected to heat stress, it did not lead to improved outcomes that were observed with EPA and DHA treatments, either (Xiao et al., 2015). Finally, a diet with 7 E% from walnut oil, but not one with soybean or sunflower oil, protected from increased permeability in DSS-colitis (Bartoszek et al., 2020). Overall, these studies indicate that high-LA feeding may result in harm in specific settings, *i.e.*, in response LPS exposure or during development. However, the latter appears to be

reversible, and dietary LA has little effect on intestinal permeability in healthy adult animals.

In addition to paracellular permeability, the amount and source of dietary fat can influence the translocation of endotoxins such as LPS through lipid rafts (Ghoshal et al., 2009). In pigs, postprandial plasma LPS activity was higher after a coconut oil-containing meal when compared to a no-fat control meal or a fish oil-containing meal (Mani, 2013). While the *ex vivo* permeability to LPS was higher in ileal segments treated with micellized coconut oil, TEER did not change, suggesting that the oil did not influence paracellular permeability. As the pigs only received a single meal, the effect on LPS is likely not due to different microbiotas and thus, these results indicate that especially SFAs may promote LPS translocation through the transcellular pathway. In mice fed a diet with 33.7 E% from fat, palm oil increased the plasma concentration of LPS-binding protein but did not lead to elevated LPS activity (Laugerette et al., 2012). Milkfat, also high in SFAs, did not increase LPS-binding protein nor LPS activity. In fact, LPS activity was higher in groups receiving sunflower or rapeseed oil, and the rapeseed oil also led to elevated levels of sCD14. These studies did not measure paracellular permeability. The microbiota of the palm oil group had elevated levels of LPS-containing *Escherichia coli* which may also have contributed to the results. However, this was not observed in the milkfat group. Overall, these results indicate that high-SFA diets may allow for increased LPS translocation, that the source of SFAs may modulate the response, and that the increased translocation does not necessarily lead to higher plasma LPS activity.

High-fat diets and dietary fat sources in intestinal inflammation

HFDs are generally considered to be pro-inflammatory (Duan et al., 2018). However, the data from animal experiments on HFDs and colitis are contradictory and there are reports of both benefit and harm. A diet with 41.2 E% from either milkfat or lard protected from DSS-induced colitis when compared to a low-fat diet (Papoutsis et al., 2022), indicating that a higher fat intake may actually be protective. Three similar findings (Lee et al., 2022; Stenman et al., 2013b; Zhang et

al., 2022) but also one contrary (Cheng et al., 2016) have been reported with diets with 60 E% mainly from lard. Therefore, most of the evidence suggests that HFDs may offer protection from the DSS-induced inflammation. Unlike in the DSS-model, HFD has been shown to only worsen TNBS-induced colitis. In mice, a diet with 60 E% from fat from an unknown source exacerbated TNBS-colitis (Li et al., 2019; Wu et al., 2022). Thus, the effects of HFD may depend on the model used. Overall, the impact of HFDs on experimental colitis is unclear and may depend on not only the specific composition of the diet but also on factors outside the diet.

Again, the influence of the diet may be affected by its fat source. Animal studies comparing the impact of different fat sources on colitis are listed in Table 4. In a diet with 7 E% fat, walnut oil protected from DSS-induced colitis to a greater extent than a high-oleic acid sunflower oil and soybean oil performed the worst (Bartoszek et al., 2020). In another study, fish oil, high in EPA and DHA, aggravated DSS-colitis more than lard (Stenman et al., 2013b). Indeed, these findings indicate that the severity of DSS-colitis is modulated by the fat source of the diet. On the other hand, Papoutsis et al. (2022) did not observe a difference between milkfat and lard, suggesting that not all fat sources perform differently in this model. Olive oil, rich in oleic acid; corn oil, high in LA; milkfat, rich in SFAs; and a Mediterranean blend (a combination of olive oil, milkfat, and fish oil) were compared in Muc2$^{-/-}$ mice fed a diet with 40 E% from fat (Haskey et al., 2022). Of these, milkfat and corn oil worsened outcomes; the former resulted in highest disease activity and histopathological score whereas the latter had mixed effects on mRNA expression of inflammatory cytokines and resulted in increased permeability to FITC-dextran. While olive oil did not offer any significant protection, the Mediterranean blend was effective in alleviating colitis when compared to the other groups. These results suggest that the dietary fatty acid composition can influence colitis also in a genetic model. Finally, dietary fats may alter the degree of intestinal inflammation in healthy animals. In the previously described study where female rats and their pups were fed a LA-rich diet, negative effects of LA were also observed in inflammatory markers at three months of age in line with the increased permeability (Marchix et al., 2020). Again, these changes had reversed by six months, suggesting that the LA-induced inflammation subsides by itself. When HFDs with 40 E% from fat from

Table 4. Effects of dietary interventions comparing different fat sources on colitis in animal models. The direction of colitis is compared to controls with colitis unless otherwise stated. DSS = dextran sodium sulfate, E% = energy percentage, HFD = high-fat diet, TNF-α = tumor necrosis factor α.

Model	Dietary intervention	Main methods	Main results	Colitis	Ref.
DSS-treated C57BL/6J male mice	HFD with 60 E% from lard or lard + fish oil for 4 weeks Diets compared to LFD with 10 E% from fat	Macroscopic analyses Colonic TNF-α expression	Macroscopic changes ↓ with lard Mortality ↓ with lard and ↑ with fish oil	Lard ↓ Lard + fish oil ↑	Stenman et al., 2013b
DSS-treated C57BL/6 male mice	Diet with 7 E% from soybean oil, sunflower oil, or walnut oil for 15 weeks Diets compared against each other	Macroscopic and histological analyses Colonic expression of inflammatory marker genes	Macroscopic and histological changes ↓ with sunflower oil and ↓↓ with walnut oil Expression of inflammatory markers ↓ with sunflower oil and ↓↓ with walnut oil	Soybean oil ↑ Sunflower oil ↓ Walnut oil ↓↓	Bartoszek et al., 2020
DSS-treated C57BL/6JRj male mice	HFD with 41.2 E% from milkfat or lard for 6 weeks Diets compared to LFD with 10.3 E% from fat	Macroscopic analysis Colonic expression of inflammatory marker genes Fecal lipocalin	Macroscopic changes, expression of inflammatory markers, and lipocalin ↓ with milkfat or lard	Milkfat ↓ Lard ↓	Papoutsis et al., 2021
Muc2$^{-/-}$ female and male mice	HFD with 40.8 E% from corn oil, olive oil, milkfat or Mediterranean blend (corn oil + olive oil + milkfat + fish oil) for 84 days Diets compared against each other	Macroscopic and histological analyses Colonic expression of inflammatory marker genes Lamina propria immunophenotyping Serum cytokines	Macroscopic and histological changes ↓ with Mediterranean blend and ↑ with corn oil and milkfat Inflammatory immune cell profile ↓ with Mediterranean blend and ↑ with milkfat	Milkfat ↑ Corn oil ↑ Olive oil ↔ Mediterranean blend ↓	Haskey et al., 2022

either LA-rich maize oil, oleic acid-rich rapeseed oil, or a combination of maize oil and fish oil were fed to aged mice, increased infiltration of macrophages and neutrophils in ileum was observed in animals consuming maize oil and rapeseed oil

(Ghosh et al., 2013). Fish oil supplementation protected from immune cell infiltration and promoted the recruitment of immunosuppressive T-regulatory cells. Altogether, the results of these studies are conflicting and, such as in the case of fish oil, suggest that the effects of the fat type on intestinal inflammation are context dependent.

Modulation of gut microbiota by high-fat diets

Diet can alter the composition of the microbiota in as short a time as a day (Faith et al., 2011; Turnbaugh et al., 2009) and most of the inter-individual variation in the microbiota is explained by dietary factors (David et al., 2014). Thus, the effects of a HFD on the intestine may be mediated through changes in the gut microbiota. However, as was demonstrated by Muller et al. (2016), microbiota-modifying environmental factors independent of the diet, like housing conditions, can also determine the effects of a HFD on the intestine. Additionally, since there is no universal formula for a HFD, the differences in diet composition can result in significant between-study divergences. While there is little uniformity between studies, HFDs generally increase the ratio of *Firmicutes* to *Bacteroidetes* (Candido et al., 2018). An elevation of this ratio has been linked to obesity and poor metabolic health which often result from eating a regular HFD. These diets may also decrease genera considered beneficial such as *Lactobacillus* and *Bifidobacteria*, as well as promote those classified as harmful, like *Desulfovibrio* (Candido et al., 2018). Thus, it is possible that the typical HFDs result in a more pro-inflammatory gut microbial profile which contributes to the adverse systemic effects.

In addition to other dietary factors, the fat source of the diet may partly explain the genus-level differences. In a study investigating this phenomenon, mice were fed a low-fat control diet or a diet with 40 E% from fat sourced from milkfat, olive oil, or corn oil after weaning to compare how the diets affect mucosal microbiota and proteome (Abulizi et al., 2019). Milkfat and corn groups had higher α-diversity, *i.e.*, richness of species. All the HFDs had elevated the *Firmicutes* to *Bacteroidetes* ratio when compared to the control group but when the HFDs were compared, there were

significant genus-, class, and family-level differences within these phyla, supporting the concept that microbiota can be impacted by the dietary fat source. In a comprehensive study on the effect of the dietary fatty acid composition on the microbiota, seven groups of mice were given diets with 42 E% from fat from varying sources containing different ratios of fatty acids, and were compared to a diet with milkfat as the only fat source (Schoeler et al., 2023). Intriguingly, the diets that mostly differed from the milkfat diet were also high in saturated fat. Both had cacao butter as the main fat source with some added fish oil with one having only those two fat sources and the other having some of the cacao butter replaced by coconut oil. Thus, both diets were enriched with the long-chain SFA stearic acid. Indeed, these diets resulted in lower species count and diversity but no reduction in the bacterial load when compared to the milkfat diet. At the genus level, there was greater abundance of *Akkermansia* and *Bacteroides*. A diet high in MCT oil and olive oil had a distinct microbiota profile with decreased levels of *Bifidobacteria* and *Lactobacillus*. This may be explained by the increased metabolism of MCTs to ketone bodies which inhibit the growth of *Bifidobacteria*, as is discussed further below. Overall, the diet's fat source clearly influences the composition of the gut microbiota.

2.4.2 Ketogenic diets and exogenous ketones in intestinal health

Effects of ketogenic diets and exogenous ketones on the intestinal barrier

There are limited data on the impact of KD on intestinal permeability in human subjects. An increased lactulose–mannitol ratio, indicative of intestinal permeability, was observed in obese patients after eight weeks of consumption of a very-low calorie KD (800 kcal/day) despite a reduction in the level of systemic inflammation (Linsalata et al., 2023). Serum levels of LPS were also higher. However, the extreme caloric restriction, a stressor to the body, is a major confounding factor in this study, in addition to which there was no control group, but instead, the end values were compared to the baseline situation. In a short-term study, endurance trained male subjects completed a 25-km race walk before and

after following a KD for six days. When compared to pre-diet values, the KD led to increased I-FABP, sCD14, and LPS-binding protein levels as a response to the walk but there were no differences when compared to other groups (McKay et al., 2023). While this may be suggestive of epithelial damage, it should, again, be emphasized that epithelial I-FABP expression rises as a response to increased fat content of the diet (Lau et al., 2016; Mahmood et al., 2023) and thus, the plasma I-FABP concentration may be higher due to elevated intestinal expression despite a similar level of enterocyte shedding. As was also discussed previously, LPS-binding protein and sCD14 levels are not necessarily indicators of epithelial injury but may reflect increased LPS translocation as a response to higher fat intake which is likely in the context of a KD. Regardless, a six-day diet probably does not allow the body to fully adapt to ketogenic metabolism (Sherrier & Li, 2019) which may manifest as negative effects especially upon strenuous exercise. Altogether, the evidence of the effects of KDs on intestinal permeability in humans is lacking but the existing data suggest that a short-term KD may have negative effects on barrier function in physiologically stressful situations.

Preclinical studies have assessed true permeability as a response to a KD in healthy mice and upon experimental colitis (Table 5). In these experiments, a KD high in LA did not increase intestinal permeability to FITC-dextran in healthy animals (Li et al., 2021a; Olson et al., 2018) even though a regular HFD can lead to increased permeability depending on the baseline microbiota (Muller et al., 2016) as was discussed previously. In induced inflammation, the effects of a KD on intestinal barrier function are in line with the diet's overall effect on inflammation; in an animal study where a KD reduced inflammation, TJ protein expression was normalized by the diet (Kong et al., 2021), and the opposite was true if the KD aggravated inflammation (Li et al., 2021a). While Kong et al. (2021) did not assess permeability, Li et al. (2021a) detected an increase as a response to the KD in line with a worsening of inflammation. Exogenously administered BHB has been reported to prevent the DSS- or LPS-induced increases in intestinal permeability to FITC-dextran (Li et al., 2021b; Wang et al., 2024). In addition to permeability, KDs also influence other barrier properties: while some data suggest that typical HFDs may lead to defects in the intestinal mucus layer predisposing to microbial

Table 5. Effects of dietary interventions with ketogenic diets and exogenous ketones on intestinal permeability in animal models. The direction of permeability is compared to healthy controls. BHB = β-hydroxybutyrate, FITC-dextran = fluorescein isothiocyanate dextran, KD = ketogenic diet, LPS = lipopolysaccharide, TJ = tight junction.

Model	Dietary intervention	Main methods	Main results	Permeability	Ref.
Healthy Swiss Webster female and male mice	KD for 14 days Diet compared to LFD	*In vivo* intestinal permeability to FITC-dextran (4 kDa)	Permeability ↔ on KD	KD ↔	Olson et al., 2018
DSS-treated C57BL/6 male mice	KD for 30 days Diet compared to LFD	*In vivo* intestinal permeability to FITC-dextran (4 kDa) Colonic TJ protein levels and distribution	Permeability upon DSS treatment ↑ on KD TJ protein levels with and without DSS exposure ↓ on KD	KD ↔ KD + DSS ↑	Li et al., 2021a
DSS-treated C57BL/6 mice	Intragastric BHB (20, 100, or 500 mg/kg/day) for 7 days BHB compared to saline	*In vivo* intestinal permeability to FITC-dextran (4 kDa)	Permeability upon DSS treatment ↔ with BHB	BHB + DSS ↔	Li et al., 2021b
LPS-treated ICR male mice	BHB administered intragastrically (30 mg/kg/d) or in drinking water (3 g/kg/day) for 18 days BHB compared to regular drinking water	*In vivo* intestinal permeability to FITC-dextran (70 kDa) Colonic expression of TJ protein-coding genes Colonic TJ protein levels and distribution	Permeability upon LPS treatment ↔ with BHB	BHB + LPS ↔	Wang et al., 2024

penetration (Schroeder et al., 2018), a KD does not exert the same effect (Ang et al., 2020). In a rat model of irritable bowel syndrome, a KD promoted the expression of barrier-sealing small intestinal TJ proteins claudin-1 and occludin on both

protein and mRNA level even above the levels of healthy controls (Gigante et al., 2021).Since intestinal permeability was not assessed, it is unclear whether the changes in TJ proteins translate into lower permeability. Overall, KDs do not negatively alter paracellular permeability in healthy rodents.

Ketogenic diets and exogenous ketones in intestinal inflammation

Even though research on the effects of nutritional ketosis on other conditions is accumulating, clinical studies on its impact on intestinal diseases are still scarce. In colon biopsies taken from IBD patients, the tissue expression of the ketogenic enzymes acetyl-CoA acetyltransferase, hydroxymethylglutaryl-CoA synthase, and D-β-hydroxybutyrate dehydrogenase were downregulated at both the mRNA and protein level (Huang et al., 2022). Colonic BHB levels were also lower when compared to healthy controls. This indicates that local ketone body metabolism may be impaired upon intestinal inflammation.

The results from studies on experimental colitis, listed in Table 6, are not uniform with two studies reporting benefit and one showing harm. A KD has been shown to alleviate DSS-induced colitis through reducing the levels of colonic group 3 innate lymphoid cells in mice (Kong et al., 2021). In a rat study, a KD evoked a protective effect related to the maintenance of autophagy and the mitigation of apoptosis via the inhibition of the NLPR3 inflammasome (Abdelhady et al., 2023). In contrast, one group described a KD to exacerbate inflammation in DSS-colitis (Li et al., 2021a). Intriguingly, both the beneficial and the harmful effects have been associated with changes in the gut microbiota. A KD-related human gut microbiota decreases intestinal pro-inflammatory T helper 17 cells (Ang et al., 2020), a finding more consistent with the results of Kong et al. (2022) and Abdhelhady et al. (2023). Previous research has also shown BHB to protect from the DSS-induced intestinal inflammation by itself either when administered via an orogastric gavage (Li et al., 2021b), intraperitoneally (Abdelhady et al., 2023), or intrarectally (Huang et al., 2022). Similar to a KD, BHB blocks the NLPR3 inflammasome and maintains normal levels of autophagy (Abdelhady et al., 2023). In addition, orally supplemented ketone body derivatives poly-D-3-hydroxybutyric acid and (R)-3-

hydroxybutyl (R)-3-hydroxybutyrate, both of which increase plasma BHB levels, protect from DSS-induced colitis (Mohammed et al., 2024; Suzuki et al., 2023). The only report comparing a KD and BHB showed the latter to be slightly superior with less macroscopic inflammation and lower expression of IL-1β, IL-8, and NLPR3 in response to BHB (Abdelhady et al., 2023). BHB supplementation resulted in higher plasma levels of the compound than the KD which may explain the result. At present, there are no studies examining the effects of KDs or exogenous ketones on other models of colitis. Altogether, KDs and BHB may reduce intestinal inflammation, but the data on KDs are not consistent.

Modulation of gut microbiota by ketogenic diets and ketone bodies

Most of the human reports on the effect of KDs on intestinal health have focused on changes in the gut microbiota. While the reported changes vary between studies, which likely is a result of how the diet is constructed as discussed below, it is evident that a KD results in drastic shifts in the composition of the microbiota. A systematic review concluded that bacterial richness declined slightly on a short-term diet (two to twelve weeks) (Rew et al., 2022). However, this effect might be reversed by long-term adherence. In patients with multiple sclerosis, microbial mass and diversity were initially reduced by a KD but began to recover after twelve weeks and after 24 weeks exceeded the baseline values (Swidsinski et al., 2017). As opposed to a regular HFD, a KD does not increase *Firmicutes* to *Bacteroidetes* ratio but may even decrease it in humans (Lim et al., 2022). A KD generally results in increases in *Akkermansia muciniphila*, whereas species of the genus *Bifidobacterium* decrease (Santangelo et al., 2023). The loss of *Bifidobacteria* may be responsible for the KD-related decrease in the intestinal T helper 17 cell population (Ang et al., 2020); this is likely attributable to the increased levels of luminal ketone bodies. The levels of other carbohydrate-metabolizing bacteria of genera like *Roseburia*, *Eubacterium*, and *Ruminococcus* also tend to be reduced as a response to a KD. Since the diet is low in carbohydrates, the decrease in the levels of bacteria involved in their metabolism is logical.

Table 6. Effects of dietary interventions with ketogenic diets and exogenous ketones on colitis in animal models. The direction of colitis is compared to controls with colitis. BHB = β-hydroxybutyrate, DSS = dextran sodium sulfate, KD = ketogenic diet, LFD = low-fat diet.

Model	Dietary intervention	Main methods	Main results	Colitis	Ref.
DSS-treated C57BL/6 male mice	KD for 16 weeks KD compared to LFD	Macroscopic and histological analyses	Macroscopic and histological changes, expression of inflammatory markers, and mortality ↓ with KD	KD ↓	Kong et al., 2021
DSS-treated C57BL/6 male mice	KD for 30 days KD compared to LFD	Macroscopic and histological analyses Colonic expression of inflammatory marker genes Serum cytokines	Macroscopic and histological changes, expression of inflammatory markers, and serum cytokines ↑ with KD	KD ↑	Li et al., 2021a
DSS-treated C57BL/6 mice	Intragastric BHB (20, 100, or 500 mg/kg/day) for 7 days BHB compared to saline	Macroscopic and histological analyses Spleen immunophenotyping Serum cytokines	Macroscopic and histological changes, inflammatory immune cell profile, and serum cytokines ↓ with BHB	BHB ↓	Li et al., 2021b
DSS-treated C57BL/6J male mice	Intrarectal BHB (15 mg/day) for 18 days BHB compared to saline	Macroscopic and histological analyses Colonic expression of inflammatory marker genes	Macroscopic changes and expression of inflammatory markers ↓ with BHB	BHB ↓	Huang et al., 2022
DSS-treated Sprague Dawley male rats	KD or intraperitoneal BHB (300 mg/kg/day) for 25 days KD and BHB compared to LFD and against each other	Macroscopic and histological changes Colonic expression of inflammatory markers	Macroscopic changes and expression of inflammatory markers ↓ with KD or BHB	KD ↓ BHB ↓↓	Abdelhady et al., 2023
DSS-treated C57BL/6J male mice	Ketone polyester-supplemented (2 %) diet for 25 days Ketone polyester compared to standard diet	Macroscopic and histological changes Colonic expression of inflammatory marker genes	Macroscopic changes and expression of inflammatory markers ↓ with ketone polyester	Ketone polyester ↓	Suzuki et al., 2022
DSS-treated Sprague Dawley male rats	Intraperitoneal BHB (200 mg/kg/day) or ketone monoester-supplemented (4 %) diet for 25 days BHB and ketone monoester compared to standard diet	Macroscopic and histological changes Colonic expression of inflammatory markers	Macroscopic changes and expression of inflammatory markers ↓ with BHB or ketone monoester	BHB ↓ Ketone monoester ↓↓	Mohammed et al., 2024

The effects of KDs can differ depending on what foods the diet is composed of. A KD based on only animal products with no fiber resulted in a significant increase in β-diversity of the microbiota, indicating a within-subject difference in the microbial

diversity before and after the diet. This diet induced the expected shift towards more bile-tolerant organisms when compared to a non-ketogenic, high-fiber vegan diet (David et al., 2014). On the other hand, a KD and a high-carbohydrate, vegan diet resulted in similar changes in the microbiota compared to baseline when both diets were rich in fiber (Link et al., 2024). While the first study had a small number of participants and there were confounding factors, such as medications, that may have affected the result, the contrast between these studies suggests that the effect of a KD on microbiota may be dependent on the foods the diet is comprised of. Thus, while the exact macronutrient composition of the diet may influence its effects on microbiota, it also matters from which foods those macronutrients originate.

Since KDs are used as a treatment for drug-resistant epilepsy, many of the studies examining the effects of the diet on other parameters have been conducted in patients with this condition. Even though KDs do not change the α-diversity of the microbiota in patients with epilepsy, taxonomic and functional differences have been detected. These include a decrease in *Bifidobacteria* and an increase in *Escherichia* accompanied with reduced carbohydrate metabolism (Lindefeldt et al., 2019). Interestingly, in preclinical models of epilepsy, the protective effect of a KD on seizures is dependent on gut microbiota, specifically the presence of *Akkermansia muciniphila* and *Parabacteroides*, which mediate a decrease in circulatory γ-glutamylated amino acids and an increase in hippocampal ratio of the neurotransmitters gamma-butyric acid and glutamate (Olson et al., 2018). In contrast, the KD does not protect from seizures in germ-free or antibiotic-treated mice, highlighting the importance of the microbiota. In addition, the transfer of the fecal microbiota from KD-treated epilepsy patients to germ-free mice resulted in a higher seizure threshold which is indicative of a protective effect from the KD-related microbiota (Lum et al., 2023). These studies suggest that also the extraintestinal effects of KDs on diseases such as epilepsy may be mediated by the gut microbiota.

2.4.3 Ketogenic diets – effects on the systemic and intestinal renin-angiotensin-aldosterone system

Surprisingly little is known about the effect of a KD on RAAS despite the diet's recognized stimulatory effect on natriuresis, *i.e.* renal sodium and fluid loss (Kirkpatrick et al., 2019). Since insulin promotes sodium retention in the kidney, the KD-related natriuresis is likely related to low insulin levels (DeFronzo et al., 1975). However, according to a systematic review, KDs do not appear to lower blood pressure (Amini et al., 2024). In overweight and obese adults, a hypocaloric KD either by itself or supplemented with ketone salts, providing 23.2 g of BHB and 3.8 g of sodium a day for six weeks, lead to increased plasma aldosterone despite no change in renin nor blood pressure and electrolytes (Belany et al., 2023). Intriguingly, ketone levels were positively correlated with higher aldosterone levels and the group with the higher sodium intake from ketone salts experienced an even larger increase from the baseline situation. The study suggests a unique feature of ketosis to promote aldosterone production independent of renin, since subjects consuming a low-fat, non-ketogenic diet did not exhibit increased levels of aldosterone despite achieving a similar weight loss. While ketosis leads to accelerated natriuresis, the mechanism at play here is at least partly independent of electrolyte balance since an increased sodium intake did not lower aldosterone levels.

In animal models, KDs affect locally expressed renin-angiotensin system components. When compared to standard chow, a KD increases AT2R expression in rat subcutaneous and epididymal adipose tissue (Da Eira et al., 2023). It downregulates ACE2 protein expression and MasR mRNA expression in subcutaneous adipose tissue whereas in epididymal adipose tissue it decreases ACE protein levels. In rat lung, a KD lowers ACE protein levels when compared to standard chow (Da Eira et al., 2021). These findings indicate that KDs may promote the alternative arm of the renin-angiotensin system. Levels of aldosterone synthase, CYP11B2, or aldosterone were not analyzed in these studies. Since no causal relationships were investigated, this may either be a downstream effect of the anti-

inflammatory effects of KDs or another mechanism through which KDs regulate inflammation.

The amount and source of dietary fat may influence intestinal permeability and inflammation. However, there are contradicting results from both HFDs and KDs which can be either promote or alleviate intestinal inflammation depending on the context. Many of these effects can be mediated by the gut microbiota which is influenced by dietary components.

3 Aims of the study

Dietary fat quantity and source influence intestinal health through diverse mechanisms such as inflammation, intestinal permeability, and the microbiota. This thesis examined how high-fat, low-carbohydrate ketogenic diets (KDs) with different fat sources and exogenous β-hydroxybutyrate (BHB) affect intestinal health in healthy mice and mice with colitis. Study I characterized the impact of KDs with different fat sources on paracellular permeability and inflammation in healthy animals. Study II explored whether the fat source of the KD would determine its effects on colitis induced with dextran sodium sulfate (DSS). Dietary interventions for colitis were further investigated in Study III, which focused on the effects of supplemental BHB in the same model. Study IV defined how the KDs with different fat sources impact the components of the local renin-angiotensin-aldosterone system (RAAS) in the intestine. The hypotheses were that the fat source of the KD would determine its effects on intestinal permeability and inflammation, BHB would alleviate colitis alike the KDs, and the KDs would promote the expression of the components of the alternative RAAS arm.

The specific aims of the thesis were to:

1. Define the effects of KDs with two different fat sources, one high in saturated fatty acids or and the other rich in polyunsaturated linoleic acid, on intestinal permeability and barrier function in healthy and inflamed mouse intestine. (Studies I and II)

2. Compare the effects of the KDs with the two different fat sources and exogenous BHB supplementation on intestinal inflammation in a mouse model of experimental colitis. (Studies II and III)

3. Characterize the effects of the KDs with the two fat sources on selected local RAAS components in healthy and inflamed mouse intestine. (Study IV)

4 Materials and methods

4.1 Experimental animals

Experiments were conducted with nine-week-old C57BL/6J male mice obtained from Scanbur (Karlslunde, Denmark). The studies were approved by the animal research board of the Regional State Administrative Agency for Southern Finland (ESAVI/9377/2019). The mice were housed individually in a specific-pathogen free animal facility under a 12 h light–dark cycle, at 20 ± 2 ◦C in Studies I and II and at 25 ± 1 °C in Study III, and 50–60% humidity with *ad libitum* access to food and water.

4.2 Study diets

The study diets were custom-made (Envigo, Indianapolis, IN, USA) and matched for protein (9.4-9.7 E%) and micronutrients. The macronutrient profiles of the diets are presented in Table 7. In all studies, the control animals were fed a low-fat control diet (CD) (77.8 E% from carbohydrate and 12.5 E% from fat). In Studies I and II, the animals in the KD groups were given low-carbohydrate, high-fat diets (90.1 E% from fat, 0.4 E% from carbohydrate) rich in either saturated fatty (SFA-KD) acids or linoleic acid (LA-KD). In Study III, animals received a low-fat diet in which 12 E% from carbohydrates was replaced with BHB. The KD used in Study III was LA-KD.

Table 7. The macronutrient compositions of the diets. Values are expressed as a percentage of energy. CD = control diet, BHB = β-hydroxybutyrate-supplemented diet, LA-KD = ketogenic diet with linoleic acid, SFA-KD = ketogenic diet with saturated fatty acids.

	CD	SFA-KD	LA-KD	BHB
Protein (E%)	9.7	9.4	9.4	9.7
Carbohydrate (E%)	77.8	0.5	0.5	65.8
Fat (E%)	12.5	90.1	90.1	12.5
Saturated fat (E%)	2.6	56.6	22.8	2.6
Monounsaturated fat (E%)	3.0	26.0	17.9	3.0
Polyunsaturated fat (E%)	6.9	4.5	49.3	6.9
Linoleic acid (E%)	6.4	4.0	44.5	6.4
BHB (E%)	0	0	0	12

4.3 Study designs

4.3.1 Study I

The designs of the animal experiments are illustrated in Figure 9. In study I, male mice were acclimatized for 17 days and then randomly allocated into three groups: control group (n = 10) fed with CD, and two intervention groups receiving either SFA-KD (n = 9) or LA-KD (n = 9). The dietary interventions were continued for four weeks, during which intestinal permeability to iohexol was measured at two time points: after two and twenty-six days of feeding. On day twenty-eight, animals were sacrificed under isoflurane anesthesia for sample collection.

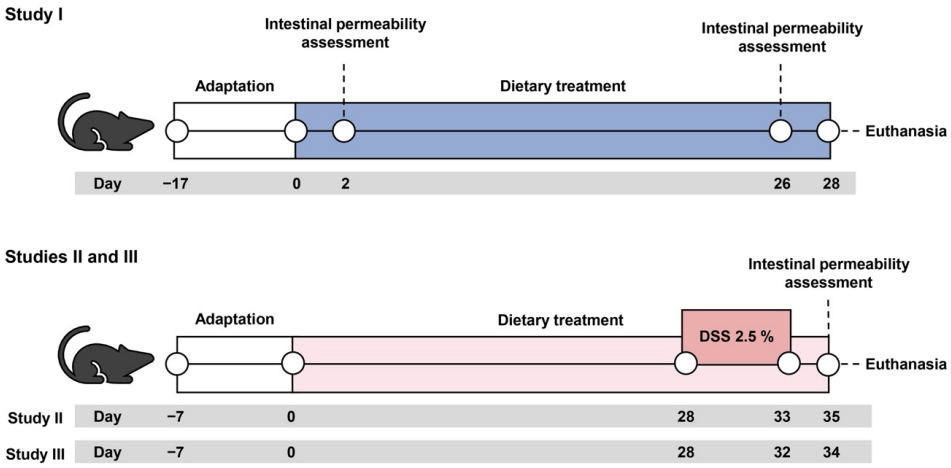


Figure 9. Designs of the animal experiments. All experiments consisted of four-week dietary interventions. Study I was terminated after this period. In Studies II and III, experiments were further continued for another week with DSS administration to induce colitis. Dietary treatments were maintained throughout this period. DSS = dextran sodium sulfate.

4.3.2 Studies II and III

In Studies II and III, mice were acclimatized for seven days before the start of the dietary interventions. Animals were randomized into healthy controls and DSS groups. In Study II, there were four groups: healthy group fed CD (n = 8), DSS group fed CD (n = 8), DSS group fed SFA-KD (n = 10), and DSS group fed LA-KD (n = 10). The KDs were only administered to DSS mice since the effects of the diets on healthy intestines had already been examined in Study I. In Study III, all diets were given to both healthy and DSS mice and there were 6 groups in total: healthy (n = 8) and DSS (n = 8) group fed CD, healthy (n = 6) and DSS (n = 8) group fed KD, and healthy (n = 6) and DSS (n = 8) group fed a BHB-supplemented diet.

The mice were fed for four weeks, after which colitis was induced with a 2.5 % (w/v) DSS (DB001, TdB Labs, Uppsala, Sweden) solution administered in place of regular drinking water. Dietary interventions were continued throughout this period. Animals consumed the solution for five days in Study II and for four days in Study III followed by two days of tap water before sacrifice. During the DSS period, animals were weighed and examined daily to calculate the Disease Activity Index, a

score composed of weight loss, diarrhea, and rectal bleeding. Intestinal permeability to FITC-dextran was assessed on the day of the sacrifice. If an animal reached the humane endpoint prior to the end of the experiment, it was euthanized.

4.3.3 Study IV

Jejunal and colonic samples for Study IV were collected from the mice used in Studies I and II.

4.4 Intestinal permeability measurements

4.4.1 Permeability to iohexol

Iohexol (10 ml/kg, Omnipaque 300®, GE Healthcare, Oslo, Norway) was used to measure intestinal permeability in Study I. The mice were weighed, and the solution was administered by gastric gavage. For the urine collection, the animals were placed individually in metabolic cages for 24 h. The amount of the urine collected was measured and the concentration of iohexol in the sample was determined with enzyme-linked immunosorbent assay (ELISA) (FIT-0515, BioPAL Inc., Worcester, MA, USA). The amount of iohexol recovered was calculated as a percentage of the amount given.

4.4.2 Permeability to fluorescein isothiocyanate dextran

In Studies II and III, animals were given FITC-dextran (4 kDa, FD4, TdB Labs) solution (600 mg/kg, 125 mg/ml) by gastric gavage four hours before sacrifice. The plasma FITC-dextran concentration was analyzed with a fluorescence spectrophotometer at the excitation wavelength of 495 nm and the emission wavelength of 525 nm. To determine the concentration in the samples, FITC-dextran was diluted in PBS-T to obtain standard curves.

4.5 Sample collection

Fecal samples were collected before the DSS administration in Study III. The mice were placed alone in empty cages for 4 hours, after which fecal pellets were collected and frozen in −80 °C.

On the last day of each experiment, animals were sacrificed under isoflurane anesthesia by exsanguination. Blood was drawn from the *vena cava*. The entire intestine was removed and in Studies II and III, colon was photographed for macroscopic evaluation and its length was measured. The tissue pieces collected for histological analyses from jejunum for Studies I and IV and colon for all studies were fixed in 4 % buffered paraformaldehyde solution for 36 hours and then stored at 4 °C in 70 % ethanol. Specimens for biochemical analyses were frozen in liquid nitrogen, and stored in −80 °C.

4.6 Macroscopical evaluation

In Studies II and III, the DSS-induced colonic inflammation and damage were evaluated from photographs taken with a handheld device. Three parameters were scored: the presence of diarrhea, visible fecal blood, and inflammation (edema and/or ulceration). Based on the severity, each parameter was given a score from 0 to 3 and the final score was calculated as their average. The evaluation was performed blinded to the treatments independently by three different researchers.

4.7 Histological analyses

The intestinal samples were cut into longitudinal halves, dehydrated, embedded in paraffin, and cut into 4 μm-thick sections. The sections were stained with hematoxylin and eosin stain. In Study I, selected jejunal sections were stained with Alcian blue–Periodic Acid-Schiff to detect intestinal mucins (goblet cells) and

polysaccharides. The slides were read and evaluated blinded to the treatments by an experienced veterinary pathologist.

In Study I, epithelial vacuolation in jejunum was graded semi-quantitively as follows: 0 = absent; 1 = mild, 2 = moderate, and 3 = marked. Edema in villus *lamina propria* was graded as absent (0) or present (1). In Studies II and III, the slides were assessed for the severity of tissue damage and inflammation. The scoring principally followed the system described by Wirtz et al. (2017), where tissue damage and inflammation were separately scored from 0 to 3 and then summed to a score ranging from 0 to 6. In Study II, the scoring was modified due to the relatively mild histological changes in most DSS-KD animals with the mildest DSS-induced changes receiving a tissue damage score of 0.5 and mild *lamina propria* mononuclear infiltrates a score of 0.5. Mucosal tissue damage without marked surface epithelial erosion or ulceration was scored as 1.5 and non-extensive damage extending beyond mucosa as 2.5. Inflammatory cell infiltration limited to the inner layer *muscularis externa* received the score of 2.5.

4.8 Biochemical analyses

4.8.1 Enzymatic assays

Plasma BHB levels were determined with a commercial enzymatic kit (700190, Cayman Chemicals, Ann Arbor, MI, USA) in Studies I, II, and III. Limulus Amebocyte Lysate Assay (A39553, Pierce™ Chromogenic Endotoxin Quant Kit, Thermo Fisher Scientific, Waltham, MA, USA) was used to detect LPS activity in plasma samples in Study I. In Study III, fecal calprotectin levels were analyzed as a marker of intestinal inflammation with Mouse S100A8/S100A9 Heterodimer DuoSet ELISA (DY8596-05, R&D Systems, Minneapolis, MN, US) and normalized against the protein concentration with Pierce™ BCA Protein Assay Kit (23225, Thermo Fisher Scientific).

4.8.2 Gene expression analysis

RT-qPCR was used to analyze the expression of selected TJ-protein-coding genes, inflammatory marker genes, IAP-subtype-coding gene *Akp6*, and RAAS component genes. The primer sequences are listed in Table 8. RNA was extracted from tissue pieces with NucleoSpin RNA Kit (740955.250, Macherey Nagel, Duren, Germany) and transcribed into complementary DNA with iScript™ cDNA Synthesis Kit (1708891, Bio-Rad). RT-qPCR was run with LightCycler® 480 SYBR Green Master (04887352001, Roche Diagnostics Corp., Indianapolis, IN, USA). The gene expression of target genes was calculated as relative quantities by normalizing the values for each sample against the geometric mean of the housekeeping genes *Eef2*, *Rplp0* and either *Actb* (Study I) or *18S* (Studies II, III, and IV).

4.8.3 Protein expression analysis

Western blot was used to analyze the relative levels of intestinal TJ proteins claudin-1, -2, and -4, as well as occludin in Studies I and II, and those of RAAS components ACE, AT1R, and AT2R in Study IV. Samples were prepared from supernatants collected from tissue homogenates by diluting them to the same total protein concentration with PBS-T and Laemmli sample buffer (161-0747, Bio-Rad, Hercules, CA, USA) containing 5 % 2-mercaptoethanol followed by denaturing the proteins on a heat block.

After the SDS-PAGE run, the proteins were transferred to a nitrocellulose membrane. In Study II, total protein concentration was determined with a commercial kit (926-11010, Revert™ 700 Total Protein Stain Kit, LI-COR, Lincoln, NE, USA). The primary antibodies used were claudin-1 (166338, 1:200; Santa Cruz Biotechnology, Dallas, TX, USA), claudin-2 (293233, 1:200; Santa Cruz Biotechnology), claudin-4 (376643, 1:200; Santa Cruz Biotechnology), occludin (91131, 1:10000, Cell Signaling Technology, Danvers, MA, USA), and β-actin (3700, 1:3000; Cell Signaling Technology). For the detection, membranes were incubated in fluorescence-labeled secondary antibody solution (1:10000 (926-68050, IRDye

Table 8. Primer sequences used in RT-qPCR analyses. If no reference is listed, the primer pair was designed using NBCI Primer-BLAST (Ye et al., 2012).

Gene	Forward primer	Reverse primer	Ref.
Actb	CTGAATGGCCCAGGTCTGAG	AAGTCAGTGTACAGGCCAGC	
18S	AACGAACGAGACTCTGGCAT	ACGCCACTTGTCCCTCTAAG	
Eef2	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA	(Eissa et al., 2016)
Rplp0	TAACCCTGAAGTGCTCGACA	GGTACCCGATCTGCAGACA	(Salmenkari et al., 2018)
Cldn1	AGACCTGGATTTGCATCTTGGTG	TGCAACATAGGCAGGACAAGAGTTA	(Li et al., 2012)
Cldn2	GCAAACAGGCTCCGAAGATACT	GAGATGATGCCCAAGTACAGAG	(Zhang et al., 2015)
Cldn4	TGAGCGATGGCGTCTATGG	GATGTTGCTGCCGATGAAGG	
Ocln	CGGTACAGCAGCAATGGTAA	CTCCCCACCTGTCGTGTAGT	(Song et al., 2018)
Il1b	CTCCAGCCAAGCTTCCTTGT	TCATCACTGTCAAAAGGTGGCA	(Salmenkari et al., 2019)
Il6	ATCGTGGAAATGAGAAAAGAGTTGT	CTGCAAGTGCATCATCGTTGT	
Tnf	TGGCACCACTAGTTGGTTGTCT	AGCCTGTAGCCCACGTCGTA	
Lcn2	CCACCACGGACTACAACCAG	AGCTCCTTGGTTCTTCCATACAG	(Nakano et al., 2020)
Akp6	ACCGAAGCTCAGAGTGTTGAT	GCAAATATGGCCACGTCCTC	
Agt	CTTCCAAGGAACGATGAGAGGTT	ACAGACACCGAGATGCTGTT	(Salmenkari et al., 2018)
Ace	GCTGGAGGGTCTTTGATGGA	AGTCACCTTGGGATCTTGGC	(Salmenkari et al., 2018)
Ace2	GGATACCTACCCTTCCTACATCAGC	CTACCCCACATATCACCAAGCA	(Oudit et al., 2009)
Agtr1	AGTCGCACTCAAGCCTGTCT	ACTGGTCCTTTGGTCGTGAG	(Wang et al., 2015)
Agtr2	GAAGCTCCGCAGTGTGTTTA	TGGCTAGGCTGATTACATGC	(Gao et al., 2012)
Mas1	CGGTCTACATTACCCACTTGTC	CCCGTGTTGTAGCCAAATAGA	(Hurst, 2020)

680LT goat anti-mouse, or 926-32211, IRDye 800CW goat anti-rabbit, LI-COR)). The β-actin bands were detected with the Odyssey CLx infrared Imaging system (LI-

COR) and analyzed with the Image Studio program (LI-COR). The intensities of the target protein bands were normalized against the intensity of β-actin in Study I and against the intensity of total protein in Study II.

4.9 Fecal microbiota analysis

In Study III, pre-DSS fecal microbiota was analyzed through 16S rRNA sequencing in The Translational Genomics Research Institute (Phoenix, AZ, US). Fecal pellets were lysed and DNA extracted and purified from the supernatant using the KingFisher Apex Purification System (Thermo Fisher Scientific) followed by the MagMAX™ Microbiome Ultra Nucleic Acid Isolation protocol. Paired-end DNA libraries were created using 16S rRNA V4 dual-index primers. After library quality assessment, the sequencing was performed using the MiSeq Reagent Kit v2 on the Illumina MiSeq platform. QIIME2 (Bolyen et al., 2019) was used for primary sequence analysis with differentially abundant taxa assessed using Analysis of Composition of Microbiomes with Bias Control (ANCOM-BC) (Lin & Peddada, 2020). α-Diversity was determined with Shannon diversity and Faith Phylogenetic Diversity analyses and β-diversity with Unweighted and Weighted Unifrac analyses.

4.10 Statistical analyses

GrapPad Prism 10 (Dotmatics, La Jolla, CA, USA) was used for statistical analyses and figures. Weight changes and Disease Activity Index were analyzed with a mixed-effects model followed by Tukey's multiple comparisons test. Permeability to iohexol was analyzed using a mixed-effects model followed by Sidak's post-hoc test. For other parameters, normality was tested with Shapiro-Wilk test, based on which the data were analyzed either using one-way ANOVA followed by Tukey's post-hoc test, or Kruskal-Wallis H test followed by Dunn's post-hoc test. In the pooled analyses for this thesis, data from Studies I-III were combined and analyzed according to the same principles. The level of statistical significance was set at $p < 0.05$.

5 Results

Mouse groups with similar dietary interventions were used in Studies I-III. Therefore, the data on parameters that were analyzed in at least two of these studies were pooled for the analyses presented in this thesis. All data used for the new analyses were derived from the original publications. Data from different studies are indicated with the following colors in the figures unless stated otherwise: ● = Study I, ● = Study 2, and ● = Study 3.

5.1 Effects of ketogenic diets on metabolic parameters

5.1.1 Ketogenic diets lead to normal weight development in healthy mice despite increased energy intake

Healthy animals initially lost weight on the KDs but regained it within two weeks from the start of the experiments in all studies, *i.e.*, in Study I and before the DSS intervention in Studies II and III (Figure 10A). From that point onwards, the between-group body weight gain was similar. However, the LA-KD-fed mice had a significantly elevated energy intake compared to the CD-fed mice in all experiments (Figures 10B and 10C). In Study I, the energy consumption of mice fed the SFA-KD did not differ from the CD but was higher in Study II and when data from Studies I and II were combined. Energy consumption in Study III was not suitable for comparison with the previous studies since the mice were housed at a higher temperature which decreases energy expenditure and intake. Energy intake in the BHB-fed mice was similar to those fed the CD in Study III (Figure 10C).

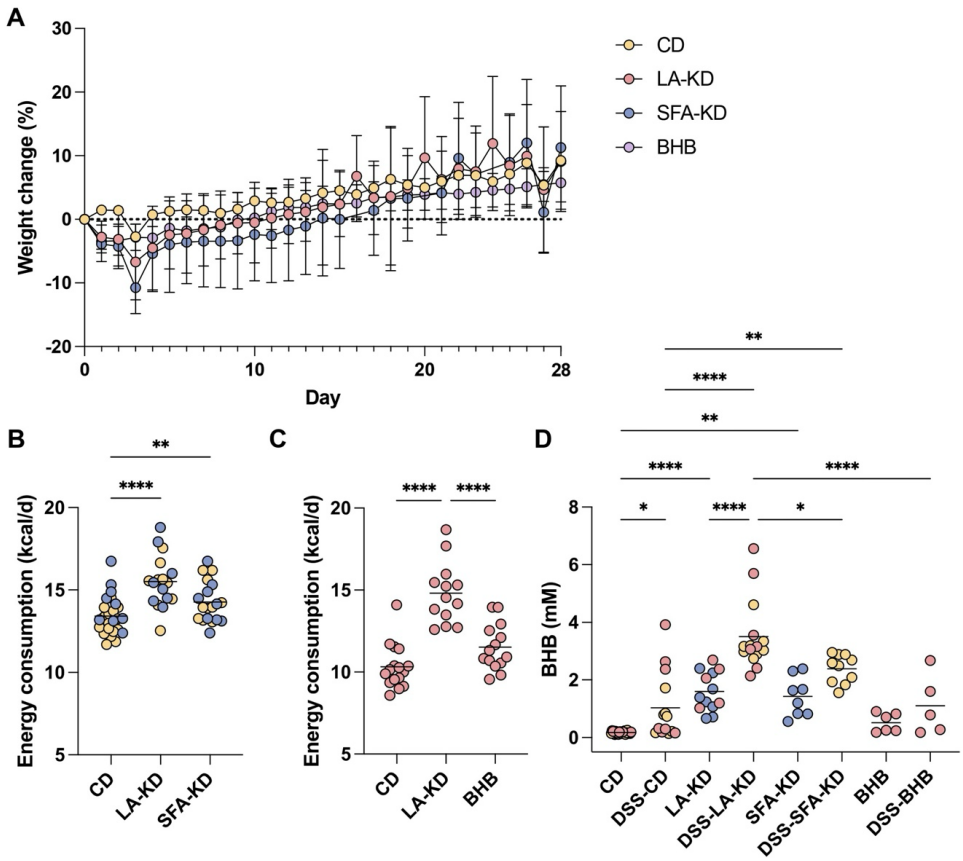


Figure 10. The KDs led to normal weight development despite increased energy intake. A. Weight gain during the first four weeks of feeding showing an initial weight loss with the KDs after which development continues similar to CD-fed animals. B. Energy consumption in Studies I and II showing increased intake in the KD groups. C. Energy consumption in Study III illustrating higher intake in the LA-KD group. D. Plasma BHB levels across studies showing elevation by the KDs which is accentuated by DSS exposure. In A and D, data are pooled from studies I-III, and in B from I and II. In B-C, ● = Study I, ● = Study 2, and ● = Study 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** < p 0.0001. BHB = β-hydroxybutyrate-supplemented diet, CD = control diet, DSS= dextran sodium sulfate, DSS-CD = control diet with DSS, DSS-LA-KD = linoleic acid-rich ketogenic diet with DSS, DSS-SFA-KD = saturated fat-rich ketogenic diet with DSS, DSS-BHB = β-hydroxybutyrate-supplemented diet with DSS, LA-KD = linoleic acid-rich ketogenic diet, and SFA-KD = saturated fat-rich ketogenic diet.

5.1.2 Ketogenic diets increase plasma β-hydroxybutyrate levels while exogenous ketone supplementation does not

Plasma BHB was analyzed as an indicator of ketone body production. As expected, higher levels of plasma BHB, indicative of elevated ketogenesis, were detected in animals consuming the KDs (Figure 10D). In addition, DSS exposure led to slightly elevated BHB levels, typical of inflammatory conditions where macronutrient intake and absorption is decreased. Interestingly, in DSS-induced colitis, the LA-KD-fed mice had higher levels than the SFA-KD-fed mice. This difference was not observed in healthy animals. Unlike the KDs, supplementation with BHB did not result in significant elevation in plasma BHB in healthy animals nor upon DSS exposure.

5.2 Alleviation of intestinal inflammation by ketogenic diets

5.2.1 Ketogenic diets induce small inflammation-related changes in healthy intestine

First, the effects of the KDs were characterized in the healthy intestine. In the jejunum, minimal to mild villous subepithelial edema was observed as a response to both KDs (Figure 11A and 11C). The KDs also resulted increased jejunal vacuolation (Figure 11B). Since no changes were observed in the colon, the vacuolation was likely indicative of an increased small intestinal fatty acid uptake due to the higher intake of dietary fat While there was no significant difference between the groups upon scoring, the vacuolation was pronounced in the SFA-KD-fed mice. There were no differences in the jejunal inflammatory marker mRNA expression as a response to the KDs when compared to the CD mice, whereas *Il1b* was slightly higher in the SFA-KD group when compared to the LA-KD group (Figure 12D). In the original analyses, the KDs produced small but statistically significant changes in colonic transcription inflammatory markers. In the LA-KD group, the colonic expression of *Tnf* was elevated when compared to the CD group.

In comparison to the SFA-KD group, colonic *Il6* was higher in the LA-KD-fed mice in the original analysis, but these differences disappeared in the pooled analysis (Figure 12D).

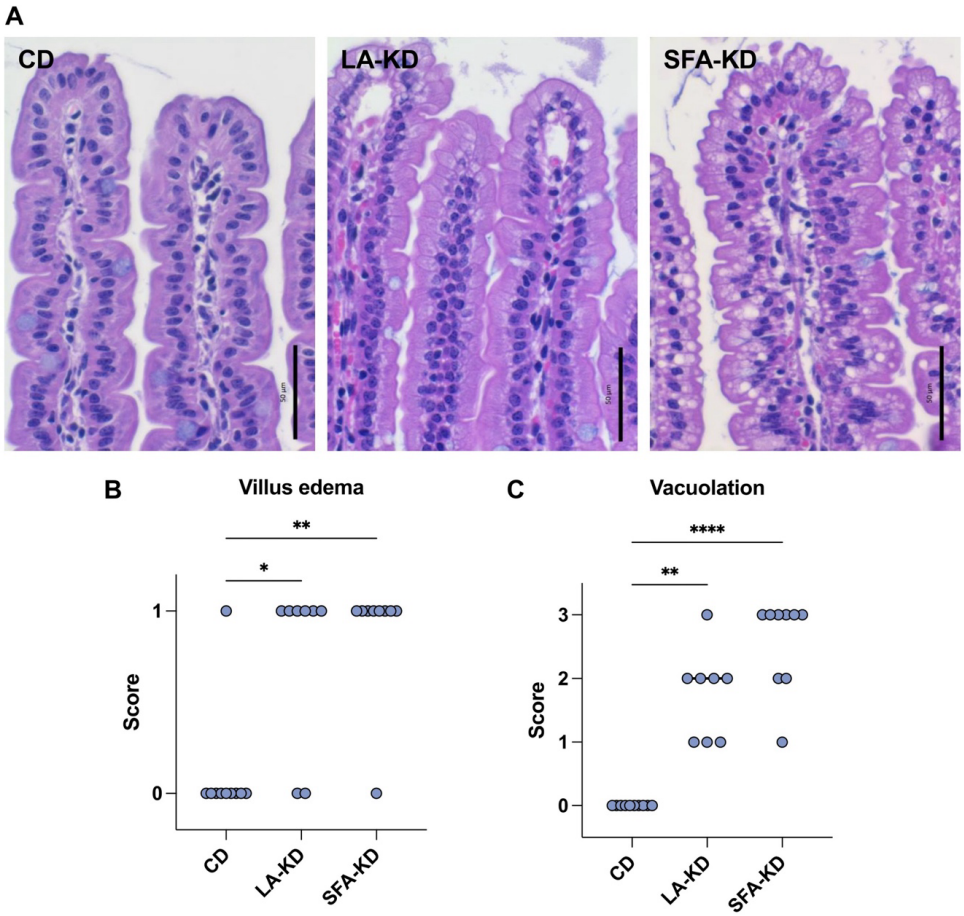
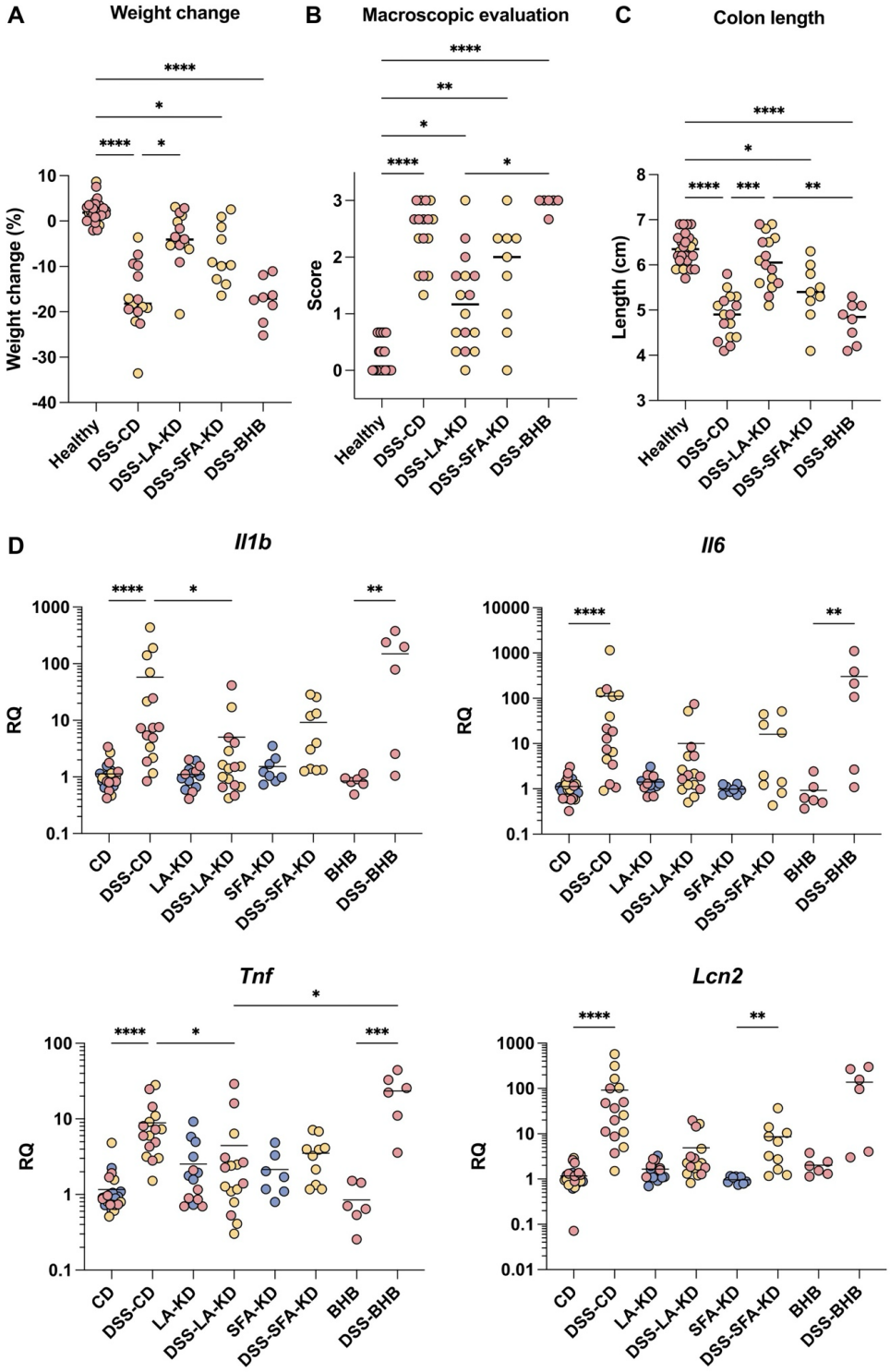


Figure 11. The KDs increased epithelial vacuolation in jejunal villi. A. Microphotographs of hematoxylin and eosin-stained jejunum sections from Study I showing increased vacuolation and minimal to mild edema in the KD groups. Adapted from the original publication under the Creative Commons license. B. Vacuolation score demonstrating the presence of vacuoles only in the KD-fed mice. C. Villus edema score illustrating the presence of villous subepithelial edema in the KD groups but not in the CD group. In B-C, ● = Study I. * $p < 0.05$, ** $p < 0.01$, **** $< p\ 0.0001$. CD = control diet, LA-KD = linoleic acid-rich ketogenic diet, and SFA-KD = saturated fat-rich ketogenic diet.

5.2.2 Ketogenic diets alleviate dextran sodium sulfate-induced colitis

The KDs were further studied after the induction of intestinal inflammation. Colitis was induced with DSS and manifested as weight loss, macroscopic and histological damage, and an elevated expression of inflammatory marker genes. Both KDs alleviated these DSS-induced changes. Weight loss, calculated as a percentage from the start of DSS administration, was mitigated (Figure 12A) and the severity of the macroscopic changes in the colon, *i.e.*, visible diarrhea, fecal blood, and inflammation, as well as colon shortening were reduced in the KD-fed animals (Figure 12B and 12C). However, the protection from the macroscopically visible damage disappeared in the pooled analysis (Figure 12B). While both KD groups exhibited improved colitis outcomes, the overall protective effect was pronounced with the LA-KD. Mice fed this diet had significantly lower mRNA levels of inflammatory markers than the DSS-CD mice (Figure 12D) and little to no histologically detectable damage or inflammation, defined by the levels of intestinal lesions and immune cell infiltration, respectively (Figure 13). While the DSS-SFA-KD mice were protected from colitis compared to the DSS-CD group, they had more histological lesions and immune cell infiltration than the DSS-LA-KD mice. Colonic levels of *Il1b* and *Lcn2* were modestly elevated in the DSS-SFA-KD group when compared to healthy controls in the original analysis, whereas in the pooled analyses, a difference was only observed in *Lcn2* expression. However, there was a lack of significant changes between the two DSS-KDs in all analyzed parameters.

Due to the protective effect of the KDs in DSS-induced colitis, the effect of exogenous BHB was tested to examine whether it would provide the same benefits. As opposed to the KDs, there was no benefit from continuous feeding with BHB. BHB administration increased mortality upon DSS exposure with three out of eight animals reaching the humane endpoint before the end of the study. BHB also aggravated the colitis-related weight loss and disease activity. Inflammatory marker gene transcription and histological changes were at similar as in the DSS-CD mice (Figures 12 and 13). No inflammation-related changes were detected in healthy mice administered BHB.


A
Weight change
Weight change (%)
10
0
-10
-20
-30
-40
****
*
****
*
Healthy
DSS-CD
DSS-LA-KD
DSS-SFA-KD
DSS-BHB
B
Macroscopic evaluation
Score
3
2
1
0
****
**
*
****
*
C
Colon length
Length (cm)
8
7
6
5
4
3
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*
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**
D
Il1b
RQ
1000
100
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1
0.1
****
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CD
DSS-CD
LA-KD
DSS-LA-KD
SFA-KD
DSS-SFA-KD
BHB
DSS-BHB
Il6
10000
1000
100
10
1
0.1
Tnf
100
10
1
0.1
*
***
Lcn2
0.01

Figure 12. The KDs protected from DSS-induced macroscopic changes and downregulated the mRNA expression of inflammatory markers. A. DSS-induced weight loss as a percentage showing the LA-KD to be protective. B. Macroscopic evaluation score indicating DSS-induced macroscopic damage in all groups. C. Colon length demonstrating the LA-KD to alleviate inflammation-related colon shortening. D. Colonic mRNA expression of inflammatory markers showing a protective effect from the KDs which is pronounced in the LA-KD mice. In A, B, and C, data are pooled from Studies II and III, and in C from Studies I-III. In the group Healthy, data are pooled from all animals without DSS exposure to obtain a clearer illustration as there are no differences between these groups. ● = Study I, ● = Study 2, and ● = Study 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $< p$ 0.0001. BHB = β-hydroxybutyrate-supplemented diet, CD = control diet, DSS= dextran sodium sulfate, DSS-CD = control diet with DSS, DSS-LA-KD = linoleic acid-rich ketogenic diet with DSS, DSS-SFA-KD = saturated fat-rich ketogenic diet with DSS, DSS-BHB = β-hydroxybutyrate-supplemented diet with DSS, LA-KD = linoleic acid-rich ketogenic diet, RQ = relative quantity, and SFA-KD = saturated fat-rich ketogenic diet.

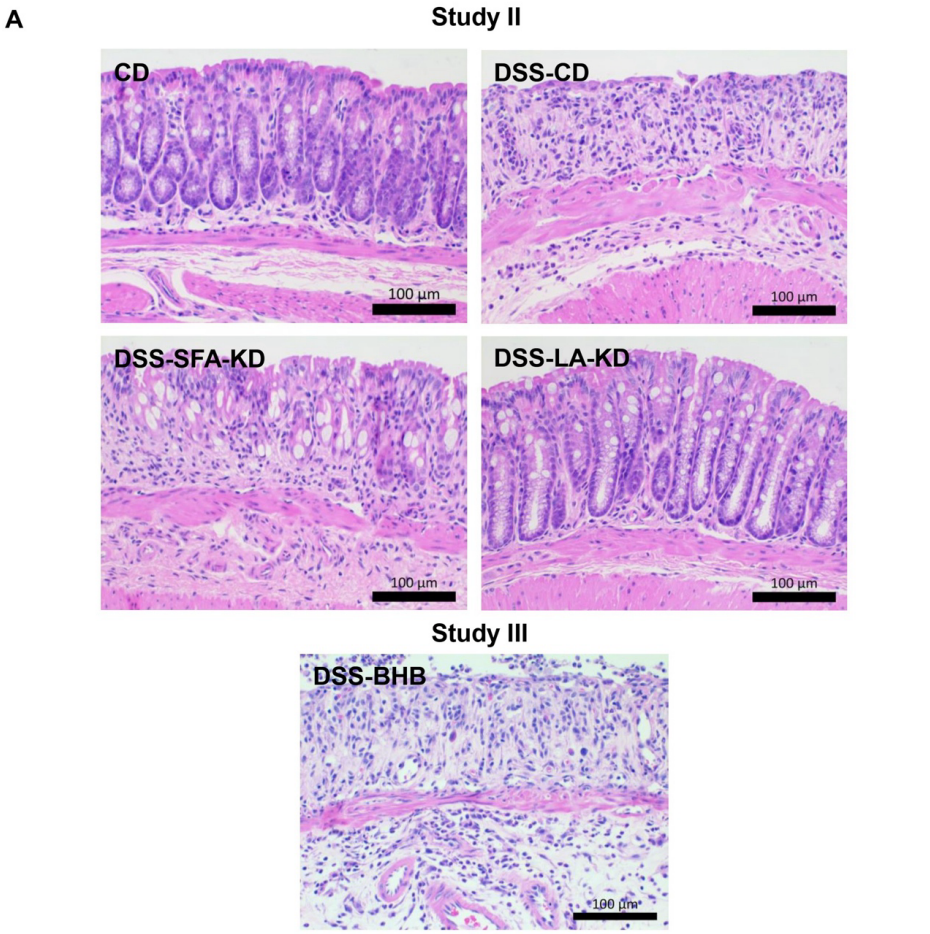
A
Study II
CD
DSS-CD
DSS-SFA-KD
DSS-LA-KD
100 µm
Study III
DSS-BHB
B
Tissue damage
C
Inflammation
D
Sum score
Score
Healthy
DSS-CD
DSS-SFA-KD
DSS-LA-KD
DSS-BHB

Figure 13. The KDs protected from DSS-induced histological damage. A. Microphotographs of hematoxylin and eosin-stained colon sections from Studies II and III. Adapted from the original publication under the Creative Commons license. B. Tissue damage score showing notable changes as a response to DSS to be mitigated by the KDs but not BHB. C. Inflammation score indicating prominent infiltration of inflammatory cells upon DSS administration to be alleviated by the KDs. D. Combined score of tissue damage and inflammation demonstrating extensive DSS-induced histopathological changes in the CD-fed and BHB-supplemented mice which are abated by the KDs. In B-D data are pooled from Studies II and III. In the group Healthy, data are pooled from all animals without DSS exposure for clearer illustration as there are no differences between these groups. In B-C, ○ = Study 2, and ○ = Study 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $< p\ 0.0001$. DSS-CD = control diet with DSS, DSS-LA-KD = linoleic acid-rich ketogenic diet with DSS, DSS-SFA-KD = saturated fat-rich ketogenic diet with DSS, DSS-BHB = β-hydroxybutyrate-supplemented diet with DSS.

5.3 Impact of ketogenic diets on intestinal barrier

5.3.1 Ketogenic diets do not influence intestinal permeability in healthy or in inflamed intestine

To determine whether intestinal permeability is affected by the KDs and their fat sources in healthy animals, iohexol was administered in Study I. Mice were given iohexol on days two and twenty-six to assess permeability upon the adaptation to the diet and after a longer-term feeding. No differences in permeability were found between the groups at either time point nor within the groups between the time points (Figure 14A).

As iohexol has only been validated for analyzing intestinal permeability from urine, FITC-dextran was used to measure permeability from plasma upon DSS exposure. DSS led to increased intestinal permeability to FITC-dextran (Figure 14B). Despite the overall protective effect of the KDs in experimental colitis, there were no differences in permeability between the DSS-CD and DSS-KD groups in the original analysis. This was also observed in Study III, where the LA-KD did not prevent the DSS-induced increase in permeability to FITC-dextran. Furthermore, no differences were detected in the pooled analysis (Figure 14B). In addition to

paracellular permeability, the activity of LPS in plasma was assessed in healthy animals as an indicator of its translocation from gut lumen to the systemic circulation. No significant differences between the groups were detected (Figure 14C).

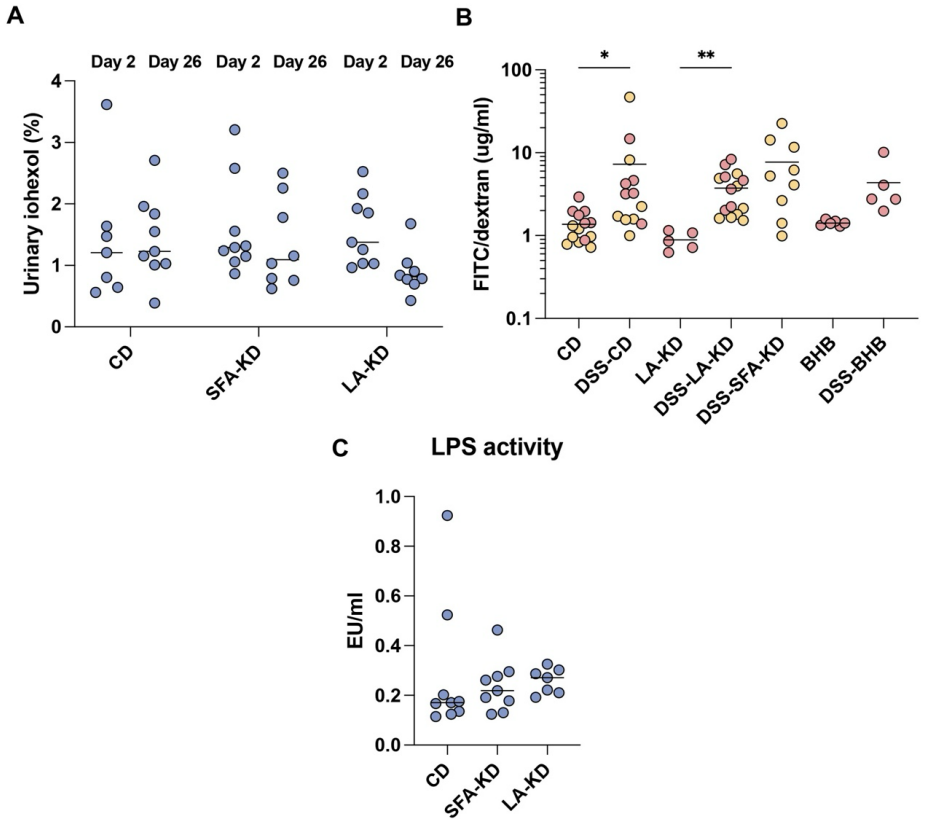

Figure 14. Intestinal permeability remained unchanged as a response to the KDs. A. Intestinal permeability measured as the percentage of iohexol in the urine after 24-h collection showing no between- or within-group differences. B. Intestinal permeability measured as the plasma concentration of FITC-dextran showing an increase upon DSS-exposure regardless of the diet. C. Plasma LPS activity indicating no significant difference as a response to the KDs. In B, data are pooled from Studies II and III. ● = Study I, ● = Study 2, and ● = Study 3. * $p < 0.05$, ** $p < 0.01$. BHB = β-hydroxybutyrate-supplemented diet, CD = control diet, DSS= dextran sodium sulfate, DSS-CD = control diet with DSS, DSS-LA-KD = linoleic acid-rich ketogenic diet with DSS, DSS-SFA-KD = saturated fat-rich ketogenic diet with DSS, DSS-BHB = β-hydroxybutyrate-supplemented diet with DSS, FITC-dextran = fluorescein isothiocyanate dextran, LA-KD = linoleic acid-rich ketogenic diet, LPS = lipopolysaccharide, and SFA-KD = saturated fat-rich ketogenic diet.

5.3.2 Ketogenic diets alter the expression of tight junction proteins

To characterize the effects of the KDs on other barrier properties, the expression of TJ proteins and TJ-protein coding genes was determined with Western blot and RT-qPCR, respectively. Although there were no differences in intestinal permeability, alterations were observed in the expression of colonic TJ proteins in both healthy animals and those with colitis, possibly indicative of early changes in barrier function that did not yet translate to increased permeability. In healthy animals, no differences were detected in the jejunum, but the SFA-KD induced a significant increase in colonic protein expression of claudins 1 and 4 when compared to both CD and LA-KD (Figure 15A). No differences in mRNA expression were evident in either part of the intestine (Figure 15C). While claudins 1 and 4 are considered to be barrier-sealing, the expression of both proteins was elevated also upon exposure to DSS. The LA-KD prevented the increase in their levels (Figure 15B). While the expression of claudin-2 and occludin was similar in healthy animals regardless of the diet, DSS induced a decrease in their mRNA and protein levels, respectively. For claudin 2, this was reflected as lower mRNA levels but not in the protein expression. This may be a result of decreased transcription that is not yet reflected in translation or of rapid protein turnover. Claudin-2 transcription remained at a healthy level as a response to the KDs, especially to the LA-KD. Unlike the LA-KD, the SFA-KD did not prevent the downregulation of occludin expression in Study II. While *Ocln* transcription remained at a relatively constant level despite DSS administration, the DSS-LA-KD mice had significantly higher levels than the DSS-CD mice in the original analysis for Study II. However, the significant result was not replicated in the new analysis. Protein expression data (Figures 15A and 15B) could not be pooled due to between-study differences in the method.

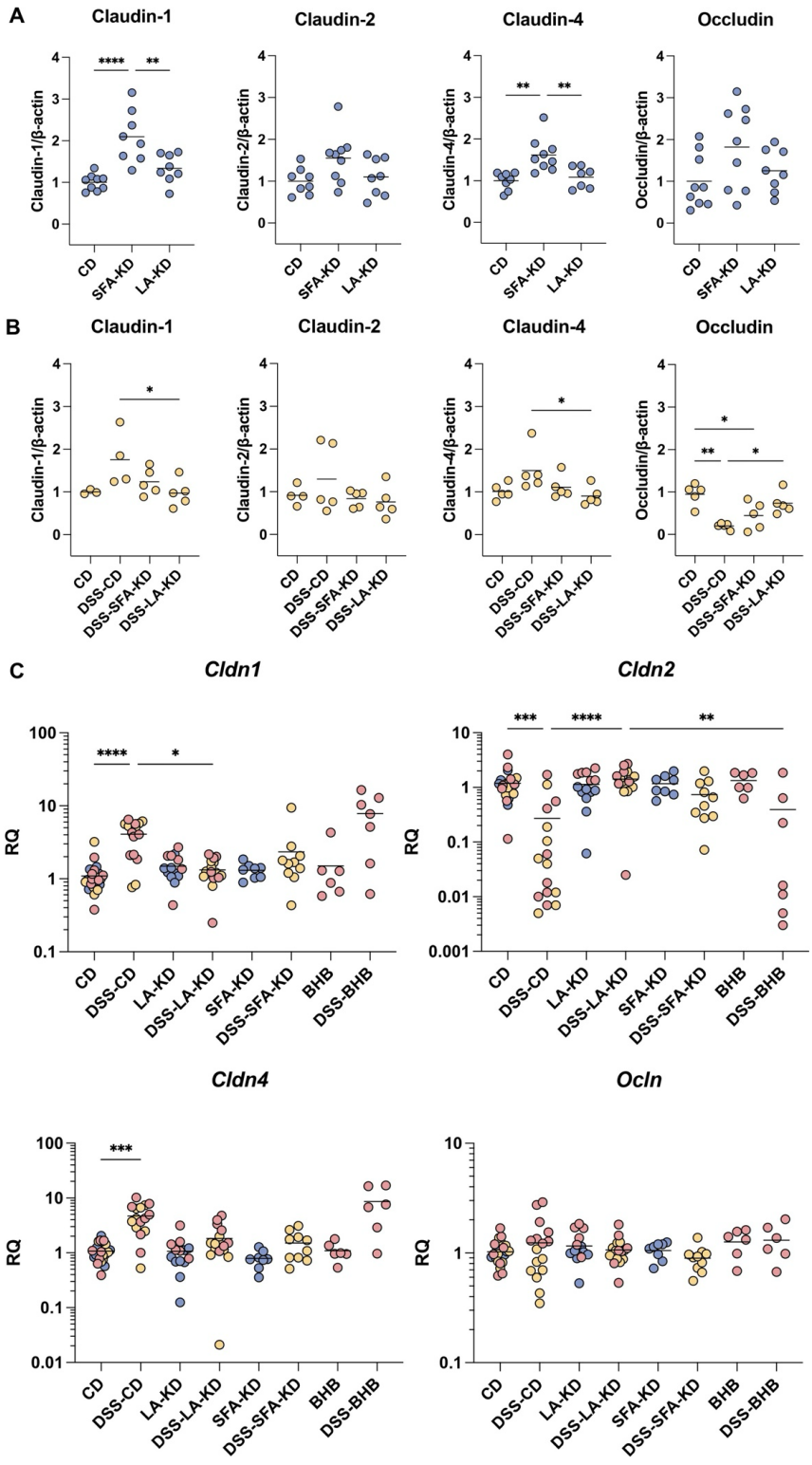
A
Claudin-1
Claudin-2
Claudin-4
Occludin
Claudin-1/β-actin
Claudin-2/β-actin
Claudin-4/β-actin
Occludin/β-actin
CD
SFA-KD
LA-KD
B
DSS-CD
DSS-SFA-KD
DSS-LA-KD
C
Cldn1
Cldn2
Cldn4
Ocln
RQ
LA-KD
DSS-LA-KD
SFA-KD
DSS-SFA-KD
BHB
DSS-BHB

Figure 15. The KDs influenced the colonic expression of TJ proteins. A. TJ protein expression in healthy animals analyzed with Western blot showing elevated expression of claudins 1 and 4 as a response to the SFA-KD. B. TJ protein expression in experimental colitis analyzed with Western blot showing the DSS-induced changes to be alleviated by the LA-KD. C. Transcription of TJ-protein-coding genes indicating the DSS-induced alterations to be mitigated by the KDs. In C, data are pooled from Studies I-III. ● = Study I, ● = Study 2, and ● = Study 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** < p 0.0001. BHB = β-hydroxybutyrate-supplemented diet, CD = control diet, DSS= dextran sodium sulfate, DSS-CD = control diet with DSS, DSS-LA-KD = linoleic acid-rich ketogenic diet with DSS, DSS-SFA-KD = saturated fat-rich ketogenic diet with DSS, DSS-BHB = β-hydroxybutyrate-supplemented diet with DSS, LA-KD = linoleic acid-rich ketogenic diet, RQ = relative quantity, and SFA-KD = saturated fat-rich ketogenic diet.

5.4 Modulation of gut microbiota by ketogenic diet

5.4.1 Ketogenic diet changes the composition of fecal microbiota

To determine whether the gut microbiota would affect the outcomes of colitis, 16S rRNA sequencing was used to characterize the composition of the gut microbiota before the DSS administration in Study III. Based on the Shannon entropy score, the LA-KD-feeding resulted in a higher α-diversity, *i.e.*, overall microbial diversity based on richness and evenness, but, according to the Faith Phylogenetic Diversity score, a less phylogenetically diverse microbiome (Figures 16A and 16B). There were no differences between the CD and BHB groups.

In the Unifrac analyses that measure β-diversity, the Unweighted Unifrac analysis revealed differences between all groups, suggesting different microbiotas when the presence or absence of unique lineages is considered (Figure 16C). However, the difference between the CD and BHB groups disappeared in the Weighted Unifrac analysis, indicating that their differences were not as distinct when relative abundance of the unique lineages was taken into account (Figure 16D). However, in terms of both lineage presence and their relative abundance, the LA-KD-feeding resulted in a distinct microbiota composition in comparison to the other groups.

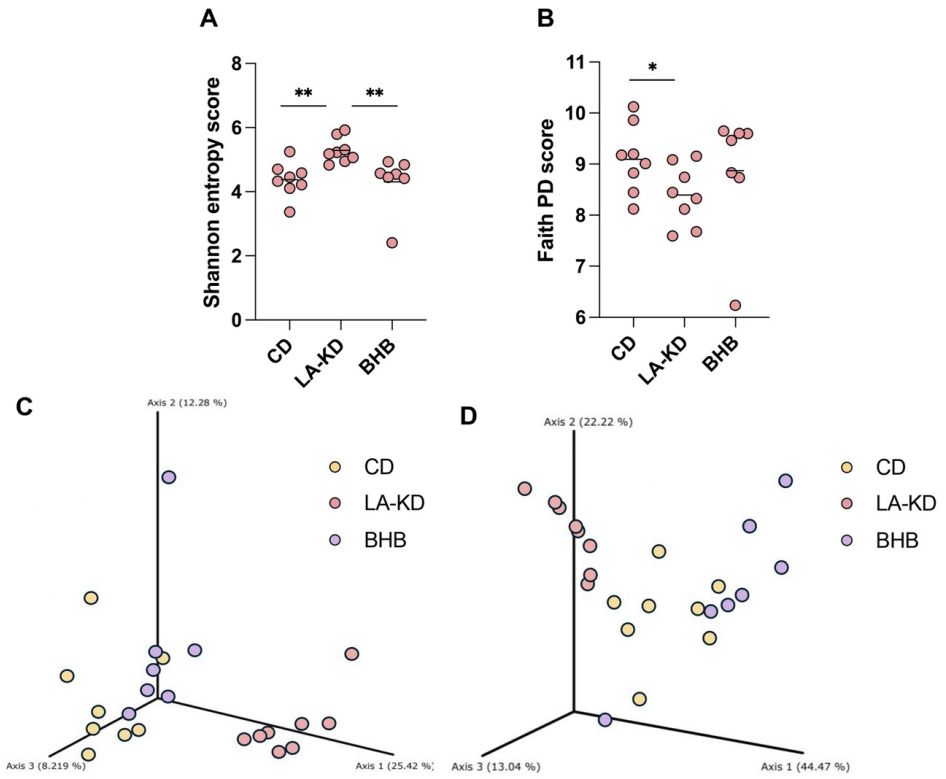

Figure 16. The KDs resulted in a distinct microbiota composition analyzed with 16S rRNA sequencing. A. Shannon diversity analysis indicating that the LA-KD group has a higher overall microbial diversity than CD and BHB groups. B. Faith phylogenetic diversity analysis suggesting that the LA-KD group to have a gut microbiome that is less phylogenetically diverse than in the CD and BHB groups. C. Unweighted Unifrac dissimilarity analysis demonstrating differences in microbial community composition between the diet groups. D. Weighted Unifrac dissimilarity analysis showing that the LA-KD group has a distinct microbial community composition compared to the CD and BHB groups. In A and B, ○ = Study 3. * $p < 0.05$, ** $p < 0.01$. BHB = β-hydroxybutyrate-supplemented diet, CD = control diet, LA-KD = linoleic acid-rich ketogenic diet.

5.4.2 Ketogenic diet promotes distinct genus-level differences in fecal microbiota

ANCOM-BC analysis was used to identify genus-level differences between the groups in the fecal microbiota in Study III. The differences here describe the features in which there is a statistically significant difference in the relative abundance larger than $\log_{10}2$. The LA-KD upregulated the levels of *Coprococcus*,

Peptococcaceae, *Defluviitalea*, while downregulating those of *Sutterella*, and, as expected, *Bifidobacterium* (Figure 17). Compared to the CD, the LA-KD also increased *Clostridia*, *Anaerotruncus*, *Enterobacteriaceae*, *Biophila*, and *Ruminococcus* but downregulated *Anaeroplasma*. BHB supplementation downregulated *Lactobacillus* and the butyrate-producing *Butyricicoccus* when compared to both the CD and LA-KD. Expectedly, the levels of *Bifidobacterium* and *Anaeroplasma* were lower in the BHB group than in the CD group, whereas those of *Enterococcus* were higher. Compared to the LA-KD, the animals consuming BHB exhibited lower levels of *Lactococcus*.

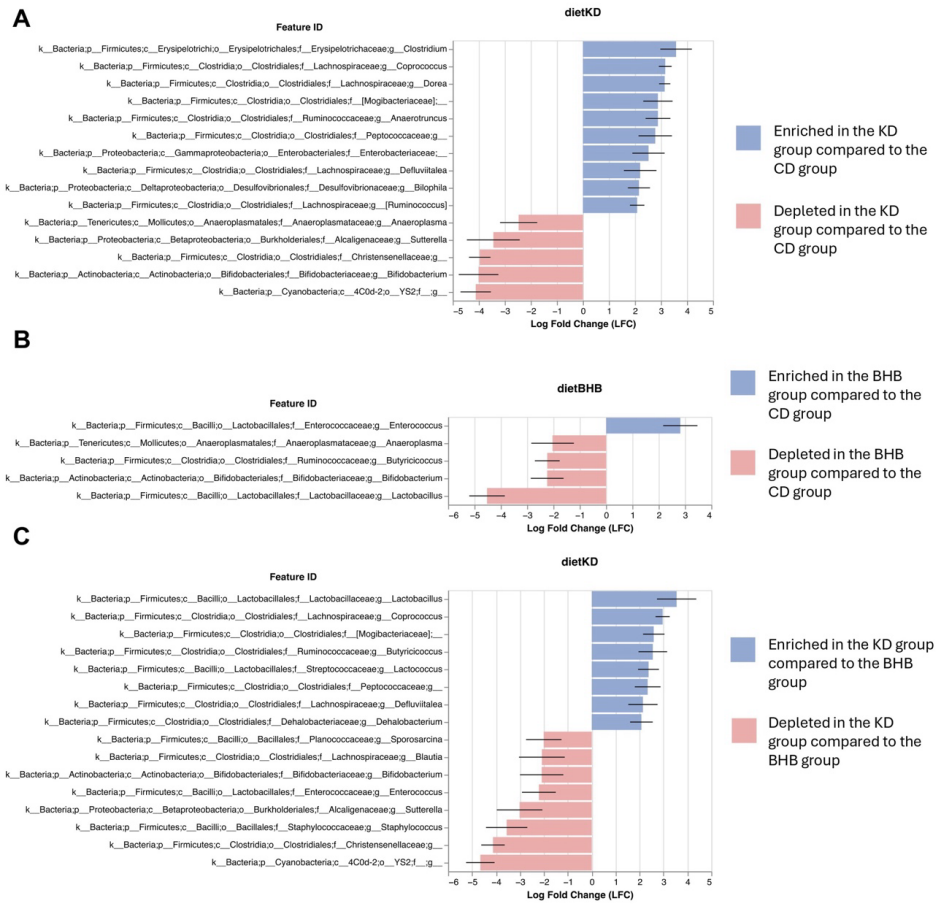

Figure 17. The KD changes gut microbiota composition based on ANCOM-BC analysis results of A. CD group vs. LA-KD group, B. CD group vs. BHB group, and D. KD group vs. BHB group. ANCOM-BC = Analysis of Composition of Microbiomes with Bias Control, BHB = β-hydroxybutyrate-supplemented diet, CD = control diet, LA-KD = linoleic acid-rich ketogenic diet.

5.5 Ketogenic diet-related alterations in the intestinal renin-angiotensin-aldosterone-system

Samples for Study IV were obtained from animal experiments in Studies I and II. Thus, in this section, samples from the different experiments are distinguished with the previously used colors: ● = Study I and ● = Study 2.

5.5.1 Ketogenic diets upregulate AT2R expression in healthy jejunum

As KDs can influence RAAS-mediated intestinal processes, such as fluid, electrolyte, and nutrient transport, the impact of the KDs on the expression RAAS components on protein and mRNA levels was first characterized in the healthy intestine. The results from the Western blot and RT-qPCR analyses indicated that the KDs exerted no influence on the expression of the studied components of the classical RAAS pathway in healthy jejunum or colon. However, changes in the alternative pathway were observed in jejunum: *Ace2* mRNA expression was lower in the KD groups when compared to controls (Figure 18A). The KDs also upregulated jejunal AT2R expression regardless of the dietary fat source without differences in *Agtr2* transcription (Figure 18B). In the colon, the components of the alternative RAAS pathway were unaffected by the dietary interventions.

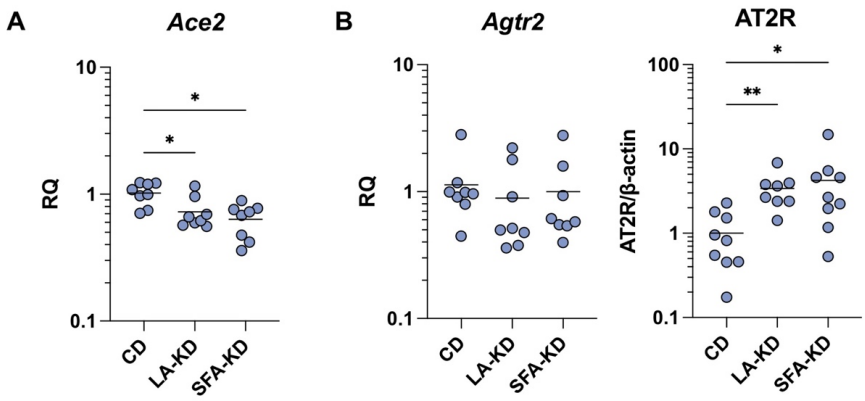

Figure 18. The KDs modulate the jejunal expression of selected RAAS components. A. mRNA expression of *Ace2* indicating decreased transcription as a response to the KDs. B.

mRNA and protein expression of AT2R showing elevated protein expression but not transcription in the mice consuming the KDs. ● = Study I. * $p < 0.05$, ** $p < 0.01$. CD = control diet, LA-KD = linoleic acid-rich ketogenic diet, RQ = relative quantity, and SFA-KD = saturated fat-rich ketogenic diet.

5.5.2 Ketogenic diets modify the expression of the intestinal renin-angiotensin-aldosterone-system components in inflammation

In inflammatory conditions, the balance between the RAAS pathways is inclined towards the classical pathway with the alternative one being less active. The impact of the KDs on RAAS pathways components was investigated also upon intestinal inflammation to clarify whether their protective effects extended to this system. DSS administration upregulated the mRNA expression of the angiotensinogen-coding gene *Agt* and this was prevented in the DSS-SFA-KD mice (Figure 19A). While the transcription of the classical pathway component *Ace1*, coding for ACE protein, was not elevated in the DSS-CD group, the KDs resulted in divergent changes in its levels with the DSS-LA-KD mice exhibiting higher levels than the DSS-SFA-KD group (Figure 19B). The levels in the DSS-LA-KD group were also significantly higher than in the DSS group. However, DSS resulted in elevated protein expression of ACE which was not observed in the DSS-KD groups despite the differences in mRNA expression. For the KDs, this may reflect a delay in translation or indicate rapid protein turnover. In line with the hypothesis, the DSS-SFA-KD mice exhibited lower expression of AT1R at both the protein and gene levels in the original analyses, but the difference in protein expression disappeared in the pooled analysis (Figure 19D). In the original analysis, the DSS-LA-KD group showed lower *Cyp11b2* mRNA expression than DSS-CD group, but this difference no longer reached significance in the pooled analysis. With respect to the alternative pathway components, DSS resulted in lower levels of *Ace2* mRNA expression, and this was prevented in the DSS-LA-KD mice but not in the DSS-SFA-KD group (Figure 19C). The transcription of *Agtr2* was also lower as a response to DSS but both KDs maintained its levels (Figure 19E). AT2R protein levels were similar in all groups. In the DSS-CD group, the discrepancy between the mRNA and protein levels may reflect a slow protein turnover.

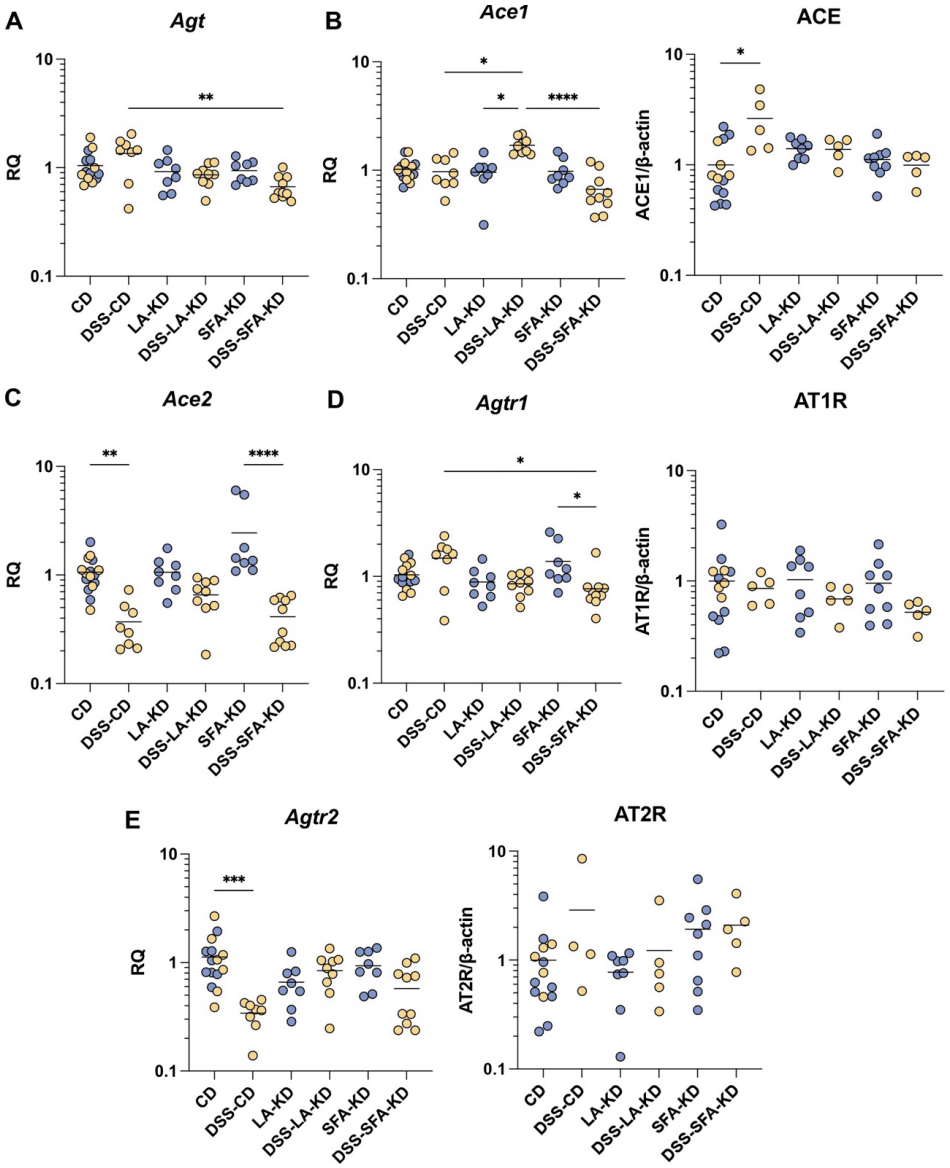

Figure 19. The KDs influence the colonic expression of selected RAAS components. A. mRNA expression of *Agt*. B. mRNA and protein expression of ACE. C. mRNA expression of ACE2. D. mRNA and protein expression of AT1R. E. mRNA and protein expression of AT2R. ● = Study I and ○ = Study 2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** < p 0.0001. CD = control diet, DSS= dextran sodium sulfate, DSS-CD = control diet with DSS, DSS-LA-KD = linoleic acid-rich ketogenic diet with DSS, DSS-SFA-KD = saturated fat-rich ketogenic diet with DSS, LA-KD = linoleic acid-rich ketogenic diet, RQ = relative quantity, and SFA-KD = saturated fat-rich ketogenic diet.

6 Discussion

In this thesis, consisting of four studies, the effects of KDs, their fat source, and exogenous ketones on intestinal inflammation, barrier function, and local RAAS expression were investigated. The studies were conducted in healthy mice and in the DSS model of intestinal inflammation using male C57BL/6J mice that were exposed to dietary interventions for four to five weeks. In DSS-induced colitis, the KDs alleviated inflammation and there was a pronounced benefit from a diet rich in LA compared to a diet high in SFAs. Intestinal permeability remained unchanged as a response to the dietary interventions in both healthy and diseased animals. However, the KDs prevented specific DSS-related changes in the expression of intestinal RAAS. Thus, the KDs mitigate DSS-induced colitis without influencing intestinal permeability and the magnitude of the effect depends on the fat source of the diet with an added benefit from the LA-rich diet.

6.1 Ketogenic diets lead to increased energy intake but not weight gain

A KD is an effective weight loss strategy for humans (Bueno et al., 2013), often attributed to the diet's beneficial effects on satiety (Gibson et al., 2015). Although weight management was not the focus of this thesis, here, after an initial weight loss, healthy mice fed the KDs were observed to have an equal weight gain in comparison to those consuming the CD. At odds with to these results, some earlier studies reported weight to drop at the beginning of a KD and to remain at a level 15 % lower than the initial weight (Jornayvaz et al., 2010; Kennedy et al., 2007). In these diets, only ~ 5 % of the energy was derived from protein. It was later discovered that the protein content of the diet is a determining factor in the weight

change as KDs with 10 % or more of energy from protein lead to similar or higher weight gain as a low-fat, control diet (Newman et al., 2017; Stemmer et al., 2015). The KDs used in this thesis had 9.4 % of energy from protein which was still sufficient for normal weight development.

KDs have been postulated to influence energy expenditure by first increasing and later decreasing it (Basolo et al., 2022). In this thesis, despite displaying a similar weight gain, healthy mice fed the LA-KD had a significantly higher energy intake than controls. The same was observed for the SFA-KD in Study II and when results of the studies were pooled. In mice, KDs containing up to 8.6 % protein have been studied in the context of energy expenditure and have been shown to elevate it (Kennedy et al., 2007; Pissios et al., 2013). An elevated expenditure would explain why the increased energy intake did not lead to a greater body weight gain in the KD-fed animals in comparison to controls. However, adaptation to the ketogenic metabolism happens over time. As the mice in this thesis were only fed for four weeks, it might be that after a longer exposure to a KD, energy expenditure lowers, and intake drops to a level comparable to controls. In addition to changes in expenditure, KDs can also decrease energy absorption (Li et al., 2024). Mechanistically, the KD-induced changes in gut microbiota result in elevated levels of specific bile acids in serum, the downstream effect of which is to reduce the absorption of energy. Thus, the KD-related changes in energy absorption might also explain the discrepancy between the higher energy intake but the similar weight gain compared to controls.

6.2 Ketogenic diets protect from colitis with a more pronounced benefit from a linoleic acid-rich diet

Regular high-fat feeding may lead to pro-inflammatory changes in the intestine even without baseline inflammation. In this thesis, no histologically detectable changes were observed in the colon in healthy mice consuming the KDs, but jejunal enterocytes exhibited increased vacuolation and minimal to mild edema was evident in the subepithelial villi. As the diets resulted in little change in the transcription of inflammatory markers in healthy jejunum, the vacuolation detected

was likely a physiological response since the formation of lipid droplets and chylomicrons is upregulated after an increased intake of dietary fat (D'Aquila et al., 2016; Zhu et al., 2009). Since only mRNA but not protein expression of inflammatory cytokines was quantified, pro-inflammatory changes in the intestine upon the KDs cannot be fully excluded. Nonetheless, it is more likely that the observed mild subepithelial edema was a result of the flow of chylomicrons towards the villus lacteals as a response to high-fat feeding (Ko et al., 2020; Li et al., 2023). This observation corroborates previous findings where male mice fed a KD had an increased jejunal triglyceride mass upon fasting and refeeding caused an upregulation of triglyceride secretion (Morrow et al., 2022). The SFA-KD resulted in a higher amount of vacuoles than the LA-KD, which may be a result of a more efficient secretion of LA- than SFA-rich triglycerides from enterocytes as had been observed in Caco-2 cells (van Greevenbroek et al., 1996; van Greevenbroek et al., 1995). Overall, in healthy intestine, the KDs result in changes reflecting increased fatty acid transport, a logical finding after high-fat feeding, without having pro-inflammatory effects.

The DSS-induced colitis resembles human IBDs with regard to some key immunological and histopathological features and thus, was chosen to model intestinal inflammation. The DSS administration resulted in weight loss, macroscopical and histological damage to the colon, upregulation of colonic mRNA expression of inflammatory markers, increased intestinal permeability, and abnormal expression of TJ proteins. The KDs alleviated the DSS-induced inflammation, observed as less histological damage and a lower expression of inflammatory markers. These findings are in line with Kong et al. (2021) and Abdelhady et al. (2023) who also observed KDs to improve outcomes in experimental colitis and contrast those of Li et al. (2021a) who reported a KD to lead to the aggravation of intestinal inflammation. These contradictory results from previous investigations led this thesis to explore whether the direction of the effect could be dependent on the fat source of the diet. However, both KDs led to improved outcomes, weakening the hypothesis that the worsening of colitis by a KD could be attributed to the type of fat used. Nevertheless, the protective effect was more pronounced with the LA-KD as compared to the SFA-KD. In healthy animals, the

LA-KD resulted in a slight upregulation in the colonic transcription of *Il6* and *Tnf* when compared to the CD and SFA-KD, respectively. It should be noted that the findings at the level of transcription level do not necessarily translate to protein expression (Kosti et al., 2016) and thus, there might have been little between-group difference in the inflammatory profiles at the protein level. Nevertheless, this small elevation in the transcription of inflammatory cytokines did not lead to worse outcomes upon DSS exposure. In a preclinical study, supplementation with LA protected from aggravation of colitis with *Porphyromonas gingivalis* by decreasing the ratio between T regulatory cells and T helper 17 cells (Jia et al., 2024). As a KD has also been demonstrated to decrease the levels of intestinal T helper 17 cells (Ang et al., 2020), the synergistic effect of LA and a KD on T cell-mediated inflammation may be one differentiating factor between the two KDs.

Many of the anti-inflammatory properties of KDs have been attributed to BHB which can block the NLPR3 inflammasome (Youm et al., 2015). Indeed, this is one of the pathways through which both KD and BHB may protect from the DSS-induced colitis as was observed in a rat model (Abdelhady et al., 2023). Thus, an elevation in ketone body levels may be one of the mechanisms through which KDs confer protection from colitis. While there were no differences between the KDs in plasma levels of BHB in healthy animals, higher levels were observed in the LA-KD-fed mice compared to those given the SFA-KD upon DSS exposure. This could partly explain the differences in outcomes between the KDs in Study II. However, contrary to the possible protection from endogenously produced BHB and previous observations with exogenous administration, Study III detected no evidence protection and even signs of harm, such as increased mortality, from BHB supplementation. This contrasts with the findings of others where intragastric, intraperitoneal, or rectal administration of BHB has led to protection or improved recovery from colitis in rodents (Abdelhady et al., 2023; Huang et al., 2022; Li et al., 2021b) These discrepancies might be a result of the different administration regimes as animals in Study III had constant access to BHB-enriched food whereas in other studies the compound was given once a day. Furthermore, the form of BHB may influence the outcomes. This study used the free acid form of R-BHB which is the exact same form the body endogenously produces. In healthy humans, free acid

R-BHB is well-tolerated (Pimentel-Suarez & Soto-Mota, 2023). However, the relative dose used in Study III was notably higher and the compound was administered to sick animals. While the dose was determined based on previous studies using BHB salts and esters, these experiments were not conducted on models of intestinal inflammation. On the other hand, BHB supplementation did not cause a significant elevation in plasma levels in Study III. An intraperitoneal BHB injection once a day resulted in levels higher than a KD at eight hours post-administration and an alleviation of colitis (Abdelhady et al., 2023). The pharmacodynamics of orally supplemented free acid BHB have not been studied and hypothetical features of the specific form, such as poor absorption or rapid degradation, might explain the lack of effect. It will be essential to perform pharmacodynamic studies on this form of BHB to clarify the results presented here. In conclusion, these results do not exclude the possibility of benefits from increased levels of circulating ketone bodies via endogenous production, such as obtained with a KD, or via exogenous administration.

6.3 Ketogenic diets protect from colitis independently of changes in intestinal permeability

Intestinal permeability *in vivo* can be measured with different, orally administered probes (Vancamelbeke & Vermeire, 2017). Study I used iohexol as a probe to assess paracellular permeability in healthy animals fed the KDs, whereas FITC-dextran was chosen for Studies II and III. The concentration of iohexol reliably indicates the level of permeability when measured from urine (Frias et al., 2012; Halme et al., 2000), but its assessment from plasma has not been validated for intestinal permeability measurements. The measurement from urine becomes problematic in the DSS model due to diarrhea which increases the risk of fecal contamination. Thus, FITC-dextran was used as the permeability probe and analyzed from plasma in Studies II and III.

Previous reports have shown regular HFDs (Cani et al., 2008; Stenman et al., 2012) and specific fats (Lam et al., 2015) to increase intestinal permeability in mice. However, in Study I, the KDs did not influence paracellular permeability to iohexol

in healthy animals regardless of the fat source. The systemic impacts of HFDs are generally inflammatory (Duan et al., 2018) which may explain the difference compared KDs which tend to be more anti-inflammatory (Ji et al., 2024). In addition, the HFD-induced intestinal permeability is dependent on the microbiota (Muller et al., 2016). As the microbiota can influence intestinal permeability and the HFDs and KDs lead to different microbiota compositions (Ang et al., 2020), this is another possible factor explaining the differences. Finally, iohexol was used to assess permeability of the whole intestine whereas permeability in individual parts of the intestine was not determined here. Multi-sugar tests *in vivo* or *ex vivo* Ussing chamber experiments could further elucidate whether the diets altered permeability in specific regions of the intestine. Overall, the results of this thesis indicate that fat quantity or quality *per se* do not drive intestinal permeability, but their effects depend on the overall composition of the diet.

Intestinal inflammation is associated with elevated intestinal permeability (Halme et al., 2000) and treatments that alleviate the inflammation can also lower permeability (Suenaert et al., 2002). Interestingly, no differences in permeability to FITC-dextran were observed in Studies II and III between the DSS groups and there was even a trend towards increased permeability as a response to the KDs in the presence of inflammation. Again, the permeability in individual regions of the intestine was not assessed. As the DSS-induced inflammation mainly affects colon, more specific analyses of colonic permeability could be informative. However, FITC-dextran is often used as the permeability probe in the DSS model and between-group differences are readily observed (Li et al., 2021a). Thus, it is unlikely that a different method would drastically alter the results that suggest that the protective effect of the KDs is not linked to paracellular permeability.

In addition to intestinal permeability, increased LPS translocation through the intestinal epithelia followed by elevated plasma LPS activity can result from high-fat feeding, especially if the diet is high in coconut or palm oil derived SFAs (Candido et al., 2020). As this has been mainly studied in the context of regular HFDs, Study I investigated whether elevated LPS activity would be observed also in response to the KDs. LPS activity remained unchanged between groups which may

indicate equal between-group levels of LPS translocation from the gut lumen to the systemic circulation and/or an upregulation in LPS detoxification in response to the KDs. Again, the KDs elicit an effect different from regular HFDs, highlighting that the effects of dietary fat on LPS functions are context dependent.

TJs are key regulators of intestinal permeability and thus, the expression and function of TJ proteins provide valuable information on barrier function. Therefore, their expression was quantified to further characterize the effects of the KDs on the properties of the intestinal barrier. In healthy animals, the SFA-KD but not the LA-KD promoted the colonic protein expression of barrier-sealing claudins 1 and 4 despite there being no differences in paracellular permeability to iohexol. However, this is logical as claudins are more intimately involved in the proper function of the pore pathway as opposed to the leak pathway utilized by iohexol. Intriguingly, the protein and mRNA expression of these claudins was upregulated upon DSS-induced inflammation in Study II and this was confirmed for mRNA expression in Study III. The elevation was mitigated by the KDs. It should be noted that the expression of only a selected few TJ proteins and the genes coding for them were analyzed here and that paracellular permeability is regulated not only via the expression of TJ proteins but also through their dynamics. Thus, higher throughput methods, such as mass spectrometry or microarrays, and analyses on structural changes in TJs would provide added information on TJ function as a response to the consumption of the KDs. Nevertheless, in line with the results obtained here, the expression of claudins 1 and 4 were elevated in active IBD, and the levels of claudin-1 correlated with the extent of inflammatory activity (Weber et al., 2008). This indicates that the elevated claudin expression might be a compensatory response as opposed to a beneficial change also in the healthy, SFA-KD fed animals. Moreover, the overexpression of claudin-1 not only increases susceptibility to but also impairs recovery from the DSS-induced colitis by decreasing the expression of mucin-2 (Pope et al., 2014), indicating that an increase in its expression might not be advantageous. Based on these findings, elevated baseline expression of claudins might be a factor related to the smaller protective effect from the SFA-KD than obtained from the LA-KD.

6.4 Changes in gut microbiota are linked to the beneficial effects of the ketogenic diet on colitis

IBDs are linked to compositional and metabolic alterations in the intestinal microbiota (Ni et al., 2017). Since these diseases also respond to antibiotic treatments (Wang et al., 2012), it is likely that gut microbiota contributes to the development of intestinal inflammation. As the DSS-induced colitis is also dependent on the microbiota (Hernandez-Chirlaque et al., 2016; Kitajima et al., 2001), in Study III, the composition of the fecal microbiota prior to the DSS administration was mapped to investigate whether differences in microbiota would predict the outcomes of colitis. Indeed, it was found that the KD led to a notably different microbiota composition as opposed to the low-fat control diet with or without BHB. This microbiota was characterized by elevated levels of *Coprococcus*, *Peptococcaceae*, *Defluviitalea* and lower levels of *Sutterella*, and *Bifidobacterium* when compared to the two other groups. As others have reported the pre-DSS microbiota to predict colitis outcomes (Forster et al., 2022), the aim here was to study whether that would hold true in the context of a KD as well. However, the post-DSS microbiota was not characterized. Therefore, the extent to which the microbiotas between the groups differed after the DSS administration was left unclear. Furthermore, analyses on microbial metabolites were not conducted. As the metabolite profile of the microbiota mostly mediates its effects on the host's physiology (Blakeley-Ruiz et al., 2019), characterization of the metabolites would provide a deeper understanding of the connections between KDs, microbiota, and colitis. Nevertheless, based on the previous literature, the effects of a KD on experimental colitis are related to the microbiota as a fecal microbiota transplant from KD-fed mice alleviates inflammation also in mice fed a low-fat control diet (Kong et al., 2021), suggesting that the protective effects reported in this thesis may also be a result of the changes in the microbiota.

The aim was to compare the fecal microbiota between the SFA-KD- and LA-KD-fed animals, but multiple samples were lost in transit, leaving the sample size of the KD groups too small to be analyzed. However, the type of dietary fat influences microbiota composition (Schoeler et al., 2023). Thus, while it cannot be concluded

that the differences in microbiota between mice fed the SFA-KD and the LA-KD led to the differing outcomes in colitis, it is a possibility that warrants further investigation.

A KD and exogenous BHB inhibit the growth of *Bifidobacteria* as BHB is directly toxic to these bacteria (Ang et al., 2020). In line with this finding, in Study III, both the diet and BHB resulted in a decrease in *Bifidobacteria,* and the effect was more potent in mice consuming the KD, suggesting that the bacteria would also respond to carbohydrate restriction. This is logical as these bacteria mainly metabolize carbohydrates (Pokusaeva et al., 2011). The loss of *Bifidobacteria* as a response to a KD or exogenous ketones decreases the numbers of intestinal inflammatory T helper 17 cells (Ang et al., 2020) which has been postulated to be one of the protective mechanisms of the diet. On the other hand, in Study III, the LA-KD and BHB had contrary effects on experimental colitis despite both decreasing the levels of *Bifidobacteria* and thus, this change does not completely explain the results related to colitis. Others have observed that a KD increases the abundance of genera considered beneficial such as *Akkermansia* and *Roseburia* and lead to protection from colitis (Kong et al., 2021). However, no such changes were detected in Study III. These discrepancies suggest that the microbiota-related effects of KDs on experimental colitis are likely not a result of changes in the individual genera of bacteria but more likely an outcome of the diet-induced changes in the microbial community and its metabolites as a whole.

6.5 Ketogenic diets maintain a more anti-inflammatory local renin-angiotensin-aldosterone system expression in colitis

Dietary factors can influence the function of RAAS and KDs direct the expression of local renin-angiotensin system in the adipose tissue and lung towards the alternative pathway (Da Eira et al., 2021; Da Eira et al., 2023). The main components of RAAS are also expressed in the intestine (Fändriks, 2011) where the system participates in several physiological processes such as the transport of fluid,

electrolytes, and nutrients. As KDs influence fluid and electrolyte balance and the glucose supply of the diet is restricted, Study IV investigated whether these diets would change the expression of RAAS components in the healthy intestine. The KDs induced small changes in the jejunum where both diets upregulated *Ace2* transcription and AT2R protein expression. While ACE2 is involved in amino acid uptake (Hashimoto et al., 2012), AT2R activity appears to enhance jejunal fluid absorption (Jin et al., 1998). As the KDs lead to elevated renal excretion of fluid and electrolytes, this increase in AT2R levels may represent a compensatory mechanism. While the KDs had little overall impact on intestinal RAAS expression, the changes observed may be reflective of compensatory mechanisms against the KD-related changes in fluid and nutrient homeostasis.

The activity of the classical RAAS pathway is increased in inflammatory conditions and the alternative pathway becomes less active (Fändriks, 2011). In the DSS-induced colitis, the KDs helped to maintain the levels of specific RAAS components at a level comparable to healthy controls. The *Agt* gene codes for the angiotensinogen peptide which is the precursor for all downstream RAAS peptides. DSS increased the transcription of this gene which may be indicative of overall upregulated RAAS activity. This increase was prevented in the SFA-KD-fed mice. While this might be a result of the anti-inflammatory effect of the diet, mice deficient in *Agt* develop less severe colitis (Inokuchi et al., 2005), and thus, the decrease in its expression may also be a contributing factor in the protective effect of the diet. With respect to the classical RAAS components, both KDs prevented the DSS-induced increase in the protein levels of ACE despite its gene expression being elevated in DSS-LA-KD. Again, the lower protein expression of ACE may be a consequence of the anti-inflammatory effects of the KDs. However, this enzyme is recognized to exacerbate intestinal inflammation via the production of angiotensin II (Shi et al., 2016; Spencer et al., 2007) and thus, its downregulation may also have contributed to the alleviation of colitis. AT1R expression was downregulated at both the protein and gene level in the DSS-SFA-KD group. As most of the inflammatory effects of the classical pathway are downstream of this receptor (Cantero-Navarro et al., 2021), this likely is a consequence of the overall anti-inflammatory effects of the SFA-KD diet. With respect to the anti-alternative pathway components, the

DSS-LA-KD mice maintained the level of *Ace2* expression, but the DSS-SFA-KD animals did not. While ACE2 activity is considered to be anti-inflammatory and *Ace2* knockout leads to aggravation of colitis (Hashimoto et al., 2012), pharmacological inhibition of ACE2 alleviates it (Byrnes et al., 2009). Thus, the role of ACE2 in colitis is unclear but lower *Ace2* expression may reflect more severe inflammation. Finally, the KDs prevented the downregulation of *Agtr2* expression. Overall, the KDs maintain a more anti-inflammatory RAAS profile upon colitis with some of the effects dependent on the dietary fat source. While some of the differences between the KDs with different fat sources correlated with the colitis outcomes, this was not consistently observed. Based on these findings, it is unlikely that the KD-related changes in intestinal RAAS expression had a significant effect on the prevention of colitis. Rather, the changes in RAAS component expression were more likely a result of lower DSS-induced inflammation in the KD groups.

6.6 Clinical relevance and future directions

IBDs significantly decrease the life quality and expectancy of patients (Kuenzig et al., 2020). These diseases are becoming increasingly common (Wang et al., 2023) and while pharmacotherapy is effective in most patients, many individuals experience difficult adverse effects or fail to respond to treatment (Alsoud et al., 2021). Thus, the development of novel therapies is important. Currently, KDs are being studied as a treatment for a variety of metabolic and neurological disorders. However, there are no published or registered clinical trials on the effects of KDs on intestinal diseases at the moment of writing. While the findings of this preclinical study cannot be extrapolated to humans, they do hold promise that a KD may represent a potential way to alleviate IBDs. As KDs appear to be a safe intervention for many chronic diseases and there is no strong contraindication for studying their effects in patients with IBD, human trials testing the safety and efficacy of KDs for this condition are warranted.

This thesis studied experimental colitis in young adult male mice. As the DSS-induced inflammation is influenced by sex, with females being more resistant to colitis than males (Bábíčková et al., 2015; Mähler et al., 1998), it is important to

characterize the effects of KDs, their fat sources, and exogenous ketones also in females. In addition, the metabolic effects of KDs may differ between females and males (Smolensky et al., 2023), which further highlights the importance of conducting studies in both sexes. In addition to sex, age influences the severity of colitis with older mice developing more severe DSS-induced inflammation (Liu et al., 2020). On the other hand, IBDs are not only diseases of the elderly but are often diagnosed in early adulthood and, increasingly, in childhood (Wang et al., 2023). Thus, it is crucial to define the effects of KDs on intestinal inflammation across the lifespan.

This study highlights the possible importance of the dietary fat source in IBD as the LA-rich diet exerted a greater protective effect than a diet high in SFAs. At present, there is little information on the impact of the type of dietary fat on intestinal inflammation and even less in the context of KDs. Thus, it is unclear whether other fats such as MCT oil or olive oil would result in similar effects. This thesis focused only on two fat sources as studying liquid oils is challenging in this setting as KDs for rodents require a solid base and the aim was to maximize the amount of specific fatty acid types. An attempt should be made to develop more suitable diet formulas to enable research on colitis to be conducted with other fat types. Furthermore, the effects of different fat sources may differ between species. While Study II revealed a pronounced benefit from a LA-rich KD, in an observational setting, high LA consumption in humans in a regular diet was strongly associated with an increased risk of developing ulcerative colitis (Tjonneland et al., 2009). The association, however, disappeared in a more recent meta-analysis which also did not find any correlation with ulcerative colitis and other fat sources (Wang et al., 2017). In addition to these observational reports, there are little data elucidating the effects of different dietary fat sources on IBD and this area warrants further research.

In addition to the KDs, this study tested the effects of exogenous supplementation of ketone bodies on experimental colitis as this would be a less restrictive, more feasible dietary approach for patients with IBD. No benefit and even harm was observed from the supplementation. While these results do not favor the testing of BHB supplementation for patients with IBD, others have observed experimental

colitis to be alleviated by BHB (Abdelhady et al., 2023; Huang et al., 2022; Li et al., 2021b). Thus, research comparing the differences between BHB forms, doses, and administration routes should be conducted to draw more informed conclusions.

Finally, the results indicate that KDs can prevent inflammation-induced changes in the expression of intestinal RAAS components. While it is more likely that these changes represent a downstream effect of lower inflammation as opposed to a mechanism underpinning the protective effect of the KDs, causative relationships were not investigated in this study. In order to determine whether changes in RAAS could actually contribute to the protective effect of the KDs, research in the DSS-model utilizing RAAS inhibitors or genetically modified mice with RAAS defects should be conducted. In addition, the fat source of the KD influences RAAS expression in the intestine. Future studies should investigate whether the anti-inflammatory effects of KDs on RAAS could be potentiated with the incorporation of specific fats in the diet.

7 Conclusions

This thesis investigated the effects of dietary fat source, ketogenic diets (KDs), and exogenous ketone supplementation on intestinal permeability, inflammation, and the local renin-angiotensin-aldosterone system (RAAS) in mice. Based on the findings, the following conclusions are drawn:

1. Regardless of the fat source of the diet, KDs do not alter intestinal permeability in healthy mouse intestine nor prevent its increase in inflamed intestine. Thus, the effect of KDs on intestinal inflammation is not linked to permeability.

2. KDs confer protection from experimental colitis with the magnitude of the effect being dependent on the fat source of the diet with linoleic acid offering greater protection than saturated fatty acids. The effects of KDs cannot be matched by supplementation with exogenous β-hydroxybutyrate, suggesting that other features, such as the gut microbiota, alongside the increased supply of ketone bodies are responsible for the impact of the diet.

3. KDs have only a marginal influence on the expression of local intestinal RAAS components in healthy mouse intestine. However, in mice with experimental colitis, KDs prevent inflammation-related changes in the expression of the RAAS components. Thus, the anti-inflammatory effects of KDs extend to RAAS expression in intestinal inflammation.

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Ketogenic Diet High in Saturated Fat Promotes Colonic Claudin Expression without Changes in Intestinal Permeability to Iohexol in Healthy Mice

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Abstract: Ketogenic diets (KDs) have been studied in preclinical models of intestinal diseases. However, little is known of how the fat source of these diets influences the intestinal barrier. Herein, we studied the impact of four-week feeding with KD high either in saturated fatty acids (SFA-KD) or polyunsaturated linoleic acid (LA-KD) on paracellular permeability of the intestine to iohexol in healthy male C57BL/6J mice. We investigated jejunal and colonic tight junction protein expression, histological changes, and inflammatory markers (*Il1b*, *Il6*, *Tnf*, and *Lcn2*), as well as the activity and expression of intestinal alkaline phosphatase (IAP) in feces and jejunal tissue, respectively, and plasma lipopolysaccharide. KDs did not change intestinal permeability to iohexol after two or twenty-six days of feeding regardless of fat quality. SFA-KD, but not LA-KD, upregulated the colonic expression of tight junction proteins claudin-1 and -4, as well as the activity of IAP. Both KDs resulted in increased epithelial vacuolation in jejunum, and this was pronounced in SFA-KD. Jejunal *Il1β* expression was lower and colonic *Il6* expression higher in LA-KD compared to SFA-KD. In colon, *Tnf* mRNA was increased in LA-KD when compared to controls. Overall, the results suggest that KDs do not influence intestinal permeability to iohexol but elicit changes in colonic tight junction proteins and inflammatory markers in both jejunum and colon. Future research will show whether these changes become of importance upon proinflammatory insults.

Keywords: intestinal permeability; ketogenic diet; dietary fat; tight junction proteins

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1. Introduction

Proper function of the intestinal barrier is crucial for the absorption of dietary nutrients and the prevention of harmful compounds entering the systemic circulation. While most nutrients are taken up transcellularly, other substances, such as microbial and dietary antigens, mainly pass the epithelial layer through the paracellular pathway only when the integrity of the tight junctions (TJs) connecting adjacent epithelial cells is compromised. This unwanted passage can lead to a vicious cycle where inflammatory cytokines, like tumor necrosis factor α, interleukin 1β, and interleukin 6 are produced, and they further exacerbate barrier dysfunction [1].

Dietary factors can influence the integrity of the epithelial layer and, therefore, the paracellular passage. Some amino acids such as glutamine can decrease intestinal permeability [2–4] while gliadin, a component of gluten, may induce dysfunction of the epithelial barrier [5]. Some studies have shown a regular high-fat diet (HFD), which also contains a substantial amount of carbohydrates, to negatively alter the function of the intestinal epithelium [6], and a carbohydrate-free diet with 72% fat to increase permeability [7], although this seems to be dependent on the composition of the microbiota [8]. Regardless, these findings suggest that the amount of dietary fat might be an important nutritional factor in the pathogenesis of barrier dysfunction.

The ketogenic diet (KD) is a dietary approach fundamentally different from regular HFDs in its metabolic effects. While in KD most of the energy is obtained from fat, the amount of carbohydrates is limited (less than 10% of energy). This diet has been used to treat drug-resistant epilepsy for a century [9], in addition to which it is studied for conditions ranging from neurodegenerative diseases [10] to diabetes [11] with encouraging results. The diet might also improve intestinal health through several mechanisms, such as by increasing the circulating levels of the anti-inflammatory ketone body β-hydroxybutyrate (BHB) [12], which is readily used as an energy source by intestinal cells [13], and modulating the gut microbiota [14,15]. Interestingly, the therapeutic effect of KD on epilepsy might also be mediated by its effects on intestinal microbiota [16].

Despite this, preclinical studies on KD and intestinal inflammation have produced inconsistent results. KD promoted the expression of intestinal TJ proteins in a rat model of irritable bowel syndrome [17]. In mice, it protected from experimental colorectal cancer [18] and alleviated dextran sodium sulphate (DSS)-induced colitis [19]. Contrary to this, KD worsened intestinal inflammation in another study with the same model of experimental colitis [20]. The evidence on the impact of different fat sources on intestinal permeability is also mixed. There are studies suggesting that large quantities of the omega-6 fatty acid, linoleic acid (LA), might prime the intestinal barrier for damage upon assault, and that saturated fatty acids (SFAs), especially medium-chain triglycerides, could be less harmful [21,22], while others have reported no or mixed effects [23,24]. It has been theorized that high dietary LA but not SFA may be harmful to the intestine due to increased lipid peroxidation [22]. On the other hand, SFAs have been shown to promote postprandial endotoxemia via increased lipopolysaccharide (LPS) translocation from the gut lumen to the systemic circulation in both animal models [25] and humans [26], which is considered deleterious. While the evidence is inconclusive, it seems that different fat sources have dissimilar, context-dependent effects on intestinal permeability and inflammation.

Our aim was to study whether KDs with different fat sources would have an impact on paracellular permeability and inflammation of the intestine. We fed healthy mice for four weeks with KD comprised of either milkfat high in SFAs, or vegetable fat sources abundant in LA, or with a low-fat control diet. Intestinal permeability to iohexol was determined in vivo at two time points to assess possible differences between two days and four weeks of ketosis and impacts of the diets.

2. Materials and Methods

2.1. Animal Experiment

The study was approved by the animal research board of the Regional State Administrative Agency for Southern Finland (ESAVI/9377/2019). Seven-week-old male C57BL/6J mice (n = 28) were purchased from Scanbur (Karlslunde, Denmark). The study began after 17 days of acclimatization when the animals were nine weeks old. They were kept under a 12 h light–dark cycle at 20 ± 2 °C and 50–60% humidity with *ad libitum* access to food and water.

The mice were housed individually and randomly allocated to three groups based on the dietary intervention: control group (CD) (n = 10), high-SFA ketogenic diet group (SFA-KD) (n = 9), and high-LA ketogenic diet group (LA-KD) (n = 9, of which one had to be euthanized during the study due to progressive weight loss). The diets were custom-

made (Envigo, Indianapolis, IN, USA) and matched for protein and micronutrients. The macronutrient compositions of the diets are presented in Table 1.

Table 1. The macronutrient compositions of the diets. Values are presented as percentage of total energy. CD = control diet, SFA-KD = ketogenic diet with saturated fatty acids, LA-KD = ketogenic diet with linoleic acid.

	CD	SFA-KD	LA-KD
Protein (E%)	9.7	9.4	9.4
Carbohydrate (E%)	77.8	0.5	0.5
Fat (E%)	12.5	90.1	90.1
Saturated fat (E%)	2.6	56.6	22.8
Monounsaturated fat (E%)	3.0	26.0	17.9
Polyunsaturated fat (E%)	6.9	4.5	49.3
Linoleic acid (E%)	6.4	4.0	44.5

Food and water consumption was monitored daily. For the first week, the mice were weighed every day, after which their weight was checked every other day. After 28 days of feeding, the mice were euthanized under isoflurane (4%, Vetflurane, Virbac, Carros, France) anesthesia. The set-up of the study is illustrated in Figure 1**.** The duration of the intervention was chosen based on previous experiments showing deleterious effects of high-fat feeding for intestinal barrier after four weeks [7].

Figure 1. The set-up of the animal experiment.

2.2. Measurement of Intestinal Permeability

Paracellular permeability of the intestine was assessed in vivo with iohexol (10 mL/kg, Omnipaque 300®, GE Healthcare, Oslo, Norway) after two and twenty-six days of feeding. The mice were weighed, and the solution was administered via a gastric gavage. After this, the animals were placed individually in metabolic cages for 24 h for urine collection. The amount of collected urine was measured, and samples were stored in −80 °C. If fecal contamination was observed, the sample was discarded.

The concentration of iohexol in the urine was measured by enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (BioPAL Inc., Worcester, MA, USA). The amount of recovered iohexol was determined as a percentage of the administered amount according to the following equation:

$$\text{Iohexol } (\%) = \frac{\text{amount of iohexol recovered in urine in 24 h } (\text{mg})}{\text{amount of administered iohexol } (\text{mg})} \times 100$$

2.3. Collection of Samples

At study termination on day 28, anesthetized animals were sacrificed by drawing blood from *vena cava* into EDTA-tubes (Kisker, Steinfurt, Germany), resulting in the death of the animal. To separate the plasma, samples were centrifuged at 2000× *g* for 15 min at 4 °C, frozen in liquid nitrogen, and stored at −80 °C.

After the euthanasia the entire intestine was removed. 1 cm-long samples were collected from the middle section of jejunum and the proximal and middle section of colon for histological and biochemical analyses. Other samples were opened longitudinally, but one sample from each part of the intestine was left unopened for histological analyses. Colonic pellets were harvested, and the remaining intestinal contents were flushed off with

ice cold 0.9% NaCl solution. Except for samples for histological analyses, tissues were snap-frozen in liquid nitrogen and stored at −80 °C.

2.4. Histological Analyses

Tissue pieces from jejunum (opened and unopened) and colon (unopened) were fixed in 4% buffered paraformaldehyde (Thermo Fisher Scientific, Waltham, MA, USA) solution for 36 h and then stored at 4 °C in 70% ethanol. The opened jejunal samples were cut into longitudinal halves, and the unopened jejunal parts and colon samples trimmed transversally. The fixed samples were dehydrated, embedded in paraffin, and cut into 4 µm thick sections. All sections were stained with hematoxylin and eosin (HE) stain, and selected jejunal sections with Alcian blue–Periodic Acid–Schiff (AB-PAS) stain, which detects intestinal mucins (goblet cells) and polysaccharides.

A veterinary pathologist (J.L.) read and evaluated the HE-stained slides blinded to the treatments. Vacuolation of the villus epithelium in jejunum was semi-quantitively graded employing three tiers: 0 = no epithelial vacuolation (EV); 1 = mild EV, present only in the basal half of the villi and partly occupying the epithelial cell cytoplasm; 2 = moderate EV, either present only in the basal half of the villi or partly occupying the epithelial cell cytoplasm; 3 = marked EV, present throughout the villi and fully occupying the epithelial cell cytoplasm. Villus *lamina propria* edema was graded as absent (0) or present (1).

2.5. Biochemical Assays

The state of ketosis was confirmed by measuring plasma BHB levels with a commercial enzymatic kit (Cayman Chemicals, Ann Arbor, MI, USA). A Limulus Amebocyte Lysate Assay (Pierce™ Chromogenic Endotoxin Quant Kit, Thermo Fisher Scientific) was used to detect LPS activity in plasma samples.

2.6. Western Blot

The relative quantities of jejunal and colonic TJ proteins claudin-1, -2, and -4, as well as occludin, were assessed with Western Blot (WB). The tissue samples were homogenized in PBS-T (136 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 4.46 mM KH_2PO_4, 0.1% Tween, pH 7.4) containing protease inhibitor (Pierce™ Protease Inhibitor Mini Tablets, Thermo Fisher Scientific) with Precellys 24 homogenizer (Bertin Technologies, Montigny le Bretonneux, France) for 3 × 20 s at 5500 rpm at 4 °C. The homogenates were sonicated for 12 s at 21% of the maximal power (VC 505 Ultrasonic Processor, Sonics, Newtown, CT, USA) and centrifuged for 15 min at 12,000× *g* at 4 °C. The protein concentrations of the supernatants were assessed with a commercial kit (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific). The supernatants were diluted to the same total protein concentration using PBS-T and Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) with 5% 2-mercaptoethanol. The proteins were denatured on a heat block at 95 °C for 5 min.

Samples containing 30 µg total protein were loaded in 4–20% Mini-PROTEAN® TGX™ Precast gels (Bio-Rad). The gels contained 4–5 samples from each group. After the SDS-PAGE run, the proteins were transferred to a nitrocellulose membrane (Bio-Rad), which was blocked for 1 h with a commercial buffer (Odyssey blocking buffer (TBS), LI-COR, Lincoln, NE, USA) at RT and incubated in primary antibody solution overnight at 4 °C. The primary antibodies used were claudin-1 (sc-166338, 1:200; Santa Cruz Biotechnology, Dallas, TX, USA), claudin-2 (sc-293233, 1:200; Santa Cruz Biotechnology), claudin-4 (sc-376643, 1:200; Santa Cruz Biotechnology), and occludin (#91131, 1:1000, Cell Signaling Technology, Danvers, MA, USA). After washing, the membranes were incubated in fluorescence-labeled secondary antibody solution (1:10,000 (IRDye 680LT goat anti-mouse or IRDye 800CW goat anti-rabbit, LI-COR)) for 1 h at RT, protected from light. The bands were detected with the Odyssey CLx infrared imaging system (LI-COR) and analyzed with the program Image Studio (LI-COR). The protein quantities were normalized to the quantity of the loading control, β-actin (#3700, 1:3000; Cell Signaling Technology).

2.7. Reverse Transcription Quantitative Polymerase Chain Reaction

The mRNA expression of the TJ protein-coding genes *Cldn1*, *Cldn2*, *Cldn4*, and *Ocln* and inflammatory marker genes *Tnf*, *Il1b*, *Il6*, and *Lcn2* in jejunal and colonic tissue were analyzed with reverse transcription quantitative polymerase chain reaction (RT-qPCR). The expression of intestinal alkaline phosphatase (IAP)-subtype-coding gene *Akp6* was analyzed from jejunum. Total RNA was extracted from the tissue samples with a commercial kit (NucleoSpin RNA Kit, Macherey Nagel, Duren, Germany) and its concentration was analyzed with NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). The samples were diluted to the same concentration, and RNA was reverse transcribed to complementary DNA with iScript™ cDNA Synthesis Kit (Bio-Rad). RT-qPCR was run with LightCycler® 480 SYBR Green Master (Roche Diagnostics Corp., Indianapolis, IN, USA). The amplification protocol was: 10 min at 95 °C, 40 cycles of denaturation (15 s, 95 °C), annealing (30 s, 60 °C), and elongation (30 s, 72 °C). The melt curves were analyzed at the end of the experiment. The primer sequences used are listed in Table 2.

The results were calculated as relative quantities (RQ) of messenger RNA (mRNA) normalized against the mRNA expression of three housekeeping genes according to the Vandesompele method [27]. The housekeeping genes used were *Actb*, *Eef2*, and *Rplp0.*

Table 2. Primer sequences used in RT-qPCR analyses. If no reference is cited, the primer pair is designed by the authors.

Gene	Forward Primer	Reverse Primer	Ref.
Actb	CTGAATGGCCCAGGTCTGAG	AAGTCAGTGTACAGGCCAGC	[28]
Eef2	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA	[29]
Rplp0	TAACCCTGAAGTGCTCGACA	GGTACCCGATCTGCAGACA	[30]
Cldn1	AGACCTGGATTTGCATCTTGGTG	TGCAACATAGGCAGGACAAGAGTTA	[31]
Cldn2	GCAAACAGGCTCCGAAGATACT	GAGATGATGCCCAAGTACAGAG	[32]
Cldn4	TGAGCGATGGCGTCTATGG	GATGTTGCTGCCGATGAAGG	
Ocln	CGGTACAGCAGCAATGGTAA	CTCCCCACCTGTCGTGTAGT	[33]
Il1b	CTCCAGCCAAGCTTCCTTGT	TCATCACTGTCAAAAGGTGGCA	[28]
Il6	ATCGTGGAAATGAGAAAAGAGTTGT	CTGCAAGTGCATCATCGTTGT	
Tnf	TGGCACCACTAGTTGGTTGTCT	AGCCTGTAGCCCACGTCGTA	[34]
Lcn2	CCACCACGGACTACAACCAG	AGCTCCTTGGTTCTTCCATACAG	
Akp6	ACCGAAGCTCAGAGTGTTGAT	GCAAATATGGCCACGTCCTC	

2.8. Intestinal Alkaline Phosphatase Activity

Fecal pellets were suspended in alkaline phosphatase extraction buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, pH 8.0) containing protease inhibitor (cOmplete™, EDTA-free Protease Inhibitor Cocktail, Roche Diagnostics Corp.) with a pipette tip, followed by vortexing for 10 min. Samples were centrifuged for 5 min at 5000× *g* at 4 °C for the removal of insoluble matrix, after which the supernatants were transferred into new tubes and centrifuged for 15 min at 12,000× *g* at 4 °C.

IAP activity was assayed from the supernatants using a *p*-nitrophenyl phosphate (pNPP)-based assay. A standard curve was generated for the assay by incubating known amounts of pNPP (Sigma-Aldrich, St. Louis, MO, USA) with commercial calf IAP. All dilutions were made in assay buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, pH 10). The samples were incubated in 0.45 mM pNPP solution for 30 min at 37 °C. The absorbances were read at 405 nm and the amount of formed *p*-nitrophenol (pNP) was read from the standard curve. IAP activity was calculated as pNP formed in μmol*min^{-1} per g of protein (units per g) in the sample. Protein concentrations were assayed using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

2.9. Statistical Analyses

Statistical analyses were conducted, and figures drawn with GrapPad Prism 8 (Dotmatics, La Jolla, CA, USA). The level of statistical significance was set at $p < 0.05$. Permeability to iohexol was analyzed using mixed-effects model followed by Sidak's post-hoc test. Based

on normal distribution determined by Shapiro–Wilk test, other data were analyzed either using one-way ANOVA followed by Tukey's post-hoc test, or Kruskal–Wallis followed by Dunn's post-hoc test. Unless stated otherwise, data are presented as mean except for RT-qPCR results that are expressed as geometric mean.

3. Results

3.1. Metabolic Parameters

At the start of the experiment, there were no significant differences in body weights between the three groups. Initially, animals in both KD groups lost weight but fully regained it after two weeks on the diet (Figure 2A). From that point onwards, the weight changes followed the same trajectory as controls. All groups lost weight upon metabolic caging (days two and 26) but regained it within two days after being returned to home cages.

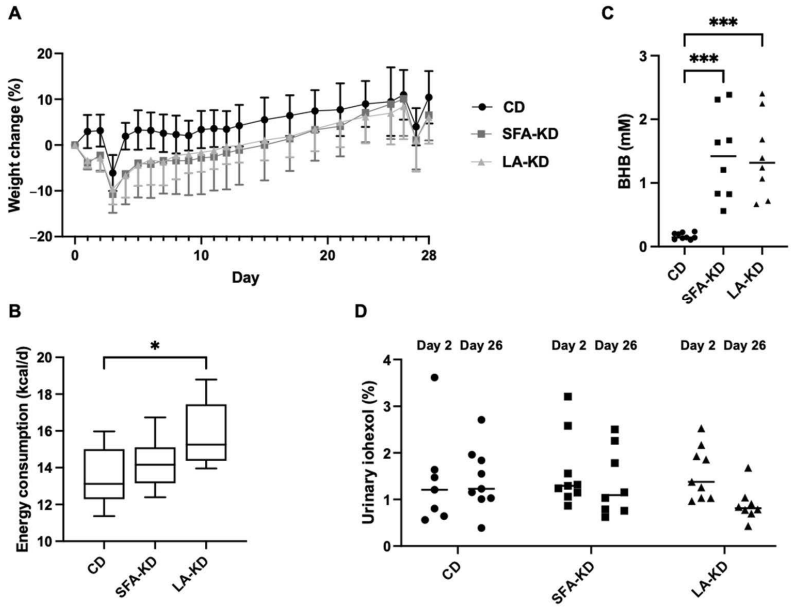

Figure 2. Metabolic parameters and intestinal permeability to iohexol. (**A**). Body weight change as a percentage from the baseline (mean ± SD, n = 8–10/group). (**B**). Average energy consumption (mean ± SD, n = 8–10 per group) showing higher energy consumption in LA-KD. (**C**). Plasma BHB levels showing elevated levels in KD groups. (**D**). Intestinal permeability measured as the percentage of iohexol in the urine after 24-h collection showing no between- or within-group differences. * $p < 0.05$, *** $p < 0.001$. BHB = β-hydroxybutyrate, CD = control diet, LA-KD = ketogenic diet with linoleic acid, SFA-KD = ketogenic diet with saturated fatty acids.

The average energy consumption in LA-KD was slightly higher than in CD despite no differences in weight gain (Figure 2B). Energy intake in SFA-KD did not differ from other groups. The initial loss of body weight seen in both KD groups was not related to lower energy intake. The effect of metabolic caging was reflected in energy consumption, which was lower on the days of these experiments.

We analyzed plasma BHB to confirm the level of nutritional ketosis. Generally, the state of ketosis is defined as plasma BHB level of 0.5 mM or above [35]. As expected, all animals on KDs showed elevated BHB levels with no difference between the two groups (Figure 2C). Animals on CD were not in ketosis.

3.2. Intestinal Permeability

3.2.1. Permeability to Iohexol

On day two and day twenty=six of the experiment, we orally administered iohexol to the animals to compare paracellular permeability of the intestine between groups. There were no significant between-group differences in the percentual 24-h urinary recovery of administered iohexol after two or twenty-six days of dietary intervention (Figure 2D), indicating that intestinal permeability to this compound was not altered at either time point. While there was no statistical change within groups between two and twenty-six days of feeding, a non-significant ($p = 0.097$) decrease of 43% was observed in LA-KD.

3.2.2. Tight Junctions

To investigate other changes in the epithelial barrier, we assessed the relative levels of TJ proteins claudin-1, -2, and -4, and occludin with WB and the expression of TJ protein-coding genes determined with RT-qPCR. While there were no differences in jejunum (Figure 3), the analyses revealed a statistically significant increase in the colonic expression of the barrier-sealing TJ proteins claudin-1 and claudin-4 in SFA-KD in comparison to CD and LA-KD (Figure 4). The relative level of claudin-1 was doubled in SFA-KD when compared to CD and was 57% higher in comparison with LA-KD. The expression of claudin-4 in SFA-KD was 62% higher with comparison to CD and 50% higher than in LA-KD. Levels of claudin-2 and occludin were not statistically different between groups in either part of the intestine. The mRNA expressions of *Cldn1*, *Cldn2*, *Cldn4*, and *Ocln* were not different between groups in either jejunum or colon (Figures 3 and 4).

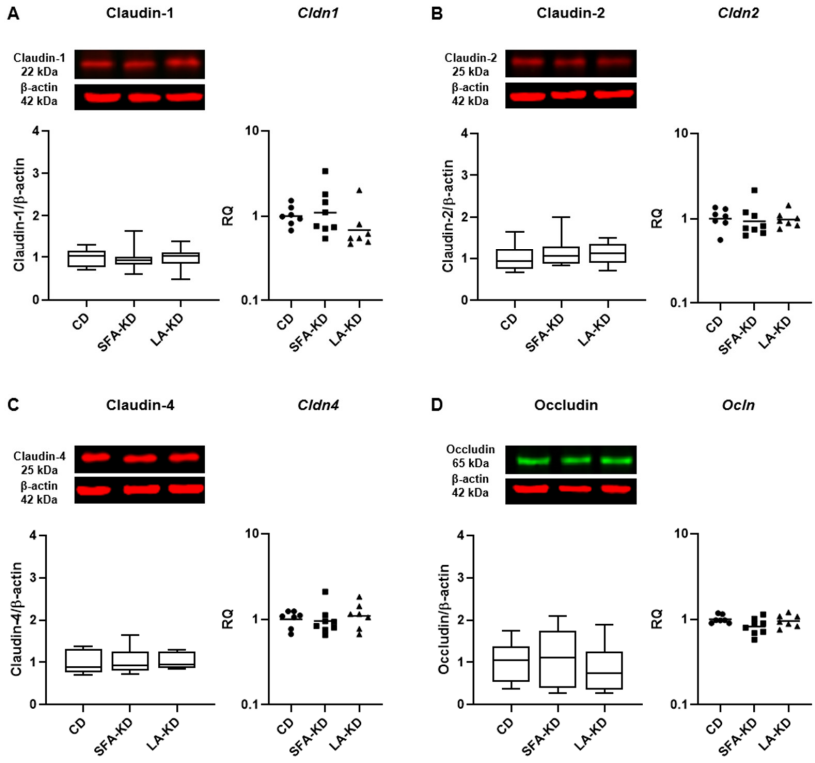

Figure 3. Jejunal tight junction protein and mRNA expressions of (**A**). claudin-1, (**B**). claudin-2, (**C**). claudin-4, and (**D**). occludin analyzed with Western Blot ($n = 7$–9) and RT-qPCR showing no

differences in the expression of these proteins. Images of original blots are presented in Figure S1. CD = control diet, LA-KD = ketogenic diet with linoleic acid, RQ = relative quantity, SFA-KD = ketogenic diet with saturated fatty acids.

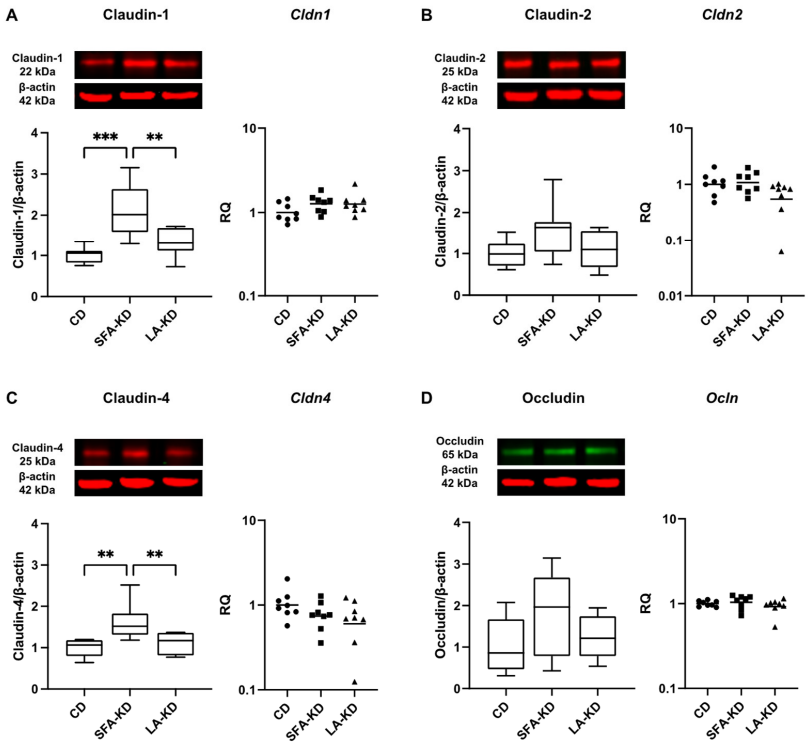

Figure 4. Colonic tight junction protein and mRNA expressions of (**A**). claudin-1, (**B**). claudin-2, (**C**). claudin-4, and (**D**). occludin analyzed with Western Blot (n = 7–9) and RT-qPCR showing increased protein expression of claudin-1 and -4 in SFA-KD. Images of original blots are presented in Figure S2. ** $p < 0.01$, *** $p < 0.001$. CD = control diet, LA-KD = ketogenic diet with linoleic acid, RQ = relative quantity, SFA-KD = ketogenic diet with saturated fatty acids.

3.3. Epithelial Cell Vacuolation

Jejunum and colon samples showed no definite histological lesions. However, jejunal villus epithelial cells in both KD groups exhibited vacuolation, which was more pronounced in SFA-KD than in LA-KD (Figure 5). There was no vacuolation in the colon. The extent of jejunal vacuolation varied from basal 1/3 of the villus length to diffuse involvement, and the intensity from single vacuoles in apical cytoplasm to foaminess and/or marked vacuolation throughout the cell. In addition, minimal to mild villous subepithelial edema, manifested as sparse lamina propria and/or focal detachment of the epithelial cells from the underlying lamina propria in sporadic villi, was present in SFA-KD and LA-KD mice (Figure 5). The CD mice showed no epithelial vacuolation (EV grade = 0) and only one animal exhibited villous edema. In contrast, in LA-KD the average EV grade was 1.8 and 6 of the 8 samples analyzed showed villus edema, and in SFA-KD the average EV grade was 2.6 and villus edema was present in 8 of 9 samples. The vacuoles did not contain simple polysaccharides or mucins as shown by the AB-PAS staining, and no vacuolation or edema was observed in colon, consistent with the hypothesis that jejunal vacuoles resulted from absorbed dietary fat.

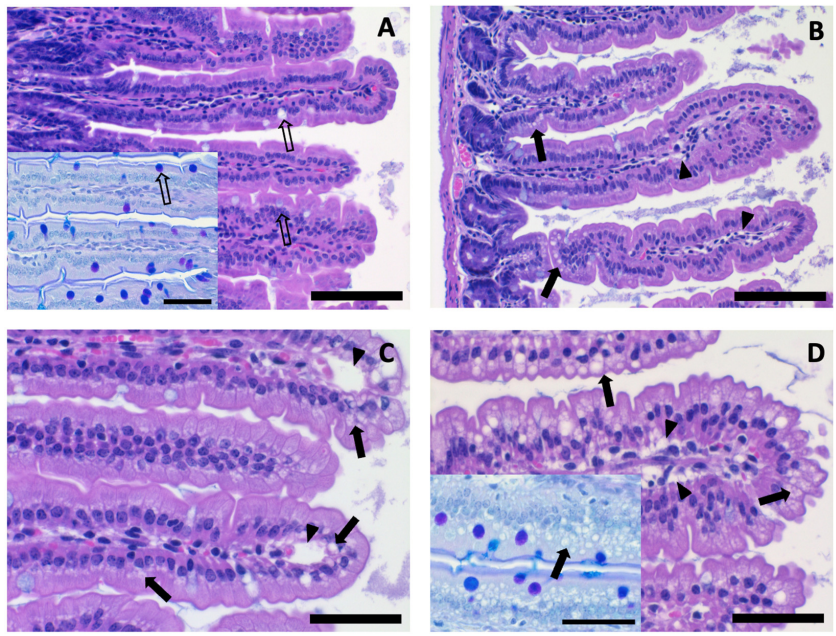

Figure 5. Representative microphotographs of HE- and AB-PAS-stained (insets) sections of jejunal villi. (**A**). No epithelial vacuolation in CD. Goblet cells (open arrows). Bar 100 μm, 20× objective magnification. Inset: Close-up of an AB-PAS-stained section. Goblet cell mucins stain bluish with AB and or purple with PAS. Bar 50 μm, 40× obj. mag. (**B**). Grade 1 vacuolation in LA-KD. Single small, clear apical vacuoles (arrows) present in the epithelial cells in the basal half of the villi and slight subepithelial edema (arrowheads). Bar 100 μm, 40× obj. mag. (**C**). Grade 2 vacuolation in LA-KD. Moderate vacuolation (arrows) partly occupying most epithelial cells and extending to villus tips that also show subepithelial blebs (arrowheads; artefactual change accentuated by edema). Bar 50 μm, 40× obj. mag. (**D**). Grade 3 vacuolation in SFA-KD. Marked vacuolation (arrows) present throughout the villi and extensively occupying epithelial cell cytoplasms, and subepithelial edema (arrowheads). Bar 50 μm, 40× obj. mag. Inset: Close-up of an AB-PAS-stained section. The epithelial vacuoles are AB-PAS negative. Bar 50 μm, 40× obj. mag. AB = Alcian blue, CD = control diet, HE = hematoxylin and eosin, LA-KD = ketogenic diet with linoleic acid, PAS = Periodic acid–Schiff, SFA-KD = ketogenic diet with saturated fatty acids.

3.4. Inflammation

We used RT-qPCR to analyze the gene expression levels of the markers of inflammation *Tnf*, *Il1b*, *Il6*, and *Lcn2* in the intestine. Small but statistically significant changes were detected: in jejunum, *Il1b* mRNA levels were slightly higher in SFA-KD than in LA-KD without differences when compared to CD (Figure 6), while in colon, LA-KD promoted the expression of *Tnf* relative to CD and *Il6* to SFA-KD.

3.5. Intestinal Alkaline Phosphatase

We examined the activity of IAP, an anti-inflammatory and TJ protein level-promoting enzyme, in the feces in order to explore potential contributors to the increased TJ protein expression. Activity of the enzyme was increased in SFA-KD but not in LA-KD when compared to CD (Figure 7A). The activity between the two KD groups did not differ significantly.

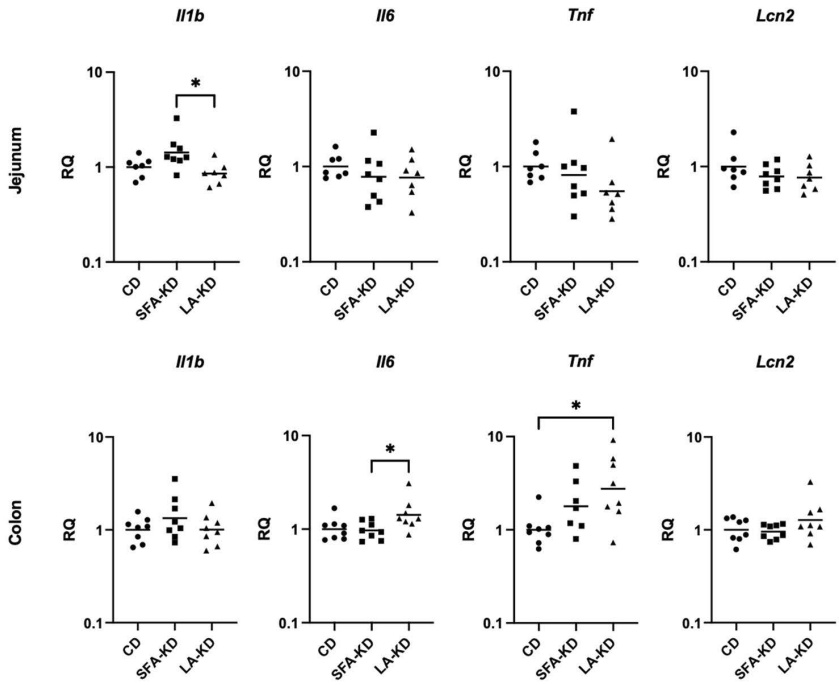

Figure 6. Jejunal and colonic mRNA expressions of inflammatory markers. SFA-KD promoted the expression of *Il1b* in jejunum and LA-KD *Il6* and *Tnf* expression in jejunum. * $p < 0.05$. CD = control diet, LA-KD = ketogenic diet with linoleic acid, RQ = relative quantity, SFA-KD = ketogenic diet with saturated fatty acids.

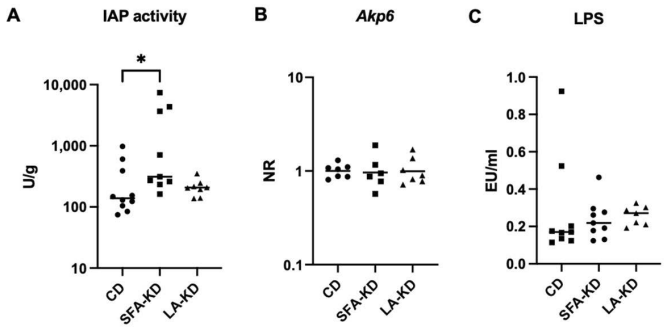

Figure 7. IAP activity, *Akp6* expression and LPS. (**A**) IAP activity (U/g) measured from feces showing increased activity in SFA-KD. Data is presented as median. (**B**) *Akp6* expression in jejunum showing no differences. (**C**) LPS activity (EU/mL) in plasma showing no differences. Data is presented as median. * $p < 0.05$. CD = control diet, IAP = intestinal alkaline phosphatase, LA-KD = ketogenic diet with linoleic acid, LPS = lipopolysaccharide, RQ = relative quantity, SFA-KD = ketogenic diet with saturated fatty acids.

Due to the increased IAP activity in feces, we also analyzed the jejunal expression of *Akp6*, which encodes for a mouse-specific IAP subtype evenly expressed throughout the intestine. However, increased activity did not correspond to changes in mRNA expression in the tissue (Figure 7B).

3.6. Lipopolysaccharide

In addition to markers of paracellular permeability, we investigated whether the study diets influence LPS translocation from the gut lumen to circulation. Plasma LPS activity, however, was not significantly increased in either of the KD groups (Figure 7C). Removal of outliers did not change the result.

4. Discussion

The aim of this study was to investigate whether ketogenic diets (KDs) with disparate fat sources impact paracellular permeability or cause inflammation in the intestine differently. We fed healthy mice with two KDs—one group a diet high in saturated fatty acids SFAs from milkfat (SFA-KD) and the other group a diet abundant in linoleic acid from vegetable sources (LA-KD). Intestinal permeability to iohexol was measured at two time points: after two and twenty-six days from the start of the dietary intervention. As other parameters related to permeability, we analyzed jejunal and colonic TJ protein expression, histological changes, and inflammatory markers, as well as changes in the activity and expression of IAP. Moreover, LPS activity levels were determined in plasma samples. KDs induced small changes in intestinal TJ proteins, inflammatory parameters, and fecal IAP activity, without a significant impact on paracellular permeability to iohexol.

We determined intestinal permeability to iohexol, which is a stable, low-molecular weight (821 Da) compound validated for the assessment of paracellular permeability of the whole intestine in mice [36]. As a marker of permeability, iohexol has been compared to ^{51}Cr-EDTA with high correlation between the two [37], and permeability to iohexol is a superior predictor of disease activity in inflammatory bowel disease patients when compared to lactulose-mannitol ratio [38]. In our study, permeability to iohexol differed neither between groups at either time point nor within groups between time points. Mice enter ketosis after 24–48 h of severe carbohydrate restriction [39]. Here we show that short-term (two days) or longer-term (four-week) feeding with KD does not influence paracellular permeability to iohexol and, thus, it does not seem to change upon the shift from carbohydrate-based to ketone-based metabolism or as a result of four weeks of ketosis. These results illustrate that while some studies have shown barrier function to be impaired by HFD for durations ranging from one to fifteen weeks [6,7,40] or specific fats fed for eight weeks [23], the same does not seem to apply in the context of four-week KD in mice. Our findings are also in line with the observation that even though a low-fiber HFD can lead to the bacterial degradation of the colonic mucus layer in mice [41], this does not happen with high-fat KDs despite the lack of fermentable fiber [15]. KD is distinctly different from HFDs regularly used in animal experiments due to its lack of carbohydrates and the resulting state of ketosis. It is possible that the effects of dietary fat and its source on paracellular permeability may differ depending on the composition of the diet as a whole.

Despite no differences in permeability, we observed SFA-KD but not LA-KD to promote the colonic expression of TJ proteins claudin-1 and claudin-4 on protein but not on mRNA level. Claudins comprise a family of 27 known adhesion proteins, with key roles in the paracellular barrier and channel functions of TJs [42]. While we saw no changes in jejunal TJ protein expression, others have reported KD to increase the expression of small intestinal claudin-1 protein but not gene expression in conjunction with lowering inflammation and restoring crypt length in a rat model of irritable bowel syndrome [17]. Even though these claudins are considered to be barrier-sealing [43,44], in human biopsies their expression is elevated in active inflammatory bowel disease, and claudin-1 levels correlate with inflammatory activity [45]. In addition, claudin-1 overexpression results in greater susceptibility to and poorer recovery from DSS-induced colitis [46], making it unclear whether the increase in claudin-1 in our study is a beneficial change or a compensatory mechanism.

KDs influenced the gene expression of inflammatory markers *Il1b*, *Il6*, and *Tnf* in the intestine. Intriguingly, jejunal *Il1b* levels were elevated in SFA-KD when compared to LA-KD, but colonic *Il6* was upregulated in LA-KD in comparison to SFA-KD and *Tnf* in comparison to CD. While statistically significant, these between-group differences were

small, bringing into question their physiological meaning. Nevertheless, it is possible that upon assault, these changes might become of importance. Based on previous studies, high LA in the context of a diet rich in both fat and carbohydrate does not increase permeability [23] but might compromise the intestinal barrier upon exposure to LPS [21] or alcohol [22]. KDs have been studied as an intervention for DSS-induced experimental colitis, but the results are contradictory—one group reported KD to ameliorate colonic inflammation [19], whereas another noted the diet to worsen it [20]. While there are several differences between these studies, one contributing factor might be the fat source of the diet. Here, we saw that SFA- and LA-rich KDs had dissimilar impacts on both small and large intestine and the differences might become more pronounced upon induced intestinal inflammation. Since we only observed subtle differences in the intestine, plasma levels of these markers were not assessed.

Triglyceride consumption has been shown to induce lipid droplet and chylomicron formation in enterocytes in various species, including mice, with well-known mechanisms [47,48]. However, to our knowledge, we are the first to report high-fat KDs to result in histologically detectable increased, in all probability lipid, vacuolation in jejunal enterocytes and minimal to mild subepithelial villous edema. While investigating the nature of the vacuoles in depth was outside the scope of this study, we found them to be AB-PAS negative, excluding polysaccharide or mucin content, which corroborates the lipid presumption and is logical in the context of a diet high in fat. We propose that the observed histological findings indicate an adaptive response and might be a natural consequence of drastically increased fat intake in a species adapted to eat a low-fat diet. The enterocyte vacuoles and foaminess suggestively denote the extremely high number of enterocyte lipid droplets acting as triacylglycerol storage pool and the chylomicrons, and the subepithelial edema the chylomicron flow towards villus lacteals (see [49,50] for recent reviews). Consistent with the modest protein and mRNA expression changes, we detected no further histological alterations in the jejunum and no histological changes in the colon. Long-term HFDs from eight to fourteen weeks of length have been reported to shorten villus length and crypt depth as well as to reduce goblet cell numbers [50]. The number of vacuoles was markedly higher in SFA-KD than in LA-KD. This could be explained by the finding that at least in Caco-2 cells, LA is more potent than SFAs in stimulating the synthesis of triglycerides and secretion of chylomicrons [51,52], and thus, is likely to be more efficiently cleared from the cells, albeit in in vivo studies such differences have not been reported [49,50].

A novel finding was that a KD high in SFAs promotes the fecal activity of IAP, despite not changing its expression on a transcriptional level in jejunum. This enzyme produced by enterocytes is generally recognized to be beneficial for intestinal health for its anti-inflammatory, LPS-detoxifying properties [53]. Importantly, IAP influences the expression of TJ proteins, and has specifically been shown to upregulate claudin-1 mRNA levels in a mouse model of sepsis [54]. The increase we observed in the expression of claudins, especially claudin-1, could be partly a result of the higher activity of IAP. HFDs and omega-3 fatty acids have been shown to promote the expression and activity of IAP [55]. With the former, this is thought to be a response against increased diet-induced LPS translocation and resulting endotoxemia, whereas omega-3s upregulate IAP production through different mechanisms, such as the production of inflammation-resolving resolvins [56]. SFAs in the context of a regular diet can increase LPS transport, especially when derived from palm or coconut oil [26]. However, we did not observe higher plasma LPS activity as a response to a SFA-rich KD with milkfat as the fat source nor to a LA-rich KD, indicating that LPS translocation might not increase as a response to KD similarly to a non-ketogenic HFD [7]. This could also be a result of upregulated detoxification or an effect of the fat source since milk-derived phospholipids seem to protect from HFD-induced increases in LPS-binding protein [57]. The ketone body BHB has been reported to increase IAP expression in vitro in both LS174T and HT29 cell lines and in vivo in mouse small intestine [58]. Thus, increased BHB levels might also be an explanatory factor for the higher activity in SFA-KD. However,

while SFA-KD promoted IAP activity, LA-KD did not. Interestingly, DeCoffe et al. [59] observed no differences in IAP activity between diets containing 20% SFA or corn oil, high in LA, after infecting rats with *Citrobacter rodentium*. The addition of 1% of fish oil to the diet increased the activity and provided protection from the infection when given with SFA but had detrimental effects with the high-LA diet. Thus, it is possible that SFAs paired with an anti-inflammatory agent can promote IAP activity and, in our setting, the combination of SFAs and BHB, a molecule shown to attenuate inflammation [60], could function similarly. In line with the results of DeCoffe et al., a LA-rich diet does not seem to have the same effect.

The systemic effects of the diets, except for plasma LPS activity, were not included in the frame of this study since mice are poor models for cardiovascular effects of HFDs. Dietary fat quality and quantity are generally recognized to influence cardiovascular disease, and while KDs have been studied in the context of cardiovascular disease risk (see [61] for a recent review), the research on the effects of the fat source of these diets is scarce. Since we did not assess the systemic effects of these diets, predicting the cardiovascular influence of the diets used was not possible. It also remains to be investigated whether other fat sources, such as those high in monounsaturated fat, influence barrier function, inflammation, and IAP activity in the intestine differently in the context of KD. The challenge with this is diet formulation; KDs for rodents require a solid base which makes studying liquid oils, such as olive oil, difficult in this setting. In addition, it is not possible to isolate the effects of specific fatty acids with these diets. Most fat sources are comprised of a variety of different fatty acids and thus definitive conclusions on their effects cannot be drawn. On the other hand, human diets—whether ketogenic or not—usually contain fat from a variety of sources, which might either counteract or enhance each other's effects.

5. Conclusions

Our study indicates that KDs, regardless of fat source, do not directly influence intestinal permeability to iohexol in healthy mice after two days or four weeks of feeding. However, we saw KDs to cause modest changes in indirect measures related to intestinal permeability, altering colonic TJ protein levels and cytokine expression in both jejunum and colon. In addition, we detected increased IAP activity and jejunal vacuolation as a response to KD high in SFAs. Future research is warranted to show whether these changes become of significance in a proinflammatory setting.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16010018/s1, Figure S1: Original and unmodified Western Blot images of jejunal tight junction proteins. Figure S2: Original and unmodified Western Blot images of colonic tight junction proteins.

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Ketogenic Diet Protects from Experimental Colitis in a Mouse Model Regardless of Dietary Fat Source

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Abstract: While ketogenic diets (KDs) may have potential as adjunct treatments for gastrointestinal diseases, there is little knowledge on how the fat source of these diets impacts intestinal health. The objective of this study was to investigate how the source of dietary fat of KD influences experimental colitis. We fed nine-week-old male C57BL/6J mice ($n = 36$) with a low-fat control diet or KD high either in saturated fatty acids (SFA-KD) or polyunsaturated linoleic acid (LA-KD) for four weeks and then induced colitis with dextran sodium sulfate (DSS). To compare the diets, we analyzed macroscopic and histological changes in the colon, intestinal permeability to fluorescein isothiocyanate−dextran (FITC–dextran), and the colonic expression of tight junction proteins and inflammatory markers. While the effects were more pronounced with LA-KD, both KDs markedly alleviated DSS-induced histological lesions. LA-KD prevented inflammation-related weight loss and the shortening of the colon, as well as preserved *Il1b* and *Tnf* expression at a healthy level. Despite no significant between-group differences in permeability to FITC–dextran, LA-KD mitigated changes in tight junction protein expression. Thus, KDs may have preventive potential against intestinal inflammation, with the level of the effect being dependent on the dietary fat source.

Keywords: intestinal inflammation; ketogenic diet; dietary fat; colitis

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1. Introduction

Inflammatory bowel diseases (IBDs), namely, Crohn's disease and ulcerative colitis, are chronic conditions characterized by inflammation of the gastrointestinal tract. Symptoms of these diseases include abdominal pain, chronic diarrhea, rectal bleeding, and weight loss. There is no known cure for IBDs, and their prevalence is rising worldwide [1]. They decrease both life quality and expectancy of patients [2]—thus, new forms of treatment are needed.

A low-carbohydrate, high-fat ketogenic diet (KD) is a possible adjunct treatment for IBDs [3]. On KD, the body enters a state of ketosis wherein the levels of circulating ketone bodies, such as β-hydroxybutyrate (BHB), increase. Most tissues of the body can utilize these molecules for energy production. While this is particularly important for the brain in low-glucose states, the intestine also uses ketone bodies readily as an energy source [4]. Ketone bodies function not only as energy sources, but they are also anti-inflammatory [5] and decrease oxidative stress [6]. KD has shown efficacy in preclinical models of intestinal

diseases. In experimental settings, the diet can protect from colon cancer [7], improve irritable bowel syndrome [8], and ameliorate dextran sodium sulfate (DSS)-induced colonic inflammation [9,10]. However, the results are not entirely uniform. In one study, KD worsened experimental colitis by modifying microbiota and its metabolites and increasing intestinal permeability [11].

In addition to the amount of dietary fat, the fat source and the fatty acid composition of the diet can also influence intestinal physiology. Some studies indicate that linoleic acid (LA), an essential omega-6 polyunsaturated fatty acid, can, in large quantities, predispose the intestinal barrier to insults, whereas saturated fatty acids (SFAs), specifically medium-chain triglycerides, may not have the same effect [12,13]. According to one hypothesis, this might be due to differences in lipid peroxidation [13]. Previously, we have observed that, in the context of KD, intestinal permeability in healthy animals remains unchanged after four weeks of feeding regardless of the fat source but KD high in SFAs upregulates colonic tight junction (TJ) proteins and intestinal alkaline phosphatase activity whereas a LA-rich KD does not [14]. However, it is unknown whether these are compensatory mechanisms or beneficial changes.

Due to the discrepancy between animal studies on KD and intestinal inflammation, we aimed to explore how KD affects DSS-induced colitis and whether the effect is dependent on the dietary fat source. The mice were fed either a low-fat control diet or one of the two KDs—high in SFAs (SFA-KD) or high in LA (LA-KD)—for four weeks before the induction of colitis. To compare the effects of the diets, we analyzed macroscopic and histological changes in the colon, colonic expression of inflammatory markers and TJ proteins, and intestinal permeability.

2. Materials and Methods

2.1. Animal Experiment

The experiment was conducted under the approval of the animal research board of the Regional State Administrative Agency for Southern Finland (ESAVI/9377/2019). Thirty-six eight-week-old male C57BL/6J mice were purchased from Scanbur (Karlslunde, Denmark). Dietary interventions were started after seven days of acclimatization. The mice were housed individually and kept under a 12 h light–dark cycle, at 20 ± 2 °C and 50–60% humidity. Access to food and water was unrestricted.

Before the start of the study, the mice were randomly allocated to four groups based on the diet and the DSS treatment: healthy on a control diet (CD) (n = 8), DSS on a control diet (DSS-CD) (n = 8), DSS on a SFA-rich KD (DSS-SFA-KD) (n = 10), and DSS on a LA-rich KD (DSS-LA-KD) (n = 10, one of which had to be sacrificed after three weeks of the intervention due to progressive weight loss). The study diets were custom-made (Envigo, Indianapolis, IN, USA) and matched for protein and micronutrients. Their macronutrient compositions are presented in Table 1, and the exact diet formulations are provided in Table S1.

Table 1. The macronutrient compositions of the diets. Values are expressed as a percentage of energy. CD = control diet, SFA-KD = ketogenic diet with saturated fatty acids, and LA-KD = ketogenic diet with linoleic acid.

	CD	SFA-KD	LA-KD
Protein (E%)	9.7	9.4	9.4
Carbohydrate (E%)	77.8	0.5	0.5
Fat (E%)	12.5	90.1	90.1
Saturated fat (E%)	2.6	56.6	22.8
Monounsaturated fat (E%)	3.0	26.0	17.9
Polyunsaturated fat (E%)	6.9	4.5	49.3
Linoleic acid (E%)	6.4	4.0	44.5

On day 28, colitis was induced in the three DSS groups by replacing regular drinking water with DSS (40 kDa, TdB Labs, Uppsala, Sweden) solution (2.5% *w/v*). After five days

of DSS administration, the animals were given regular water for two days, and sacrificed on day 35. From the start of the administration, weight, stool consistency, and visibility of fecal blood were monitored daily. Based on a previously described scoring system [15], each of these parameters was given a score from 1 to 4, from which disease activity index (DAI) was calculated as an average. Food and fluid consumptions were measured daily. Figure 1 illustrates the set-up of the animal experiment.

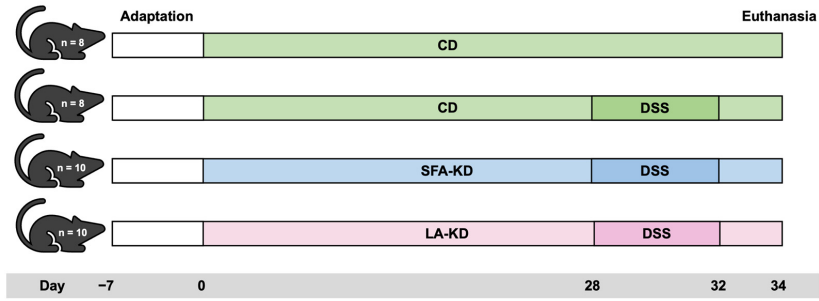

Figure 1. Animal experiment set-up. CD = control diet, DSS = dextran sodium sulfate, LA-KD = ketogenic diet with linoleic acid, and SFA-KD = ketogenic diet with saturated fatty acids.

2.2. Collection of Samples

On day 35, animals were killed under isoflurane (4%, Vetflurane, Virbac, Carros, France) anesthesia by exsanguination. Blood was drawn from *vena cava* into EDTA tubes (Kisker, Steinfurt, Germany). Samples were centrifuged at 2000× *g* for 15 min at 4 °C. The separated plasma was snap-frozen in liquid nitrogen and stored in −80 °C.

After confirming the death of the animal, the colon was removed, and its length (from cecocolic orifice to rectum) was measured. Both before and after opening it longitudinally, the colon was photographed for macroscopic evaluation. Intestinal contents were flushed off with ice cold 0.9% NaCl solution. Three 0.5–1-cm-long samples were collected from the distal part of the colon for histological and biochemical analyses. Samples for the latter were frozen in liquid nitrogen and stored at −80 °C.

2.3. Measurement of Intestinal Permeability

Four hours before euthanasia, animals were given fluorescein isothiocyanate−dextran (FITC−dextran) (4 kDa, TdB Labs) solution (600 mg/kg, 125 mg/mL) via a gastric gavage. For the analysis, plasma was diluted in PBS-T (136 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 4.46 mM KH_2PO_4, 0.1% Tween, pH 7.4) and analyzed for FITC–dextran concentration with a fluorescence spectrophotometer at the excitation wavelength of 495 nm and the emission wavelength of 525 nm. Standard curves for determining the concentration in the samples were obtained by diluting FITC–dextran in PBS-T.

2.4. Macroscopical Evaluation

The level of colonic inflammation and damage was evaluated from photographs based on three parameters: presence of diarrhea, visible fecal blood, and colon wall damage (edema and/or ulceration). The evaluation was performed independently by three researchers blinded to the treatments. Based on the severity, scores from 0–3 were given for each parameter, and the final colitis score was calculated as their average as described previously [16].

2.5. Plasma β-Hydroxybutyrate

As a marker of ketosis, the level of plasma BHB was determined with a commercial enzymatic kit (Cayman Chemicals, Ann Arbor, MI, USA).

2.6. Histological Analyses

Tissue sections from the distalmost part of the colon were fixed in 4% paraformaldehyde (Thermo Fischer Scientific, Waltham, MA, USA) solution for 36 h and then stored at 4 °C in 70% ethanol until histological processing. The fixed samples were cut into two halves resulting in two longitudinal pieces that were embedded in paraffin and sectioned at 4 µm thickness. The slides were stained with hematoxylin and eosin (HE) dye and evaluated for histological changes. The slides were read blinded to the treatments.

The HE-stained slides were evaluated for the severity of tissue damage and inflammation, which were separately scored. The scoring mainly followed the system described in [15], which employs separate integer scores from 0 to 3 for tissue damage and inflammation summed to a combined score ranging from 0 to 6. In this study, the scoring was modified to account for the generally mild histological alterations in DSS-KD groups. The mildest DSS-induced changes consisting of minimal or mild mucosal damage received a tissue damage score of 0.5 and a mild *lamina propria* mononuclear infiltrate a score of 0.5. Correspondingly, mucosal tissue damage that did not include marked surface epithelial erosions or ulceration received a damage score of 1.5 and non-extensive damage extending beyond mucosa a score of 2.5. Inflammatory cell infiltration limited to the inner circular layer of *muscularis externa* was scored as 2.5.

2.7. Reverse Transcription Quantitative Polymerase Chain Reaction

The expression of the TJ protein-coding genes *Cldn1*, *Cldn2*, *Cldn4*, and *Ocln* and inflammatory marker genes *Tnf*, *Il1b*, *Il6*, and *Lcn2* in colonic tissue was analyzed with reverse transcription quantitative polymerase chain reaction (RT-qPCR) according to a previously described protocol [14]. In brief, RNA was extracted from colonic samples with NucleoSpin RNA Kit (Macherey Nagel, Duren, Germany). RNA concentration was determined, samples were diluted to the same concentration, and RNA was reverse transcribed to complementary DNA with iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). RT-qPCR was run with LightCycler® 480 SYBR Green Master (Roche Diagnostics Corp., Indianapolis, IN, USA) with the following amplification protocol: 10 min at 95 °C, 40 cycles of denaturation (15 s, 95 °C), annealing (30 s, 60 °C), and elongation (30 s, 72 °C). The melt curves were analyzed at the end of the experiment. The primer sequences used are listed in Table 2. The results were calculated as relative quantities (RQs) of messenger RNA (mRNA) according to the Vandesompele method [17]. The housekeeping genes used for normalization were *18S*, *Eef2*, and *Rplp0*.

Table 2. Primer sequences used in RT-qPCR analyses. If no reference is cited, the primer pair is designed by the authors.

Gene	Forward Primer	Reverse Primer	Ref.
18S	AACGAACGAGACTCTGGCAT	ACGCCACTTGTCCCTCTAAG	
Eef2	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA	[18]
Rplp0	TAACCCTGAAGTGCTCGACA	GGTACCCGATCTGCAGACA	[19]
Cldn1	AGACCTGGATTTGCATCTTGGTG	TGCAACATAGGCAGGACAAGAGTTA	[20]
Cldn2	GCAAACAGGCTCCGAAGATACT	GAGATGATGCCCAAGTACAGAG	[21]
Cldn4	TGAGCGATGGCGTCTATGG	GATGTTGCTGCCGATGAAGG	
Ocln	CGGTACAGCAGCAATGGTAA	CTCCCCACCTGTCGTGTAGT	[22]
Il1b	CTCCAGCCAAGCTTCCTTGT	TCATCACTGTCAAAAGGTGGCA	[23]
Il6	ATCGTGGAAATGAGAAAAGAGTTGT	CTGCAAGTGCATCATCGTTGT	
Tnf	TGGCACCACTAGTTGGTTGTCT	AGCCTGTAGCCCACGTCGTA	[24]
Lcn2	CCACCACGGACTACAACCAG	AGCTCCTTGGTTCTTCCATACAG	

2.8. Western Blot

Western Blot (WB) was used to analyze the relative quantity of jejunal and colonic TJ proteins claudin-1, -2, and -4, as well as occludin. Samples were prepared according to a previously described protocol [14]. Briefly, the tissue pieces were homogenized, sonicated, and centrifuged. Supernatants were collected and their protein concentrations determined with a commercial kit (Pierce™ BCA Protein Assay Kit, Thermo Fischer

Scientific). Samples for analysis were prepared by diluting supernatants to the same total protein concentration with PBS-T and Laemmli sample buffer (Bio-Rad) containing 5% 2-mercaptoethanol followed by the denaturation of the proteins on a heat block.

From each group, 2–3 samples (30 µg total protein) were loaded in 4–20% Mini-PROTEAN® TGX™ Precast gels (Bio-Rad). After the SDS-PAGE run, the proteins were transferred to a nitrocellulose membrane (Bio-Rad), and total protein concentration was determined with a commercial kit (Revert™ 700 Total Protein Stain Kit, LI-COR, Lincoln, NE, USA). Afterwards, the membranes were blocked (Odyssey blocking buffer (TBS), LI-COR) for 1 h and incubated overnight in a primary antibody solution. The primary antibodies used were claudin-1 (sc-166338, 1:200; Santa Cruz Biotechnology, Dallas, TX, USA), claudin-2 (sc-293233, 1:200; Santa Cruz Biotechnology), claudin-4 (sc-376643, 1:200; Santa Cruz Biotechnology), and occludin (#91131, 1:1000, Cell Signaling Technology, Danvers, MA, USA). The next day, the membranes were incubated in fluorescence-labeled secondary antibody solution (1:10,000 (IRDye 680LT goat anti-mouse, or IRDye 800CW goat anti-rabbit, LI-COR)) for 1 h. The bands were detected with the Odyssey CLx Infrared Imaging system (LI-COR) and analyzed with the Image Studio program (LI-COR). The intensities of the target protein bands were normalized against the intensity of the total protein.

2.9. Statistical Analyses

GrapPad Prism 10 (Dotmatics, La Jolla, CA, USA) was used for conducting the statistical analyses and creating the figures. Weight change and DAI were analyzed with mixed-effects model followed by Tukey's multiple comparisons test. For other parameters, Shapiro–Wilk test was used to determine normal distribution, based on which the data were analyzed either using one-way ANOVA followed by Tukey's post hoc test or Kruskal–Wallis H test followed by Dunn's post hoc test. RT-qPCR results are expressed as geometric mean and other data as mean unless stated otherwise. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Ketogenic Diets Mitigate DSS-Induced Weight Loss and Macroscopic Signs of Inflammation

After the induction of colitis, both KDs alleviated inflammation-induced weight loss when compared to DSS-CD in which the mice had lost 20% of their pre-DSS weight at the end of the experiment (Figure 2A). The weight loss in the DSS-SFA-KD group (10% of the pre-DSS weight) was significant when compared to healthy controls which did not lose weight, whereas the DSS-LA-KD group only lost 5% of its pre-DSS weight and the difference to healthy mice did not reach significance. However, there was no significant difference between the KD groups. Weight development in KD groups was comparable to controls before DSS administration, and there were no significant differences in average daily energy consumption (13.0 $\pm$ 0.8 kcal/d in CD and DSS-CD, 14.4 $\pm$ 1.2 kcal/d in DSS-SFA-KD, and 15.3 $\pm$ 1.4 kcal/d in DSS-LA-KD). Compared to DSS-CD, mice in both KD groups exhibited fewer visible signs of colitis, indicated by the lower DAI scores with significant differences between DSS-CD and DSS-LA-KD from day five of DSS administration onwards and between DSS-CD and DSS-SFA-KD on day four (Figure 2B). DSS-CD mice began to display signs of colitis on day four (difference in DAI compared to CD), and this was delayed in DSS-SFA-KD and DSS-LA-KD groups by one and two days, respectively. As expected, on the day of sacrifice, all mice in KD groups showed significantly elevated plasma BHB levels (Figure 2C) which were significantly higher in DSS-LA-KD when compared to DSS-SFA-KD. Some animals in DSS-CD also showed increased levels of BHB. While macroscopic changes in the colon were clearly visible in DSS-CD and DSS-SFA-KD groups, LA-KD-fed mice were protected from these changes (Figure 2D,E). In DSS-LA-KD, DSS-induced colon shortening was prevented (Figure 2D,F). DSS-SFA-KD mice showed a decrease of 13% in the mean colon length, whereas in DSS-CD group, colons were 21% shorter than in healthy controls.

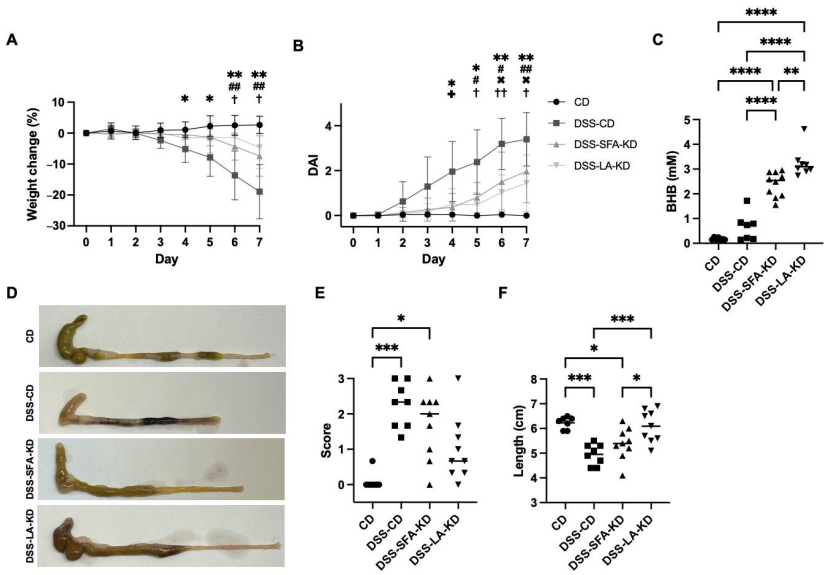

Figure 2. Macroscopic parameters of DSS-induced inflammation and plasma BHB levels. (**A**) Weight change (mean ± SD, n = 8–10/group) as a percentage from the start of DSS administration. (**B**) DAI scores (mean ± SD, n = 8–10/group) from the start of DSS administration. (**C**) Plasma BHB. (**D**) Representative photographs of colons after DSS period. (**E**) Colitis score from the macroscopic evaluation of the colon. (**F**) Length of the colons after DSS treatment. In (**A**,**B**), * = CD vs. DSS-CD, # = CD vs. DSS-SFA-KD, × = CD vs. DSS-LA-KD, + = DSS-CD vs. DSS-SFA-KD, and † = DSS-CD vs. DSS-LA-KD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, †† $p < 0.01$, and ## $p < 0.01$. BHB = β-hydroxybutyrate, DAI = disease activity index, DSS = dextran sodium sulfate, CD = healthy group with control diet, DSS-CD = DSS group with control diet, DSS-LA-KD = DSS group with diet high in linoleic acid, and DSS-SFA-KD = DSS group with diet high in saturated fatty acids.

3.2. Ketogenic Diets Protect from DSS-Induced Mucosal Damage

Both KDs substantially alleviated DSS-induced histological lesions and reduced the depth of the tissue damage and inflammation (Figure 3A), with a statistically significant effect in the LA-KD-fed mice (Figure 3B–D). The DSS-CD mice exhibited, in general, marked tissue damage and inflammatory cell infiltration, whereas the DSS-SFA-KD mice showed mild to moderate and the DSS-LA-KD mice showed minimal or mild changes, generally limited to mucosa and submucosa. In the DSS-CD group, the lesions extended to submucosa and muscular layers in four and throughout the intestinal wall in two samples. Typical findings were abundant crypt loss, fibroplasia, and diffuse to focally extensive erosion of the surface epithelium with degeneration and dysplasia of the remaining crypts and epithelium. Moderate to marked mixed (macrophages, neutrophils, and lymphocytes) inflammatory cell infiltrate was present.

The mildly affected six DSS-SFA-KD mice showed minimal to mild degenerative changes in the surface epithelium and variable degeneration and dysplasia of the crypt epithelium, as well as goblet cell hypertrophy and/or slight crypt elongation, dilatation, and increased number of mitoses. In the moderately affected four mice, variable crypt loss and few epithelial erosions were present, and surface epithelial cells showed marked degeneration and dysplasia. The *lamina propria* contained minimal to moderate mononuclear cells (macrophages and lymphocytes) or mixed inflammatory infiltrates, and the mixed inflammation extended to submucosa in two mice and to *muscularis externa* in two mice. In the DSS-LA-KD mice, the minimal or mild histopathological changes were limited to mucosa. The surface epithelium exhibited focal to diffuse and minimal to mild degen-

erative changes, and the crypt epithelium exhibited minimal to mild degeneration and individual cell loss as well as mild crypt dilatation and/or mild elongation and goblet cell hyperplasia. Minimal to mild mononuclear cell inflammatory infiltrate was present in the *lamina propria*. In one mouse, basophilic, low crypt epithelium, and increased number of goblet cells suggested minimal crypt hyperplasia.

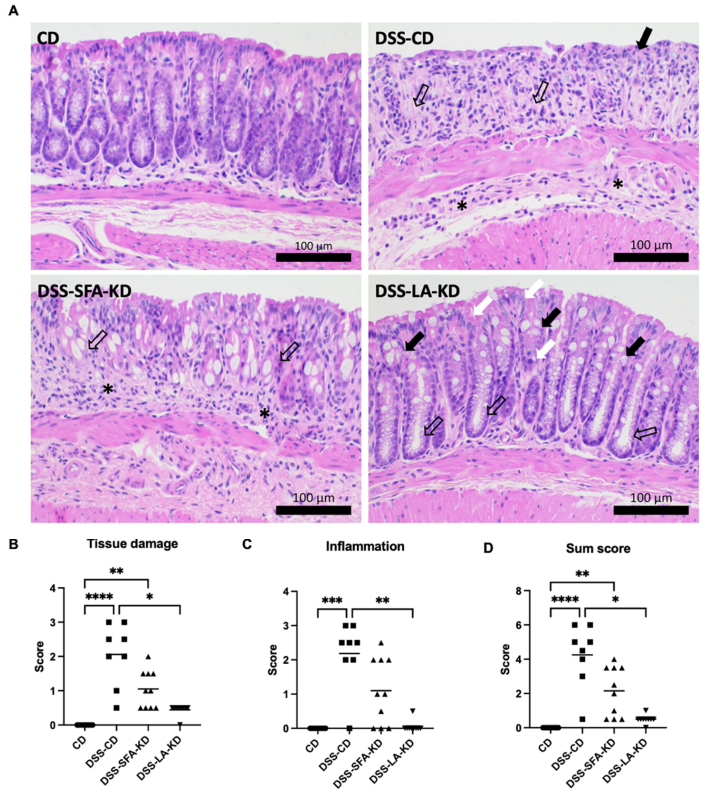

Figure 3. Histological findings and histological lesion scoring of DSS-induced colitis. (**A**) Microphotographs of HE-stained colon sections. Bars 100 µm. CD. No histopathological findings. DSS-CD. Crypt loss and lamina propria fibroplasia (open arrows). Surface epithelium erosion (arrow) and moderate neutrophil and macrophage infiltration in the lamina propria and submucosa (asterisks). DSS-SFA-KD. Marked degeneration and dysplasia of the surface and crypt epithelium and goblet cell hypertrophy (open arrows). Moderate mononuclear cell inflammatory infiltrate in the lamina propria (asterisks). DSS-LA-KD. Minimal degenerative changes in the surface and crypt epithelium (white arrows) and goblet cell hyperplasia (arrows), as well as mild crypt elongation and dilatation (open arrows). (**B**) Tissue damage score. (**C**) Inflammation score. (**D**) Combined sum score of tissue damage and inflammation. Original microphotographs are presented in Figure S1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. DSS = dextran sodium sulfate, CD = healthy group with control diet, DSS-CD = DSS group with control diet, DSS-LA-KD = DSS group with diet high in linoleic acid, DSS-SFA-KD = DSS group with diet high in saturated fatty acids, and HE = hematoxylin and eosin.

3.3. Ketogenic Diet High in Linoleic Acid Prevents Increases in Inflammatory Markers

The transcription of all analyzed inflammatory markers was significantly increased in DSS-CD. LA-KD protected from DSS-induced increases in the mRNA expression of *Il1b* and *Tnf*, and there was no statistical difference in any of the inflammatory markers between DSS-LA-KD and CD (Figure 4). While there were no significant differences between DSS-

SFA-KD and CD in *Il6* and *Tnf*, DSS-SFA-KD exhibited a modest increase in *Il1b* and *Lcn2*. There were no statistically significant differences between the two DSS-KD groups.

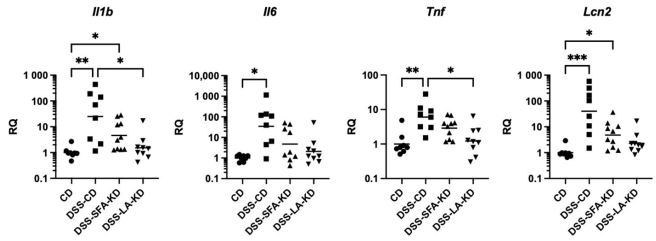

Figure 4. Colonic mRNA expression of inflammatory markers *Il1b*, *Il6*, *Tnf*, and *Lcn2*. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. DSS = dextran sodium sulfate, CD = healthy group with control diet, DSS-CD = DSS group with control diet, DSS-LA-KD = DSS group with diet high in linoleic acid, DSS-SFA-KD = DSS group with diet high in saturated fatty acids, and RQ = relative quantity.

3.4. Ketogenic Diets Normalize DSS-Induced Abnormalities in Tight Junction Protein Expression

DSS increased permeability to FITC–dextran (4 kDa) in all groups and its level did not differ significantly between the DSS groups (Figure 5). In DSS-CD, the expression of claudin-1 and claudin-4 was increased both on mRNA and protein level compared to CD and DSS-LA-KD, respectively (Figure 5). Interestingly, *Cldn2* expression was depressed in DSS-CD, but LA-KD preserved its expression. DSS-associated decrease in occludin levels was prevented by LA-KD, whereas in SFA-KD, its expression was modestly lower.

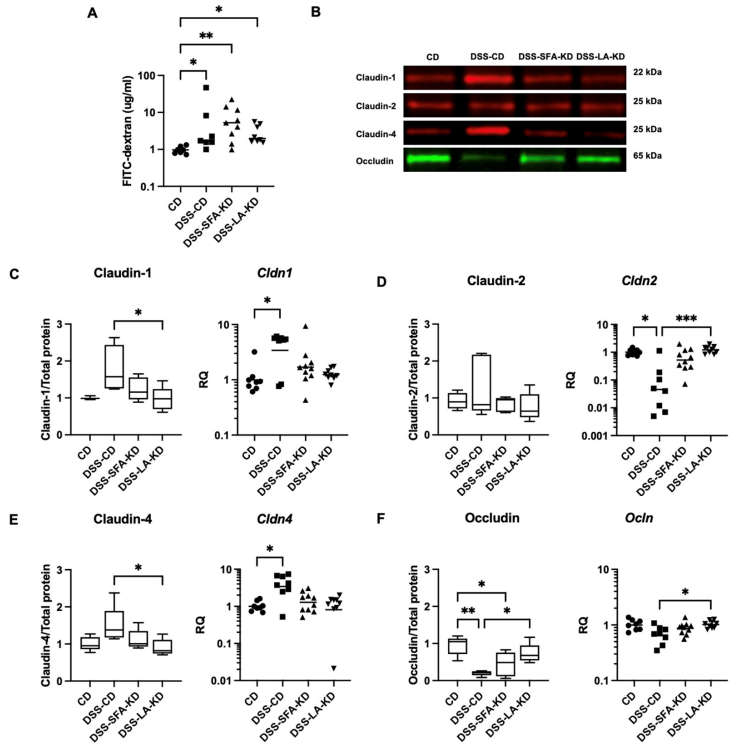

Figure 5. Intestinal permeability to FITC–dextran and colonic tight junction protein expression on protein and mRNA level. (**A**) Intestinal permeability to FITC–dextran (4 kDa) measured as the plasma

concentration of the compound. Data is expressed as median. (**B**) Representative Western Blot bands for the analyzed proteins. (**C**) Claudin-1, (**D**) claudin-2, (**E**) claudin-4, and (**F**) occludin. For Western Blot analyses, n = 3–5 per group. Images of original blots are presented in Figure S2. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. DSS = dextran sodium sulfate, CD = healthy group with control diet, DSS-CD = DSS group with control diet, DSS-LA-KD = DSS group with diet high in linoleic acid, DSS-SFA-KD = DSS group with diet high in saturated fatty acids, and FITC–dextran = fluorescein isothiocyanate dextran.

4. Discussion

We investigated the impact of KDs with two different fat sources on DSS-induced experimental colitis. The KDs were high in either SFAs or polyunsaturated LA. We analyzed histological changes, intestinal permeability, TJ protein levels, and inflammatory markers. In our setting, both KDs were protective against inflammation, and the effect was more pronounced with LA-KD. To our knowledge, this is the first time KDs with different fat sources have been compared in the context of experimental colitis. Histological damage and inflammatory markers, *Il6*, *Il1b*, *Tnf*, and *Lcn2*, were markedly reduced particularly in LA-KD-fed mice. Colitis-induced derangements in TJ protein and mRNA expression were normalized by LA-KD. Although SFA-KD led to the amelioration of colitis, the effect was lesser than with LA-KD. In addition, LA-KD resulted in higher plasma levels of the ketone body BHB. Here, we show that KDs reduce the severity of DSS colitis, and their preventive potential is dependent on the dietary fatty acid composition.

DSS administration induced weight loss and visible signs of colitis, as well as macroscopic damage and shortening of the colon in animals fed the control diet. Both KDs alleviated inflammation-related weight loss, and the DAI scores were lower in both KDs when compared to DSS-CD. However, only LA-KD was protective against DSS-induced colon shortening and macroscopic changes. In our previous research, BHB levels were not different between healthy mice on SFA-KD or LA-KD [14], whereas in the present study, we saw significantly higher plasma concentrations in DSS-LA-KD than in DSS-SFA-KD. BHB administered either orally [9,25] or rectally [26] has been reported to alleviate DSS-induced colitis, and it is possible that, in our setting, the greater protective effect from LA-KD is partly a result of higher BHB availability. However, in these studies, plasma BHB levels were not reported, and it is unclear whether the magnitude of the difference in our study is large enough to produce a physiologically meaningful effect. Some animals in DSS-CD also showed elevated levels of plasma BHB which is likely due to inadequate food intake and absorption as a response to the DSS-induced inflammation.

Severe DSS-induced histological lesions, damage, and inflammation were observed in DSS-CD. KDs, especially LA-KD, protected the mice from these changes. This is in line with the findings of Kong et al. [10] and Abdelhady et al. [9] who reported less inflammatory cell infiltration in DSS-treated rodents fed KDs with unknown fat sources. On the other hand, Li et al. [11] showed LA-rich KD to promote immune cell infiltration and epithelial damage in DSS colitis. As expected, in DSS-CD, the transcription of *Tnf*, *Il1b*, *Il6*, and *Lcn2* was upregulated. LA-KD prevented the DSS-induced increase in all the inflammatory markers in colon and lowered *Il1b* and *Tnf* compared to DSS-CD. While SFA-KD maintained the levels of *Il6* and *Tnf* with no differences to healthy controls, the diet was unable to fully protect from the elevation in *Il1b* and *Lcn2*. These results contradict those of Li et al. [10] who reported KD to increase colonic mRNA expression of *Il1b*, *Il6*, and *Tnf* upon exposure to DSS. In our prior research, we saw LA-KD to induce slight increase in colonic *Il6* and *Tnf* in healthy animals [14]. However, based on the present study, it seems that these changes do not translate into pronounced inflammation upon a proinflammatory insult.

After the induction of colitis, both KDs preserved claudin expression on a level similar to the healthy controls. Significant changes were observed in DSS-CD and, interestingly, the mRNA and protein expression of the barrier-sealing claudins 1 and 4 was increased, whereas the transcription of pore-forming claudin-2 was decreased. Accordingly, previous research suggests that, even though claudins 1 and 4 are recognized to be barrier-forming TJ proteins [27,28], their protein expression is higher in biopsies from patients with ac-

tive inflammatory bowel disease, and claudin-1 level is correlated with the severity of inflammation [29]. In addition, claudin-1 overexpression causes increased susceptibility to DSS-induced colitis and inferior recovery from it [30]. Thus, the upregulation of these claudins in DSS-CD is likely a compensatory mechanism. This compensation might be an explanatory factor for why there were no significant differences between DSS groups in intestinal permeability to FITC–dextran despite the overall protective effects of KDs. The protein expression of occludin was depressed in DSS-CD and DSS-SFA-KD, whereas LA-KD preserved a level of expression equal to healthy controls. Previously, we have demonstrated four-week feeding with SFA-KD but not LA-KD to promote the protein expression of claudins 1 and 4 in healthy animals [14], but based on the current study, this does not appear to be meaningful upon DSS-induced inflammation.

The gut microbiota is an important mediator of the DSS-induced inflammation and the effect of KD, whether protective or predisposing, might be related to its composition [10,11]. While the microbiota was not analyzed in this study, it is possible that changes in its composition are responsible for the different outcomes between groups. As a whole, the KD-related gut microbiota seems to decrease the levels of pro-inflammatory Th17 cells in the intestine [31]. These cells contribute to the severity of colitis [32], and while analyzing immune cell subpopulations was outside the scope of this study, KD-mediated reduction of Th17 cells might be one of the mechanisms behind the protective effect. The fat source of the diet also has an impact on the gut microbiota. In mice fed a diet with 42 E% from fat, the fatty acid composition of the diet determines the composition of the microbiota [33], and the possible differences between the two KD groups in our study might explain the greater protective effect from LA-KD. LA supplementation was recently reported to protect from *Porphyromonas gingivalis*-induced aggravation of DSS-induced colitis by decreasing the Th17/Treg cell ratio [34]. The combined effect of KD and LA on T cell subpopulations may be a factor behind the pronounced benefit from LA-KD.

Overall, our results align with the observations of Kong et al. [10] who reported KD to alleviate DSS-induced colitis via microbiota-dependent reduction in colonic group 3 innate lymphoid cells, and Abdelhady et al. [9] who observed benefits associated with NLRP3 inflammasome inhibition, decreased apoptosis, and increased autophagy. Thus, our findings contradict those of Li et al. [11] who reported KD to worsen outcomes in the same model of colitis. Our study challenges the hypothesis of the difference being related to the duration of the diet intervention, since our experiment was closer in length to that of Li et al. (thirty days) than Kong et al. (16 weeks). While the protocol of DSS administration was slightly different in duration and the concentration of the compound, our design was, again, more resembling that of Li et al. in these aspects. Importantly, our findings indicate that the differences observed between studies may not be a result of the fat source of the diet—while the effect was more pronounced in LA-KD, we saw both KDs to offer protection when compared to the non-ketogenic control diet. Moreover, the KD formula used by Li et al. closely resembled LA-KD which we showed to be more protective than SFA-KD. While investigating the cause of these between-study discrepancies warrants further research, this study supports the notion of KDs holding potential in alleviating intestinal inflammation.

5. Conclusions

We observed KDs to reduce the severity of DSS colitis, and the preventive effect seems to be affected by dietary fatty acid composition. KDs alleviated inflammation and maintained colonic TJ protein levels, indicating the prevention of colitis development altogether. KD rich in polyunsaturated fatty acid LA was consistently more effective in alleviating colitis than KD high in SFAs. Thus, KDs may have preventive potential against intestinal inflammation, with the level of effect being dependent on the dietary fat source.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16091348/s1, Table S1: Formulations of study diets. Figure S1: Original microphotographs of HE-stained colon sections. Figure S2: Original and unmodified Western Blot images of colonic tight junction proteins.

Author Contributions: Conceptualization, L.T., H.S. and R.K.; methodology, L.T. and H.S.; formal analysis, L.T. and J.L.; investigation, L.T. and J.L.; resources, R.K.; writing—original draft preparation, L.T.; writing—review and editing, L.T., H.S., M.L. and R.K.; visualization, L.T.; supervision, H.S. and R.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol is approved by the Ethics Committee of the Regional State Administrative Agency for Southern Finland (ESAVI/9377/2019), the approval date is 14 May 2019.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data used in this study are available from the corresponding author upon reasonable request due to privacy.

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Free acid β-hydroxybutyrate supplementation does not ameliorate dextran sodium sulfate-induced colitis similar to ketogenic diet in male mice

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ABSTRACT

High-fat, low-carbohydrate ketogenic diets have been found to alleviate experimental colitis in rodents. These diets lead to increased endogenous production and utilization of ketone bodies, such as β-hydroxybutyrate (BHB), and supplementation with exogenous ketones has arisen as a potential alternative to ketogenic diets. This study aimed to investigate how continuous high-dose feeding with free acid BHB influences experimental colitis compared to a ketogenic diet with the hypothesis that BHB would also alleviate the inflammation. We fed nine-week-old C57BL/6 J male mice for four weeks with one of three diets: a low-fat control diet, a ketogenic diet, or a low-fat diet supplemented with free acid R-BHB and then induced colonic inflammation with dextran sodium sulfate (DSS). We assessed macroscopic and histological changes in the colon, intestinal permeability to fluorescein isothiocyanate dextran, colonic mRNA expression of tight junction proteins and inflammatory markers, fecal calprotectin, and microbiota composition. While the ketogenic diet alleviated DSS-induced weight loss, macroscopic changes, and histological lesions, the BHB-supplemented diet did not have the same effect. The pre-DSS composition of the microbiota was drastically different between the diet groups which may partly explain the different outcomes. In conclusion, high-dose supplementation with free acid BHB may not produce the same benefits as ketogenic diet in the context of colonic inflammation.

1. Introduction

Inflammatory bowel diseases (IBDs) are chronic, debilitating conditions of the intestine to which there is no known cure. IBDs comprise of Crohn's disease and ulcerative colitis, and the clinical presentations of these diseases include abdominal pain, diarrhea, rectal bleeding, and weight loss. In the current care, IBDs are treated with pharmacotherapies, such as aminosalicylates, corticosteroids, immunomodulators, and biologics, especially tumor necrosis alpha-inhibitors [1]. However, a substantial number of patients fails to respond to these therapies [2] which calls for novel treatment strategies.

High-fat, low-carbohydrate ketogenic diets have arisen as potential adjunct therapies for chronic diseases [3,4]. On such a diet, ketogenesis is upregulated in the liver due to the decreased availability of glucose, which leads to increased circulating levels of ketone bodies acetoacetate and β-hydroxybutyrate (BHB). Most tissues of the body, including the intestine [5], can use these metabolites as an energy source. In addition to providing energy, BHB has anti-inflammatory properties [6] and it can decrease oxidative stress [7]. Recently, supplementation with exogenous ketone bodies has emerged as an alternative strategy to increase ketone levels without carbohydrate restriction [8].

Previously, we and others have observed ketogenic diets to ameliorate experimental colitis in rodents and it seems that the effect of the diet is mediated through alterations in the intestinal microbiota [9–11]. One mechanism through which these diets may modify the microbiota is via ketone bodies, specifically BHB which is also metabolized by the gut

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microbiota [12]. BHB appears to alter gut microbiota in a way that reduces pro-inflammatory Th17 cells in the intestine. In line with this, previous studies have shown BHB to alleviate dextran sodium sulfate (DSS)-induced colitis in mice when administered rectally [13], via a gastric gavage [14], or intraperitoneally [10,15]. Injected BHB was reported to abate DSS-induced changes in the microbiota in a similar fashion as a ketogenic diet [10]. In addition, continuous feeding with a ketone monoester exhibited a pronounced benefit over intraperitoneal BHB injection on colitis in rats [15], indicating that the effects of ketone bodies in the gut lumen, such as interaction with the gut microbiota, may be of importance. As a treatment for chronic diseases such as IBDs, ketone body supplementation would be more feasible and sustainable when compared to a ketogenic diet and therefore, warrants further research. However, besides the increased supply of ketone bodies, other features of ketogenic diets such as the macronutrient composition, can also be responsible for the diet's effect on microbiota and thus, the alleviation of colitis.

As previous studies have not compared the effects of oral BHB supplementation and ketogenic diets on colitis, we set out to investigate whether BHB administration would alleviate colonic inflammation alike the diet. To investigate this, we fed mice a low-fat diet either with or without free acid R-BHB, the bioidentical form of the compound, or a ketogenic diet for four weeks, after which colitis was induced with DSS. We compared macroscopic and histological changes in the colon, fecal calprotectin, colonic mRNA expression of inflammatory markers and tight junction (TJ) proteins, intestinal permeability, and microbiota composition with the aim to clarify if continuous high-dose feeding with BHB recapitulates the beneficial effects of ketogenic diets in experimental colitis.

2. Materials and methods

2.1. Animal experiment

The experiment was approved by the animal research board of the Regional State Administrative Agency for Southern Finland (ESAVI/9377/2019) and conducted according tho the ARRIVE guidelines [16]. Eight-week-old male C57BL/6 J mice (n = 44) were obtained from Scanbur (Karlslunde, Denmark) and allowed to acclimatize for seven days before the start of the dietary interventions. The animals were housed in individual cages under a 12 h light–dark cycle, at 25 ± 1 °C and 50–60 % humidity with unrestricted access to food and water. Sample size was determined based on our previous experience on the DSS model of experimental colitis [11].

The mice were randomly divided into three groups based on the diet: low-fat control diet group (CD) (n = 16), ketogenic diet group (KD) (n = 14), and BHB-supplemented diet group (BHB) (n = 14). One animal in KD had to be euthanized after three weeks of the intervention due to progressive weight loss. Weight and consumption of food and fluid were measured daily. The study diets were custom-made (Envigo, Indianapolis, IN, USA) and matched for protein and micronutrients. Free acid R-BHB incorporated into the diet was supplied by NNB Nutrition (Nanjing, China). In the product, acidity was neutralized with KOH. The diet formulations are provided in Table 1 and macronutrient compositions of the diets in Table 2. The percentage of BHB (12 E%), accounting in the diet was determined based on previous literature [17,18]. In human studies, doses up to 752 mg/kg of BHB, possibly accounting for 8–12 % of daily energy intake, as ketone esters have been tested without serious adverse effects [19]. Duration of the experiment was based on our previous research using ketogenic diets in the same model [11].

DSS treatment was started on day 28 of the experiment by replacing regular drinking water with DSS (40 kDa, TdB Labs, Uppsala, Sweden) solution (2.5 % w/v). From each diet group, eight animals were randomly allocated to DSS groups: DSS-treated control diet group (DSS-CD), ketogenic diet group (DSS-KD), and BHB-supplemented diet group (DSS-BHB) (Fig. 1). DSS was administered for four days, after which regular water was given for two days before sacrifice. In addition to weight, food, and fluid consumption, consistency of stool, and presence of blood in the feces were monitored daily during this period. Based on these parameters, disease activity index (DAI) was determined by following a previously described scoring system [20] where scores from 0 to 4 are given for each of the following: weight loss, stool consistency, and the degree of intestinal bleeding, and the final score for each animal is calculated as their average. If the animal reached the humane endpoint during the experiment, it was euthanized.

Table 1
Formulations of study diets.

	CD	KD	BHB
	TD.220115	TD.220116.PWD	TD.230257
Ingredient	**g/kg**	**g/kg**	**g/kg**
Casein	100.0	180.0	100.0
DL-Methionine	1.6	2.88	1.6
Corn Starch	512.46	0	412.46
Sucrose	100.0	0	100.0
Maltodextrin	155.0	0	155.0
Vegetable Shortening	25.0	590.0	25.0
Safflower Oil	25.0	85.0	25.0
3-Hydroxybutyric Acid, liquid, customer supplied	0	0	100.0
Cellulose	44.648	70.164	44.648
Mineral Mix, w/o Ca & P (98057)	13.4	24.8	13.4
Calcium Phosphate, dibasic	10.1	18.6	10.1
Calcium Carbonate	5.0	9.4	5.0
Vitamin Mix, w/o choline, A, D, E (83171)	5.0	13.875	5.0
Choline Bitartrate	2.5	4.625	2.5
Vitamin E, DL-alpha tocopheryl acetate (500 IU/g)	0.225	0.41	0.225
Vitamin A, palmitate (500,000 IU/g)	0.027	0.05	0.027
Vitamin D3, cholecalciferol (50,000 IU/g in sucrose)	0.03	0.056	0.03
TBHQ, antioxidant	0.01	0.14	0.01

TD.230257 is modified from TD.220115 to replace 10 % cornstarch with 3-Hydroxybutyric Acid (BHB). The formula shows 10 % inclusion, reflecting the net BHB addition. The preparation contains 50 % water, so the actual inclusion of the ingredient is 20 %. The 10 % water from the BHB ingredient contributes to the water that is added in order to pellet the diet. BHB contains ~4.6 kcal/g. When 10 % is added, BHB account for ~12 % of total energy.
BHB = β-hydroxybutyrate supplemented diet, CD = control diet, KD = ketogenic diet.

Table 2
Dietary compositions. Values are expressed as a percentage of energy.

	Control diet	Ketogenic diet	BHB-supplemented diet
Energy (kcal/g)	3.7	6.8	3.7
Protein (E%)	9.7	9.4	9.7
Carbohydrate (E %)	77.8	0.5	65.8
Fat (E%)	12.5	90.1	12.5
BHB (E%)	0	0	12

BHB = β-hydroxybutyrate.

2.2. Sample collection

Fecal samples were collected prior to DSS administration on day 27 of the experiment. For sample collection, animals were placed in empty cages and after 4 hours, fecal pellets were collected and frozen.

Animals were sacrificed under isoflurane (4 %, Vetflurane, Virbac, Carros, France) anesthesia by drawing blood from *vena cava* into EDTA-tubes (Kisker, Steinfurt, Germany). Blood samples were centrifuged at 2000 g for 15 min at 4 °C to separate plasma which was then frozen in

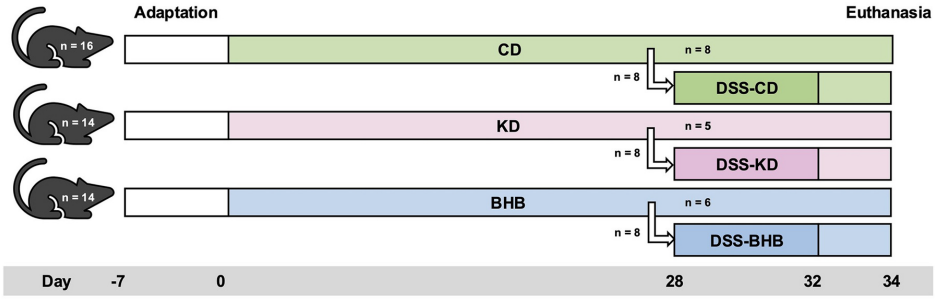

Fig. 1. The set-up of the animal experiment.

liquid nitrogen.

The entire intestine was removed, and the length of the colon (from cecocolic orifice to rectum) measured. The colon was photographed for macroscopic evaluation before and after opening it longitudinally. Intestinal contents were collected, residues of which were flushed off with 0.9 % NaCl solution. Three 0.5–1 cm-long tissue sections were collected from the distal part of the colon. They were frozen in liquid nitrogen for biochemical analyses or fixed for histological analyses as described below. Samples were also collected from prematurely killed animals and used for analyses.

2.3. Intestinal permeability measurement

Fluorescein isothiocyanate–dextran (FITC–dextran) (4 kDa, TdB Labs) solution (600 mg/kg, 125 mg/ml) was administered to the animals 4 hours before the euthanasia via a gastric gavage. FITC-dextran concentration was analyzed from plasma diluted in PBS-T (136 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 4.46 mM KH_2PO_4, 0.1 % Tween, pH 7.4) with a fluorescence spectrophotometer at the excitation wavelength of 495 nm and the emission wavelength of 525 nm. The standard curve for determining the concentration in the samples was obtained by diluting known amounts of FITC–dextran in PBS-T.

2.4. Macroscopical evaluation

Photographs of colon were used to evaluate the extent of colitis based on three parameters: presence of diarrhea, visible fecal blood, and inflammation (edema and/or ulceration). Based on the severity, scores from 0 to 3 were given for each parameter by following a system described previously [21]. The scoring was performed blinded.

2.5. Histological analyses

Tissue sections from the most distal part of the colon were fixed in 4 % paraformaldehyde solution (Thermo Fisher Scientific, Waltham, MA, USA) for 36 h after which they were transferred to 70 % ethanol and stored at 4 °C. The fixed samples were cut into 2 halves to obtain 2 longitudinal pieces that were embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin (HE) dye.

The HE-stained slides were evaluated for histological changes and scored for severity of tissue damage and inflammation. The scoring followed a previously described system where separate scores from 0 to 3 for tissue damage and inflammation were summed to a combined score ranging from 0 to 6 [20]. The scoring was modified due to the generally mild histological alterations observed in DSS-KD group. The mildest DSS-induced changes with minimal to mild mucosal damage received a tissue damage score of 0.5 and mild *lamina propria* mononuclear infiltrates a score of 0.5. The minimal to mild mucosal damage was defined as slight elongation and/or dilatation of the crypts and goblet cell hyperplasia as well as mild degenerative changes in the surface and crypt epithelium. Mucosal tissue damage (marked degeneration, flattening and/or dysplastic crypt epithelium) that did not include marked surface epithelial erosions or ulceration was scored as 1.5, and non-extensive damage extending beyond mucosa as 2.5. Inflammatory cell infiltration limited to the inner layer of *muscularis externa* received a score of 2.5. The scoring was performed blinded.

2.6. Biochemical analyses

Plasma BHB concentration was analyzed with a commercial enzymatic kit (Cayman Chemicals, Ann Arbor, MI, USA) after diluting the samples to the assay buffer. Levels of fecal calprotectin were analyzed with Mouse S100A8/S100A9 Heterodimer DuoSet ELISA (R&D Systems, Minneapolis, MN, US). Results were normalized against total protein determined with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

2.7. Targeted gene expression analysis

The expression of the TJ protein-coding genes *Cldn1, Cldn2, Cldn4,* and *Ocln;* and inflammatory marker genes *Tnf, Il1b, Il6,* and *Lcn2* in colonic tissue was analyzed with reverse transcription quantitative polymerase chain reaction (RT-qPCR) according to a protocol previously described [22]. In brief, RNA was extracted from colonic samples with NucleoSpin RNA Kit (Macherey Nagel, Duren, Germany). RNA concentration was determined, samples were diluted to the same concentration, and RNA was reverse transcribed to complementary DNA with iScript™ cDNA Synthesis Kit (Bio-Rad). RT-qPCR was run with LightCycler® 480 SYBR Green Master (Roche Diagnostics Corp., Indianapolis, IN, USA) with the following amplification protocol: 10 min at 95 °C, 40 cycles of denaturation (15 s, 95 °C), annealing (30 s, 60 °C) and elongation (30 s, 72 °C). The primer sequences used are listed in Table 3. The results were calculated as relative quantities (RQ) of messenger RNA (mRNA) according to the Vandesompele method [23]. The housekeeping genes used for normalization were *18S, Eef2,* and *Rplp0*.

2.8. Fecal microbiota analysis

The composition of the fecal microbiota prior to DSS administration was analyzed through 16S rRNA sequencing. A single fecal pellet was transferred to Lysing Matrix E tubes (MP Biomedicals, Santa Ana, California, USA) containing lysis buffer (MagMAX™, Thermo Fisher Scientific). Samples were lysed using the TissueLyser II (Qiagen, Hilden, Germany), and the supernatant was transferred to a deep well block (Thermo Fisher Scientific). DNA extractions and purifications were carried out using the KingFisher Apex Purification System (Thermo Fisher Scientific) following the MagMAX™ Microbiome Ultra Nucleic Acid Isolation protocol. Paired-end DNA libraries with a 350 bp insert

Table 3
Primer sequences used in RT-qPCR analyses.

Gene	Forward primer (5´-3´)	Reverse primer (5´-3´)	Gene ID
18S	AACGAACGAGACTCTGGCAT	ACGCCACTTGTCCCTCTAAG	19791
Eef2	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA	13629
Rplp0	TAACCCTGAAGTGCTCGACA	GGTACCCGATCTGCAGACA	11837
Cldn1	AGACCTGGATTTGCATCTTGGTG	TGCAACATAGGCAGGACAAGAGTTA	12737
Cldn2	GCAAACAGGCTCCGAAGATACT	GAGATGATGCCCAAGTACAGAG	12738
Cldn4	TGAGCGATGGCGTCTATGG	GATGTTGCTGCCGATGAAGG	12740
Ocln	CGGTACAGCAGCAATGGTAA	CTCCCCACCTGTCGTGTAGT	18260
Il1b	CTCCAGCCAAGCTTCCTTGT	TCATCACTGTCAAAAGGTGGCA	16176
Il6	ATCGTGGAAATGAGAAAAGAGTTGT	CTGCAAGTGCATCATCGTTGT	16193
Tnf	TGGCACCACTAGTTGGTTGTCT	AGCCTGTAGCCCACGTCGTA	16193
Lcn2	CCACCACGGACTACAACCAG	AGCTCCTTGGTTCTTCCATACAG	16819

size were prepared using 16S rRNA V4 region primers (515F-806R) with dual indexing. Library quality was assessed with Tapestation, Qubit, and KapaQuant, and sequencing was performed using the MiSeq Reagent Kit v2 (500 cycles) on the Illumina MiSeq platform. The demultiplexed sequences were quality-controlled using DADA2. Microbiome bioinformatics, including alpha and beta diversity and relative abundance of specific taxa, were analyzed using QIIME2 [24], with differentially abundant taxa assessed using Analysis of Composition of Microbiomes with Bias Control (ANCOM-BC) [25].

2.9. Statistical analyses

The data was analyzed, and figures created with GraphPad Prism 10 (Dotmatics, La Jolla, CA, USA). For fecal microbiota data, the figures were generated with QIIME2 [24]. Mixed-effects model followed by Tukey's multiple comparisons test was used for weight change and DAI. For other analyses, normal distribution was determined by Shapiro-Wilk test. Based normality, the data was analyzed either using one-way ANOVA followed by Tukey's post-hoc test, or Kruskal-Wallis H test followed by Dunn's post-hoc test. The level of statistical significance was

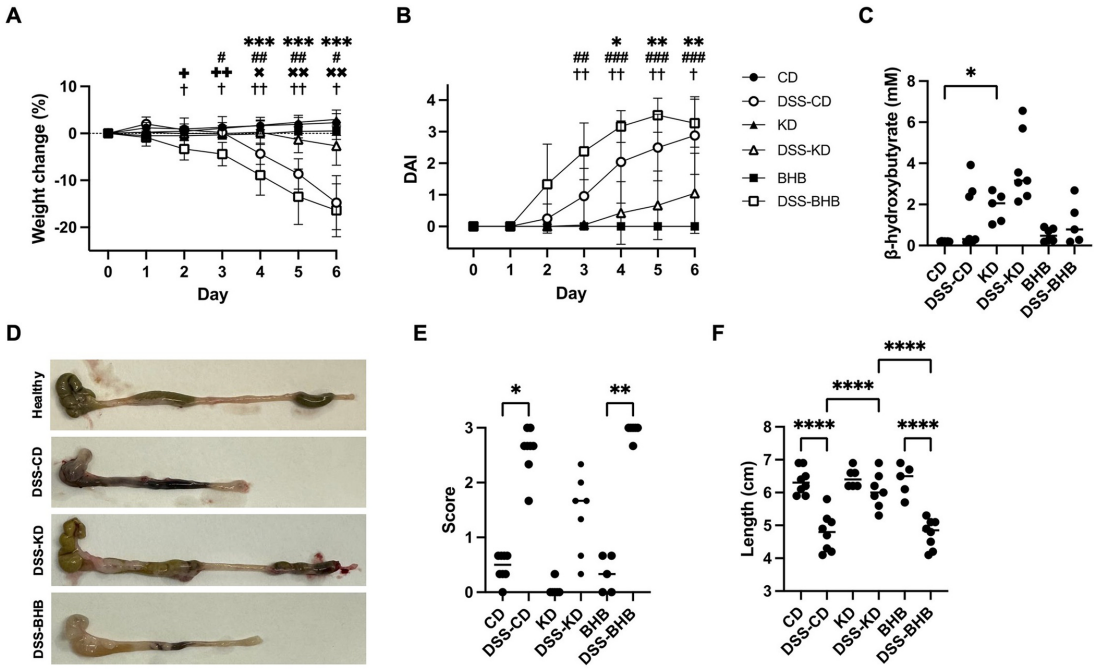

Fig. 2. Macroscopic parameters of DSS-induced inflammation and plasma BHB levels. A. Weight change (mean ± SD, $n = 6–8$/group) as a percentage from the start of DSS administration demonstrating substantial weight loss in DSS-CD and DSS-BHB but not in DSS-KD. B. DAI scores (mean ± SD, $n = 6–8$/group) from the start of DSS administration showing substantial disease activity induced by DSS that was prevented by a ketogenic diet. C. Plasma β-hydroxybutyrate showing elevated levels upon a ketogenic diet but not β-hydroxybutyrate supplementation. D. Representative photographs of colons after DSS period showing marked macroscopic damage in DSS-CD and DSS-BHB but not in DSS-KD. E. Colitis score from the macroscopic evaluation of the colon suggestive of DSS-induced damage that is alleviated by a ketogenic diet. F. Length of the colon after DSS treatment showing significant shortening in DSS-CD and DSS-BHB but not in DSS-kD. Results for A. and B. are analyzed with mixed-effects model followed by Tukey's multiple comparisons test, for C. and F. with Kruskal Wallis H test followed by Dunn's post-hoc test, and for E. with one-way ANOVA followed by Tukey's post-hoc test. In A. and B., *= CD vs. DSS-CD, # = BHB vs. DSS-BHB, x = DSS-CD vs. DSS-KD, + = DSS-CD vs. DSS-BHB, † = DSS-KD vs. DSS-BHB. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *** * < p 0.0001. DAI = disease activity index, DSS = dextran sodium sulfate, CD = healthy group with control diet, DSS-CD = DSS group with control diet, KD = healthy group with ketogenic diet, DSS-KD = DSS group with ketogenic diet, BHB = healthy group with β-hydroxybutyrate-supplemented diet, DSS-CD = DSS group with β-hydroxybutyrate-supplemented diet.

set at $p < 0.05$. The data is illustrated as mean except for RT-qPCR results which are shown as geometric mean. In the analyses, each DSS group was compared to a healthy group on the same diet and all DSS groups were compared against each other. For microbiota data, pairwise Kruskal-Wallis H test was used for α-diversity analyses (Shannon entropy score and Faith PD score) and pairwise Permanova test for β-diversity analyses (Unifrac analyses).

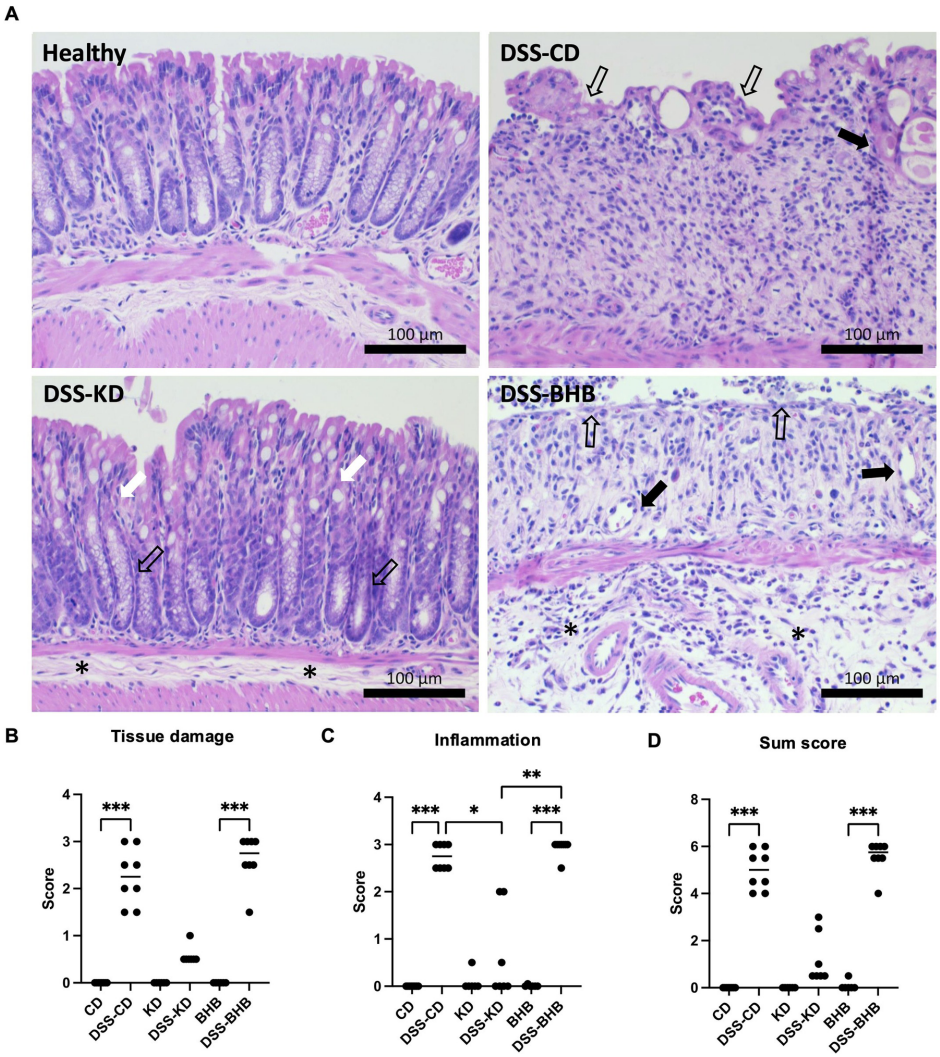

Fig. 3. Histological findings of DSS-induced colitis. A. Microphotographs of hematoxylin-eosin-stained colon sections. Bars 100 µm. Control. No histopathological findings. DSS-CD. Crypt loss and lamina propria fibroplasia (astrisks). Surface epithelium (open arrows) and remaining crypt epithelium (arrow) show marked degeneration and dysplasia. DSS-KD. Minimal degenerative changes in the crypt epithelium and goblet cell hyperplasia (white arrows). Crypt elongation, epithelial lowering, and basophilia point to crypt hyperplasia (open arrows). No cellular infiltrate in the submucosa (asterisks). DSS-BHB. The surface epithelium is eroded and covered with purulent infiltrate (open arrows). Lamina propria shows fibroplasia and apparent neovascularization (arrows) with plump endothelial cells. Moderate neutrophil and macrophage infiltration in the lamina propria and marked infiltration in the edematous submucosa (asterisks). B. Tissue damage score showing extensive damage in DSS-CD and DSS-BHB with little change in DSS-KD. C. Inflammation score demonstrating ample infiltration of inflammatory cells in DSS-CD and DSS-BHB which is ameliorated in DSS-KD. D. Combined sum score of tissue damage and inflammation indicating extensive DSS-induced histopathological changes in DSS-CD and DSS-BHB which are alleviated by ketogenic diet. Results are analyzed with Kruskal Wallis H test followed by Dunn's post-hoc test. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$. DSS = dextran sodium sulfate, CD = healthy group with control diet, DSS-CD = DSS group with control diet, KD = healthy group with ketogenic diet, DSS-KD = DSS group with ketogenic diet, BHB = healthy group with β-hydroxybutyrate-supplemented diet, DSS-CD = DSS group with β-hydroxybutyrate-supplemented diet.

3. Results

3.1. Ketogenic diet alleviates DSS-induced macroscopic changes while BHB supplementation aggravates them

While all animals in DSS-CD and DSS-KD survived to the end of the experiment, in DSS-BHB two mice died and one had to be sacrificed prematurely. DSS administration resulted in significant weight loss which was ameliorated in DSS-KD group that did not lose weight (Fig. 2A). DSS-BHB group exhibited a significant decrease in weight when compared to other DSS groups already on days two and three but the difference to DSS-CD lost significance on day four. DAI was lower in DSS-KD when compared to other DSS groups that exhibited visible signs of inflammation (Fig. 2B). However, the difference was significant only between when DSS-KD and DSS-BHB. As expected, BHB levels were elevated in KD groups (Fig. 2C). BHB supplementation resulted in only a small increase in plasma levels of the compound and the difference to animals on a control diet was not significant. DSS induced drastic macroscopic changes in the colon when compared to healthy controls in DSS-CD and DSS-BHB, whereas in DSS-KD there was no significant difference to KD (Fig. 2D and E). While DSS-CD and DSS-BHB exhibited DSS-induced reduction in colon lengths, animals in DSS-KD were

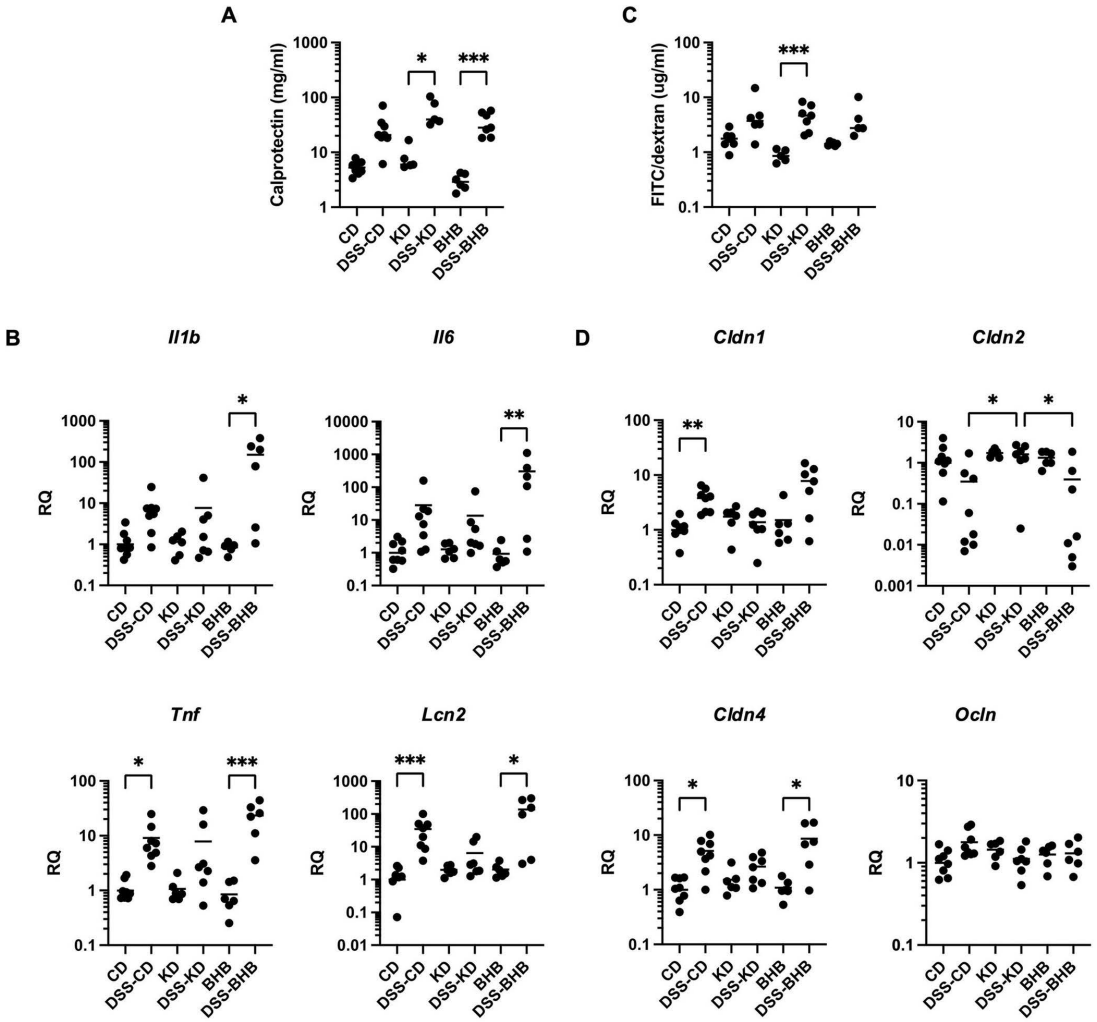

Fig. 4. Selected markers of intestinal inflammation and permeability. A. Fecal calprotectin levels suggestive of a DSS-induced increase in all groups that is not prevented by a ketogenic diet. B Colonic mRNA expression of *Il1b*, *Il6*, *Tnf*, and *Lcn2* showing upregulation of transcription as a response to DSS to be aggravated in DSS-BHB group and alleviated in DSS-KD group. C. Intestinal permeability to FITC-dextran (4 kDa) measured as the plasma concentration of the compound indicating increased permeability upon DSS-induced inflammation. D. Colonic mRNA expression of *Cldn1*, *Cldn2*, *Cldn4*, and *Ocln* suggestive of DSS-induced changes in TJ protein expression that are mitigated with a ketogenic diet. Results are analyzed with Kruskal Wallis H test followed by Dunn's post-hoc test. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$. DSS = dextran sodium sulfate, CD = healthy group with control diet, DSS-CD = DSS group with control diet, KD = healthy group with ketogenic diet, DSS-KD = DSS group with ketogenic diet, BHB = healthy group with β-hydroxybutyrate-supplemented diet, DSS-CD = DSS group with β-hydroxybutyrate-supplemented diet.

protected from colon shortening (Fig. 2D and F).

3.2. Ketogenic diet protects from DSS-related mucosal damage

DSS administration induced marked crypt loss, fibroplasia, and mucosal neovascularization, and in half of the samples, diffuse to focally extensively erosion of the surface epithelium with the remaining crypts having flat, degenerated, or dysplastic epithelium, and the surface epithelial cells showing marked degeneration and dysplasia (Fig. 3). This was ameliorated in DSS-KD where the histopathological changes were minimal to moderate and mostly limited to the mucosa. Indeed, there was no statistically significant difference in tissue damage between KD and DSS-KD. On the other hand, in DSS-BHB, the histological alterations were qualitatively similar to those in DSS-CD but appeared to be more intense. Diffuse to focally extensive erosion of the surface epithelium was present in six of the seven samples. However, there was no significant difference in tissue damage score between DSS-CD and DSS-BHB.

In DSS-CD, moderate to marked mixed inflammatory cell infiltrate was present in the *lamina propria*. The submucosal inflammatory infiltrate consisted of macrophages and neutrophils. In most DSS-KD animals, there was no inflammatory infiltrate with three mice exhibiting minimal mononuclear cell or mixed cell inflammatory infiltrate. Again, the most severe changes appeared in DSS-BHB where several mice exhibited purulent infiltrate on the mucosal surface. Mixed inflammatory cell infiltrate filled the lamina propria and tissue damage extended throughout the intestinal wall in four mice. While no statistical difference was observed between DSS-CD and DSS-BHB, both groups exhibited a marked increase when compared to DSS-KD.

3.3. BHB supplementation worsens DSS-induced increase in inflammatory marker gene expression

As a marker of DSS-induced inflammation, we determined fecal calprotectin levels. All DSS groups displayed an increase in calprotectin levels when compared to their respective controls and differences were significant between KD and DSS-KD and BHB and DSS-BHB groups (Fig. 4A). No differences between the DSS groups were found. We also analyzed the gene expression of inflammatory markers *Il1b*, *Il6*, *Tnf*, and *Lcn2* from colonic tissue. Based on the expression of these genes, DSS induced increased inflammation in all groups (Fig. 4B). However, the elevation was more pronounced in DSS-CD and DSS-BHB which displayed statistically significant increases in *Il1b* and *Il6* when compared to CD and BHB groups, respectively. The level of inflammatory marker gene expression did not reach statistical difference in DSS-KD when compared to KD. Despite this, there were no significant differences in the expression of these genes between DSS groups.

3.4. Ketogenic diet normalizes the expression of tight junction protein-coding genes despite DSS-induced increased intestinal permeability

To assess intestinal permeability, the levels of orally administered FITC-dextran were analyzed from plasma. There was a trend towards lower permeability in healthy KD group when compared to other healthy groups. DSS induced an increase in FITC-dextran levels with all diets, indicating increased permeability, but the difference to their respective controls reached significance only in DSS-KD group (Fig. 4C).

The expression of TJ-protein coding genes *Cldn1*, *Cldn2*, *Cldn4*, and *Ocln* was assessed to further evaluate barrier function (Fig. 4D). In DSS-CD, the expression of *Cldn1* and *Cldn4* was elevated when compared to CD. *Cldn4* levels were also significantly higher in DSS-BHB when compared to BHB group. Both DSS-CD and DSS-BHB displayed a decrease in *Cldn2* when compared to DSS-KD. In DSS-KD, the expression of all TJ-protein coding genes was preserved at a level similar to healthy controls.

3.5. Ketogenic diet shifts the composition of fecal microbiota

The composition of the microbiota was analyzed from the three diet groups before DSS administration to investigate whether the diet-related changes in gut ecology might predict colitis severity. Shannon entropy score showed no differences in α-diversity between CD and BHB groups, but the score was higher for KD, indicating a higher overall microbial diversity based on richness and evenness (Fig. 5A, Table S2). However, KD exhibited a lower Faith PD score compared to other groups, implying a less phylogenetically diverse microbiome (Fig. 5B, Table S3). Unweighted Unifrac analysis showed significant differences in the composition of the microbial community between all groups, indicating different compositions even when the presence or absence of unique lineages is considered (Fig. 5C, Table S4). Weighted Unifrac analysis revealed no significant difference between CD and BHB, suggesting that while there may be differences in the presence of specific lineages, these differences are not as pronounced when relative abundance of these lineages is considered (Fig. 5D, Table S5). KD, however, differed from both groups, suggesting a distinct microbial community composition compared to both CD and BHB groups in terms of both lineage presence and their relative abundance.

Notable differences in relative abundances of taxa between groups were observed (Fig. S1). While CD and BHB group resembled each other, there was a marked reduction in the relative abundance of taxonomic features associated with the genus *Allobaculum*, the family S24–7, and the genus *Parabacteroides* in KD group. Conversely, in KD group there was an increase in taxonomic features linked to the *Clostridiales* order, the *Oscillospira* genus, and the *Lachnospiraceae* family.

Discriminating features between groups were identified with ANCOM-BC analysis. Fig. S2 shows the features that the relative abundance difference is larger than $\log_{10}2$. In BHB group, *Enterococcus* was enriched whereas *Butyricoccus* and *Lactobacillus* were decreased when compared to both CD and KD (Figs. S2A and S2C). *Anaeroplasma* and *Bifidobacterium* were also lower in comparison to CD and *Coprococcus*, *Lactococcus*, and *Dehalobacterium* less abundant when compared to KD. KD depleted *Sutterella* and *Bifidobacterium* and increased *Coprococcus* and *Defluviitalea*, when compared to both CD and BHB. Also, KD increased the abundance of *Clostridium*, *Dorea*, *Anaerotruncus*, *Bilophila*, and *Ruminococcus* when compared to CD (Fig. S2B).

4. Discussion

We and others have previously found ketogenic diets to protect from DSS-induced colitis [9,11]. The aim of this study was to explore whether these protective effects could be achieved with exogenous ketone body supplementation alone as a more sustainable option to a ketogenic diet. Thus, we investigated the effect of high-dose supplementation with exogenous free acid BHB in the same model of colitis. We fed mice with three diets: a low-fat control diet with or without BHB or a ketogenic diet. After four weeks of feeding, we treated mice with DSS to induce colitis and assessed histological changes, intestinal permeability, tight junction protein levels, inflammatory markers, and microbiota composition. We found colitis and histopathological lesions to be alleviated by the ketogenic diet, but, against the hypothesis, the effects were not recapitulated by high-dose feeding of exogenous free acid BHB on a low-fat diet.

In mice fed the control diet, DSS administration resulted in weight loss, visible signs of colitis, colon shortening, and macroscopically detectable colonic damage. While mice in DSS-KD were protected from these changes, DSS-BHB mice were not. DSS-CD presented typical DSS-related histopathological lesions and damage alongside elevated inflammatory transcription of *Tnf* and *Lcn2* while the ketogenic diet alleviated these changes. This corroborates the earlier findings by us and others where ketogenic diets have protected from DSS-induced inflammation and damage [9–11]. We saw DSS to induce a significant elevation in calprotectin levels in DSS-BHB and, interestingly, also in DSS-KD.

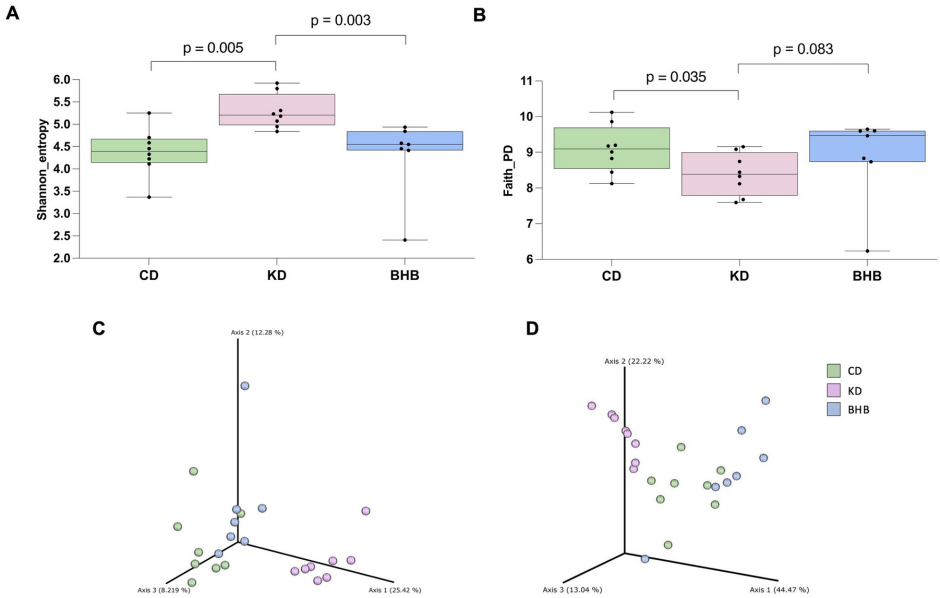

Fig. 5. Diet-induced differences in fecal microbiota analyzed using 16S rRNA sequencing. A. Shannon diversity analysis indicating that KD group has a higher overall microbial diversity than CD and BHB groups. B. Faith phylogenetic diversity analysis suggesting KD group to have a gut microbiome that is less phylogenetically diverse than CD and BHB groups. C. Unweighted Unifrac dissimilarity analysis demonstrating differences in microbial community composition between the diet groups. D. Weighted Unifrac dissimilarity analysis showing that KD group has a distinct microbial community composition compared to CD and BHB groups. Results for A. and B. are analyzed with Kruskal-Wallis H test and for C. and D. with Permanova test. CD = healthy group with control diet, KD = healthy group with ketogenic diet, BHB = healthy group with β-hydroxybutyrate-supplemented diet.

Therefore, protective effects of the ketogenic diet may not extend to calprotectin. As opposed to the ketogenic diet, BHB administration did not protect the mice from colitis – in fact, BHB seemed to even aggravate inflammation and resulted in death or euthanasia of several mice in DSS-group earlier than planned. These findings are not in line with others who have reported BHB administration to alleviate DSS-induced histopathological damage in the colon when administered intraperitoneally [10,15], rectally [13], or via a gastric gavage [14] which suggest that the administration route, dose, and form of BHB, *i.e.* ester, salt, or free form, may determine the outcome of supplementation.

Despite a clearly milder colitis phenotype in KD mice, the DSS-induced intestinal permeability to FITC-dextran was similar in all diet groups. Interestingly, the permeability to FITC-dextran in healthy KD mice was lower than in CD or BHB groups. However, the ketogenic diet maintained the transcription of all measured TJ proteins on a level comparable to healthy animals. We saw DSS to induce an elevation in *Cldn4* levels in DSS-CD and DSS-BHB, whereas *Cldn2* expression was reduced in these groups. DSS-KD was protected from these changes. While claudin-4 is considered as a barrier-sealing TJ protein and claudin-2 pore-forming, we have previously observed similar changes in experimental colitis [11]. Claudin-4 protein expression is also elevated in IBD [26] . Indeed, the increased expression of barrier-forming and the decreased expression of pore-forming TJ proteins may be a compensatory mechanism which could also explain why there were no differences in permeability to FITC-dextran between DSS groups.

Based on our study, high-dose continuous feeding with free acid in the context of a low-fat diet does not seem protect against DSS-induced colitis alike ketogenic diets, raising the question of the protective mechanism of KD. Ketone bodies themselves have anti-inflammatory properties [27]. Here, as expected, the plasma levels of BHB were elevated in mice on the ketogenic diet, but in mice supplemented with BHB, the levels of the compound did not increase in plasma despite the significant dietary dose. In other colitis studies on exogenous BHB, plasma levels increased when the compound was administered intraperitoneally [10,15]. In addition, feeding rats with a ketone monoester resulted in plasma BHB levels equal to those achieved by BHB injection and protected from DSS-induced colitis [15]. A beneficial effect on DSS-colitis was also achieved with feeding mice with poly-D-3-hydroxybutyric acid, another BHB derivative which consumption increases plasma BHB levels [28]. Thus, the levels of circulating ketones may influence the development of intestinal inflammation. On the other hand, as we did not observe BHB supplementation to elevate plasma levels of the compound, supplementation-induced ketoacidosis likely did not contribute to the results.

Development of intestinal inflammation and lesions is associated with gut microbiota, and therefore diet-induced changes in gut microbial composition can have significant consequences on the outcome of colitis. Indeed, microbiota appears to be a critical determinant of the severity of DSS-induced inflammation [29]. In addition, the effects of KD on DSS-induced colitis seem to be related to changes in the composition of microbiota [9]. In our study, the BHB group had a microbiota distinctly different from the KD group, which was protected from colitis, suggesting that changes in its composition may have mediated the differing outcomes. In the other mouse studies on BHB and experimental colitis, microbiota was not analyzed and thus, comparisons cannot be made. In rats administered DSS, KD and intraperitoneally administered BHB both mitigated decreases in *Fusobacterium* spp., and *Lactobacillus* spp. BHB also helped maintain *Clostridium* spp. levels [10]. It is possible that the different BHB form, dose, or administration regime may have modified the gut microbiota to a direction that worsened colitis in our setting. On the other hand, the protective effect of the ketogenic diet can also be mediated through the gut microbiota.

Our results contravene those of others who have reported BHB to

alleviate DSS-induced colitis when administered via a gastric gavage [14], or intraperitoneally [10]. In addition, BHB enemas seem to improve recovery from DSS-induced inflammation rectally [13]. However, there are several differences between the studies which may explain the contradicting outcomes. Our mice had constant access to BHB-supplemented food whereas Li et al. [14] orally administered BHB once a day. While the dose we used was determined based on previous literature, these studies were not done on models of intestinal inflammation: even though high-dose continuous feeding with diet containing 10–20 % exogenous ketones has shown benefit in rodent models of cancer [17] and epilepsy [30], a resembling dose seems to be detrimental in DSS-induced colitis based on our findings. In addition, we used free acid BHB which may have different effects from other forms of BHB. As opposed to BHB salts and esters, free acid R-BHB is molecularly identical to the endogenously produced BHB, and in healthy humans, 10 g/d of the free form seems to be tolerated well [31]. However, our relative dose was orders of magnitude higher and administered to diseased animals. Overall, the contrast between our results and those of others highlights the importance of the administration route, dose, and form BHB in the context of intestinal inflammation. On the other hand, this study corroborates the previous findings on the benefits of a ketogenic diet in experimental colitis [9–11]. This suggests that other KD-related metabolic changes or features of the diet, not only the increased supply of BHB, may be responsible for the protective effect of the diet.

5. Conclusions

While ketogenic diet ameliorates experimental DSS-induced colitis in mice, high-dose continuous feeding with free acid BHB seems to aggravate it. Ketogenic diet and BHB supplementation led to drastically different microbiota compositions which may partly explain the outcomes. Our results indicate that the protective effect of ketogenic diet might not be mediated by increased supply of BHB alone. Overall, replacing ketogenic diet with high-dose exogenous ketone supplementation might not offer benefit in the context of intestinal inflammation.

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CRediT authorship contribution statement

Lehto Markku: Writing – review & editing. **Salmenkari Hanne:** Writing – review & editing, Supervision, Conceptualization. **Korpela Riitta:** Writing – review & editing, Supervision, Resources, Conceptualization. **Toivio Lotta:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Toivio Jyri:** Writing – original draft, Visualization, Investigation, Formal analysis. **Lindén Jere:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Lee Keehoon:** Writing – review & editing, Visualization, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phanu.2025.100437.

Data availability

Data will be made available on request.

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Ketogenic diet counteracts the proinflammatory alterations in the renin- angiotensin-aldosterone system in murine experimental colitis

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Abstract

Inhibiting the overexpression of the renin-angiotensin-aldosterone system (RAAS) alleviates intestinal inflammation. Recently, we and others reported that a high-fat, low carbohydrate, ketogenic diet (KD), shown to downregulate the conventional RAAS components in rat lung and adipose tissue, can protect mice from colitis. Here we assessed whether the proinflammatory angiotensin-converting enzyme - angiotensin receptor type 1 (ACE-AT1R) axis and the anti-inflammatory angiotensin-converting enzyme 2- MAS1 receptor (ACE2-MAS1) axis RAAS components are influenced by the consumption of a KD rich either in saturated fatty acids (SFA-KD) or polyunsaturated linoleic acid (LA-KD) in healthy and inflamed intestine of C57BL/6J male mice. In healthy jejunum, KD increased the AT2R protein level and decreased *Ace2* level regardless of the fat source, whereas in the healthy colon, the RAAS components were unaffected by the dietary interventions. In colon, administration of 2.5 % (w/v) dextran sodium sulfate (DSS) for 5 days upregulated ACE protein while downregulating *Agtr2* gene expression. Both KDs antagonized these changes. Furthermore, the DSS-SFA-KD group exhibited lower angiotensinogen gene expression than in the DSS animals and DSS-LA-KD inhibited the DSS-induced decrease in *Ace2* gene expression. In conclusion, KDs modulate intestinal RAAS component expression, partly counteracting the DSS-induced proinflammatory RAAS changes in the colon.

Key words: renin-angiotensin-aldosterone system, intestinal inflammation, ketogenic diet, mouse

1. Introduction

The renin-angiotensin-aldosterone system (RAAS) is not only in charge of blood pressure homeostasis but is also involved in the regulation of inflammation [1]. The inflammatory effects of RAAS are determined by the balance between the proinflammatory angiotensin-converting enzyme - angiotensin receptor type 1 (ACE-AT1R) and the anti-inflammatory angiotensin-converting enzyme 2- MAS1 receptor (ACE2-MAS1) pathways [2]. In addition to the systemic RAAS, this multi-peptide system functions locally in several vital organs, including human and mouse intestine, where it plays several roles *e.g.* in motility and secretion [3–7]. In the gut, the activation of RAAS drives the development of inflammation by promoting epithelial cell apoptosis, colonic myofibroblast production, and oxidative stress [8,9]. With age and after exposure to some dietary factors, such as salt loading in mice and rats, the balance of local intestinal RAAS components tends to shift towards the proinflammatory pathway [10,11]. This shift has been observed also in pathological conditions in humans, such as in inflammatory bowel disease (IBD) [7,12]. For example, IBD patients exhibit higher concentrations of angiotensin II (Ang II), the key effector peptide of the ACE-AT1R axis, in colonic mucosa than healthy volunteers [13]. On the contrary, the levels of Ang (1-7), the main effector peptide in the ACE2-MAS1 axis are reduced [12]. Interestingly, ACE inhibitors and angiotensin receptor blockers have been demonstrated to alleviate colitis in preclinical models [9] as well as improving clinical outcomes in IBD patients [14,15], highlighting the role of RAAS in the modulation of inflammatory responses.

There has been speculation about the potential therapeutic benefits of a high-fat, low-carbohydrate ketogenic diet (KD) to reduce the symptoms of colitis [16] to this diet's anti-inflammatory effects, such as its ability to lower the levels of inflammatory cytokines [17]. Indeed, we recently reported that a KD rich in saturated fatty acids (SFA-KD) or in polyunsaturated linoleic acid (LA-KD) alleviated colitis in C57BL/6J mice and inhibited the increase of colonic *Tnf* and *Il-6* induced by dextran sodium sulfate (DSS) [18]. Others have observed that consumption of a KD was able to relieve DSS-induced colitis in mice and lower the levels of the pro-inflammatory interleukin-class of cytokines in colon tissue [16].

A KD promoted the expression of anti-inflammatory RAAS components and decreased the expression of the pro-inflammatory counterparts in rat adipose tissue [19]. Furthermore, in rat lung, a KD was reported to lower ACE levels [20]. Based on these findings, we hypothesized that a KD could possibly downregulate the ACE-AT1R RAAS axis and upregulate the ACE2-MAS1 axis in the intestine and this might help to alleviate the symptoms of colitis. The objective of this study was to examine the impacts of SFA-KD and LA-KD on the balance between the ACE-AT1R and ACE2-MAS1 RAAS axes components in healthy and inflamed mouse intestine.

2. Materials and methods

2.1. Animal experiments

The animal experiments were conducted under the permission of the Regional State Administrative Agency for Southern Finland (ESAVI/9377/2019) using nine-week-old male C57BL/6J mice (Scanbur, Karlslunde, Denmark). The animals were kept under a 12 h light–dark cycle, at 20 ± 2°C and 50–60 % humidity with unlimited access to food and water.

The two animal experiments were conducted as presented in Figure 1 and the diets' effects on metabolic parameters and colitis were previously described in [18,21]. In both experiments, mice were randomly allocated into groups that were fed during four (experiment I) or five weeks (experiment II) with one of the three different diets: a low-fat control diet (CD) in a pellet form, a KD high in saturated fatty acids from milk fat (SFA-KD), and a KD high in linoleic acid from vegetable fat (LA-KD) in paste form (Envigo, Indianapolis, IN, USA) (Table 1). The formulations of the study diets are presented in Table S1. The fiber content of each diet was standardized. The formulations and a detailed analysis of the fatty acid compositions of the study diets are presented in table S1 and S2.

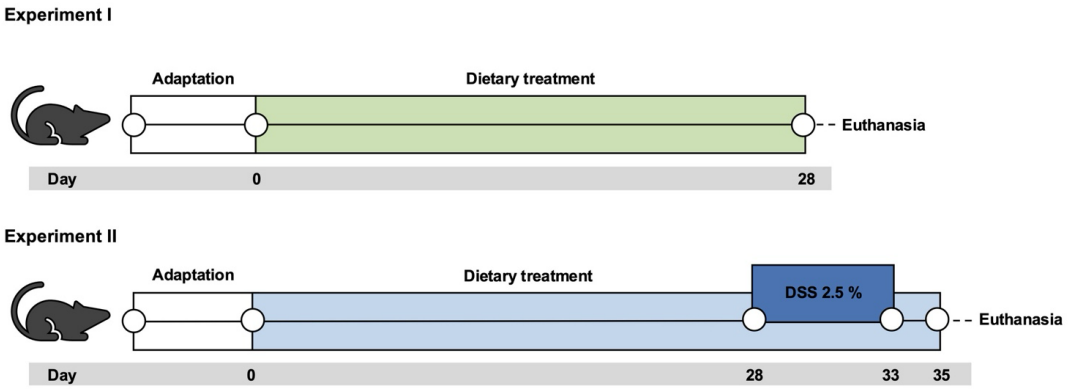

Figure 1. Designs of the animal experiments. DSS = dextran sodium sulfate.

First, the impact of the KDs was investigated in healthy intestine by feeding mice (n= 27) with experimental diets for four weeks after which the animals were sacrificed for sample collection. Next, intestinal RAS was investigated in a pathophysiological setting. Following the four-week feeding, the mice (n = 35) were further divided into four groups: untreated control group (CD) and three groups (DSS-CD, DSS-SFA-KD and DSS-LA-KD) that were administered dextran sodium sulfate (DSS) (40 kDa, TdB Labs, Uppsala, Sweden) to induce intestinal inflammation. Inflammation was induced by changing the drinking water to a 2.5 % (w/v) DSS solution for five days, after which water was given for a final two days.

Table 1. The macronutrient compositions of the diets expressed in energy percent as described by the producer. CD = control diet, LA-KD = ketogenic diet with linoleic acid, SFA-KD = ketogenic diet with saturated fatty acids.

	CD	SFA-KD	LA-KD
Protein (E%)	9.7	9.4	9.4
Carbohydrate (E%)	77.8	0.5	0.5
Fat (E%)	12.5	90.1	90.1
Saturated fat (E%)	2.6	56.6	22.8
Monounsaturated fat (E%)	3.0	26.0	17.9
Polyunsaturated fat (E%)	6.9	4.5	49.3
Linoleic acid (E%)	6.4	4.0	44.5

At euthanasia, unfasted animals were sacrificed under isoflurane (4 %, Vetflurane, Virbac, Carros, France) anesthesia by exsanguination. Blood samples were drawn into EDTA-tubes from *vena cava* and plasma was separated by centrifuging the samples at 2000× g for 15 min in 4˚C. The intestine was removed, opened longitudinally, and flushed free of any intestinal content with ice cold 0.9 % saline. Samples of 0.5 to 1 cm in length from the middle section of jejunum and colon were collected, snap-frozen in liquid nitrogen, and stored at -80˚C.

2.2. Reverse transcription quantitative polymerase chain reaction

The intestinal mRNA expression of RAAS component genes *Agt, Ace*, *Ace2*, *Agtr1*, *Agtr2*, and *Mas1* were analyzed with RT-qPCR. RNA was extracted from jejunal and colonic samples with NucleoSpin RNA Kit (Macherey Nagel, Duren, Germany) and diluted to the same concentration. RNA was reverse-transcribed to complementary DNA with iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). RT-qPCR was run with LightCycler® 480 SYBR Green Master (Roche Diagnostics Corp., Indianapolis, IN, USA) with the amplification protocol: 10 min at 95˚C, 40 cycles of denaturation (15 s, 95˚C), annealing (30 s, 60˚C) and elongation (30 s, 72˚C). Melt curves were analyzed at the end of the experiment. The primer sequences used are listed in Table 2. For *Cyp11b2*, mRNA expression was analyzed with Taqman gene expression assay Mm00515624_m1 (Thermo Fischer Scientific, Waltham, MA, USA) with LightCycler® Multiplex RNA Virus Master mix (Roche Diagnostics Corp.) All results were calculated as relative quantities (RQ) of mRNA based on the Vandesompele method [22]. The genes used for normalization were *18S, Eef2,* and *Rplp0*.

Table 2. Primer sequences used in RT-qPCR analyses.

Gene	Forward primer	Reverse primer	Ref.
18S	AACGAACGAGACTCTGGCAT	ACGCCACTTGTCCCTCTAAG	[23]
Eef2	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA	[24]
Rplp0	TAACCCTGAAGTGCTCGACA	GGTACCCGATCTGCAGACA	[23]
Ace	GCTGGAGGGTCTTTGATGGA	AGTCACCTTGGGATCTTGGC	[23]
Ace2	GGATACCTACCCTTCCTACATCAGC	CTACCCCACATATCACCAAGCA	[25]
Agtr1	AGTCGCACTCAAGCCTGTCT	ACTGGTCCTTTGGTCGTGAG	[26]
Agtr2	GAAGCTCCGCAGTGTGTTTA	TGGCTAGGCTGATTACATGC	[27]
Mas1	CGGTCTACATTACCCACTTGTC	CCCGTGTTGTAGCCAAATAGA	[28]
Agt	CTTCCAAGGAACGATGAGAGGTT	ACAGACACCGAGATGCTGTT	[23]

2.3. Western blot

For Western blotting, the tissue pieces were homogenized in phosphate-buffered saline with Tween 20 (PBS-T) (136 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.46 mM KH_2PO_4, 0.1 % Tween, pH 7.4) containing an inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) using a Precellys homogenizer (Bertin Technologies, Montigny le Bretonneux, France). After homogenization, the samples were sonicated, centrifuged and the supernatants were collected. A commercial kit (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) was used to determine the total protein concentration in the samples. The supernatants were diluted to the same protein concentration with PBS-T and denatured in a heat-block for 5 min at 95˚C in Laemmli sample buffer (Bio-Rad) including 5 % of β-mercaptoethanol (Bio-Rad).

Samples containing 30 µg/µl protein were separated using 4–20 % Mini-PROTEAN® TGX™ Precast gels (Bio-Rad). After the SDS-PAGE separation, proteins were transferred onto a nitrocellulose membrane (Bio-Rad) *via* semi-dry blotting and blocked in a commercially available blocking buffer (LI-COR, Lincoln, NE, USA) for 2h at RT. The membranes were probed with the following monoclonal antibodies: AT1R (ab124734, 1:1000, Abcam, Cambridge, UK), AT2R (ab92445, 1:1000, Abcam) as well as ACE (ab254222, 1:100, Abcam) and normalized against β-actin (#3700, 1:10000, Cell Signaling Technologies, Danvers, MA, USA) at 4˚C. Finally, a fluorescence-labeled secondary antibody (IRDye680LT or IRDye800CW, LI-COR) was bound to the primary antibody and the results were detected using Odyssey CLx InfraRed analyzer (LI-COR).

2.4. Immunohistochemistry

The immunohistochemical expression patterns of ACE and ACE2 in the colon were studied employing four representative colon samples from each group (CD, DSS-CD, DSS-SFA-KD and DSS-LA-KD) in experiment II [18]. In the immunohistochemistry experiments, the colon sections were first incubated for 20 min at 99°C in 10 mM Tris-EDTA buffer (pH 9). For antigen retrieval, endogenous peroxidase activity was blocked with 3 % H_2O_2 and nonspecific staining with 5 % bovine serum albumin (BSA, Merck, Darmstadt, Germany) in Tris-buffered saline with Tween 20 (TBS-T) (140 mM NaCl, 3.0 mM KCl, 25 mM $C_4H_{11}NO_3$, 0.05 % Tween). The primary rabbit monoclonal ACE (ab254222, 1:500, Abcam) and ACE2 (ab108252, 1:400, Abcam) antibodies were incubated at RT for 60 min. Vectastain ABC-HRP kit (Vector Laboratories, Burlingham, CA, USA) with biotinylated anti-rabbit antibody (1:200) and DAP substrate were used for detection of the primary antibody according to the manufacturer's instructions.

2.5. Statistical analyses

The data was analyzed, and the figures were plotted with GraphPad Prism software version 9 (San Diego, CA, USA). Statistical differences between the groups were analyzed based on the result of Shapiro-Wilk normality test using either one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or Kruskal-Wallis test followed by Mann-Whitney test. P-values smaller than 0.05 were deemed significant.

3. Results

This study builds upon a larger research project on the impact of KDs on intestinal barrier and experimental colitis, where plasma β-hydroxybutyrate, body masses of the animals, energy intake, the expression inflammation-related genes as well as the macroscopic and histological inflammatory changes have been reported [18,21]. Here, we focus on the effect of KDs on the intestinal RAAS components.

3.1. KD increased AT2R protein expression and decreased Ace2 gene expression in healthy jejunum

The effects of KDs with different fat sources were first studied in the jejunums and colons of healthy mice. In jejunum, KD did not affect the pro-inflammatory RAAS axis proteins, ACE and AT1R (Figure 2A and 2C). However, both KDs evoked changes in the anti-inflammatory RAAS axis. They increased the protein levels of ATR2 regardless of the dietary fat source (Figure 2C) and lowered the Ace2 mRNA expression (Figure 2D). The mRNA expression levels of Cyp11b2, the rate-limiting enzyme in aldosterone synthesis (Figure 2G) as well as those of *Agt* (Figure 2F) and *Mas1* (Figure

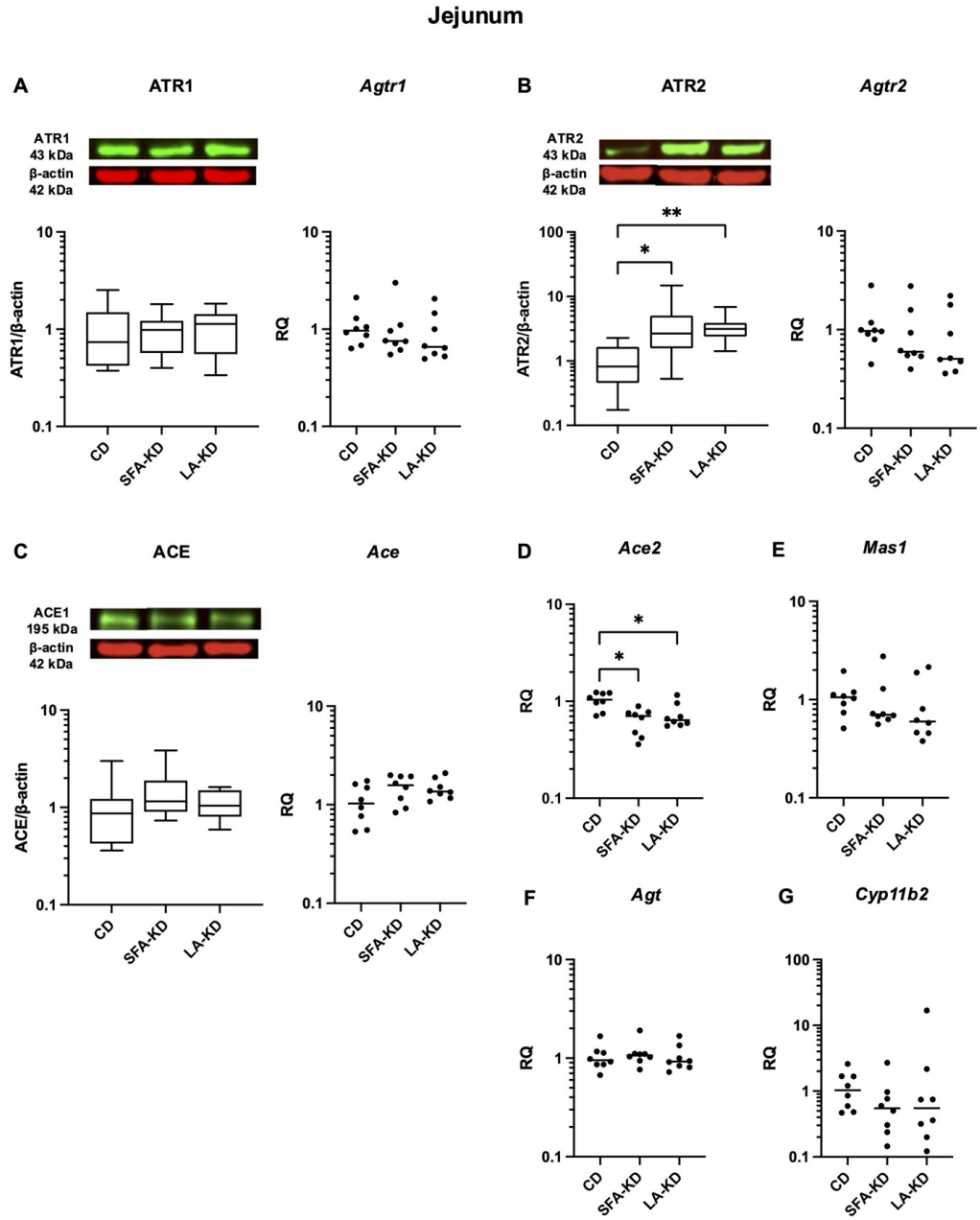

Figure 2. Expression of local renin-angiotensin-aldosterone system components in healthy mouse jejunum. Protein and mRNA expression of A. angiotensin II type 1 receptor (AT1R), B. angiotensin II type 2 receptor (AT2R), and C. angiotensin-converting enzyme (ACE) and mRNA expression of D. angiotensin-converting enzyme 2 (Ace2), E. Mas receptor (Mas1), F, angiotensinogen (Agt), and G. aldosterone synthase (Cyp11b2). In the Western blot analyses, n = 5–9 per group. * $p < 0.05$ and ** $p < 0.01$. CD = control diet, LA-KD = ketogenic diet with linoleic acid, SFA-KD = ketogenic diet with saturated fatty acids, and RQ = relative quantity.

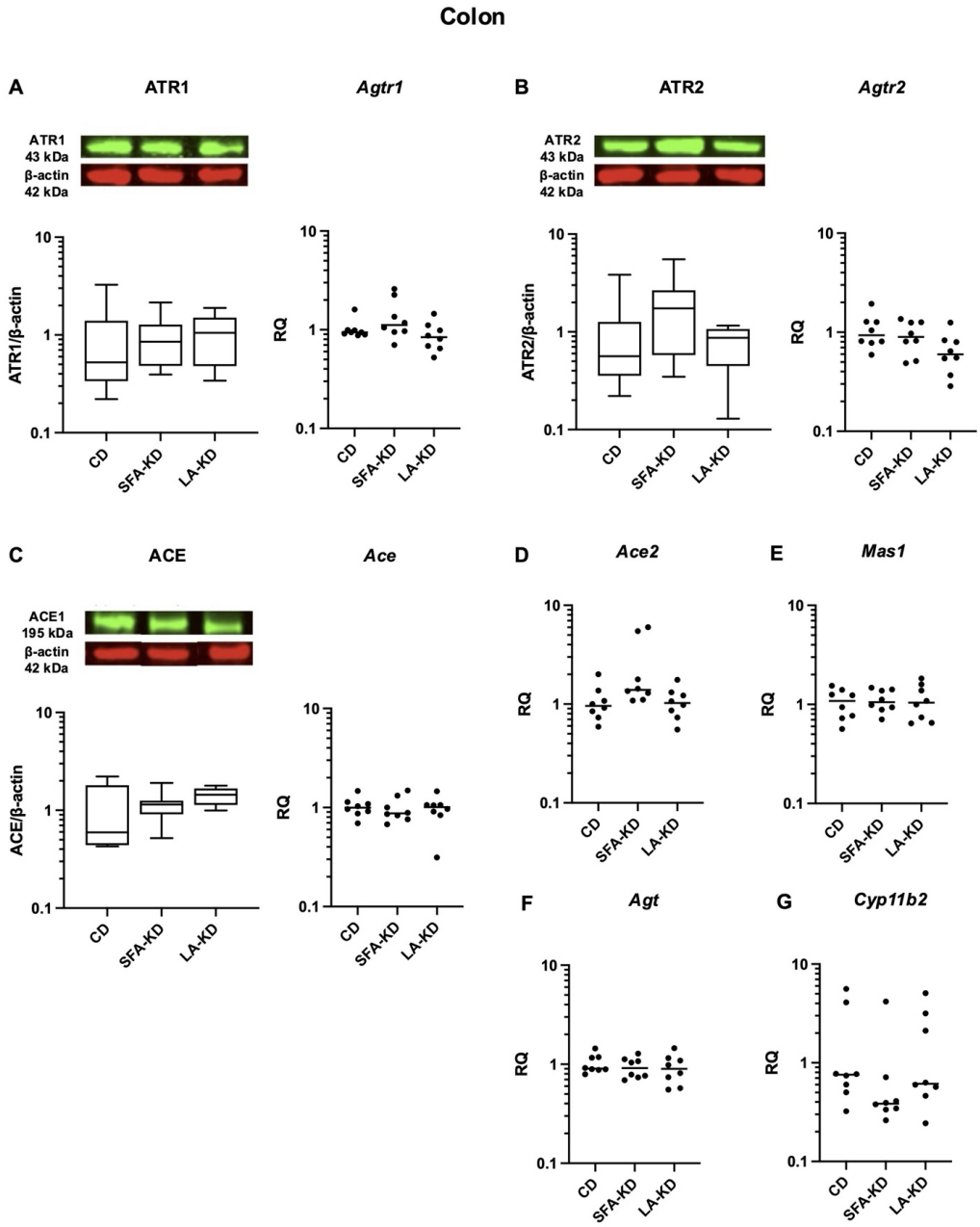

Figure 3. Expression of local renin-angiotensin-aldosterone system components in healthy mouse colon. Protein and mRNA expression of A. angiotensin II type 1 receptor (AT1R), B. angiotensin II type 2 receptor (AT2R), and C. angiotensin-converting enzyme 1 (ACE) and mRNA expression of D. angiotensin-converting enzyme 2 (Ace2), E. Mas receptor (Mas1), F, angiotensinogen (Agt), and G. aldosterone synthase (Cyp11b2). For Western blot analyses, n = 5–9 per group. CD = control diet, LA-KD = ketogenic diet with linoleic acid, SFA-KD = ketogenic diet with saturated fatty acids, and RQ = relative quantity.

2E) were not affected by diet. The original Western blot images are shown in the supplementary data (Figure S1, S2 and S3).

In healthy colon, the amounts of AT1R and ACE were unaffected by the dietary interventions at both the protein and mRNA level (Fig 3A and 3B). SFA-KD doubled the AT2R protein amount when compared to the control mice but this difference did not reach statistical significance (Figure 3C). The transcription levels of *Ace2,* Agt, Agtr2, Mas1, and Cyp11b2 remained stable irrespective of the KD interventions.

3.2. KD partly reversed the DSS-induced inflammatory changes in colonic RAAS components

Since the intestinal RAAS regulates the extent of inflammation in the intestine, next we investigated how the RAAS is affected by DSS colitis and whether KDs modulate this effect. DSS induced typical changes in colon, including ulceration and heavy inflammation (Figure 5A and C), while the DSS-SFA-KD mice showed mild to moderate and DSS-LA-KD mice displayed minimal to mild histological lesions (Figures 5H and 5I as well as 6D and 6F) [18]

In inflamed colon, the DSS-SFA-KD mice exhibited lower AT1R levels than the controls (Figure 4). Consistent with these findings, the DSS-SFA-KD group had the lowest *Agtr1* relative mRNA expression with a significant difference to the control group. While no changes in the AT2R protein expression were detected, DSS downregulated the *Agtr2* gene expression and consumption of the KDs, regardless of the fat source, antagonized this decrease. Administration of DSS increased the ACE protein level in colon and both KDs inhibited this increase (Figure 4). Interestingly, there was a difference in the *Ace* gene expression between the diets, with DSS-LA-KD group exhibiting higher levels than DSS-SFA-KD group. The DSS-LA-KD group also demonstrated a higher *Ace* mRNA level than evident in the DSS group. The gene expression of *Ace2* was lower in the DSS-CD and DSS-SFA-KD groups compared to the healthy controls. LA-KD prevented this decrease. The DSS-SFA-KD group exhibited a lower *Agt* gene expression than mice administered DSS alone. Dietary interventions did not affect *Mas1* gene expression. However, DSS-LA-KD exhibited lower *Cyp11b2* mRNA expression than the DSS-CD group.

Immunohistochemical staining of ACE in the colon corroborated the Western blot findings (Figure 5). In the healthy control mice, colonic ACE showed a marked expression in the blood vessel endothelium and moderate expression in the lymphatic vessel endothelium. A minimal amount of ACE was also present on the surfaces of crypt epithelial cells in the upper half of the crypts and on surface epithelium immediately adjacent to the openings of the crypts. In contrast, the DSS-CD mice,

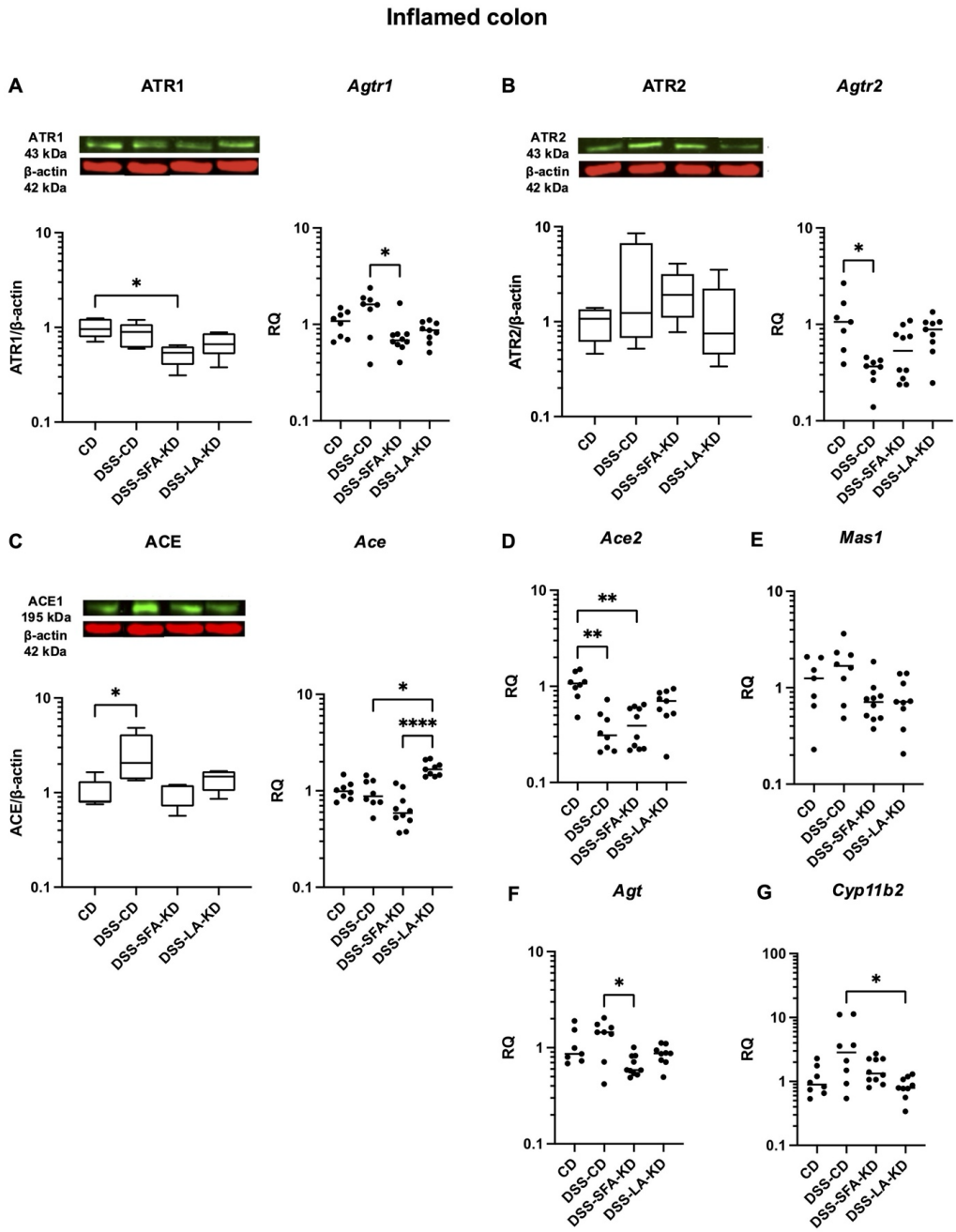

Figure 4. Expression of local renin-angiotensin-aldosterone system components in inflamed mouse colon. Protein and mRNA expression of A. angiotensin II type 1 receptor (AT1R), B. angiotensin II type 2 receptor (AT2R), and C. angiotensin-converting enzyme 1 (ACE) and mRNA expression of D. angiotensin-converting enzyme 2 (Ace2), E. Mas receptor (Mas1), F, angiotensinogen (Agt), and G. aldosterone synthase (Cyp11b2). In the Western blot analyses, n = 5–9 per group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. DSS = dextran sodium sulfate, CD = healthy group with control diet, CD = DSS group with the control diet, DSS-SFA-KD =

DSS group with a diet high in saturated fatty acids, DSS-LA-KD = DSS group with a diet high in linoleic acid, and RQ = relative quantity.

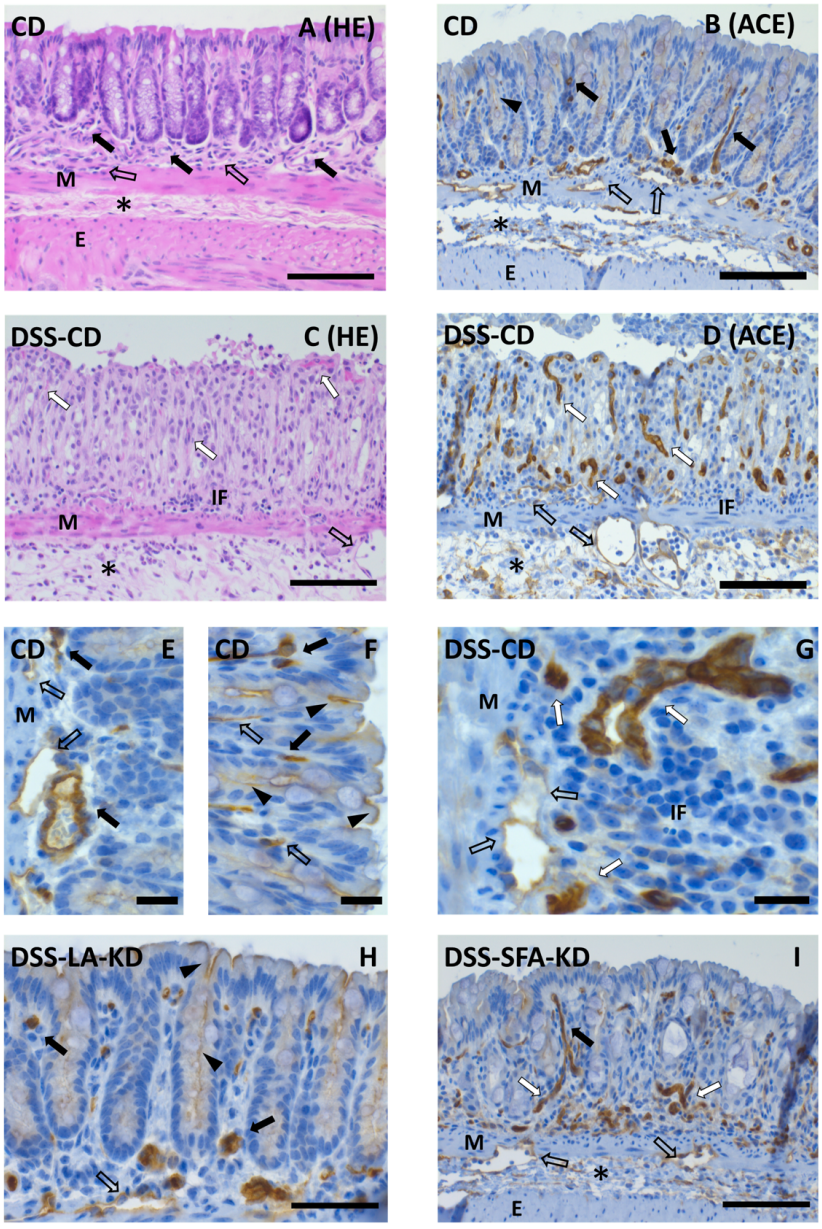

Figure 5. Hematoxylin-eosin (HE) staining of the colon wall of CD and DSS-CD mice and immunohistochemical staining of ACE in the colon wall of CD, DSS-CD, DSS-LA-KD and DSS-SFA-KD mice. A, B) HE and ACE staining in CD mice. Muscularis mucosae (M), submucosa (asterisk), inner part of the external muscle layer (E). In HE staining, the capillaries (arrows) and lymphatic vessels (open arrows) are inconspicuous, while in the immunohistochemistry evaluations, the capillaries show strong while the lymphatic vessels display modest ACE expression. Epithelial cell surfaces (arrowheads) in the crypts and on the surface

exhibit slight ACE staining. Scale bars 100 µm. C, D) HE and ACE staining in DSS-CD mice mucosa. Muscularis mucosae (M), submucosa (asterisk). The mucosa shows surface epithelial erosion, marked epithelial degeneration and crypt loss, as well as ample neovascularization (white arrows) that is poorly discernible in HE staining but clear evidence of abundant ACE expression. Epithelial ACE expression is lost. Submucosa and basal lamina propria display dilated lymphatic vessels (open arrows) and inflammatory cell infiltration (IF). Scale bars 100 µm. E, F, G) ACE staining in basal and superficial mucosa in a CD mouse and in basal mucosa in a DSS-CD mouse. In the CD mouse, the capillary endothelium (arrows) displays strong, lymphatic vessel endothelium (open arrows) exhibit modest, while the epithelial surfaces (arrowheads) show only slight ACE expression. In the DSS-CD mouse, tortuous newly-formed capillaries (white arrows) with plump endothelial cells which exhibit an intense ACE expression. A dense inflammatory cell infiltrate (IF) in the lamina propria replaces lost crypts. Scale bars 20 µm. H, I) ACE staining in DSS-LA-KD and DSS-SFA-KD mice mucosa. Muscularis mucosae (M), submucosa (asterisk), inner part of the external muscle layer (E). In the DSS-LA-KD mouse, mild mucosal degenerative changes appear to have no effect on ACE expression in epithelial cells (arrowheads), capillaries (arrows) or lymphatic vessels (open arrows). In the DSS-SFA-KD mouse showing marked epithelial cell degeneration and dysplasia, the epithelial ACE expression is reduced while there are some seemingly newly-formed capillaries (white arrows). H) Scale bar 50 µm. I) Scale bar 100 µm. CD = healthy group with control diet, DSS-CD = DSS group with control diet, DSS-LA-KD = DSS group with diet high in linoleic acid, DSS-SFA-KD = DSS group with diet high in saturated fatty acids.

where DSS administration triggered severe mucosal damage and crypt loss [18], showed a prominent increase of ACE expression in the endothelium of the most likely newly formed vasculature. In general, the eight SFA-KD and LA-KD -fed and DSS-dosed mice showed a comparable ACE expression pattern as the healthy control mice: The minimal damage in DSS-LA-KD mice (Figure 5H) seemed to induce no change on the mucosal ACE expression, whereas moderate histological lesions in DSS-SFA-mice (Figure 5I) appeared to reduce the epithelial surface ACE expression while tending to increase endothelial ACE expression.

Colonic ACE2 expression in healthy controls was confined to single cells, generally among the basal crypt epithelium, and the positive cells showed a distinctive infundibular shape with an intensely staining apical droplet facing the crypt lumen (Figure 6A, 6B and 6C). Typical minimal histological lesions or epithelial hyperplasia in DSS-LA-KD mice evoked no change in the intensity of ACE2 staining (Figure 6D) while moderate degenerative and dysplastic changes in a DSS-SFA-KD and mice appeared to result in the degeneration and apoptosis of the ACE2 positive cells (Figure 6F and 6G). In the DSS-CD mice, the total loss crypt cells also led to ACE2 positive cell loss (Figure 6E), albeit some cells seemed to remain although with a markedly degenerated epithelium.

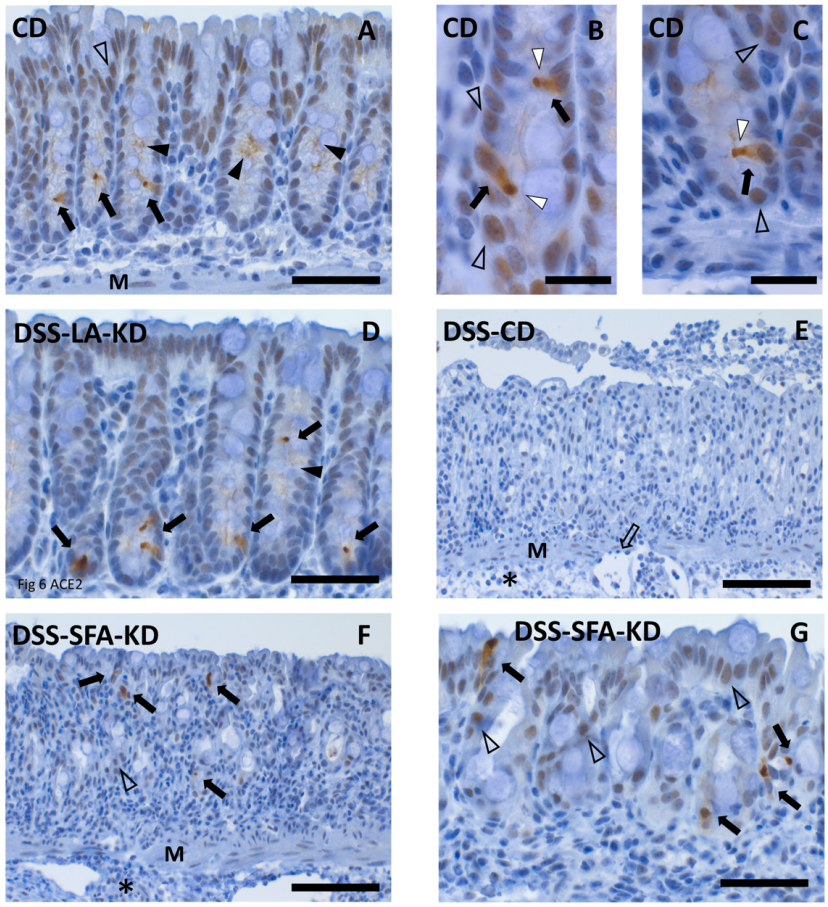

Figure 6. Immunohistochemical staining of ACE2 in the colon wall of CD, DSS-CD, DSS-LA-KD and DSS-SFA- KD mice. A, B, C) CD mouse mucosa (muscularis mucosae, M) contains ACE2-expressing funnel-shaped cells (arrows) amongst the epithelial cells at basal–middle crypt height. Crypt lumens show some minute, loose accumulations of ACE2-reactive material (arrowheads). Epithelial cell nuclei display variable, most likely nonspecific, reactivity (open arrowheads). The infundibular ACE2-expressing cells (arrows) in basal crypt epithelium exhibit intensely staining apical droplets (white arrowheads), which appear to be releasing their contents into crypt lumen. A) Scale bar 50 µm. B, C) Scale bars 20 µm. D, E) DSS-LA-KD and DSS-CD mice mucosa. Muscularis mucosae (M), submucosa (asterisk) lymphatic vessel (open arrow). In the DSS-LA-KD mouse, mild mucosal degenerative changes appear to have no effect on the ACE2-expressing cell (arrows) numbers or morphology. In contrast, the DSS-CD mouse mucosa contains no ACE2 expressing cells. D) Scale bar 50 µm. E) Scale bar 100 µm. F, G) DSS-SFA-KD mouse mucosa (muscularis mucosae, M) shows marked epithelial cell degeneration but little evidence of necrosis. Some degenerated or apoptotic ACE2-expressing cells (arrows) are present generally in superficial mucosa amidst the degenerated and dysplastic crypt epithelium. Nonspecific reactivity in the epithelial cell nuclei (open arrowheads). F) Scale bar 100 µm. G) Scale bar 50 µm. CD = healthy group with control diet, DSS-CD = DSS group with control diet, DSS-LA-KD = DSS group with diet high in linoleic acid, DSS-SFA-KD = DSS group with diet high in saturated fatty acids.

4. Discussion

This study set out to investigate the effect of KD on the components of the intestinal ACE-AT1R and ACE2-Mas1 RAAS axes, and their involvement in the regulation of intestinal inflammation. Previously, we have demonstrated that KDs rich in either saturated fatty acids (SFA-KD) or polyunsaturated linoleic acid (LA-KD) can alleviate the manifestations of DSS-induced colitis in mice [18]. Additionally, in rat lung and adipose tissue, KD seems to downregulate the expression of the pro-inflammatory ACE-AT1R RAAS axis [19,20]. Therefore, we measured the key RAAS proteins from colon and jejunum in mice fed two KDs with or without DSS administration. Here, we report that KDs partly prevented the pro-inflammatory changes on intestinal RAAS components evoked by DSS administration.

In healthy jejunum, the dietary interventions increased AT2R protein levels, exposing the small intestine to more anti-inflammatory conditions and demonstrating that KDs can modulate intestinal RAAS locally. Interestingly, both diets decreased the expression of the *Ace2* gene. Similar KD-induced alterations of AT2R and ACE2 have been reported to occur in the rat inguinal adipose tissue [19] but not in the lung [20], indicating that the impact of KD on the local RAAS is tissue-specific. KDs did not induce any changes in healthy colon; this is logical as fat absorption mostly takes place in the jejunum. Therefore, the diet-related changes in intestinal RAAS components in healthy animals might be a result of a direct interaction of the food-derived fatty acids with the epithelium as opposed to an indirect, more systemic effect of the diets and their fat source. In contrast, in the case of DSS-induced inflammation, the systemic diet-induced changes seem to become meaningful, also resulting in changes in the RAAS components in the colon [18,21].

Intestinal RAAS expression is modulated by inflammation [4,7,13]. In line with a previous observation [29], DSS-induced intestinal inflammation upregulated the protein expression of ACE, which appeared to result from neovascularization displaying abundant endothelial ACE. As a novel finding, ACE was detected also in the lymphatic vessels. Here, we found that KDs, regardless of the fat source, prevented the inflammation-induced increase in the level of ACE. This could be attributed to the ability of KDs to alleviate intestinal inflammation. However, given the importance of ACE as a driver and exacerbator of intestinal inflammation via Ang II production [29,30], it is possible that the lowered ACE levels could also be an underlying mechanism contributing to the alleviation of colitis. In addition to stimulating apoptotic and fibrotic changes [4], Ang II induces Th17 cell-mediated responses leading to the production of inflammatory cytokines in the intestinal mucosa [31]. Conversely, KD reduces the level of these cells [32]. Thus, it is possible that besides modulating the levels of intestinal RAAS components, KDs can also alleviate the proinflammatory effects of Ang II. Consistent with this hypothesis, the DSS-SFA-KD group exhibited lower colonic *Agt* gene expression

compared to the DSS group and both KD groups mitigated the DSS-induced decrease in the *Agtr2* mRNA level. These changes could have significance in the progression of colitis when one considers that the breakdown of angiotensinogen to angiotensin I is the first step in the cascade producing active angiotensin peptides, and the level of *Agt* gene expression correlates with the severity of the symptoms of colitis [33]. This, in addition to the normalization of anti-inflammatory Agtr2 mRNA levels upon DSS exposure, may be related to the protective capacity of KDs. To further investigate the role of intestinal RAAS in alleviating colitis with KD, gene-modified animals and a chronic colitis model could be used.

Previously, KDs have been reported to alleviate intestinal inflammation in rodent models with the protective effect being associated with changes in the gut microbiota [16,34] apoptosis and autophagy [34]. Moreover, we have previously observed the level of protection to be dependent on the fat source in the diet, with a greater benefit conferred by LA-KD than SFA-KD [18]. Here, we investigated whether these diets could also influence intestinal RAAS in different ways. In line with our previous findings, we observed that LA-KD could lower *Cyp11b2 and* maintain *Ace2* mRNA expression when there was the presence of intestinal inflammation. Conversely, *Ace* mRNA expression was elevated by LA-KD when compared to the SFA-KD and furthermore the SFA-KD animals showed a lower colonic *Agt* content than the DSS animals. These results, partly in accordance with our earlier findings, suggest that the fat source of the diet influences the regulation of intestinal RAAS components. However, the literature is scanty on the mechanistic effects of different fatty acids and fat sources on systemic or local RAAS systems and the mechanisms involved warrant further research.

While we observed LA-KD to offer greater protection from the colitis associated with higher Ace2 mRNA expression, the role of ACE2 intestinal inflammation is not entirely clear [35,36]. Even though it enhances the anti-inflammatory Mas1 signalling by converting Ang II to Ang (1-7), in a rat model it was observed that administration of an ACE2 blocker could alleviate the manifestations of DSS-induced colitis [36]. On the contrary, an ACE2 deficiency has been reported to increase the vulnerability of mice to develop colitis; the authors related this finding to its role in dietary amino acid homeostasis, innate immunity, and gut microbial ecology [35,37]. We detected the presence of ACE2 protein in healthy colon in a small number of distinctive infundibular-shaped cells with an intensely ACE2-staining apical droplet facing the crypt lumen, which appears to suggest that the cells primarily release ACE2 into the crypt and gut lumen. Intriguingly, this pattern of secretion and the cell morphology resemble enteroendocrine cells [38,39] which control digestive and defensive gut functions. In contrast to our findings and the low colon expression reported by Hashimoto et al [35], the human intestine exhibits the highest Ace2 gene expression of any organ in the body [40],

highlighting the importance of considering differences between species both in physiology and in pathological conditions.

In conclusion, KDs alter the intestinal RAAS component expression and partly diminish the pro-inflammatory changes in local RAAS components in DSS colitis.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Conceptualization, H.L., L.T. and R.K.; investigation H.L., L.T. and J.L.; formal analysis, H.L., L.T and J.L.; resources, R.K.; writing—original draft preparation, H.L and L.T; writing—review and editing, H.L., L.T., J.L., H.S and R.K.; visualization H.L., L.T. and J.L.; supervision H.S. and R.K.

Study data availability

The data from this study can be acquired from the corresponding author upon reasonable request.

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