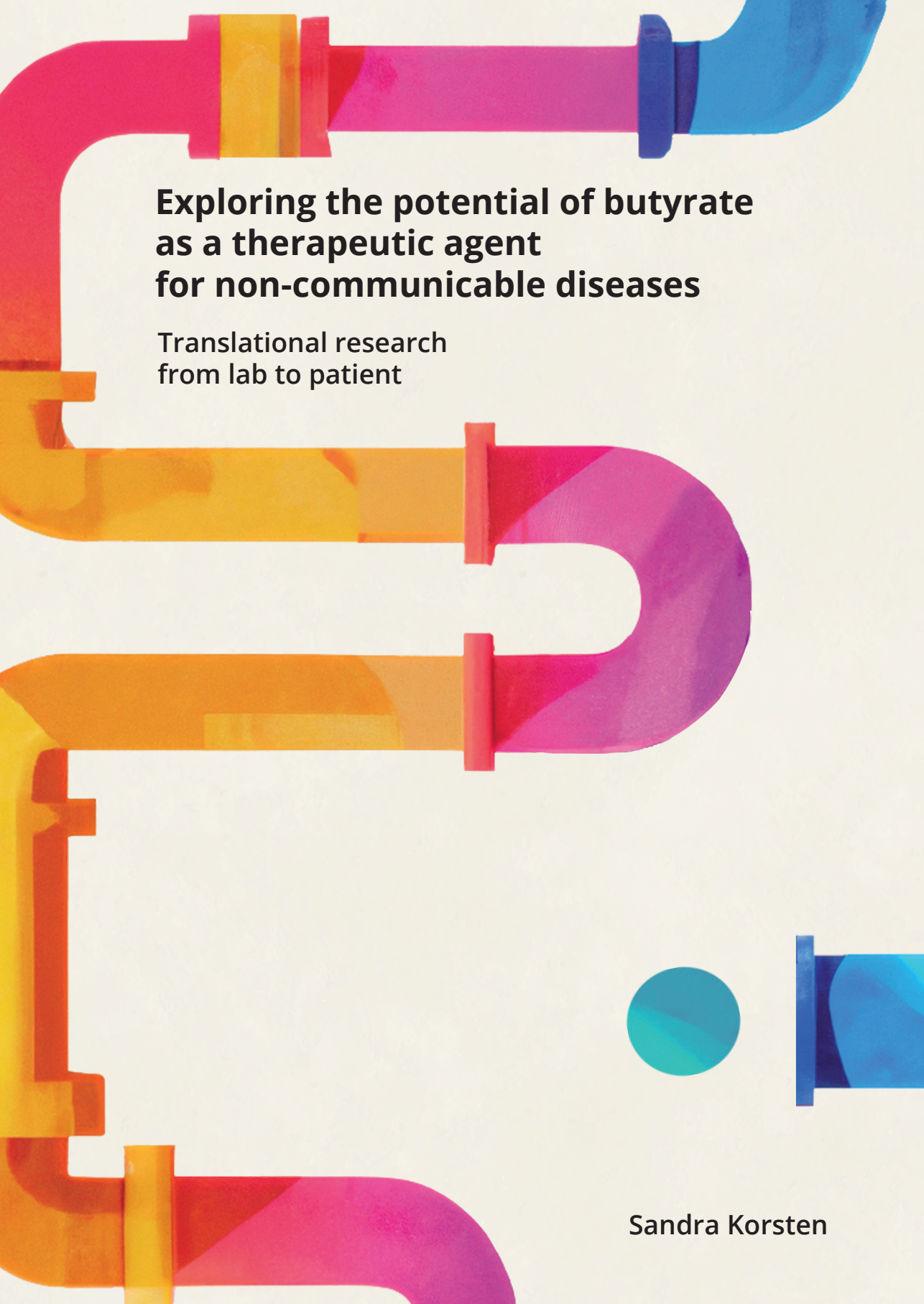

Exploring the potential of butyrate as a therapeutic agent for non-communicable diseases

Translational research from lab to patient

Sandra Korsten

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Onderzoek naar de potentie van butyraat als therapeutisch middel tegen niet-overdraagbare aandoeningen

Translationeel onderzoek van lab naar patiënt

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NOTHING IS MORE MEMORABLE THAN A SMELL

~Diane Ackerman

CONTENT

1

GENERAL INTRODUCTION

NON-COMMUNICABLE DISEASES

Non-communicable diseases (NCDs) are responsible for 74% of all deaths worldwide and are the leading cause of mortality [1]. NCDs are chronic metabolic and/or immune disorders that are not caused by infectious agents. Examples of NCDs include diabetes, inflammatory diseases, cardiovascular diseases and chronic respiratory diseases. Unhealthy dietary habits, physical inactivity, and tobacco and alcohol use are validated risk factors of NCDs. These chronic diseases are characterized by low grade inflammation both systemically as well as locally in the gastro-intestinal tract.

It is hypothesized that NCDs are associated with dysbiosis of the microbiome and a disrupted intestinal barrier [2–10]. Dysbiosis of the microbiome results in an increased influx of macromolecules such as lipopolysaccharides (LPS) through the disrupted intestinal barrier, causing local inflammation in the gut but also low grade systemic inflammation [11,12]. Furthermore, inflammation may as well establish a feedback loop by maintaining the disrupted intestinal barrier, leading to an ongoing influx of macromolecules and consequently resulting in chronic inflammation and the development of NCDs, see Figure 1 [13–18].

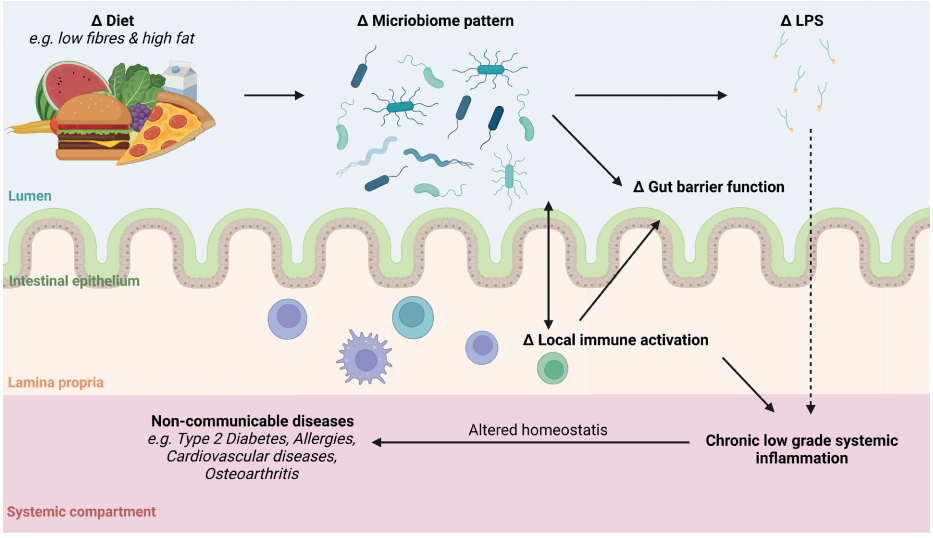

Figure 1. *Schematic overview of the development of Non-communicable diseases, adjusted from [26]. LPS: lipopolysaccharides. Created with Biorender.com.*

Additionally, the microbiome produces biologically active molecules such as short chain fatty acids (SCFAs) through fermentation of fibers [19,20]. SCFAs production might be altered by dysbiosis of the microbiome [21,22]. While SCFA concentrations in the intestine may be reduced and/or ratios of these SCFAs might be changed in patients with

NCDs, these compounds have shown beneficial effects which includes epithelial barrier protection and immunoregulatory and anti-inflammatory effects. Therefore, SCFAs could be a possible treatment option for patients with NCDs by restoring intestinal homeostasis including intestinal barrier function [23–25].

WHAT IS BUTYRATE?

Butyrate is a SCFA which can naturally be found in the gut since it is produced by the microbiome together with several other SCFAs, propionate and acetate. The SCFA acetate is most abundantly present in the gut followed by propionate and butyrate. Acetate, propionate and butyrate are produced in the colon of healthy adults in a molar ratio of approximately 60:20:20 [27–29]. The ratio of these SCFAs in the small intestine has not been described in literature so far. SCFAs concentrations are highest in the colon, because the microbiome necessary to ferment fibers into SCFAs is mainly found in the colon, while in other parts of the gastro-intestinal tract they are present to a lesser extent. The microbiome abundance in the small intestine is five percent of the microbiome in the large intestine, also different kind of bacteria are present [30].

Butyrate was first isolated from cow's butter and identified by a French chemist named Michel Eugène Chevreul in the early 19th century [31]. In the early 20th century it was discovered to be present in the human gut as well. The important role that butyrate can play in human and animal health became of interest later on during the mid-20th century. It was not only discovered that butyrate is a major energy source for intestinal epithelial cells, but it was also observed that butyrate has anti-inflammatory properties, giving it potential as therapeutic agent. Although butyrate has not been marketed as a drug product yet and is only available in the clinic as a compounded enema for active Ulcerative Colitis.

Butyrate would not be the first substance that originates from the human body, having beneficial health effects and developed into a commercially available drug product. This is for example also the case for insulin, corticosteroids, dopamine, erythropoietin and epinephrine.

But, could butyrate be applied as drug for its therapeutical potential as well?

THE USE OF BUTYRATE IN THE ANIMAL INDUSTRY

Butyrate is already widely used as a feed additive in the animal industry, as an alternative for in-feed antibiotics and to promote gut health and improve animal growth and productivity in among others poultry and pigs. In poultry, different forms of butyrate have been used as a feed additive, including capsulated or non-capsulated sodium butyrate and butyrate glycerides [32]. The capsulated forms are developed to deliver the butyrate along the whole intestinal tract and the glycerides deliver specifically to the colon. Sodium

butyrate increased DNA, RNA and proteins concentrations in the duodenal mucosa of poultry, implicating that butyrate stimulated the growth of the duodenum [33]. Others observed that sodium butyrate supplementation in an unprotected form and encapsulated in a vegetable fat were effective in preventing colonization in the crop and cecum and in preventing inflammation in the liver of birds challenged with for example *Salmonella*. The encapsulated form of butyrate was more effective than the unprotective form [34,35]. Additionally, the anti-inflammatory effects of butyrate were observed in poultry, as LPS challenged birds fed butyrate showed reduced levels of IL-6 and TNF-α in the serum [36] and birds fed tributyrin showed reduced levels of IL-1β in the jejunum and ileum, IL-6 in the duodenum and jejunum, and prostaglandin E_2 in the duodenum [37]. Concentrations of butyrate used in poultry are in the range of 0.5-1 g/kg.

In pigs, butyrate supplementation is mainly used to improve the development of the gastro-intestinal tract of weaning pigs. It was observed that butyrate supplementation or cecal infusion increased the mucosal thickness in the stomach, jejunum, ileum, colon and cecum [38,39].

THE USE OF BUTYRATE AS A FOOD SUPPLEMENT IN HUMANS

There are various butyrate containing food supplements available on the market. Each product with a different dosage, different form of butyrate and release characteristic. Also their health claims differ, as can be seen in Table 1 and it should be noted that most claims are not evidence based. Although some of these health claims might be described for butyrate in literature and further along in this thesis, the effectiveness of these products are not fully proven, as almost all of them have not been tested in a clinical study.

CLINICAL STUDIES INVESTIGATING THE EFFECT OF BUTYRATE

Only a few clinical studies are available investigating the effects of butyrate in humans. Most studies investigate the effect of butyrate enemas in patients with active inflammatory bowel diseases (Table 2). The butyrate enemas used contain a dosage of 418 mg up until more than 3 g a day and sometimes even twice a day. Almost all studies showed an improvement in inflammatory markers, stool frequency, quality of life and blood in feces. Only the study with the lowest dosage used, 418 mg butyrate per day, showed no effect. In addition, efficacy of butyrate enemas was investigated in patients with diversion colitis showing reduced atrophy and endoscopic scores [48], in patients with radiation proctitis showing reduced clinical scores in one study [49] and no effect in the other [50]. In healthy volunteers and patients with inflammatory bowel diseases reduced nociception was observed [51,52].

In addition to enemas, butyrate has been administered orally as well in a few different clinical trials, as summarized in Table 3. Placebo-controlled trials mainly focused

Table 1. *Overview of various butyrate or tributyrin containing food supplements.*

Brand	Form	Recommended dosage	Release	Health claims	Reference
ButyrAid	Tributyrin	1-3 x daily 1 capsule with 200 mg tributyrin	Sustained release	• Supporting colon health and integrity	[40]
ButyrEN	Tributyrin	1-3 x daily 1 capsule with 200 mg tributyrin	Sustained release	• Colon lining nutrition • Support gut health	[41]
Bodybio	Calcium and magnesium salts or sodium salt	1x daily 1-2 capsules with 600 mg butyrate salt	Direct release	• Fuel gut cells, strengthen the gut lining and healing leaky gut	[42]
Debutir	Sodium salt	2 x daily 1 capsule with 150 mg sodium butyrate	Sustained release	• Product is developed to be used by among others people with irritable bowel syndrome, inflammation in the intestine, intestinal infection and diarrhea	[43]
Butycaps	Tributyrin	1 x daily 1-2 capsules with 450 mg tributyrin	Sustained release	• Supports intestinal transit and promotes intestinal function	[44]
Probutyrate	Cyloc™alpha dextrin fiber matrix butyric acid complex	2 x daily 1-3 capsules with 300 mg butyric acid in dextrin matrix	Sustained release	• Stabilize the gut microbiome	[45]
Butyflam	Sodium salt	3 x daily 2 tablets with 500 mg butyrate	Gastric resistant	• Immune modulating (Treg + IL-10 anti-inflammation) • Remodeling intestinal barrier function	[46]
Ostrovit sodium butyrate	Sodium salt	2 x daily 1 capsule with 600 mg sodium butyrate	Gastric resistant and sustained release	• Supports proper functioning of the microbiome • Supports proper functioning of the intestine • Has anti-inflammatory effects	[47]

on changes in disease activity and stool habits. Whereas the study observing anti-inflammatory effects in the treatment group have been compared with the status before start of the trial, but did not include a proper placebo group. None of these studies looked into the effects of butyrate on intestinal barrier function, while these effects are mainly described when looking into the effects of butyrate as described in scientific literature.

THE CONCEPT OF DRUG DEVELOPMENT

Drug products are developed for a certain pharmacotherapy, meaning the treatment of diseases with medicine. Pharmacotherapy is based on the assumption that there is a relationship between the dosage regimen, the exposure time and place, and the effects of the drug. The required dose and its response are determined by the pharmacodynamics and the pharmacokinetics of the molecule.

Pharmacodynamics of butyrate

Pharmacodynamics is the study of how drugs interact with their targets in the body to produce a pharmacological response. Several studies have shown the beneficial effects of butyrate on intestinal barrier, inflammation and the microbiome, although the exact mechanisms involved have not been fully elucidated yet and may even contain multiple mechanism. The two main mechanisms of action described for butyrate are histone deacetylase (HDAC) inhibition and binding to G protein-coupled receptors (GPCR), GPR41, GPR43 and GPR109A.

Barrier improving and protective effects of butyrate

In Caco-2 cells it was observed that butyrate enhanced transepithelial electrical resistance (TEER) and decreased the paracellular permeability of fluorescein isothiocyanate (FITC)-labeled inulin [62]. This positive effect on the barrier of the epithelial cell layer was not related to changes in expression level of the tight junction proteins occludin, claudin-1, claudin-4 and Zonula occludens-1 (ZO-1). However a time-dependent increase in the levels of phosphorylated AMP-activated protein kinase (AMPK), which reflects AMPK activity was observed [63]. AMPK is involved in tight junction assembly [64] and butyrate accelerated the relocation of ZO-1 and occludin after disruption of the tight junction structure by calcium depleted cell culture conditions.

In Caco-2 BBE cells butyrate increased TEER measurements as well, although butyrate did not affect permeability/translocation of ^{14}C-mannitol. In this model butyrate, contrary to the previous study, affected protein expression levels of tight junction proteins in claudin-7 (+376%), occludin (+115%), claudin-1 (-39%) and claudin-2 (-90%). The changes in claudin-2 and claudin-7 expression levels were not correlated with changes in their gene expression, suggesting butyrate affected post-translational regulation of these tight junction proteins [65].

Table 2. *Summary of clinical studies performed with sodium butyrate enemas.*

Study design	Disease	N	Treatment	Additional medication?	Duration	Results*	Reference
Cross-over	Active Ulcerative Colitis	10	100 mL of 100 mM sodium butyrate, twice daily	Oral medication continued as usual	2 weeks	Stool frequency ↓ Blood in feces ↓ Inflammatory markers ↓	[53]
Placebo-controlled	Active Ulcerative Colitis	19 vs 19	60 mL of 80 mM sodium butyrate, placebo consisted of 0.8 mM sodium butyrate, once daily	Oral medication continued as usual and kept constant	6 weeks	Inflammatory markers X Disease activity index X	[54]
Placebo-controlled	Ulcerative Colitis	6 vs 5	60 mL of 100 mM butyrate or saline placebo, twice daily	Oral medication continued as usual and kept constant	8 weeks	Inflammatory markers ↓ Disease activity index ↓	[55]
Placebo-controlled	Active Ulcerative Colitis	24 vs 27	80 mL of 80 mM butyrate or saline placebo, once daily	Combined with 4 g topical mesalazine	6 weeks	Urge to defecate ↓ Stool frequency ↓ Self-assessment ↑	[49]
Placebo-controlled	Ulcerative Colitis in remission	17 vs 18	60 mL of 100 mM butyrate or saline placebo		20 days	Minor effects on inflammatory and oxidative stress parameters ↓	[56]

*↓ indicates decrease in observed parameter, ↑ indicates increase in observed parameter, X indicates no change in observed parameter

Table 3. *Summary of clinical studies performed with orally administered butyrate.*

Study design	Disease	N	Treatment	Additional medication?	Duration	Results*	Reference
Placebo-controlled	Moderate Ulcerative Colitis	12 vs 13	5 x 800 mg sodium butyrate tablets with colon targeting coating or placebo, once a day	Combined with 2.4 g oral mesalazine daily	6 weeks	Disease activity index ↓ Clinical index ↓	[57]
Placebo-controlled	Irritable Bowel Syndrome	41 vs 38	2 x 150 mg sodium butyrate (Debutir) or placebo, once a day	Oral medication continued as usual	12 weeks	Urge to defecate ↓ Pain during defecation ↓	[58]
Placebo-controlled	Diverticulosis	30 vs 22	2 x 150 mg sodium butyrate (Debutir) or placebo, once a day	Not reported	52 weeks	Episodes ↓ Quality of life ↑	[59]
Uncontrolled	Crohn's Disease	13	2 g butyrate as enteric-coated tablet designed to release in the terminal ileum and colon, twice a day	2.4 g oral mesalazine daily	8 weeks	Endoscopic score ↓ Histological score ↓ Inflammatory markers ↓	[60]
Uncontrolled	Lean versus Metabolic Syndrome	9 vs 10	4 g butyrate, once a day		4 weeks	Glucose metabolism in lean ↑	[61]

*↓ indicates decrease in observed parameter, ↑ indicates increase in observed parameter

The barrier improving effects of butyrate were not just observed in *in vitro* models, but in animal experiments as well. Wistar rats suffering from dextran sulfate-induced colitis and treated daily with 3 mL of aqueous saline pH 7.4 with or without 25 mM butyrate showed an improvement of barrier function and colon cell viability in the butyrate treated group. In addition butyrate reduced the infiltration of neutrophils in the lamina propria [66].

Butyrate can stimulate the secretion of mucin also, which may contribute to intestinal barrier function. This was observed in tissue cultures of colon biopsies from patients undergoing colectomy for cancer, colonic inertia or Ulcerative Colitis [67] and in isolated vascular perfused rat colon [68]. In the distal colon of BALB/c mice which were given daily 0.1 mL enemas of 100 mM butyrate the adherent mucus layer was decreased, but mucin 1 (MUC1), MUC2 and MUC3 expression in the distal colon and MUC1, MUC2 and MUC4 expression in the proximal colon was increased [69]. In T84 human enterocytes and LS174T human goblet like cells, butyrate increased MUC2 mRNA and/or protein expression as well, most probably via HDAC inhibition [70,71].

The effects of butyrate on the barrier of the cell monolayer and the mucus layer were seen with concentrations in the range of 2-5 mM butyrate. In contrast, 8-100 mM in some cases did not lead to any positive effect and was even sometimes hinting to negative effects indicating the complexity of the dose response relationship.

Anti-inflammatory effects of butyrate

Butyrate can modulate the cytokine release of different cell types and thereby regulating the activation of among others epithelial cells, macrophages and dendritic cells, stimulate the maturation from naïve T-cells into regulatory T-cells (Treg) and affect activation and polarization of T helper (Th) cells. The overall profile is in general anti-inflammatory, and is mediated at least in part by the activation of GPCRs (GPR41, GPR43, and GPR109a) and inhibition of HDAC [72].

The effect of butyrate on the cytokine release of immune cells has been extensively studied. Table 4 summarizes literature related to this topic. These data show overall a decrease in inflammatory cytokines and an increase in anti-inflammatory cytokines.

Addition of SCFAs to co-cultures of mice Treg and Th effector cells increased the Treg suppressive capacity towards Th effector cells. Incubation of colon Treg cells with 0.1 mM propionate for 24 h resulted in increased proliferation and in enhanced acetylation of histone H3, suggesting involvement of HDAC inhibition in the effect of SCFAs on Treg cells. Similar experiments using GPR43-/- mice or colon Treg cells from GPR43-/- mice demonstrated that all these findings were at least partly dependent on GPR43 [83].

Table 4. *Evaluated cytokines released by immune cells after butyrate exposure.*

Cytokine measured*	Cell type studied	Effective butyrate concentration	Reference
IL-10 ↑ IL-12 ↓ IL-4 ↑ IL-2 ↓ IFN- y ↓	• Human PBMCs • Monocytes isolated from PBMCs	• Inhibition of IL-2 and IFN-y release by PBMCs: 0.125 mM • Induction of IL-4 and IL-10 release by PBMCs: 0.06-0.25 mM • Inhibition IL-12p40 and TNF-α release by monocytes: >0.03 mM • Induction IL-10 release by monocytes: optimum at 0.25 mM	[73]
IL-1β ↓ IL-6 ↓ TNF-α ↓ IFN-y ↓ IL-17 ↓	• Human PBMCs • T cells isolated from PBMCs	• Inhibition of TNF-α release by PBMCs: IC_{50} = 0.13 mM • Inhibition of IL-6 release by PBMCs: IC_{50} = 2.44 mM • Inhibition of IL-1β release by PBMCs: IC_{50} = 0.44 mM • Inhibition of IFN-y release by T cells: IC_{50} = 0.05 mM • Inhibition of IL-17 release by T cells: IC_{50} = 0.06 mM	[74]
IL-1β ↓ IL-2 ↓ IL-6 ↓ IL-10 ↑ IL-17 ↓ IL-21 ↓ IL-23 ↓ TGF-β =	• Human PBMCs	• 1, 1.5 and 2 mM	[75]
IL- β 1 ↓ IL-6 ↓ TNF-α ↓ IL-10 ↑	• RAW264.7 macrophage- like cells	• Inhibition of IL-1β release by macrophages: 1.2 mM • Inhibition of IL-6 release by macrophages: 0.1, 1, 1.2 mM • Inhibition of TNF-α release by macrophages: 1 and 1.2 mM-Inhibition of IL-10 release by macrophages: 1 and 1.2 mM	[76]
PGE_2 ↑ MCP-1 ↓ IL-10 ↑ IFN- y ↓ TNF-α ↓	• Human PBMCs • Monocytes isolated from PBMCs	• Inhibition of TNF-α and IFN-y release by PBMCs: > 0.2 mM • Induction of PGE_2 release by monocytes: 2 mM • Inhibition of IL-10 release by monocytes: > 0.2 mM	[77]

Table 4. *Continued*.

Cytokine measured*	Cell type studied	Effective butyrate concentration	Reference
TNF-α ↓ PGE_2 ↑	• Human PBMCs	• Inhibition of TNF-α release by PBMCs: 0.5-10 mM • Induction PGE_2 release by PBMCs: 1-2 mM	[78]
TNF-α ↓ NO ↓	• Rat neutrophils	• Inhibition of TNF-α release by neutrophils: 0.4-3.2 mM-Inhibition of NO release by neutrophils: 1.6-3.2 mM	[79]
TNF-α ↓ MCP-1 ↓ IL-6 ↓	• Rat lymphocytes isolated from lymph nodes	• 0.2-1 mM	[80]
TNF-α ↓	• PBMCs	• 2-10 mM	[81]
IL-2 ↓ IFN-y ↓ IL-10 ↓	• Rat lymphocytes isolated from lymph nodes	• 1.5 mM	[82]

*↓ indicates that the cytokines release decreased after exposure to butyrate, ↑ indicates that the cytokines release increased after exposure to butyrate and = indicates that no changes were observed after butyrate exposure.. IC_{50}: Half-maximal inhibitory concentration; PBMC: Peripheral blood mononuclear cells.

In germ-free mice treated with butyrate (100 mM), propionate (150 mM), acetate (150 mM) or a mix of SCFAs (total 150 mM) in drinking water for 3 weeks significant immune effects have been demonstrated. The treatment led to an increase in Treg cells in colon lamina propria, but not in the spleen, mesenteric lymph node or thymus. The increase in colon Treg cells was accompanied by an increase in anti-inflammatory IL-10, while TGF-ß was unaffected [83].

In male Wistar rats suffering from dinitrobenzene-induced colitis, colonic instillation of calcium butyrate (30 mg/day) 3 days before and 3 days after colitis induction prevented loss of body weight and significantly reduced colon oedema and the area of mucosal damage [84].

Butyrate administered by oral gavage to male Sprague-Dawley rats with trinitrobenzene sulfonic acid-induced colitis improved body weight gain, reduced inflammation and leukocyte infiltration in colon, and regulated Treg/Th17 balance [64]. Treg cells were enhanced by butyrate in peripheral blood, accompanied by increased levels of anti-inflammatory IL-10. IL-17 (in colon and plasma), IL-23 (in plasma) and RORγt (in mesenteric lymph node) were reduced by butyrate.

Pharmacokinetics of butyrate

Pharmacokinetics is the field of study that describes what the body does with the drug substance. A key part of pharmacokinetics is the description of the ADME characteristics of the substance. ADME stands for absorption, distribution, metabolism and excretion.

Absorption

Absorption of a drug substance is the process by which the substance enters the body. This process is influenced by several aspects including the route of administration and the physicochemical properties of the substance. SCFAs consist of carbon atoms and a carbon acid group. Acetate consists of two carbon atoms, propionate consists of three carbon atoms and butyrate consists of four carbon atoms. These compounds have an approximate pKa of 4.8 which means that with a pH of 5.5 to 6.5 in the lumen of the intestinal tract, a great portion of the SCFAs in the lumen are ionized on the carbon-acid group [85]. Absorption of a compound in the intestine can take place via two different mechanisms, passive diffusion and/or active transport. For a compound to cross the epithelial barrier in the gut via diffusion, it must be lipophilic enough to pass the membranes of the epithelial cells, meaning that mainly uncharged compounds can diffuse easily. Unionized butyrate can, due to its high lipophilicity and its low molecular mass, diffuse readily. Only a small percentage of butyrate in the gut lumen is present in its unionized form. Therefore, a large portion of the SCFAs in the gut cannot diffuse easily over the epithelial membrane, and require active transport via transporters for their uptake as well. There are two important transporters located on the epithelial cells for the uptake of SCFAs, namely monocarboxylate transporter 1 (MCT-1) and sodium-coupled monocarboxylate transporter 1 (SMCT-1) [24]. MCT-1 and SMCT-1 are highly expressed on the epithelial cells along the gastro-intestinal tract [86,87].

Distribution

When the drug substance is absorbed from the intestine it will distribute throughout the body. Table 5 gives an overview of the butyrate concentration at different locations in the human body, measured in human victims of sudden death.

Metabolism

Metabolism describes the process by which the drug substance is transformed into metabolites. Metabolism mainly takes place in the liver, but can also take place in other organs such as the kidneys, lungs and the intestine.

Metabolism of butyrate takes places in majority at three different sites in the body, namely the colonocytes, the liver and muscle tissue [88]. In the intestinal epithelial cells butyrate is partly used as an energy source and only a small percentage of the butyrate available in the lumen of the intestine will reach the portal vein. In the liver virtually all of the butyrate available in the portal vein is metabolized to produce cholesterol, fatty acids and ketone bodies, causing very low butyrate availability in the systemic circulation

[25,89]. The part of butyrate that reaches the systemic circulation is metabolized in the peripheral muscle tissue or is being excreted [90].

Table 5. *Butyrate concentrations in the small intestine, large intestine and in blood measured in victims of sudden death [28].*

Compartment		Concentration (±SEM)
Small intestine	Jejunum	-
	Ileum	2.3 (±1.3) mmol/kg
	Caecum	26.1 (±3.8) mmol/kg
Large intestine	Ascending	24.5 (±4.2) mmol/kg
	Transverse	24.4 (±2.2) mmol/kg
	Descending	14.7 (±2.9) mmol/kg
	Rectum	17.9 (±5.6) mmol/kg
Blood	Portal	29 (±7) µM
	Hepatic	12 (±4) µM
	Peripheral	4 (±1) µM

Excretion

Finally, the drug substance is eliminated from the body through excretion. Only a very small percentage (0.0006%) of the dosed butyrate will be excreted via urine [91].

Because of butyrate's fast absorption from the lumen of the intestine and its high metabolism in the intestinal epithelial cells and the liver it is hypothesized that an oral drug product containing butyrate should focus mainly on a local effect in the intestine and lamina propria and not on direct systemic effects.

AIM AND OUTLINE OF THIS THESIS

A good understanding of the pharmacodynamics and pharmacokinetics of butyrate is essential in the development of a drug product containing and releasing effective butyrate levels in the small intestine. Therefore, this thesis does not only describe the developed butyrate drug product, but focusses on butyrate's pharmacokinetics and pharmacodynamic effect in different *in vitro* models and in a clinical study as well. An overview of all elements studied in this thesis is presented in Figure 2.

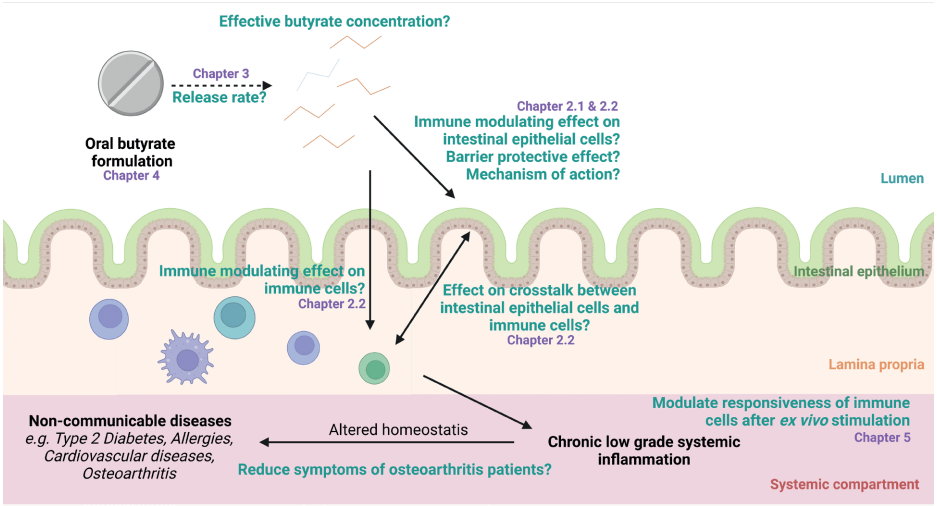

Figure 2. *Overview of the different aspects studied in this thesis. A mathematical model was made to calculate the required butyrate release rate out of an oral formulation to achieve pharmacologically active concentration. In various in vitro models the effect of butyrate was further elucidated on intestinal epithelial cells and peripheral blood mononuclear cells (PBMCs), investigating the effect of butyrate on intestinal barrier, immune modulation of intestinal epithelial cells and immune cells and the cross talk between these two cell types. The designed drug product was tested in a placebo-controlled trial with osteoarthritis patients, studying systemic inflammation, intestinal barrier, pain reduction and responsiveness of whole blood and isolated PBMCs to ex vivo stimulations.*

In **Chapter 2** we investigated the effects of butyrate on intestinal epithelial cells and immune cells in greater details to create a better understanding of the pharmacodynamics and to address gaps in knowledge regarding mechanisms involved and effects in more advanced *in vitro* models. In **Chapter 2.1** the effects of butyrate in activated intestinal epithelial cells HT-29 was studied while investigating the involvement of HDAC inhibition. In **Chapter 2.2** the effects of butyrate in different models using intestinal epithelial cells (Caco-2) with and without PBMCs activated with LPS or αCD3/CD28. This model mimics the cross talk between epithelial cells and activated immune cells, and it was unknown whether butyrate would still have beneficial effects in immune mediated barrier disruption as may occur in the human intestine.

The pharmacokinetic characteristics of butyrate together with the known pharmacodynamic effects and pharmacologically active concentrations were used to design a mathematical model. This model formed the basis to develop a pharmaceutical formulation containing butyrate with the potential to achieve clinical responses, such as barrier improvement and reduction of inflammation locally in the small intestine and lamina propria. This mathematical model is described in **Chapter 3** of this thesis and the drug product, the sustained release butyrate (as calcium) tablet, is described in **Chapter 4**.

A lot of *in vitro* studies and animal studies were already conducted showing the anti-inflammatory and barrier protective effects of butyrate. Furthermore, the type of drug product plays an important role in the potential effect. Therefore it was decided to perform a clinical study with the sustained release butyrate formulation described in **Chapter 4**, to study its effectiveness in patient with osteoarthritis. Osteoarthritis is characterized by degeneration of the cartilage in the joints of the hands which leads to pain, stiffness and reduced functioning of the hand. These symptoms are thought to be related to chronic inflammation and alterations in the intestinal microbiome and therefore it was thought to be a suitable model disease to investigate the effects of butyrate on NCDs. Patients with this disease are mainly treated with pain medication, but there is no drug product available that modulates the cause. In **Chapter 5** the randomized double-blind placebo-controlled clinical study was described in which patients with hand osteoarthritis were given sustained release butyrate. Blood was drawn from these patients and immune assays were performed to investigate the effect of butyrate on systemic inflammation, LPS leakage markers and *ex vivo* immune activation. The results of all chapters will be discussed and summarized in the summarizing discussion.

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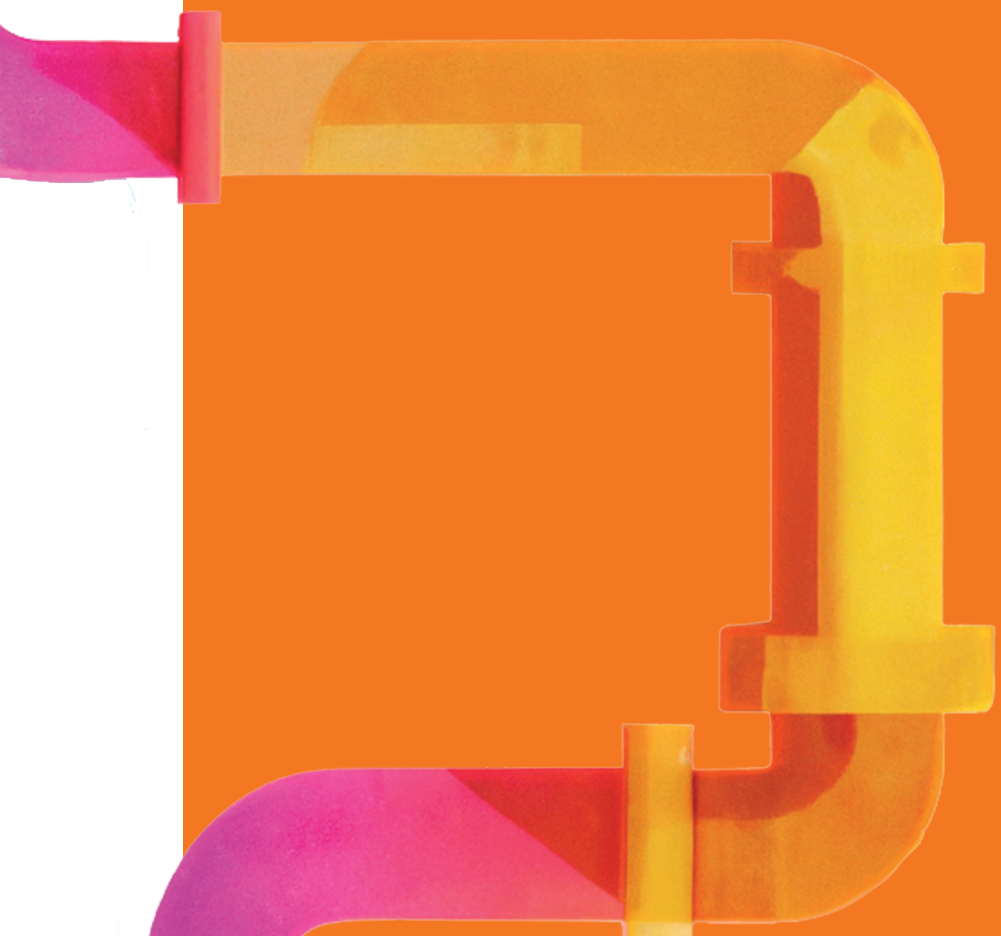
PHARMACODYNAMICS - *IN VITRO*

CHAPTER

2.1



Butyrate prevents induction of CXCL10 and non-canonical IRF9 expression by activated human intestinal epithelial cells via HDAC inhibition

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ABSTRACT

Non-communicable diseases are increasing and have an underlying low-grade inflammation in common, which may affect gut health. To maintain intestinal homeostasis, unwanted epithelial activation needs to be avoided. This study compared the efficacy of butyrate, propionate and acetate to suppress IFN-γ+/-TNF-α induced intestinal epithelial activation in association with their HDAC inhibitory capacity, while studying the canonical and non-canonical STAT1 pathway. HT-29 were activated with IFN-γ+/-TNF-α and treated with short chain fatty acids (SCFAs) or histone deacetylase (HDAC) inhibitors. CXCL10 release and protein and mRNA expression of proteins involved in the STAT1 pathway were determined. All SCFAs dose-dependently inhibited CXCL10 release of the cells after activation with IFN-γ or IFN-γ+TNF-α. Butyrate was the most effective, completely preventing CXCL10 induction. Butyrate did not affect phosphorylated STAT1, nor phosphorylated NFκB p65, but inhibited IRF9 and phosphorylated JAK2 protein expression in activated cells. Additionally, butyrate inhibited *CXCL10*, *SOCS1*, *JAK2* and *IRF9* mRNA in activated cells. The effect of butyrate was mimicked by class I HDAC inhibitors and a general HDAC inhibitor Trichostatin A. Butyrate is the most potent inhibitor of CXCL10 release compared to other SCFAs and acts via HDAC inhibition. This causes downregulation of *CXCL10*, *JAK2* and *IRF9* genes, resulting in a decreased IRF9 protein expression which inhibits the non-canonical pathway and CXCL10 transcription.

INTRODUCTION

The increase in the number of non-communicable diseases among humans is one of the major global health challenges of this century. A high-risk factor for non-communicable diseases is the consumption of a Western diet consisting of high fat and low fibers. Such a diet can result in changes in microbiome diversity, leading to dysbiosis [1,2]. Dysbiosis is associated with a disrupted intestinal barrier, leading to increased systemic endotoxin levels and low-grade inflammation both locally in the gut and systemically [3-7]. Low-grade inflammation causes neurological, metabolic and other subsequent immunological changes which are involved in the development of non-communicable diseases, such as cardiovascular diseases, diabetes, inflammatory diseases, asthma, allergies and even cancer. The development of these diseases may relate to the insufficient intake of fermentable fibers which serve as a substrate for the gut microbiota and are converted into short chain fatty acids (SCFAs), such as butyrate, propionate and acetate. These SCFAs improve intestinal barrier function [8-13] and have anti-inflammatory properties [5,11,14-20]. Preclinical studies have shown that reduced levels of SCFAs can result in intestinal barrier disruption and local inflammation, causing the passage of endotoxins over the intestinal barrier, leading to systemic low-grade inflammation [21,22]. The intestinal barrier and local systemic immune homeostasis may be improved by supplementation with SCFAs. However, more insight is required regarding the mechanisms by which SCFAs act to protect against unwanted inflammatory responses in mucosal tissues via their impact on the intestinal epithelial cells (IECs).

The intestinal barrier is disrupted and the intestinal epithelium is activated by several pro-inflammatory cytokines, including interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) [23-26]. IFN-γ activates the signal transducer and activator of transcription 1 (STAT1) signaling cascade upon binding to its receptor and signals via the canonical or non-canonical cascade [27]. In the canonical cascade, the dimerized IFN-γ-receptor is activated by phosphorylation of Janus kinase 1 (JAK1) and Janus kinase 2 (JAK2) domains which downstream facilitates dimerization of intracellular STAT1 monomers. The STAT1 dimer is phosphorylated and travels into the nucleus to act as a transcription factor via gamma-activated sequences, resulting in the expression of target genes, such as C-X-C motif chemokine ligand 10 (CXCL10). In the non-canonical cascade, the STAT1 dimer will not phosphorylate but bind to interferon regulatory factor 9 (IRF9). This complex will travel to the nucleus to act as a transcription factor via interferon-stimulated response elements, also resulting in the expression of target genes, such as CXCL10 [28]. In both activated cascades, suppressors of cytokine signaling 1 (SOCS1) can directly inhibit JAK1 and JAK2, ensuring a negative feedback mechanism [29]. The activation of these cascades and release of pro-inflammatory CXCL10 will accelerate Th-1 type inflammatory responses [30]. Blockage of CXCL10 release reduces this Th-1 type response, reduces inflammation and, in turn, could be beneficial in maintaining immune homeostasis and intestinal barrier integrity.

Another common pro-inflammatory mediator, TNF-α, activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway which, amongst others, will result in the release of pro-inflammatory C-X-C motif chemokine ligand 8 (CXCL8). Moreover, TNF-α is known to intensify IFN-γ-induced epithelial activation, resulting in a synergetic effect on CXCL10 release [31]. This synergy can be explained by either an upregulation of the IFN-γ-receptor by TNF-α [32,33], an increased IFN-γ induced STAT1 phosphorylation by TNF-α [33-35] or the enhanced recruitment of the STAT1 dimer to the promotor site by TNF-α [33].

SCFAs are known inhibitors of CXCL10 release and the STAT1 signaling cascade [36,37,38,39,40,41]. The effect of SCFA butyrate has already been studied in IFN-γ-activated intestinal epithelial cells; however, it is unknown whether propionate and acetate are capable of inhibiting IFN-γ-mediated signal transduction in intestinal epithelial cells as well. In addition, it is unknown whether SCFAs are still effective in inhibiting CXCL10 release when IECs are exposed to both IFN-γ and TNF-α instead of IFN-γ alone. Research related to how butyrate may affect CXCL10 release mainly focusses on the canonical STAT1 signaling cascade [37,39]. Whether butyrate affects CXCL10 release via the non-canonical STAT1 signaling cascade is largely unknown.

SCFAs inhibit histone deacetylase (HDAC) [15]. HDAC can be subdivided into class I (HDAC1, HDAC2, HDAC3 and HDAC8), class IIa (HDAC4, HDAC5, HDAC7 and HDAC9), class IIb (HDAC6 and HDAC10) and class IV (HDAC11) [42]. HDAC inhibitors inhibit histone deacetylase and can regulate gene expression. Butyrate is a potent class I and class IIa HDAC inhibitor [43]. Propionate is a similar or slightly less potent HDAC inhibitor [44,45] while the effects of acetate are unclear, with studies reporting only an effect in high concentrations or no effect at all [45-47]. It is known that a nonspecific HDAC inhibitor reduces CXCL10 release, and that HDAC is of importance for gut homeostasis and can interact with the canonical and non-canonical STAT1 signaling cascade [38,48-53]. It is unknown whether the HDAC inhibitory properties of butyrate are strong enough to affect these signaling cascades in a similar way and whether more specific HDAC inhibitors are capable of reducing CXCL10 release.

In this study, we compared the efficacy of butyrate, propionate or acetate to suppress IFN-γ and IFN-γ+TNF-α-induced intestinal epithelial activation in association with their capacity to inhibit HDAC, while studying the canonical and non-canonical STAT1 signaling cascade. In addition, we will investigate the effect of more specific HDAC inhibitors on CXCL10 release to better understand which HDACs are involved in the effect of SCFAs. We hypothesize that SCFAs, and in particular butyrate, affect the STAT1 signaling cascade and CXCL10 release via HDAC inhibition.

MATERIALS AND METHODS

Intestinal epithelial cell culture

Human IECs, HT-29 cells (ATCC, HTB-38, Manassas, VA, USA; passages 150–175) were grown in McCoy's 5A medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin (100 U/mL and 100 ug/mL) (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured in 75 cm2 flasks (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) and passaged once a week. Cells were kept in an incubator at 37 °C in the presence of 5% CO_2.

Epithelial activation and SCFAs and HDAC inhibitor treatment

HT-29 were seeded in 48 or 96 well plates (Corning, New York, NY, USA) and grown till confluency before they were used to perform experiments. The medium was changed every 2–3 days and 1 day prior to the experiment. After reaching confluence, cells were activated with IFN-γ (100 IU/mL) or IFN-γ (100 IU/mL) + TNF-α (1 ng/mL) (Invitrogen) and simultaneously treated with one of the SCFAs or HDAC inhibitors for 1, 4, 16 or 24 h. As SCFAs, either sodium butyrate (2, 4, 8 mM), sodium propionate (2, 4, 8 mM) or sodium acetate (4, 8, 16 mM) (Sigma-Aldrich) were used dissolved in culture medium, and as HDAC inhibitors, either Trichostatin A (TSA) (10, 100 µM), Tinostamustine (1, 5, 15 µM), Tacedinaline (1, 10 µM), Droxinostat (10, 50 µM), RGFP966 (5, 20 µM), Cay10683 (5, 20 µM) or TMP269 (10, 20, 50 µM) (MedChemExpress, Sollentuna, Sweden) were used dissolved in DMSO and further diluted in culture medium.

Table 1. *HDAC inhibition by specific HDAC inhibitors, categorized per class. Intensity in color indicates half-maximal inhibitory concentration of the HDAC inhibitor in mM, µM, nM or pM range (light grey to black).*

	Class I				Class IIb		Class IIa			
HDAC	1	2	3	8	6	10	4	5	7	9
TSA										
Butyrate										
TMP269										
Tinostamustine										
Tacedinaline										
Droxinostat										
RGFP966										
Cay10683										

mM µM nM pM

Table 1 indicates the HDAC inhibitor specificity. A quantity of 0.5% DMSO in culture medium served as a control. After 1, 4, 16 or 24 h of incubation, supernatants were collected for enzyme-linked immunosorbent assay (ELISA) and cells were either lysed in LBP lysis buffer (Macherey-Nagel, Düren, Germany) for quantitative polymerase chain reaction (qPCR) analysis or in RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with protease and phosphatase inhibitors (Roche, Diagnostics, Mannheim, Germany) for Western blot analysis. At 24 h, the viability of the cells was assessed.

HDAC activity assay

To determine the effect of SCFAs on HDAC activity in HT-29 cells, an HDAC activity assay (In Situ Histone Deacetylase Activity Fluorometric Assay Kit, Sigma-Aldrich) was performed according to the manufacturer's protocol. In short, cells were grown in white clear bottom 96 well plates. After reaching confluence, cells were treated with either sodium butyrate (2, 4 mM), sodium propionate (2, 4 mM) or sodium acetate (4, 8 mM). HDAC inhibitor TSA (10 μM) served as positive controls. After 24 h substrate was added, and cells were incubated for 2 h. Subsequently, developer mix was added, and cells were incubated for 30 min, after which fluorescence was read at Excitation/Emission 368/442 nm in a microplate reader (GloMax Discover, Promega Corporation, Madison, WI, USA).

ELISA

CXCL10 and CXCL8 secretion was measured in the supernatant with the use of a commercially available ELISA kit (CXCL10: BioLegend, San Diego, CA, USA or R&D Systems, Minneapolis, MN, USA; CXCL8: Invitrogen, Thermo Fisher Scientific) both according to the manufacturer's protocol. In short, high-binding 96-well plates were coated with capture antibody and incubated overnight. Non-specific binding was blocked for 1 h at room temperature. After washing, the samples or the standard were added for 2 h at room temperature. Then, plates were washed and incubated with streptavidin-horseradish peroxidase for 1 h at room temperature. Subsequently, the plates were washed and incubated in the dark with substrate solution at room temperature. The reaction was stopped with 1 M H2SO4 and absorbance was measured at 450 nm in a microplate reader (iMark, Bio-Rad Laboratories, Hercules, CA, USA or GloMax Discover, Promega Corporation, Madison, WI, USA).

Western blot

First, protein concentration was determined using a BCA protein assay kit (Thermo fisher Scientific) to ensure equal protein loading across samples. Bromophenol blue and 1,4-Dithiothreitol were added to the samples to denature the proteins. Protein samples were then added to a CriterionTM 4–20% Tris-HCl gel (Bio-Rad, Veenendaal, The Netherlands) for separation with electrophoresis. Thereafter the proteins were transferred to a polyvinylidene difluoride membrane (Transblot Turbo, Bio-Rad). The membrane was blocked using 5% milk protein in phosphate-buffered saline containing 0.05% Tween-

20. After blocking, the membrane was incubated with primary antibodies overnight at 4 °C. As primary antibodies phosphorylated JAK2 (1:200, Thermo Fisher Scientific), phosphorylated STAT1 (1:200, R&D systems), phosphorylated NF-kappaB p65 (1:1000, Cell signaling, Danvers, MA, USA), IRF9 (1:1000, Cell signaling) and β-actin (1:1000, Cell signaling) were used. After incubation, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Dako, Santa Clara, CA, USA) for 2 h. Membranes were again washed and the proteins on the membranes were visualized using ECL reagent (Bio-Rad) for phosphorylated NF-kappaB p65 and β-actin or ECLTM Prime (Cytiva, Marlborough, MA, USA) for the other proteins of interest that were assessed. Data was analyzed using Image J (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). When the same membrane was used for the analysis of multiple proteins with different sizes, the membrane was stripped using Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific).

qPCR

First, RNA was isolated from lysed cell homogenates using a NucleoSpin® RNA Plus kit (Macherey-Nagel) according to the manufacturer's protocol. DNAse (Qiagen, Hilden, Germany) was used to remove contaminated DNA. Second, complementary DNA was synthesized using an iScriptTM cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. Quantitative analysis was performed on a CFX96 real-time PCR detection system with the use of IQTM SYBR® Green Supermix (both from Bio-Rad). Commercially available primers for *SOCS1, JAK2, CXCL10* and *IRF9* were obtained as genes of interest and *ribosomal protein S13* (*RPS13*) was obtained as a reference gene (all from Qiagen). Relative mRNA expression was calculated as 100 × 2[Ct reference-Ct gene of interest] [78].

Viability assay

After 24 h, cell viability was determined using a WST-1 assay (Roche) according to the manufacturer's protocol. In short, WST was diluted in culture medium (1:10 dilution) and added to each well for 30 min at 37 °C in the presence of 5% CO2. Subsequently, 100 µL of each well was transferred to a clear 96 well plate and absorbance measured at 450 nm with a microplate reader (iMark, Bio-Rad Laboratories, Hercules, CA, USA or GloMax Discover, Promega Corporation, Madison, WI, USA).

Statistical analysis

The data is expressed as mean ± standard error of the mean (SEM) of 3 or 4 independent measurements. All statistical analyses were performed using GraphPad Prism software version 8.4.3. (GraphPad Software, San Diego, CA, USA). Data was normally distributed and statistical significance was tested using the repeated measures one-way ANOVA analysis, followed by Bonferroni's post hoc test with selected pairs or, if indicated, with a paired two-sample t-test. Results were considered statistically significant when $p < 0.05$.

RESULTS

SCFAs prevent the release of CXCL10 by activated IECs

IECs were treated with butyrate, propionate or acetate and activated with IFN-γ or IFN-γ+TNF-α. CXCL10 and CXCL8 were measured in the supernatant collected after 1, 4, 16 and/or 24 h. After 24 h CXCL10 release of IFN-γ-activated cells was increased compared to medium control. CXCL10 release of combined IFN-γ+TNF-α-activated cells was increased by almost 15-fold compared to IFN-γ-activated cells. CXCL10 release of IFNγ and IFNγ+TNFα-activated cells was inhibited by all SCFAs in a dose-dependent manner. Acetate was the least effective, followed by propionate and butyrate. Butyrate was most effective, and completely prevented the induced CXCL10 release at a dose of only 2 mM, not only of IFN-γ-activated cells but of IFN-γ+TNF-α-activated cells as well (Figure 1A,B).

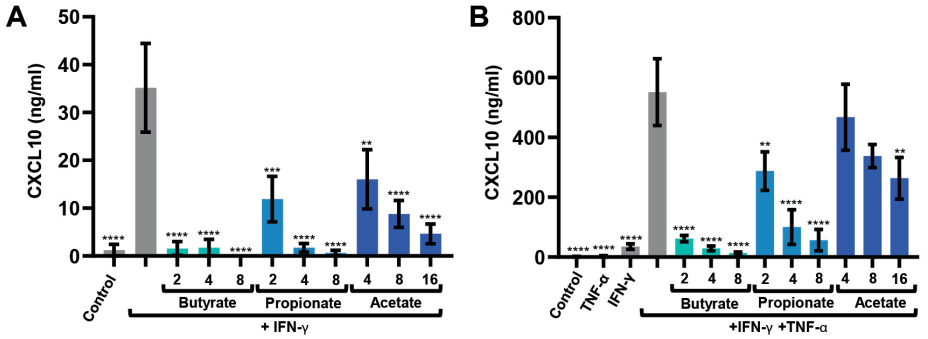


Figure 1. *CXCL10 release by intestinal epithelial cells after 24 h, activated with IFN-γ (A) or IFN-γ+TNF-α (B) and treated with 2, 4, 8 and 16 mM butyrate, propionate or acetate. Data are represented as mean ± SEM (N = 3). Significant differences are shown as ** p < 0.01, *** p < 0.001, **** p < 0.001 compared to IFN-γ (A) or IFN-γ+TNF-α (B) activated cells.*

The kinetics of the release of CXCL10 and CXCL8 was studied and the inhibitory effect of butyrate was further investigated. CXCL10 was detected after 4 h of IFN-γ+TNF-α exposure and after 16 h of exposure to IFN-γ alone. An amount of 2 mM butyrate reduced CXCL10 at 4 h in IFN-γ+TNF-α-activated IECs and at 16 h in IFN-γ and IFN-γ+TNF-α-activated IECs (Figure 2A,C,E). IFN-γ+TNF-α-activated cells additionally released CXCL8, which was not affected by 2 mM butyrate (Figure 2B,D,F). None of the treatments affected the viability of the cells (Supplemental Figure S1).

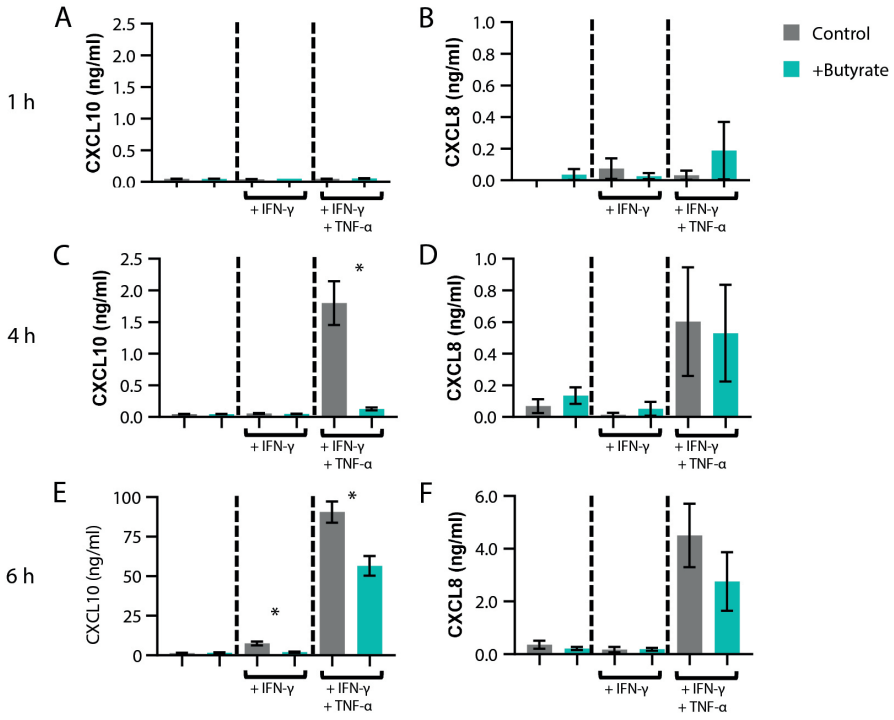
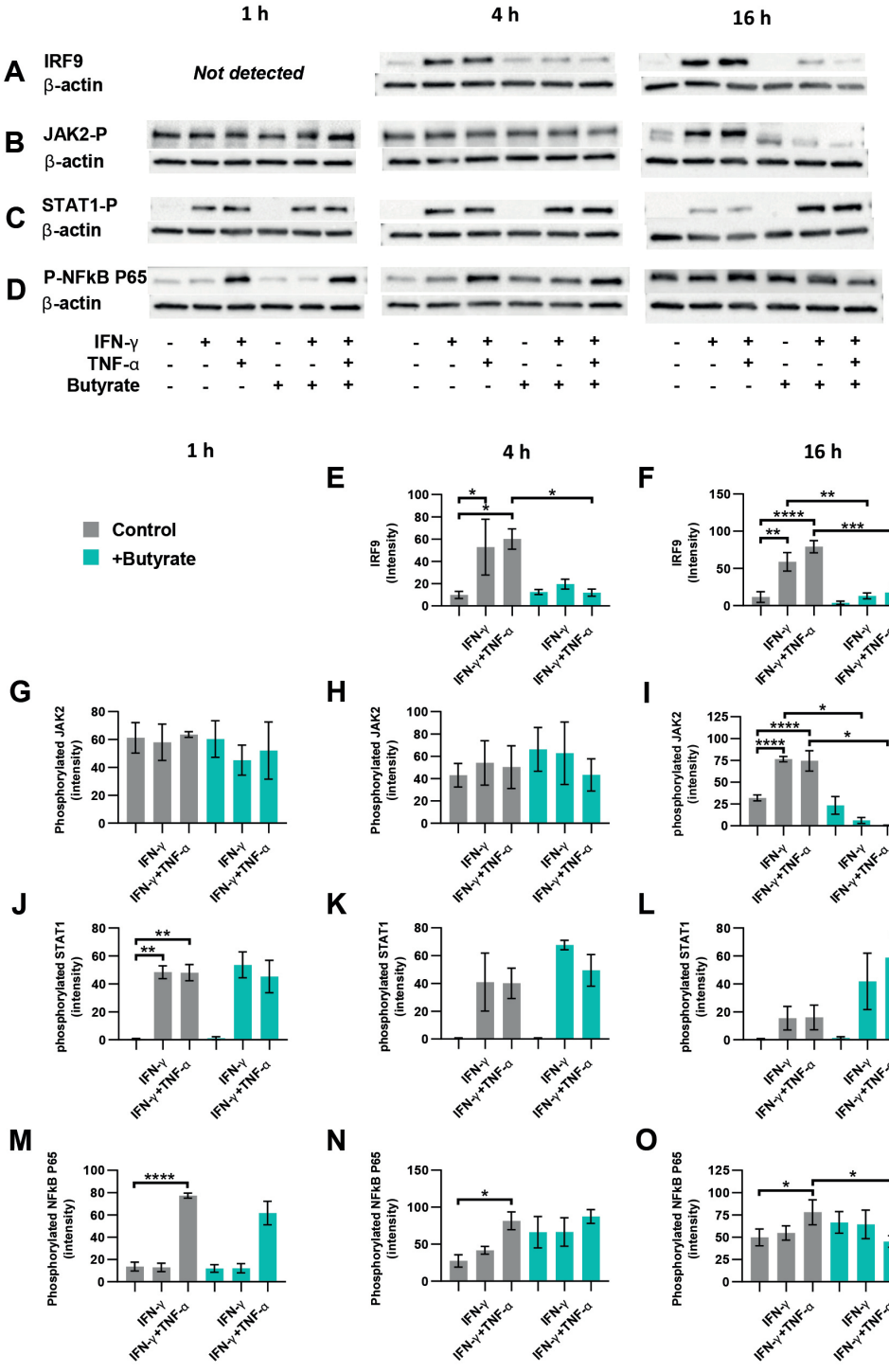


Figure 2. *Chemokine release in time by intestinal epithelial cells activated with IFN-γ or IFN-γ+TNF-α and treated with 2 mM butyrate (green bars). CXCL10 (A,C,E) and CXCL8 (B,D,F) release was measured after 1, 4 and 16 h incubation. Data are represented as mean ± SEM (N = 4). Significant differences are shown as * $p < 0.05$ tested with a paired t-test between control and butyrate treated cells.*

Butyrate reduces proteins related to the non-canonical STAT1 signaling cascade

IECs were activated with IFN-γ or IFN-γ+TNF-α and treated with 2 mM butyrate because this concentration showed to be effective in the prevention of induced CXCL10 release. After 1, 4 and 16 h, cells were lysed and IRF9, phosphorylated JAK2, phosphorylated STAT1 and phosphorylated NFκB p65 protein levels were quantified; β-actin served as a control for equal protein loading (Figure 3A–D).


1 h
4 h
16 h
A
IRF9
β-actin
Not detected
B
JAK2-P
β-actin
C
STAT1-P
β-actin
D
P-NFkB P65
β-actin
IFN-γ
TNF-α
Butyrate
Control
+Butyrate
E
F
G
H
I
J
K
L
M
N
O
IRF9 (Intensity)
Phosphorylated JAK2 (intensity)
phosphorylated JAK2 (intensity)
phosphorylated STAT1 (intensity)
Phosphorylated NFkB P65 (intensity)
IFN-γ
IFN-γ+TNF-α

< Figure 3. *Canonical or non-canonical STAT1 pathway protein expression. Cells were activated with IFN-γ or IFN-γ+TNF-α and treated with 2 mM butyrate (green bars) for 1 h, 4 h or 16 h. IRF9 (A,E,F), phosphorylated JAK2 (B,G–I), phosphorylated STAT1 (C,J–L) and phosphorylated NFkB p65 (D,M–O) protein expression was determined. Representable blots of proteins of interest and β-actin control are shown in A-D. Data are represented as mean ± SEM (N = 3). Significant differences are shown as * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.001, medium control as compared to 2 mM butyrate control or to IFN-γ or IFN-γ+TNF-α-activated intestinal epithelial cells in absence of butyrate. Activated cells were also compared to activated cells treated with butyrate.*

IRF9 expression increased in IFN-γ and IFN-γ+TNF-α-activated cells at 4 h and 16 h of incubation, while at 1 h, IRF9 could not be detected. A quantity of 2 mM butyrate inhibited IRF9 induction at 4 and 16 h, maintaining similar levels as for the control cells (Figure 3A,E,F). Phosphorylated JAK2 increased in IFN-γ and IFN-γ+TNF-α-activated cells at 16 h of incubation and 2 mM butyrate decreased JAK2 phosphorylation at this timepoint (Figure 3B,G–I).Phosphorylated STAT1 increased in IFN-γ and IFN-γ+TNF-α-activated cells at 1 h of incubation and 2 mM butyrate did not affect this (Figure 3C,J–L). Phosphorylated NFκB p65 increased only in IFN-γ+TNF-α-activated cells at 1, 4 and 16 h of incubation, and 2 mM butyrate inhibited this only at 16 h of incubation (Figure 3D,M–O).

SCFAs inhibit HDAC activity in HT-29

SCFAs, and in particular butyrate, are known HDAC inhibitors [54]. Here, their inhibitory capacity was studied in IECs HT-29. All SCFAs reduced HDAC activity compared to medium control. Acetate was the least effective, followed by propionate and butyrate. Quantities of 2 and 4 mM butyrate reduced HDAC activity similar to 10 μM Trichostatin A (TSA), a general HDAC inhibitor (Figure 4A).

HDAC inhibitors mimic the effect of butyrate in activated IECs

It was investigated whether HDAC inhibitors mimic the anti-inflammatory effects of butyrate. Again, CXCL10 release of IFN-γ-activated cells after 24 h was increased compared to medium control. CXCL10 release of combined IFN-γ+TNF-α-activated cells was increased by almost 10-fold compared to IFN-γ-activated cells (Figure 4B–E). CXCL10 release of IFN-γ or IFN-γ+TNF-α-activated IECs was inhibited by general HDAC inhibitor TSA and a higher concentration of the class I and IIb HDAC inhibitor tinostamustine. In contrast, CXCL10 release was not inhibited by the class IIa HDAC inhibitor TMP269. Except for IFN-γ+TNF-α-activated cells treated with 50 μM TMP269, which showed a significant reduction in CXCL10 release, as well as the DMSO control (Figure 4B,C). HDAC class I consists of HDAC 1, 2, 3, 8 and class IIb consists of HDAC 6 and 10. The more specific HDAC inhibitors droxinostat (which inhibits HDAC 3, 6 and 8), RGFP966 (which inhibits HDAC 3 and to a lesser extent 1 and 2) and tacedinaline (which inhibit HDAC 1, 2 and 3) inhibited CXCL10 release of IFN-γ-activated cells, but only 20 μM of RGFP966 inhibited CXCL10 release of IFN-γ+TNF-α-activated (Figure 4C,D). Cay10683 (which inhibits HDAC 2 and 6) did not inhibit CXCL10 release of activated IECs.

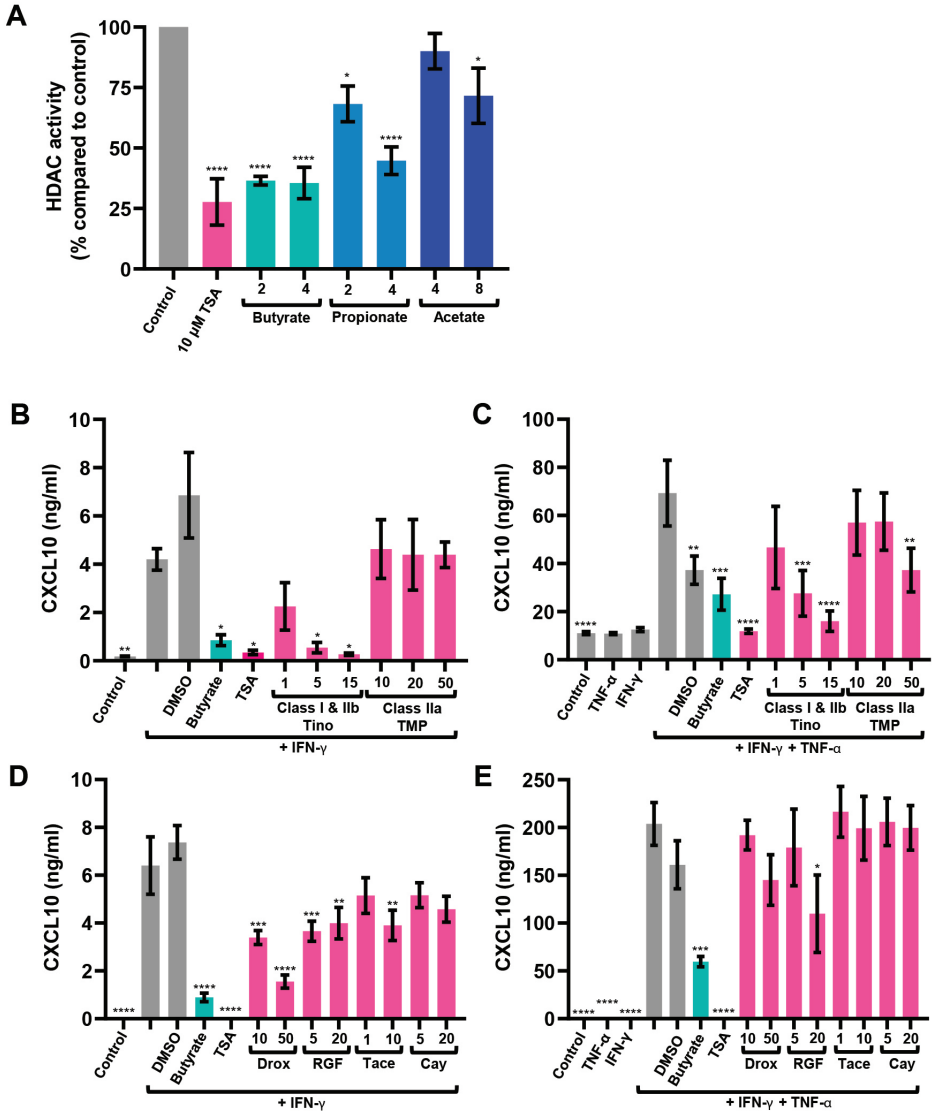


Figure 4. *The effect short chain fatty acids on histone deacetylase (HDAC) activity (A) and the effect of HDAC inhibitors on CXCL10 release (B–E). HDAC activity of IECs after 24 h exposure to butyrate (2, 4 mM), propionate (4, 8 mM), acetate (4, 8 mM) or Trichostatin A (TSA) (10 µM) (A). Data are represented as mean ± SEM (N = 3). Significant differences are shown as * p < 0.05, **** p < 0.001 compared to control. CXCL10 release by intestinal epithelial cells after 24 h, activated with IFN-γ (B,C) or IFN-γ+TNF-α (D,E) and treated with 2 mM butyrate; 10 µM TSA; 1, 5, 15 µM tinostamustine (Tino); 10, 20, 50 µM TMP269 (TMP) (B,C) or with 2 mM butyrate; 10 µM TSA; 10, 50 µM droxinostat (Drox); 5, 20 µM RGFP966 (RGF); 1, 10 µM tacedinaline (Tace); 5, 20 µM Cay10683 (Cay) (D,E). 0.5% DMSO served as a control. Data are represented as mean ± SEM (N = 3). Significant differences are shown as * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.001 compared to IFN-γ-activated cells (B,D) or IFN-γ+TNF-α-activated cells (C,E).*

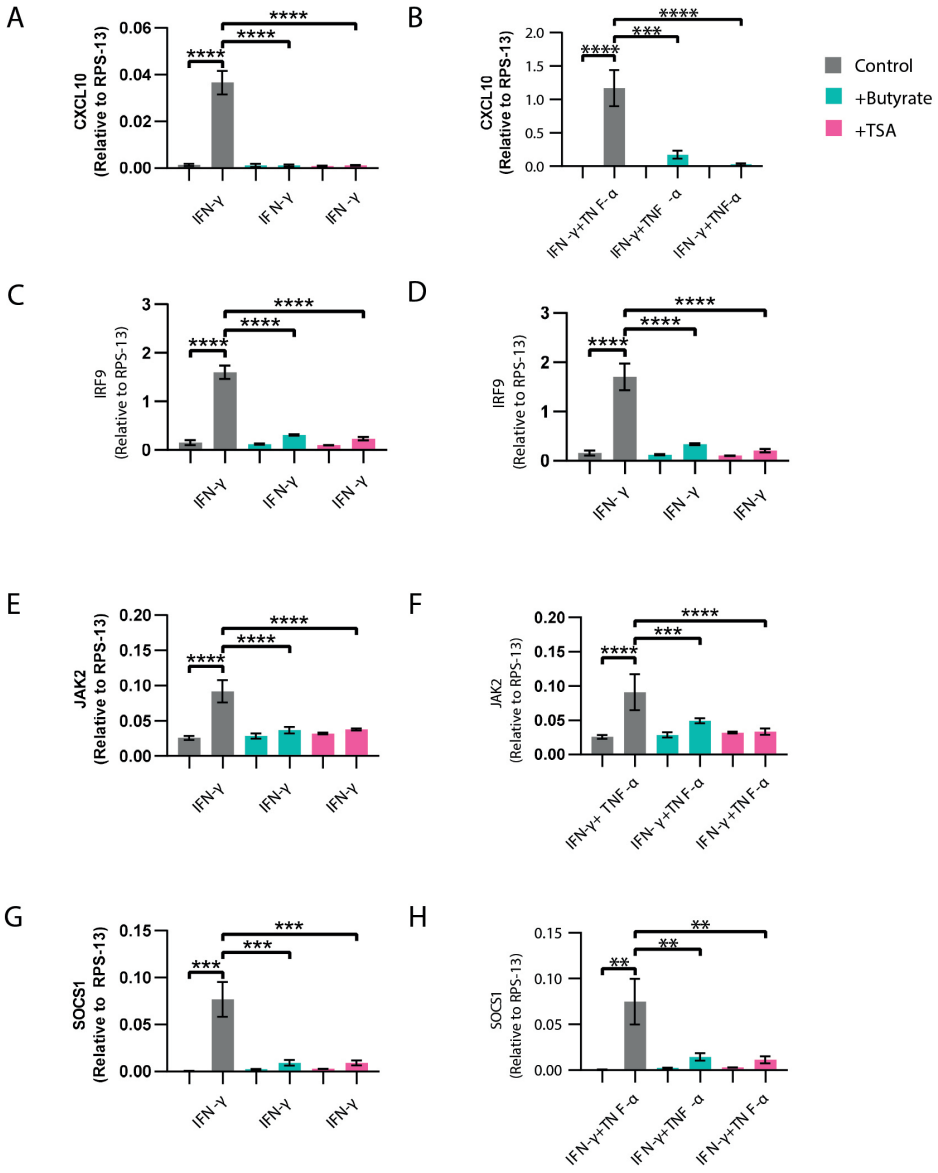


Figure 5. *The effect of butyrate and histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) on mRNA expression of genes related to the STAT1 signaling cascade. Intestinal epithelial cells were activated with IFN-γ or IFN-γ+TNF-α and treated with 2 mM butyrate (green bars) or 10 μM TSA (pink bars) for 4 h. mRNA expression was measured in CXCL10 (A,B), IRF9 (C,D) JAK2 (E,F) and SOCS1 (G,H). Data are represented as mean ± SEM (N = 4). Significant differences are shown as ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$ Control compared to butyrate, TSA, IFN-γ (A,C,E,G) or IFN-γ+TNF-α (B,D,F,H) activated cells and activated cells compared to butyrate or TSA treated activated cells.*

Butyrate inhibits CXCL10 transcription in activated IECs

HDAC and HDAC inhibitors affect gene transcription; therefore the mRNA expression of *CXCL10*, *IRF9*, *JAK2* and *SOCS1* genes in IFN-γ or IFN-γ+TNF-α-activated cells were studied at 4 h incubation with 2 mM butyrate or 10 μM TSA. *RPS-13* served as a household gene. *CXCL10* (Figure 5A,B), *IRF9* (Figure 5C,D), *JAK2* (Figure 5E,F) and *SOCS1* (Figure 5G,H) gene expression was increased in IFN-γ and IFN-γ+TNF-α-activated cells, which was completely prevented by both butyrate and TSA (Figure 5A–F).

HDAC inhibitor TSA prevents induced IRF9 expression similar to butyrate

To investigate whether HDAC inhibitors affect the STAT1 signaling cascade similarly to butyrate, protein expression and phosphorylation of proteins related to the cascade were determined. Therefore, cells were activated with IFN-γ or IFN-γ+TNF-α and treated with 10 μM TSA. TSA prevented the induction of IRF9 in IFN-γ and IFN-γ+TNF-α-activated cells (Figure 6A,E) and did not affect the expression of phosphorylated JAK2 (Figure 6B,F) and phosphorylated NFκB p65 (Figure 6D,H). TSA significantly induced phosphorylation of STAT1 only in IFN-γ-activated cells (Figure 6C,G).

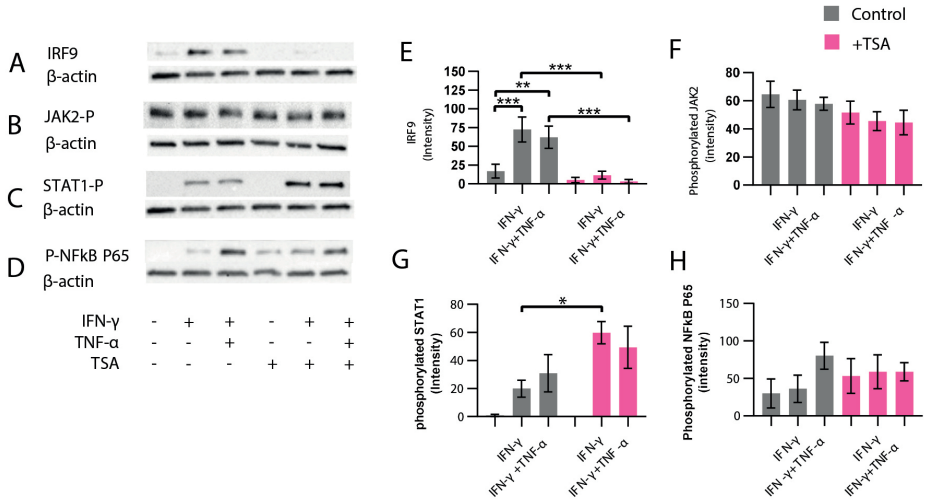


Figure 6. *The effect of histone deacetylase inhibitor Trichostatin A (TSA) on downstream proteins of the STAT1 signaling cascade. Protein expression in IFN-γ or IFN-γ+TNF-α-activated intestinal epithelial cells (IECs) after 4 h incubation with 10 μM TSA (pink bars). Proteins measured were IRF9 (A,E), phosphorylated JAK2 (B,F), phosphorylated STAT1 (C,G), phosphorylated NFκB p65 (D,H). Data are represented as mean ± SEM (N = 3). Significant differences are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, control compared to IFN-γ-activated IECs, IFN-γ+TNF-α-activated IECs or 10 μM TSA control. Activated cells were compared to activated cells treated with TSA.*

DISCUSSION

SCFAs are known to improve intestinal barrier function and to act anti-inflammatory. In the present study, IFN-γ-activated IECs in the presence or absence of TNF-α were used as a model to compare the efficacy of SCFAs in inhibiting CXCL10 release. In addition, their capacity to affect the canonical and non-canonical STAT1 signaling cascade was studied while investigating the role of HDAC inhibition.

SCFAs, and most prominently butyrate, completely prevented the cytokine-induced CXCL10 release, not only of IFN-γ-activated IECs but also of IFN-γ+TNF-α-activated IECs. Importantly, IFN-γ+TNF-α induced CXCL10 release up to 10–15-fold higher concentrations when compared to IFN-γ-activation alone. The synergy between these two pro-inflammatory cytokines, IFN-γ and TNF-α, is a known phenomenon with different proposed underlying mechanisms [31-33,35,53]. The inhibitory effect of butyrate on the CXCL10 release induced by IFN-γ+TNF-α to our knowledge has not been studied before and indicates the strong anti-inflammatory efficacy of butyrate. Acetate was least effective in preventing CXCL10 release, while butyrate completely blocked CXCL10 release of both IFN-γ and IFN-γ+TNF-α-activated cells at a concentration of only 2 mM. These findings are in line with previous research where CXCL10 release was reduced in mature dendritic cells, LPS stimulated whole blood and IFN-γ-stimulated human colonic subepithelial myofibroblasts, after treatment with butyrate or propionate, but not after treatment with acetate [38,40,41]. TNF-α is known to induce CXCL8 release by IECs via NFκB signaling. Even though butyrate is known as an NFκB inhibitor [55,56], 2 mM butyrate did not affect CXCL8 release at 1, 4 and 16 h of incubation, nor did it affect TNF-α-induced phosphorylated NFκB p65 at 1 and 4 h of incubation. Therefore, butyrate did not block TNF-α-induced epithelial activation at these early time points, and thus the selective preventive effect of butyrate on synergistically induced CXCL10 release by IFN-γ+TNF-α-activated IECs could not be explained by the early blockage of the NFκB signaling cascade. However, butyrate did inhibit NFκB p65 phosphorylation at 16 h of incubation. In other studies, butyrate was found to inhibit CXCL8 release of endothelial and intestinal epithelial cells after pretreatment for at least 24 h [57,58], and butyrate was found to inhibit NFκB activity after 24 and 48 h, but not after 4 and 8 h of incubation in HT-29 cells [56]. This may indicate that the NFκB inhibitory effects of butyrate might set in at a later timepoint in the current model or that pretreatment is required. The CXCL10 release was completely blocked after only 4 h of incubation in IFN-γ+TNF-α-activated IECs, and therefore butyrate blocked the synergistical effect of TNF-α on CXCL10 release independent of NFκB.

While butyrate did not affect STAT1 phosphorylation, it clearly prevented the induction of IRF9 expression at 4 h and 16 h, and phosphorylation of JAK2 at 16 h in IFN-γ and IFN-γ+TNF-α-activated IECs, therefore blocking the non-canonical STAT1 signaling cascade. At 4 h, butyrate not only prevented the induction of IRF9 expression, but it also prevented

transcription of several signaling proteins, such as JAK2. As a consequence, this may have resulted in lower JAK2 protein expression at 16 h of incubation, which would also affect its phosphorylation. The importance of the non-canonical signaling cascade and IRF9 on the expression of CXCL10 in intestinal cells [59] and the influence of HDAC inhibition on IRF9 induction was studied before [51]. Nevertheless, to our knowledge, this is the first study to show that butyrate is capable of having a strong inhibitory effect on IRF9 induction as well and, therefore, we provide novel molecular insights into the anti-inflammatory effect of butyrate as a potent HDAC inhibitor.

However, the effect of butyrate on other proteins related to the canonical signaling cascade and the non-canonical signaling cascade, such as phosphorylated JAK2, JAK1 and STAT1, has been studied before. Butyrate reduced JAK2 phosphorylation, JAK1 phosphorylation, STAT1 phosphorylation and its nuclear translocation and DNA binding activity in IFN-γ-activated HCT116 or Hke-3 colon carcinoma cell lines. In these cell lines, butyrate affected the canonical signaling cascade, resulting in the prevention of induced CXCL10 release [36,37]. However, phosphorylation of STAT1 and its nuclear translocation was not always observed, for example, in IFN-β-activated lung carcinoma epithelial cells [39]. Even though we did not investigate nuclear translocation of the phosphorylated STAT1 dimer, in contrast to previous studies, STAT1 phosphorylation was not suppressed by butyrate in the current study. Although we confirmed butyrate blocks the non-canonical pathway, we cannot exclude that butyrate blocks the canonical signaling cascade in our model as well. Reduced translocation of phosphorylated STAT1 could result in the accumulation of phosphorylated STAT1 in the cell cytoplasm and we hypothesize that this underlies the inclining pattern of phosphorylated STAT1 protein expression in butyrate treated activated IECs at 16 h of incubation in the current study. Butyrate most prominently inhibits HDAC activity in HT-29 cells, while acetate was least effective. This pattern is similar to the effects of these SCFA on CXCL10 release. Therefore, it was considered that HDAC inhibition plays a role in the mechanism of the effect of SCFAs on the STAT1 signaling cascade. It was observed before that HDAC, and in particular HDAC1, HDAC2 and HDAC3, are required for STAT signaling [48,50]. For example, silencing of HDAC1, HDAC2 and HDAC3 decreased IFN-γ-driven gene activation in Hke-3 cells and overexpression of these HDACs enhanced STAT1-dependent transcription activity [36]. Moreover, a general HDAC inhibitor, similar to SCFAs, also reduced CXCL10 release by IFN-γ-activated human colonic subepithelial myofibroblasts and cervical cancer cells (HeLa cells) and 2fTGH cells [38,48,53]. In the present study, several HDAC inhibitors mimic the effect of SCFAs by inhibiting the induced CXCL10 release in activated IECs. It was not only the general HDAC inhibitor TSA that reduced CXCL10 release, but more specific HDAC inhibitors which inhibit class I and IIb HDAC, did so too, while an HDAC inhibitor which inhibits class IIa HDACs was not effective. Butyrate is known not to inhibit class IIb HDAC [43], so only HDAC from class I could be inhibited by butyrate to prevent induced CXCL10 release. More specific inhibitors of HDAC within class I were studied and all of them inhibited CXCL10 release by IFN-

γ-activated IECs, while in IFN-γ+TNF-α-activated IECs, only a class I HDAC1,2,3 inhibitor affected CXCL10 release. Although HDAC inhibitor concentrations used were based on concentrations from previous studies [60-72], the concentrations used in these studies might not be sufficient to inhibit the strong synergistic effect of IFN-γ+TNF-α. However, higher concentrations could interfere with their selectivity; therefore, we adhered to this concentration range. Overall, our data suggest that butyrate inhibits a combination of HDACs, and that specifically HDAC 1, 2, 3 and 8 (class I) inhibition may have resulted in the suppression of CXCL10 release. HDAC inhibitors mimic the effect of butyrate on induced CXCL10 release; therefore, the effect of either butyrate or TSA on the transcription of genes related to the STAT1 signaling cascade was further investigated. Butyrate indeed prevented IFN-γ and IFN-γ+TNF-α-induced gene expression of *CXCL10, JAK2, SOCS1* and *IRF9* in the activated IECs. Butyrate prevented gene transcription in all studied genes at the early timepoint of 4 h, not only of CXCL10 itself but also of the genes related to its upstream signaling cascade, *JAK2, SOCS1* scand *IRF9*, which implies gene silencing via an overall blocking mechanism, such as HDAC inhibition. This was further confirmed by the HDAC inhibitor TSA, which showed a similar effect when compared to butyrate. This is in line with previous studies that show the importance of HDAC inhibition in IFN gene suppression [39,48,50]. The present study is performed in human colorectal adenocarcinoma cell line HT-29. Although these cells are generally used as an intestinal epithelial model, it would be of additional value to confirm these effects in primary intestinal cells or intestinal biopsies.

In conclusion, butyrate is the most potent inhibitor of IFN-γ as well as IFN-γ+TNF-α-induced CXCL10 release of activated IECs when compared to propionate and acetate. Butyrate already completely blocks CXCL10 release at 2 mM. Butyrate blocks CXCL10 release via HDAC inhibition and its efficacy is comparable to known HDAC inhibitors, such as TSA. The HDAC inhibition causes downregulation of the expression of genes and proteins related to the non-canonical STAT1 pathway, including IRF9 and CXCL10 itself, resulting in the prevention of induced CXCL10 release. HDAC inhibitors are getting more and more attention as potential therapeutic agents because of their anti-inflammatory properties [73]. Our findings show that butyrate is a very effective HDAC inhibitor, which demonstrates the importance of assuring sufficient butyrate levels in the intestine by means of diet or, perhaps, by supplementation or pharmaceutical therapy. The essential role for butyrate in controlling homeostasis and maintaining gut health has been shown in experimental models, while in addition, butyrate may contribute to the prevention of systemic diseases via its immunoregulatory effects [74-77]. The current manuscript further highlights the great potential of butyrate to act as a controller of intestinal homeostasis by blocking unwanted epithelial activation, which may contribute to lowering the risk of local and systemic inflammation, and which are both understood to contribute to development of non-communicable diseases.

SUPPLEMENTAL FIGURES

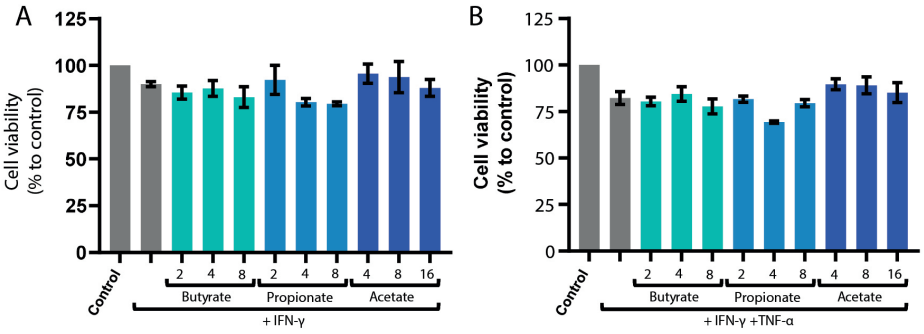


Figure S1. *Viability of intestinal epithelial cells after 24 h, activated with IFN-γ (A) or IFN-γ+TNF-α (B) and treated with 2, 4, 8 and 16 mM butyrate, propionate or acetate. Data are represented as mean ± SEM (N = 3).*

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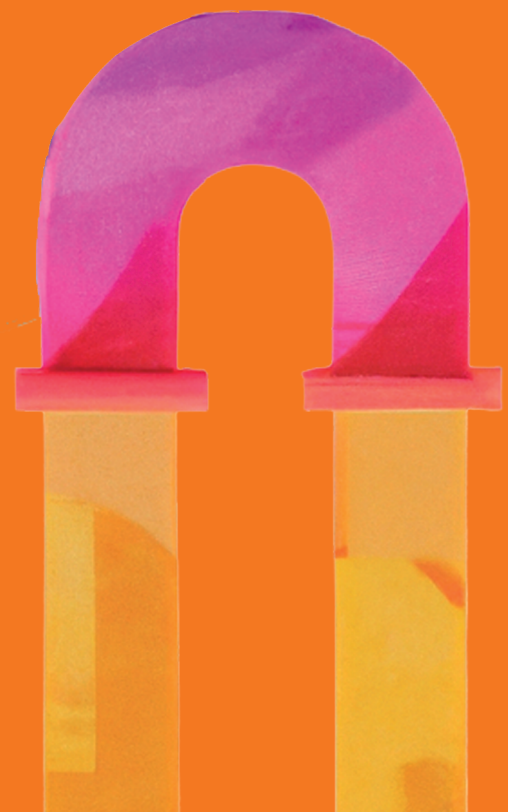
CHAPTER

2.2



Butyrate protects barrier integrity and suppresses immune activation in a Caco-2/PBMC co-culture model while HDAC inhibition mimics butyrate in restoring cytokine-induced barrier disruption

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ABSTRACT

Low-grade inflammation and barrier disruption are increasingly acknowledged for their association with non-communicable diseases (NCDs). Short chain fatty acids (SCFAs), especially butyrate, could be a potential treatment because of their combined anti-inflammatory and barrier- protective capacities, but more insight into their mechanism of action is needed. In the present study, non-activated, lipopolysaccharide-activated and αCD3/CD28-activated peripheral blood mononuclear cells (PBMCs) with and without intestinal epithelial cells (IEC) Caco-2 were used to study the effect of butyrate on barrier function, cytokine release and immune cell phenotype. A Caco-2 model was used to compare the capacities of butyrate, propionate and acetate and study their mechanism of action, while investigating the contribution of lipoxygenase (LOX), cyclooxygenase (COX) and histone deacetylase (HDAC) inhibition. Butyrate protected against inflammatory-induced barrier disruption while modulating inflammatory cytokine release by activated PBMCs (interleukin-1 beta↑, tumor necrosis factor alpha↓, interleukin-17a↓, interferon gamma↓, interleukin-10↓) and immune cell phenotype (regulatory T-cells↓, T helper 17 cells↓, T helper 1 cells↓) in the PBMC/Caco-2 co-culture model. Similar suppression of immune activation was shown in absence of IEC. Butyrate, propionate and acetate reduced inflammatory cytokine-induced IEC activation and, in particular, butyrate was capable of fully protecting against cytokine-induced epithelial permeability for a prolonged period. Different HDAC inhibitors could mimic this barrier-protective effect, showing HDAC might be involved in the mechanism of action of butyrate, whereas LOX and COX did not show involvement. These results show the importance of sufficient butyrate levels to maintain intestinal homeostasis.

INTRODUCTION

Non-communicable diseases (NCDs) are a leading cause of mortality, responsible for approximately 74% of all deaths worldwide [1]. NCDs are chronic metabolic or immune disorders, including diabetes, inflammatory diseases, cardiovascular diseases and chronic respiratory diseases, that are not caused by infectious agents. They are characterized by low-grade inflammation, systemically and locally in the gut, and it is hypothesized that these diseases are associated with dysbiosis of the microbiome and a disrupted intestinal barrier [2,3,4,5,6,7,8,9]. Dysbiosis of the microbiome leads to increased influx of macromolecules through the intestinal barrier, such as lipopolysaccharides (LPS), leading to local inflammation in the gut and low-grade inflammation systemically [10,11]. Furthermore, inflammation may establish a positive feedback loop by maintaining the disrupted intestinal barrier, leading to an ongoing influx of macromolecules and consequently resulting in chronic inflammation and the development of NCDs [12,13,14,15,16,17].

The microbiome produces biologically active molecules such as short chain fatty acids (SCFAs) by fermentation of fibers, which may be hampered in NCDs due to dysbiosis [18,19,20,21,22]. SCFAs, especially butyrate, are known for their beneficial effects on systemic inflammation and gut health, including anti-inflammatory and barrier-improving effects. Therefore, SCFAs could be a possible treatment option for patients with NCDs by restoring intestinal homeostasis and intestinal barrier function [23,24,25]. SCFAs have been extensively studied in various *in vitro* models with intestinal epithelial cells (IEC) or in immune assays using peripheral blood mononuclear cells (PBMCs) revealing the diverse effects of SCFAs. However, these effects have not been studied in Caco-2/PBMC co-culture models allowing the cross talk between IEC and immune cells mimicking their proximity at the mucosal surface while studying immune-mediated barrier disruption.

In IEC models, in particular, butyrate altered the assembly and expression of tight junction proteins and was able to enhance barrier function [26,27,28,29]. Additionally, butyrate reduced the activation status of IEC, thus contributing to homeostasis, by lowering the expression of pro-inflammatory cytokines and chemokines, including Interleukin (IL)-8 and C-X-C motif chemokine ligand 10 (CXCL10) [30,31,32,33]. Butyrate is known as a histone deacetylase (HDAC) inhibitor, which was found to contribute to its anti-inflammatory and barrier-protective effect [34]. Butyrate-induced activation of lipoxygenase was also found to contribute to barrier protection [26].

In PBMC models, butyrate inhibited the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), IL-1β and IL-6, while increasing the production of anti-inflammatory cytokines such as IL-10 [34,35,36,37,38,39,40]. Moreover, butyrate could modulate the differentiation of T-cells

within the PBMC models into different subtypes, such as the promotion of regulatory T-cells [35].

The current study aimed to investigate the effect of butyrate using a Caco-2/activated PBMC co-culture model which makes it possible to study the cross talk between IEC and effector immune responses. This type of research can help to provide a more complete understanding of the complex interactions between these different cell types and how they are affected by butyrate. In this transwell co-culture model, IEC were grown on inserts and PBMCs were added to the basolateral compartment and LPS or αCD3/CD28 were used to activate the PBMCs in the presence or absence of butyrate. Next, the protective effect of butyrate, or the other SCFAs, propionate and acetate, in cytokine-mediated barrier disruption, and the potential involvement of LOX, COX or HDAC inhibition in their mechanism of action was investigated.

MATERIALS AND METHODS

Cell culture

Intestinal epithelial cell culture

Human colorectal adenocarcinoma Caco-2 cells (ATTC, HTB-38, Manassas, VA, USA; passage 36–45) were used as intestinal epithelial cells (IEC) and were maintained in 75 cm^2 culture flasks (Greiner Bio-One, Kremsmünster, Austria) using culture medium. Culture medium consisted of high glucose Dulbecco's Modified Eagle Medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively) (Sigma-Aldrich), 1% non-essential amino acids (Gibco) and 2 mM L-Glutamine (Gibco). Medium was refreshed every 2–3 days and cells were passaged when a confluency of 70–90% was reached. Cells were kept at 37 °C and 5% CO_2 in an incubator.

PBMC isolation

Healthy donor buffy coats (Blood Bank, Amsterdam, The Netherlands) were used to isolate human PBMCs by density gradient centrifugation using pre-filled Leucosep™ tubes (Greiner Bio-One). Tubes were centrifuged for 13 min at 1000× g after which the PBMC fraction was isolated and washed with phosphate buffered saline (PBS) (Lonza, Basel, Switzerland) supplemented with 2% heat-inactivated FCS and the remaining erythrocytes were lysed using a lysis buffer (4.14 g NH_4Cl, 0.5 g $KHCO_3$, 18.6 mg Na_2EDTA in 500 mL demineralized water, sterile filtered, pH = 7.4) for 5 min on ice. Finally, the PBMCs were resuspended in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively). All blood donors, who can volunteer without any selection on age, ethnicity or sex, gave informed consent for the use of their blood for scientific research purposes.

In addition, strict conditions for use were set, for which permission was obtained by our research group.

In vitro models

Co-Culture model and PBMC stimulation

The Caco-2 cells were seeded in a 12-transwell system with a pore size of 0.4 μm (Costar, Corning Incorporated, Corning, NY, USA) and grown for 3 weeks until they were differentiated to small intestinal epithelial cells before the start of the experiment, as described elsewhere [41]. For the co-culture experiment, the culture medium was changed to RPMI 1640 supplemented with 2.5% heat-inactivated FCS and 1% penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively). In the basolateral compartment, PBMCs at a concentration of 2 × 106 cells/mL were seeded non-activated or activated with 1 µg/mL LPS or a combination of αCD3 and αCD28 (clone CLB-T3/2 and clone CLB-CD28, both 1:10,000, Sanquin, Amsterdam, The Netherlands). In the apical compartment, sodium butyrate (Sigma-Aldrich) was added in a concentration of 0.5, 2 or 8 mM. At t = 24 h, complete apical medium was replaced with fresh culture medium with or without butyrate and 0.5 mL of the basolateral medium was replaced with fresh culture medium without any additions. At t = 48 h, the experiment was ended and the basolateral medium was stored at –20 °C until further analysis.

In addition, PBMCs were incubated with butyrate in the absence of Caco-2 cells at a concentration of 0.125, 0.5, 2 mM butyrate in a 12-well plate. At t = 24 h, 1 mL medium was replaced with 0.5 mL fresh culture medium and 0.5 mL fresh culture medium with or without 0.5, 2 or 8 mM butyrate, to simulate the co-culture experiment. At t = 48 h, the experiment was ended and medium was stored at –20 °C up until further analysis. During the experiment, the cells were kept at 37 °C and 5% CO_2 in an incubator. See Figure 1A,B for an overview of the co-culture and PBMC model.

IEC model

The Caco-2 cells were seeded in a 12-transwell system with a pore size of 0.4 μm and grown for 3 weeks until they were differentiated to small intestinal epithelial cells before the start of the experiment, as described elsewhere [41]. At t = 0 h, the SCFAs, sodium butyrate (Sigma-Aldrich), sodium propionate (Sigma-Aldrich) and sodium acetate (Emsure, MI, USA) were added to the apical side of the cells and a mixture of pro-inflammatory cytokines, 10 ng/mL IL-1β (Preprotech, London, UK), 100 U/mL IFN-γ (Preprotech) and 10 ng/mL TNF-α (Sigma-Aldrich) was added to the basolateral side of the cells. The SCFAs were dissolved and diluted to the working concentration in culture medium. Butyrate was used at a concentration of 0.5, 1, 2, 4 and 8 mM, propionate was used at a concentration of 2 and 4 mM and acetate was used at a concentration of 4 and 8 mM. The pro-inflammatory cytokines were dissolved in PBS and diluted to the working concentration in culture medium. At t = 24 h, complete apical medium was replaced with

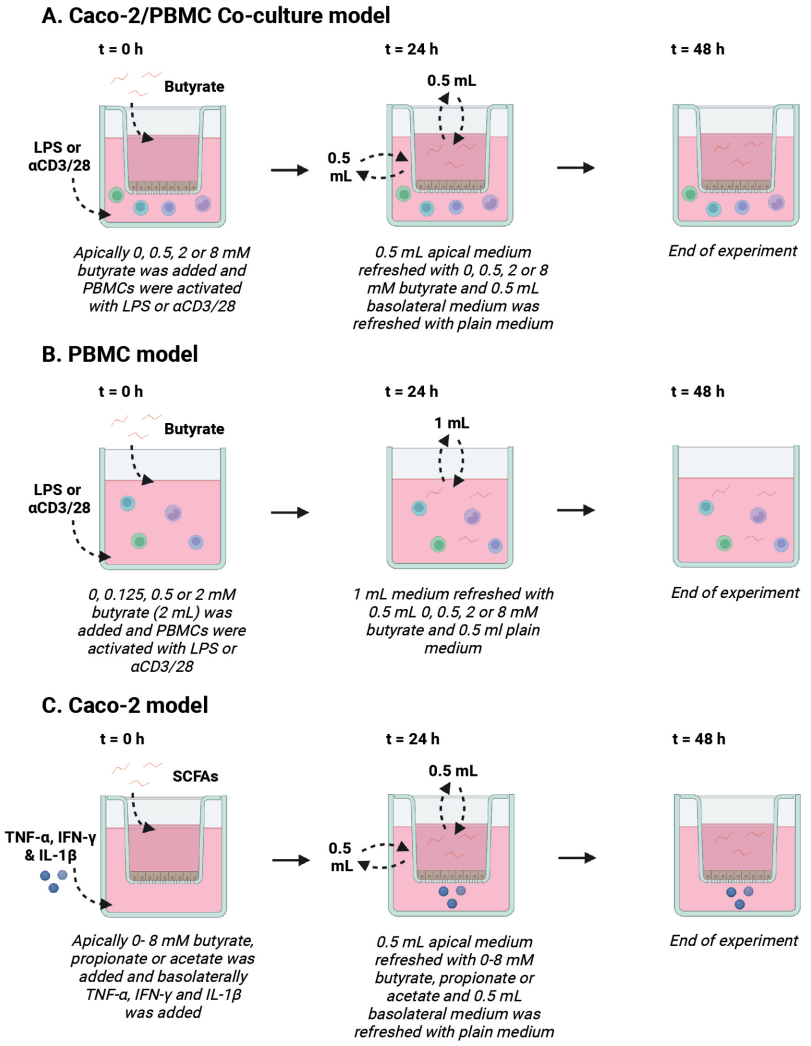

Figure 1. *Schematic overview of Caco-2/peripheral blood mononuclear cells (PBMCs) co-culture model (A), PBMC model (B) and Caco-2 model (C). (A) In the Caco-2/PBMC co-culture model, 0–8 mM butyrate was added to the apical side of the Caco-2 cells and PBMCs were activated with either lipopolysaccharide (LPS) or αCD3/CD28. After 24 h, apical medium was refreshed with fresh medium containing 0–8 mM butyrate and 0.5 mL of the basolateral medium was refreshed with medium without butyrate. At 48 h, the experiment was ended. (B) In the PBMC model, 0–2 mM butyrate was added to the PBMCs, which were activated with either LPS or αCD3/CD28. After 24 h, 1 mL of medium was refreshed with 0.5 mL medium containing 0–8 mM butyrate and 0.5 mL medium without butyrate, to mimic the co-culture model. At 48 h, the experiment was ended. (C) In the Caco-2 model, 0–8 mM butyrate, propionate or acetate was added to the apical side of the Caco-2 cells and TNF-α, IFN-γ and IL-1β was added to the basolateral compartment to activate the cells. After 24 h, apical medium was refreshed with fresh medium containing 0–8 mM butyrate, propionate or acetate and 0.5 mL of the basolateral medium was refreshed with medium without short chain fatty acids (SCFAs). At 48 h, the experiment was ended. Created with BioRender.com.*

fresh culture medium with or without SCFAs and 0.5 mL of the basolateral medium was replaced with fresh culture medium without any additions. At t = 48 h, the experiment was ended and cells were stored in RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) at –20 °C until further analysis, even as basolateral medium. During the experiment, the cells were kept at 37 °C and 5% CO2 in an incubator. See Figure 1C for an overview of the IEC model.

To investigate the mechanism of action of the short chain fatty acids (SCFAs), LOX inhibitor nordihydroguaiaretic acid (10 μM) or COX inhibitor indometacine (1 μM) (Sigma-Aldrich) were added to the experiment described above. In addition, HDAC inhibitors trichostatin A (1 μM), tacedinaline (2.5, 25, 250 μM), tinostamustine (0.1, 1, 10 μM) and TMP269 (0.1, 1, 10 μM) (MedChemExpress, Sollentuna, Sweden) were added as a treatment instead of the SCFAs to see whether these inhibitors could mimic the effect of the SCFAs.

Barrier assessment

Barrier integrity of the Caco-2 monolayer was assessed with two different methods, transepithelial electrical resistance (TEER) and a 4 kDa fluorescein isothiocyanate-dextran (FD4) permeability assay.

TEER

The TEER of the monolayer was measured using a Millicell ERS-2 Volt-ohm meter (Merck Millipore, Burlington, MA, USA) at t = 0 h, t = 24 h and t = 48 h. Data were shown as a percentage compared to the TEER of the monolayer at t = 0 h.

4 kDa FITC-Dextran permeability assay

At t = 48 h, a FD4 (Sigma-Aldrich) permeability assay was performed to assess paracellular permeability of the cell monolayer. First, the phenol red-containing medium was discarded, the cells were washed with PBS and the same culture medium without phenol red (Gibco) was added. Before the start of the assay, the cells were left in the incubator for 1 h to become stable again. A concentration of 1.6 mg/mL FD4 was added to the apical side of the IEC and 100 uL samples of the basolateral medium were taken 1, 4 or 24 h after the addition of the FD4 and collected in a white 96-well plate (Corning). The taken samples were measured at Ex/Em = 492/518 nm with Fluorskan Ascent FL (Thermo Labsystems, Waltham, MA, USA).

Viability assay

After the FD4 permeability assay, the cells were washed again with PBS and the viability of the cells was assessed using the cell proliferation Reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Data were shown as a percentage compared to the control cells.

Enzyme-Linked immunosorbent assay (ELISA)

Basolateral supernatant was used to measure the IL-8, IL-17a, IFN-γ, IL-10, TNF-α, IL-1β and IL-6 concentrations present in the medium as a measure for epithelial or immune cell activation. Cytokine and chemokine concentrations were measured using commercially available ELISA kits (Thermo Fischer scientific, Waltham, MA, USA). ELISA was performed according to the manufacturer's instructions. In short, high-binding 96-well plates (Corning) were coated with capture antibody and incubated overnight at 4 °C; all of the following steps were performed at room temperature. Non-specific binding was blocked for 1 h. After washing, the samples or the standard were added for 2 h. Then, plates were washed and incubated with streptavidin–horseradish peroxidase for 1 h. Subsequently, the plates were washed and incubated in the dark with substrate solution. The reaction was stopped with 1 M H_2SO_4 and absorbance was measured at 450 nm in a microplate reader (iMark, Bio-Rad Laboratories, Hercules, CA, USA or GloMax Discover, Promega Corporation, Madison, WI, USA).

Flow cytometry

At the end of the cell experiments, PBMCs were transferred to a 96-well plate with a U bottom (Corning) for flow cytometry. First, cells were blocked with PBS supplemented with 5% heat-inactivated FCS to prevent unspecific binding, dyed with Fixable Viability Dye 780-APC Cyanine 7 (1:2000; eBioscience, Thermo Fisher Scientific) for 5 min in the dark and washed with FACS buffer (PBS + 2.5% heat-inactivated FCS). Second, cells were incubated for 30 min on ice with titrated volumes of the following antibodies: CD4-PerCP Cy 5.5, CD69-PE, CD25-FITC, CD127-PE Cy 7, CRTH2-APC (all eBioscience), CXCR3-FITC (BD Bioscience, San Jose, CA, USA). After incubation, cells were washed again using FACS buffer and fixed to store them overnight at 4 °C. Cells that were only stained with extracellular antibodies were fixed with IC fixation buffer 1:4 in PBS and cells that were stained extracellularly and intracellularly were fixated and permeabilized with Fix/Perm buffer (Thermo Fisher Scientific). The next day panels with only extracellular staining were washed, resuspended in FACS buffer and measured using a BD FACS Canto II (Becton Dickinson, Franklin Lakes, NJ, USA). Panels with extracellular and intracellular staining were washed and incubated for 30 min on ice with titrated volumes of the following antibodies: FoxP3-eFluor660, RORγ-PE, Tbet-eFluor660 and GATA3-PE (all eBioscience). After incubation, cells were washed, resuspended in FACS buffer and measured using a BD FACS Canto II. Acquired data were analyzed using FlowLogic software (Inivai Technologies, Mentone, Australia).

Western blot

At t = 48 h, the cells were collected in RIPA lyses buffer supplemented with protease inhibitors (Roche). First, protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific) and protein concentrations were equalized across all samples. Bromophenol blue and 2-mercaptoethanol were added to the samples to denature the proteins. Protein samples were then added to a Criterion™ 4–20% Tris-HCl gel

(Bio-Rad, Veenendaal, The Netherlands) for separation with electrophoresis. Thereafter, the proteins were transferred from the gel to a polyvinylidene difluoride membrane (Transblot Turbo, Bio-Rad). The membrane was blocked using 5% milk protein (Nutricia protifar, Danone, Hoofddorp, The Netherlands) in PBS containing 0.05% Tween-20. After blocking, the membrane was incubated with primary antibodies overnight at 4 °C. As primary antibodies, zonula occludens-1 (ZO-1) (1:1000, Thermo Fisher Scientific), occludin (1:1000, Thermo Fisher Scientific) (1:1000) and β-actin (1:1000, cell signaling) were used. After incubation, the membranes were washed and incubated with horseradish peroxidase conjugated secondary antibodies (Dako, Santa Clara, CA, USA) for 2 h. Membranes were again washed in PBS containing 0.05% Tween-20 and the proteins on the membranes were visualized using ECL reagent (Bio-Rad). Data were analyzed using Image J version 1.52a.

Statistical analysis

Results are presented as means (± SEM) as the experiments were performed as at least 4 individual experiments. Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA). The statistical significance of normally distributed data was assessed with the repeated measures one-way ANOVA analysis, followed by Bonferroni's post hoc test with selected pairs. Statistical significance of not normally distributed data was assessed with the Friedman test with selected pairs, followed by Dunn's multiple comparisons test. Data presented in the figures are, in some cases, divided into different subsets for statistical analysis. The different subsets are divided by dotted lines. Within these subsets, selected pairs were used for statistical analysis, in which the activated or non-activated condition without butyrate, propionate or acetate was compared with the different concentrations of butyrate, propionate or acetate within the same subset. A paired Student's t-test (for normally distributed data) or Wilcoxon matched pairs signed rank test (for not normally distributed data) was used to compare positive and negative controls, namely, non-activated cells with LPS, αCD3/CD28 or cytokine mix-activated cells. Results were considered statistically significant when $p < 0.05$. Significant differences are shown in the figures as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

RESULTS

Butyrate improves intestinal barrier function in a Caco-2/PBMC co-culture model

To investigate the potential of butyrate to improve the intestinal barrier function, a co-culture model of intestinal epithelial cells (IEC) combined with activated immune cells simulating an effector immune response was used. The PBMCs were activated with either LPS or a combination of αCD3 and αCD28 to provoke a cytokine response contributing to barrier disruption. Both LPS and αCD3/CD28 activation significantly reduced barrier function, as TEER was reduced by approximately 20% (Figure 2A,B). Butyrate protected

against inflammatory-induced barrier disruption (TEER) in the activated models. In the LPS-activated model, butyrate protected at a concentration of 2 and 8 mM and in the αCD3/CD28 activated model at a concentration of 8 mM at 24 h and 48 h (Figure 2A,B). In the non-activated model, 2 and 8 mM butyrate slightly reduced TEER values at t = 48 h (Figure 2B). The functional FD4 paracellular permeability in the activated models was not significantly increased as compared to controls (IEC/non-activated PBMCs), but butyrate (8 mM) lowered the basal permeability (Figure 2C). Furthermore, 8 mM butyrate also reduced permeability in the LPS-activated model and the αCD3/CD28-activated model showed a similar pattern (Figure 2C).

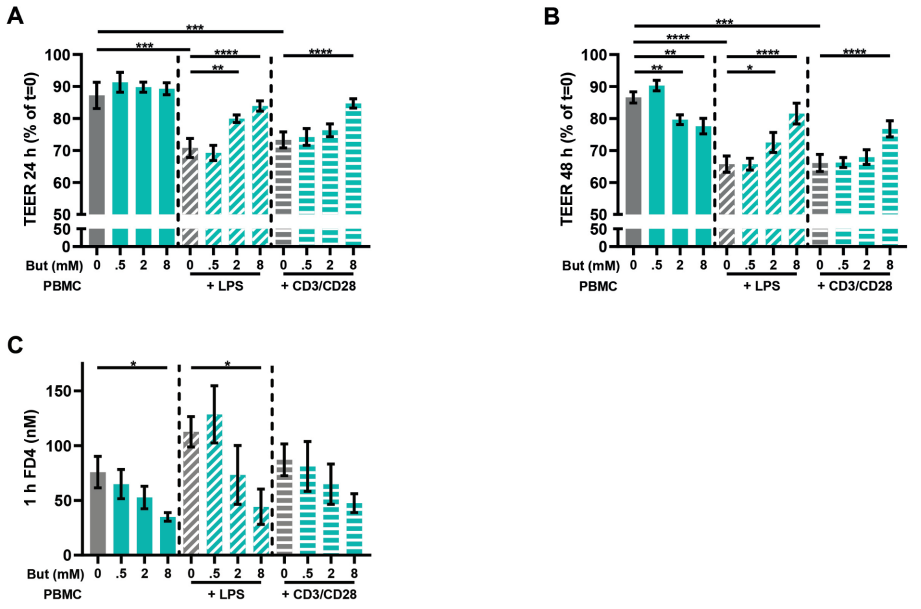

Figure 2. *Butyrate protects against barrier disruption in a Caco-2/PBMC co-culture model with LPS- and αCD3/CD28-activated PBMCs. Cells were treated with 0–8 mM butyrate. (A) Transepithelial electrical resistance (TEER) values at t = 24 h expressed as percentage of the initial TEER at t = 0. (B) TEER values at t = 48 h expressed as percentage of the initial TEER at t = 0. (C) 4 kDa fluorescein isothiocyanate dextran (FD4) permeability assay performed at the end of the experiment. Results show the FD4 concentration in the basolateral compartment after 1 h of incubation. Data are presented as mean ± SEM and the datasets used for statistical analysis are divided by dotted lines (N = 6 individual experiments). Significant differences are shown as * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared to control. But: butyrate; PBMC: peripheral blood mononuclear cells.*

Butyrate modulates pro-inflammatory and regulatory cytokines release

In order to investigate the effect of butyrate on immune activation, various cytokines were measured in the basolateral supernatant of the co-culture model and in the supernatant of the PBMC model. Similar patterns were observed in the results of both models. LPS induced the release of IL-1β, IL-6, TNF-α and IL-10, but not of IL-17A and IFN-γ in both models, although, in some cases, not significantly in one of both models. The induced release of TNF-α and IL-10 was prevented by 8 mM butyrate in the co-culture model and 2 mM butyrate in the PBMC model (Figure 3). Butyrate did not influence the release of IL-6 in both models, while low concentrations of butyrate (0.125 and 0.5 mM) reduced the release of IL-1β in the PBMC model, but not in the co-culture model. However, 8 mM butyrate further enhanced the release of IL-1β in the co-culture model (Figure 3A). αCD3/CD28 induced the release of IL-1β, TNF-α, IFN-γ, IL-17A and IL-10, but not of IL-6 and IL-1β in both models, although, in some cases, not significantly in one of both models. Moreover, in this model, the induced release of TNF-α and IL-10 was prevented by 8 mM butyrate in the co-culture model and 2 mM butyrate in the PBMC model, similar to the LPS-activated models. Butyrate did not influence the release of Il-1β in both models, while 2 mM butyrate reduced the release of IFN-γ and IL-17A in the PBMC model, but had no statistically significant effect in the co-culture model (Figure 3).

Butyrate modulates T-cell phenotypes

PBMCs were analyzed by means of flow cytometry to identify whether butyrate modulated the phenotype of the PBMCs. The LPS and αCD3/CD28-activated Caco-2/PBMC or PBMC model showed an increase in CD25+ activated T-cells and CD25+FoxP3+ regulatory T-cells, while only the αCD3/CD28 activated models showed an increase in RORγ+ T helper (Th)17-cells. Butyrate largely prevented these changes at concentrations of 2 and/or 8 mM. The percentages of CD25+ activated T-cells and CD25+FoxP3+ regulatory T-cells were even reduced by 2 mM butyrate in the control conditions using non-activated PBMCs (Figure 4).

Th1-cells were analyzed in two panels, using CXCR3 or Tbet as Th1-type marker. In the panel with CXCR3 as Th-1 type marker, the αCD3/CD28-activated models showed an increase in both CD69+ activated CXCR3+ and CXCR3– Th-cells. Butyrate (8 mM) decreased the percentage of CD69+CXCR3+ Th1-cells, while increasing the percentage of CD69+CXCR3– Th-cells (Figure 5). In addition, butyrate showed a slight increase in CD69+CXCR3– cells in the LPS-activated PBMC model and a slight decrease in the non-activated models (Figure 5).

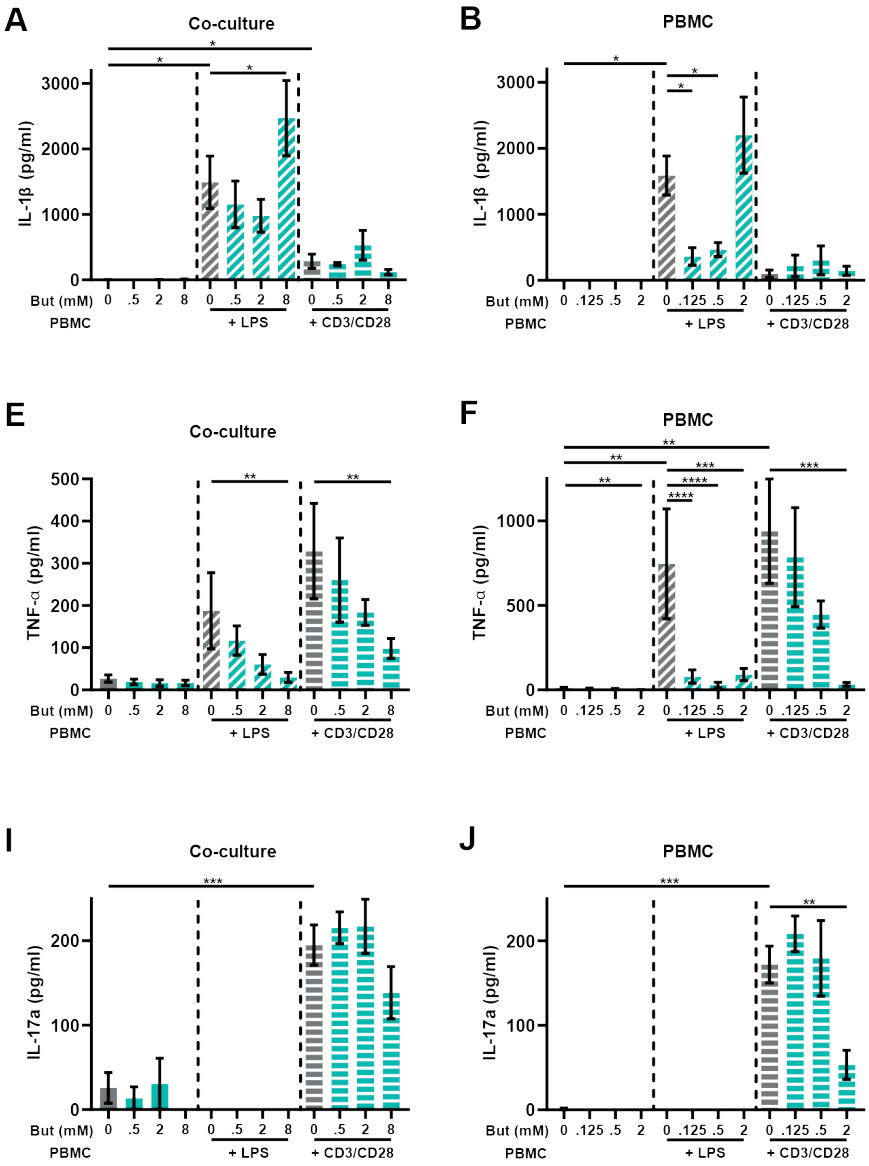

Figure 3. *Butyrate modulates cytokine release in a Caco-2/PBMC co-culture model (A,C,E,G,I,K) and PBMC model (B,D,F,H,J,L). PBMCs were activated with LPS or αCD3/CD28 while cells were treated with 0–8 mM butyrate. IL-1β (A,B), IL-6 (C,D), TNF-α (E,F), IFN-γ (G,H), IL-17a (I,J) and IL-10 (K,L) were measured in the supernatant. Data are presented as mean ± SEM and the datasets used for statistical analysis are divided by dotted lines (N = 5–6 individual experiments). Significant differences are shown as * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared to control. But: butyrate; PBMC: peripheral blood mononuclear cells.*

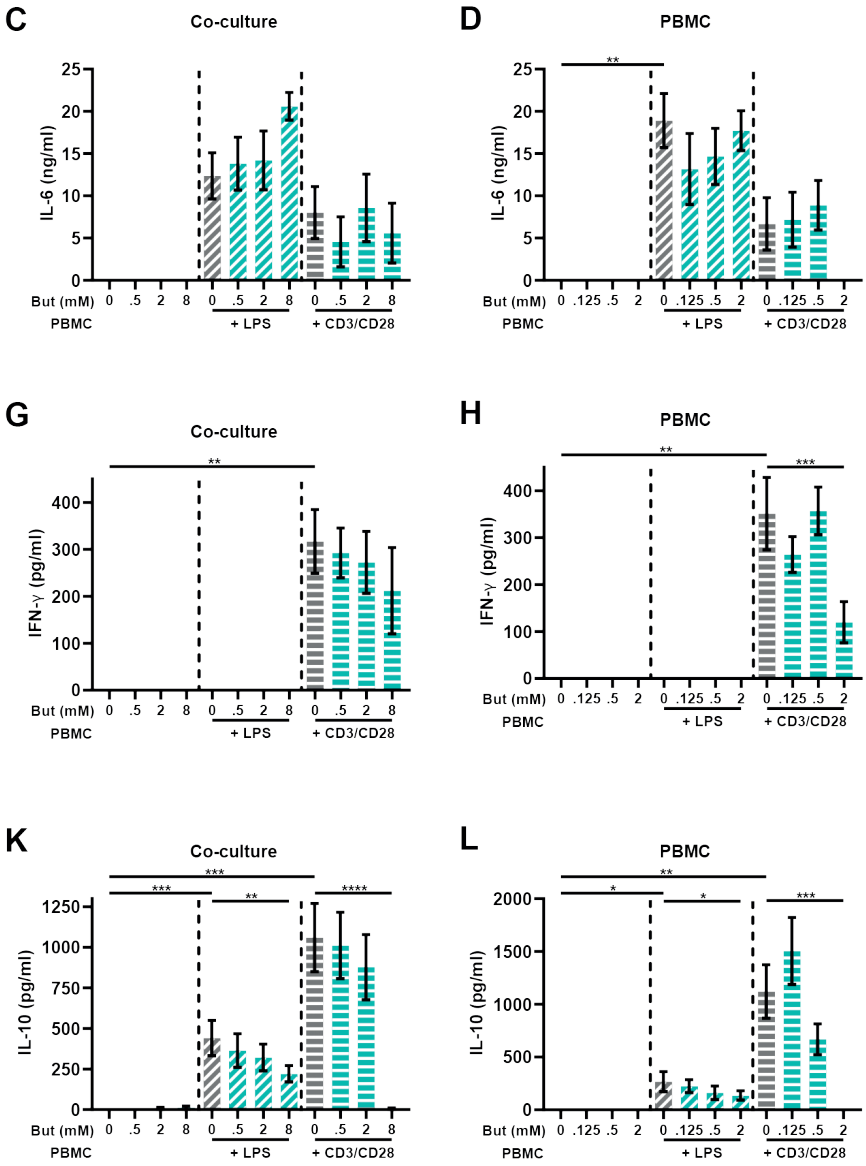

Figure 3. *Continued*.

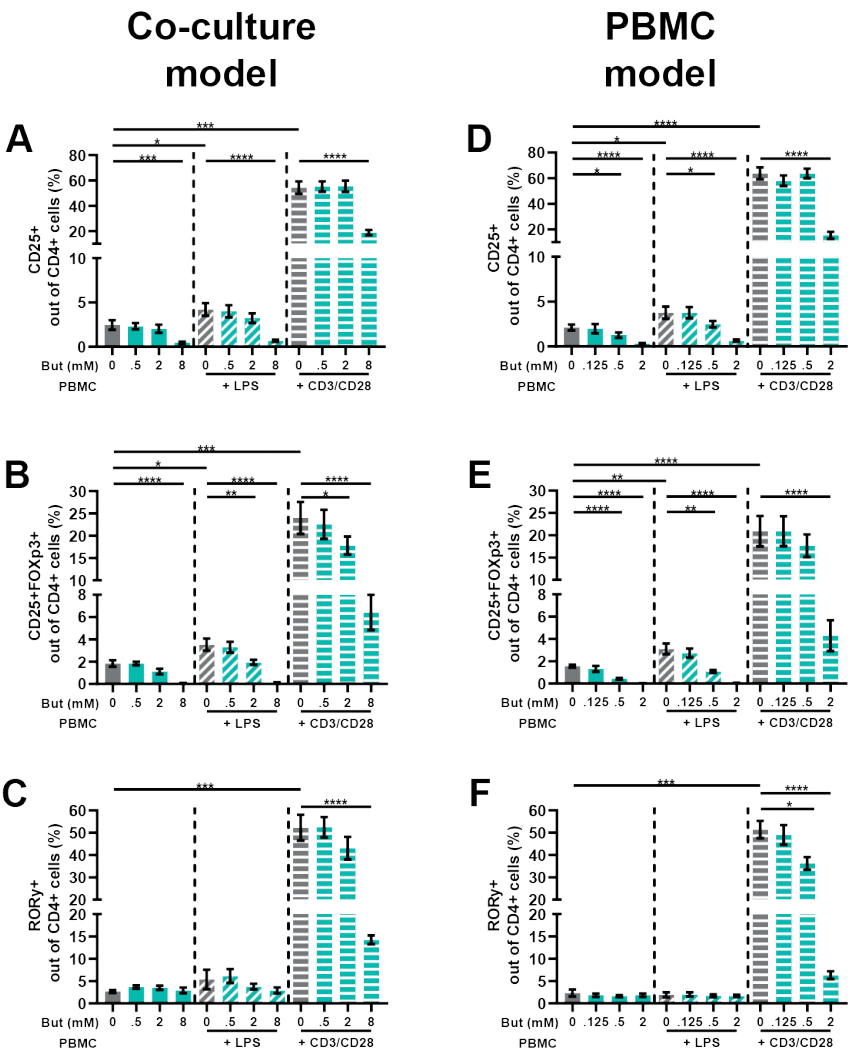

Figure 4. *Butyrate modulates CD25+, CD25+FoxP3+ and RORγ+ cells in a Caco-2/PBMC co-culture model (A–C) and PBMC model (D–F). PBMCs were activated with LPS or αCD3/CD28 and cells were treated with 0–8 mM butyrate. (G) Used gating strategy of a representative sample with corresponding fluorochrome minus one (FMO) sample, single cells gating > viable cells gating > CD4+ cells gating followed by CD25+ or CD25+FoxP3+ or RORγ+ gating. (H) CD25+ gating of a representative sample including FMO sample of CD25. (I) CD25+FoxP3+ gating of a representative sample including FMO sample of CD25 and FoxP3. (J) RORγ+ gating of a representative sample including FMO sample of RORγ. Data are presented as mean ± SEM and the datasets used for statistical analysis are divided by dotted lines (N = 6 individual experiments). Significant differences are shown as * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared to control. But: butyrate; PBMC: peripheral blood mononuclear cells.*

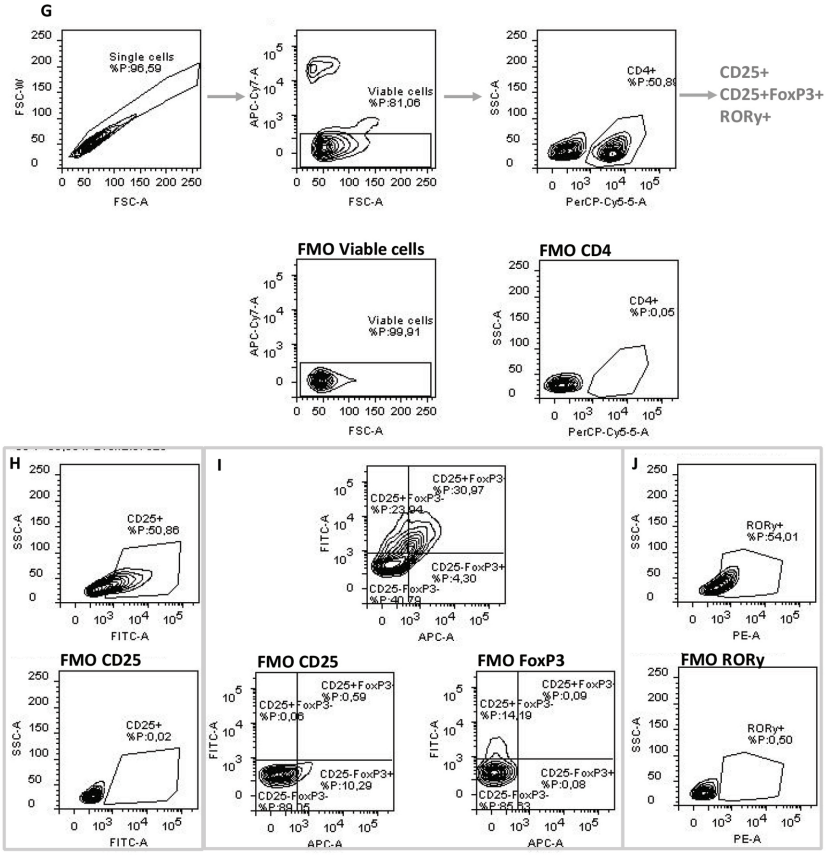

Figure 4. *Continued*.

In the panel with Tbet as Th1-type marker, the αCD3/CD28-activated models showed an increase in both CD69+ activated Tbet+ and Tbet– Th-cells as well. Contrary to the CXCR3+ panel, butyrate did not decrease the percentage of CD69+Tbet+ Th1-cells. Although, also here, the percentage of CD69+Tbet– Th-cells increased in the 8 mM butyrate conditions. In addition, butyrate showed a slight increase in CD69+Tbet+ and CD69+Tbet– cells in the LPS-activated models (Figure S1).

SCFAs reduce epithelial activation and improve barrier in a caco-2 monolayer

The previous results using the co-culture model of IEC with activated PBMCs raised the question of whether the effect of butyrate on the barrier of the Caco-2 cells was an indirect effect via a decreased immune activation, less inflammation and thus reduced barrier disruption, or whether butyrate could protect against the barrier disruptive effect of the inflammatory mediators TNF-α, IFN-γ and IL-1β, which are known for their barrier disrupting capacities [42,43,44]. Caco-2 cells were incubated with different concentrations

of butyrate, propionate and acetate. In addition to barrier function, epithelial activation was measured by means of IL-8 release in the basolateral compartment. Cells activated with the cytokine mix released a higher amount of IL-8 compared to controls and all concentrations (0.5–8 mM) of butyrate reduced IL-8 release; 2 and 4 mM propionate were equally able in reducing IL-8 release followed by 8 mM acetate; 4 mM acetate did not affect IL-8 release (Figure 6A,B).

Caco-2 cells activated with the cytokine mix resulted in decreased TEER at 24 and 48 h and an increased FD4 permeability. Butyrate only improved TEER in the activated cells at 24 and not at 48 h, but showed a dose-dependent reduction of FD4 permeability in the non-activated and activated cells. Propionate did not improve TEER at both timepoints, but reduced FD4 permeability in activated cells as well. Acetate increased TEER of activated and non-activated cells and reduced FD4 permeability of activated cells. Butyrate reduced FD4 permeability with more than 60%, while acetate only improved FD4 permeability by approximately 30%. FD4 permeability is a direct measure of how leaky the intestinal barrier is for antigen leakage from the gut lumen, while TEER is an indirect measure since it measures ion flux across tight junctions.

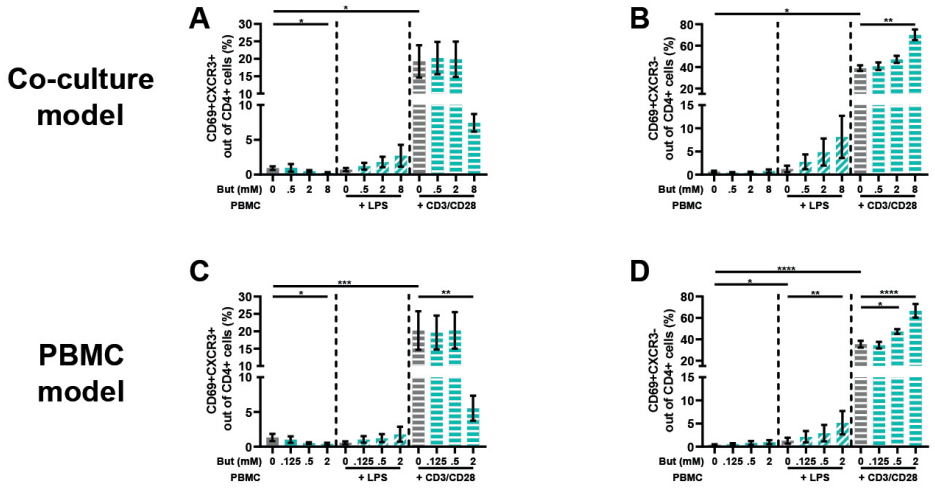

Figure 5. *See figure legend on page 75.*

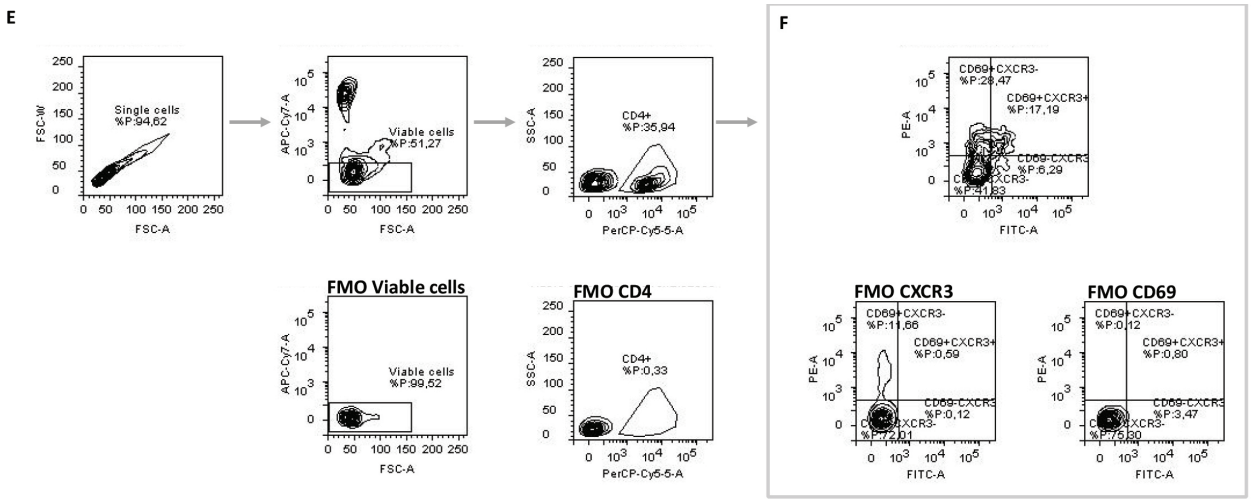

Figure 5. *Butyrate modulates CD69+CXCR3+ and CD69+CXCR3− cells in a Caco-2/PBMC co-culture model (A,B) and PBMC model (C,D). PBMCs were activated with LPS or αCD3/CD28 and cells were treated with 0–8 mM butyrate. (E) Used gating strategy of a representative sample with corresponding fluorochrome minus one (FMO) sample, single cells gating > viable cells gating > CD4+ cells gating followed by (F) CD69+CXCR3+ and CD69+CXCR3− gating. Data are presented as mean ± SEM and the datasets used for statistical analysis are divided by dotted lines (N = 6 individual experiments). Significant differences are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to control. But: butyrate; PBMC: peripheral blood mononuclear cells.*

FD4 permeability was also sampled at the earlier timepoint of 4 h (Figure S4A,B); these results show similar trends to the results of 24 h (Figure 7E,F). The effect of the SCFAs on the barrier cannot be explained by an increased expression in tight junction proteins occludin and ZO-1 (Figure S2). The WST assay showed that the treatments did not affect the viability of the cells (Figure S3A,B).

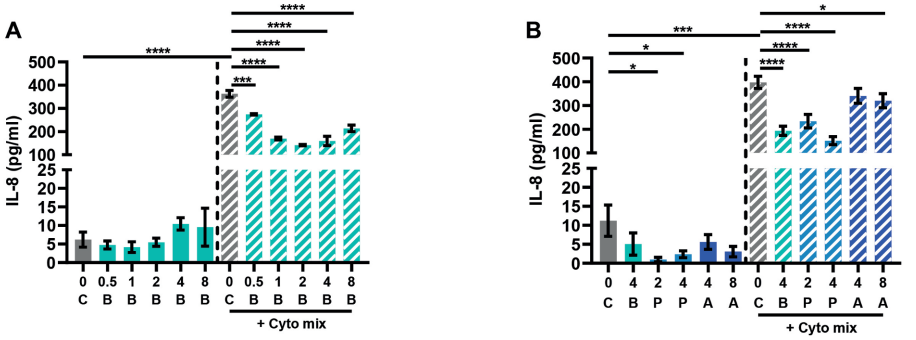

Figure 6. *Butyrate (A,B), propionate and acetate (B) reduce IL-8 release of Caco-2 cells upon stimulation with pro-inflammatory cytokines. Data are represented as mean ± SEM of N = 4 individual experiments. * p < 0.05, *** p < 0.001, **** p < 0.0001 as compared to the control cells or cells exposed to the cyto mix (10 ng/mL TNF-α, 100 U/mL IFN-γ and 10 ng/mL IL-1β). The concentration of SCFAs is expressed in mM. A: acetate; B: butyrate; C: control; P: propionate.*

HDAC inhibitors mimic the protective effects of butyrate on IL-8 release and FD4 permeability

LOX, COX or HDAC inhibition might be involved in the mechanism of action of butyrate [26]. Therefore, we investigated whether this also applied in our model by adding a LOX inhibitor or COX inhibitor to butyrate-exposed cells or an HDAC inhibitor to mimic effects of butyrate which is known for its HDAC inhibitory capacities. Again, the cytokine mixture induced IL-8 release and enhanced FD4 permeability in the Caco-2 monolayer which were both largely prevented by 4 mM butyrate; 4 mM propionate lowered cytokine- induced IL-8 release similar to butyrate and 8 mM acetate protected against cytokine-induced increase in epithelial permeability. The LOX inhibitor nor COX inhibitor could counteract the protective effect of the SCFAs. However, HDAC inhibitor TSA mimicked the effect of butyrate by lowering the cytokine-induced IL-8 release and FD4 permeability (Figure 8A–F). Tinostamustine, a more selective HDAC inhibitor, which inhibits HDACs from class I and IIb showed a similar pattern. HDAC inhibitor TMP269, which inhibits HDACs from class IIa, was not effective. Tacedinaline, which inhibits HDACs from class I, decreased the cytokine-induced rise in FD4 permeability, but in contrast to butyrate, it increased IL-8 release at this concentration (Figure 8E,F). The different treatments did not affect viability of the cells (Figure S3C–E) and TEER was also not affected by the different treatments (Figure S4C,D).

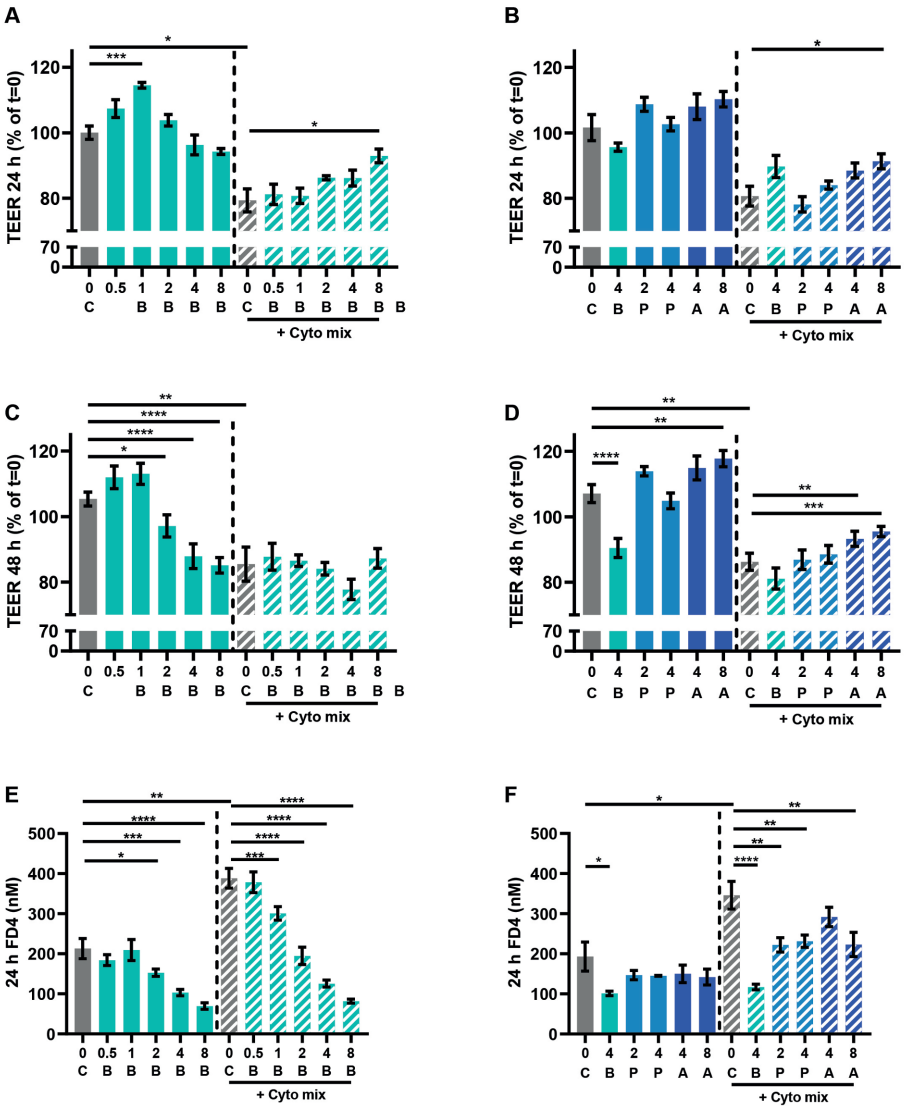

Figure 7. *The effect of SCFAs on TEER measurements after 24 h (A,B) and 48 h (C,D) and FD4 permeability (E,F). Data are represented as mean ± SEM of N = 4 individual experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 as compared to the control cells or cells exposed to the cyto mix (10 ng/mL TNF-α, 100 U/mL IFN-γ and 10 ng/mL IL-1β). The concentration of SCFAs is expressed in mM. A: acetate, B: butyrate, C: control, P: propionate.*

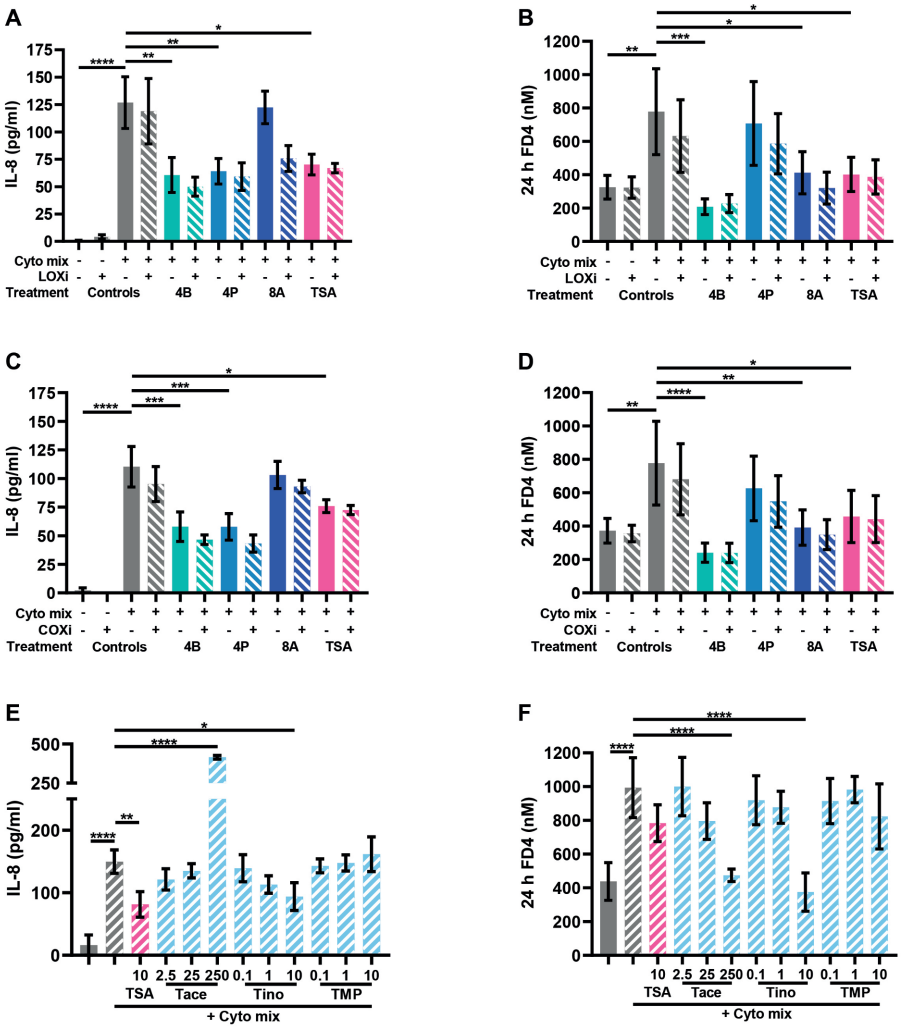

Figure 8. *The effect of a lipoxygenase (LOX) inhibitor, cyclooxygenase (COX) inhibitor and histone deacetylase (HDAC) inhibitors on epithelial activation (A,C,E) and FD4 permeability (B,D,F). Data are represented as mean ± SEM of N = 4 individual experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 as compared to cells exposed to the cyto mix (10 ng/mL TNF-α, 100 U/mL IFN-γ and 10 ng/mL IL-1β). The concentration of SCFAs is expressed in mM and of HDAC inhibitors in μM. A: acetate; B: butyrate; COXi: COX inhibitor indometacine; LOXi: LOX inhibitor nordihydroguaiaretic acid; P: propionate; Tace: tacedinaline; Tino: tinostamustine; TMP: TMP269; TSA: trichostatin A.*

DISCUSSION

The intestinal barrier is one of the main defense mechanisms in the human body. It is getting more and more attention because it is shown that an impaired intestinal barrier is a common feature in non-communicable diseases (NCDs) [45,46,47,48]. The intestinal barrier consists of different layers including a mucosal layer, an epithelial cell monolayer and the lamina propria. The lamina propria lies beneath the epithelium and contains various effector immune cells, such as T-cells, B-cells, dendritic cells and macrophages [43]. The mucosal tissue plays a critical role in maintaining intestinal homeostasis. Intestinal epithelial cells prevent non-specific leakage of immunogenic agents such as endotoxin LPS by providing a barrier. In addition, the epithelial cells may regulate responses of the underlying immune cells. Immune cell activation in the lamina propria, however, can affect intestinal epithelial cell homeostasis since several cytokines, such as TNF-α, IL-1β, IFN-γ and IL-17a can activate epithelial cells and/or affect their barrier function [49]. Short chain fatty acids (SCFAs), in particular butyrate, have been shown to be able to contribute to intestinal homeostasis because they are known for their anti-inflammatory and intestinal barrier-supporting properties [24]. These effects were observed in *in vitro* models using intestinal epithelial cells or using immune cells alone, but it is unknown whether butyrate's effect is strong enough to protect against immune-mediated barrier disruption in a co-culture model of intestinal epithelial cells (IEC) and activated immune cells (PBMCs). In the present study, PBMCs were activated by LPS or αCD3/CD28. LPS is recognized by immune cells through the pattern recognition receptor Toll-like receptor 4 (TLR4) expressed on the surface of mainly innate immune cells such as monocytes, macrophages and dendritic cells of which only monocytes are present in the PBMC mixture. Binding of LPS to TLR4 triggers signaling cascades that lead to the activation of transcription factors like nuclear factor kappa B (NFκB) and the production of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and regulatory cytokine IL-10 [50].

αCD3/CD28 are monoclonal antibodies that bind to a surface receptor on T-cells. Anti-CD3 binds to the T cell receptor complex, while anti-CD28 binds to a co-stimulatory molecule. This combined activation leads to activation and the production of various cytokines such as TNF-α, IFN-γ, IL-17a and regulatory IL-10 by T-cells [51]. In the co-culture model, butyrate dose-dependently improved the intestinal barrier when the Caco-2 cell monolayer was disrupted by cytokine release from PBMCs activated with LPS or αCD3/28. Butyrate protected against decrease in TEER and suppressed cytokine responses in both situations, indicating the generic anti-inflammatory potency of butyrate on immune cells activated via different pathways. The barrier-protective effect of butyrate was observed before in various monoculture experiments using Caco-2 or T-84, while investigating its effect on the basal barrier or with different methods to disrupt barriers such as ethanol or inflammatory cytokines [27,28,29,52,53,54,55,56,57,58]. Only three other studies using co-culture models investigated the barrier-protective effect of butyrate.

However, to our knowledge, we are the first to show barrier-protective effects at lower concentrations of butyrate (2 and 8 mM) in immune-mediated barrier disruption. The first study showed no statistically significant effect of butyrate in a model with Caco-2 and LPS-activated macrophages. However, the barrier was only assessed after 6 h [59], while in the present study, we showed the protective effect of butyrate after 24 and 48 h. The second study showed butyrate to improve the barrier in a model with Caco-2 and non-activated macrophage-like cells, but did not investigate the effect of butyrate in immune-mediated barrier disruption [60]. The third study showed only barrier-protective effects of high doses of butyrate (20 mM) in a model of Caco-2 cells with LPS-stimulated whole blood samples [61]. The current study focused on butyrate as it was observed before to be most beneficial in maintaining barrier properties during inflammation as compared to propionate and acetate [56,62,63]. For future studies, it would be interesting to compare the efficacy of butyrate, propionate and acetate alone as well as using these SCFAs combined in specific ratios known to be present in the intestinal lumen. This should be studied in *in vitro* models for inflammatory-induced barrier disruption combining IEC and immune cells, including the current LPS- or αCD3/28-activated PBMC/Caco-2 co-culture model or alternatively, instead of PBMCs, lamina propria-derived mononuclear cells may be used [64]. In addition, effects of SCFAs could be further studied in a co-culture model with Caco-2 and LPS-activated macrophages to further mimic the *in vivo* situation in the gut.

Although butyrate reduced barrier function as determined with TEER measurements in the Caco-2/non-activated PBMC model, it decreased functional permeability as indicated by a reduction in FD4 permeation into the basolateral compartment. TEER is an indirect indicator of barrier function since it measures ion fluxes over the tight junctions, and this can be disturbed, for example, by chloride secretion. FD4 is an inert direct marker of tight junction permeability and is used to confirm the TEER measurements. In this case, it shows that butyrate already affects basic barrier properties in absence of any inflammatory insult. This may also underly the protective effect of butyrate on barrier function when Caco-2 were exposed to activated PBMCs. However, beyond acting directly on the epithelial barrier, butyrate was also capable of lowering immune activation.

PBMCs activated with LPS induced the release of IL-1β, IL-6, TNF-α and IL-10, whereas butyrate could only inhibit the release of TNF-α and IL-10. IL-1β and TNF-α are known to disrupt barriers [42,43,44], while IL-10 is known to promote barrier function [54]. In addition, TNF-α is found to synergize with other pro-inflammatory cytokines, such as IL-1β and IFN-γ, which could further enforce barrier disruption [65,66]. Butyrate-induced reduction in TNF-α by activated PBMCs may therefore be essential for its barrier-protective effects. Typically though, at the highest dose of butyrate, IL-1β release increased, while its barrier-protective effects were maintained. IL-10 is known for its barrier-protective effects, however, butyrate also lowered IL-10 secretion thus this could

not explain the protective effect. Future studies should therefore focus on the underlying mechanism involved in butyrate's barrier-protective effect in the higher dose range.

When compared to LPS, in addition to IL-6, TNF-α and IL-10, αCD3/CD28-activated PBMCs also induced IFN-γ and IL-17a. This is coherent as LPS mainly activates monocytes and αCD3/CD28 activates T-cells, which are capable of producing IFN-γ and IL-17a. Butyrate also inhibited the release of TNF-α and IL-10 by αCD3/CD28-activated cells similar to LPS-activated cells. In addition, butyrate inhibited IFN-γ and IL-17a release in the PBMC model, while in the Caco-2/PBMC co-culture, a similar declining pattern was observed. Therefore, like in the LPS-activated conditions, also here, the butyrate-induced suppression of TNF-α secretion by the activated immune cells may have largely contributed to the barrier-protective effects of butyrate.

The PBMC mixture contains monocytes, which is in contrast to the lamina propria which contains macrophages. The presence of inflammatory-type macrophages (M1) in the lamina propria can disturb immune homeostasis. Monocytes derived from the bone marrow can differentiate into macrophages in the intestinal tissue [67] and a reduction of TNF-α producing monocytes may suggest that butyrate may also control M1-type activation hereby contributing to intestinal homeostasis. This is in line with previous research showing that butyrate inhibits NFκβ activation in macrophages in the lamina propria of patients with ulcerative colitis [68]. Furthermore, macrophages exposed to butyrate showed induced antimicrobial activity, contributing to maintain intestinal homeostasis [69].

In the αCD3/CD28-activated PBMCs, butyrate reduced Th1- and Th17-type cytokines IFN-γ and IL-17A secretion. Indeed, butyrate lowered the frequency of activated Th1 cells as shown by the percentage of CD4+CD69+CXCR3+ cells, although the percentage of CD4+CD69+Tbet+ cells remained unaltered. The reduction in IL-17a, however, was associated with the butyrate-induced reduction in the percentage of CD4+RORγ+ cells, which links to reduced Th17 activation.

The inhibitory effect of butyrate on Th1-cells, Th17-cells and pro-inflammatory cytokines secretion was observed previously, but the reduction in regulatory T-cells and IL-10 was different from most other publications [34,35,36,37,39]. However, depending on the dose, differential immune effects of butyrate have been shown previously. For example, IL-10 release was induced by 0.25 mM butyrate in αCD3-activated PBMCs, while IL-10 release was inhibited by 1 mM butyrate [36]. Contrary, 1–2 mM butyrate induced the release of IL-10 and the percentage of regulatory T-cells in LPS-activated PBMCs (5 μg/mL), while 0.2–20 mM butyrate inhibited IL-10 release in LPS-activated monocytes and PBMCs (0.5 μg/mL), showing no statistically significant effect on non-activated cells [35,37]. This suggests that the effect of butyrate not only is dependent on the concentration of butyrate present, but also on the type and strength of immune activation.

Typically though, butyrate increased expression of activation marker CD69+ on CD4 cells, which could not be linked to either Foxp3+ natural regulatory T-cells, Th1 nor Th17 cells. On the other hand, the expression of CD25 was reduced. Beyond FoxP3+ regulatory T-cells, other types of regulatory T-cells exist. In this respect, CD4+CD25−CD69+ cells have been previously indicated as a novel type of regulatory T-cells and can suppress T-cell proliferation in a cell–cell contact manner, which may explain the reduced percentages of Th-cells and regulatory T-cells (CD4+CD25+FoxP3+) [70]. Although, not a lot is known about this novel type of regulatory T-cells therefore more research would be needed to identify if butyrate affects these types of cells and how they can contribute to intestinal immune homeostasis.

LPS and αCD3/CD28 activation are complementary since the immune cascade is initiated from either the innate or the adaptive immune compartment. LPS and αCD3/CD28 both showed to produce different inflammatory cytokine patterns which could be suppressed by butyrate. Low-grade inflammation can lead to leaky gut and LPS leakage. LPS may activate innate immune cells which further contributes to chronic low-grade inflammation increasing the risk of NCDs. In immune-mediated NCDs, T-cell activation also increases inflammatory cytokines that may also contribute to a leaky gut [45,46,47,48]. Therefore, both activation pathways used in our studies are relevant when considering immune activation in NCDs. The present study shows that butyrate suppressed LPS and αCD3/CD28 induced immune activation and protected against inflammatory-induced barrier disruption, which shows the broad anti-inflammatory potential of butyrate. Similar effects were observed in different murine models of NCDs. Mice treated with butyrate in drinking water showed improved skin and intestinal barriers, which contributed in reducing the development of diseases such as arthritis and atopic dermatitis [71,72]. In addition, dietary supplementation of butyrate to mice fed with a high-fat diet proved to act as an anti-inflammatory [73,74].

TNF-α, IL-1β and IFN-γ are known to disrupt the intestinal barrier and IL-10 can promote barrier function [42,43,44,54]. As discussed before, butyrate partially reduced the release of these cytokines by activated PBMCs, and therefore the effect of butyrate on the intestinal barrier in the co-culture model might be indirect via its anti-inflammatory properties. To further investigate the direct barrier-protective effect of butyrate, Caco-2 were exposed to a cytokine mixture of TNF-α, IFN-γ and IL-1β. In this model, butyrate again proved its barrier-protective effects and its anti-inflammatory capacity by inhibiting IL-8 release, similar to previous observations [27,28,31,53,75]. Compared to propionate and acetate, butyrate was most effective since it both improved barrier function under inflammatory conditions and suppressed epithelial cell activation which is important to maintain homeostasis.

Cytokine-mediated barrier disruption often results in reduced expression of tight junction proteins like ZO-1 and occludin [76]. In our study, the cytokine mixture did not decrease

the level of these tight junction proteins and butyrate, propionate and acetate also did not affect these protein levels. It could be that the barrier-supportive effects of the SCFAs butyrate and acetate result from more dense tight junction reassembly [28] or that the expression of other tight junction proteins is affected. For example, it was shown that butyrate increased Claudin-1 expression in the cdx2-IEC cell line and that it induced redistribution of ZO-1 and Claudin-1 [75].

Butyrate is the most potent HDAC inhibitor, which suggests the potential involvement of HDAC in the mechanism of action of these SCFAs [24,30,77,78]. Another study described the potential involvement of LOX and COX in the mechanism of action of butyrate [26]. In the present study, LOX and COX were not involved in the anti-inflammatory effects or the mechanism of barrier protection by the SCFAs. Contrary, general HDAC inhibitor TSA showed similar effects on barrier improvement and reduction in inflammatory-induced IL-8 release as butyrate, indicating that HDAC inhibition might indeed be involved in the mechanism of action of the SCFAs. More specific HDAC inhibitors showed various effects. Class IIa (HDAC 4, 5, 7, 9) HDAC inhibitor TMP269 showed no statistically significant effect on barrier integrity and IL-8 release, while the highest concentration of class IIb (HDAC 1, 2, 3, 8) and I (HDAC 6, 10) HDAC inhibitor tinostamustine showed a protective effect on barrier integrity and, albeit not significant, a similar pattern in IL-8 release as compared to butyrate. Strikingly, HDAC inhibitor tacedinaline, specifically inhibiting HDAC 1, 2 and 3 also had barrier-protective effects similar to butyrate, but it increased IL-8. Butyrate, propionate, TSA and a class II HDAC inhibitor previously have been shown to lower IL-8 release in stimulated Caco-2 cells [79,80]. However, general HDAC inhibitor TSA was also found to increase IL-8 levels in some conditions [81]. To our knowledge, here it is shown for the first time that HDAC inhibition can protect against inflammatory-induced barrier disruption similar to butyrate. Butyrate is known as a potent inhibitor of HDAC 2, 3 and 8, followed by 1, 4 and 5 to a lesser extent. In comparison to the HDAC inhibitors used to mimic the effect of butyrate, inhibition of HDAC 1, 2, 3, 4 and 5 did not show similar effects to butyrate and are therefore most probably not involved in its barrier-protective effect. While in comparison to the HDAC inhibitors used, a HDAC inhibitor inhibiting HDAC 8 did show similar effects to butyrate. Our results suggest that the beneficial effect of butyrate on intestinal barrier function and epithelial activation is likely mediated by the inhibition of specific HDACs, of which HDAC 8 would be the most promising candidate. The effect of specific HDAC 8 inhibitors has recently been investigated in a murine model of colitis showing barrier-improving effects via upregulation of occludin [82]. To the best of our knowledge, specific HDAC inhibitors were not investigated in immune-mediated barrier disruption *in vitro* and the potential involvement of HDAC 8 was shown here for the first time.

Butyrate concentrations in the small intestine range from 0 to 26 mM from beginning to end and are highly impacted by diet and bacterial fermentation [83,84]. A change in diet or bacterial composition could therefore potentially result in reduced butyrate

concentrations. In the present study, butyrate was found to have anti-inflammatory and barrier-protective effects in the range of 4–8 mM. Our work shows the importance of sufficient butyrate levels. This can be achieved by consuming a diet rich in fermentable fibers or by butyrate supplementation via nutritional supplements or by a pharmaceutical drug product releasing sufficient amounts of butyrate.

CONCLUSIONS

These findings highlight that butyrate plays an important role in maintaining both barrier integrity as well as immune homeostasis. This emphasizes the importance of having sufficient intestinal butyrate levels. Here, the essential role for butyrate in controlling gut health was shown in an experimental Caco-2/PBMC co-culture model allowing for the cross talk between epithelial cells and activated immune cells. In addition, butyrate directly inhibited the inflammatory response of activated PBMCs. Butyrate proved not only to reduce barrier disruption via lowering local inflammatory responses, but also had a direct protective effect on cytokine-mediated barrier disruption. The effect of butyrate is most probably mediated via HDAC, of which HDAC 8 inhibition may be the main target in controlling both barrier as well as inflammation. In conclusion, the HDAC inhibitory effect of butyrate may protect against both inflammatory-induced barrier disruption as well as immune activation and can therefore have a protective role in NCDs.

SUPPLEMENTAL FIGURES

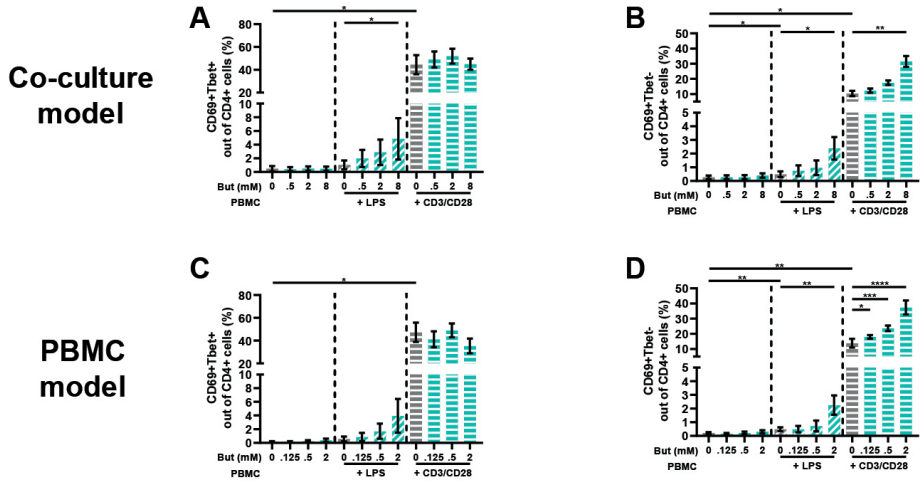

Figure S1. *See figure legend on page 85.*

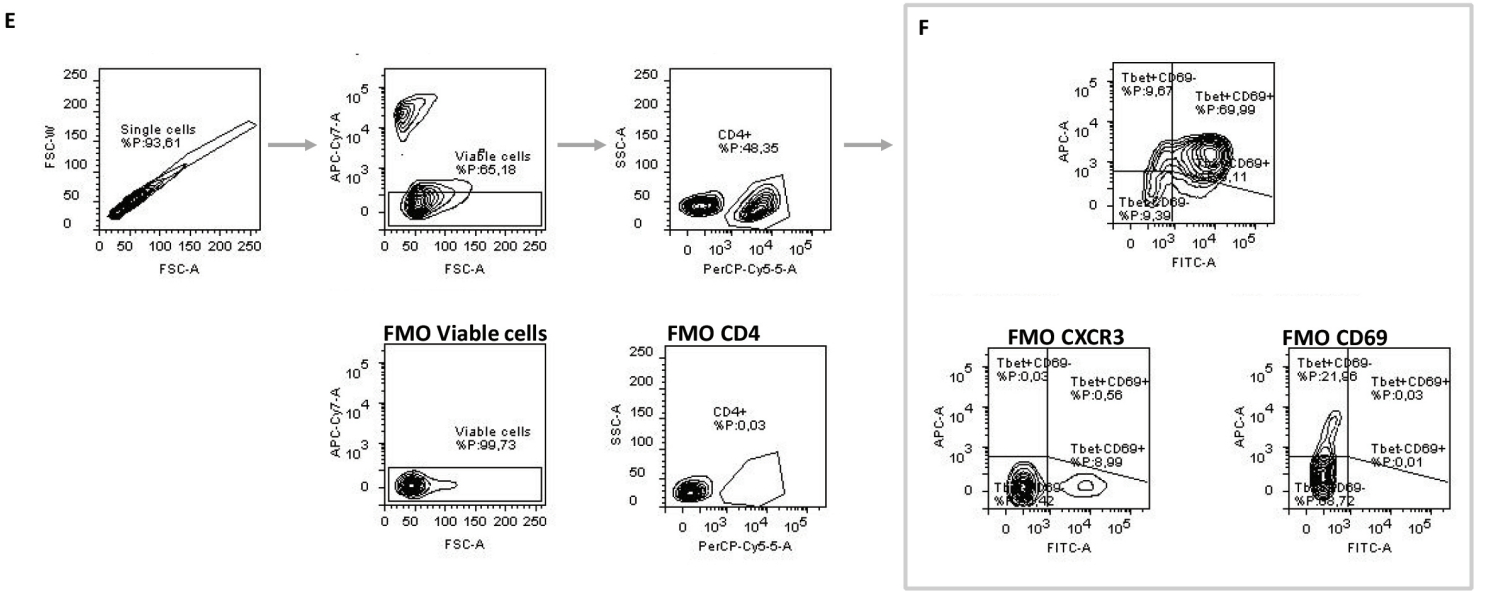

Figure S1. *Butyrate modulates CD69+Tbet+ and CD69+Tbet− cells in a Caco-2/PBMC co-culture model (A,B) and PBMC model (C,D). PBMCs were activated with LPS or anti-CD3/CD28 and cells were treated with 0–8 mM butyrate. (E) Used gating strategy of a representative sample with corresponding fluorochrome minus one (FMO) sample, single cells gating > viable cells gating > CD4+ cells gating followed by (F) CD69+Tbet+ and CD69+Tbet− gating. Data are presented as mean ± SEM and the datasets used for statistical analysis are divided by dotted lines (N = 6 individual experiments). Significant differences are shown as * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.001 compared to control. But: butyrate; PBMC: peripheral blood mononuclear cells.*

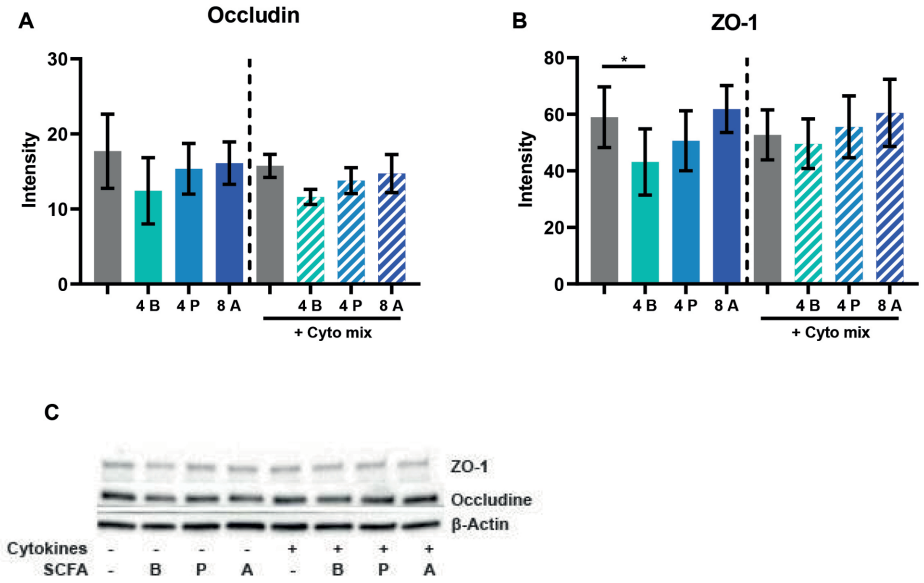

Figure S2. *The effect of SCFAs on occludin (A) and ZO-1 (B) protein levels, including a representative western blot image (C). Data are represented as mean ± SEM of N = 4 individual experiments. Significant difference is shown as * $p < 0.05$ compared to control. Cells were treated with 4 mM butyrate, 4 mM propionate or 8 mM acetate and activated with a cytokine mix (10 ng/mL TNF-α, 100 U/mL IFN-γ and 10 ng/mL IL-1β). A: acetate; B: butyrate; P: propionate.*

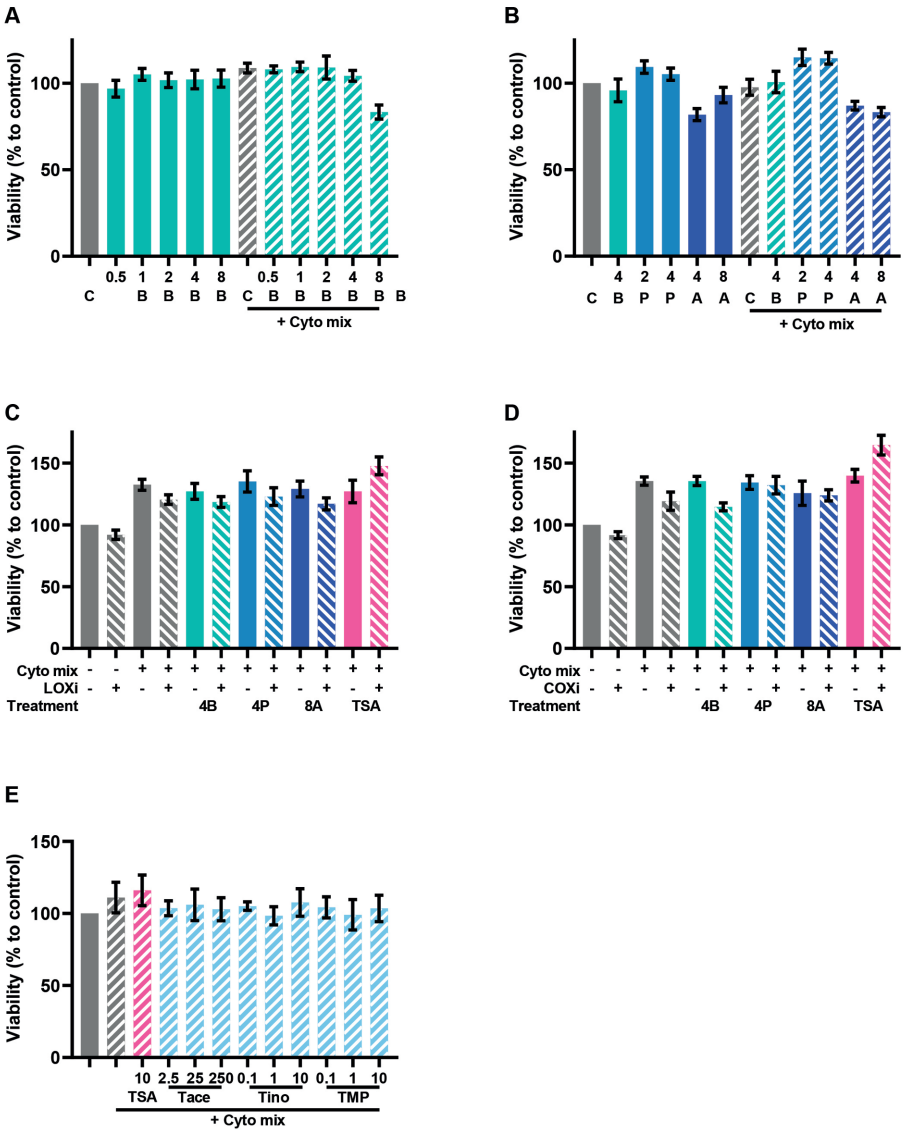

Figure S3. *Viability of the Caco-2 cells after the experiments with butyrate (A), SCFA (B), a LOX inhibitor (C), a COX inhibitor (D) and the different HDAC inhibitors (E). Data are represented as mean ± SEM of N = 4 individual experiments. The concentration of SCFAs is expressed in mM, the concentrations of HDAC inhibitors in µM. A: acetate; B: butyrate; C: control; P: propionate; COXi: COX inhibitor indometacine; LOXi: LOX inhibitor nordihydroguaiaretic acid; Tace: tacedinaline; Tino: tinostamustine; TMP: TMP269; TSA: trichostatin A.*

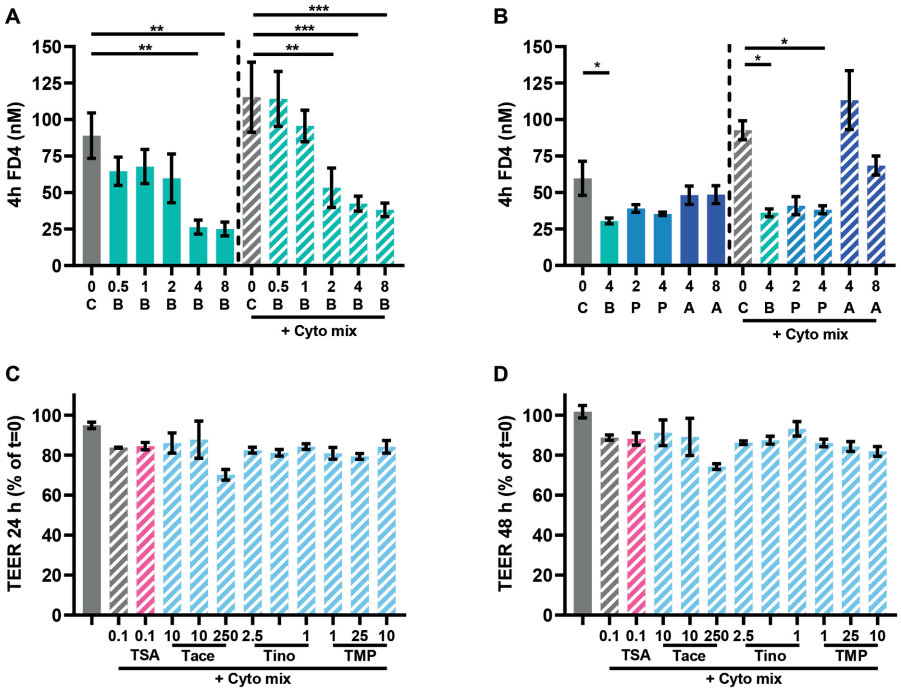

Figure S4. *Additional barrier function measurements. (A,B) FD4 permeability sampled after 4 h in the experiment with Caco-2 cells activated with a cytokine mix (10 ng/mL TNF-α, 100 U/mL IFN-γ and 10 ng/mL IL-1β) and treated with butyrate, propionate or acetate. Data are represented as mean ± SEM of N = 4 individual experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to the control cells or cells exposed to the cyto mix. (C,D) TEER values after 24 h and 48 h measured in Caco-2 cells activated with a cytokine mix and treated with different HDAC inhibitors. Data are represented as mean ± SEM of N = 3 individual experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to the cells exposed to the cyto mix. The concentration of SCFAs is expressed in mM, the concentrations of HDAC inhibitors in μM. A: acetate; B: butyrate; C: control; P: propionate; COXi: COX inhibitor indometacine; LOXi: LOX inhibitor nordihydroguaiaretic acid; Tace: tacedinaline; Tino: tinostamustine; TMP: TMP269; TSA: trichostatin A.*

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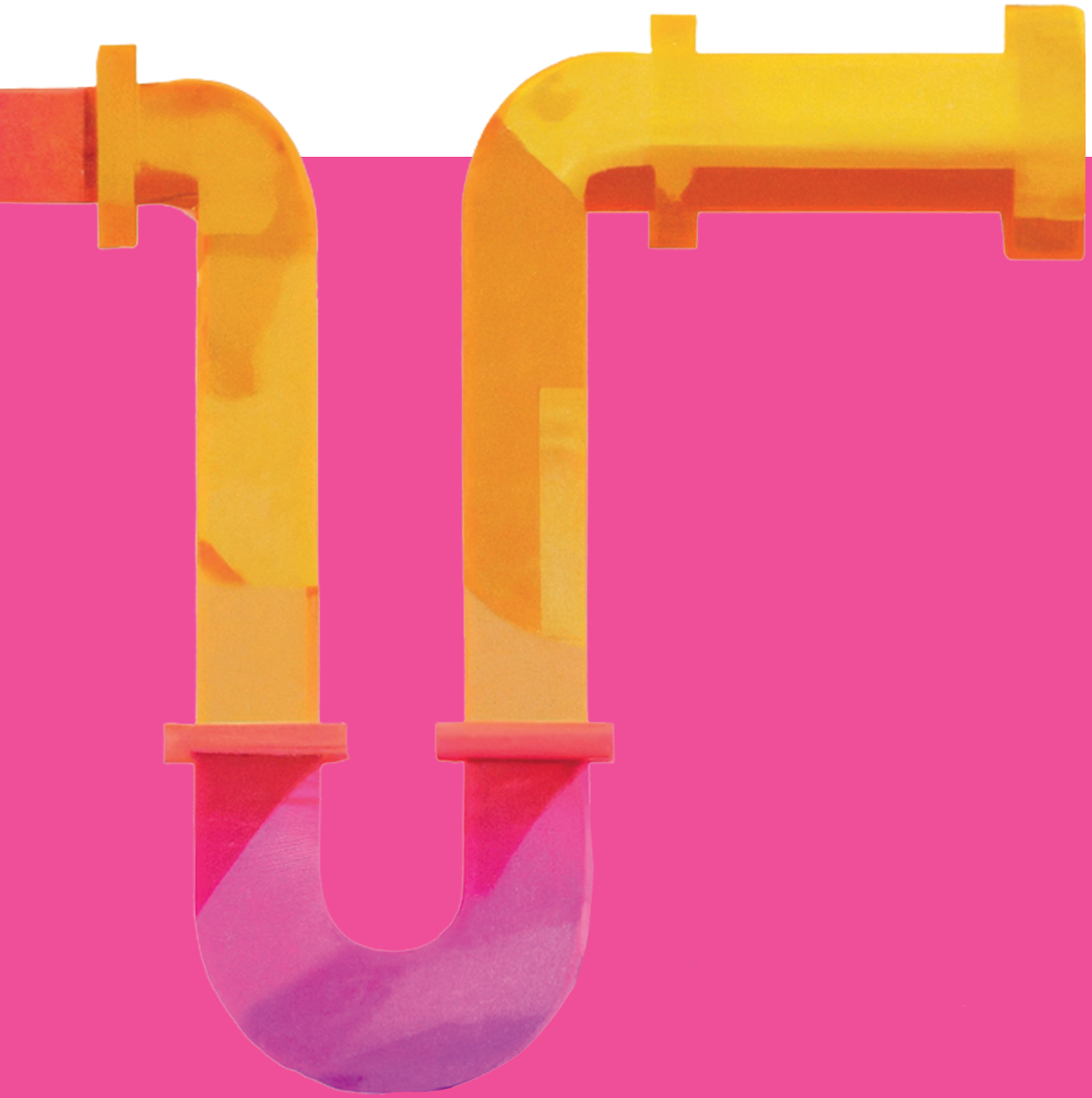
PHARMACOKINETICS



CHAPTER

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Modeling of the luminal butyrate concentration to design an oral formulation capable of achieving a pharmaceutical response

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ABSTRACT

Butyrate concentrations required for a direct effect on intestinal epithelial cells lie between 2–5 mM. In order for butyrate to affect the small intestine the local pharmacokinetics need to be understood. We used a mathematical approach to model the luminal butyrate concentration after oral administration of an immediate release formulation or a sustained release formulation to humans. This model was used to design an oral formulation capable of achieving a local pharmaceutical response in the small intestine. The model showed that an immediate release formulation is only capable of maintaining pharmacologically active concentrations during the first half hour after the formulation has entered the small intestine. A sustained release formulation is capable of maintaining pharmacologically active concentrations for hours and thus throughout the whole small intestine. To reach these concentrations the sustained release formulation requires a zero order release rate of 0.08-0.2 mmol/h. The anticipated release rates are expected to result in luminal butyrate concentrations that are high enough at the surface of the epithelial cells to improve the intestinal barrier and to have anti-inflammatory properties. However, it is uncertain if the duration of exposure, and quantity of exposed epithelial cells is adequate to have a clinical effect.

INTRODUCTION

Butyrate is a promising therapeutic agent which is thought to be of benefit in the treatment of several noncommunicable diseases (NCDs) when administered to the small intestine. NCDs are diseases in which chronic low-grade inflammation plays an important role. This low-grade inflammation could be caused by damage of the gastrointestinal barrier which is pivotal for efficient host defense [1]. Butyrate might influence gut integrity via different mechanisms of action. Direct interaction with the intestinal epithelial cells (IECs) but also direct interaction with immune cells present within and just below the mucosal membrane. Several studies indicated earlier that butyrate can improve the intestinal barrier and modulate local immune responses in the small intestine [2-4].

Butyrate can be produced by the bacteria in the gastrointestinal tract as a result of fiber fermentation [5-7]. Butyrate-producing bacteria are abundantly present in the colon, so butyrate levels are high there. Butyrate-producing bacteria are less abundantly present in the small intestine, so butyrate levels are relatively lower there. The upper part of the small intestine in particular lacks butyrate [8,9]. Therefore, the small intestine would be a highly interesting target for novel butyrate containing drug formulations.

The small intestine is part of the gastrointestinal tract and extends from the stomach to the beginning of the colon. From the inside to the outside, the intestinal wall consists of a mucus layer, epithelial cells and the lamina propria with immune cells [10].

Since we focus on how butyrate affects IECs, which are present along the whole small intestine, it could be speculated that butyrate needs to be available throughout all the IECs along the small intestinal wall. Additionally, butyrate should be present in a concentration that results in a pharmacological response. The pharmacologically active concentration of butyrate to affect IECs lies between 2–5 mM [11-14]. At these concentrations, the intestinal barrier is improved and the local mucosal immune response seemed to be affected. Therefore our goal is to develop a butyrate formulation that reaches these concentrations at the surface of the IECs, preferably along the entire length of the small intestine.

Butyrate is a drug with a high solubility, causing it to dissolve very fast in the luminal fluid. The luminal fluid is not distributed homogeneously over the small intestine, but is present in separated pockets with variable volumes [15,16]. Therefore butyrate will only dissolve in the volume available in one pocket, the pocket where the oral formulation is present, and not in the total volume available in the whole small intestine.

Once butyrate is dissolved, it starts to diffuse across the mucus and is subsequently absorbed by the IECs. The remaining butyrate concentration in the lumen at a certain moment is determined by both the amount that is released and the amount that is diffused

and absorbed. In other words, the concentration is determined by the balance between influx and outflux. The overall absorption rate of butyrate in the small intestine has been studied by Schmitt et al. [17]. They investigated the disappearance rate of butyrate out of the lumen making it possible to model the butyrate concentration in the lumen after intake of an oral immediate release or sustained release butyrate formulation with specified release characteristics.

This study aims to model the butyrate concentration in the small intestine after oral administration of a butyrate formulation to humans. With the help of this mathematical model we anticipate developing a formulation suitable to maintain pharmacologically active butyrate concentrations in the small intestine to treat NCDs. We consider knowledge of a dosing regimen as a biopharmaceutical prerequisite to perform clinical studies.

METHODS

Schematic representation of the small intestine

A mathematical approach was used to model the luminal butyrate concentration after intake of an oral formulation by humans. To build this model we made several assumptions, which will be described below.

As described before, small intestinal fluid is present in several fluid pockets. The total volume of these pockets and the volume distribution across these pockets has been studied before [16,18]. Although the volume of one pocket is variable, in this model the volume of luminal fluid present for butyrate to dissolve in was set constant. This volume was used to calculate the available butyrate concentration in one pocket. The mean volume in one pocket is reported to be 6 ± 2 mL after intake of 240 mL of water [16]. Therefore a constant volume of 6 mL was used in our model.

To the best of our knowledge the origin and the exact kinetics of a pocket containing an oral formulation are unknown. Therefore the assumption was made that the butyrate formulation when entering the small intestine gets captured in a fluid pocket and the luminal fluid surrounding the formulation and the formulation itself move forward in the small intestine simultaneously (Figure 1). The butyrate formulation will thus be present in one fluid pocket during the intestinal transit from stomach to the colon and for this specific pocket the butyrate concentration will be calculated. As the pocket transits through the small intestine, the location of the formulation changes in time, as illustrated by the red rectangle in Figure 1.

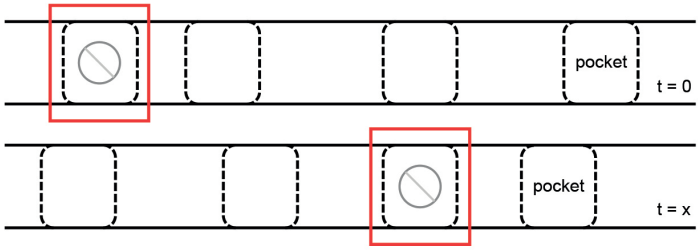

Figure 1. *Representation of the movement of the formulation containing pocket through the small intestine. Concentrations will be calculated for the area within the red rectangle. Different time points therefore also reflect different positions in the small intestine.*

The butyrate concentration was modelled in the luminal fluid of one pocket. The butyrate that is present in the luminal fluid diffuses across the mucus to the surface of the intestinal epithelial cells (IECs) where it can exert its effect. We assume that the butyrate concentration in the luminal fluid will be equal to the butyrate concentration at the surface of the IECs, because butyrate is a small and hydrophilic molecule and because the mucus layer in the small intestine is very thin [18].

Additionally, the dimensions of one pocket in the model were set, namely the diameter and the length available for absorption. A pocket can be described as a cylinder with a diameter of 2.5 cm [23]. The length of one pocket was calculated to be 1.2 cm based on the volume of 6 mL, see Figure 2.

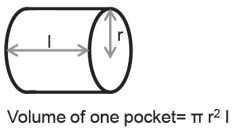

Figure 2. *Schematic representation of a pocket in the small intestine. r = radius of the small intestine: 1.25 cm [23]; l = length of the pocket: 1.2 cm. The volume of one pocket in our model is 6 cm³.*

The absorption of butyrate

The absorption of butyrate was studied by Schmitt et al. and was used to model the absorption rate of butyrate [17]. It was shown that the absorption rate of butyrate is concentration dependent and can be described by Michaelis-Menten kinetics, see Figure 3 [17]. The Michaelis-Menten equation, Equation 1, was used to reproduce the data of Schmitt et al. with regard to the absorption rate corresponding to the concentration range of interest, the linear part of the curve in Figure 3:

$$V = V_{max}\frac{C}{C + K_M} \quad (1)$$

where V is the absorption rate of butyrate in mmol/h/cm, V_{max} is the maximum absorption rate of butyrate (0.820 mmol/h/cm), K_M is the Michaelis constant of butyrate in the small intestine (25.6 mM) and C is the butyrate concentration available in the small intestine in mM. Michaelis-Menten kinetics indicate that the absorption of butyrate is driven by active transport, especially at the concentration range of our interest. At higher concentrations active transport is saturated and diffusion of butyrate will further increase the absorption rate of butyrate.

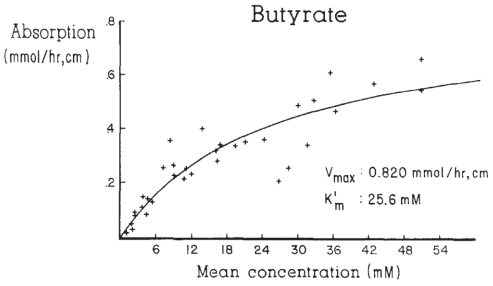

Figure 3. *Absorption data obtained from Schmitt et al. with permission [17].*

As already indicated, the anticipated pharmacologically active concentration is 2–5 mM. The absorption rate corresponding to this concentration range, can be found in the linear part of the curve. The values on the y-axis of Figure 3 were converted from mmol/h/cm to mmol/h/the length of one pocket and the values on the x-axis of Figure 3 were converted from mM to mmol in the volume of one pocket, as shown in Figure 4. By this conversion the data were adapted to the situation in one pocket and first order kinetics could be used to describe the absorption. The absorption of butyrate in one pocket of the small intestine can be described by the following equation:

$$Rate\,of\,absorption\,of\,butyrate = -k_{abs} \times A(t) \quad (2)$$

where A(t) is the amount of butyrate in one pocket of the small intestine in mmol, t is time in h and k_{abs} is the first order absorption rate constant of butyrate in h^{-1}. The data was fitted linearly from 0 to 0.09 mmol butyrate in one pocket with a R^2 of 1. k_{abs} equals 4, as can be seen in Figure 4.

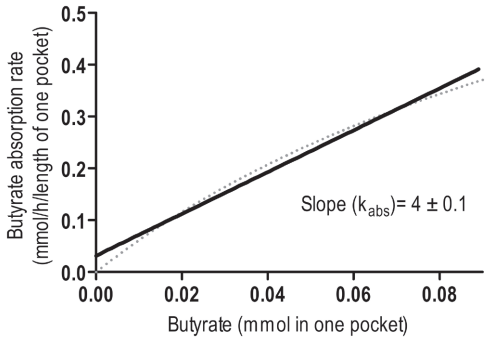

Figure 4. *The dotted grey line shows the converted data of Schmitt et al. to the situation in one pocket (volume=6 mL, length = 1.2 cm). Data were linearly fitted to obtain the slope (k_{abs}) [17].*

Model of sustained release formulation

The butyrate concentration in the lumen was first calculated for a sustained release (SR) formulation. This was done by subtracting the amount of butyrate absorbed out of the lumen from the amount of butyrate released into the lumen, as shown in Equation 3.

$$\underbrace{Rate\ of\ change\ of\ butyrate\ in\ the\ small\ intestine}_{} = \underbrace{Rate\ of\ release\ of\ butyrate\ from\ formulation}_{\text{Part 1}} - \underbrace{Rate\ of\ absorption\ of\ butyrate}_{\text{Part 2}} \quad (3)$$

Part 1 of Equation 3 shows the rate of butyrate released from a SR formulation. The release of butyrate can be adequately described by zero order kinetics. The equation of a zero order release formulation is shown in Equation 4:

$$Rate\ of\ release\ of\ butyrate\ from\ formulation = R_{rel} \quad (4)$$

where R_{rel} is the zero order release rate from the formulation in mmol/h.

Part 2 of Equation 3 is described previously in Equation 2. Equation 3 can be translated to Equation 5 by combining Equation 2, the absorption of butyrate, and Equation 4, the release of butyrate from a formulation.

$$\frac{dA(t)}{dt} = R_{rel} - k_{abs} \times A(t) \quad (5)$$

Because we were interested in the butyrate concentration in the lumen, we substituted A(t) by C(t) times V, yielding Equation 6:

$$\frac{d[C(t) \times V]}{dt} = R_{rel} - k_{abs} \times C(t) \times V \quad (6)$$

where V is the mean constant volume of one pocket in the small intestine, 6 mL. This resulted in the following equation for the change of butyrate concentration in the lumen with time (Equation 7).

$$\frac{dC(t)}{dt} = \frac{1}{V} \times R_{rel} - k_{abs} \times C(t) \quad (7)$$

Equation 7 is solved over time, yielding the following equation for C(t):

$$C(t) = \frac{1}{V} \times \frac{R_{rel}}{k_{abs}} + cst_{SR} \times e^{-k_{abs} \times t} \quad (8)$$

where cst_{SR} is the constant of integration, which was calculated by solving Equation 8 for a known concentration at a known timepoint, namely a concentration of 0 mM at t = 0 h. At t = 0 h, the formulation will enter the upper small intestine of which it is known that it lacks butyrate [9].

Butyrate concentrations in the pocket containing the formulation were modelled using Equation 8. We aim to reach a concentration of 2–5 mM, 15 min after the formulation enters the small intestine to ensure that a pharmacologically active concentration of butyrate gets available to the IECs from the beginning of the small intestine until the end of the small intestine. Therefore, we calculated the R_{rel} to find the release rate needed to reach pharmacologically active concentrations.

Model of immediate release formulation

Contrary to a SR formulation, an immediate release (IR) formulation has no constant supply of butyrate into the lumen. We assumed that the butyrate present in an IR formulation will instantly dissolve in the volume of a pocket. The dissolved butyrate will subsequently be absorbed out of the lumen. This means that only absorption will play a role in the rate at which the butyrate concentration changes in the small intestine, see Equation 9.

$$Rate\ of\ change\ of\ butyrate\ in\ the\ small\ intestine = -Rate\ of\ absorption\ out\ of\ butyrate \quad (9)$$

Equation 9 can be translated to Equation 10:

$$\frac{dA(t)}{dt} = -k_{abs} \times A(t) \quad (10)$$

where A(t) is the amount of butyrate in one pocket of the small intestine in mmol, t is time in h and k_{abs} is the first order absorption rate constant of butyrate in h^{-1}. Because we were interested in the concentration we substituted A(t) by C(t) times V, yielding Equation 11:

$$\frac{d[C(t) \times V]}{dt} = -k_{abs} \times C(t) \times V \quad (11)$$

where C(t) is the butyrate concentration in one pocket of the small intestine and V is the mean constant volume of one pocket in the small intestine, 6 mL. This resulted in the following equation for the change of butyrate concentration in the lumen with time Equation 12.

$$\frac{dC(t)}{dt} = -k_{abs} \times C(t) \quad (12)$$

Equation 7 is solved over time, yielding the following equation for C(t):

$$C(t) = cst_{IR} \times e^{-k_{abs} \times t} \quad (13)$$

where cst_{IR} is the constant of integration, which was calculated by solving Equation 13 for a known concentration at a known time point, namely the butyrate concentration available directly after dissolving the total amount of butyrate present in the IR formulation in the volume of one pocket at t = 0 h, (C(0)). Meaning that cst_{IR} equals the dosage of butyrate in the formulation divided by the volume of the pocket.

Butyrate concentrations in the lumen were modelled using Equation 13. We aim to reach a concentration of 2–5 mM, 15 min after the formulation entered the small intestine. Therefore, we varied C(0) in the equation to find the dosage needed to reach pharmacologically active concentrations. Additionally, Equation 13 gives information about the contact time of butyrate with the IECs after a SR formulation left the pocket and a butyrate concentration will stay present at the surface of the mucus layer. In this case C(0) is the butyrate concentration available at the surface of the mucus layer after the SR formulation left the pocket.

Data analysis

Graphpad Prism 5.0 was used to fit and plot the data.

RESULTS

Sustained release formulation

A butyrate concentration of 2–5 mM is needed to achieve a pharmacological response [11-14]. Our aim was to obtain these concentrations within 15 min after the formulation

reaches the small intestine. The butyrate concentration after oral administration of a sustained release (SR) formulations was modelled using Equation 8 where $k_{abs} = 4\ h^{-1}$, V = 0.006 L and C(0.25) = 2 mM or 5 mM. The R_{rel} corresponding to C(0.25) = 2 mM is 0.08 mmol/h and the R_{rel} corresponding to C(0.25) = 5 mM is 0.2 mmol/h. Figure 5 shows the concentration-time curves for SR formulations with these release rates. As can be seen, the steady state concentrations in the pocket will be reached within one hour, reaching levels of approximately 3 mM when C(0.25) = 2 mM and of approximately 8 mM when C(0.25) = 5 mM.

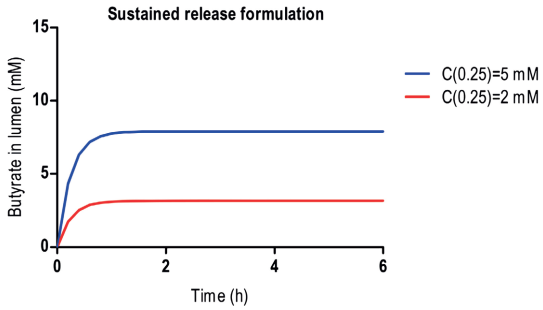

Figure 5. *Concentration-time curve of butyrate in a pocket passing the lumen of the small intestine after intake of two different sustained release formulations with a zero order release rate of 0.08 mmol/h (red) and 0.2 mmol/h (blue).*

Immediate release formulation and local concentration after passage of a pocket

As stated, an immediate release (IR) formulation will immediately deplete its butyrate content. The IR results in high concentrations in the pocket containing the formulation. For example, a dose of 10 mg butyrate would yield a concentration of 19.14 mM in a pocket of 6 mL.

The concentration-time profile of such a pocket was calculated using Equation 13 where $k_{abs} = 4\ h^{-1}$, V = 0.006 L and C(0.25) = 2 mM or 5 mM, see Figure 6. The C(0) corresponding to C(0.25) = 2 mM is 5.4 mM and the C(0) corresponding to C(0.25) = 5 mM is 13.6 mM. In addition, these concentrations can be converted to the amount of butyrate in the volume of one pocket, consequently being the amount of butyrate in the IR formulation. This resulted in IR formulations with a dosage of 33 µmol butyrate to reach a concentration of 2 mM after 15 min and of 82 µmol butyrate to reach a concentration of 5 mM after 15 min.

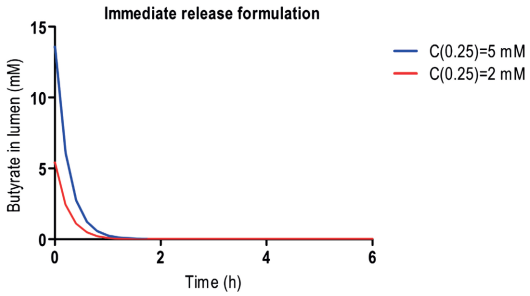

Figure 6. *Concentration–time curve of butyrate in a pocket passing the lumen of the small intestine after intake of two different immediate release formulations with a dosage of either 33 µmol (red) or 82 µmol (blue).*

The butyrate concentration rapidly drops and within one hour hardly any butyrate is left in the pocket because of the relatively high absorption rate. This means a high exposure of butyrate to the upper part of the small intestine while the lower part of the intestine will have no exposure to butyrate at all. Figure 6 represents the concentration profile in a pocket that is transiting the small intestine while containing an IR formulation or its content. However, principally the same profile reveals at any spot at the surface of the mucus when a SR formulation containing pocket transits through the small intestine. The butyrate containing pocket is in contact with the apical side of the mucus layer causing butyrate to diffuse for the lumen to the surface of the mucus. After the SR formulation containing pocket transits further along the small intestine, butyrate will still be present in the mucus and the butyrate diffuses from the surface of the mucus to the IECs. The butyrate concentration in a pocket where the SR was present will rapidly fall when considering Figure 6. In this situation a pharmacologically active concentration can be reached along the whole small intestine in the pocket where the formulation was present, although this is only valid for a period of one hour at maximum.

DISCUSSION

Sustained release versus immediate release

In this study we determined the required release rate of an oral butyrate sustained release (SR) formulation to achieve pharmacologically active concentrations in the small intestine. The model provided by this study helps to develop the first butyrate containing oral drug product to treat noncommunicable diseases (NCDs) in which mucosal barrier disturbance are involved.

A SR formulation is capable of maintaining pharmacologically active concentrations in the pocket where the formulation is present for multiple hours throughout the whole small intestine. SR formulations reach a steady state concentration after approximately 2 h and the level of this steady state concentration depends on the release rate of the

formulation in relation to the absorption rate. Immediate release (IR) formulations are only capable of maintaining pharmacologically active concentrations within the first half hour after the formulation has reached the small intestine and thus will not target the whole small intestine. A higher immediate butyrate dose would be capable of maintaining concentrations above the minimal required concentration of 2 mM throughout larger parts of small intestine, but this would at the same time result in a very high concentration in the beginning of the small intestine of which toxicity is unknown. In short, a SR formulation is most promising to treat NCDs via the small intestine without toxicity. In order to maintain pharmacologically active concentrations throughout the small intestine, it requires a release rate of 0.08 to 0.2 mmol/h and sufficient butyrate to be able to release butyrate for approximately 3 h [19].

In our model the formulation and the luminal fluid surrounding it simultaneously move forward in the small intestine. As a consequence, the butyrate concentration in the lumen is calculated for the pocket in the small intestine in which the formulation is present. The duration a formulation stays in one pocket in the small intestine is unknown. The length of time that butyrate will be exposed to the intestinal epithelial cells stays thus also unknown, but will be limited. The length of time butyrate stays present in the mucus at the surface of the epithelial cells after the formulation moves forward in the intestine can be estimated by considering Figure 7. The butyrate in the mucus will be only taken up by the cells, which makes the process comparable to an IR formulation in other words; only absorption (k_{abs}) plays a role. Figure 7 shows that the butyrate concentration decreased to almost 0 mM approximately half an hour after a pharmacologically active butyrate concentration was present in a pocket. This indicates that butyrate has to act on the intestinal epithelial cells in a short period of time. At this moment it is unclear if pharmacological response is to be expected under these conditions. Pharmacological experiments are normally carried out at constant concentrations.

A formulation passes through the stomach before it enters the small intestine, meaning that some butyrate is released in the stomach before it reaches the small intestine. There are two strategies to ensure that enough butyrate reaches the small intestine: the formulation can either be coated with a gastric resistant coating to prevent butyrate release in the stomach or the formulation can be left uncoated and consist of sufficient butyrate for release in the stomach and the small intestine. Because the upper part of the small intestine lacks butyrate, we prefer an uncoated formulation which will immediately start to release butyrate in the small intestine, while a coated formulation will release a limited amount of butyrate in the upper part of the small intestine because the coating first needs to dissolve [20,21]. The gastric emptying time of an oral formulation is highly variable and depends on the fasted or fed state. The median gastric emptying time of a non-disintegrating capsule or tablet of at least 5 mm in the fasted state is less than 30 min and in the fed state around 5 h [22]. To make sure that the SR formulation consists of sufficient butyrate to have butyrate release along the whole small intestine, the SR formulation

should consist of sufficient butyrate for an 8 h release in total, more specific a 5 h release in the stomach and a 3 h release in the small intestine [19,22]. The SR formulation requires a butyrate release rate of 0.08-0.2 mmol/h and a butyrate release of 8 h. This results in a total dosage of 0.64–1.6 mmol butyrate, which equals 55.7–139.4 mg butyrate.

Limitations

Our model is designed based on several assumptions and thus has some limitations. During the calculation of the available butyrate concentration in the small intestine, the volume and length of one pocket were estimated. We used the mean volume after intake of a glass of water, 6 mL. Nevertheless, this volume is susceptible to interindividual and intraindividual variability. In 60% of the pockets the volume ranges from 0.5 to 2.5 mL, and in 10% of the pockets the volume is larger than 20 mL [16]. The volume of one pocket will influence the performance of the oral formulation, because a different volume results in a different luminal butyrate concentration. A smaller pocket with a lower fluid volume results in a higher butyrate concentration and a larger pocket with a higher fluid volume results in a lower butyrate concentration than calculated by our model.

Another factor that influences the performance of the oral formulation is the diameter of the small intestine, because it influences the area available for absorption. In the model we assigned the small intestine a diameter of 2.5 cm. The diameter of the small intestine differs per segment and ranges from 2 to 4 cm [23]. This diameter influences the absorption rate constant, because it changes the length of one pocket in our model (see Figure 3). A thinner small intestine increases the k_{abs} and a thicker small intestine decreases the k_{abs}. A change in k_{abs} results for the oral formulation in a different luminal butyrate concentration. Namely, a thinner small intestine results in a lower butyrate concentration and a thicker small intestine result in a higher butyrate concentration.

The fluid in the small intestine does not move with a constant flow from beginning to end, but moves forward with a wave like motion, called peristaltic movement. Peristalsis can have different propagation velocities and distances of spread depending on the segment of the small intestine. The luminal fluid spreads with a high velocity over a long distance in the duodenum, which makes it unclear to what extent mass transfer between the fluid pocket and the mucus takes place. Furthermore, it may be questionable whether butyrate comes into contact with the intestinal wall throughout the whole duodenum. The luminal fluid spreads with a moderate velocity over a shorter distance in the jejunum, which makes it more likely that butyrate comes into contact with a large area of the jejunum wall. The luminal fluid spreads with a slow velocity and a short distance in the ileum, which makes it likely that butyrate comes into contact with a large area of the ileum wall [22,24].

The incidence of an oral formulation passing intestinal segments without fluid pockets is unknown, while in our model we assume that the formulation is always present in a pocket with 6 mL fluid [15]. To build a more accurate model information is needed on the

interaction between oral formulations and the intestine which accurately reveals how an oral formulation moves through the small intestine and how the fluid in the small intestine moves through the small intestine in relation to the oral formulation.

CONCLUSION

Butyrate is thought to offer promising properties to treat noncommunicable diseases in which mucosal barrier disturbances are involved. Pharmacological data indicate that intestinal epithelial cells should be exposed to concentration in the range of 2–5 mM. This paper applies a mathematical model which enables estimation of the concentrations in the small intestine when administering either an immediate release or a sustained release (SR) oral formulation. The model shows that only SR formulations will be able to yield pharmacologically active concentrations along the whole small intestine, although these concentrations will get in contact with the intestinal epithelial cells relatively short.

The results can be used to develop an oral butyrate formulation with the right release characteristics to reach pharmacologically active concentrations. The SR formulation should have a release rate of 0.08 to 0.2 mmol/h and does not need a gastric resistant coating. This information is necessary to develop an oral formulation that has a chance to succeed in a clinical study. The anticipated release rates are expected to result in luminal butyrate concentrations that are high enough at the surface of the epithelial cells to improve the intestinal barrier and to have anti-inflammatory properties. However, it is uncertain if the duration of exposure, and quantity of exposed epithelial cells is adequate to have the desired clinical effect.

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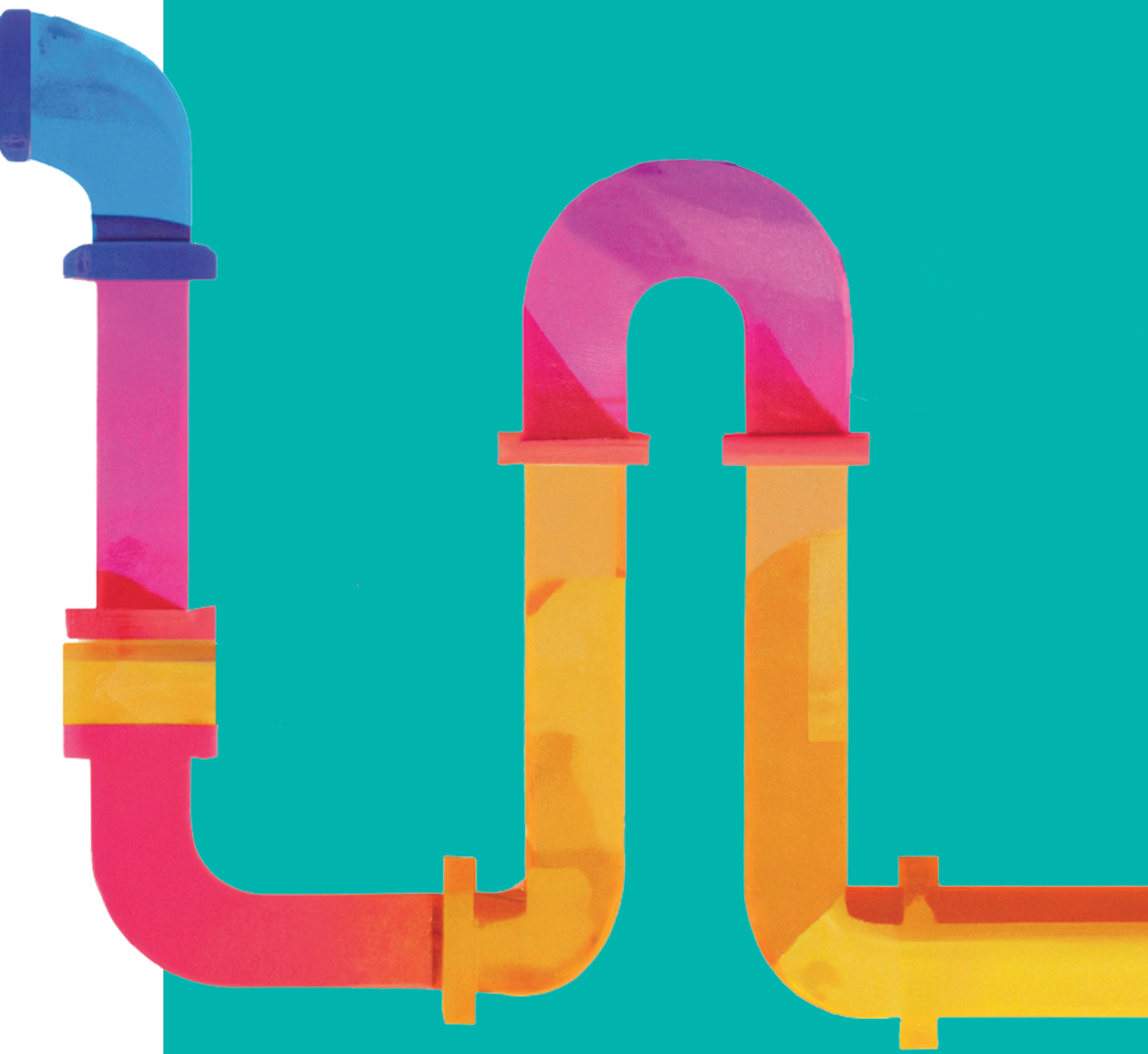
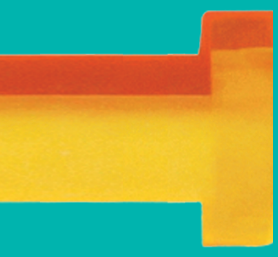
PHARMACEUTICAL DEVELOPMENT

CHAPTER

4



Characteristics of the drug product and the production process



Drug substance

As the drug substance in the sustained release butyrate tablet it was decided to use the calcium salt of butyrate and not the sodium salt. The sodium salt is more hygroscopic compared to the calcium salt which could cause problems during the tableting process.

Formulation

Based on the knowledge obtained in Chapter 2 and 3 of this thesis a butyrate containing drug product was developed, which effects were investigated in a clinical study. The qualitative and quantitative composition of butyrate (as calcium) 150 mg sustained release tablets is listed in Table 1. The tablets were coated with a taste masking coating, the composition of the taste masking coating is listed in Table 2. The taste masking coating dissolves at low pH in the stomach and therefore does not influence the dissolution of the butyrate. The tablets are round, shallow convex and have a diameter of 10.75 mm.

Table 1. *Composition of butyrate (as calcium) 150 mg sustained release tablets.*

Substance	Mass per tablet (mg)	Function
Calcium Butyrate*	192.51	Active ingredient
Hydroxypropyl Methylcellulose (Methocel E4M)	73.80	Matrix for sustained release
Silicified Microcrystalline Cellulose** (Prosolv SMCC90)	99.01	Filler, glidant
Magnesium Stearate Veg	3.69	Lubricant
Tablet cores Total	369.01	-
Taste masking coating (see Table 2)	10	Taste masking
Coated tabletsTotal	379.01	-

* Equals 150 mg butyrate and may be adjusted according to the results of the HPLC assay test in which mg butyrate per g raw material calcium butyrate is determined. The amount of silicified microcrystalline cellulose will be adjusted accordingly. ** The sum of the amount of calcium butyrate and silicified microcrystalline cellulose needs to be 291.52 mg per tablet.

Table 2. *Composition of taste masking coating.*

Substance	Mass per tablet (mg)*	Function
Talc	13.97	Anti-tacking
Titanium dioxide	7.50	Pigment
PEG6000	2.81	Plasticizer
Simethicon emulsion	0.47	Prevent foaming
Purified water**	139.20	Solvent
Eudragit RL 30 D**	40.00	Polymer
Triethylcitrate	2.40	Plasticizer

*An excessive amount of coating liquid was weighed for production to coat the tablets to a weight gain of 10 mg. ** Volatile excipients evaporate from the coating during the coating process. As such, 10 mg/tablet of nonvolatile excipients remain.

Description of manufacturing process and process controls

The formulations and the manufacturing of the products are standard procedures for oral solid formulations, using a direct compression method and coating process, see Figure 1.

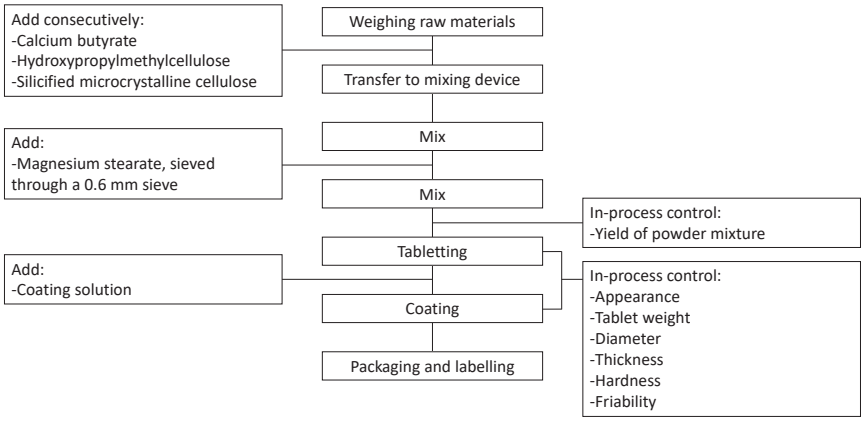


Figure 1. *Flowchart of the manufacturing process and process controls of butyrate (as calcium) 150 mg sustained release tablets.*

Drug product specifications

Table 3 shows the product specifications, test method, acceptance criteria and the test results of the clinical batch.

Table 3. *Overview of product specifications, test methods, acceptance criteria and the test results of butyrate (as calcium) 150 mg sustained release tablets.*

Test item	Method	Acceptance criteria	Test results
Appearance	Visual observation	Tablet, white to off white, 10.75 mm, round, biconvex, coated	Complies
Uniformity of weight (coated)	Ph. Eur.	379 mg (95 -105%)	381.2 mg (100.6%)
Identification: • Butyrate • Calcium	 • HPLC • Color reaction	 • Conform reference • Complies with Ph. Eur. 2.3.1.	 • Positive • Positive
Impurities: • Crotonic acid • Max. individual unknown impurities • Total impurities	HPLC	 • NMT 0.5% • NMT 0.2% • NMT 2.0%	 • <0.05% • 0.17% • 0.22%
Content uniformity	HPLC	• 85 -115% of declaration • Acceptance value L1 ≤15, T=100 Complies with Ph. Eur. 2.9.40	• 101.28% • L1=3.3
Assay butyrate	HPLC	• 95.0 – 105.0% of declaration • 142.5 – 157.5 mg/tablet	• 98.1% • 147.1 mg/tablet
Dissolution	HPLC	2 – 6 h: ≥0.08 mmol/h	0.15 mmol/h

HPLC: High Performance Liquid Chromatography; NMT: Not more than.

Justification specification dissolution

The proposed investigational medicinal product will be tested in a clinical study with osteoarthritis patients. It is hypothesized that these patients lack butyrate in the small intestine resulting in local inflammation in the gut and a reduced intestinal barrier. By giving these patients butyrate (as calcium) sustained release tablets we hypothesis that we can improve these symptoms. As described in Chapter 3 a sustained release formulation is capable of maintaining pharmacologically active concentrations for hours and thus throughout the whole small intestine. To reach these concentrations the sustained release formulation requires a zero order release rate of 0.08-0.2 mmol/h. The anticipated release rates are expected to result in luminal butyrate concentrations that are high enough at the surface of the epithelial cells to improve the intestinal barrier and to have anti-inflammatory properties.

When a sustained release tablet is ingested by a patient it will first reach the stomach and stay there for approximately 2 h, second the tablet will reach the small intestine which it will transit in approximately 4, thereafter the tablet will reach the colon. The pharmacologically relevant timepoint to study dissolution is therefore between 2 and 6 h.

When we link these two aspect, the desired release rate and the pharmacologically relevant timepoint, to a dissolution test performed as a quality control for the tablets after production we can set a specification for this dissolution test. As mentioned before the release rate of the tablets should be at least 0.08 mmol/h between 2 h and 6 h after the start of the dissolution.

PHARMACODYNAMICS – *IN VIVO & EX VIVO*

CHAPTER

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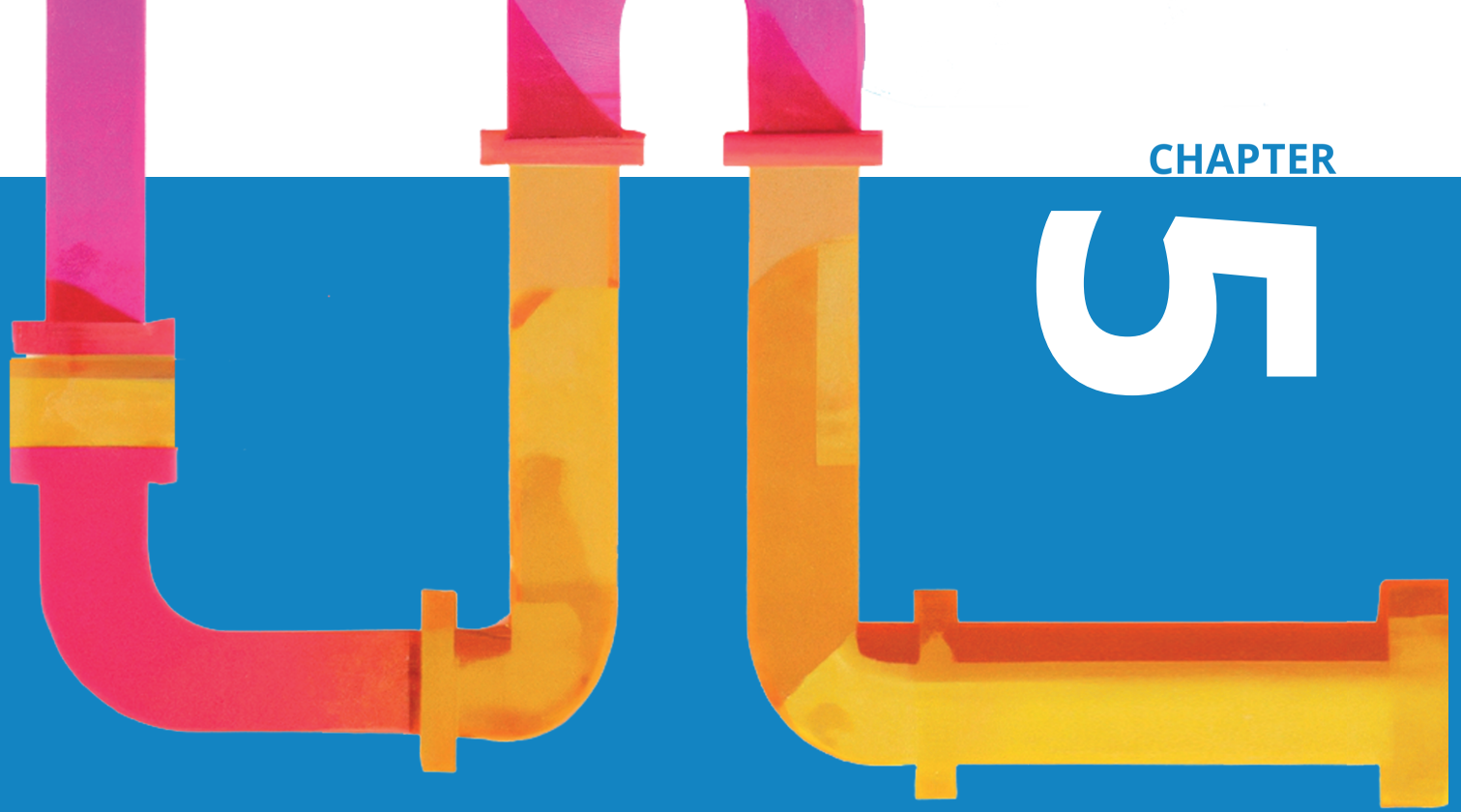

A sustained release butyrate tablet suppresses *ex vivo* T helper cell activation of osteoarthritis patients in a double blind placebo-controlled randomized trial

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ABSTRACT

Osteoarthritis (OA) is a degenerative joint disease which is characterized by degeneration of cartilage, synovial inflammation and low grade systemic inflammation. It is frequently associated with microbial dysbiosis and intestinal barrier defects. Butyrate is known for its anti-inflammatory and gut barrier protective effects and therefore it might be a good treatment option for OA patients, as currently no disease modifying treatment is available.

The aim of this study was to investigate the effects of four-five weeks oral treatment with sustained release butyrate tablets (600 mg/day) on systemic inflammation and immune function of patients with hand OA in a double blind placebo-controlled randomized trial. This was studied by measuring plasma or serum levels of systemic inflammation markers, lipopolysaccharide (LPS) leakage markers and *ex vivo* stimulation of whole blood or peripheral blood mononuclear cells (PBMCs). All parameters were measured at baseline and the end of the study.

Butyrate treatment did not affect markers of systemic inflammation nor LPS leakage, although these levels might have been too low to measure any effect. Neither any effect was observed of the butyrate treatment on *ex vivo* LPS stimulation of whole blood or PBMCs (TNF-α, IL-6, IFN-γ and/or IL-10 release) nor on restimulated monocytes (CD14+TNF-α+, CD14+IL-10+ and CD14+TLR4+). By contrast, butyrate treatment reduced the percentage of activated T helper (Th) cells (CD4+CD69+, CD4+CD25+ and CD4+CD25+FoxP3-) and lowered the Th17/Treg ratio in αCD3/CD28-activated PBMCs, although their cytokine release upon stimulation remained unaffected. Nevertheless, the percentage of CD4+IL9+ cells was reduced in the group of patients treated with butyrate. In both the butyrate and placebo group the frequency of Th1, Treg, Th17, activated Th17, CD4+IFNγ+ and CD4+TNFα+ cells was reduced.

This study shows proof of principle of some immunomodulatory effects using sustained release butyrate treatment by hand OA patients. The inflammatory potential of Th cells was reduced as indicated by a reduced percentage of activated Th cells and Th9 cells, and by an improved Th17/Treg balance in *ex vivo* αCD3/CD28 activated PBMCs. Future studies are warranted to further optimize the butyrate dose regime to ameliorate inflammation in OA patients.

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease with rising prevalence [1]. OA can be classified as a non-communicable disease and is characterized by degeneration of cartilage, synovial inflammation and low grade systemic inflammation [2]. Clinical evidence shows that this inflammation is associated with microbial dysbiosis and intestinal barrier defects [1,3–7]. It has been hypothesized that dysbiosis of the microbiome leads to local inflammation in the gut and increased intestinal permeability leading to influx of bacterial components such as lipopolysaccharides (LPS) into the bloodstream, resulting in systemic low grade inflammation [8,9]. This systemic inflammation might attribute to the development of OA resulting in joint pain [10–15]. Furthermore, the inflammation might cause a positive feedback loop by maintaining the intestinal barrier defect, thereby facilitating a continuous influx of LPS and consequently sustaining inflammation and in this way driving the pathology of OA. Markers for systemic LPS leakage are amongst others LPS-IgG and LPS binding protein (LBP). A rise in serum LBP is associated with increased knee OA progression [9]. Additionally, the microbiome is known for its capacity to produce biologically active immunomodulatory molecules such as short chain fatty acids (SCFAs) by fermentation of fibers [1,4,10]. Dysbiosis of the microbiome might lead to altered production of SCFAs, although the latter has not been confirmed in OA patients yet [16–18].

In the joints of OA patients, activated cells of the innate and adaptive immune system are found, and the synovial fluid contains increased concentrations of inflammatory mediators which can affect homeostasis in the cartilage [19–21]. In particular, activated macrophages and T helper (Th)1, Th17 and Th9 cells play a role in the pathophysiology of OA [22,23], with LPS being the main driver of macrophage activation. Increased levels of IL-17a, IL-9, and Th17 and Th9 cells can be observed in the circulation of OA patients and are associated with disease activity [23–26]. In addition, regulatory T-cell (Treg) function and Treg/Th17 balance may be disturbed [25,27].

Currently, no disease modifying treatment is available for OA patients and the current standard therapy consists of patient education, exercise therapy and pain medication [28,29]. The SCFA butyrate is known for its anti-inflammatory and barrier improving properties and could therefore be a possible treatment for patients with OA [30–32]. In particular when considering intestinal dysbiosis, which might cause butyrate shortage in the intestine of these patients [16-18].

The direct effects of butyrate on peripheral blood mononuclear cells (PBMCs) or innate immune cells like monocytes, which are pre-cursors for tissue-resident macrophages, have previously been studied. In these studies butyrate was shown to reduce the release of various pro-inflammatory cytokines by activated PBMCs and to modulate immune cell phenotypes [33–41]. In addition, butyrate can improve the intestinal epithelial barrier,

protect against inflammatory mediated barrier disruption and suppress activation of epithelial cells *in vitro* [36,42–45]. It should be realized that in these models there was direct and constant contact of butyrate with the cells. So far it is unknown whether oral pharmacotherapy with butyrate can establish an anti-inflammatory response in human extraintestinal pathologies associated with intestinal barrier defects such as patients with OA.

The aim of this study was to investigate the anti-inflammatory properties of sustained release butyrate tablets in patients with hand OA, within a double blind placebo-controlled randomized trial. The sustained release butyrate tablet was developed to release sufficient amounts of butyrate that in theory should be able to achieve pharmacologically active concentrations along the small intestine [46]. We hypothesized that this dosage form and dose of butyrate will have beneficial effects on the intestinal barrier and via this way on the influx of LPS and systemic inflammation which would affect the inflammatory potential of monocytes and Th cells and the Th17/Treg cell balance as well. In this manuscript we outline the effect of the sustained release butyrate tablet on *in vivo* and *ex vivo* immune parameters.

METHODS

Participants

Thirty three patients participated in this study, after giving their informed consent. The clinical study was approved by the Ethical Committee of the Radboud University Medical Center (Nijmegen, The Netherlands, protocol number: NL73382.091.21) and was conducted in full accordance with the principles of the Declaration of Helsinki. The clinical trial was registered in the European Union Clinical Trials Register with reference code 2020-001071-33 and conducted at the Sint Maartenskliniek (Ubbergen, the Netherlands). To be eligible for inclusion in the study, participants had to be ≥ 50 and ≤ 80 years of age, have a Body Mass Index (BMI) >20 and <30 kg/m2 and have hand OA according to the 1990 ACR diagnostic criteria for hand OA in both hands. Pain scored by the numeric pain rating scale (NRS) during hand activity needed to be ≥4 ≤8 (scale 0-10), during 15 of the last 30 days. Exclusion criteria were use of antibiotics within three months before the start of the study, use of NSAIDs, use of immunosuppressants, previous surgery of one of the hands, a cerebro- or cardiovascular incident within 6 months before the start of the study. Furthermore, diabetes or other chronic inflammatory diseases or autoimmune diseases, cognitive deficits affecting the scoring process, fibromyalgia or any other syndrome or condition that could interfere with the assessment of pain. In addition, severe current psychiatric disorders assessed by a physician, self-reported consumption of >2 units of alcohol per day, intramuscular or intraarticular corticosteroid injections within four weeks before the start of the study, or estimated glomerular filtration rate (eGFR)<30 mL/min/1.73m2 and alanine aminotransferase (ALAT) < 1.5 ULN were exclusion criteria.

Design double blind placebo-controlled randomized clinical trial

The patients participated in a double blind randomized placebo-controlled study and were randomly allocated to either 150 mg butyrate (as calcium) sustained release tablets (Tiofarma B.V., Oud-Beijerland, The Netherlands) or matching placebo tablets (Tiofarma B.V.). Twice a day two tablets (thus 600 mg butyrate/day in total) were taken for approximately four weeks (26-35 days). Excipients used in the tablet core were hydroxypropyl methylcellulose, silicified microcrystalline cellulose and magnesium stearate and the tablets were coated with a taste masking coating consisting of talc, titanium dioxide, polyethylene glycol 6000, simethicone emulsion, Eudragit RL 30 D and triethyl citrate. The tablet was formulated to release >0.08 mmol of butyrate per h within the time frame of 2 to 4 h post-ingestion, corresponding with the time frame the tablet is present in the small intestine [46]. Whole blood was collected in 4x10 mL heparin tubes, 1x3 mL heparin gel tube and 1x10 mL clot tube at the beginning (visit 1) and at the end of the study (visit 2) (Figure 1A). The blood in the 3 mL heparin gel tube was used to measure plasma levels of high sensitive C-reactive protein (hsCRP). The clot tubes were spun down and serum was stored in cryovials at -80°C until further basal serum measurements. The blood in the 10 mL heparin tubes was used for two follow up experiments. First, the whole blood was stimulated with LPS for 24 h and IL-10 and TNF-α were measured in the blood plasma. Second, PBMCs were isolated from the whole heparin blood and used for experiments in which the PBMCs were stimulated to measure cytokine release and to identify immune cell phenotypes (see experimental scheme Figure 1B-D). The effects on clinical parameters and the intestinal microbiome will be presented in another manuscript.

Basal serum and plasma hsCRP measurements

Serum was collected at baseline (visit 1) and end of the study (visit 2) to measure LPS-binding protein (LBP), LPS IgG, soluble CD14 (sCD14), soluble TNF-receptor 1 (sTNFR1), soluble TNF-receptor 2 (sTNFR2), nitrite, nitrate, total nitric oxide (NO), IL-6 and IL-1β. LBP (Thermo Fisher Scientific), IgG LPS (Hycult Biotech, Uden, The Netherlands) and sCD14 (R&D systems (Minneapolis, MN, USA) were measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturers instruction. Optical density was measured using a CLARIOstar Microplate Reader (BMG Labtech, Ortenberg, Germany). IL-6, IL-1β (Millipore, Milliplex human bone magnetic bead panel), sTNFR1 and sTNFR2 (Millipore, Milliplex human soluble cytokine receptor magnetic bead panel) were measured using an Antibody-Immobilized Beads immunoassay according to the manufacturers instruction and analyzed using a Bio-Plex 200 (Bio-Rad, Hercules, CA, USA). hsCRP was determined using the chemical analyzer Olympus type AU400.

Whole blood stimulation

On the same day as the whole heparin blood samples were drawn from the patients, whole blood samples were diluted 1:1 with plain RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) or 2 μg/mL LPS (#tlrl-3pelps, Invivogen, Toulouse, France) in RPMI-1640 in triplo

in a autoclaved screw cap micro tube (Sarstedt, Nümbrecht, Germany). Each tube was closed and gently mixed, thereafter the cap was unscrewed a quarter turn, to make sure the tubes were not completely closed. Tubes were incubated for 24 h in an incubator at 37 °C and 5% CO_2 (Figure 1B). After 24 h stimulation the tubes were centrifuged for 10 min at 150 x g at 20 °C followed by 10 min at 700 x g at 20 °C in a Eppendorf centrifuge 5424 R. Blood plasma was transferred to a clean tube and stored at -80 °C until measurement of IL-10 and TNF-α using ELISA.

PBMC isolation

On the same day as the whole heparin blood samples were drawn from the patients, PBMCs were isolated. First, the whole blood was diluted 1:1 with Phosphate buffered Saline (PBS) (Lonza, Basel, Switserland) supplemented with 2% heat inactivated Fetal Calf Serum (FCS) (Biowest, Ennigerloh, Germany) at room temperature. Second, the diluted blood was carefully dripped on the porous membrane of the leucosep tubes (Greiner Bio-One, Kremsmünster, Austria) followed by 13 min centrifugation at 1000 x g at 20°C using a Eppendorf centrifuge 5810 R with the acceleration and deceleration set at 4. Third, the enriched cell fraction of PBMCs was washed twice using PBS+2%FCS. PBMCs were resuspended in RPMI-1640+20%FCS and diluted with an equal volume of ice cold RPMI-1640+20%FCS+20%DMSO (Sigma-Aldrich) in a cryovial (maximum of $3x10^7$ PBMCs/mL per cryovial). Cryovials (Corning, NY, USA) were frozen using a CoolCell® LX (Corning) and stored at -80 °C until used in the PBMC stimulation experiments.

PBMC stimulation

PBMCs were quickly thawed and washed with culture medium consisting of RPMI-1640 supplemented with 2.5% FCS and 1% Penicillin/Streptomycin (10000U/mL/10000 µg/mL) (Gibco, Invitrogen, Carlsbad, CA, USA). After washing, cells were counted and diluted to a final concentration of $1x10^6$ cells/mL and transferred to a 12 well suspension plate (Greiner). Visit 1 and visit 2 of the same patient were kept in the same well plate and for each patient two plates were prepared. Cells were let to rest for 1 h in an incubator at 37 °C and 5% CO_2 before stimulations. Cells were stimulated with 1 µg/mL LPS or αCD3 (150 ng/mL) combined with αCD28 (100 ng/mL) (BD Biosciences, San Jose, CA, USA). After 19 h one plate of each patient was spun down at 1200 rpm for 5 min at 20 °C in a Eppendorf centrifuge 5810 R and the supernatant was carefully discarded. Cells were restimulated with 1 µg/mL golgiplug (BD Biosciences), 5 ng/mL phorbol myristate acetate (PMA) (Sigma-Aldrich) and 750 ng/mL ionomycin (Sigma-Aldrich) in culture medium, while plain culture medium was added to control cells for an additional 5 h at 37 °C and 5% CO_2. At 24 h the experiments were ended and both plates were spun down at 1200 rpm for 5 min at 20°C. Supernatant of the plates without restimulation were stored at -80°C until measurement of IL-6, IL-10, IL-17a, TNF-α, IFN-γ and IL-9 using ELISA. Cells were resuspended in cold PBS and transferred to a U bottom 96 wells plate (Corning, Falcon) for FACS staining and analysis (Figure 1C,D). Left over cells were pooled and used for fluorochrome minus one (FMO) controls.

FACS analysis

Four FACS panels were used. In panel 1 PBMCs were stained with CD4 PerCP-Cyanine5.5, CD25 Alexa Fluor 488, CD127 PE-Cyanine7, FOXP3 eFluor 660, RORγ (t) PE (All Thermo Fisher Scientific, Waltham, MA, USA) and matching isotypes. In panel 2 PBMCs were stained with CD4 PerCP-Cyanine5.5, CD69 PE, CD196 (CCR6) APC (All Thermo Fisher Scientific), CD183 (CXCR3) Alexa Fluor 488 (BD Biosciences), and matching isotypes. In panel 3 PBMCs were stained with CD14 APC (Thermo Fisher Scientific), TLR4 PE (BD Biosciences), IL-10 Brilliant Violet 421, TNF-α Brilliant Violet 510 (both BioLegend, San Diego, CA, USA) and matching isotypes. And in panel 4 PBMCs were stained with CD4 PerCP-Cyanine5.5 (Thermo Fisher Scientific), IL-17a Alexa Fluor 488, IL-9 PE (both BD Biosciences), IFN-γ Alexa Fluor 647 (all three BioLegend), IL-10 Brilliant Violet 421, TNF-α Brilliant Violet 510 and matching isotypes. PBMCs in the U bottom 96 well plate were first washed with PBS and incubated for 30 min at 4 °C with Fixable Viability dye eFluorTM 780 (Thermo Fisher Scientific) in PBS. This was followed by blocking the cells with Fc block (BD Biosciences) for 10 min at 4 °C. After blocking the cells were incubated for 45 min with appropriate extracellular antibody solution at 4°C protected from light and washed with 1% bovine serum albumin (Roche) in PBS. Panel 1 was fixed overnight at 4°C protected from light with FOXP3 fixation/permeabilization buffer (Thermo Fisher Scientific). Panel 2 was fixed with 1:4 diluted intracellular fixation buffer (Life Technologies, Themo Fisher Scientific) in PBS. And Panel 3 and 4 were fixed with undiluted intracellular fixation buffer. The next day, PBMCs were washed and blocked for 10 min at 4 °C protected from light. After blocking appropriate intracellular antibody solution was added for 45 min at 4 °C protected from light and washed. All panels were measured with a BD FACS Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and data was analyzed using Flowlogic software Version 8 (Inivai Technologies, Mentone, Australia). In addition, Compensation beads (UltraComp eBeadsTM Plus, Life technologies) were stained with 1 μL of each antibody for 45 min at 4°C and washed twice with FACS buffer. After washing, beads were measured with the flow cytometer to be used for compensation in the analysis. See supplemental Table S1 for the titrated dilutions of the antibodies used.

ELISA of whole blood plasma and PBMC supernatant

TNF-α, IL-10, IL-6, IL-9, IL-17a and IFN-γ ELISA (Thermo Fisher Scientific) was performed according to the manufacturers instruction. In short, high binding 96 wells plates (Corning Costar 9018) were coated with capture antibody and incubated overnight at 4 °C. The next day, plates were washed, blocked and samples and standard were incubated for 2 h. After washing, detection antibody was added to the wells and incubated for 1 h, followed by (strept)avidin-HRP for 30 min protected from light. After another round of washing, TMB solution was added and the color reaction was stopped with 2N H2SO4. Optical Density was measured using a Glomax® Discover Microplate Reader (Promega Corporation, Madison, WI, USA).

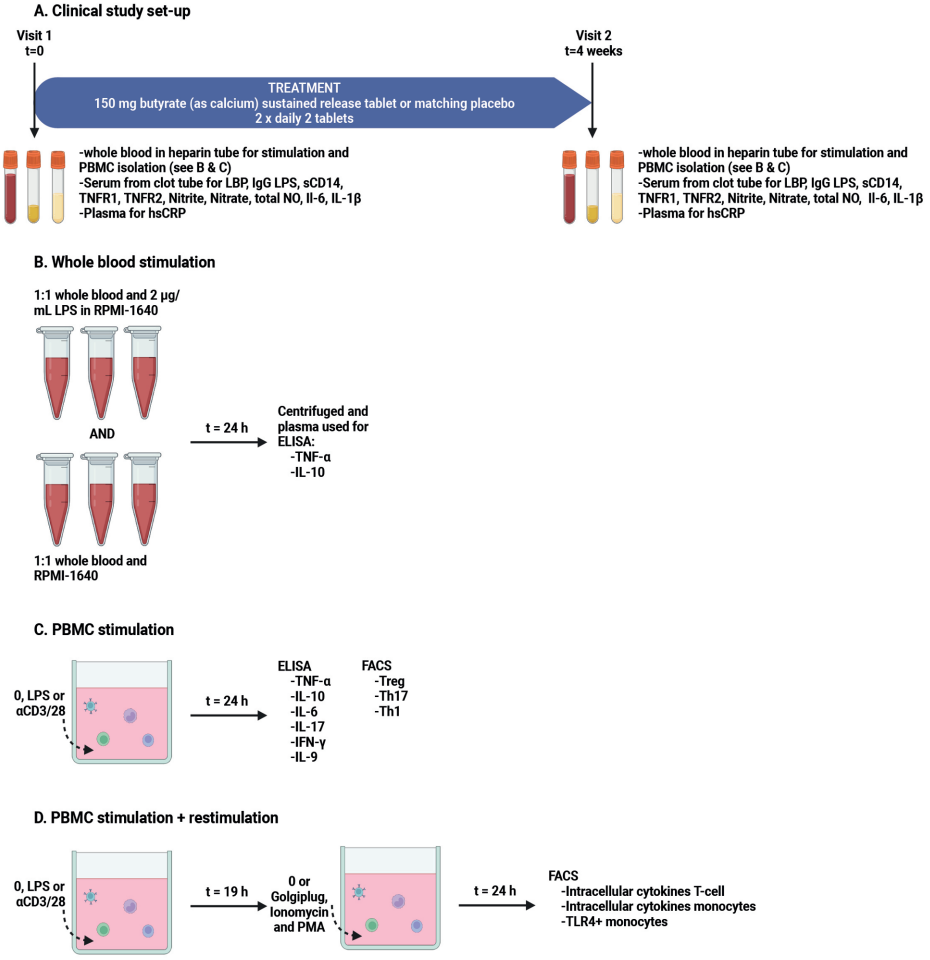


Figure 1. *Clinical study set-up (A) and set-up of the methods used to stimulate and analyze the whole blood (B) and peripheral blood mononuclear cells (C,D). ELISA: enzyme-linked immunosorbent assay, hsCRP: high sensitive C-reactive protein, IFN-γ: interferon-gamma, IL: interleukin, LPS: lipopolysaccharides, LBP: LPS binding protein, NO: Nitric oxide, PBMC: peripheral blood mononuclear cell, PMA: phorbol myristate acetate, Th: T helper cell, TNF-α: Tumour Necrosis Factor alpha , TNFR: TNF receptor, Treg: regulatory T-cell.*

Statistical analysis

Differences between visit 1 of the placebo group and visit 1 of the butyrate treated group, and between visit 2 of the placebo group and visit 2 of the butyrate treated group, were assessed using an Ordinary one-way ANOVA, with selected pairs followed by Bonferroni's post hoc test for normally distributed data or a Kruskal-Wallis test with selected pairs for not normally distributed data. Differences within the

treatment groups, thus between visit 1 and visit 2 for the placebo or butyrate treated group were assessed using a paired Student's t-test if data was normally distributed. If not normally distributed a Wilcoxon matched pairs signed rank test was used. In addition, of each parameter the Δvisit 2-visit 1 was calculated. Differences between Δvisit 2-visit 1 of the placebo group and Δvisit 2-visit 1 of the butyrate treated group were assessed using an unpaired Student's t-test for normally distributed data or the Mann-Whitney test for not normally distributed data. Results are presented as means ± SEM. Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA). Results were considered statistically significant when $p < 0.05$. Significant differences are shown in the figures as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$.

RESULTS

Baseline characteristics

Thirty-three patients (27 females and 6 males, age 50 to 74 years) participated in this study of which sixteen received sustained release butyrate tablets and seventeen placebo tablets. Patient characteristics at baseline are described in Table 1. In the sustained release butyrate treated group half of the patients had hand OA for less than five years, whereas this was a quarter of the group given placebo.

Table 1. *Patient characteristics at baseline, if applicable values are given as the mean +/- SD K&L: Kellgren&Lawrence.*

	Placebo (N = 17)	Sustained release butyrate (N = 16)
Age (years)	63.3 ± 8.3	61.6 ± 5.0
BMI (kg/m^2)	25.5 ± 2.7	26.0 ± 2.5
Female (N)	13	14
Disease duration <5 years (N)	4	8
Number of joints K&L≥2 (0-30 joints)	8 ± 5	8 ± 4

Effect of butyrate supplementation on systemic inflammation and LPS influx

It was hypothesized that butyrate treatment could improve the intestinal barrier, which potentially might lead to a decreased influx of LPS and as a consequence reduced systemic inflammation. To test this hypothesis, systemic levels of hsCRP, LBP and LPS IgG were measured at visit 1 and visit 2. No significant changes in hsCRP, a measure for systemic inflammation, were observed during the study period in both treatment groups (Figure 2A). Similar results were obtained for LBP and LPS IgG, measures for LPS

leakage (Figure 2B). Soluble CD14 (sCD14), TNF receptor 1 (TNFR1), TNF receptor 2 (TNFR2), nitrite, nitrate and total nitric oxide (NO) are biomarkers shown to be elevated in association with chronic systemic inflammation. These levels were not affected by treatment with butyrate nor placebo tablets (Supplemental Figure S1), whereas IL-6 and IL-1β were not detectable.

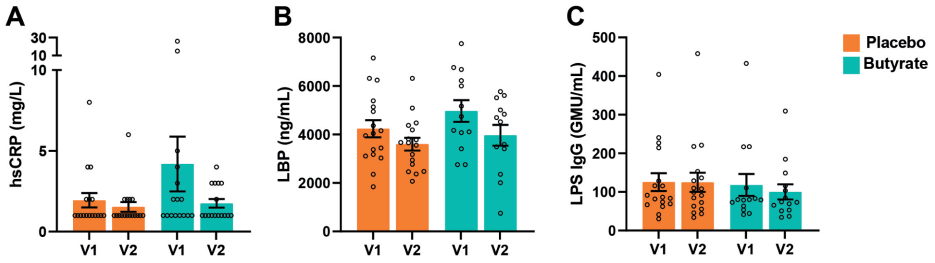


Figure 2. *(A) hsCRP levels in the plasma of patients, (B) LBP levels and (C) LPS IgG levels in the serum of patients at baseline (V1) and the end of the study (V2). Orange bars indicate serum levels from patients who received placebo and blue bars indicate patients who received butyrate. Data are presented as mean ± SEM (N = 17 placebo group, N = 13-16 butyrate group).*

Effect of butyrate supplementation on LPS induced activation of whole blood and PBMCs

The effect of four weeks oral sustained release butyrate supplementation on *ex vivo* LPS stimulation of whole blood and PBMCs was evaluated. To monitor basal effects on monocytes, intracellular IL-10 and TNF-α measurements were performed in the monocytes within the ionomycin-PMA-golgiplug restimulated PBMC. LPS stimulation of the whole blood induced the release of IL-10 and TNF-α compared to the negative control (Supplemental Table S2). IL-10 and TNF-α release were both not affected by butyrate treatment for four weeks, neither did the placebo (Figure 3). To study intracellular IL-10 and TNF-α in monocytes, PBMCs were restimulated with golgiplug, ionomycin and PMA. All used stimulations and restimulations did not affect cell viability (Supplemental Figure S2). The percentage of monocytes in the PBMC mixture was approximately 1%, which was lower than expected, although still clearly visible as a separate population in the FACS gating (Figure 4E). LPS stimulation impacted the monocyte gating and was therefore not used (data not shown). Butyrate treatment did not affect intracellular IL-10 and TNF-α expression in the ionomycin-PMA-golgiplug restimulated monocytes, neither did the placebo (Figure 4A,B,E). In addition, the expression of LPS receptor TLR4 on monocytes was investigated. Both, the percentage of TLR4+ monocytes and the mean fluorescence intensity of TLR4, which indicated the level of TLR4 expression on monocytes, were not affected by butyrate treatment nor placebo (Figure 4C-E).

Also isolated PBMCs were stimulated with LPS which induced the release of IL-10, TNF-α, IL-6 or IFN-γ, but not IL-9 or IL-17a, compared to non-stimulated PBMC (Supplemental Table S2). Sustained release butyrate treatment did not affect *ex vivo* IL-10, TNF-α, IL-6 or IFN-γ release of LPS-stimulated PBMCs, neither did the placebo (Figure 5).

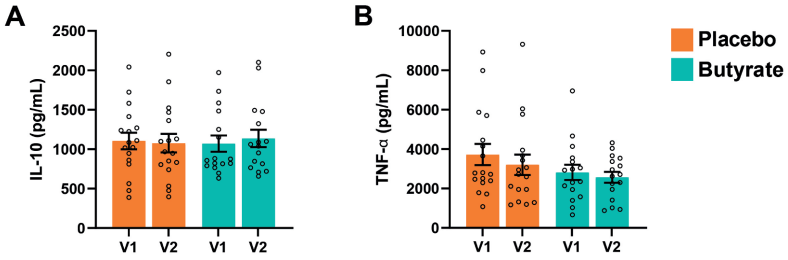


Figure 3. *Ex vivo LPS stimulation of whole blood samples to induce IL-10 (A) and TNF-α (B) plasma concentrations. Orange bars indicate whole blood samples from patient who received placebo and blue bars indicate patients who received butyrate. Data are presented as mean ± SEM (N = 17 placebo group, N = 16 butyrate group). V1: Visit 1 (baseline), V2: Visit 2 (end of study).*

Effect of butyrate supplementation on Th cells

To study the effect of sustained release butyrate supplementation on the *ex vivo* T-cell activation, PBMCs were stimulated with αCD3/CD28. Within the CD4+ Th cell subset, only αCD3/CD28 stimulation of PBMCs induced the percentage of RORγ+ Th17 cells, CD25+FoxP3+ Treg, and CD25+ and CD25+FoxP3- activated effector Th cells compared to non-stimulated PBMCs (Supplemental Table S3). LPS stimulation of PBMCs did not induce these T-cell phenotypes (Supplemental Table S3). The frequencies of RORγ+ Th17 cells and CD25+FoxP3+ Treg were reduced at visit 2 compared to visit 1 after sustained release butyrate treatment and placebo use. Whereas the frequencies of CD25+ and CD25+FoxP3- activated Th cells were only reduced after butyrate treatment and not after placebo (Figure 6). However, no significant difference was observed between the butyrate group and the placebo group when comparing ΔV2-V1 of both groups (Supplemental Table S3). In addition, the Th17/Treg balance was only reduced in the patient group treated with sustained release butyrate which was not observed after placebo use. Although, again no significant difference was observed comparing ΔV2-V1 of the butyrate and the placebo treated groups (Supplemental Table S3).

Furthermore, αCD3/CD28 stimulation of PBMCs enhanced the frequency of CD69+ activated Th cells, CCR6+CXCR3- Th17 cells, CD69+CCR6+CXCR3- activated Th17 cells, CD69+CXCR3+ Th1 cells within the CD4+ population compared to non-stimulated PBMCs (Supplemental Table S3). LPS stimulation of PBMCs did not induce these T-cell phenotypes (Supplemental Table A3).

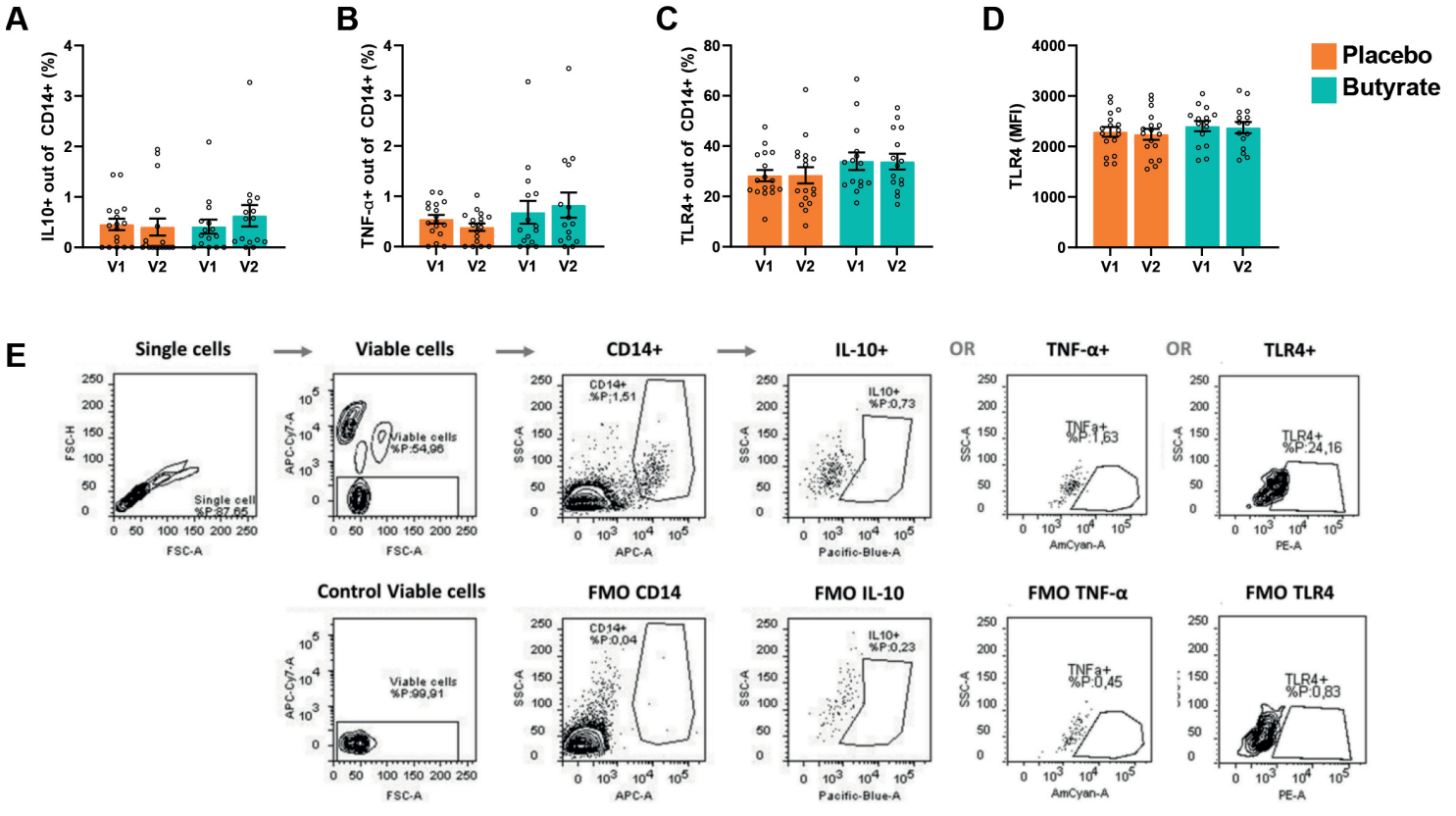


Figure 4. *FACS analysis of restimulated PBMCs. (A) percentage of IL-10+ cells out of CD14+ cells, (B) percentage of TNF-α+ cells out of CD14+ cells, (C) percentage of TLR4+ cells out of CD14+ cells, (D) Mean fluorescence intensity (MFI) of TLR4 and (E) the used gating strategy with corresponding fluorescence minus one (FMO) controls for a representative sample. Orange bars indicate whole blood samples from patient who received placebo and blue bars indicate patients who received butyrate. Data are presented as mean ± SEM (N = 17 placebo group, N = 15 butyrate group). V1: Visit 1 (baseline), V2: Visit 2 (end of study).*

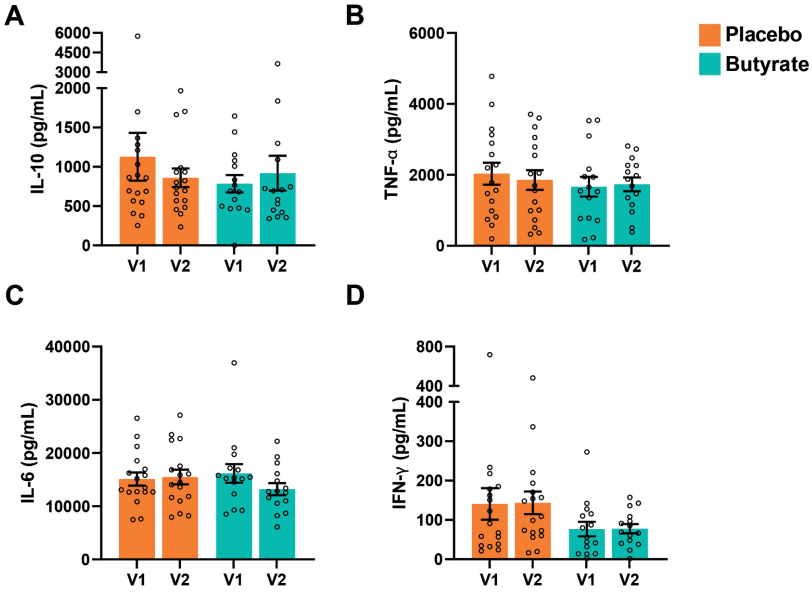


Figure 5. *IL-10 (A), TNF-α (B), IL-6 (C) and IFN-γ (D) release of LPS-stimulated PBMCs measured with ELISA. Orange bars indicate whole blood samples from patient who received placebo and blue bars indicate patients who received butyrate. Data are presented as mean ± SEM (N = 17 placebo group, N = 15 butyrate group). V1: Visit 1 (baseline), V2: Visit 2 (end of study).*

The percentage of CD69+ activated Th cells was reduced after use of sustained release butyrate tablet and not after use of placebo (Figure 7A,E). However, no significant effect was observed comparing ΔV2-V1 of CD69+ activated Th cells between the placebo and the butyrate group (Supplemental Table S2). Similar to RORγ+ T-helper (Th) 17 cells, the percentages of CCR6+CXCR3- Th17 cells and CD69+CCR6+CXCR3- activated Th17 cells were reduced at visit 2 compared to visit 1 in the *ex vivo* αCD3/CD28-activated PBMC of OA patients receiving sustained release butyrate supplementation and after placebo use as well (Figure 7C,D). In addition, the frequency of CD69+CXCR3+ activated Th1 cells was reduced both after use of sustained release butyrate as well as the placebo (Figure 7B).

Even though some T-cell phenotypes were affected by the treatments, no effects of sustained release butyrate nor placebo were observed on the release of regulatory cytokine IL-10 and inflammatory cytokines TNF-α, IL-6, IFN-γ, IL-9 or IL-17a by αCD3/CD28-stimulated PBMCs (Supplemental Table S3). However, when studying intracellular cytokine expression in CD4+ T-cells within the αCD3/CD28-stimulated PBMCs significant effects were observed (Figure 8). These PBMCs were restimulated with ionomycin and PMA in the presence of golgiplug enabling the intracellular measurement of IL-10+, TNF-α+, IL-6+, IFN-γ+, IL-9+ or IL-17a+ in Th cells compared to non-activated PBMCs (Supplemental Table S3). Sustained release butyrate nor placebo affected the percentage of IL-10+ or IL-17a+ cells, but both reduced the percentage of IFN-γ+ and TNF-α+ Th cells. In addition, sustained release butyrate, but not placebo, reduced the percentage of IL-9+ Th cells. However, no significant effect was observed comparing ΔV2-V1 of the percentage IL-9+ Th cells between the placebo and the butyrate group (Supplemental Table S2).

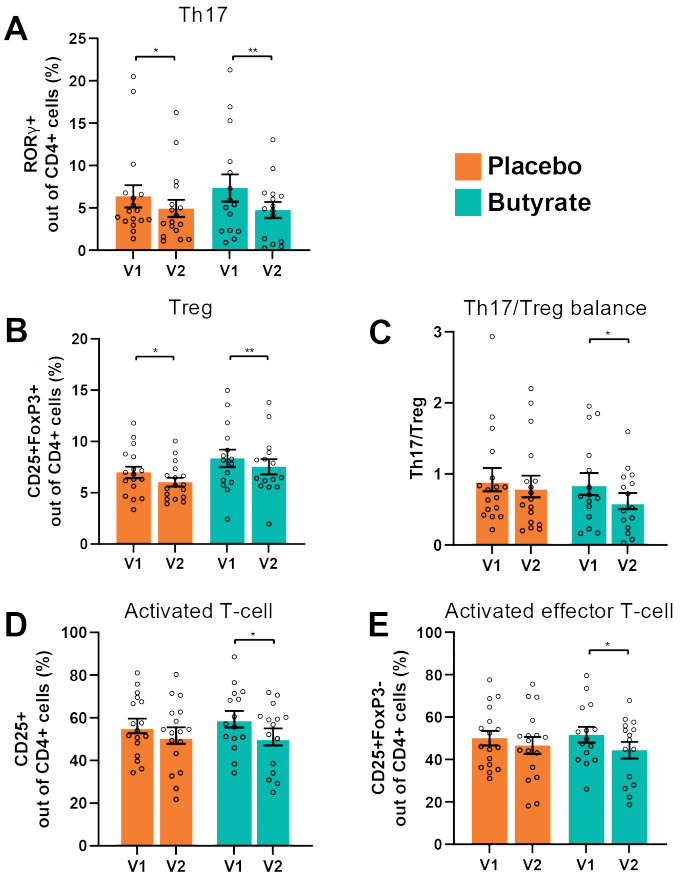


Figure 6. *See figure legend on page 141.*

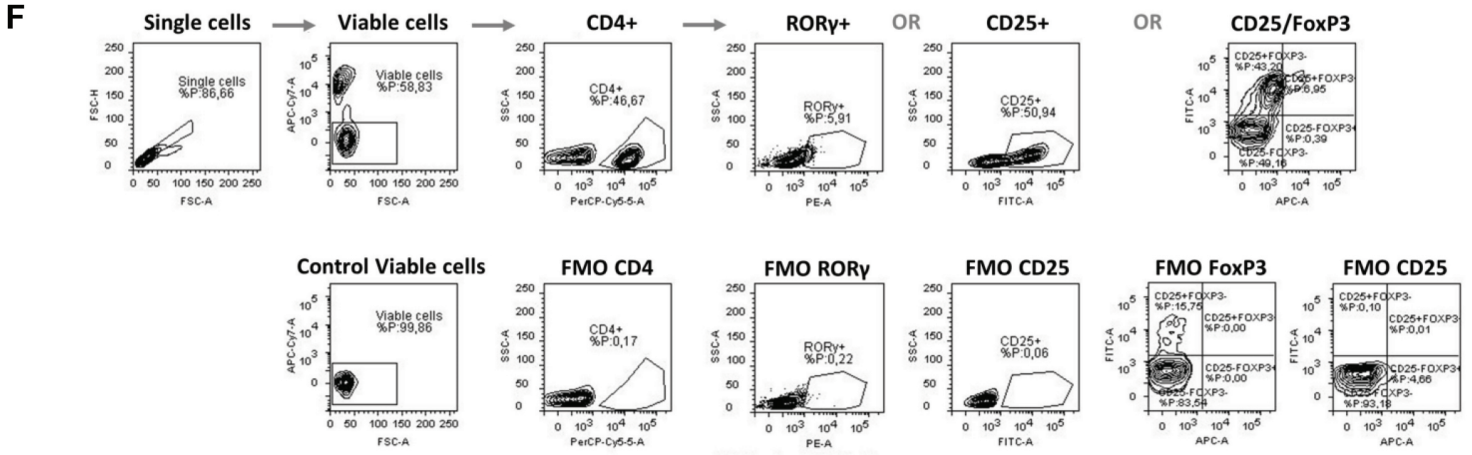


Figure 6. *FACS analysis of αCD3/CD28-stimulated PBMCs. (A) percentage of RORγ+ cells out of CD4+ cells (Th17 cells), (B) percentage of CD25+FoxP3cells out of CD4+ cells (Treg cells), (C) Th17/Treg balance, (D) percentage of CD25+ cells out of CD4+ cells (activated T-cells), (E) percentage of CD25+FoxP3- cells out of CD4+ cells (activated T-cells), and (F) the used gating strategy with corresponding fluorescence minus one (FMO) controls for a representative sample. Orange bars indicate whole blood samples from patient who received placebo and blue bars indicate patients who received butyrate. Data are presented as mean ± SEM (N = 17 placebo group, N = 15 butyrate group). Significant differences are shown as * $p < 0.05$, ** $p < 0.01$. V1: Visit 1 (baseline), V2: Visit 2 (end of study).*

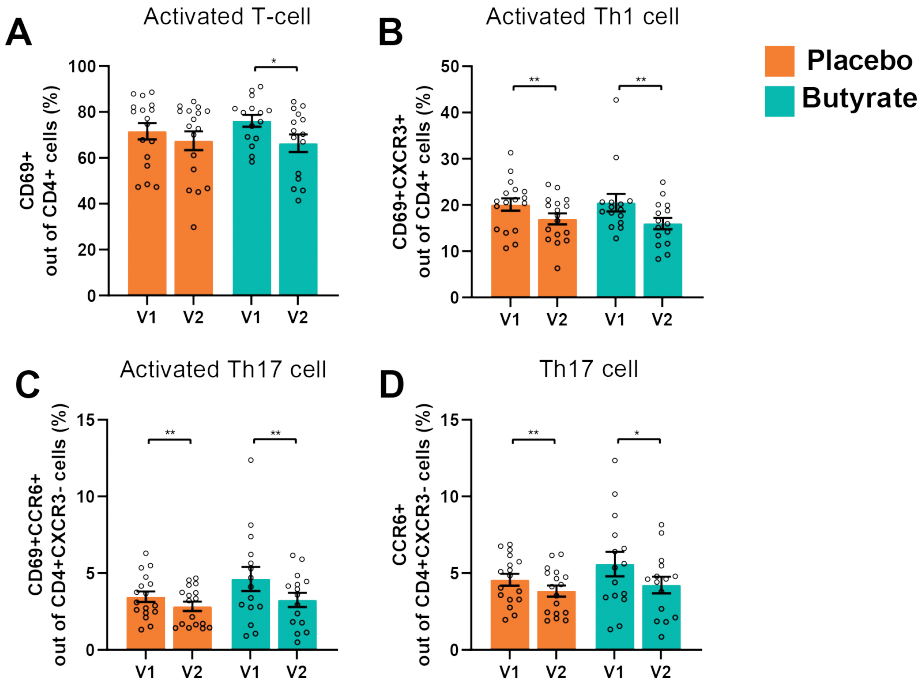


Figure 7. *FACS analysis of αCD3/CD28-stimulated PBMCs. (A) percentage of CD69+ cells out of CD4+ cells (Activated T-cells), (B) percentage of CD69+CXCR3+ cells out of CD4+ cells (activated Th1 cells), (C) percentage of CD69+CCR6+ cells out of CD4+CXCR3- cells (activated Th17 cells), (D) percentage of CCR6+ cells out of CD4+CXCR3- cells (Th17 cells), and (E) the used gating strategy with corresponding fluorescence minus one (FMO) controls for a representative sample. Orange bars indicate whole blood samples from patient who received placebo and blue bars indicate patients who received butyrate. Data are presented as mean ± SEM (N = 17 placebo group, N = 15 butyrate group). Significant differences are shown as * p < 0.05, ** p < 0.01. V1: Visit 1 (baseline), V2: Visit 2 (end of study).*

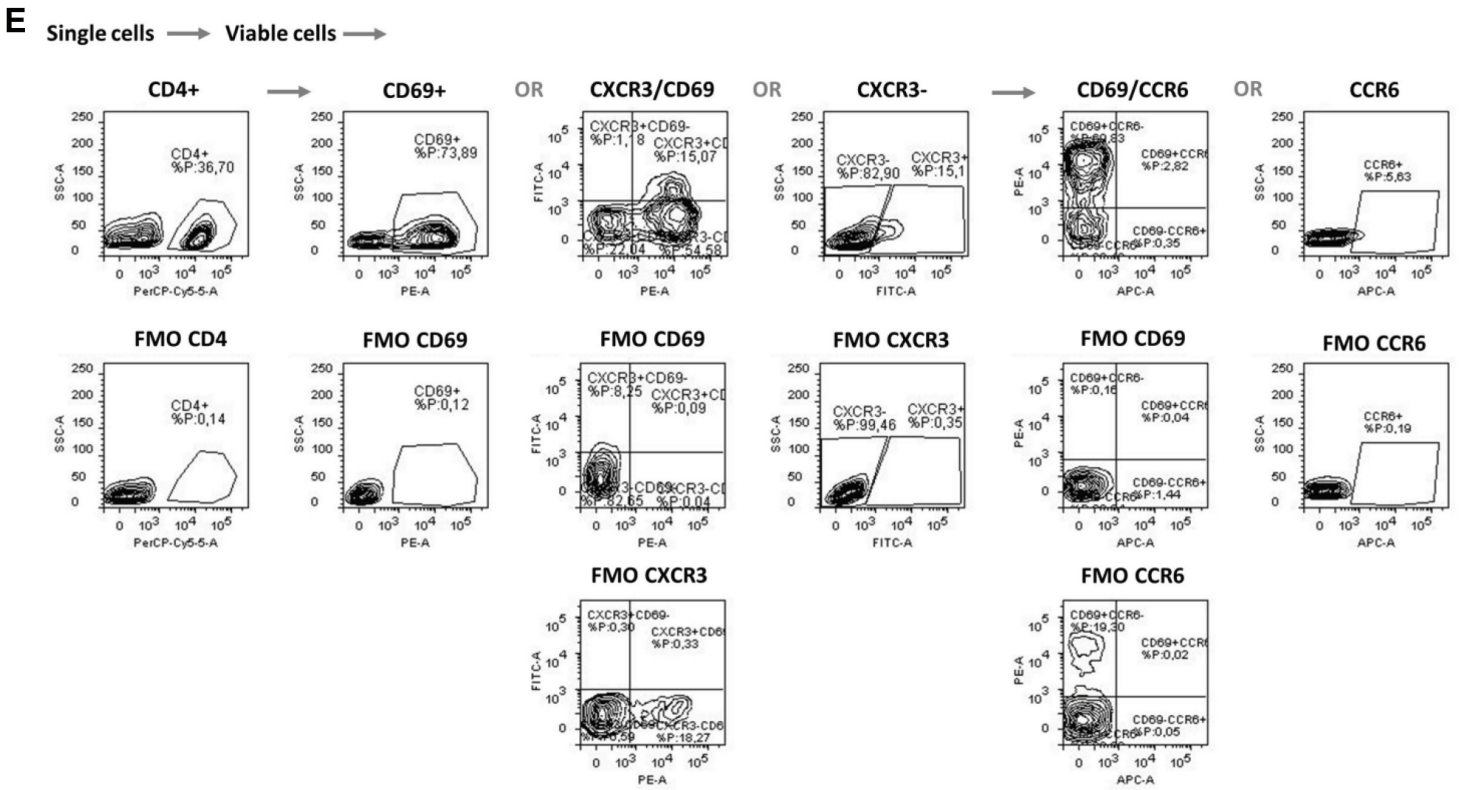


Figure 7. *Continued*.

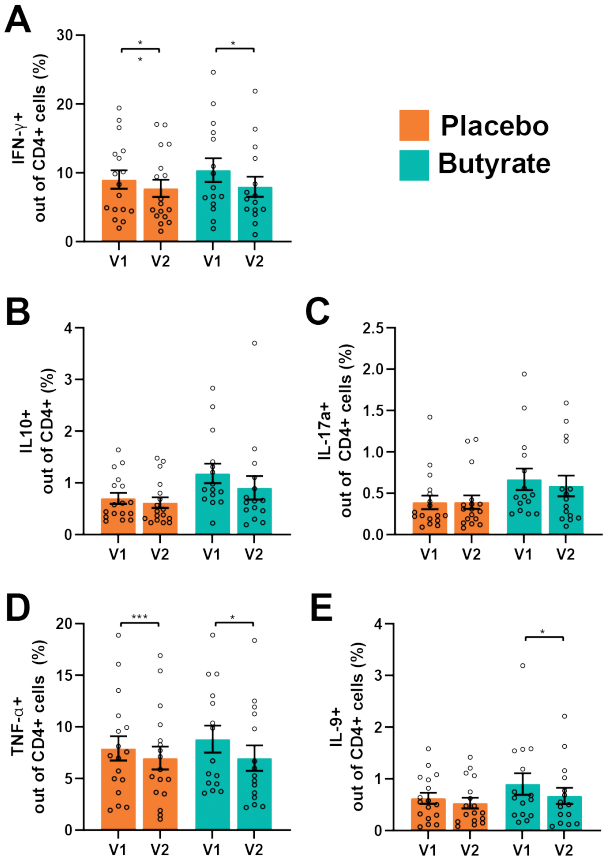


Figure 8. *See figure legend on page 145.*

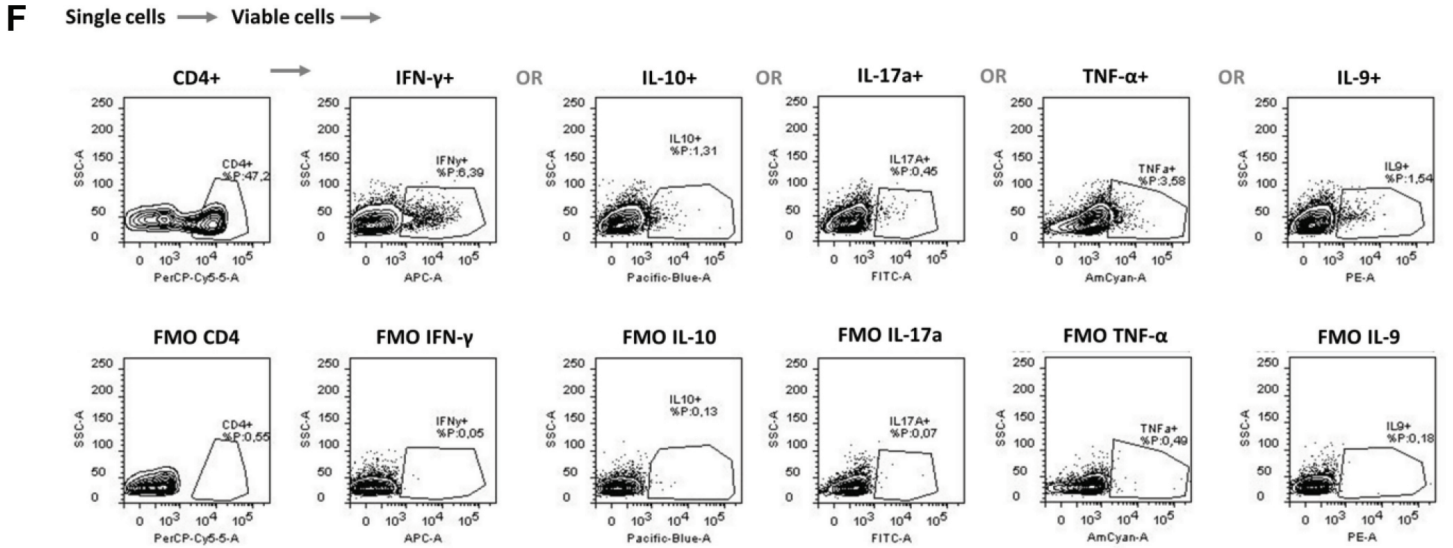


Figure 8. *FACS analysis of αCD3/CD28-stimulated PBMCs, which were restimulated with PMA, ionomycin and golgiplug. (A) percentage of IFN-γ+ cells out of CD4+ cells, (B) percentage of IL-10+ cells out of CD4+ cells, (C) percentage of IL-17a+ cells out of CD4+ cells, (D) percentage of TNF-α+ cells out of CD4+ cells, (E) percentage of IL-9+ cells out of CD4+ cells and (E) the used gating strategy with corresponding fluorescence minus one (FMO) controls for a representative sample. Orange bars indicate whole blood samples from patient who received placebo and blue bars indicate patients who received butyrate. Data are presented as mean ± SEM (N = 17 placebo group, N = 15 butyrate group). Significant differences are shown as * $p < 0.05$, *** $p < 0.001$. V1: Visit 1 (baseline), V2: Visit 2 (end of study).*

DISCUSSION

The purpose of this study was to investigate the effects of four-five weeks sustained release butyrate treatment on systemic inflammation and immune function as studied using *ex vivo* stimulation of whole blood and PBMCs of hand OA patients.

The pathophysiology of hand OA involves multiple factors, with increasing evidence suggesting a supporting role of a reduced intestinal barrier and low grade inflammation, not only in the synovial fluid, but in the circulation as well [2,7]. In the current study, hand OA patients were provided with sustained release butyrate tablets and it was aimed to deliver a butyrate concentration in the intestinal lumen high enough for a pharmacological effect leading to reduced systemic inflammation via improvement of among others the intestinal barrier function. However, in the present study, we did not observe an effect of butyrate on the systemic inflammation marker hsCRP, neither did we observe an effect on serum LBP or IgG LPS concentrations, which both are an indirect measure for intestinal LPS leakage. LBP is a protein which is synthesized by the liver in response to inflammatory stimuli, particularly LPS, whereas IgG LPS is an antibody which is produced in response to LPS. The effect of butyrate treatment on hsCRP, LBP or IgG LPS, hasn't been studied before in clinical trials. Among markers associated with low grade systemic inflammation such as IL-6, IL-1β and hsCRP, in this study only hsCRP was detectable, although in low concentrations. Systemic low grade inflammation, as determined by hsCRP levels, becomes more pronounced in patients having more severe OA [10]. It can be hypothesized that for this reason it was not possible to detect an effect of the intervention on low grade inflammation in this patient category. In addition, even though hsCRP levels can be elevated in OA patients, the increase compared to healthy controls is relatively small with a mean difference of 1.19 mg/L [10]. The same accounts for LBP, with levels similar or even lower than measured in healthy controls [47–49]. Beyond these parameters, other OA associated systemic inflammation markers like NO, TNFR and LPS leakage markers like sCD14 remained unaffected. Therefore, the main objective to use a dose of butyrate high enough to suppress LPS leakage and thus systemic inflammation may have failed in the current study group with patients receiving 600 mg butyrate per day. Other clinical trials have used dosages up to 4 g butyrate per day, indicating that it would be safe to increase the dosage of our treatment in future studies, although it should be noted that the formulations used in the other trials were completely different, namely colon targeted or immediate release. We developed a sustained release butyrate tablet, which slowly releases sufficient amounts of butyrate along the whole small intestine to reach a local pharmacologically active concentration. Nonetheless, it is important to note that we cannot prove that this concentration was actually reached. Additionally, it remains to be revealed if indeed a pharmacologically active concentration was present and whether the exposure time was sufficient to achieve a pharmacological effect on systemic inflammation markers. The absence of an effect on systemic inflammation markers and LPS leakage markers might suggest that the

designed formulation was ineffective, possibly due to one of the aforementioned reasons. Although, it might also be that the four-week treatment duration was too short to achieve these effects.

In addition the systemic immunomodulatory effect of butyrate treatment was studied in *ex vivo* stimulated whole blood or PBMCs. These stimulations were performed to give more insight in the potential of butyrate as a treatment for patients with OA, because these stimulations show the inflammatory potential of different immune cells, such as monocytes and T-cells. Monocytes are key effector cells of the immune system and precursors of macrophages such as present in inflamed synovia of OA patients. Furthermore, emerging evidence shows that infiltration of monocytes into the synovial tissues of knee OA patients is part of the pathogenesis [50], as well as elevated monocyte activation systemically [51]. In different *in vitro* studies it was observed that butyrate inhibits the release of pro-inflammatory cytokines and induced the release of regulatory cytokines by monocytes, which could be beneficial in OA patients [37,52]. Similar to macrophages, monocytes are very sensitive to LPS activation. In the present study we did not observe any effect of the butyrate treatment *ex vivo* LPS stimulation of whole blood cells nor PBMCs. Both in the whole blood plasma as well as in LPS activated PBMC, no effect was found on TNF-α, IL-6, IFN-γ and/or IL-10. Also other cells can be sensitive for LPS, thus these results cannot exclude an effect on monocytes yet. Although, LPS receptor TLR4 expression on monocytes, and intracellular TNF-α and IL-10 expression in ionomycin-PMA-golgiplug restimulated monocytes also remained unaffected in the butyrate group. Contrary, another study showed that *in vivo* butyrate treatment did decrease *ex vivo* oxLDL or β-glucan trained immunity in monocytes of obese males [53]. Even though this study did not concern OA patients and the monocytes were first trained, before LPS or PAM3CSK4 activation, the main difference may be the dose of butyrate given. Since in the other study twice daily intake of 4 grams butyrate for four weeks was studied, which is approximately 13 times higher than the dosage used in the present study. Although the release characteristics of butyrate from the formulations were different between both studies as well. From animal studies and *in vitro* studies it is known that short chain fatty acids can affect the balance of pro-inflammatory M1 and anti-inflammatory M2 type macrophages in favor of M2, which might ameliorate inflammatory effects [54]. Additionally, eight weeks of treatment with 100 mM butyrate enemas decreased nuclear translocation of NK-kappaB in macrophages of patients with ulcerative colitis [55]. We hypothesize that a higher dosage of sustained release butyrate might have anti-inflammatory effects on monocytes and macrophages, which needs to be confirmed in future studies.

T-cells play a central role in the adaptive immune system. Similar to monocytes, T-cell infiltration into the synovial tissues of OA patients contributes to the disease's pathogenesis, as well as T-cell subset imbalances and altered production of cytokines by T-cells systemically. For example, it was observed that the percentage of Treg

(IL17R-CD25+) cells is decreased in peripheral blood of patients with inflammatory knee OA [56] and the percentage of CD4+CD8-IL9+, CD4+CD8-IFNγ, CD4+CD8-IL17a+ cells and serum levels of IL-9, IFN-γ, IL-17a and IL-6 were increased [24,57]. In the current study serum levels of IL-6 remained below detection and the PBMCs of this moderate hand OA patient group did not secrete any of the measured cytokines in absence of stimulation. However, upon stimulation with αCD3/CD28 we were able to study the effect of four weeks sustained release butyrate treatment on *ex vivo* T-cell activation. Butyrate treatment reduced the percentage of activated CD69+CD4+, CD25+CD4+ and activated effector CD25+FoxP3- Th cells, and had a lowered Th17/Treg ratio shifting the balance in favor of Treg. Although no significant difference was observed when comparing ΔV2-V1 between the butyrate and placebo groups. Therefore, studies in bigger patient groups are required to fully exclude involvement of a placebo effect. Reduced generic T-cell activation and an improved Th17/Treg balance could be beneficial for OA patients in whom Th1, Th17 and Th9 activation is known to contribute to disease pathology along with an increased Th17/Treg balance [23,25]. Even though the activation status of the Th cells was suppressed in the butyrate group, the cytokine release of the activated PBMC remained unaffected. It may be that the effect on activation was too small to also suppress cytokine secretion or that beyond Th cells, also other cells like natural killer cells and cytotoxic T-cells contributed to the secretion of these cytokines [58]. However, when studying intracellular cytokine expression within the Th cells of αCD3/CD28-activated PBMC also the percentage of IL9+ cells was reduced by the butyrate treatment. Intracellular IFN-γ and TNF-α expression were also reduced, but this also applied for the placebo group. While the lowering of IL-9+ Th9 cells was selective for the butyrate group, although a placebo effect cannot be excluded since ΔV2-V1 between the butyrate and placebo group did not differ. Beyond pro-inflammatory cytokines like IFN-γ, IL-17a and TNF-α, also IL-9 is an important player in OA disease progression. IL-9 is being recognized as systemic biomarker in knee OA severity. It was shown that the number of Th9 cells in the circulation was positively associated with elevated CRP levels and that the number of Th9 cells and serum IL-9 concentrations in patients with OA were positively related with loss of daily functioning [24]. Therefore, these results may imply that our sustained release butyrate treatment does have a beneficial effect, reducing the state of inflammation in OA patients at least at the level of the T-cells.

In addition to the parameters mentioned above, butyrate and placebo treatment both lowered, the frequency of Treg cells, activated Th1 cells, Th17 cells, activated Th17 cells, IFNγ+CD4+ cells and TNFα+CD4+ cells. The patient inclusion of the butyrate and placebo group was done on regular bases during the year at one location, so the inclusion period and location were similar between groups. Also the general patient characteristics did not differ between groups. The excipients used in the formulation are not known to have any pharmacological effects, however interference of the excipients with the intestinal microbiome can be possible. We cannot exclude that some of the ingredients, amongst which nonfermentable fiber hydroxypropyl methylcellulose, may

have beneficially affected the microbiome [59–61], which may have caused some indirect beneficial immunomodulatory effects.

The present study has several limitations, which should be taken into account. The study was set-up as a proof-of-concept study, and the study groups may have been too small limiting the statistical power for these secondary parameters determining immune function. Furthermore, OA patients were given two 150 mg butyrate (as calcium) sustained release tablets twice daily which may have been too limited for full pharmacological effectiveness. The sustained release butyrate tablets used in this study should theoretically be able to release enough butyrate for a pharmacological response in the small intestine, but local butyrate concentrations were not measured. It could be that the butyrate dosage was too low to exert the optimal effect, that the release characteristics of the tablet or the formulation itself were not optimal and/or it could be that the exposure time of butyrate was too low. This should therefore be investigated further in future studies.

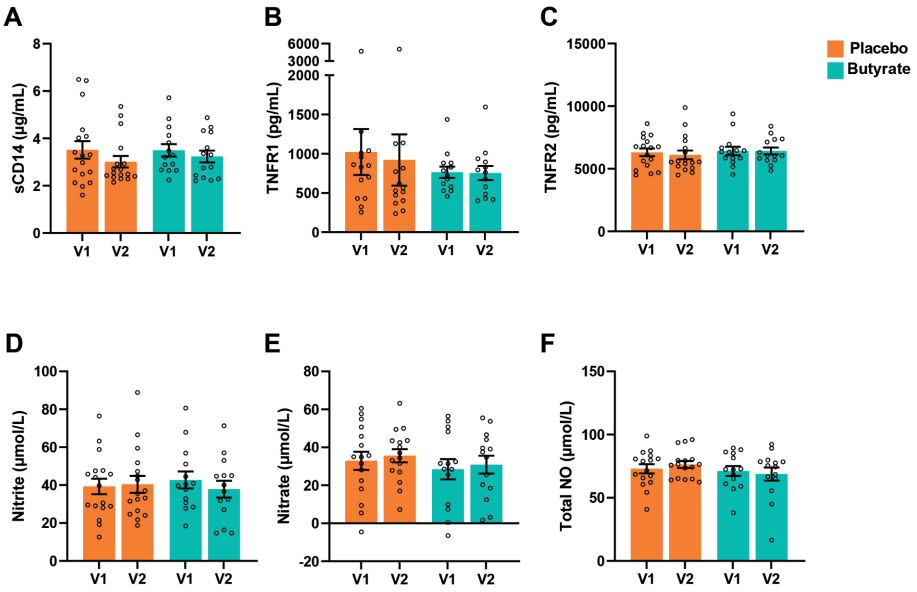
CONCLUSION

This double-blind placebo-controlled randomized clinical trial showed that the sustained release butyrate treatment reduced the inflammatory potential of Th cells in hand OA patients, as indicated by a reduced percentage of activated Th cells, IL-9 expressing Th9 cells and an improved Th17/Treg balance within *ex vivo* αCD3/CD28 activated PBMCs. This could contribute to restoring the immune balance in hand OA patients, which might benefit the patient by reducing their inflammatory status.

SUPPLEMENTAL FIGURES AND TABLES

Supplemental Table S1. *Overview titrated dilution of FACS antibodies.*

Antibody	Brand	Dilution
CD4 PerCP-Cyanine5.5	Thermo Fisher Scientific	1:80
CD25 Alexa Fluor 488	Thermo Fisher Scientific	1:40
CD127 PE-Cyanine7	Thermo Fisher Scientific	1:80
FOXP3 eFluor 660	Thermo Fisher Scientific	1:320
RORγ (t) PE	Thermo Fisher Scientific	1:640
CD69 PE	Thermo Fisher Scientific	1:80
CD196 (CCR6) APC	Thermo Fisher Scientific	1:160
CD183 (CXCR3) Alexa Fluor 488	BD Biosciences	1:40
CD14 APC	Thermo Fisher Scientific	1:160
TLR4 PE	BD Biosciences	1:80
IL-10 Brilliant Violet 421	Biolegend	1:160
TNF-α Brilliant Violet 510	Biolegend	1:50
IL-17A Alexa Fluor 488	BD Biosciences	1:40
IL-9 PE	BD Biosciences	1:320
IFN-γ Alexa Fluor 647	Biolegend	1:120



Supplemental Figure S1. *(A) soluble CD14 (SCD14), (B) TNF receptor 1 (TNFR1), (C) TNF receptor 2 (TNFR2), (D) Nitrite, (E) Nitrate and (F) total nitric oxide (NO) at baseline (V1) and the end of the study (V2). Orange bars indicate serum levels from patients who received placebo and blue bars indicate patients who received butyrate. Data are presented as mean ± SEM (N = 14-17 placebo group, N = 13-16 butyrate group).*

Supplemental Table S2. *ELISA data of LPS-stimulated whole blood and negative control and FACS data of restimulated PBMCs and negative control. Data are presented as mean ± SEM. Significant differences are shown as * p < 0.05, ** p < 0.01. V1: Visit 1 (baseline), V2: Visit 2 (end of study).*

Whole blood										
	Negative control				**LPS-stimulated**				**LPS-stimulated**	
	Placebo		Butyrate		Placebo		Butyrate		Placebo	Butyrate
	Visit 1	Visit 2	Visit 1	V2	Visit 1	Visit 2	Visit 1	Visit 2	V2-V1	V2-V1
IL-10(pg/mL)	6.245 (±1.321)	4.024** (±0.913)	8.526 (±1.891)	3.992* (±0.741)	1104.4 (±105.60)	1076.8 (±117.71)	1071.0 (±102.91)	1136.4 (±111.18)	-27.65 (±64.72)	65.46 (±102.2)
TNF-α(pg/mL)	21.43 (±6.708)	19.32** (±6.771)	24.764 (±6.951)	21.976* (±6.796)	3724.9 (±538.58)	3206.0 (±518.69)	2814.1 (±388.74)	2571.5 (±276.45)	-518.9 (±258.6)	-242.7 (±239.6)

PBMC										
	Negative control				**Restimulated**				**Restimulated**	
	Placebo		Butyrate		Placebo		Butyrate		Placebo	Butyrate
	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	V2-V1	V2-V1
IL-10+ out of CD14+ (%)	0.55 (±0.18)	0.30 (±0.10)	0.22 (±0.04)	0.34 (±0.14)	0.46 (±0.11)	0.40 (±0.17)	0.41 (±0.14)	0.63 (±0.21)	-0.05 (±0.10)	0.22 (±0.26)
TNF-α+ out of CD14+ (%)	0.40 (±0.13)	0.10* (±0.03)	0.25 (±0.06)	0.17 (±0.06)	0.54 (±0.08)	0.39 (±0.07)	0.68 (±0.23)	0.83 (±0.25)	-0.16 (±0.09)	0.14 (±0.15)
TLR4+ out of CD14+ (%)	38.57 (±3.85)	38.86 (±4.71)	35.79 (±2.85)	33.81 (±3.92)	28.29 (±2.22)	28.41 (±3.22)	34.01 (±3.49)	33.83 (±3.11)	0.12 (±2.13)	-0.18 (±2.63)
TLR4 (MFI)	2509.0 (±109.5)	2414.6* (±124.6)	2485.3 (±136.9)	2449.1 (±140.3)	2288.4 (±98.07)	2239.3 (±107.7)	2402.6 (±100.9)	2372.9 (±110.6)	-49.03 (±23.49)	-26.69 (±44.45)

Supplemental Table S3. *FACS and ELISA data of LPS-stimulated, αCD3/CD28-stimulated, αCD3/CD28-stimulated+restimulated PBMCs and negative control. Data are presented as mean ± SEM. Significant differences are shown as * p < 0.05, ** p < 0.01, *** p < 0.001. V1: Visit 1 (baseline), V2: Visit 2 (end of study).*

PBMC (FACS)														
	Negative control				**LPS-stimulated**				**αCD3/CD28-stimulated**				**αCD3/CD28-stimulated**	
	Placebo		Butyrate		Placebo		Butyrate		Placebo		Butyrate		Placebo	Butyrate
	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	V2-V1	V2-V1
CD25+FoxP3+out of CD4+ (%)	0.78 (±0.11)	0.64 (±0.09)	1.14 (±0.20)	1.03 (±0.23)	1.45 (±0.16)	1.22* (±0.11)	1.63 (±0.23)	1.59 (±0.22)	6.99 (±0.56)	6.04* (±0.43)	8.36 (±0.85)	7.53** (±0.75)	-0.95 (±0.41)	-0.83 (±0.26)
CD25+FoxP3-out of CD4+ (%)	1.23 (±0.27)	1.05 (±0.15)	1.43 (±0.33)	1.17** (±0.26)	2.65 (±0.44)	2.42 (±0.37)	2.76 (±0.48)	2.81 (±0.53)	50.06 (±3.41)	46.64 (±3.98)	51.64 (±3.70)	44.36* (±3.92)	-3.42 (±2.07)	-7.28 (±2.97)
CD25+ out of CD4+ (%)	1.63 (±0.23)	1.37 (±0.15)	2.29 (±0.48)	1.91* (±0.44)	2.93 (±0.18)	2.65 (±0.19)	3.50 (±0.50)	3.59 (±0.50)	56.20 (±3.40)	51.65 (±3.89)	59.34 (±3.85)	51.04* (±4.03)	-4.55 (±2.27)	-8.30 (±3.11)
RORγ+out of CD4+ (%)	0.46 (±0.10)	0.73 (±0.45)	0.43 (±0.07)	0.32* (±0.05)	0.49 (±0.10)	0.33* (±0.06)	0.43 (±0.07)	0.36 (±0.07)	6.37 (±1.31)	4.94* (±1.01)	7.34 (±1.60)	4.75** (±0.94)	-1.43 (±0.52)	-2.59 (±1.05)
Th17/Treg	2.22 (±1.50)	1.68 (±1.11)	0.62 (±0.19)	0.46 (±0.10)	0.41 (±0.12)	0.29 (±0.05)	0.35 (±0.08)	0.26 (±0.06)	0.92 (±0.16)	0.82 (±0.15)	0.86 (±0.16)	0.62 (±0.11)	-0.10 (±0.07)	-0.24 (±0.09)

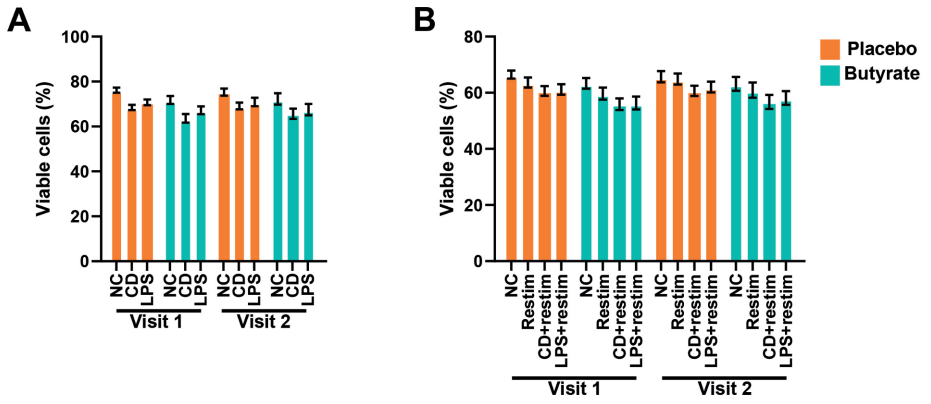
Supplemental Table S3. *Continued*.

PBMC (FACS)														
	Negative control				**LPS-stimulated**				**αCD3/CD28-stimulated**				**αCD3/CD28-stimulated**	
	Placebo		Butyrate		Placebo		Butyrate		Placebo		Butyrate		Placebo	Butyrate
	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	V2-V1	V2-V1
CD69+ out of CD4+ (%)	0.41 (±0.05)	0.36 (±0.05)	0.65 (±0.17)	0.41 (±0.06)	3.91 (±0.63)	3.54 (±0.56)	3.63 (±0.44)	2.99 (±0.30)	71.60 (3.55)	67.45 (±4.09)	76.10 (±2.60)	66.35* (±3.83)	-4.16 (±2.42)	-9.76 (±3.41)
CD69+CXCR3+ out of CD4+ (%)+	0.19 (±0.02)	0.18 (±0.02)	0.32 (±0.11)	0.17 (±0.02)	0.65 (±0.09)	0.63 (±0.11)	0.56 (±0.05)	0.58 (±0.07)	20.11 (±1.35)	17.00** (±1.17)	20.51 (±1.88)	16.01** (±1.24)	-3.11 (±0.88)	-4.50 (±1.35)
CD69+CCR6+ out of CD4+CXCR3- (%)	0.04 (±0.00)	0.03 (±0.00)	0.05 (±0.01)	0.04 (±0.01)	0.47 (±0.09)	0.43 (±0.08)	0.36 (±0.06)	0.34 (±0.05)	3.46 (±0.35)	2.83** (±0.31)	4.62 (±0.79)	3,26** (±0.46)	-0.63 (±0.20)	-1.37 (±0.55)
CCR6+ out of CD4+CXCR3- (%)	4.45 (±0.45)	3.69*** (±0.38)	4.19 (±0.55)	3.56 (±0.46)	5.69 (±0.64)	5.23 (±0.72)	4.69 (±0.60)	4.18 (±0.60)	4.57 (±0.38)	3.83** (±0.36)	5.59 (±0.80)	4.23* (±0.54)	-0.73 (±0.21)	-1.36 (±0.56)

PBMC (FACS)														
	Negative control				**Restimulated**				**αCD3/CD28-stimulated + restimulated**				**αCD3/CD28-stimulated + restimulated**	
	Placebo		Butyrate		Placebo		Butyrate		Placebo		Butyrate		Placebo	Butyrate
	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	V2-V1	V2-V1
IL-17a+ out of CD4+ (%)	0.13 (±0.03)	0.23 (±0.08)	0.15 (±0.04)	0.31** (±0.11)	0.32 (±0.05)	0.34 (±0.07)	0.48 (±0.11)	0.44 (±0.09)	0.39 (±0.08)	0.39 (±0.08)	0.67 (±0.13)	0.59 (±0.13)	0.002 (±0.03)	-0.08 (±0.08)
IFN-γ+ out of CD4+ (%)	0.12 (±0.03)	0.17 (±0.05)	0.11 (±0.03)	0.18 (±0.07)	5.28 (±1.38)	4.31* (±1.10)	5.10 (±1.49)	4.26 (±1.58)	9.02 (±1.34)	7.74** (±1.26)	10.38 (±1.73)	7.98* (±1.46)	-2.40 (±1.29)	-1.28 (±0.42)
IL-9+ out of CD4+ (%)	0.11 (±0.04)	0.20 (±0.08)	0.12 (±0.04)	0.26* (±0.11)	0.13 (±0.05)	0.19 (±0.08)	0.19 (±0.09)	0.24 (±0.08)	0.62 (±0.11)	0.53 (±0.10)	0.90 (±0.21)	0.67* (±0.16)	-0.23 (±0.11)	-0.09 (±0.05)
IL-10+ out of CD4+ (%)	0.17 (±0.04)	0.32** (0.08)	0.21 (±0.04)	0.39*** (±0.11)	0.24 (±0.06)	0.29 (±0.08)	0.29 (±0.09)	0.42 (±0.13)	0.70 (±0.11)	0.62 (±0.10)	1.18 (±0.19)	0.90 (±0.23)	-0.28 (±0.28)	-0.08* (±0.06)
TNF-α+ out of CD4+ (%)	0.24 (±0.05)	0.38** (±0.09)	0.31 (±0.06)	0.45 (±0.12)	3.37 (±0.96)	2.86 (±0.86)	3.86 (±1.31)	3.38 (±1.24)	7.92 (±1.18)	6.98*** (±1.12)	8.82 (±1.31)	6.98* (±1.23)	-1.84 (±0.75)	-0.93 (±0.23)

Supplemental Table S3. *Continued*.

PBMC (ELISA)														
	Negative control				LPS-stimulated				αCD3/CD28-stimulated				αCD3/CD28-stimulated	
	Placebo		Butyrate		Placebo		Butyrate		Placebo		Butyrate		Placebo	Butyrate
	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2	V2-V1	V2-V1
IL-17a (pg/mL)	1.80 (±0.15)	1.98 (±0.26)	2.19 (±0.15)	1.89 (±0.22)	32.34 (±5.88)	29.11 (±5.22)	40.19 (±7.50)	32.45 (±6.56)	1.78 (±0.10)	2.08 (±0.42)	1.60 (±0.17)	3.55 (±1.04)	-3.230 (±4.061)	-7.745 (±6.199)
IFN-γ (pg/mL)	2.74 (±0.51)	2.59 (±0.41)	3.17 (±0.73)	2.63 (±0.51)	638.55 (±86.95)	572.32 (±73.10)	470.00 (±60.35)	573.93 (±147.44)	140.72 (±40.02)	143.38 (±28.86)	76.83 (±18.29)	77.66 (±11.68)	-66.23 (±55.14)	-103.9 (±108.1)
IL-9 (pg/mL)	0.39 (±0.00)	0.39 (±0.00)	0.50 (±0.11)	0.39 (±0.00)	27.05 (±6.63)	23.60 (±5.25)	22.42 (±4.56)	20.73 (±5.07)	0.39 (±0.00)	0.39 (±0.00)	0.41 (±0.02)	0.50 (±0.08)	-3.443 (±3.176)	-1.684 (±3.473)
IL-10 (pg/mL)	1.17 (±0.00)	1.28 (±0.11)	1.17 (±0.00)	1.17 (±0.00)	665.61 (±178.03)	513.59 (±107.31)	492.42 (±90.63)	414.99 (±71.79)	1126.8 (±303.70)	859.77 (±117.79)	784.93 (±109.29)	918.68 (±221.55)	-152.0 (±93.16)	-77.44 (±90.58)
TNF-α(pg/mL)	3.40 (±1.41)	3.96 (±2.10)	2.96 (±0.70)	1.95 (±0.00)	1104.4 (± 152.18)	844.59 (±151.62)*	1058.6 (±163.36)	966.73 (±159.06)	2031.14 (±310.28)	1855.66 (±279.22)	1664.44 (±278.88)	1732.84 (±194.78)	-259.8 (±113.6)	-91.85 (±190.2)
IL-6 (pg/mL)	26.89 (±8.67)	22.67 (±9.71)	62.70 (±20.95)	36.76 (±11.22)	344.05 (±111.92)	277.16 (±73.36)	252.04 (±65.15)	280.25 (±130.42)	15118.2 (±1230.5)	15497.2 (±1379.5)	16167.1 (±1750.4)	13218.1 (±1121.2)	-66.88 (±77.83)	28.20 (±110.2)



Supplemental Figure S2 *Viability of PBMCs after stimulation with either LPS or αCD3/CD28 with and without restimulation (PMA, Ionomycine, golgiplug) and the negative control. Data are presented as mean ± SEM (N = 17 placebo group, N = 15 butyrate group). CD: αCD3/CD28, NC: Negative control, Restim:restimulation.*

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CONFLICT OF INTEREST

JPWV is a major shareholder of Birr Beheer BV. SGPJK and HV were employed by Tiofarma. JG is part time employed by Danone Nutricia Research B.V. Birr Beheer B.V. and Tiofarma are involved in the development of sustained release forms of butyrate for the treatment of immune related chronic diseases. The others authors reported no conflict of interest.

SUMMARIZING DISCUSSION

NON-COMMUNICABLE DISEASES

Non-communicable diseases (NCDs) are chronic diseases including cardiovascular diseases, chronic respiratory diseases, diabetes and immune disorders such as inflammatory and allergic diseases. These diseases kill over 41 million people each year and are accountable for 74% of all deaths worldwide [1]. NCDs emerge a significant global health challenge and therefore both prevention as well as treatment of NCDs are part of the 2030 Agenda for Sustainable Development. The 2030 Agenda for Sustainable Development represents a worldwide initiative that serves as a blueprint for peace and prosperity for both humanity and the planet now and in the future. With this initiative governments commit to develop plans that reduce one third of premature mortality from NCDs through prevention and treatment by 2030.

It has been hypothesized that NCDs are associated with dysbiosis of the gut microbiome, a disrupted intestinal barrier leading to increased leakage of macromolecules such as lipopolysaccharides (LPS) from the intestines to the systemic compartment, and low grade inflammation systemically and locally in the gut [2–18]. Although quite some literature is available on the different aspects separately, no research is available that connects all aspects such as, microbial dysbiosis, disrupted intestinal barrier, LPS leakage and intestinal and systemic inflammation. Another question to be answered would be whether the NCD starts with dysbiosis of the microbiome or with one of the other aspects such as local or systemic inflammation (in other words, what is the chicken and what is the egg). Nonetheless, that there is a connection between these different aspects is considered plausible and has increasingly gained interest among scientists.

In NCDs dysbiosis could lead to changes in the presence of butyrate-producing bacteria and consequently to reduced butyrate levels in the intestine [19–21]. Butyrate is known for its anti-inflammatory and barrier protective effects [22–24] and reduced butyrate levels in the intestine could impact intestinal homeostasis and integrity. Therefore, butyrate supplementation in the intestine via a pharmaceutical drug product could be beneficial for patients suffering from a NCD.

The aim of this thesis was to explore butyrate's potential as a therapeutic agent for NCDs, in the lab as well as in patients. For this purpose, the pharmacodynamics and pharmacokinetics of butyrate were further investigated and a pharmaceutical drug product with butyrate as the active substance was developed and tested in patients having hand osteoarthritis (OA) in a double-blind randomized placebo-controlled clinical trial.

PHARMACODYNAMICS OF BUTYRATE; WHAT WE HAVE LEARNED IN THE LAB

Chapter 2 focusses on getting a better understanding on the effects of butyrate and its mechanism of action in different *in vitro* cell models, namely two different epithelial cell lines HT-29 and Caco-2, peripheral blood mononuclear cells (PBMCs) and a transwell co-culture model in which the Caco-2 cells and PBMCs were combined.

In **Chapter 2.1** intestinal epithelial cells HT-29 were activated with interferon-gamma (IFN-γ) or IFN-γ combined with tumor necrosis factor-alpha (TNF-α) and treated with butyrate to study its mechanism of action. Butyrate was added to the cells in solution for 1 to 24 h. IFN-y and TNF-α are both known activators of intestinal epithelial cells and can disrupt the intestinal barrier as well [25–28]. IFN-γ activates the signal transducer and activator of transcription 1 (STAT1) pathway which results in the release of C-X-C motif chemokine ligand 10 (CXCL10). TNF-α combined with IFN-γ has a synergistic effect on this pathway [29]. CXCL10 release was completely prevented by butyrate, not only in cells activated with IFN-γ, but also in cells activated with the combination of IFN-γ and TNF-α. This shows the strong anti-inflammatory efficacy of butyrate. To better understand how butyrate affects CXCL10 release, its effect on proteins and genes of the canonical and non-canonical STAT1 pathway were investigated as well. We showed that butyrate blocks CXCL10 release via the non-canonical pathway, similar to the known histone deacetylase (HDAC) inhibitor Trichostatin A (TSA). HDAC inhibition causes downregulation of the expression of genes and proteins related to the non-canonical STAT1 pathway, including interferon regulatory factor 9 (IRF9) and CXCL10 itself, resulting in the prevention of induced CXCL10 release. The effect of butyrate on IRF9 was never studied before, and even though a lot of research is available on the effects of butyrate, its effects are still not fully understood.

IRFs, including IRF9, are described to be involved in different NCDs and it has been discussed that they are potential targets in the treatment of NCDs, such as cardiovascular diseases [30,31]. Our work shows that butyrate could be an interesting drug substance for these targets.

In **Chapter 2.2** the effects of butyrate were studied in different models using intestinal epithelial cells Caco-2 with and without PBMCs activated with LPS or αCD3/CD28. In this model the PBMCs represent the immune compartment of the lamina propria. The lamina propria is the effector immune compartment in the intestinal mucosa containing both innate as well as adaptive immune cells, both in close proximity of a monolayer of intestinal epithelial cells. The co-culture model was set up to mimic the cross talk between epithelial cells and activated immune cells, and to investigate if butyrate has beneficial effects in immune mediated barrier disruption as may occur in the human intestine of patients suffering from NCDs. The intestinal barrier is one of the main

defense mechanisms in the human body and consists of different layers including a barrier forming mucus layer and intestinal epithelial cell monolayer, which are connected to each other by tight junctions. The lamina propria lies beneath the epithelium and contains various effector immune cells, such as T-cells, B-cells, dendritic cells and macrophages [27]. The mucosal tissue plays a critical role in maintaining intestinal homeostasis. Intestinal epithelial cells prevent non-specific leakage of immunogenic agents such as endotoxin LPS by providing a barrier. In addition, the epithelial cells may regulate responses of the underlying immune cells. Immune cell activation in the lamina propria, however, can affect intestinal epithelial cell homeostasis since several cytokines, such as TNF-α, interleukin (IL)-1β, IFN-γ and IL-17a can activate epithelial cells and/or affect the mucosal barrier function [32]. In **Chapter 2.2** butyrate was found to inhibit IL-10 and TNF-α release, while the highest dose of butyrate induced IL-1β release by LPS-activated PBMCs. Furthermore, in αCD3/CD28-activated PBMCs butyrate inhibited IL-10, TNF-α, IFN-γ and IL-17a release. The effect of butyrate on cytokine release was in line with its effect on T-cell phenotypes, as butyrate lowered the percentage of activated T-helper (Th) 1 cells, Th17 cells and regulatory T (Treg) cells. In the co-culture model of Caco-2 cells and PBMCs activated with LPS or αCD3/CD28, the Caco-2 barrier function was disrupted due to cytokines released by the activated PBMCs. This was dose-dependently prevented by butyrate. Butyrate also inhibited the release of pro-inflammatory cytokines by the PBMCs, therefore we hypothesized that the effect of butyrate on cytokine release was linked to its barrier protective effect. However, the barrier protective effect of butyrate was additionally studied in a Caco-2 model in which inflammatory cytokines TNF-α, IFN-γ and IL-1β were added to the basolateral compartment. In this model, butyrate again showed to have a barrier protective effect, implicating also a direct protective effect on inflammatory induced epithelial barrier disruption. In **Chapter 2.1** we observed a role for HDAC inhibition in the protective effects in cytokine induced epithelial activation, similar to the effect of butyrate and therefore it was studied if HDAC inhibition also could mimic butyrate's barrier protective effect in inflammatory induced barrier disruption. The general HDAC inhibitor TSA showed similar effects compared to butyrate, related to barrier improvement and reduction of inflammatory-induced IL-8 release by Caco-2 cells. Again indicating that HDAC inhibition might be involved in the mechanism of action of butyrate. The effect of HDAC inhibitors on PBMCs activation or protection against inflammatory induced barrier disruption in the Caco-2/PBMC co-culture model was not studied, but it would be interesting to investigate this in future studies in our models. A more complete understanding of butyrate's mechanisms of action on different cell types and especially in models that mimic the real situation in the intestine more closely are necessary to develop the most optimal butyrate containing drug product. Overall **Chapter 2** shows the importance of sufficient butyrate levels in the intestine to ensure intestinal integrity and homeostasis, both of the epithelial cells as well as underlying immune cells.

In general two mechanisms of action are described for butyrate, namely HDAC inhibition or binding to G protein-coupled membrane receptors (GPCR), GPR41, GPR43 and

GPR109. In our work we mainly show the potential involvement of HDAC inhibition in the effect of butyrate, but it is of importance to note that these conclusions were drawn from the fact that known HDAC inhibitors mimicked butyrate's effects. HDAC can be subdivided into class I (HDAC1, HDAC2, HDAC3 and HDAC8), class IIa (HDAC4, HDAC5, HDAC7 and HDAC9), class IIb (HDAC6 and HDAC10) and class IV (HDAC11) [33]. HDAC inhibitors inhibit histone deacetylase and can regulate gene expression. Butyrate is a potent class I and class IIa HDAC inhibitor [34]. Based on the results from **Chapter 2** we have drawn the conclusion that in particular HDAC class I (HDAC6 and 10) are involved in butyrate's inhibitory effect on CXCL10 release and HDAC8 in butyrate's barrier protective effects. We did not study the involvement of GPCRs and therefore it cannot be excluded that they play an additional role in butyrate's mechanism of action in the used models. However, in general HDAC inhibitors could mimic the protective effect of butyrate, indicating the importance of this mechanism in the anti-inflammatory and barrier protective effects.

In our studies we observed effects of butyrate in the range of 1-8 mM depending on the cell model used, which is in line with other research, showing anti-inflammatory and barrier protective effects in the range of 2-5 mM on intestinal epithelial cells [35–38], and in the range of 0.05-10 mM on immune cells [39–48]. It should be noted that in our transwell experiments the butyrate concentration was expressed as the concentration of butyrate added to the apical compartment. The basolateral compartment was free of butyrate, however due to butyrate's physicochemical properties it will freely diffuse from the apical compartment to the basolateral compartment until a homogenous solution across both compartments is reached. As 0.5 mL of the butyrate solution was added to the apical compartment and 1.5 mL is present in the basolateral compartment, it could be that effective butyrate concentrations for a pharmacological effect in our models were four times lower during the experiments using transwell systems, than the concentrations added to the apical compartment.

Chapter 2.1 and 2.2 did not only focus on the effects of butyrate, but compared its effects to those of two other short chain fatty acids (SCFAs), propionate and acetate, as well. In **Chapter 2.1** propionate and acetate were less effective in inhibiting the release of CXCL10. And in **Chapter 2.2** 2 and 4 mM propionate were equally able to inhibit IL-8 release of Caco-2 cells activated with a cytokine mixture of TNF-α, IFN-γ and IL-1β compared to 4 mM butyrate, followed by 8 mM acetate. Both propionate and acetate, also improved the barrier of the Caco-2 cell monolayer as measured with the 4kDa FITC-dextran permeability assay. Butyrate being the most effective followed by propionate and acetate is in line with other research [49–53]. This is in most studies linked to the HDAC inhibitory potency of these three SCFAs, which we also showed by studying HDAC activity of HT-29 cells treated with these SCFAs in **Chapter 2.1**.

PHARMACOKINETICS OF BUTYRATE

Butyrate concentrations required for a direct effect on intestinal epithelial cells or immune cells in our research were between 1–8 mM. For the development of a drug with butyrate as the active substance, it is important to understand the local pharmacokinetics of butyrate in the small intestine. In **Chapter 3** we present a mathematical approach to model the luminal butyrate concentration after oral administration of an immediate release (IM) or a sustained release (SR) butyrate formulation to humans.

Butyrate can be produced by the bacteria in the gastrointestinal tract as a result of fiber fermentation [54–56]. Butyrate-producing bacteria are abundantly present in the colon, so butyrate levels are high and constantly present there. Butyrate-producing bacteria are less abundantly present in the small intestine, so butyrate levels are relatively lower there. The upper part of the small intestine especially lacks butyrate [57,58]. Therefore, we selected the small intestine in particular as the most interesting target for a novel butyrate containing drug formulation.

Once butyrate is released and dissolved from the drug formulation, it starts to diffuse across the mucus and is subsequently absorbed by the intestinal epithelial cells. The remaining butyrate concentration in the lumen at a certain moment, is determined by both the amount that is released from the formulation and the amount that is diffused and absorbed through the intestinal epithelium. In other words, the concentration is determined by the balance between influx from the tablet into the lumen and outflux from the lumen to the circulation. The overall absorption rate of butyrate in the small intestine has been studied by Schmitt et al. [59]. They investigated the disappearance rate of butyrate out of the lumen, making it possible to model the butyrate concentration in the lumen after intake of an oral butyrate formulation with specified release characteristics. The mathematical model shows that an IR formulation is only capable of maintaining pharmacologically active concentrations within the first half hour after the formulation has reached the small intestine and thus will not target the whole small intestine, because all available butyrate will be absorbed in the very beginning of the small intestine. Contrary, a SR formulation will be able to yield pharmacologically active concentrations along the whole small intestine, although these concentrations will be locally maintained for only a short duration, because butyrate will be absorbed quickly and the formulation will travel through the small intestine. Some parts of the epithelial cell lining might not even get in contact with butyrate, because of the peristaltic movement of intestinal content and thus the formulation [60,61].

Based on the calculations from the mathematical model, the sustained release formulation requires a release rate in the range of 0.08-0.2 mmol/h in order to achieve pharmacologically active concentrations. In addition, it should contain a sufficient amount of butyrate to be able to release the butyrate along the whole small intestine. Both the

release rate and a sufficient amount of butyrate will contribute to reach pharmacologically active concentrations along the small intestine, but it is questionable whether the exposure time of this butyrate concentration is long enough to achieve an effect.

DEVELOPMENT OF A SUITABLE DRUG PRODUCT

Based on the knowledge obtained in **Chapter 2 and 3** a butyrate containing drug product was developed as described in **Chapter 4**. In short, the calcium salt of butyrate was used as drug substance. The calcium salt was preferred above the sodium salt, because it is less hygroscopic which is preferrable for the tableting process. In the sustained release core hydroxypropyl methylcellulose, silicified microcrystalline cellulose and magnesium stearate were used as excipients. The tablet cores were coated with a taste masking coating consisting of talc, titanium dioxide, PEG6000, simethicone emulsion, Eudragit RL 30 D and triethyl citrate. The coating is a shield for the bad taste of butyrate and stays intact at the higher pH in the mouth. As soon as the tablet reaches the stomach with a low pH the coating starts to dissolve. Consequently, butyrate release from the formulation will start in the stomach, but a sufficient amount is available in the tablet core to release butyrate along the whole small intestine. It was decided not to use a gastric resistant coating, which would protect the butyrate from being released in the stomach, because it takes time for this coating to dissolve in the first part of the small intestine and consequently no butyrate will be released from the formulation in this part of the small intestine [62,63].

When a SR tablet is ingested by a patient it will first reach the stomach and stay there for approximately 2 h, second the tablet will reach the small intestine which it will transit in approximately 4 h and thereafter the tablet will reach the colon. The pharmacologically relevant timepoint to study dissolution is therefore between 2 and 6 h. According to the outcome of the mathematical model in **Chapter 3**, the tablet was developed to release 0.08-0.2 mmol butyrate per hour during this time period, which was tested as a release and shelf-life specification of the product. Each tablet consists of 150 mg (equals 1.72 mmol) of butyrate (as calcium), which is sufficient for the desired release time. The release characteristics of commercially available food supplements are unknown and therefore it cannot be concluded if they are able to reach pharmacologically active concentrations. Furthermore, in the agriculture sector butyrate is used as a feed additive. This implies that these animals consistently have access to butyrate and consequently the frequency of interaction between butyrate and their intestinal mucosa increases, which will increase the chance to obtain a pharmacological effect.

SUSTAINED RELEASE BUTYRATE TABLETS & HAND OSTEOARTHRITIS PATIENTS

Osteoarthritis (OA) of the hand is a NCD which is characterized by degeneration of the cartilage in the joints of the hands which leads to pain, stiffness and reduced functioning. These symptoms are related to alterations in the microbiome, intestinal barrier defects, synovial inflammation and low grade systemic inflammation [64–70]. Therefore, OA was thought to be a suitable model disease to investigate the effects of the sustained release butyrate tablets on NCDs in a randomized double-blind placebo-controlled clinical trial. In **Chapter 5**, the effects of butyrate treatment on systemic inflammation markers, LPS leakage markers and *ex vivo* stimulations of whole blood or PBMCs are shown.

Sustained release butyrate did not affect systemic inflammation markers, such as high sensitive C-reactive protein (hsCRP), IL-6, IL-1β, or LPS leakage markers such as IgG LPS and LPS binding protein (LBP). IL-6 and IL-1β were not detectable and hsCRP and LBP were in the range or close to values of healthy controls. For this reason it might be difficult to measure significant differences in a small study group as is the case in the current study. However, the systemic immunomodulatory effect of butyrate treatment was additionally studied *ex vivo*. *Ex vivo* stimulations of whole blood and PBMCs were performed to give more insight in the potential of butyrate as a treatment, because these stimulations show the inflammatory potential of different immune cells, such as monocytes and T-cells.

From the clinical trial results it can be concluded that sustained release butyrate tablets did not affect the phenotype of monocytes nor their function. Neither in the LPS-stimulated whole blood plasma nor in the PBMC supernatant, an effect was found on TNF-α, IL-6, IFN-γ and/or IL-10 release. Also other cells can be sensitive for LPS, thus these results cannot fully exclude an effect on monocytes. However, in ionomycin-PMA-golgiplug restimulated monocytes also the intracellular TNF-α and IL-10 expression and surface receptor TLR4 remained unaffected in the butyrate treated group.

Nevertheless, the sustained release butyrate tablets did affect Th-cell activation. In αCD3/CD28-activated PBMCs the percentage of activated CD69+CD4+, CD25+CD4+ and activated effector CD25+FoxP3- Th cells was reduced and Th17/Treg ratio shifted in favor of Treg. Even though the activation status of the Th cells was suppressed in the butyrate group, the cytokine release of the activated PBMC remained unaffected. However, intracellular IL-9 expression within the Th cells of αCD3/CD28-activated PBMC was reduced by the butyrate treatment. IL-9 is being recognized as systemic biomarker in OA severity. It was shown that the number of Th9 cells in the circulation was positively associated with elevated CRP levels and that the number of Th9 cells and serum IL-9 concentrations in patients with OA were positively related with the Western Ontario and McMaster Universities Osteoarthritis arthritis index (WOMAC), which is

a self-administered questionnaire used to asses pain, stiffness and physical functioning [71]. In addition to the Th cell parameters mentioned above, butyrate treatment but also placebo treatment, both lowered the percentage of activated Th1 cells, Th17 cells, activated Th17 cells, Treg cells, IFNγ+CD4+ cells and TNFα+CD4+ cells. Therefore it cannot be excluded that the excipients used in the formulation, such as hydroxypropyl methyl cellulose (HPMC) have an effect as well. The nonfermentable fiber HPMC may have beneficially affected the microbiome, which indirectly may have exerted the immunomodulatory effects observed [72-74].

Observing an effect on Th cell level, but not on the level of LPS leakage and monocytes, might implicate that the SR butyrate tablet did not have a barrier protective effect on the intestinal epithelial cells in the small intestine resulting in reduced LPS leakage and therefore monocyte activation. However, the suppressive effect of the butyrate SR tablet on *ex vivo* Th cell activation may relate to a barrier protective effect of butyrate, although this could not be confirmed via indicators of LPS leakage and systemic inflammation markers since these levels were relative low in the study population. Reduction of systemic inflammation could affect the sensitivity of Th cells to get activated upon antigen presentation, meaning that the sustained release butyrate treatment may have had an indirect effect via lowering systemic inflammation. Another possibility would be that, SR butyrate treatment had a direct effect on antigen presenting cells in the lamina propria that instruct Th cell development in the gut associated lymphoid tissue. These Th cells traffic via the bloodstream and home back to the lamina propria, however they may also act in systemic tissues such as the joints [75,76]. Thus, butyrate may have affected Th cell development in the gut resulting in decreased systemic activation of Th cells.

The present study is not the first study investigating butyrate's pharmacological effects in human. Previously, several studies investigated the effect of butyrate enemas on active ulcerative colitis showing the anti-inflammatory potential of butyrate in the intestine [77–79]. These studies provided enemas with a dosage of 60-100 mL of 80-100 mM sodium butyrate once or twice daily. Compared to the calculated concentration of butyrate present as a result of our sustained release butyrate formulation, the concentrations in the enemas are relatively high and depending on the volume contact area and contact time will be higher. Only one other study investigated the anti-inflammatory effect of oral butyrate treatment and showed a reduction of inflammatory markers, but this study was not placebo-controlled [80]. Here, we showed for the first time that oral sustained release butyrate treatment can have an anti-inflammatory effect systemically, although the formulation is not meeting its potential as compared to effects observed *in vitro*. The clinical study presented in **Chapter 5** provided 600 mg sustained release butyrate (as calcium) per patient per day. Compared to other clinical studies investigating oral butyrate treatment, 600 mg per day is relatively low [80–84]. These studies investigated other study parameters and the drug formulations were different, but it is an indication that it would be safe to increase the dosage of our treatment in future studies. There are

several options that could improve our butyrate treatment. First, by increasing the number of tablets a day per timepoint or by increasing the butyrate release rate from the tablet, which would result in higher concentrations in the small intestine. Second, by increasing the dosage frequency, which would result in more frequent exposure of the cells in the small intestine to the butyrate concentration.

FUTURE PERSPECTIVES

There is not a lot of knowledge available on how an oral formulation travels through the intestine and how the intestine, the intestinal fluid, the oral formulation and their (peristaltic) movement interact. Especially for an oral formulation with a local effect in the gastro-intestinal tract this knowledge is crucial to fully understand its potential. The mathematical model that we developed included quite some assumptions which would affect the calculated required release rate for butyrate from the formulation. Although the model is helpful in getting a better understanding of the potential of IM and SR release formulations, as well as giving a good insight in the approximate range the release rate has to lie around, it should be subject for future studies. The available butyrate concentration in the intestine could be studied with aspiration of human gastro-intestinal fluids, which could sample luminal fluid after intake of an oral formulation [85].

Second, as mentioned before every *in vitro* experiment has been done with a constant concentration of butyrate for a certain time period, which as discussed is not representing the real situation in the intestine. Therefore, I would recommend to repeat *in vitro* experiments using intestinal epithelial cells and include conditions with shorter contact time of the butyrate with the cells. Because of the shorter contact time, I would include higher concentrations than used before as well, because toxicity might be lower with shorter contact time.

These experiments could help to further optimize the dosage regimen of the developed tablets for future clinical studies, for example by increasing the dosage frequency or if needed to further optimize the formulation, by adjusting the butyrate release rate. The optimal sustained release butyrate tablet would not only affect Th-cell activation, but would affect intestinal barrier properties as well. In addition to LPS leakage markers, such as LBP and IgG LPS, a direct measure for the intestinal barrier should be included in future clinical studies as well. This could be done with an *in vivo* permeability assay, which uses orally ingested solutes such as lactulose/mannitol or different sizes of polyethylene glycol that are excreted and measured in urine [86].

In addition, the effect of the placebo tablets on the microbiome should be studied before a new placebo-controlled trials is initiated. The results of our clinical trial showed that the placebo tablets had immunomodulatory effects similar to the sustained release butyrate tablets, which could indicate a placebo effect or that another component in the tablets has

an immunomodulatory effect as well. It is known that HPMC can affect the microbiome, but not whether the amount of HPMC in our placebo tablets was high enough to achieve these effects.

Something not previously addressed in this thesis but worth noting is that butyrate may directly affect the microbiome. This aspect should be explored in future studies because the existing research on butyrate and the intestinal microbiome predominantly concentrates on the microbiome's production of butyrate rather than the direct effect of butyrate on the microbiome. By influencing the balance and diversity of the microbiome in the intestine, butyrate could potentially affect the production of short-chain fatty acids (SCFAs) by the microbiome, introducing an additional source of butyrate alongside sustained-release tablets.

DOES BUTYRATE HAVE POTENTIAL AS A THERAPEUTIC AGENT FOR NCDS?

Based on our *in vitro* experiments with butyrate and those of others, as well as animal experiments and the animal industry as explained in the introduction of this thesis, the potential of butyrate has already been proven to a certain extent. Butyrate is very effective in improving intestinal homeostasis and intestinal barrier, which is beneficial for NCD patients. A major challenge is whether we can get enough butyrate in the right place for the right time period. The short contact time of butyrate with the intestinal epithelial cells in the small intestine might not be enough to create a barrier protective effect. Although, we did observe some effects on Th cells in OA patients treated with the sustained release butyrate tablets, which is an indication that a short contact time at least at the level of the Th cells in circulation would be sufficient. In this thesis the first steps are made towards the development of a sustained release butyrate formulation to treat NCDs.

In conclusion, this thesis gains more insight regarding the pharmacodynamics of butyrate in various *in vitro* models. In a model with activated HT-29 cells butyrate inhibited IRF9 and CXCL10 gene and protein expression, which resulted in the prevention of CXCL10 release. In a Caco-2 model with and without activated PBMCs, butyrate protected against immune-mediated barrier disruption and acted anti-inflammatory. These *in vitro* studies show the strong anti-inflammatory and barrier protective effect of butyrate, with effective concentrations ranging between 1-8 mM. Based on a mathematical model we calculated which release rate is required for a sustained release butyrate formulation to achieve pharmacologically active concentrations in the intestinal lumen, namely 0.08-0.2 mmol butyrate/h. This knowledge was used to develop a 150 mg butyrate (as calcium) sustained release tablet, which was studied in patients having hand OA in a double-blind randomized placebo-controlled clinical trial. The butyrate treatment lowered the percentage of activated Th cells, activated effector Th cells and Th9 cells, as well as shifting the Th17/Treg balance in favor of Treg after ex vivo stimulation of PBMCs.

All these effects are presented and summarized in Figure 1. Overall, butyrate as a drug substance has shown high potential as a therapeutic agent for NCDs, but more research is needed on butyrate in a drug product.

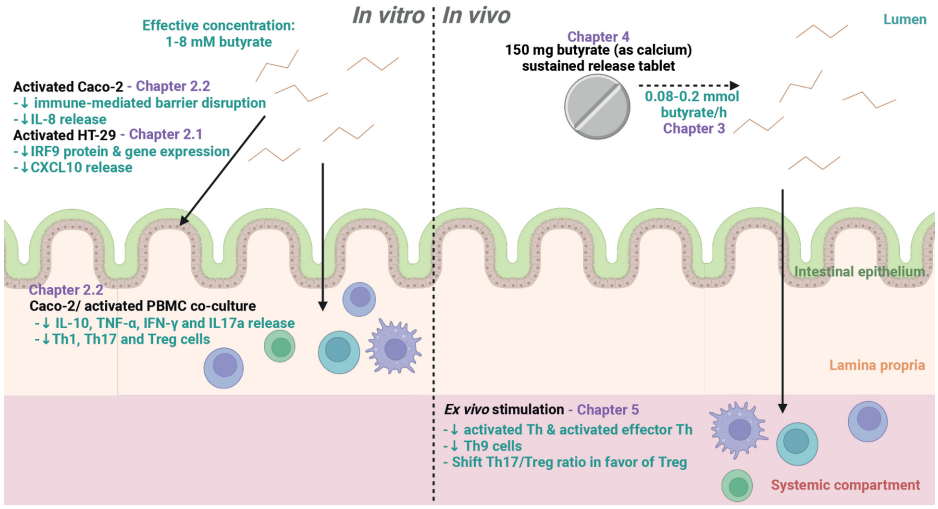

Figure 1. *This figure summarizes the results obtained in this thesis. In various in vitro models butyrate protected against immune mediated barrier disruption, inhibited the release of IL-8 and CXCL10 by intestinal epithelial cells, inhibited IRF9 protein and gene expression, inhibited the release of IL-10, TNF-α, IFN-γ and IL-17a by PBMCs, and lowered the percentage of Th1, Th17 and Treg cells. A mathematical model was developed to calculated the desired butyrate release rate (0.08-0.2 mmol/h) to achieve pharmacologically active concentrations in the intestinal lumen. This knowledge was used to develop a 150 mg butyrate (as calcium) sustained release tablet. In a randomized placebo-controlled trial in hand OA patients this tablet showed to decrease activated Th, activated effector Th cells and Th9 cells, and shifted the Th17/Treg ratio in favor of Treg.*

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NEDERLANDSE SAMENVATTING

Niet-overdraagbare aandoeningen ('non-communicable diseases' of NCDs) zijn chronische ziekten, zoals bijvoorbeeld hart- en vaatziekten, chronische luchtwegaandoeningen, diabetes en immuunstoornissen. Elk jaar eisen deze ziekten meer dan 41 miljoen levens en zijn ze verantwoordelijk voor 74% van alle sterfgevallen wereldwijd. NCDs worden onder andere geassocieerd met een verstoorde samenstelling van het darmmicrobioom en een verstoorde darmbarrière. Dit leidt tot een verhoogde doorlaatbaarheid van macromoleculen zoals lipopolysacchariden (LPS) en een laaggradige ontsteking, zowel systemisch als lokaal in de darm.

Bij NCDs zou een verstoorde samenstelling van het darmmicrobioom kunnen leiden tot veranderingen in de aanwezigheid en activiteit van bacteriën die butyraat produceren en daardoor mogelijk tot verlaagde butyraatconcentraties in de darm. Butyraat staat bekend om zijn ontstekingsremmende en barrière-beschermende effecten. Verminderde butyraatconcentraties in de darm zouden invloed kunnen hebben op de darmhomeostase en integriteit. Om deze reden zou suppletie van butyraat in de darm via een geneesmiddel voordelig kunnen zijn voor patiënten met een NCD.

Het doel van dit proefschrift was om het potentieel van butyraat als therapeutisch middel voor chronische ziekten te onderzoeken, zowel in het laboratorium als bij patiënten. Hiervoor werden de farmacodynamiek en farmacokinetiek van butyraat verder onderzocht en werd een geneesmiddel met butyraat als actieve stof ontwikkeld en getest bij patiënten met handartrose in een gerandomiseerde, dubbelblinde, placebogecontroleerde klinische studie.

De farmacodynamiek van butyraat

In **hoofdstuk 2.1** werden darmepitheelcellen HT-29 geactiveerd met interferon-gamma (IFN-γ) of IFN-γ gecombineerd met tumor necrosis factor-alpha (TNF-α) en behandeld met butyraat om het werkingsmechanisme te bestuderen. IFN-γ en TNF-α, beide ontstekingsmediatoren, staan bekend als activators van darmepitheelcellen en kunnen ook de darmbarrière verstoren. IFN-γ activeert de signaaltransducer- en activator van transcriptie 1 (STAT1) route, wat resulteert in de afgifte van C-X-C-motief chemokine ligand 10 (CXCL10). TNF-α gecombineerd met IFN-γ heeft een synergetisch effect op deze route. De afgifte van CXCL10 werd volledig geblokkeerd door butyraat, niet alleen in cellen geactiveerd met IFN-γ, maar ook in cellen geactiveerd met de combinatie van IFN-γ en TNF-α. Dit toont de sterke ontstekingsremmende effectiviteit van butyraat aan. Om beter te begrijpen hoe butyraat de afgifte van CXCL10 beïnvloedt, werden ook eiwitten en genen van zowel de kanonieke als de niet-kanonieke STAT1-route onderzocht. We toonden aan dat butyraat de afgifte van CXCL10 blokkeert via de niet-kanonieke route, vergelijkbaar met de bekende histondeacetylase (HDAC) remmer Trichostatin A (TSA). HDAC-remming onderdrukt expressie van eiwitten en genen die gerelateerd zijn

aan de niet-kanonieke STAT1-route, waaronder interferon-regulerende factor 9 (IRF9) en CXCL10 zelf, met als gevolg de voorkoming van geïnduceerde CXCL10-afgifte.

In **Hoofdstuk 2.2** werden de effecten van butyraat bestudeerd in verschillende modellen gebruikmakend van darmepitheelcellen Caco-2, zowel met als zonder geactiveerde perifeer bloed mononucleaire cellen (PBMCs). De PBMCs werden geactiveerd met LPS of αCD3/CD28. In dit model vertegenwoordigen de PBMCs het immuuncompartiment van de lamina propria. De lamina propria is het immuuncompartiment van de darm en bevat zowel aangeboren als adaptieve immuuncellen. Het co-cultuurmodel werd opgezet om de interactie tussen darmepitheelcellen en geactiveerde immuuncellen na te bootsen, en om te onderzoeken of butyraat mogelijk gunstige effecten heeft bij immuun gemedieerde darmbarrièreverstoring, zoals dit ook kan voorkomen in de darm van NCD patiënten. De darmbarrière is een van de belangrijkste verdedigingsmechanismen in het menselijk lichaam en voorkomt lekkage van endotoxines zoals LPS. Bovendien kunnen de darmepitheelcellen de reactie van de immuuncellen in de onderliggende lamina propria reguleren. Activatie van immuuncellen in de lamina propria kan echter ook de homeostase van darmepitheelcellen beïnvloeden, aangezien verschillende cytokines, zoals TNF-α, interleukine (IL)-1β, IFN-γ en IL-17a, epitheelcellen kunnen activeren en/of de barrièrefunctie van de darm kunnen beïnvloeden. Men spreekt hier van een crosstalk tussen de darmcellen en de immuuncellen.

In **Hoofdstuk 2.2** werd aangetoond dat butyraat de afgifte van IL-10 en TNF-α remt in LPS-geactiveerde PBMCs, terwijl de hoogste dosis butyraat de afgifte van IL-1β juist induceerde. Daarnaast remde butyraat de afgifte van IL-10, TNF-α, IFN-γ en IL-17a door αCD3/CD28-geactiveerde PBMCs. Het effect van butyraat op de afgifte van cytokines kwam overeen met het effect op T-cel fenotypes, aangezien butyraat het percentage geactiveerde T-helper (Th) 1-cellen, Th17-cellen en regulatoire T (Treg) cellen verlaagde. In het co-cultuurmodel van Caco-2 cellen en LPS- of αCD3/CD28-geactiveerde PBMCs werd de barrièrefunctie van Caco-2 cellen verstoord door cytokines afgegeven door de geactiveerde PBMCs. Dit werd dosisafhankelijk voorkomen door butyraat. Butyraat remde ook de afgifte van pro-inflammatoire cytokines door de PBMCs, waardoor we veronderstelden dat het effect van butyraat op de afgifte van cytokines gekoppeld was aan het beschermende effect op de barrière. Daarom werd het beschermende effect van butyraat op de barrière ook bestudeerd in een Caco-2 transwell model waarin inflammatoire cytokines, TNF-α, IFN-γ en IL-1β, werden toegevoegd aan het basolaterale compartiment, in plaats van geactiveerde PBMCs. In dit model liet butyraat opnieuw een beschermend effect op de barrière zien, wat duidt op een direct beschermend effect op door ontstekingen geïnduceerde darmbarrièreverstoring. De algemene HDAC-remmer TSA vertoonde vergelijkbare effecten met butyraat op het gebied van barrièrebescherming en vermindering van IL-8-afgifte door geactiveerde Caco-2 cellen. Dit geeft opnieuw aan dat HDAC-remming betrokken zou kunnen zijn bij het werkingsmechanisme van butyraat. Over het algemeen toont Hoofdstuk 2 het

belang aan van voldoende butyraat in de darm om de darmintegriteit en homeostase te waarborgen, zowel van de darmepitheelcellen als van de onderliggende immuuncellen.

De farmacokinetiek van butyraat

De butyraatconcentraties die nodig zijn voor een direct effect op darmepitheelcellen of immuuncellen liggen in ons onderzoek tussen de 1-8 mM. Om een geneesmiddel te ontwikkelen met butyraat als werkzame stof en dat een farmacologisch effect kan hebben in de dunne darm, is het belangrijk dat de lokale farmacokinetiek van butyraat in de dunne darm wordt bestudeerd. In **Hoofdstuk 3** presenteren we een wiskundig model om de butyraatconcentratie in het lumen van de dunne darm te modelleren na orale toediening van een directe afgifte of vertraagde afgifte butyraatformulering aan mensen.

Zodra butyraat vrijkomt uit de formulering en oplost, zal het diffunderen door de mucuslaag om vervolgens opgenomen te worden door de darmepitheelcellen. De resterende butyraatconcentratie in het lumen op een bepaald moment wordt bepaald door zowel de hoeveelheid die vrijkomt uit de formulering als de hoeveelheid die door het darmepitheel is gediffundeerd en opgenomen. Met andere woorden, de concentratie wordt bepaald door het evenwicht tussen instroom vanuit de tablet naar het lumen en uitstroom vanuit het lumen naar de circulatie toe. Ons wiskundige model toont aan dat een directe afgifte formulering alleen in staat is om farmacologisch actieve concentraties te handhaven binnen het eerste half uur nadat de formulering de dunne darm heeft bereikt. Butyraat zal in het geval van een directe afgifte formulering niet in contact komen met de gehele dunne darm, aangezien al het in de formulering beschikbare butyraat al aan het begin van de dunne darm zal worden opgenomen. Daarentegen zal een vertraagde afgifte formulering wel in staat zijn om farmacologisch actieve concentraties in de hele dunne darm te bereiken. Hoewel deze concentraties lokaal slechts voor korte duur worden gehandhaafd, omdat butyraat snel zal worden opgenomen en de formulering door de dunne darm zal voortbewegen.

Op basis van de berekeningen uit het wiskundige model vereist de vertraagde afgifte formulering een afgiftesnelheid in de range van 0.08-0.2 mmol/uur om farmacologisch actieve concentraties te kunnen bereiken. Bovendien dient de formulering voldoende butyraat te bevatten, om afgifte door de hele dunne darm te kunnen garanderen. Het blijft echter discutabel of de blootstellingsduur van deze butyraatconcentratie aan de darmepitheelcellen lang genoeg is om een effect te bereiken.

Het geneesmiddel

Op basis van de kennis uit **Hoofdstuk 2 en 3** werd een butyraat bevattend geneesmiddel ontwikkeld. Het geneesmiddel wordt beschreven in **Hoofdstuk 4**. Als actief bestanddeel werd het calciumzout van butyraat gebruikt. In de kern van de tablet werden hydroxypropyl methylcellulose, gesilificeerd microkristallijne cellulose en magnesiumstearaat gebruikt als hulpstoffen. Deze kern is verantwoordelijk voor de vertraagde afgifte van het butyraat.

De tablettenkernen werden gecoat met een smaakmaskerende laag bestaande uit talk, titaniumdioxide, PEG6000, simeticon emulsie, Eudragit RL 30 D en tri-ethylcitraat. De coating beschermt tegen de slechte smaak van butyraat en blijft intact bij de hogere pH-waarde in de mond. Zodra de tablet de maag met een lage pH-waarde bereikt, lost de coating op. Elke tablet bestaat uit 150 mg (wat overeenkomt met 1,72 mmol) butyraat (als calcium), wat voldoende is voor de gewenste afgifteduur.

De klinische studie

Handartrose is een chronische ziekte die wordt gekenmerkt door degeneratie van het kraakbeen in de gewrichten van de handen, wat leidt tot pijn, stijfheid en verminderde functionaliteit. Deze symptomen hangen samen met veranderingen in het darmmicrobioom, defecten van de darmbarrière, synoviale ontsteking en lichte mate van systemische ontsteking. Hierdoor zagen we handartrose als een geschikt eerste exploratieve model voor NCDs om de effecten van de ontwikkelde vertraagde afgifte butyraattabletten te onderzoeken in een gerandomiseerde, dubbelblinde, placebogecontroleerde klinische studie. In **Hoofdstuk 5** worden de effecten van de behandeling met butyraat op markers van systemische ontsteking, markers van LPS-lekkage en *ex vivo* stimulaties op volbloed of PBMCs getoond.

Vertraagde afgifte butyraat had geen invloed op markers van systemische ontsteking, zoals hoog sensitief C-reactief proteïne (hsCRP), IL-6, IL-1β, of markers van LPS-lekkage zoals IgG LPS en LPS-bindend proteïne (LBP). *Ex vivo* stimulaties van volbloed en PBMCs werden uitgevoerd om meer inzicht te krijgen in het potentieel van butyraat als behandeling, omdat deze stimulaties de inflammatoire potentie van verschillende immuuncellen, zoals monocyten en T-cellen, laten zien. Uit de resultaten van de klinische studie kan worden geconcludeerd dat vertraagde afgifte butyraattabletten het fenotype van monocyten noch hun functie beïnvloedden. Ook op afgifte van TNF-α, IL-6, IFN-γ en/of IL-10 door LPS gestimuleerd volbloed en PBMCs werd geen effect gevonden. In ionomycine-PMA-golgiplug-gestimuleerde monocyten bleven ook de intracellulaire TNF-α en IL-10 expressie en LPS receptor TLR4 onveranderd in de groep behandeld met butyraat. Desondanks beïnvloedden de vertraagde afgifte butyraattabletten wel de activering van T helper (Th) cellen. In αCD3/CD28-geactiveerde PBMCs werd het percentage geactiveerde CD69+CD4+, CD25+CD4+ en geactiveerde effector CD25+FoxP3- Th cellen verminderd en de Th17/Treg-balans verschoof in het voordeel van Treg. Hoewel de activatiestatus van de Th cellen werd onderdrukt in de butyraatgroep, was er geen effect te zien op de cytokineafgifte van de geactiveerde PBMCs. Daarentegen werd wel de intracellulaire IL-9 expressie binnen de Th cellen van αCD3/CD28-geactiveerde PBMC's verlaagd door de behandeling met butyraat. IL-9 is een systemische biomarker die wordt gerelateerd aan de ernst van de handartrose. Een placebo-effect kan echter niet volledig worden uitgesloten, aangezien er geen significant verschil werd gevonden bij vergelijking van de Δvisite 2-visite 1 tussen de butyraat- en de placebogroep. Naast de

hierboven genoemde Th cel parameters, verlaagde zowel de behandeling met butyraat als de placebo-behandeling de frequentie van geactiveerde Th1 cellen, Th17 cellen, geactiveerde Th17 cellen, Treg cellen, IFNγ+CD4+ cellen en TNFα+CD4+ cellen. Hierdoor kan niet worden uitgesloten dat de hulpstoffen die in de formulering worden gebruikt ook een effect hebben.

In dit proefschrift heeft butyraat als actieve stof veel potentie getoond als therapeutisch middel voor chronische ziekten, maar er is meer onderzoek naar de farmacokinetiek van butyraattabletten om hun effectiviteit te kunnen optimaliseren voor gebruik in patiënten met NCDs.

SAMENVATTING VOOR NIET-EXPERTS

Niet-overdraagbare aandoeningen ('non-communicable diseases' of NCDs) zijn chronische ziekten, zoals bijvoorbeeld hart- en vaatziekten, chronische luchtwegaandoeningen, diabetes en immuunstoornissen. Elk jaar eisen deze ziekten meer dan 41 miljoen mensenlevens en zijn ze verantwoordelijk voor 74% van alle sterfgevallen wereldwijd. De essentie van deze aandoeningen ligt in verstoringen van het darmmicrobioom (de bacteriën in de darm) en de darmbarrière, wat resulteert in ontstekingen, zowel lokaal in de darm als door het hele lichaam. In dit proefschrift wordt onderzocht of butyraat, een stof met bekende ontstekingsremmende eigenschappen, als geneesmiddel zou kunnen werken voor mensen die een NCD hebben. Specifieker wordt onderzocht hoe butyraat effect heeft op cellen in de darm en cellen van het afweersysteem.

In laboratoriumexperimenten werd de farmacodynamiek van butyraat bestudeerd. Farmacodynamiek beschrijft simpel gezegd wat een geneesmiddel in je lichaam doet en welke effecten het geneesmiddel teweegbrengt in het lichaam of op cellen van het lichaam. Tijdens de experimenten in het laboratorium werden de effecten van butyraat op darmcellen en cellen van het afweersysteem onderzocht en werd gekeken wat het precieze werkingsmechanisme van butyraat zou kunnen zijn. In deze experimenten zagen we dat butyraat ontstekingen kon remmen en de beschermende barrièrefunctie van de darmcellen in stand kon houden.

Een tweede aspect van dit proefschrift richt zich op de farmacokinetiek van butyraat. Farmacokinetiek beschrijft simpel gezegd wat het lichaam met een geneesmiddel doet en wat het verloop is van de geneesmiddelconcentratie in verschillende delen van het lichaam. In dit proefschrift ligt de focus op de concentratie die nodig is om een positief effect in de darm te hebben. Hierbij wordt gebruikgemaakt van een wiskundig model om de butyraatconcentratie in de dunne darm te voorspellen na inname van verschillende soorten tabletten. Hierbij kun je denken aan een tablet waaruit een bepaalde hoeveelheid van het geneesmiddel heel snel wordt vrijgelaten of juist heel langzaam. Op basis van deze informatie werd een tablet ontworpen die een langzame afgifte van butyraat in de dunne darm zou bewerkstelligen. Er werd nagedacht over hoe deze tablet gemaakt moest worden en aan welke kwaliteitseisen deze tablet zou moeten voldoen. Daarnaast werd de tablet voorzien van een dun laagje, ook wel coating genoemd, om de onaangename smaak en geur van butyraat te verbergen.

Ten slotte werd de ontwikkelde butyraat tablet getest in een onderzoek met patiënten met handartrose (een NCD). Hoewel bepaalde positieve effecten worden waargenomen op cellen van het afweersysteem, lijken de tabletten geen effecten te laten zien op de beschermende barrière functie van de darm of op ontstekingen die in de bloedomloop gemeten kunnen worden.

Dit proefschrift laat voornamelijk in het laboratorium veel positieve effecten zien van butyraat. Deze effecten zijn minder terug te zien als een geneesmiddel met butyraat aan patiënten met handartrose gegeven wordt. Dit zou erop kunnen wijzen dat er meer onderzoek nodig is naar de farmacokinetiek van butyraat en dat de ontworpen tabletten op basis daarvan aangepast zouden moeten worden.

DANKWOORD

Ik heb het schrijven van dit proefschrift regelmatig vergeleken met het rennen van een marathon. De eerste kilometers gaan snel en tegen het einde, rond het 30 kilometerpunt, begin je je af te vragen of je de eindstreep ooit zult halen. Maar het schrijven van dit dankwoord betekent dat de finish in zicht is en wat een reis is het geweest. Bij de voorbereiding op het lopen van een marathon krijg je regelmatig de vraag wat je streeftijd is en tijdens het werken aan je PhD onderzoek krijg je regelmatig de vraag hoever je al bent en wanneer je klaar hoopt te zijn. Maar een marathon en een PhD onderzoek zijn meer dan alleen de finish halen, het is ook de weg daarnaartoe en het doorzettingsvermogen dat ervoor nodig is. Trainen, nieuwe vaardigheden leren, heuvels beklimmen en af en toe een dal, het is allemaal onderdeel van de route. En waar er langs de zijlijn van een marathon altijd mensen zijn om je aan te moedigen, maar ook op andere manieren te ondersteunen, is dat ook tijdens mijn PhD het geval geweest. Graag neem ik jullie mee langs de route die ik heb afgelegd om zo een ieder die het toekomt te bedanken voor zijn of haar bijdrage, op wat voor manier dan ook.

Daar gaan we dan!
Laat ik beginnen bij het begin. Het idee achter dit proefschrift, vertraagde afgifte butyraat tabletten als behandeling van lichte systemische inflammatie, darmbarrière en dysbiose van het microbioom is niet bedacht door mij, maar door Jan Vermeiden. Dankjewel Jan, dat je met jouw idee bij Tiofarma hebt aangeklopt en op die manier het startschot hebt gegeven voor mijn promotieonderzoek. We hebben samen een lange weg afgelegd met de zoektocht naar samenwerkingspartners voor de klinische studie en de ontwikkeling van de butyraat tabletten. Ik bewonder je ruimdenkende blik en de gave die je hebt om alle literatuur die je leest met elkaar te verbinden om zo jouw hypotheses te ondersteunen. Ik kijk met veel plezier terug op de autoritjes naar verschillende (potentiële) samenwerkingspartners en de samenwerking tussen ons.

Bij Tiofarma was de eerste persoon die met dit onderwerp te maken kreeg Hans Waals. Hans, zonder jouw vertrouwen in dit project en je geloof in het onderwerp had ik deze route nooit kunnen afleggen. Dankjewel dat jij en de rest van het managementteam het aan hebben gedurfd om een PhD plek in combinatie met de functie van onderzoeker op de R&D afdeling van Tiofarma aan te bieden. Ik bewonder je enthousiasme en heb de telefoontjes op willekeurige momenten om mij met iets te complimenteren erg gewaardeerd.

Het idee bestond, de mogelijkheid was gecreëerd en toen moest er nog een PhD kandidaat gevonden worden. Dat was de taak van Herman Vromans. Herman, wij kennen elkaar nu al een hele tijd. Het begon met een bachelorstage bij jou en een van jouw toenmalige PhD studenten in de ziekenhuisapotheek van het UMCU en later kwamen we elkaar nog een aantal keer tegen bij de vakken die jij doceerde als onderdeel van de studie

farmacie. Tijdens een van deze vakken gaf je aan dat je manager zou worden van de R&D afdeling bij Tiofarma en dat je nog nieuwe medewerkers zocht. We moesten je maar even mailen als we interesse hadden, dus dat deed ik. En nu 7 jaar later staan we hier. Je was mijn docent, professor, (co-)promotor en manager, maar bovenal heb ik je gezien als mentor. Door je vertrouwen in mij en de vrijheid die je mij gaf ben ik gegroeid, dankjewel daarvoor.

Al snel bleek dat dit promotieonderzoek niet af zou zijn zonder *in vitro* experimenten. De route vervolgde zich naar de Universiteit Utrecht, afdeling farmacologie. Via een van de symposia die gehouden werden binnen UIPS kwam ik in contact met Johan Garssen en Linette Willemsen. Na enkele gesprekken werden jullie naast Herman ook onderdeel van mijn promotieteam. Johan, jouw enthousiasme vanaf het eerste moment dat ik je vertelde over onze plannen was aanstekelijk. Dankjewel dat je de samenwerking met ons wilde aangaan en we er zo samen een mooi boekje van hebben kunnen maken. Linette, ooit was je mijn tutor tijdens de bachelor farmacie en nu hebben we samen een aantal mooie hoofdstukken van dit proefschrift geschreven. Dankjewel voor je kritische blik en het delen van al je kennis en kunde op het gebied van darmepitheelcellen en co-culturen.

Een ander onderdeel van dit proefschrift was het testen van de ontwikkelde butyraat tabletten in een klinische studie. Dit werd een samenwerking met de Sint Maartenskliniek, met name Els van den Ende, Merel Hartog en Calin Popa. Dank jullie wel dat jullie geloofden in de potentie van butyraat en mee wilden werken in het opzetten en uitvoeren van de klinische studie. Ik heb met plezier met jullie samengewerkt en ik vind dat we trots mogen zijn op het resultaat. Daarnaast wil ik natuurlijk alle vrijwilligers bedanken die hebben meegedaan aan dit onderzoek.

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In 7 jaar tijd hebben natuurlijk ook heel veel collega's op een manier een bijdrage geleverd. Bij Tiofarma wil ik al mijn (oud-)collega's van de R&D afdeling (Merel, Dick, Myrthe, Eva, Ton, Lydia, Sabina, Yaman, Yannick, René, Wouter, Nathan, Yvonne, Tjalke, Richard, Marc en Bahar) bedanken voor jullie support, de gezelligheid en het tolereren

van de butyraat stank. Daarnaast zijn er ook enkele Tio collega's die ik in het bijzonder wil bedanken voor hun bijdrage, in willekeurige volgorde:

- Feike: Koffie om 6:00 was nog nooit zo gezellig! Jij leerde mij hoe ik tabletten kon produceren en offerde daar zelfs je schoenen voor op (sorry!).
- Caroline: Dank voor je analytische input, maar ook de korte kletsmomenten voor of na een overleg.
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- Walter: Dankjewel voor de hulp met alle logistiek omtrent de studiemedicatie.
- Ivo en Irene: Dank voor jullie ondersteuning bij het schrijven van het IB en IMPD.
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- Alinda: Zonder jou stond ik nu nog te pipetteren, dankjewel dat je mij scherp hield op het lab.
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Nog even tot de finish, de "Hora est", wat was het een leerzame route. Als dat geen persoonlijk record is…

ABOUT THE AUTHOR

Sandra was born on March 15th 1991 in Maastricht, the Netherlands. In 2009 she graduated from high school (Bonnefanten college) and moved to Utrecht, the Netherlands to study a Bachelor's degree in Pharmacy at the Utrecht University. After successfully completing her Bachelor's degree in 2013, Sandra continued her studies with a Master's degree in Pharmacy. During her Master's program, she spent six months in Berlin for her research project that focused on investigating methods to determine the skin barrier at the Charité University in the group of Prof. Marcus Maurer, under the supervision of Dr. Joachim Fluhr. Throughout her studies, Sandra was actively participated in the study association for pharmacy students, U.P.S.V. "Unitas Pharmaceuticorum," where she served as secretary from 2012 to 2013.

During her Master's degree, Sandra developed a special interest in drug development. After obtaining her Master's degree she started working as a scientist at the R&D department of Tiofarma while pursuing her PhD in collaboration with the Pharmacology Division, Utrecht Institute of Pharmaceutical Sciences under the supervision of Prof. Johan Garssen, Dr. Herman Vromans, and Dr. Linette Willemsen. As part of her PhD she developed a sustained release tablet containing butyrate, which was tested in a placebo-controlled clinical study. Additionally, she investigated the mechanism of action of butyrate in various *in vitro* models using intestinal epithelial cells and human peripheral blood mononuclear cells.

She is currently working as an experienced clinical scientist at the Centre for Human Drug Research in Leiden, the Netherlands.

AUTHORSHIP STATEMENTS

Chapter 1

I created the idea and set up the general introduction and conducted all literature search that was needed. I designed the summarizing figure, and wrote the entire general introduction. Throughout the whole process I asked for and implemented input and feedback from the supervisory team (promotor and co-promotors).

Chapter 2.1

I contributed significantly to define the research question/goal and proposed/developed the methodology and experimental design that was needed before starting the study at UU in discussion together with the co-promotor. All experiments were performed by myself together with two MSc students, under my supervision and a lab technician. All data were carefully discussed together with the co-promotor. Additionally, I conducted the data analysis, carried out a literature search and wrote the first draft of the manuscript. Subsequently, I revised it based on input and feedback from the co-authors and external reviewers until the final publication. The role of the individual authors of this chapter have been described and published according to the guidelines of the journal in which it is published.

Chapter 2.2

I contributed significantly to define the research question/goal and proposed/developed the methodology and the experimental design that was needed before starting and during data collection of the experimental work at UU, all in discussion together with the co-promotor. I conducted the data analysis, performed a literature search, and wrote the first draft of the manuscript. The revision process involved incorporating feedback from co-authors and external reviewers until the final publication. The role of the individual authors of this chapter have been described and published according to the guidelines of the journal in which it is published.

Chapter 3

I defined the research question/goal and in close collaboration with the second author I did set up the mathematical model for calculating the luminal butyrate concentration following the oral intake of an immediate release or sustained release formulation. I conducted a literature search and wrote the first draft of the manuscript, which I revised after input and feedback from the co-authors and external reviewers up to final publication.

Chapter 4

I designed the drug product and production process of butyrate (as calcium) sustained release tablets based on the knowledge obtained from Chapter 3. I decided for the exact formulation and defined the acceptance criteria in alignment with experts at Tiofarma. I collaborated with the analytical department at Tiofarma during the product and

production process development. Additionally, I contributed to the method development of the dissolution test and characterization of an unknown impurity. I wrote the first draft of the Investigational Medicinal Product Dossier, which I revised after input and feedback from various stakeholders/experts. This drug product was studied in the clinical study described in Chapter 5.

Chapter 5

I actively participated in establishing collaboration with the Sint Maartenskliniek and in discussions regarding the clinical study presented in this chapter. I contributed to defining the research question, study endpoints and in writing the study protocol. I wrote the first draft of the Investigator's Brochure, which I revised after input and feedback from various stakeholders. The Investigator's Brochure and Investigational Medicinal Product Dossier (see statement Chapter 4) were integral parts of the submission dossier of the clinical study presented in this chapter. The methods presented in this chapter were developed collaboratively with a lab technician at UU in discussion together with the co-promotor, and documented in standard operating procedures and an analytical work plan for this clinical study. I carried out the experiments together with a lab technician. Additionally, I was responsible for data management, performed all data analysis, conducted a literature search and wrote the first draft of the manuscript. The revision process involved incorporating feedback from co-authors until submission of the manuscript for publication.

Chapter 6

I had the idea and set up of the summarizing discussion. I conducted the literature search and wrote the general discussion. Throughout this process, I actively sought and implemented input and feedback from my supervisors.

LIST OF ABBREVIATIONS

AMPK:	AMP-activated protein kinase
BMI:	Body mass index
COX:	Cyclooxygenase
CXCL8:	C-X-C motif chemokine ligand 8
CXCL10:	C-X-C motif chemokine ligand 10
eGFR:	Estimated glomerular filtration rate
ELISA:	Enzyme-linked immunosorbent assay
FD4:	4 kDa fluorescein isothiocyanate-dextran
FITC:	Fluorescein isothiocyanate
FMO:	Fluorochrome minus one
GPCR:	G protein-coupled receptor
HDAC:	Histone deacetylase
HPLC:	High-performance liquid chromatography
hsCRP:	High sensitive C-reactive protein
IECs:	Intestinal epithelial cells
IC_{50}:	Half-maximal inhibitory concentration
IFN-γ:	Interferon-gamma
IL:	Interleukin
IM:	Immediate release
IRF9:	Interferon Regulatory Factor 9
JAK1:	Janus kinase 1
JAK2:	Janus kinase 2
K&L:	Kellgren&Lawrence
LBP:	LPS binding protein
LOX:	Lipoxygenase
LPS:	Lipopolysaccharides
MCT1:	Monocarboxylate transporter 1
MUC:	Mucine
NCDs:	Non-communicable diseases
NFκB:	Nuclear factor kappa B
NMT:	Not more than
NO:	Nitric oxide
NRS:	Numeric pain rating scale
OA:	Osteoarthritis
PBMCs:	Peripheral blood mononuclear cells
PBS:	Phosphate buffered saline
PMA:	Phorbol myristate acetate
qPCR:	Quantitative polymerase chain reaction
RPS13:	Ribosomal protein S13
sCD14:	Soluble CD14

SCFAs:	Short chain fatty acids
SMCT1:	Sodium-coupled monocarboxylate transporter 1
SOCS1:	Suppressor of cytokine signaling 1
SR:	Sustained release
STAT1:	Transducer and activator of transcription 1
TEER:	Transepithelial electrical resistance
Th:	T helper
TLR4:	Toll-like receptor 4
TNFR:	TNF receptor
TNF-α:	Tumor necrosis factor-alpha
Treg:	Regulatory T-cell
TSA:	Trichostatin A
ZO-1:	Zonula occludens-1