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Short chain fatty acids and colonic health

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Contents

Chapter 1

General introduction

The large intestine, the inner tube of life

The last part of the digestive tract, the large intestine, is a tube of approximately 135 cm long that separates the external from the internal milieu. The main functions of the human large intestine are the extraction of water, electrolytes and nutrients from the luminal contents and the microbial metabolisation of undigested food components, such as dietary fibres. At the same time, the colonic mucosa forms a tight barrier that prevents the entrance of potentially toxic luminal components, such as bacteria and their metabolites, viruses and dietary antigens into the portal and systemic circulation.[1]

Barrier function

The mucosal barrier is essential for the maintenance of colonic and host health.[2] It consists of a monolayer of epithelial cells (colonocytes), which are firmly connected to each other by dynamic structures called tight junctions (TJ) that restrict the passage of even very small (2 kDa) molecules (Figure 1.1).[3]

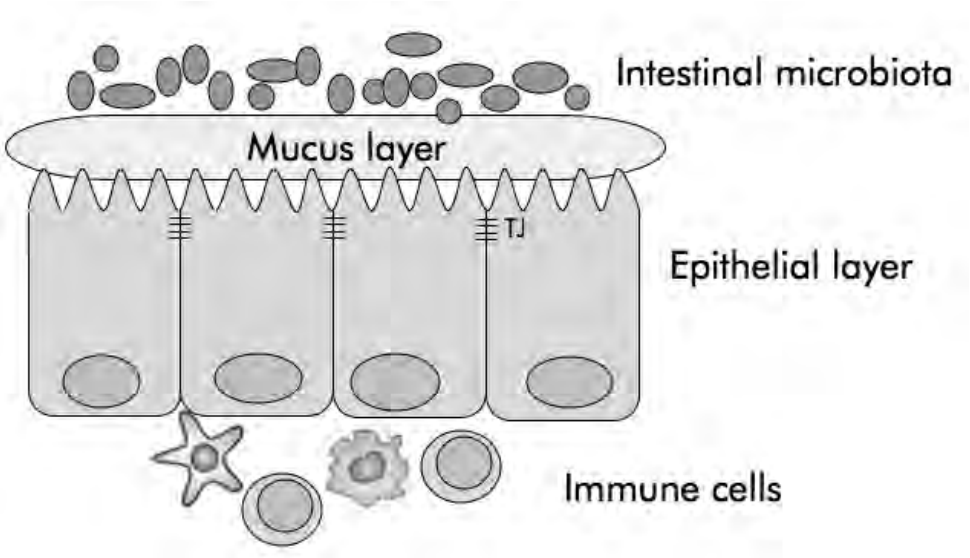

Figure 1.1 Schematic picture of the colonic epithelial barrier.

The epithelial monolayer is covered by a thick viscous mucus layer that protects the epithelial lining against luminal toxic substances and is a medium for lubrication and transport, which prevents mechanical damage.[4,5] The mucus layer mainly consists of mucin glycoproteins and trefoil factors, but also contains other proteins

secreted by the intestinal epithelium such as secretory IgA (sIgA), which prevents the epithelial adherence and penetration of bacteria.[6] Underneath the single layer of epithelial cells lies the lamina propria, which contains numerous immune cells that provide a permanent protection against invading microorganisms. The mucosal immune system can be divided into two interactive systems: the innate and the adaptive immune system.[7] The innate immune response, which is rapid but non-specific, depends largely on the recognition of conserved microbial structures by so-called pattern recognition receptors. Therefore, due to the discrimination between different bacteria, the mucosal immune system contributes to tolerance to commensal bacteria, but is concomitantly able to induce an immune response towards pathogens. Dendritic cells and macrophages play a pivotal role in this innate defence by sampling pathogens and bacteria. The adaptive immune response by T-cells and B-cells reacts slower (3-5 days), but highly specific. This response is directed against specific antigens present on pathogens. The different cells of the immune system secrete a wide variety of cytokines used for inter-cell communication.[8]

Oxidative stress

Oxidative stress is characterised by an imbalance between the generation of free radicals, mainly reactive oxygen and nitrogen species and the antioxidant defence mechanisms, leading to a cascade of reactions in which lipids, proteins and/or DNA may get damaged, which subsequently could result in a disruption of the mucosal barrier function.[10] Therefore, antioxidant defence mechanisms, including antioxidant enzymes, such as glutathione-S-transferase (GST), and non-enzymatic antioxidant molecules, such as glutathione (GSH), are essential for the maintenance of gut barrier function. During inflammation excessive production of reactive oxygen species and reactive nitrogen species generated by inflammatory cells contribute to oxidative stress. This can lead to a depletion of the antioxidant defence mechanisms and increased epithelial permeability.[9]

Colonic microbiota

The colonic lumen harbours a complex diversity of micro-organisms, called the colonic microbiota. This complex ecosystem consists of ~10^{14} bacterial cells and more than 1000 different bacterial species. The number of bacterial cells, which are mainly living within the gastrointestinal tract, is estimated to outnumber human cells by 10 to 1.[11] The colonic microbiota plays a pivotal role for the maintenance of human health as it contributes to colonization resistance, the metabolisation of non-digested nutrients, synthesis of vitamins B and K, metabolism of bile acids, and immune function of the host.[11] Nowadays, several studies indicate that the intestinal microbiota has an important role in the development of various conditions including inflammatory bowel diseases[12], cancer[13] and even obesity.[14] The

composition of the colonic microbiota and the availability of nutrients for fermentation determines for a great deal its effects on colonic (patho)physiology and host health.

Prebiotics and probiotics

There is growing interest in functional foods that affect the composition and activity of the gut microbiota. One approach is the ingestion of live microorganisms that enter the gut and persist long enough to have beneficial effects on the host (probiotics).[15] Another approach is to consume non-digestible carbohydrates (prebiotics) that are selectively fermented by indigenous beneficial bacteria, thereby resulting in increased saccharolytic fermentation.[16] One of the proposed health-promoting effects of prebiotics is the increase in the production of short chain fatty acids (SCFA) in the intestinal lumen (Figure 1.2).[17]

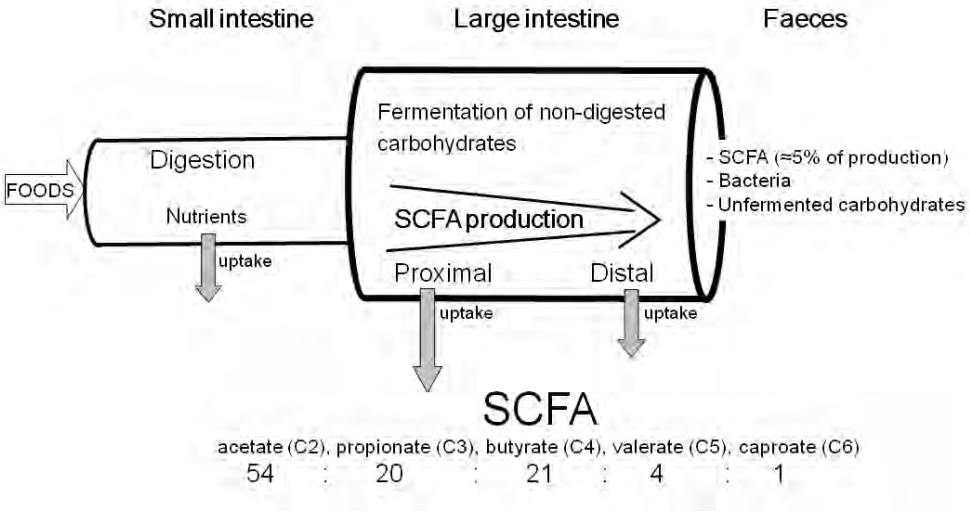

Figure 1.2 In the small intestine digestion and absorption of different nutrients occurs, whereas in the large intestine, microbial fermentation of food residues takes place. Saccharolytic fermentation of dietary fibres and resistant starches by the colonic microbiota leads to the formation of SCFA, mainly acetate, propionate and butyrate, but also valerate and caproate. SCFA are rapidly absorbed and eventually approximately only 5% of the produced SCFA ends up in the faeces.

Short chain fatty acids (SCFA)

SCFA are end-products of luminal microbial fermentation of predominantly non-digestible dietary carbohydrates. SCFA with different carbon chain lengths (acetate (C2), propionate (C3), butyrate (C4), valerate (C5) and caproate (C6)) are produced in varying amounts depending on the diet and the composition of the intestinal microbiota.[18] Most microorganisms in the colon prefer to ferment carbohydrates and switch to protein fermentation when fermentable carbohydrates are depleted.[19] While carbohydrate fermentation generally leads to health-promoting SCFA production, protein fermentation yields branched-chain fatty acids and potentially toxic metabolites (e.g. ammonia, amines, N-nitroso compounds, phenols, indoles and thiols).[20] Therefore, highest concentrations of SCFA are found in the proximal part of the colon. In autopsy samples from sudden death victims, total SCFA concentrations varied from 137 to 197 mmol/kg chyme in the caecum to 86 to 97 mmol/kg chyme in the descending colon.[21,22] In these samples, C2:C3:C4:C5:C6 was found to be present in a molar ratio of approximately 54:20:21:4:1.[21] SCFA are rapidly absorbed and have shown to have distinct bioactivity depending on their chain length. With regard to maintenance of colonic health and barrier function, butyrate has drawn most attention as this fatty acid is the major energy source for the colonocytes.[23,24] Furthermore, butyrate has been shown to have anti-inflammatory properties mainly through inhibition of nuclear factor kappa B (NF-κB) that controls inflammation by acting as a transcription factor.[25] In addition, butyrate has been shown to modulate oxidative stress[26], affect the composition of the mucus layer[27] and to have anti-carcinogenic effects mainly by affecting proliferation, differentiation and apoptosis of colonocytes.[28] The exact mechanism by which butyrate is able to induce these effects is complex and thought to involve multiple distinct mechanisms.[29] The most frequently described mechanism by which butyrate is able to induce changes in gene expression is through the inhibition of histone deacetylase. Hyperacetylation of histones disrupts their association with DNA, resulting in a more “open” chromatin structure that facilitates access of transcription factors to specific genes.[30] Although to a lesser extent than butyrate, propionate and valerate have also been demonstrated to possess the ability to induce histone hyperacetylation, while acetate and caproate show no effects on histone acetylation.[31-33] However, studies on the effects of the acetate, propionate, valerate and caproate on colonic health are limited.

Aims and outline of the thesis

Increased SCFA production in the large intestine is a possible mechanism by which prebiotics beneficially affect host health. Functional foods containing these prebiotics aim to improve the health of the consumer, focussing mainly on healthy people or patients with mild disease. The main goal of the project was to evaluate the effects of SCFA, especially butyrate on colonic health *in vitro* and *in vivo* in healthy volunteers and in patients with ulcerative colitis in clinical remission. These patients are characterised by low-grade levels of inflammation and oxidative stress of the colonic mucosa, and are therefore used as a model for patients with a mildly compromised colon. As important determinants of the colonic health status, colonic inflammation, oxidative stress and the mucus layer are object of study in the present thesis.

First, in **chapter 2** the recent knowledge regarding the role of butyrate on colonic function has been reviewed. In **chapter 3** an *in vitro* study is presented comparing the effects of different SCFA with varying chain lengths (C2-C6) on inflammation, the antioxidant status and epithelial integrity using a co-culture model that combines a human epithelial cell line with whole blood. As butyrate is regarded to be the most potent SCFA with regard to colonic health, the effects of this SCFA were studied in healthy volunteers. The effects of butyrate on the distal colon were investigated, because this way it was possible to control for the local concentration of butyrate and to collect tissue samples without prior bowel cleansing. For local distal administration of butyrate once daily rectal enemas were used for 14 days and the effects on colonic oxidative stress and inflammation were evaluated. The results of this human intervention study with a randomised double blind placebo controlled cross-over design in healthy volunteers are described in **chapter 4**. Next, this intervention with rectal butyrate administration was repeated in subjects characterised by a chronic low-grade colonic inflammation and mildly elevated levels of oxidative stress, such as patients with ulcerative colitis (UC) in clinical remission. This study with a randomised double blind placebo controlled parallel design is described in **chapter 5**. Besides inflammation and oxidative stress, the colonic mucus layer is another important part of the colonic barrier that could be affected by SCFA. **Chapter 6** describes a sampling technique to study the composition of the colonic mucus layer. In **chapter 7** the effect of butyrate on colonic mucus in healthy individuals and patients with low-grade inflammation is reported. In **chapter 8**, the major findings of all studies are reviewed and implications for future research are discussed.

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Chapter 2

Review article:
The role of butyrate on colonic function

Henrike M. Hamer, Daisy M.A.E. Jonkers, Koen Venema, Steven A.L.W. Vanhoutvin, Freddy J. Troost, Robert-Jan M. Brummer.

Aliment Pharmacol Ther 2008;27:104-119

Abstract

Background

Butyrate, a short chain fatty acid, is a main end-product of intestinal microbial fermentation of mainly dietary fibre. Butyrate is an important energy source for intestinal epithelial cells and plays a role in the maintenance of colonic homeostasis. The aim of this review is to provide an overview on the present knowledge of the bioactivity of butyrate, emphasizing effects and possible mechanisms of action in relation to human colonic function.

Methods

A PubMed search was performed to select relevant publications using the search terms: 'butyrate, short chain fatty acid, fibre, colon, inflammation, carcinogenesis, barrier, oxidative stress, permeability and satiety'.

Results

Butyrate exerts potent effects on a variety of colonic mucosal functions such as inhibition of inflammation and carcinogenesis, reinforcing various components of the colonic defence barrier and decreasing oxidative stress. In addition, butyrate may also promote satiety. Two important mechanisms include the inhibition of NF-κb activation and histone deacetylation. However, the observed effects of butyrate largely depend on concentrations and models used and human data are still limited.

Conclusion

Although most studies point towards beneficial effects of butyrate, more human *in vivo* studies are needed to contribute to our current understanding of butyrate-mediated effects on colonic function in health and disease.

Introduction

Short-chain fatty acids (SCFA), primarily acetate, propionate and butyrate, are organic acids produced within the intestinal lumen by bacterial fermentation of mainly undigested dietary carbohydrates, but also in a minor part by dietary and endogenous proteins, such as mucus, and sloughed epithelial cells.[1] Most micro-organisms prefer to ferment carbohydrate over protein and therefore saccharolytic bacterial fermentation occurs predominantly in the proximal colon, while proteolytic fermentation mainly takes place in the distal colon where fermentable carbohydrates are depleted. The latter is considered less favourable for the host because potentially toxic metabolites are formed such as ammonia, sulphur-containing compounds, indoles and phenols. Since this distal part of the colon is the predominant location of several gastrointestinal disorders, such as ulcerative colitis and colon cancer, it could be hypothesised that the production of these toxic metabolites and a lower availability of SCFA are involved in the pathogeneses of these diseases.[2, 3]

The production of SCFA allows the salvage of energy mainly from carbon sources as dietary fibre that is not digested in the small intestine. It has been estimated that SCFA can contribute to about 5-15% of the total caloric requirements of humans.[4] An important SCFA produced is butyrate that, besides being an energy source for the epithelial cells, also influences a wide array of cellular functions affecting colonic health. As such, butyrate may have an anti-carcinogenic and anti-inflammatory potential, affect the intestinal barrier and play a role in satiety and oxidative stress.

Due to this important role of butyrate and the rather low consumption of fermentable dietary fibre in today's Western diet, food manufacturers are interested in adding fibre sources to foods and beverages that rely on slow bacterial fermentation to increase distal colonic butyrate concentrations. In medical application, butyrate has also been proposed as a potential therapeutic agent for colonic inflammation.[5] In a book by Cummings *et al*. published in 1995[6], the effects of butyrate have been clearly reviewed, but the reported effects have often been based on *in vitro* and animal data. During the last decade additional human (intervention) studies have been published as well as knowledge on possible mechanisms of action is improving. This review summarises the present knowledge on the bioactivity of butyrate, emphasizing effects and possible mechanisms of action in relation to human colonic function (Figure 2.1).

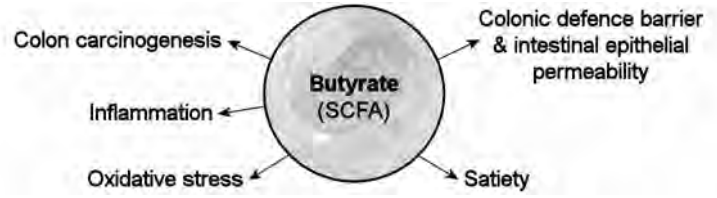

Figure 2.1 Different domains that may be affected by butyrate produced in the colonic lumen.

Production and absorption of SCFA

Total and relative molar concentrations of the main SCFA, acetate, propionate and butyrate produced in the human intestine, depend on the site of fermentation, diet and composition of the intestinal microbiota.[7] Absolute concentrations of butyrate in human faeces were found to range from 11 to 25 mmol/L[8,9], and molar ratios of acetate to propionate to butyrate varied between 48:29:23 and 70:15:15, respectively, with mean values of approximately 60:20:20.[1,9] However, the *in situ* production of total colonic SCFA is difficult to determine because more than 95% of the SCFA are rapidly absorbed and metabolised by the host.[1] Subsequently, faecal concentrations of SCFA are not necessarily representative for those in the more proximal colon and can also be affected by intestinal transit time.[10] Probably for these reasons various studies were not able to show effects of different fermentable substrates on faecal SCFA concentrations.[11,12]

Because of the difficult accessibility of the human colon, estimates of luminal SCFA concentrations are based on analyses in human gut contents of sudden-death victims[13,14] and stomal effluent of patients with transverse or sigmoid colostomy.[15] In autopsy samples, total SCFA concentrations varied from 137 to 197 mmol/kg chyme in the caecum to 86 to 97 mmol/kg chyme in the descending colon.[13,14] In transverse colostomy samples SCFA concentrations were high (1400 mol/kg dry matter) compared to those in sigmoid colostomy samples (550 mol/kg dry matter), which were similar to the concentrations in faeces from controls.[15] In line with the SCFA concentrations, the pH value is the lowest in the proximal colon (pH≈5.6) and increases towards the distal colon (pH≈6.3).[14]

As SCFA are weak acids (pKa≈4.8), more than 90% exist in the anionic, dissociated form in the colonic lumen.[16] Several different mechanisms of uptake of SCFA across the apical membrane of the colonocytes have been proposed, including diffusion of the undissociated (lipid soluble) form[16], SCFA/HCO_3^- exchange[17,18] and active transport of the dissociated form by SCFA-transporters.

Two SCFA-transporters have been reported: the monocarboxylate transporter isoform 1 (MCT1), which is coupled to a transmembrane H^+-gradient[19] and SLC5A8, which is a Na^+-coupled co-transporter and is also denoted sodium-coupled monocarboxylate transporter (SMCT1).[20] SCFA, with butyrate being the most potent one, hereby stimulate the absorption of sodium and water[21] and can be considered as anti-diarrhoeal agents.[22] The transport mechanisms involved in basolateral transport of SCFA are still unclear.

The majority of the absorbed butyrate is metabolised by the colonic epithelium, resulting in low concentrations of butyrate in portal blood. Human portal butyrate concentrations ranged from 1.3 to 14.4 μmol/L in patients during gall-bladder surgery[23] and from 14 to 64 μmol/L in sudden death victims.[14] Most likely the liver subsequently extracts the majority of the remaining butyrate, resulting in even lower venous systemic serum butyrate concentrations ranging from 0.5 to 3.3 μmol/L.[24-27] Serum concentrations of propionate and acetate in peripheral blood have been shown to range from 3.8 to 5.4 and from 98 to 143 μmol/L, respectively.[24-27]

Delivery of butyrate

An important source of butyrate is colonic fermentation of dietary fibre. The rate and amount of butyrate being produced along the colonic lumen during dietary fibre supplementation depends on its chemical structure, such as solubility and degree of polymerisation. Insoluble fibres (e.g. cellulose and lignin) have a rather low fermentability, but are associated with increased faecal mass and decreased colonic transit time. Soluble fibres are highly fermentable and hence generate greater quantities of SCFA in the colon.[28] Fibres with a higher degree of polymerization are more resistant to saccharolytic fermentation resulting in prolonged fermentation, expanding towards the distal colon.[29] Examples of fermentable dietary and chemically modified fibres that are associated with a higher production of SCFA either *in vitro* or *in vivo* are: oligofructose[30], inulin[31], psyllium[32], germinated barley foodstuff[33], hydrolysed guar gum[34], oat bran[9,35], corn starch[12], isomalt[11], gluconic acid[36] and butyrylated starch.[37] Although the beneficial effects of these fibres are often attributed to the increased butyrate production, these soluble fibres can also affect other intestinal characteristics influencing intestinal health, such as increased faecal bulk, shortened colonic transit time, changes in the composition of the gut microbiota, lowered intraluminal pH and changed bile acid profiles.[38]

Apart from dietary fibres, also other ingested substrates can contribute to an increase in the colonic butyrate concentrations by different mechanisms. An example is the oligosaccharide acarbose that increases the amount of starch entering the colon by acting as an α-glucosidase inhibitor.[8,25] In addition, tributyrin, a triglyceride containing three butyrate molecules esterified to glycerol, augments

butyrate concentrations after hydrolysation by pancreatic and gastric lipases.[39] Also butyrate tablets coated with a slow release pH dependent coating releasing butyrate in the distal ileum and proximal colon can be used.[40,41] However, these tablets may not always disintegrate and release their content at the intended location due to inter-individual differences in intra-colonic pH and transit time.[42] Finally, consumption of several types of butyrate producing probiotic bacterial strains, such as *Butyrivibrio fibrisolvens*[43] and *Clostridium butyricum*[33,44], have been studied in animal models.

Besides using oral substrates to increase colonic butyrate concentrations, a number of clinical intervention studies in patients with distal colonic inflammation have applied rectal enemas to deliver butyrate to the distal colon.[45] However, use of enemas is often hampered by a low compliance rate and a short and discontinuous exposure of the colon mucosa to butyrate.[46] These studies will be discussed in a later section.

Butyrate and colon carcinogenesis

One of the proposed beneficial effects of butyrate on human intestinal health is the prevention and inhibition of colon carcinogenesis. Although epidemiological studies are still inconclusive, the majority of these studies showed an inverse relationship between dietary fibre intake and the incidence of colorectal cancer.[40,41,47-53] Several studies hypothesised that increased colonic concentrations of butyrate are an important mediator in the observed protective effect of fermentable dietary fibre.[32,47,54,55] In many of these studies, however, the physiological properties of the ingested dietary fibres have not been considered.[56] A role for butyrate in the development of colon cancer has recently been supported by the down-regulation of butyrate transporters (MCT1 and SMCT1) in human colon cancer tissue[57,58], which results in a reduced uptake and metabolism of butyrate in the colonocytes. In addition, the SMTC1 activity was positively correlated with the disease-free survival.[59] Moreover, a lower butyrate to acetate ratio has been found in luminal samples of patients with adenomatous polyps or colon cancer versus healthy controls.[60] Although several well-designed animal models have demonstrated a protective effect of butyrate on colorectal carcinogenesis[55,61-67], direct evidence for a protective effect of butyrate on carcinogenesis in humans is still lacking.

Knowledge and hypotheses concerning the mechanisms behind the effects of butyrate on carcinogenesis are mainly based on *in vitro* cell systems. *In vitro* exposure of many tumour cell lines to butyrate leads to anti-carcinogenic effects by induction of apoptosis[68,69], inhibition of proliferation[70,71] and promotion of a more differentiated phenotype.[70,71]

In contrast to these relatively consistent findings in tumour cell lines, the observed effects of butyrate on non-carcinogenic cells are more diverse and do not always

point into the same direction as in tumour cells.[70,72] Also some data from human (intervention) studies are available, which focus predominantly on the effect of SCFA on colonocytic proliferation. Treatment with SCFA enemas for two weeks increased rectal proliferative activity in patients with a closed atrophic rectum that was deprived of its natural SCFA source.[73] In a study by Scheppach *et al.*[74], human colonic biopsies were exposed to butyrate *ex vivo* for four hours, which revealed that butyrate increased the proliferation rate at the basal 60% area of the crypt. It has been proposed that butyrate stimulates the physiological pattern of proliferation that is normally confined to the basal crypt.[75] Expansion of the proliferative zone towards the crypt surface has been considered a biomarker of increased susceptibility to cancer formation.[76] In colonic biopsies from patients with ulcerative colitis expansion of the proliferative zone has been shown and was found to be independent of the degree of inflammation.[77] Treatment with butyrate enemas for two weeks decreased the proliferation rate in the upper part of the crypt in colonic biopsies of active UC patients, resulting in values comparable to those of healthy control subjects.[45]

These apparent contradicting effects of butyrate found in normal colonocytes and in neoplastic cells are often referred to as the "butyrate paradox".[70] There are several explanations for these contrasting effects. First, they may reflect inherent differences of the cells, the cell's state of activation and their energy status. Furthermore, it may be the result of different concentrations of butyrate and the different exposure times to butyrate used in these experiments. Finally, the ability of the cells to β-oxidise butyrate may influence their response to butyrate as it can influence the rate of removal of butyrate from the cytoplasm and hence the availability of butyrate to exert its effects.[78,79]

Mechanisms of butyrate's anti-carcinogenic effect

Although the exact underlying mechanisms of action have not yet been elucidated, the ability of butyrate to influence cell function is considered to be due to its regulation of gene expression, which is often attributed to its inhibition of histone deacetylase (HDAC).[78,80] This results in hyperacetylation of histones and enhancement of the accessibility of transcription factors to nucleosomal DNA.[69,81,82] However, it is likely that butyrate has other intracellular targets, including hyperacetylation of non-histone proteins, alteration of DNA methylation, selective inhibition of histone phosphorylation and modulation of intracellular kinase signalling.[80] This multiplicity of effects may underlie the ability of butyrate to modulate gene expression and have impact on key regulators of apoptosis and cell cycle as was demonstrated for cell cycle inhibitor $p21^{Waf1/Cip1}$ and proapoptotic protein BAK.[68,69,81,83] Interestingly, cancer cells appear to be more sensitive to the actions of HDAC inhibitors than non-transformed cells, but the mechanistic basis for this apparent selectivity is poorly understood.[83]

One of the effects also demonstrated in humans is the effect of butyrate on the plasminogen/plasmin system (PPS). Increases in tumour and serum levels of several components of the PPS are found to correlate with a more invasive tumour cell phenotype and a worse prognosis in patients with colon cancer.[84] *In vitro*[85] and *in vivo* studies[86,87] have shown that butyrate is able to alter the balance of components of the PPS in a manner that favours net decreased plasminogen activator activity.
Other effects of butyrate studied in multiple colonic cancer cell lines include the enhancement of the activity of the detoxifying enzyme glutathion-S-transferase.[88] Furthermore, butyrate may have an inhibitory effect on tumour cell migration by inhibiting decay-accelerating factor (DAF) expression[89] and pro-metastatic metalloproteinase activation.[90,91] Finally, it has also been suggested that butyrate inhibits tumour-induced angiogenesis through modulation of two angiogenesis related proteins, vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1α (HIF-1α).[92]

In conclusion, butyrate may have a protective role in the prevention and progression of colorectal carcinogenesis. However, the effects on apoptosis and proliferation appear to differ between normal and neoplastic tissue. The different models and concentrations being used may partly explain this paradox. Therefore, effects and mechanisms identified by *in vitro* models have to be confirmed in humans under physiologic conditions.

Butyrate and inflammation

The intestinal epithelium, particularly in the colon, is permanently in close association with a myriad of microbes and their products. Therefore, the enterocytes must sense and respond appropriately to this potential immunological challenge of the luminal content. This leads to a normal physiological state of controlled low-grade inflammation.[93] However, mechanisms that allow individuals to tolerate commensal microbes, and thus limit the inflammatory response, are not yet completely understood. In some conditions, such as inflammatory bowel disease, this immunologic control is thought to be disturbed.[94]
Several studies, both *in vitro* and *in vivo*, indicate that bacterial metabolites such as butyrate may affect the host immune response. As butyrate is known to be an important energy source for the colonic epithelium, it has been hypothesised that a lack of luminal SCFA or the inability to oxidise butyrate leads to a nutritional deficiency of the colonic epithelium, causing mucosal atrophy in short term and “nutritional colitis” in long term.[95] Possible changes in intestinal butyrate concentrations and/or oxidation of butyrate have been reported in diversion colitis and ulcerative colitis (UC). Diversion colitis may occur as complication after a

surgically diverted intestine (e.g. Hartmann's procedure), and is characterised by severely decreased luminal concentrations of SCFA of 0, 4 and 0.8 mmol/L for acetate, propionate and butyrate, respectively, measured in the bypassed part of the rectosigmoid.[96] UC is an inflammatory bowel disease, characterised by alternating periods of flare ups and quiescent disease.[97] Increased[95,98] as well as decreased[99,100] faecal concentrations of butyrate have been reported in patients with active UC, but these were never as low as in diversion colitis.[96] In addition, a diminished capacity of the intestinal mucosa to oxidise butyrate has been reported in patients with active UC.[101,102] In patients with inactive UC, however, a normal butyrate oxidation has been found *in vivo*[101,103], which suggests that abnormal butyrate oxidation is not a primary defect in colon mucosa of UC patients. A possible explanation for the decreased oxidation in UC patients was proposed by Nancey *et al.* who showed that butyrate oxidation could be reduced by TNFα at concentrations found in inflamed human mucosa.[104] It has also been reported that butyrate oxidation in colonocytes can be inhibited by hydrogen sulphide *in vitro.*[105] Increased luminal concentrations of sulphide as well as high numbers of sulphate reducing bacteria have been reported in UC patients.[105,106] However, evidence for hydrogen sulphide as a metabolic toxin in UC *in vivo* remains limited.[105-108]

Intervention studies

Several animal studies have been performed to evaluate the effects of butyrate on inflammation and preventive as well as therapeutic effects have been reported.[109-112]

Human studies analysing the effects of SCFA were performed in patients with colonic inflammation using rectal enemas containing SCFA mixtures or butyrate alone (Table 2.1).[45,46,73,96,113-126] The majority of these studies focused on UC patients with moderately to active disease. Although some controlled studies with enemas containing butyrate or SCFA mixtures in UC patients did not find beneficial effects[113] or only trends towards clinical improvement[46,114,115], various other studies revealed a significant improvement of clinical and inflammatory parameters.[45,116-119] Studies in patients with diversion colitis reported inconsistent results with regard to improvement of clinical symptoms and inflammatory parameters in response to administration of mixtures of SCFA versus placebo.[96,120] Two other human intervention studies determined mucosal cell proliferation in patients after Hartmann's procedure and found trophic effects of SCFA mixtures in the mucosa of the closed rectal and sigmoid segment.[73,121]

The effects of butyrate containing enemas on radiation proctitis[122-125] and pouchitis[126] have been studied in small groups. Besides one report[125] that showed that butyrate was an effective treatment of radiation proctitis, other studies did not report clear-cut beneficial effects of SCFA irrigation in these two patient groups.[122-124,126]

The equivocal results in human intervention studies using enemas may partly be explained by differences in treatment duration, butyrate enemas versus SCFA enemas, differences in concentrations and volumes of these SCFA and the small number of patients included. Moreover, some studies suffered from methodological limitations such as a cross-over design with insufficient wash-out time that may have resulted in differences in pre-treatment levels between groups (Table 2.1).
As there are limitations to prolonged use of rectal enemas, especially with regard to compliance, oral ingestion of fermentable dietary fibre and the use of enteric-coated tablets containing butyrate have been explored. Administration of such tablets (4 g of butyrate daily) in combination with mesalazine versus mesalazine alone, significantly improved the disease activity score in patients with mild to moderate UC.[41] Similar butyrate tablets also resulted in a significant clinical improvement in Crohn's disease patients, but this study lacked a control group.[127]

Table 2.1 Studies performed using butyrate and/or SCFA enemas in different patient groups.

Patients	N	Enema treatment	Results	Ref
Active distal UC	10	100 mM butyrate and placebo 100 ml, pH=7, b.i.d. for 2 wks. Wash out: 2 wks	Butyrate → stool frequency↓, blood discharge↓and endoscopic and histologic scores↓	45
	45	Corticosteroid (CS), 5-ASA or 130 mM SCFA (46:23:31*) 60 ml, pH=7, b.i.d. for 6 wks	SCFA equally efficacious to CS or 5-ASA	117
	40	150 mM SCFA (53:20:27) vs. placebo 100 ml b.i.d. for 6 wks	SCFA → intestinal bleeding↓, urgency↓ and patient self-evaluation score↑	119
	47	130 mM SCFA (46:23:31) vs.100 mM butyrate vs. placebo 60 ml, pH=5.5, b.i.d. for 4-8 wks	No differences between groups. Butyrate for 8 wks → fewer colonic segments affected endoscopically	114
	38	80 mM butyrate vs. placebo 60 ml, pH=7 for 6 wks	No differences between butyrate and placebo treatment	113
	91	150 mM SCFA (53:20:27) vs. placebo 100 ml, pH=7 b.i.d. for 6 wks	SCFA enemas were not of therapeutic value	46
	35	130 mM SCFA (46:23:31) vs. 100 mM butyrate vs. placebo 60 ml, pH=5.5, b.i.d. for 4-8 wks	Butyrate → trend for reduced density of neutrophils in lamina propria. SCFA and butyrate → cell proliferation in upper crypt↓	115
	11	100 mM butyrate vs placebo 60 ml, pH=5.5 b.i.d. for 4-8 wks	Butyrate → nr of nuclear translocated NF-κB positive macrophages↓, nr of neutrophils↓ and Disease Activity Index↓	116
	51	5-ASA & saline vs. 5-ASA & 80 mM butyrate 80 ml b.i.d. for 6 wks	5-ASA in combination with butyrate → more patients into remission, bowel movements↓, urgency↓ and patient's self-evaluation↑	118
Diversion colitis	4	130 mM SCFA (46:23:31) vs. placebo 60 ml, pH=7 b.i.d. for 2-60 wks	SCFA → clinical symptoms↓and histological score↓	96
	13	130 mM SCFA (46:23:31) vs. placebo 60 ml, pH=7 b.i.d. for 2 wks	No endoscopic or histologic changes	120
After Hartmann's procedure	6	150 mM SCFA (58:27:15) 100 ml b.i.d. for 10-14 days	SCFA had a trophic and vasodilatory effect	121
	8	150 mM SCFA (50:29:21) and placebo 100 ml, pH=7.0 b.i.d. for 2 wks. No wash-out	SCFA → proliferation↑, mainly in the middle and upper thirds of the crypts	73
Radiation proctitis	7	SCFA for 4 wks	SCFA → clinical symptoms↓	122
	15	40 mM Butyrate. vs placebo b.i.d for 2 wks. Wash-out: 1 week	No differences between groups	124
	19	130 mM SCFA (46:23:31) vs. placebo 60 ml pH=7 b.i.d. for 5 wks	SCFA → rectal bleeding↓, endoscopic score↓ and hemoglobin↑	123
	20	80 mM butyrate and placebo 80 ml for 3 wks. No wash-out	Butyrate → clinical symptoms↓, endoscopic and histological scores↓	125
Pouch after UC	25	130 mM SCFA (46:23:31) 30 ml b.i.d. pH= 7 for 1-2 wks	SCFA → no effects on defaecation frequency and endoscopic and histologic scores of pouches	126

*Ratio=Acetate:Propionate:Butyrate; N=number of patients included; SCFA=short chain fatty acids; CS=corticosteroid; 5-ASA=5-aminosalicylic acid; b.i.d.=twice daily

The effects of fermentable fibre supplementations, such as germinated barley foodstuff[128-130], inulin[131,132], psyllium[133,134] and oat bran[9], that increase faecal butyrate concentrations, have also been studied in several clinical trials. Germinated barley foodstuff prolonged remission in inactive UC patients[128] and attenuated clinical activity in active UC patients.[129,130] Inulin supplementation resulted in a decreased mucosal inflammation of the ileal reservoir in patients with an ileal-anal pouch anastomosis[132] and lowered faecal calprotectin concentrations in patients with active UC.[131] Two other studies in UC patients in remission, evaluating the supplementation of psyllium and oat bran, showed that the supplementation was safe, increased faecal butyrate concentrations and were found to be effective in the maintenance of remission.[9,133,134] However, this effectiveness should be confirmed in larger clinical trials.

Although not all studies in patients with colonic inflammation confirmed the reduction of inflammation and clinical symptoms due to luminal administration of butyrate or stimulation of luminal butyrate production by the ingestion of dietary fibre, an amelioration of the inflammation and symptoms in active UC patients is strongly suggested. In addition, butyrate might also play a role in the prevention of inflammation as supported by the results with UC patients in remission.

Mechanisms of butyrate's anti-inflammatory effect

Apart from being an important energy source for the colonocytes, butyrate can exert direct immuno-modulatory effects.[111,116,135] Suppression of nuclear factor kappa B (NF-κB) activation, which may result from the inhibition of HDAC, is the most frequently studied anti-inflammatory effect of butyrate.[136,137]

NF-κB is a transcription factor that controls the expression of genes encoding pro-inflammatory cytokines, chemokines, inducible inflammatory enzymes such as inducible NO synthase and cyclooxygenase-2, adhesion molecules, growth factors, some acute phase proteins, and immune receptors.[138] In a study with UC patients, the increased mucosal levels of activated NF-κB were reduced by butyrate and this correlated with a decrease in the disease activity index and the numbers of infiltrated neutrophils and lymphocytes.[116] This anti-inflammatory effect of butyrate via NF-κB inhibition, contributing for example to decreased concentrations of myloperoxidase, cyclooxygenase-2, adhesion molecules and different cytokine levels, has been confirmed in several *in vitro* and *in vivo* studies.[109,111,112]

Apart from inhibition of NF-κB activation, butyrate may also exert an anti-inflammatory activity through inhibition of the interferon-γ -production and/or -signalling[139,140] and the upregulation of peroxisome proliferator-activated receptor-γ (PPARγ).[141-145] PPARγ is a ligand-activated transcription factor that is highly expressed in colonic epithelial cells and its activation is thought to exert anti-inflammatory effects.[146] PPARγ protein expression is 60% lower in the non-

inflamed colonic mucosa of UC patients compared to controls.[147] Modulation of PPARγ protein expression in UC may prove to be an interesting treatment for UC.
Finally, butyrate as well as other SCFA can act as signalling molecules through specific G-protein-coupled receptors, GPR41 and GPR43, identified for SCFA.[148-150] These two SCFA receptors are expressed on immune cells, particularly polymorphonuclear leukocytes, and are also highly present in the colonic mucosa.[151] It has been hypothesised that these receptors play a role in the immune surveillance of the colonic mucosa towards microbial activity.[149-151]
In conclusion, several mechanisms for the anti-inflammatory activity of butyrate have been described, which can contribute to the observed clinical effectiveness of colonic butyrate supplementation on colonic inflammation.

Butyrate and oxidative stress

As described above, butyrate may influence the inflammatory process and the initiation and progression of carcinogenesis. In both these processes, oxidative stress is involved.[152,153] Oxidative stress is the result of an imbalance between the generation of reactive oxygen and reactive nitrogen species and the antioxidant defence mechanisms, resulting in a cascade of reactions in which lipids, proteins and DNA may be damaged. Neutrophilic granulocytes are an important source of potent oxidising species in the inflamed colon.[152]
There is some evidence that butyrate is able to modulate oxidative stress. In two studies, pre-incubation of isolated rat[154] or human[155] colonocytes with butyrate showed a significant reduction of H_2O_2-induced DNA damage. In rats, resistant starch intake decreased the levels of colonocyte DNA damage induced by a high protein diet. This DNA damage correlated negatively with caecal butyrate concentrations.[156]
The mechanism by which butyrate reduces oxidative stress is unknown. Scavenger activity of butyrate is unlikely due to its chemical structure. However, butyrate may affect DNA repair systems and levels of enzymatic or non-enzymatic (anti-) oxidant systems. For example, fermentable fibre supplementation resulted in a decreased colonic myeloperoxidase activity and a restoration of the colonic concentration of the antioxidant glutathione in a rat model of TNBS-induced colitis.[157] In addition, butyrate enhanced the activity of glutathione-S-tranferase in HT-29 cells[88] and increased catalase activity in artery smooth muscle cells of rats.[158]
Although human *in vivo* data are not yet available, butyrate may enhance the protection against mucosal oxidative stress by affecting the activity of intracellular antioxidants, DNA repair systems or (anti-) oxidant enzymes.

Butyrate and the colonic defence barrier

Besides the effects of butyrate on carcinogenesis, inflammation and oxidative stress, butyrate has been shown to affect several components of the colonic defence barrier leading to enhanced protection against luminal antigens. One important component of this barrier is the mucus layer covering the epithelial lining consisting of mainly mucin glycoproteins and trefoil factors. Mucin glycoproteins are classified into neutral and acidic subtypes and the latter category further includes sulfomucins and sialomucins. Sulphated mucins are generally considered to be more resistant to bacterial degradation.[159] Several epithelial mucin (MUC)-genes have been identified in humans, of which MUC2 is predominantly expressed in the human colon.[160] Alterations in goblet cell function, composition and thickness of the intestinal mucus layer have been found in several intestinal disorders. For example, a reduced mucus thickness and a decreased MUC2 production has been reported in UC patients.[161]

In *in vitro* studies, butyrate increased the MUC2 gene expression in specific cell lines.[162-165] In addition, 0.1-1 mmol/L butyrate administered to human colonic biopsy specimens *ex vivo* stimulated mucin synthesis.[166] Luminal butyrate administration of 5 mmol/L, but not 100 mmol/L, increased mucus secretion in an isolated perfused rat colon.[167] In another rat study, caecal and faecal SCFA concentrations were found to correlate with mucus thickness. In humans effects of butyrate alone on mucus synthesis, thickness of the mucus layer and MUC expression *in vivo* have not been reported.

The effects of a number of fermentable dietary fibres on the mucus layer have been studied with varying results. For example, resistant starch increased the number of acidic mucins, but did not affect the number of goblet-cells in rats.[61] In contrast, fructo-oligosaccharides increased the number of goblet-cells in piglets.[168] In a human intervention study with patients with an ileo-anal pouch, inulin supplementation did not alter MUC2 expression nor the ratio between sulpho- and sialomucins.[169]

Trefoil factors (TFF) are mucin-associated peptides that contribute to the viscoelastic properties of the mucus layer. TFF are thought to reduce the recruitment of inflammatory cells and to be involved in the maintenance and repair of the intestinal mucosa, although the exact mechanism for this effect is not yet known.[170] Intestinal trefoil factor (ITF or TFF3) is almost exclusively secreted by the intestinal goblet cells.[171] In a rat TNBS model of colitis, TFF3 expression was decreased during active disease, and intra-colonic administration of butyrate increased TFF3 expression[112,172]. However, butyrate inhibited the expression of TFF3 in colon cancer cell lines[173,174] and in colonic tissue of newborn rats.[173]

Other components of the colonic defence barrier that are involved in the maintenance of the colonic barrier, which may be influenced by butyrate are transglutaminase, anti-microbial peptides and heat shock proteins (HSP).
The enzyme transglutaminase is actively involved in intestinal mucosal healing and correlates with the severity of inflammation in UC.[175] In a rat model of colitis, butyrate restored the colonic transglutaminase levels.[62,176] Anti-microbial peptides such as cathelicidin (LL-37) and defensins, protect the gastrointestinal mucosa against the invasion and adherence of bacteria and thereby prevent infection.[177-179] Several *in vitro* studies have shown that butyrate upregulates the expression of LL-37 in different colon epithelial cell lines as well as in freshly isolated colorectal epithelial cells.[180] HSP confer protection against inflammation by suppressing the production of inflammatory modulators.[181,182] Butyrate induced the expression of HSP70 and HSP25 in Caco-2 cells[182] and in rats.[61,181,183] However, in a study in rats with DSS induced colitis, butyrate inhibited HSP70 expression. This was related to protection against the decrease in cell viability, increase in mucosal permeability and neutrophil infiltration in DSS colitis. It was concluded that the induction of heat shock response has a protective effect before an injury, whereas activation of heat shock response leads to cytotoxic effects after a pro-inflammatory stimulus.[184]
In addition, there is evidence from *in vitro* studies with human colon cancer cell lines that butyrate is involved in repair after mucosal damage through an increase in the rate of cell migration. Efficient repair of superficial injuries and mucosal ulcers is important in maintaining and re-establishing the epithelial barrier.[185]
In summary, there are several lines of evidence suggesting that butyrate reinforces the colonic defence barrier by affecting several components of this barrier, such as the promotion of epithelial migration and the induction of mucins, trefoil factor, transglutaminase activity, anti-microbial peptides and heat shock proteins. However, most of these effects still have to be confirmed in the human situation.

Butyrate and intestinal epithelial permeability

Intestinal epithelial permeability has been widely studied as an important parameter of the intestinal defence barrier. Under normal conditions, the epithelium provides a highly selective barrier that prevents the passage of toxic and pro-inflammatory molecules from the external milieu into the submucosa and systemic circulation. Macromolecules pass the epithelial barrier mainly via the paracellular route for which tight junctions are the rate limiting structures.[186] Increased permeability, indicating impaired epithelial barrier function, is thought to be involved in the pathophysiology of several gastro-intestinal inflammatory diseases, but can either be a cause or a consequence of inflammation.[187]

Several studies have assessed the effects of butyrate on intestinal permeability *in vitro* as well as *ex vivo*. At low concentrations, butyrate (up to 2 mmol/L) induces a concentration-dependent reversible decrease in permeability in a Caco-2 and HT-29 cell lines.[188,189] This decrease in permeability may be related to the butyrate associated increased expression of tight junction proteins observed in different cultured cell lines, but this effect was shown to be cell type dependent[190,191]. At higher concentrations (8 mmol/L) however, butyrate increased the permeability in a Caco-2 cell line.[189] An *ex vivo* study, using adult rat distal colon mucosa mounted in an Ussing chamber, demonstrated that acute exposure to butyrate at a concentration of 10 mmol/L, but not 1 or 5 mmol/L increased paracellular permeability in rat colon.[192] This has also been demonstrated in rats fed a diet containing fermentable fructo-oligosaccharides (FOS). The rapid bacterial fermentation of FOS led to accumulation of high concentrations of SCFA that increased intestinal permeability and was associated with increased translocation of *Salmonella.*[193] However, in humans, daily FOS supplementation of 20 g did not increase intestinal permeability.[194]

It can be concluded that the effect of butyrate on intestinal permeability depends on its concentration and on the model system or species used. The effects of butyrate at different concentrations remain to be evaluated in the human *in vivo* situation.

Butyrate and satiety

It has been hypothesised that SCFA produced in the large intestine also can influence upper gut motility and satiety.[195] Endocrine L-cells present in large numbers in the colonic mucosa secrete peptides such as glucagon-like-peptide 1 (GLP-1), peptide YY (PYY) and oxyntomodulin, which are involved in appetite regulation.[196] In several animal studies using fermentable carbohydrates such as inulin[196], lactitol[197] and fructo-oligosaccharides[198,199], an increased satiety, decreased weight gain and increased endogenous production of GLP-1 and/or PYY was reported. In humans, fructooligosaccharides increased satiety[200] and increased plasma GLP-1 concentrations.[201] However, lactitol did not affect plasma concentrations of this gut peptide.[197]

The increased satiety is possibly promoted through the production of SCFA. This is supported by a number of studies. Butyrate increased the expression of PYY and proglucagon *in vitro* in rat epithelial cells [202] and increased PYY release, but not that of GLP-1, in the isolated colon of rats[203,204] and rabbits.[205] In addition, colonic SCFA infusion in rats stimulated PYY release.[206] However, colonic infusion with SCFA in humans did not increase plasma levels of either PYY or GLP-1.[207] Activation of the SCFA receptor GPR43 expressed in endocrine L-cells may play a role in this effect on satiety.[148]

There is increasing evidence that the effect of fermentable dietary fibre on satiety is mediated through the colonic production of SCFA. However most evidence originates from rat studies, while again human evidence remains limited.

Adverse effects of butyrate

In contrast to the wide range of positive effects of butyrate on the intestinal mucosa, a small number of studies have also shown some adverse effects. Two rat studies revealed that rectal administration of butyrate (8-1000 mmol/L), dose-dependently increased colonic visceral sensitivity.[208, 209] However, these effects have not yet been reported in humans.
In faeces of weaning children low butyrate concentrations have been measured.[210] It has been hypothesised that overproduction or accumulation of SCFA may be toxic to the intestinal mucosa of premature infants and might play a role in the pathogenesis of neonatal necrotizing enterocolitis. It has been demonstrated that the severity of mucosal injury to butyrate, measured in newborn rats, was dose dependent and also depended on the maturation of the intestine.[173,211,212] It remains to be established whether luminal butyrate in premature infants can increase towards levels that are toxic for the intestinal mucosa.[212] In addition, as mentioned before, increased permeability and *Salmonella* translocation has been found after fructo-oligosaccharide supplementation in a study with rats, which may be the result of SCFA accumulation.[193] However, this was not confirmed in the human situation.[194]

Conclusions

SCFA are important end-products of microbial fermentation. Among the SCFA produced in the human intestine, butyrate has been widely studied and has been shown to play an important role in the maintenance of colonic health. Increased butyrate production in the large intestine seems to be responsible for at least some of the protective effects of fermentable dietary fibre. However, it should be taken into account that the effects of increased butyrate production may be accompanied by other effects of dietary fibres and its fermentation, such as changes in the composition of the intestinal microbiota and increased faecal bulking.
The effects of butyrate are diverse and complex and involve several distinct mechanisms that go beyond the classical impact as an energy source for the intestinal epithelial cells. Frequently described are the effect on gene expression due the inhibition of histone deacetylase and the suppression of NF-κB activation. Hence, butyrate exerts multiple effects such as the inhibition of colonic

carcinogenesis, inflammation and oxidative stress, the improvement of the colonic defence barrier function and the promotion of satiety.
It should, however, be noted that also some equivocal results have been reported, which partly can be explained by the different butyrate concentrations and models used. In addition, a few animal and *in vitro* studies demonstrate negative effects at higher butyrate concentrations on permeability and visceral sensitivity of the large intestine.
In conclusion, in the last decade several new insights into possible mechanisms and effects revealed that butyrate is a pivotal metabolite produced within the large intestine. However, these new insights are mainly based on *in vitro* data, animal models and some clinical intervention studies. More emphasis should be put on human *in vivo* studies to elucidate the role of butyrate in health and disease.

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Chapter 3

C3-C6 but not C2 short chain fatty acids affect cytokine release in a co-culture system of Caco-2 cells and whole blood

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Submitted

Abstract

Background

Short chain fatty acids (SCFA) are often hypothesised to play a role in the maintenance of colonic health. However, most studies focus on butyrate, while the effects of other SCFA with different chain lengths are studied less extensively. In addition, effects are hard to compare as they involve separate studies and/or different models. Therefore, the aim was to compare the effects of C2 to C6 SCFA on parameters of inflammation, antioxidant capacity and epithelial integrity in one study.

Methods

Using a co-culture system combining Caco-2 cells with human whole blood cultures the effects of 1, 5, and 20 mmol/L of the different SCFA on cytokine release were evaluated. In addition, the effects of the SCFA on glutathione (GSH) and glutathione disulfide (GSSG) levels and the transepithelial electrical resistance (TEER) were analysed using Caco-2 cells.

Results

Butyrate showed a dose-dependent inhibition of several pro-inflammatory cytokines, such as IL-6, IL-17, IL-1β and TNF-α. The anti-inflammatory cytokine IL-10 was increased by 1 mmol/L butyrate, while 5 and 20 mmol/L decreased its concentration. Although less pronounced than butyrate, also various significant effects on cytokine release were observed for propionate, valerate and caproate, but not acetate. All SCFA dose-dependently increased GSH and decreased GSSG concentrations in differentiated Caco-2 cells, while only the highest concentration of butyrate improved the TEER significantly.

Conclusion

From the different SCFA produced by colonic microbial fermentation *in vivo*, not only butyrate, but also other SCFA are able to decrease pro-inflammatory cytokine production and to improve the antioxidant status.

Introduction

Short-chain fatty acids (SCFA) are important end products of luminal microbial fermentation of predominantly non-digestible dietary carbohydrates. SCFA with different carbon chain lengths (acetate (C2), propionate (C3), butyrate (C4), valerate (C5) and caproate (C6)) are produced in varying amounts depending on the diet and the composition of the intestinal microbiota.[1] More than 95% of the SCFA produced is rapidly absorbed. Besides being an important energy source for the colonocytes, SCFA have been shown to play a positive role in the maintenance of colonic health and barrier function.[2] From the different SCFA, butyrate has by far been the most extensively studied. Beneficial effects of butyrate have for example been reported in patients with inflammatory bowel disease.[3-5] Previous studies have demonstrated that such anti-inflammatory effects of butyrate could be mediated by up regulation of PPAR-γ[6] and inhibition of NF-κB activation[7], which affect the level of inflammatory mediators such as interleukins (IL), tumour necrosis factor (TNF) and interferon-γ (IFN-γ). These cytokines are secreted by a variety of different cell types that mediate and regulate immunity, inflammation and haematopoiesis. The type of cytokines produced also partly determines whether naive T-helper cells develop into T-helper 1, T-helper 2 or T-helper 17 cells, each expressing different cytokine patterns[8,9] On the other hand, regulatory T-cells are able to suppress immune activation. One proposed mechanism of this effect is via production of anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF-β).[10]
Limited numbers of studies using different models have investigated the anti-inflammatory effects of SCFA with different chain lengths. One *in vitro* study using isolated human neutrophils, colon cultures from mice and the Colo320DM cell line, has demonstrated anti-inflammatory effects of propionate and acetate, which were comparable, although slightly less potent, to those of butyrate.[11] This is in line with another *in vitro* study using human umbilical vein endothelial cells that showed less potent effects of valerate and propionate on leukocyte infiltration compared to butyrate.[12]

In addition to the anti-inflammatory properties of butyrate, a number of studies have reported that butyrate was able to modulate oxidative stress[13,14] and epithelial permeability.[15,16] Oxidative stress is involved in the inflammatory process by the generation of reactive oxygen and nitrogen species by inflammatory cells. Increased inflammation and oxidative stress have been shown to disrupt mucosal barrier function of the intestinal epithelium, resulting in increased permeability.[17] A decrease of oxidative stress by butyrate has for example been demonstrated by a reduction of H_2O_2 induced DNA damage in isolated human colonocytes preincubated with butyrate[14] and by a restoration of the levels of the antioxidant glutathione (GSH) in a rat colitis model after dietary fibre supplementation that

increased the production of butyrate.[13] The transepithelial electrical resistance (TEER) in Caco-2 cells was found to be increased by butyrate at low concentrations (2 mmol/L).[15,16] However, data on the effects of other SCFA regarding oxidative stress and permeability are hardly available.

The present study aims to compare the effects of the different SCFA with chain lengths varying from two to six carbon atoms on parameters of inflammation, antioxidant capacity and permeability in one *in vitro* model. Using a co-culture system combining Caco-2 cells with human whole blood cultures, i.e. the EDI-Co gut model[18], the influence of the different SCFA on cytokine release were evaluated. Since other parameters may also reflect the functionality of the Caco-2 cells, we further evaluated the effects on the antioxidant capacity by determination of the GSH and glutathione disulfide (GSSG) levels, the transport and metabolic use of the various SCFA as well as the effects on transepithelial electrical resistance (TEER).

Materials and Methods

SCFA

The sodium salts of acetate, butyrate and valerate were purchased from Merck (Amsterdam, the Netherlands). Sodium propionate and caproic acid were purchased from Sigma (Zwijndrecht, the Netherlands). The different SCFA were dissolved in cell culture medium, checked for the pH and neutralised with NaOH if necessary. SCFA were tested in 3 concentrations: 1, 5 and 20 mmol/L. In addition, medium without SCFA was included as control.

Co-culture of Caco-2 cells with whole blood cells

Caco-2 cells, a widely used human colonic epithelial cell line (German collection for microorganisms and cell cultures, Braunschweig, Germany), were grown in culture medium (MEM-E-with Earle's salt base (Biochrom, Berlin, Germany)) supplemented with 20% fetal calf serum (Biochrom), 1% non-essential amino acids (Biochrom), 1% glutamine (Sigma) and 1% penicillin/streptomycin (Sigma) and were maintained at 37^{o}C in a humidified 5% CO_2 atmosphere. The medium was changed every 2-3 days.
For the co-culture experiments with whole blood to evaluate the release of different cytokines (Figure 3.1), cells (passages 3-6 of stock cells) were seeded into transwell inserts (pore size 0.4 μm, diameter 6.5 mm) (Corning, NY, USA)) at 2 x 10^4 cells/well in 0.2 mL culture medium. Transwell inserts were then placed in 0.9 mL medium (i.e. at the basolateral side) in a 24-well plate (Corning). Cells were

allowed to attach, reach confluency and differentiate for 8 days. Before the start of the co-culture experiment, the TEER was measured as an indicator of epithelial integrity using the Millicell-Electrical Resistance System (Millipore, Bedford, USA). In all instances, the TEER value exerted a level of more than 300 Ω/cm^2, demonstrating an intact barrier function of the culture system. Thereafter, the medium of the transwell inserts was replaced with medium containing the SCFA with different chain lengths (C2-C6) in 3 concentrations (1, 5 or 20 mmol/L). Each concentration was tested in duplicate. Subsequently, the transwell inserts were transferred to 24 well plates containing a whole blood culture from a healthy donor. The co-culture experiment was performed with blood of 3 healthy donors (A, B and C). After 4.5 hours of co-incubation, the whole blood cultures in the lower compartment were stimulated with LPS (Calbiochem, Nottingham, UK) and *Staphylococcus aureus* enterotoxin B (SE-B; Bernhard-Nocht-Institute, Hamburg, Germany) both at 0.1 μg/mL. As compared to PBMC a higher concentration is necessary to stimulate cytokine release in the whole blood cell culture. After a total co-incubation period with SCFA of 24 hours, transwell inserts were re-transferred to the plate containing culture medium only in the lower compartment and the TEER was measured again. The whole blood cultures were further incubated for two hours until the supernatants from the lower compartment were carefully collected. Duplicates were pooled and stored at -20°C for analyses of cytokines.[18]

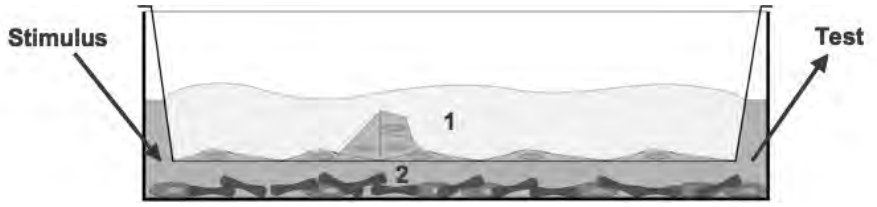

Figure 3.1 Picture of the EDI-Co gut co-culture system. Caco-2 cells were grown on the apical side in the transwell insert (1). After differentiation they were incubated with SCFA. The basolateral side (lower compartment) is filled with the whole blood culture (2). Whole blood cells were stimulated and after 24 hours of co-incubation supernatant of the lower compartment was collected for cytokine determination (see *Material and Methods*).

Determination of cytokines

The following cytokines were determined using a standard multiplexed immunoassay according to manufacturers' instructions (RBM, Austin, USA): granulocyte-macrophage colony stimulating factor (GM-CSF), IFN-γ, IL-10, IL-12p40, IL-12p70, IL-15, IL-17, IL-18, IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-23, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, monocyte chemotactic protein-1

(MCP-1), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), TNF-α and TNF-β. Concentrations of TGF-β were determined using a specific ELISA kit according to manufacturers' instructions (R&D Systems, Abingdon, UK).

Determination of transport and metabolic use of the SCFA

For the determination of the epithelial transport and metabolic use of the SCFA by the Caco-2 cells the same procedure was followed as described for the co-culture experiment above, without the addition of the whole blood. After the 24 hours of incubation with SCFA, supernatants from the upper and lower compartment were collected for SCFA analyses. Samples were stored at -20°C until analysis by GC as described before.[1]

Cell culture for the analysis of GSH and GSSG

In order to study the effects of SCFA on GSH and GSSG, Caco-2 cells (passage 6-7 of stock cells) were seeded in 6-well plates at $2x10^5$ and $8x10^5$ cells per well for experiments with proliferating cells and differentiated cells, respectively. A 24 hour incubation with SCFA in 2 mL of culture medium containing the different SCFA (C2-C6) in 3 concentrations (1, 5 or 20 mmol/L) or L-buthionine-sulfoximine (BSO, Sigma) in 3 concentrations (0.02, 0.2 or 2 mmol/L) was performed 2 days after seeding, when cells were in the proliferating state as well as 7 days after seeding when cells were in the differentiated state. At the end of the 24 hour incubation period, cells were washed with Hanks Buffered Salt Solution (Biochrom), trypsinised, washed with cold PBS and after centrifugation (5 min, 4°C, 470g), cells were lysed by addition of 1 mL cold 0.1% triton-X-100 (Sigma) and 0.6% 5-Sulfosalicilic acid (Sigma). After 3 minutes in a sonication bath with ice water, samples were centrifuged (5 min, 4°C, 3000g). Supernatants were stored at -80°C until determination of GSH and GSSG using the recycling method described by Vandeputte *et al.*[19] In brief, the increase in absorbance at 412 nm, caused by the GSH and GSSG driven reduction of 5'5'dithiobis 2-nitrobenzoic acid (Sigma) at 0.2 mmol/L in the presence of nicotinamide adenine dinucleotide phosphate (Sigma) at 0.15 mmol/L and GSSG-reductase (Sigma) at 1 U/mL, was measured for 3 minutes. The slope of the increase in absorbance of the samples was compared to the slope of GSH and GSSG standards. To measure only the GSSG concentration with the same method, cell lysates were preincubated with 2-vinylpyridine (3% final concentration) for one hour, to remove GSH. The GSH concentration was calculated by subtracting two times the GSSG concentration from the total GSH concentration. The final concentrations were adjusted for the total protein content measured using the BCA™ protein assay kit (Pierce, Rockford IL, USA).

Statistical analysis

All statistical analyses were performed with SPSS 15.0 software. All results were expressed as the stimulation index (SI) to indicate the effect of the specific SCFA compared to the effect of the medium control without SCFA, which was set equal to 1. The TEER value was expressed as the % of the TEER value at the start of the experiment. A linear mixed model with a random intercept was used to analyse the SI of the cytokine release as well as the TEER value. The Bonferroni correction was applied to correct for multiple testing, by setting the confidence interval at 99.67%. When the confidence interval did not include “1”, the effect of the intervention was considered to be significantly different. Concerning the experiment for the evaluation of the effects of the different SCFA on GSH/GSSG, no significances were calculated as the experiment was performed once in duplicate.

Results

Influence of the SCFA on cytokine release

Using a co-culture system with Caco-2 cells in combination with whole blood from healthy donors, the anti-inflammatory potential of different SCFA (C2-C6) was evaluated.

Concentrations of IL-12p70, IL-15 and IL-23 were below the detection limit and were excluded from further analyses. All other cytokines showed increased concentrations after stimulation as compared to the unstimulated control. The mean SI of all these different cytokines after incubation with the various SCFA are presented in Table 3.1 and accompanied by representative graphic examples of cytokines in Figure 3.2.

Table 3.1 The effects of incubation with different SCFA (C2-C6) on cytokine release are shown as mean values of the stimulation index from the co-cultures with blood from donors A, B and C of the different SCFA for each cytokine.

	Acetate (C2)			Propionate (C3)			Butyrate (C4)			Valerate (C5)			Caproate (C6)		
mmol/L	**1**	**5**	**20**	**1**	**5**	**20**	**1**	**5**	**20**	**1**	**5**	**20**	**1**	**5**	**20**
IL-10	1.0	1.1	1.2	1.2	1.0	0.7	**1.4***	**0.4***	**<0.1***	1.1	0.9	**0.3***	1.1	0.9	**0.6***
TGF-β	1.1	1.0	1.1	1.3	1.1	1.1	0.9	0.8	0.8	0.7	0.9	1.1	1.2	1.3	1.0
GM-CSF	1.1	1.0	1.1	1.0	1.0	0.8	1.0	0.6	**0.2***	0.9	1.2	0.8	1.1	0.9	0.9
IFN-γ	1.0	0.9	0.8	0.7	0.3	0.2	0.4	0.1	0.1	0.7	0.4	0.2	0.8	0.7	0.6
TNF-α	1.0	1.0	0.8	0.7	**0.5***	**0.4***	**0.5***	**0.3***	**0.2***	0.8	**0.6***	**0.5***	0.9	**0.7***	**0.6***
TNF-β	1.0	1.1	1.0	1.0	1.0	**0.5***	1.0	**0.3***	**0.1***	1.0	0.7	**0.5***	0.9	0.8	**0.5***
IL-1α	1.0	1.0	0.9	0.9	0.8	1.0	1.0	1.2	**0.2***	0.9	1.0	1.4	0.8	0.7	0.7
IL-1β	1.0	0.9	0.8	0.8	0.8	0.9	0.9	1.0	**0.3***	1.0	1.0	1.3	0.9	0.8	0.9
IL-1ra	1.0	1.0	1.0	1.0	1.0	0.8	0.9	**0.5***	**0.2***	0.9	0.8	**0.6***	0.9	0.9	0.8
IL-2	1.0	1.0	1.1	1.0	0.7	**0.4***	0.7	**0.4***	**0.1***	0.8	0.8	0.5	1.0	0.9	1.0
IL-3	0.9	1.0	0.9	1.0	1.3	1.1	1.5	1.1	0.7	1.1	1.6	1.2	1.2	1.0	1.1
IL-4	1.0	1.0	1.1	1.0	1.0	0.8	1.1	0.7	**0.5***	1.0	1.0	0.8	1.0	0.9	0.9
IL-5	0.9	0.9	1.2	1.1	0.9	0.5	1.2	**0.3***	**0.2***	1.3	1.0	0.4	0.8	0.7	0.9
IL-6	1.1	1.0	1.0	0.9	0.9	1.1	1.0	1.1	**0.1***	0.9	1.1	1.3	1.0	0.9	1.1
IL-7	1.0	1.0	1.0	1.1	1.1	1.1	**1.2***	**1.3***	1.1	1.2	**1.5***	**1.3***	1.1	1.1	1.2
IL-12p40	1.0	0.9	0.7	0.6	**0.3***	**0.2***	**0.3***	**0.1***	**0.1***	0.8	0.4	**0.2***	0.9	0.7	0.5
IL-17	0.9	1.1	1.3	1.2	1.1	0.9	1.1	0.7	**0.3***	1.2	1.2	0.8	1.0	1.3	1.4
IL-18	1.2	1.0	1.0	1.0	1.1	1.0	1.0	1.0	0.7	1.0	1.0	1.0	1.0	1.1	1.0
IL-8	1.1	1.3	1.4	1.3	1.8	**2.3***	1.5	2.0	0.7	1.3	**2.3***	**3.1***	1.2	1.3	**2.2***
MCP-1	1.1	1.2	1.1	1.2	0.9	0.4	0.8	**0.1***	**<0.1***	1.2	0.6	0.2	1.0	0.7	0.3
MIP-1α	1.1	1.1	1.0	0.9	0.9	1.2	0.7	1.3	0.6	1.0	1.2	**1.8***	1.0	0.9	1.2
MIP-1β	1.0	1.0	0.9	0.8	**0.7***	0.8	0.8	0.8	**0.4***	0.9	0.9	1.0	0.9	0.9	**0.6***

* Significant effect based on a linear mixed model analysis with random intercept including a Bonferroni correction for multiple testing (CI=99.67%)

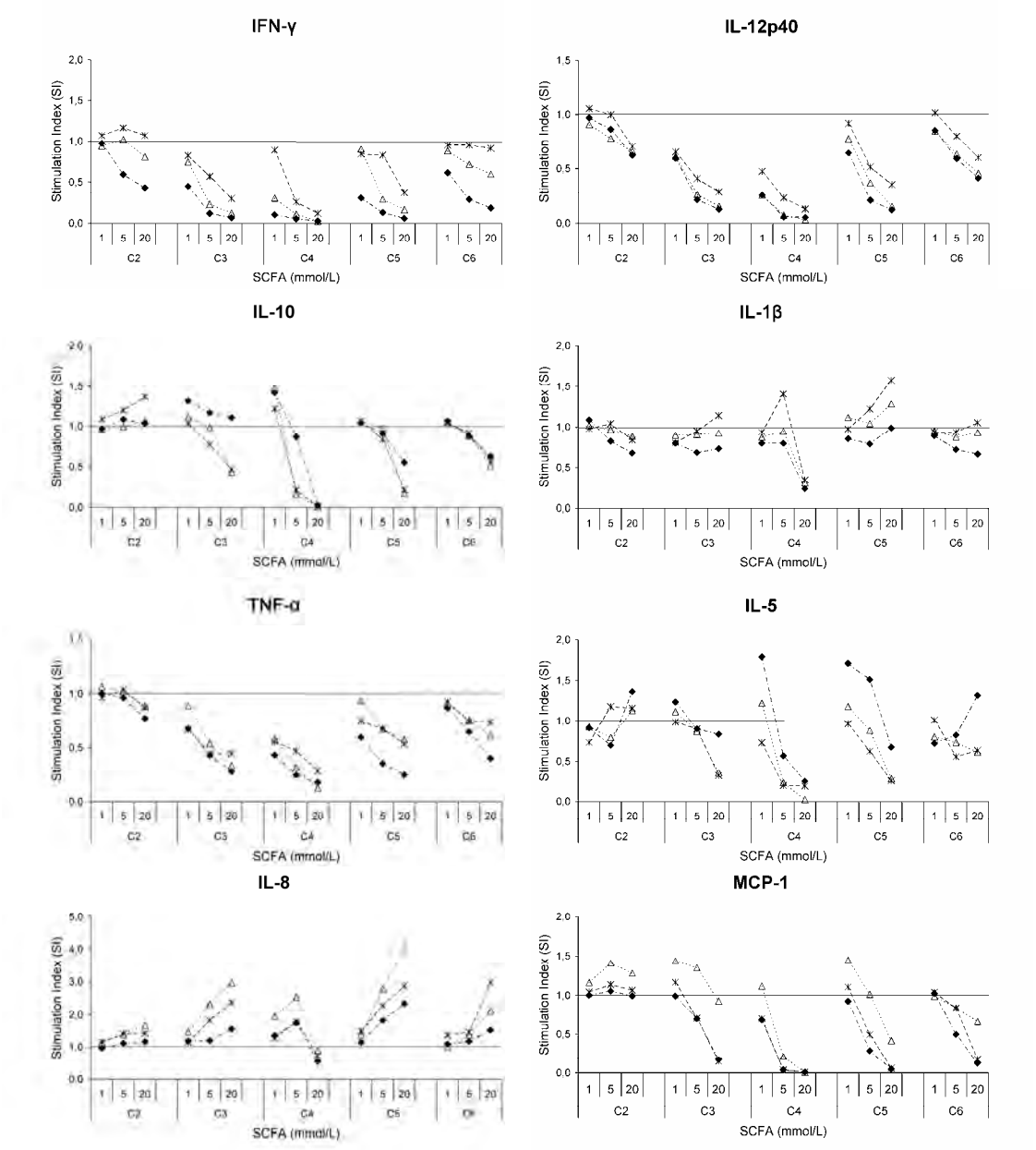

Figure 3.2 Examples of dose-dependent effect of C2-C6 SCFA on cytokine release in an *in vitro* co-culture system. The cytokine release from stimulated whole blood cells of 3 different healthy donors (3 lines; A --◆--, B --✳-- and C --△--) in the basolateral compartment is shown as stimulation index (SI). The release of the respective cytokine in the cultures without SCFA treatment was set at 1.

In the *in vitro* co-culture model, butyrate showed a dose-dependent inhibition of several of the cytokines determined, namely IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-12p40, IL-17, GM-CSF, TNF-α, TNF-β, MCP-1 and MIP-1β. Significances differed between these cytokines and are presented in Table 3.1. Several significant inhibitory effects on cytokine release were also observed for valerate, propionate, and caproate although these effects were less potent compared to those of butyrate. Acetate did not affect the release of any of the cytokines. A number of cytokines was significantly inhibited by butyrate only, such as GM-CSF, IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-17 and MCP-1. Summarizing, the findings did result in a rank order of inhibitory potency being butyrate >> valerate > propionate > caproate with regard to the inhibition of the above mentioned inflammatory cytokine release (Table 3.1 and Figure 3.2).
In contrast to the inhibition of several pro-inflammatory cytokines, incubation with some of the SCFA led to an increase of IL-10, IL-7, IL-8 and MIP-1α: The release of IL-7 was stimulated by 1 and 5 mmol/L butyrate and by 5 and 20 mmol/L valerate. MIP-1α was increased by 20 mmol/L valerate only. With respect to IL-10 and IL-8, diverse patterns occurred with significant dose-dependent dual effects: IL-10 was increased by 1 mmol/L, but decreased by 20 mmol/L butyrate. IL-8 was increased by high concentrations of propionate (20 mmol/L), valerate (5 and 20 mmol/L) and caproate (20 mmol/L), but was not significantly affected by butyrate.

Transport and metabolic use of the SCFA

After 24 hours of incubation of the Caco-2 cells with the SCFA in the transwells, the concentrations of SCFA at the apical compartment decreased due to absorption and metabolism of the SCFA (Table 3.2). All SCFA were able to pass through the Caco-2 cell layer although the barrier function of the monolayer was intact at the beginning of the cell culture. Approximately 50% of the initial 20 mmol/L of all SCFA was recovered from the basolateral side (C2: 43%; C3: 52.5%; C4: 47.5%; C5: 51.7%; C6: 49.5%). The sum of the apical and basolateral recovery of the 1 mmol/L was lowest for butyrate (29.9%) and valerate (31.2%).

Influence of the SCFA on the TEER values

Since we observed different effects of the C2-C6 SCFA on the inflammatory status, we further studied whether these SCFA influenced the TEER values and the anti-oxidant capacity of the Caco-2 cells. The change in the TEER values in the transwell system due to the 24 hours incubation with the different SCFA was determined as a parameter of the integrity of the Caco-2 cell layer. The TEER value remained unchanged during the experiment when only medium without SCFA was present at the apical and basolateral side. In contrast, the mean TEER value decreased with 35.2 % (SD: 12.2) after 24 hours of incubation of the Caco-2 cells without SCFA but with the stimulated whole blood culture on the basolateral

side. Figure 3.3 shows the mean change in TEER values due to incubation with the different SCFA (C2-C6) in 3 concentrations (1, 5 and 20 mmol/L) compared to the situation without incubation without SCFA indicated as SI (±SD). Although changes of the TEER were observed after incubation with the different SCFA, they did not reach significance except for the highest concentration of butyrate (20 mmol/L) that showed a significant increase in the TEER value compared to the TEER value of the control without butyrate (Figure 3.3).

Table 3.2 Concentrations of SCFA and percentage of recovered SCFA after 24 hours (t=24h) of incubation with the Caco-2 cells at the apical and basolateral compartment of the transwells.

	Acetate (C2)			Propionate (C3)			Butyrate (C4)			Valerate (C5)			Caproate (C6)		
Apical side t=0 (mmol/L)	1	5	20	1	5	20	1	5	20	1	5	20	1	5	20
Apical side t=24h (mmol/L)	0.06	1.07	7.43	0.23	1.02	5.73	0.09	0.89	6.18	<0.01	0.68	5.68	0.13	0.72	5.01
Basolateral side t=24h (mmol/L)	0.24	0.59	1.78	0.30	0.80	2.15	0.04	0.58	2.04	0.06	0.49	2.02	0.14	0.63	1.98
Apical side t=24h (%)	6.0	22.9	39.8	16.3	19.4	31.0	9.1	17.0	32.0	0.0	14.4	32.2	13.4	18.1	27.8
Basolateral side t=24h (%)	103.6	56.9	43.0	99.3	68.3	52.5	20.8	49.9	47.5	31.2	47.2	51.7	61.3	70.8	49.5
Total (%)*	109.6	79.8	82.8	115.6	87.7	83.5	29.9	66.9	79.5	31.2	61.6	83.9	74.7	88.8	77.3

* Total (%) reflects the % of the corresponding SCFA recovered as compared to the initial concentration (t=0).

Influence of the SCFA on GSH/GSSG

In order to study the influence of the C2-C6 SCFA on the GSH and GSSG level of the epithelial Caco-2 cells, we decided to use undifferentiated as well as differentiated cells, which are both generated in the gut and may be affected in a distinct way. In contrast to the proliferating cells that did not reach confluency when the incubation with SCFA started, the differentiated cells were confluent and dome-like structures were observed that confirmed their differentiated state (data not shown).

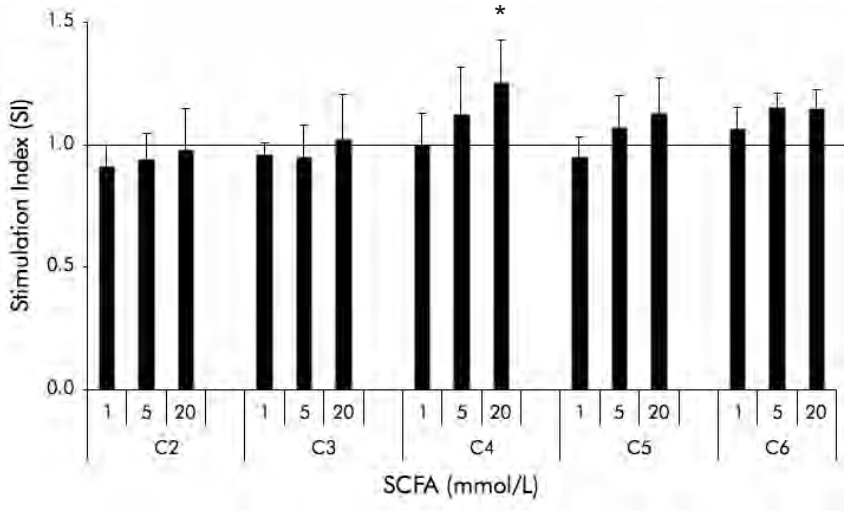

Figure 3.3 Mean change in TEER values due to incubation with the different SCFA (C2-C6) in 3 concentrations (1, 5 and 20 mmol/L) compared to the situation without incubation with SCFA indicated as SI (±SD). The TEER value of the experiment without SCFA treatment was set at 1.
*: Significant increase in the TEER value compared to the control with 0 mM of corresponding SCFA.

After incubation with medium only (control values) proliferating cells contained 33.7 μmol/g protein GSH (standard deviation (SD): 0.91) and 3.0 μmol/g protein GSSG (SD: 0.18), while the concentrations in differentiated cells were 6.5 μmol/g protein GSH (SD: 0.93) and 0.9 μmol/gram protein GSSG (SD: 0.08). In both the proliferating and the differentiated cells all concentrations of the GSH depleting L-buthionine-sulfoximine (BSO)[20], used as positive control, decreased concentrations of GSH and GSSG (Figure 3.4). The effects of the different SCFA are shown as stimulation index (SI) as compared to the control values described above, which were set at 1. In differentiated cells, all SCFA induced a dose-dependent increase of GSH with butyrate and valerate showing the highest increase. In contrast, the GSSG concentrations dramatically decreased after addition of all SCFA, leading to a 5-10 fold dose-dependent increase in the GSH/GSSG ratio (Figure 3.4). However, in proliferating cells all SCFA induced only slight increases of GSH and GSSG, resulting in no apparent effect on the GSH/GSSG ratio.

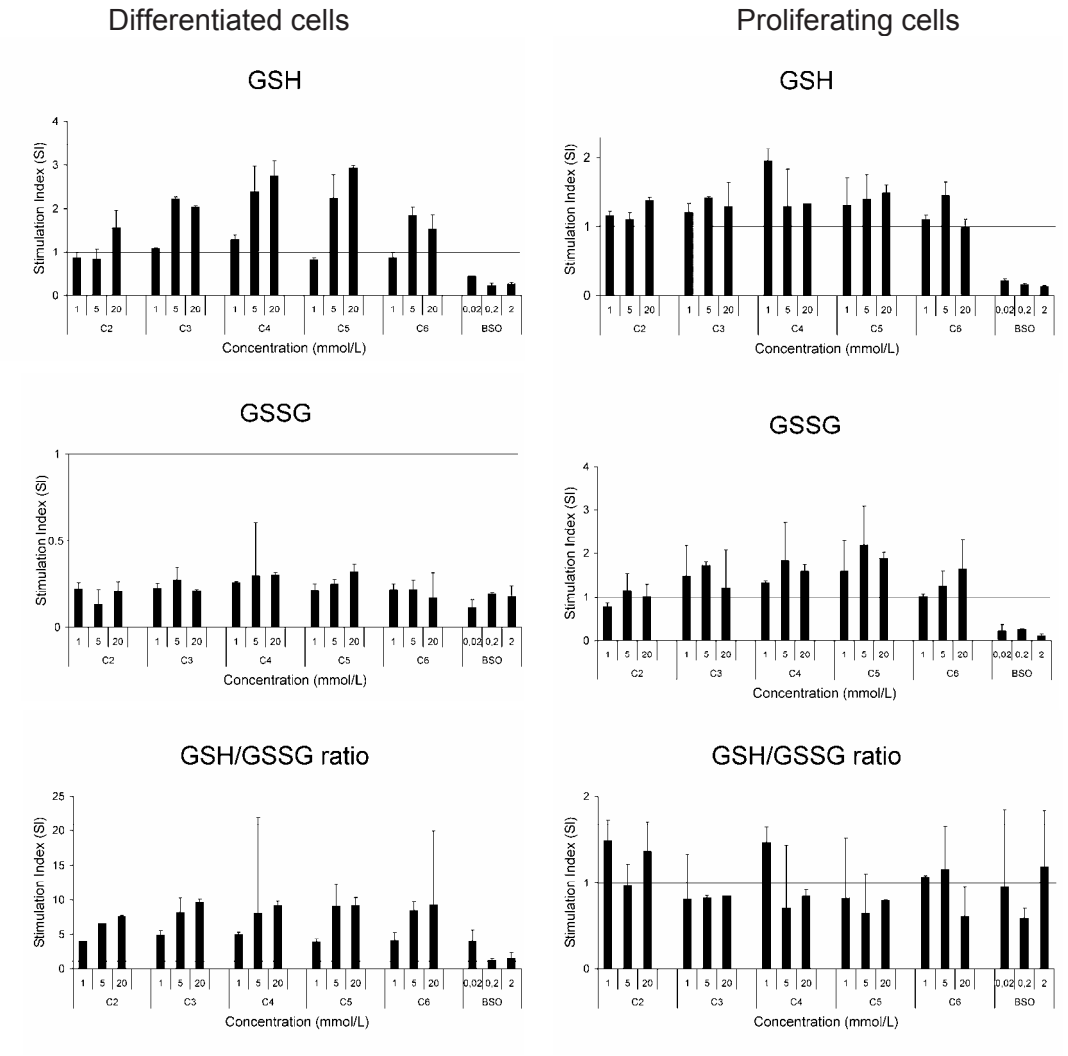

Figure 3.4 Effects of the various SCFA (C2-C6) on the level of GSH and GSSG and the GSH/GSSG ratio indicated as mean stimulation index SI (± standard deviation) compared to the control values adjusted for the protein content (based on duplicate experiments). The production of GSH and GSSG and their ratio in the cultures without SCFA treatment was set at 1. Concentrations of GSH and GSSG were measured in cell lysates from Caco-2 cells that were incubated for 24 hours with different concentrations of SCFA (1, 5 or 20 mmol/L). BSO, a GSH depleting agent, was used as a control.

Discussion

The aim of the study was to compare the effects of SCFA with different chain lengths (C2-C6) on parameters of inflammation, cellular integrity and antioxidant capacity *in vitro*. This study showed that all SCFA, except acetate, were able to modulate the cytokine response in an *in vitro* co-culture system that combines Caco-2 cells with a whole blood culture. Butyrate (C4) showed the most potent effects, but also several clear inhibitory effects on cytokine responses were observed for caproate (C6) valerate (C5) and propionate (C3). Furthermore, all of the SCFA are able to induce a dose dependent increase of the concentrations of the antioxidant GSH in differentiated Caco-2 cells. The integrity of the Caco-2 cell layer as indicated by the TEER, significantly improved only after incubation with the highest concentration of butyrate.

Anti-inflammatory properties of butyrate have previously been described in the literature[7], while little is known about the effects of the other SCFA, although they are also abundantly produced within the colonic lumen, especially after consumption of a high fibre diet.[21] In autopsy samples from sudden death victims, total SCFA concentrations varied from 137 to 197 mmol/kg chyme in the caecum to 86 to 97 mmol/kg chyme in the descending colon.[21,22] In these samples, C2:C3:C4:C5:C6 were found to be present in a molar ratio of approximately 54:20:21:4:1.[21] However, actual ratios may differ depending on the composition of the colonic microbiota and the substrates that are available for fermentation.[1] In contrast to the *in vivo* situation, the applied *in vitro* model does not contain a mucus layer. Therefore, a lower concentration range of SCFA was used in our model (1,5, 20 mmol/L), which is also in line with previous published *in vitro* data.[23]
SCFA are reported to be rapidly absorbed[24], which was also supported by the experiments in our *in vitro* study. In general, butyrate is considered to be the preferred energy source for colonocytes, while propionate is largely taken up by the liver and acetate enters the peripheral circulation to be metabolised by peripheral tissues.[25] For valerate and caproate there are hardly any human data available. One study using rat colonocytes suggested that valerate and caproate are excellent substrates for colonic oxidation, similar to butyrate.[26]
The data presented in our study may confirm these findings especially of valerate, since butyrate and valerate showed the lowest recovery rate probably suggesting a high degree of metabolism of these SCFA.

To evaluate the effects of SCFA on the release of a broad spectrum of mediators in the colon, a co-culture was used that combines differentiated human intestinal epithelial cells (Caco-2 cells) with human whole blood. Blood was derived from three different healthy donors to include inter-individual variation in the model. The major advantage of whole blood in contrast to the frequently used peripheral blood

mononuclear cell cultures is that whole blood cultures provide a more physiological environment and represent the entire spectrum of cellular as well as non-cellular components of the human immune system. In this co-culture model the gut epithelium not only serves as a natural barrier, separating non-absorbable substances from the immune cells, but also takes up and metabolises absorbable ingredients, as they would do *in vivo*. Equally important, it contributes significantly to a cytokine cross-talk comparable to what can be found in the human gut.[18] Therefore, this more complex *in vitro* model is expected to generate results that better reflect the physiologic situation, compared to mono-culture *in vitro* models, although the concentration of dendritic cells in the blood is rather low..

In the present study butyrate was found to decrease the release of a broad spectrum of cytokines including pro-inflammatory cytokines (e.g. TNF-α and IL-1β), IL-1ra, chemokines (e.g. MCP-1) and T-helper cell type 1 (e.g. IFN-γ) and type 2 (e.g. IL-5) related cytokines in a dose-dependent manner. On the other hand, valerate, propionate and caproate were also able to affect the release of several cytokines, although to a lesser extent. Acetate did not affect cytokine production. Mostly, C3, C5 and C6 mimicked the effects of butyrate, but had different effects especially on the release of IL-8 and IL-10, which will be discussed later. Only butyrate, but no other SCFA tested, was able to inhibit the release of GM-CSF, IL-1α, IL-1β, IL-4, IL-6 and IL-17.

The effects of butyrate are in line with previous studies that showed that butyrate reduced concentrations of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in patients with Crohn's disease[5], in cultured isolated human lamina propria cells and in peripheral blood mononuclear cells.[7] The most frequently reported mechanism by which butyrate exerts its anti-inflammatory effects is its ability to inhibit the activation of the transcription factor NF-κB that plays a central role in immune and inflammatory responses.[4,7] It has been described that the inhibition of NF-κB activity can be the result of butyrate's ability to inhibit histone deacetylase.[27] Hyperacetylation of histones disrupts their association with DNA, resulting in a more "open" chromatin structure that facilitates access to transcription factors of specific genes. The extent to which the other SCFA are able to inhibit NF-κB is unknown. Because propionate and valerate have also been demonstrated to possess the ability to induce histone hyperacetylation[28-30], it could be hypothesised that the observed anti-inflammatory effects of valerate and propionate are also the result of an inhibition of NF-κB activity. Butyrate is a stronger inhibitor of histone deacetylase as compared to valerate and propionate[28-30], which may explain the differences in magnitude of the effects. However, the fact that caproate does not demonstrate an effect on histone deacetylase while it is able to significantly decrease concentrations of TNF-α and TNF-β points towards the existence of an alternative mechanism, for example through alteration of the DNA methylation status.[31]

Previous *in vitro* studies have shown that butyrate is able to inhibit the pro-inflammatory IFN-γ production and signalling.[32,33] The present study does not report a significant effect of any of the SCFA on IFN-γ. However, a clear trend towards an inhibition of IFN-γ, was observed especially using butyrate (Figure 3.2). This inhibition was not considered significant due to the variation between the three blood donors and the very stringent statistical test with a confidence interval of 99.67%. However, when a confidence interval of 99% is chosen, the decrease of IFN-γ is significant for 5 and 20 mmol/L of butyrate and 20 mmol/L of propionate and valerate.

In contrast to the pronounced decrease of the secretion of a broad spectrum of pro-inflammatory and further cytokines, the secretion of the anti-inflammatory cytokine TGF-β was not significantly affected by all SCFA. Another anti-inflammatory cytokine, IL-10, was differentially affected depending upon the concentrations and the SCFA used. While incubation with butyrate at a concentration of 1 mmol/L increased IL-10, higher concentrations decreased its secretion. These results are supported by a previous study with butyrate using human peripheral blood mononuclear cells[34], which described that concentrations up to 1 mmol/L increased the concentrations of IL-10. Besides IL-10, also the secretion of IL-8 showed differences between the SCFA: IL-8 was not significantly affected by butyrate, while high concentrations of propionate, valerate and caproate increased its secretion. With regard to butyrate, inconsistent results have been reported using a variety of intestinal epithelial cell lines with an up-regulation[38] or a down-regulation[35,37] of IL-8 production depending on the duration of the incubation[36] and concentrationof butyrate.[35]

Summarizing our data on the cytokine release, the abovementioned findings indicate that SCFA, especially C3-C5, may decrease inflammation by inhibiting the release of pro-inflammatory cytokines, rather than increasing the release of the anti-inflammatory cytokines.

Besides inflammation also other processes determine the cell's functionality, such as epithelial integrity and antioxidant capacity, which may be interrelated. Therefore, the effects of the various SCFA on the TEER and glutathione levels as parameters for epithelial integrity and antioxidant status, respectively, were further evaluated.

In the literature there are several paradoxical reports concerning the effect of SCFA on epithelial barrier function.[16,39] It has been reported that butyrate at low concentrations (up to 2 mmol/L) induced a concentration-dependent reversible decrease in permeability in Caco-2 and HT-29 cell lines[16,39], while at higher concentrations (8 mmol/L), butyrate increased the permeability in Caco-2 cells, when it was exposed for more than 48 hours.[16,39] In addition, propionate and acetate also decreased permeability, with a maximal responses for propionate at 16 mmol/L, and 32 mmol/L for acetate.[39] The data presented in our study

confirmed a significantly increased TEER value of the differentiated Caco-2 cell layer, only when butyrate was used at the highest concentration (20 mmol/L) suggesting an increased epithelial integrity and improved barrier function. Because the effects of the SCFA on barrier function was not the primary aim of the present study, we only determined the TEER as a marker for permeability, it would be interesting to further study other markers such as expression of different tight junction proteins, or mannitol or horseradish peroxidase fluxes over the epithelial layer.

GSH is an important component of the antioxidant defence and a lack of GSH has been shown to result in severe degeneration of intestinal epithelial cells in mice.[40] A decrease of GSH accompanied by an increase of its oxidised form, GSSG is an indicator of oxidative stress as can also be observed in patients with inflammatory bowel disease.[41] The 24 hour incubation with all SCFA resulted in a clear increase of GSH and decrease of GSSG in the differentiated colonic epithelial cells, with butyrate and valerate showing the most pronounced effects. The increase in GSH could possibly be related to the fact that SCFA are an energy source for the colonic epithelial cells, since glutamine, the preferred energy source in the small intestine, is also able to increase GSH.[42] This may also explain why butyrate and valerate show the most pronounced effects, as these SCFA were preferentially metabolised. Due to the increase of GSH and the decrease of GSSG by the SCFA, the ratio of GSH and GSSG is increased 5-10 fold in a dose-dependent manner. These data are in line with a previous report showing an increase in GSH after incubation with butyrate using vascular smooth muscle cells.[43] In contrast, in the human colon cancer cell line HT-29, butyrate was found to decrease GSH concentrations.[20,44] These equivocal results could be explained by the different cell lines and concentrations used, but they may also be due to the state of the cells since we could demonstrate in the present study that proliferating cells contain more GSH and GSSG and do not respond as differentiated cells do to SCFA. Our observation that the effects of butyrate on GSH depend on the phenotypic state of the cell has also been reported with regard to its effect on proliferation.[45]

As the human colon is continuously exposed to a variety of toxic stimuli that are able to induce inflammatory effects, improved epithelial barrier function and antioxidant status due to the presence of increased concentrations of the different SCFA could thereby contribute to protection against inflammation.

In conclusion, this study performed with a co-culture model showed that among the various SCFA produced by luminal microbial fermentation, butyrate exerts the most potent effects on the inhibition of the release of several cytokines that have been shown to play an important role in inflammatory intestinal bowel diseases, improvement of the TEER and stimulation of the antioxidant glutathione. Remarkably, such effects were also observed for valerate, propionate and caproate with a rank order of potency being butyrate >> valerate > propionate >

caproate. Future studies could further focus on the effects of combinations of these SCFA as produced *in vivo* during colonic homeostasis.

Our study supports the proposed beneficial role of butyrate, but also of other C3-C6 SCFA on the maintenance of colonic health. If interventional dietary strategies designed to maximise colonic levels of these SCFA exert clinical and public health benefits remains to be shown.

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Chapter 4

Butyrate modulates oxidative stress in the colonic mucosa of healthy humans

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Abstract

Background

Butyrate, a short chain fatty acid produced by colonic microbial fermentation of undigested carbohydrates, has been implicated in the maintenance of colonic health. This study evaluates whether butyrate plays a role in oxidative stress in the healthy colonic mucosa.

Methods

A randomised, double blind, cross-over study with 16 healthy volunteers was performed. Treatments consisted of daily rectal administration of a 60 mL enema containing 100 mmol/L sodium-butyrate or saline for two weeks. After each treatment, a blood sample was taken and mucosal biopsies were obtained from the sigmoid colon. In biopsies, the trolox equivalent antioxidant capacity, activity of glutathione-S-transferase, concentration of uric acid, glutathione (GSH), glutathione disulphide and malondialdehyde and expression of genes involved in GSH and uric acid metabolism was determined. Secondary outcome parameters were CRP, calprotectin and intestinal fatty acid binding protein in plasma and histological inflammatory scores.

Results

Butyrate treatment resulted in significantly higher GSH ($p<0.05$) and lower uric acid ($p<0.01$) concentrations compared to placebo. Changes in GSH and uric acid were accompanied by increased and decreased expression, respectively, of their rate limiting enzymes determined by RT-PCR. No significant differences were found in other parameters.

Conclusion

This study demonstrated that butyrate is able to beneficially affect oxidative stress in the healthy human colon.

Introduction

Butyrate is a four-carbon short-chain fatty acid produced by bacterial fermentation of mainly undigested dietary carbohydrates within the colonic lumen. Increased butyrate production has often been hypothesised to be one of the beneficial effects of prebiotics. The effects of butyrate on colonic mucosal health have been widely studied. Apart from being the preferred energy source for colonic epithelial cells, butyrate can induce changes in gene expression influencing colonic function, mainly by inhibiting histone deacetylase.[1] Several studies have shown that butyrate has anti-carcinogenic and anti-inflammatory effects.[2] *In vitro*, butyrate has been shown to reduce inflammation by inhibition of NFκB activation[3] and upregulation of PPARγ.[4] *In vivo*, several studies have demonstrated a decrease in inflammation due to rectal administration of butyrate or mixtures of SCFA in patients with active ulcerative colitis[5,6] and diversion colitis[7,8], although not all studies were able to show significant effects.[8]

Oxidative stress is involved in both inflammation[9] and the process of initiation and progression of carcinogenesis.[10] During oxidative stress there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defence mechanisms, leading to a cascade of reactions in which lipids, proteins and/or DNA may get damaged. Increased oxidative stress in the intestinal epithelium has for example been shown to disrupt mucosal barrier function of intestinal epithelial cells, resulting in increased permeability.[11] In the inflamed colon, neutrophilic granulocytes are an important source of potent oxidizing metabolites such as superoxide radicals and hydroxyl radicals. To protect against ROS, multiple antioxidant defence mechanisms exist, including antioxidant enzymes, such as glutathione-S-transferase (GST) and glutathione peroxidase (GPX), and non-enzymatic antioxidant molecules, such as glutathione (GSH) and uric acid.

Limited evidence shows that oxidative stress in the colonic mucosa can be modulated by butyrate. In two *in vitro* studies, pre-incubation of isolated rat[12] or human[13] colonocytes with butyrate resulted in a significant reduction of H_2O_2-induced DNA damage. In rats, resistant starch intake decreased the levels of colonocyte DNA damage induced by a high protein diet. This DNA damage correlated negatively with caecal butyrate concentrations.[14] The mechanism by which butyrate reduces oxidative stress is unknown. Primary antioxidant capacity of butyrate is unlikely as butyrate does not act as a free-radical scavenger due to its chemical structure. However, butyrate may act as a secondary antioxidant by affecting DNA repair systems and levels of enzymatic or non-enzymatic antioxidants. For example in a rat model of TNBS-induced colitis, fermentable fibre supplementation resulted in increased colonic concentrations of butyrate, a decreased colonic myeloperoxidase (MPO) activity and a restoration of the colonic GSH concentration.[15] In addition, butyrate has been reported to enhance GST

expression in HT-29 cells.[16-18] However, these effects have not yet been demonstrated *in vivo* in humans.
In the present study the effects of butyrate on several parameters of oxidative damage and antioxidant defence are evaluated in human colonic biopsies of healthy volunteers. As secondary parameters, markers of inflammation are included.

Materials and Methods

Subjects

Sixteen healthy non-smoking volunteers (4 males, 12 females, median age 23 (18-62) years) participated in the study. The subjects were on a stable Western diet and had no history of gastrointestinal diseases. In addition, they did not use immunosuppressive drugs, antibiotics, anti-diarrhoeal drugs and laxatives during and in the last three months prior to the study. Subjects did not consume pre- or probiotics during and two weeks prior to the study. Subjects gave their written informed consent before participating. The study has been performed in accordance with the principles of the declaration of Helsinki and was approved by the Medical Ethics Committee of the University Hospital Maastricht, the Netherlands.

Study design

This study was performed according to a randomised double-blind placebo controlled cross-over design with two test periods of two weeks separated by a wash-out period of two weeks. Throughout the two test periods, subjects were instructed to maintain their habitual diet and their usual pattern of physical activity.
Volunteers self-administered a 60 mL enema once daily for two weeks containing either a sodium butyrate solution (100 mmol/L) or a placebo solution (140 mmol/L NaCl) with a neutral pH in a random order. The butyrate enemas were made isotonic by the addition of NaCl (40 mmol/L). Volunteers were instructed to self-administer the enema prior to sleeping, and to stay in a left-lateral supine position for at least 15 min. Compliance was evaluated on the basis of a questionnaire and the returned enema-bottles.
In the morning after the administration of the last enema and after an overnight fast, a sigmoidoscopy was performed without prior bowel cleansing by a gastroenterologist who was blinded for the intervention, and mucosal biopsies were taken from a standardised location of the sigmoid (approximately 20 cm from the anal sphincter at the location of the arteria iliaca communis). Two biopsies were used for analyses of oxidative stress parameters and one biopsy was used for RNA isolation. These biopsies were immediately frozen in liquid nitrogen. Two other

biopsies were fixed in formalin and embedded in paraffin for histological determination of the overall inflammatory score. After the endoscopic procedure, a blood sample was obtained and centrifuged immediately (15 min, 1750g, 4°C). Plasma was stored at -80°C until analysis. To standardise the diet prior to the sigmoidoscopy, the volunteers recorded their dietary intake three days before the first sigmoidoscopy and repeated this intake before the second sigmoidoscopy.

Analyses of colonic biopsies

Preparation of biopsy homogenates for oxidative stress analyses

Two frozen biopsies were grinded with a mortar and pastel cooled in liquid nitrogen. The powder was resuspended in 240 μL ice-cold milliQ. From this suspension 90 μL was added to 9 μL of an acidic buffer (13% 5-Sulfosalicilic acid, 100 mmol/L HCl in PBS) and was centrifuged (5 min, 14000g, 4°C). The supernatant was used for the determination of total GSH (tGSH) and glutathione disulfide (GSSG), the oxidised form of GSH. The remaining suspension was centrifuged (5 min, 14000g, 4°C) and the supernatant was stored at -80°C until the determination of GST activity, malondialdehyde (MDA), trolox equivalent antioxidant capacity (TEAC) and uric acid. Total protein content in the supernatants was quantified using a BCA^{tm} protein assay kit (Pierce, Rockford IL, USA).

GST

GST activity was determined using the GST catalysed reaction of chlorodinitrobenzene (CDNB, Sigma, Zwijndrecht, the Netherlands) with GSH resulting in glutathione-dinitrobenzene, a yellow coloured product measured at 340 nm.[19] Fifteen μl of tissue supernatant was added to 1 mmol/L CDNB and 1 mmol/L GSH in 1 mL of 100 mmol/L potassium phosphate buffer (pH 6.5). The increase in absorbance at 25°C was measured during 2 min. After correction for the non-enzymatic reaction, the activity of GST in the sample was calculated using the molar absorptivity coefficient of GSH-dinitrobenzene. One unit of GST activity is defined as the amount of enzyme catalyzing the conjugation of 1 μmol substrate per min.

TEAC

The total antioxidant capacity assay was performed in deproteinated samples as described by Fischer *et al.*[20] In short, the $ABTS^{\bullet+}$ solution was prepared by incubating 0.15 mg/mL ABTS (Sigma) and 0.53 mg/mL ABAP (Polysciences, Eppelheim, Germany) in sodium phosphate buffer (145 mmol/L, pH 7.4) at 70°C until the absorbance of the solution at 734 nm was 0.70 ± 0.02. Samples were deproteinated by adding an equal volume of 10% trichloro acetic acid to the sample. Fifty μl deproteinated tissue supernatant was mixed with 950 μl of the

$ABTS^{\bullet+}$ solution. After 5 min of incubation at 37°C, the absorbance at 734 nm was measured. The decrease in absorbance was related to that of trolox standards.

Uric acid

Uric acid was determined in the deproteinated sample by HPLC as described previously[21] with minor modifications. Ten µl of the deproteinated sample or standard was injected into a HPLC system (Agilent, Palo Alto, CA, USA) equipped with an Alltima HP C18 AQ column, 150 x 4.6mm, particle size 5µm (Grace, Deerfield, IL, USA). Samples were eluted with 0.1% trifluoric acid in milliQ. Detection was performed at 292 nm.

TBARS

The determination of TBARS is based on the formation of a coloured adduct of MDA-like breakdown products of lipids with 2-thiobarbituric acid (TBA).[22] Forty µL tissue supernatant or standard was added to 225 µL of TBA reagent (0.012 M TBA, 0.32 M H_3PO_4, 0.68 mmol/L butylated hydroxytoluene and 0.01% EDTA). The mixture was incubated in a water bath for 1 hour at 100°C. After cooling, the MDA-products were extracted with 125 µL butanol. After centrifugation (5000g), 30 µL of the butanol layer was injected into the HPLC system equipped with a fluorescence detector (set on excitation wavelength of 532 nm and emission wavelength of 553 nm) and an Alltima C18 column (150 x 3.0 mm particle size 5 µm (Grace, Deerfield, IL, USA)). Samples were eluted with 65% milliQ, 35% methanol, containing 0.05% trifluoric acid.

tGSH and GSSG

tGSH and GSSG concentrations were determined using the recycling method described by Vandeputte *et al.*[23] In short, the increase in absorbance at 412 nm, caused by the GSH and GSSG driven reduction of 5'5'dithiobis 2-nitrobenzoic acid (Sigma) at 0.2 mmol/L in the presence of nicotinamide adenine dinucleotide phosphate (Sigma) at 0.15 mmol/L and GSSG-reductase (Sigma) at 1 U/mL, was measured for 3 minutes. The slope of the increase in absorbance of the samples was compared to the slope of GSH and GSSG standards. To measure only the GSSG concentration with the same method, tissue supernatants were preincubated with 2-vinylpyridine (3% final concentration) for one hour, to remove GSH. The GSH concentration was calculated by subtracting two times the GSSG concentration from the total GSH concentration. The GSH/GSSG ratio was calculated as a marker of oxidative stress.

Real-Time PCR (RT-PCR)

The expression of all essential genes in the uric acid and GSH pathway, apart from GST that was measured at the level of activity, were determined using RT-PCR. RNA was isolated from one frozen biopsy using TRIzol reagent (Invitrogen, Carlsbad, USA). RNA was purified with an RNeasy mini kit (Qiagen, Venlo, The Netherlands) combined with a DNase treatment using the RNase-Free DNase Set (Qiagen). 500ng Total RNA was used as a template for the cDNA reaction, which was synthesised using the iScript cDNA Synthesis kit (Bio-Rad, Veenendaal, The Netherlands). The cDNA was diluted to a concentration of 0.32 ng/μL. Each reaction contained 12.5 μL iQ Sybr Green Supermix (Bio-Rad), 1 μL each of 10 μmol/L gene-specific forward and reverse primers, 4 μL cDNA template solution and 6.5 μL sterile H_2O. Primer sequences are listed in Table 4.1. Housekeeping genes included were 18SrRNA, GAPDH and CANX. Reactions were run on the My IQ Single Colour RT-PCR Detection System (Bio-Rad). PCR conditions used were 3 min at 95°C, followed by 40 amplification cycles of 10 sec at 95°C and 45 sec at 60°C.

Table 4.1 Primer information of housekeeping genes and essential genes involved in GSH and uric acid metabolism.

	Sequence ID	Forward primer (5' → 3')	Reverse primer (5' → 3')
CANX	NM_001024649	CCACTGCTCCTCCTTCATCTCC	CGGTATCGTCTTTCTTGGCTTTGG
GAPDH	NM_002046	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
18SrRNA	M10098	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
GPX1	NM_000581	CCGACCCCAAGCTCATCACC	GATGTCAGGCTCGATGTCAATGG
GPX2	NM_002083	ATCCTGAACAGTCTCAAGTATG	TGGGTCATCATAAGGGTAGG
GPX3	NM_002084	ACATGCCTACAGGTATGCGTGATTG	TGGAGTGGAGAACTGGAGAGAAAGG
GR	NM_000637	CAGGGACTTGGGTGTGATGAAATGC	GAGGTAGGGTGAATGGCGACTGTG
GS	NM_000178	AAGACACTCGTGATGAACAAGC	GGAGAGGAATGACAAATACAGAGG
GCLC	NM_001498	TGGAAGTGGATGTGGACACC	GTCTTGCTTGTAGTCAGGATGG
GCLM	NM_002061	GGCACAGGTAAAACCAAATAGTAAC	CAAATTGTTTAGCAAATGCAGTCA
XDH	NM_000379	CCTCTTCCTGGCTGCTTCTATCTTC	TGACACACAGGGTGGTGAACTTG

CANX: calnexin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; 18SrRNA: 18S ribosomal RNA; GPX: glutathione peroxidase; GR: glutathione reductase; GS: glutathione synthetase; GCLC: glutamate-cysteine ligase, catalytic subunit; GCLM: glutamate-cysteine ligase, modifier subunit; XDH: xanthine dehydrogenase.

Histology

Two mucosal biopsies were fixed separately in formaldehyde, embedded in paraffin and cut in 2 μm sections for H&E staining. Subsequently, the degree of inflammation was scored by one blinded observer on a four-point scale corresponding to none, mild, moderate or severe inflammation as previously described.[24]

Plasma markers

ELISA kits were used for calprotectin (HBT, Uden, the Netherlands) and CRP (HBT) according to manufacturer's instructions. Intestinal fatty acid binding proteins (I-FABP) are small cytoplasmic proteins expressed in the intestine. Systemic concentrations can be used as a marker for intestinal injury.[25] Concentrations of I-FABP were determined using a specific ELISA kit (HBT) according to manufacturer's instructions.

Statistical analysis

Results are expressed as median and range. The non-parametric Wilcoxon signed-rank test was used to identify significant differences. Pearsons correlations were determined between different parameters of oxidative stress. Differences were considered statistically significant when $p<0.05$. Statistical analyses were performed using SPSS 11.0 software.
RT-PCR data were analysed using a Gaussian linear regression including the repeats, house keeping genes, and the intervention period. The inference criterion used for comparing the models is their ability to predict the observed data, i.e. models are compared directly through their minimised minus log-likelihood. When the numbers of parameters in models differed, they were penalised by adding the number of estimated parameters, a form of the Akaike information criterion (AIC).[26] For each gene, the treatment group was then added to the model. The gene under consideration was found to be differentially expressed if the AIC decreased compared to the model not containing the intervention.

Results

All 16 participants completed the study and returned all enema bottles empty.
No side effects were reported.

Oxidative stress parameters in colonic biopsies

The effects of daily administration of 60 mL butyrate enemas (100 mmol/L) for 14 days on parameters of oxidative stress in colonic biopsies are shown in Table 4.2. Butyrate treatment resulted in significantly higher concentrations of tGSH (27.2 vs. 24.5 nmol/mg protein), GSH (26.5 vs. 22.9 nmol/mg protein) ($p<0.05$), and in lower uric acid levels (2.4 vs. 3.1 nmol/mg protein) ($p<0.01$) compared to placebo. In addition, a trend towards an increase in the GSH/GSSG ratio (90.8 vs. 71.1) ($p=0.07$) was found after butyrate treatment. No statistical differences were found in TEAC, GST, GSSG and MDA.

Table 4.2 Effect of butyrate enemas (100 mmol/L) once daily for 14 days on parameters of oxidative damage and antioxidant defence in colonic mucosal biopsies from 16 healthy volunteers.

	Placebo n=16	Butyrate n=16	p-value
TEAC (nmol trolox Eq/mg protein)	137.2 (112.6-210.7)	136.2 (116.2-207.6)	0.72
Uric acid (nmol/mg protein)	3.1 (1.9-3.3)	2.4 (1.6-3.7)	0.01
GST (U/mg protein)	0.34 (0.23-0.51)	0.32 (0.24-0.41)	0.55
tGSH (nmol/mg protein)	24.5 (13.4-32.4)	27.2 (21.9-35.5)	0.03
GSH (nmol/mg protein)	22.9 (12.0-31.5)	26.5 (20.4-35.2)	0.03
GSSG (nmol/mg protein)	0.4 (1.9-2.13)	0.31 (0.08-2.69)	0.18
GSH/GSSG ratio	71.1 (8.1-127)	90.8 (7.6-333.9)	0.07
MDA (nmol/mg protein)	1.09 (0.69-2.2)	0.78 (0.44-1.44)	0.13

Values expressed as medians (range)
TEAC: trolox equivalent antioxidant capacity; GST: glutathione-S-transferase; tGSH: total glutathione; GSH: reduced glutathione; GSSG: glutathione disulfide; MDA: malondialdehyde

A significant positive correlation was found between MDA and GSSG (R^2=0.35), and a significant negative correlation was found between MDA and the GSH/GSSG ratio (R^2=0.24). No significant correlation was found between MDA and GSH (Table 4.3).

Table 4.3 Correlations between parameters of oxidative stress measured in colonic mucosal biopsies.

	MDA		
	r	R^2	p-value
GSSG	0.594	0.35	0.000
tGSH	-0.146	0.02	0.425
GSH	-0.289	0.08	0.109
GSH/GSSG ratio	-0.485	0.24	0.005

GSSG: glutathione disulfide; tGSH: total glutathione; GSH: reduced glutathione; MDA: malondialdehyde

RT-PCR

Fold changes including the confidence interval of these genes are shown in Figure 4.1. mRNA expression of GPX1, GPX3 and GCLC in the mucosal biopsies were found to be significantly upregulated (1.07, 1.16 and 1.07, respectively) and mRNA expression of GPX2, GR, GS and XHD were found to be significantly downregulated (0.9, 0.93, 0.92 and 0.90, respectively).

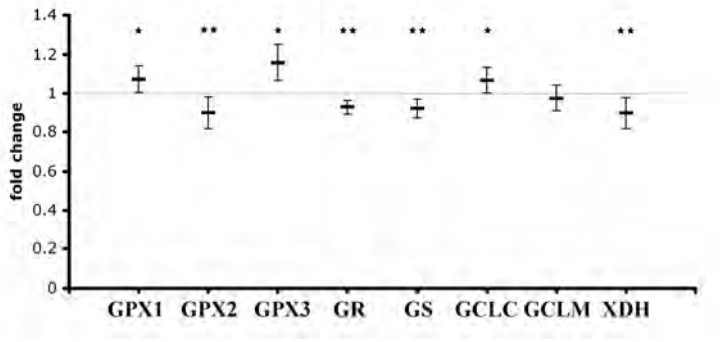

Figure 4.1: Effect of butyrate enemas (100 mmol/L) once daily for 14 days on colonic expression of genes involved in the GSH and uric acid pathway indicated as fold change and confidence interval. *: Significantly upregulated compared to placebo; **: Significantly downregulated compared to placebo.
GPX: glutathione peroxidase; GR: glutathione reductase; GS: glutathione synthetase; GCLC: glutamate-cysteine ligase, catalytic subunit; GCLM: glutamate-cysteine ligase, modifier subunit; XDH: xanthine dehydrogenase.

Histology and plasma markers

All H&E stained biopsy specimens were scored with 0 by the pathologist, indicating no increased inflammatory activity.
Plasma concentrations of calprotectin, CRP and iFABP did not differ between the two treatments.

Discussion

In this study rectal butyrate administration was shown to enhance the antioxidant capacity of the colonic mucosa in healthy volunteers by increasing the GSH concentration. Also a decrease in uric acid concentrations was found. On the level of gene expression, this increase in GSH was accompanied by an increase in GCLC, the rate-limiting enzyme in GSH production. The decrease in uric acid was accompanied by a decrease in XDH, the enzyme that converts purines into uric acid and ROS. No effect of butyrate on inflammation and intestinal injury was found in this study with healthy volunteers.

This is the first time that butyrate has been shown to increase the colonic concentrations of GSH *in vivo* in healthy humans. This increase in GSH in healthy volunteers is in line with a rat study with TNBS-induced colitis, in which a higher production of butyrate after dietary fibre supplementation resulted in an increase of colonic GSH.[15] GSH is synthesised from glutamate, cysteine and glycine by the sequential actions of glutamate cysteine ligase (GCL) and glutathione synthase (GS). The rate-limiting enzyme for GSH synthesis is GCL, which consists of a heavy catalytic subunit (GCLC) and a light regulatory subunit (GCLM), each

encoded by separate genes. GSH is used to reduce hydrogen peroxide and lipidhydroperoxides catalysed by glutathione peroxidase (GPX). The produced GSSG can be converted back to GSH through the action of glutathione reductase (GR). GSH can also be used to detoxify endogenous and exogenous electrophiles through conjugation via glutathione-S-transferases (GST).

On the level of gene expression, butyrate significantly affected several genes coding for different antioxidant enzymes, although fold changes were rather subtle. The increase in GSH was accompanied by a decrease of GS, an increase of GCLC and had no effect on GCLM. This is in accordance with other studies that have shown that an increase in GSH concentration was related to an increase in GCLC.[27,28] Furthermore, butyrate increased the expression of the so-called cytosolic GPX1 and the plasma GPX3. A decrease in the expression of GPX2 was found, which is defined as the gastrointestinal GPX. This is in line with findings after resistant starch supplementation in 1,2-dimethylhydrazine-treated rats.[29] The diverse effects on the different GPXs seem contradictory but may be explained by their distinct roles in cellular defence mechanisms.[30] The expression of GR was decreased after butyrate treatment for which an obvious explanation is lacking. However, one has to keep in mind that increased gene expression does not always result in increased enzymatic activity.

GSH is an important component of the antioxidant defence and a lack of GSH has been shown to result in severe degeneration of intestinal epithelial cells in mice.[31] In a rat model of colitis GSH administration improved the colonic histological score and reduced mucosal MDA concentrations.[32] Reduced concentrations of GSH and increased concentrations of GSSG have been reported in patients with inflammatory bowel disease.[33,34] However, in several other stress situations, an increase in GSH has been reported.[35] Damage to the intestinal epithelium results in ATP release that ultimately leads to an increase in uric acid.[36] Because in the present study a decrease in uric acid was found after butyrate treatment, damage to the epithelium is unlikely. This is further supported by the fact that no effects were seen on the included parameters of inflammation and epithelial damage. In addition, the GSSG concentrations slightly decreased, which resulted in a trend towards an increase of the GSH/GSSG ratio. MDA, the parameter of lipid peroxidation, also slightly decreased after butyrate supplementation although this was not statistically significant.

In the present study a decrease in the colonic uric acid concentration was found after the butyrate intervention. In plasma, uric acid accounts for about half of the antioxidant capacity, while in colonic biopsies, it was found to have only a small contribution (<5%) to the total antioxidant capacity, supporting that this is not the major antioxidant in the colonic mucosa as previously reported.[37]

In humans, uric acid is the final oxidation product of purine catabolism with xanthine dehydrogenase (XDH) being the rate-limiting enzyme. XDH can be

converted into xanthine oxidase by proteolytic activity that can be decreased by GSH. While XDH requires NAD^+ and produces uric acid and NADH, xanthine oxidase requires oxygen and produces uric acid and ROS (Figure 4.2). Therefore, a decrease in uric acid production may also contribute to a decrease in ROS production and subsequently less oxidative damage.[38] This may be of clinical relevance as in a previous study using a rat model of colitis, the xanthine oxidase inhibitor oxypurinol resulted in reduced concentrations of MDA.[39]
The decrease in uric acid concentrations in the present study was accompanied by a decrease in expression of XDH. A direct effect of butyrate on xanthine oxidase activity could not be demonstrated *in vitro* (data not shown).

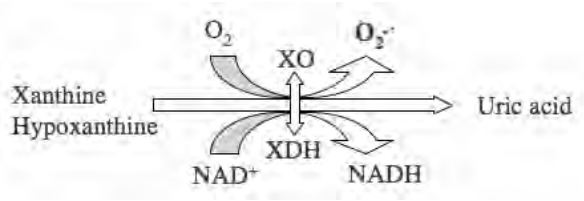

Figure 4.2 Production of uric acid catalised by xanthine oxidase (XO) and xanthine dehydogenase (XDH).

Butyrate administration did not affect the activity of GST in the present study. GSTs are a family of over 21 structurally different phase II enzymes, which are crucial in the cellular protection against oxidative stress.[40] Previously, some cell culture studies have shown that butyrate upregulates several GST subclasses, such as GST-A3, GST-P1, and GST-T1.[16-18] However, this did not always result in an increase of the GST activity.[41] Therefore, it is possible that subtypes of GSTs were affected by the butyrate intervention without influencing its overall activity.

We have demonstrated significant positive correlations between MDA and GSSG and negative correlations between MDA and the GSH/GSSG ratio. Increased concentrations of GSSG are an indicator of oxidative stress and have shown to correlate significantly with the disease activity index in patients with ulcerative colitis.[33] The observed correlations support the validity of our assessments in human colonic biopsy samples from healthy individuals.

Enemas were chosen for colonic administration of butyrate in order to standardise local concentrations. Another way to increase colonic butyrate concentrations would be through consumption of a high fibre diet. However, thereby local colonic concentrations of butyrate remain unknown and may differ between subjects depending on the fermentation capacity of their microbiota. Furthermore, after consumption of dietary fibre, butyrate is mainly produced in the proximal colon. By

using rectal enemas, butyrate reaches the distal colon, which is relatively easy accessible, and biopsies could be taken at this location without prior bowel cleansing in order to maintain the physiological situation. In a prior pilot study (data not shown), the volume of the 60 mL enemas was found to reach the sigmoid colon. The concentration of 100 mmol/L of butyrate was used as this is the expected local concentration after consumption of a high fibre diet. Previously, similar concentrations were used in studies with UC patients.[5] A single enema only once daily before going to sleep was chosen to maximise the time that content of the enema is retained, as well as the compliance. A wash out period of two weeks was assumed to be long enough as butyrate is quickly absorbed and the intestinal mucosa undergoes rapid cell turnover.

In conclusion, in the present study in healthy humans it has been demonstrated that locally administered butyrate in physiological concentrations increased the antioxidant GSH and possibly decreased ROS production, as indicated by a decreased uric acid production. As the human colon is continuously exposed to a variety of toxic stimuli, enhanced butyrate production in the colon could hereby result in an enhanced resistance against toxic stimuli and as such improve the barrier function. This might be relevant for both the pathophysiology as well as the treatment of gastrointestinal disorders such as post infectious irritable bowel syndrome, microscopic colitis, inflammatory bowel disease and diversion colitis.

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Chapter 5

Effect of butyrate enemas on inflammation and antioxidant status in the colonic mucosa of patients with ulcerative colitis in remission

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Submitted

Abstract

Background

Butyrate, produced by colonic fermentation of dietary fibres is often hypothesised to beneficially affect colonic health. This study aimed to assess the effects of butyrate on inflammation and oxidative stress in subjects with chronically mildly elevated parameters of inflammation and oxidative stress in colonic mucosa.

Methods

Thirty-five patients with ulcerative colitis in clinical remission daily administered 60 mL rectal enemas containing 100 mmol/L sodium-butyrate (n=17) or saline (n=18) during 20 days. Before and after each treatment 24-hour faeces was collected, a blood sample and mucosal biopsies from the unprepared sigmoid were obtained. In colonic biopsies, parameters of antioxidant defence and oxidative damage were determined as well as concentrations of myeloperoxidase and several pro and anti-inflammatory cytokines. In addition, faecal calprotectin and plasma CRP were determined.

Results

Butyrate enemas induced minor effects on colonic inflammation and oxidative stress. Only a significant increase of the colonic IL-10/IL-12 ratio was found in butyrate treated patients ($p=0.02$), and colonic mucosal concentrations of CCL5 were increased after butyrate compared to placebo treatment ($p=0.03$). Although in general butyrate did not affect colonic glutathione levels, the effects of butyrate enemas on total colonic glutathione appeared to be inversely related to the level of inflammation at the start of the study.

Conclusion

Although UC patients in clinical remission were characterised by low-grade oxidative stress and inflammation in the colonic mucosa, rectal butyrate enemas showed only minor effects on inflammatory and oxidative stress parameters. Possibly, higher concentrations than can be obtained by dietary means may be needed to obtain beneficial effects.

Introduction

As several epidemiological studies show protective effects of dietary fibre intake on intestinal inflammation, colorectal cancer and cardiovascular disease[1-4], there is increasing interest in adding indigestible fibres to dietary products. Short-chain fatty acids (SCFA), mainly acetate, propionate and butyrate, are important end-products of luminal microbial fermentation of these fibres and have been shown to play a role in the maintenance of colonic health and barrier function.[5] Moreover, butyrate is the major energy source for colonocytes[6] and several *in vitro* and animal studies have shown that butyrate is able to reduce inflammation mainly by inhibition of NFκB activation.[7] Previous *in vitro* studies have demonstrated that from the different SCFA, butyrate showed the most potent inhibitory effects on pro-inflammatory cytokine production[8](submitted for publication). In addition, an *in vivo* study in healthy human volunteers showed that butyrate supplementation increased the colonic levels of the antioxidant glutathione (GSH).[9] Other studies have also shown positive effects of butyrate on oxidative stress. In isolated human colonocytes, preincubation with butyrate was associated with a reduction of H_2O_2-induced DNA damage.[10] In a rat colitis model, increased colonic butyrate production after dietary fibre supplementation resulted in a restoration of colonic GSH levels.[11] This indicates that besides anti-inflammatory properties, butyrate may also play a role in the modulation of oxidative stress.

Most of the human intervention studies with butyrate administration published so far focused on overall clinical outcome parameters in patients with diversion colitis and active ulcerative colitis (UC). UC is a chronic inflammatory disorder of the colon that is characterised by alternating periods of flare-ups and quiescent disease. It is generally hypothesised that UC is the result of an exaggerated intestinal host response against luminal bacteria or their components, particularly in genetically susceptible individuals. Another factor that has been proposed to play a role in the pathophysiology of UC is oxidative stress, which is the result of excess production of reactive oxygen species due to aberrant cellular metabolism and increased activation of phagocytic leucocytes in the inflamed colon.[12,13] In active UC patients, the administration of rectal enemas containing butyrate or mixtures of SCFA have been proven effective in lowering clinical symptoms and overall inflammatory scores.[14,15]

In addition to this clinical therapeutic setting, it is also of interest whether butyrate, being an important end-product of dietary fibre fermentation, could have beneficial effects in a mildly disturbed gut health situation, such as in patients with UC in remission. These patients have no or minimal clinical symptoms during quiescent disease, but are still reported to maintain a chronic low-grade inflammation and oxidative stress of the colonic mucosa.[16-18] Possible beneficial effects of butyrate administration in this patient group can be expected as two studies reported that

fermentable dietary fibre supplementation, which resulted in increased faecal butyrate levels, was effective in maintaining remission in UC patients.[19,20]
The present study aimed to determine whether increasing intraluminal colonic butyrate levels could improve a low-grade level of colonic inflammation and/or oxidative stress in humans.

Methods

Patients

UC patients in remission were recruited from the South Limburg IBD registry.[21] Patients had a well-defined diagnosis of UC established by clinical, endoscopical, histological and/or radiological criteria. Patients were in clinical remission and were excluded from participation if the Endoscopic Grading System (EGS)[22] score was higher than 5 at the first endoscopy. The patients were between 18-65 years of age and consumed a stable Western diet. Exclusion criteria comprised: proctitis only, pregnancy, lactation, changes in medication and/or pre- and probiotic intake 2 weeks prior to and during the study, the use of corticosteroids, enemas or suppositories two weeks prior to or during the study, the use of antibiotics 3 months prior to or during the study, other clinically significant systemic diseases and previous radiotherapy or chemotherapy. All patients gave their written informed consent before participation. The study was performed in accordance with the principles of the declaration of Helsinki and was approved by the Medical Ethics Committee of the University Hospital Maastricht, the Netherlands.

Study design

This study was performed according to a randomised double-blind placebo-controlled parallel design. During an intervention period of 20 days patients self-administered a 60 mL rectal enema once daily containing either a sodium butyrate solution (100 mmol/L) or a placebo solution (140 mmol/L NaCl) with a neutral pH. The butyrate enemas were made isotonic by the addition of NaCl (40 mmol/L). Patients were instructed to self-administer the enemas prior to sleeping, and to remain in a left-lateral supine position for at least 15 minutes. Compliance was evaluated on the basis of a questionnaire and the returned enema-bottles. One week prior to the intervention period and during the study, patients completed a daily questionnaire regarding use of medication, bowel habits (defaecation frequency and faeces consistency score according to the Bristol scale[23]) and the following symptoms scored as mild (1) moderate (2) or severe (3): nausea, abdominal cramps, abdominal pain, bloating, flatulence, blood in the faeces, visible mucus in the faeces and other). The mean daily scores of defaecation frequency,

faecal consistency and symptoms were calculated from both the week before the intervention period as well as from the 20-day intervention period.
Before the start and the day after the intervention period, a sigmoidoscopy was performed in the morning after an overnight fast and without prior bowel cleansing. At each time point the gastroenterologist filled in two scoring systems, the Colitis Activity Index (CAI)[24] and the EGS.[22] During the sigmoidoscopy, mucosal biopsies were taken from a standardised location in the sigmoid (approximately 20-25 cm from the anal verge at the crossing with the arteria iliaca communis). Two biopsies were used for analyses of oxidative stress parameters, one biopsy for inflammatory parameters and one for gene expression analysis by real time PCR (RT-PCR). These biopsies were snap-frozen in liquid nitrogen. Two other mucosal biopsies were fixed in formalin and embedded separately in paraffin for histological evaluation of the overall inflammatory score.[25] After the endoscopic procedure, a fasting blood sample was obtained for determination of C-reactive protein (CRP) by the department of Clinical Chemistry of the Maastricht University Medical Centre.
In addition, each patient collected 24-hour faeces before the start of the intervention period and during the day before the last sigmoidoscopy. The faeces was weighed, homogenised and aliquots were stored at -80°C for determination of faecal calprotectin. The patients recorded their dietary intake three days before the first sigmoidoscopy and consumed the same diet during the three days before the second sigmoidoscopy in order to standardise the diet prior to the sigmoidoscopy.

Inflammatory and oxidative stress parameters in colonic mucosal biopsies

One frozen biopsy intended for cytokine determinations was grinded with a mortar and pastel cooled in liquid nitrogen and resuspended in 100 μL ice-cold PBS containing 10 μL/mL of a cocktail of protease inhibitors (Sigma, Zwijndrecht, the Netherlands). The homogenate was centrifuged for 5 minutes (21000 g, 4°C) and the supernatant was stored at -80°C until analyses of myeloperoxidase (MPO) and cytokines. The following cytokines were determined using a multiplex immunoassay kit[26] according to manufacturer's instructions (Bio-Rad, Veenendaal, the Netherlands): interleukin (IL)-1β, IL-5, IL-6, IL-8, IL-10, IL-12(p70), interferon (IFN)-γ, monocyte chemotactic protein-1 (MCP-1), and chemokine CCL5 (C-C motif ligand 5, also known as RANTES). In short, 50 μL of the undiluted biopsy supernatant or standard was incubated with the antibody-coated beads in a 96 well 1.2 μm filter plate. After 30 minutes of incubation, a cocktail of biotinylated secondary antibodies was added to each well and incubated for another 30 minutes. After subsequent incubation for 10 minutes with streptavidin-PE, the fluorescence intensity of the beads was measured using the Bio-Plex system in combination with the Bio-Plex Manager software version 3.0 (Bio-Rad). In the remaining supernatant myeloperoxidase was determined using a specific ELISA kit

(HBT, Uden, the Netherlands) according to manufacturers' instructions. Two other frozen biopsies intended for oxidative stress analyses were grinded and resuspended in 240 μL ice-cold milliQ water. From this suspension 90 μl was added to 9 μL of an acidic buffer (13% 5-Sulfosalicilic acid, 100 mmol/L HCl in PBS) and centrifuged for 5 minutes (21000 g, 4°C). The supernatant was used for the determination of total GSH (tGSH) and glutathione disulfide (GSSG), the oxidised form of GSH using the recycling assay described by Vandeputte et al.[27] with minor modifications as described previously.[9] The remaining original suspension was centrifuged for 5 minutes (21000 g, 4°C) and the supernatant was stored at -80°C for the determination of glutathione-S-transferase (GST) activity, malondialdehyde (MDA) concentrations, trolox equivalent antioxidant capacity (TEAC) and uric acid concentrations as described previously.[9] Total protein content in the supernatants was quantified using a BCA^{tm} protein assay kit (Pierce, Rockford IL, USA).

Real-Time PCR (RT-PCR)

The expression of glutamate-cysteine ligase, catalytic subunit (GCLC) and xanthine dehydrogenase (XDH) were determined using RT-PCR, which are the rate limiting enzymes for GSH and uric acid synthesis, respectively. RNA was isolated from a frozen biopsy using TRIzol reagent (Invitrogen, Carlsbad, USA) and purified with the RNeasy mini kit (Qiagen, Venlo, The Netherlands) combined with a DNase treatment using the RNase-Free DNase Set (Qiagen). 500 ng total RNA was used as a template for the cDNA reaction, which was synthesised using the iScript cDNA Synthesis kit (Bio-Rad). The cDNA was diluted to a concentration of 0.32 ng/μL. Each reaction contained 12.5 μL iQ Sybr Green Supermix (Bio-Rad), 1 μL of 10 μmol/L gene-specific forward and reverse primers, 4 μL cDNA template solution and 6.5 μL sterile H_2O. As housekeeping genes, 18SrRNA, GAPDH and CANX, were used. Primer sequences are given in Table 5.1. Reactions were run on the My IQ Single Colour RT-PCR Detection System (Bio-Rad). PCR conditions used were 3 minutes at 95°C, followed by 40 amplification cycles of 10 seconds at 95°C and 45 seconds at 60°C.

Table 5.1 Primer information of housekeeping genes and genes that are essential in GSH and uric acid metabolism.

	Sequence ID	Forward primer (5' → 3')	Reverse primer (5' → 3')
CANX	NM_001024649	CCACTGCTCCTCCTTCATCTCC	CGGTATCGTCTTTCTTGGCTTTGG
GAPDH	NM_002046	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
18SrRNA	M10098	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
GCLC	NM_001498	TGGAAGTGGATGTGGACACC	GTCTTGCTTGTAGTCAGGATGG
XDH	NM_000379	CCTCTTCCTGGCTGCTTCTATCTTC	TGACACACAGGGTGGTGAACTTG

CANX: calnexin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; 18SrRNA: 18S ribosomal RNA; GCLC: glutamate-cysteine ligase, catalytic subunit; XDH: xanthine dehydrogenase.

Faecal calprotectin

Approximately 100 mg of wet faeces was diluted 50 times in extraction buffer (0.1 mol/L Tris, 0.15 NaCl, 1 mol/L Urea, 10 mmol/L $CaCl_2$, 0.1 mol/L Citric acid, 5 g/L BSA, pH; 8.0).[28] Samples were shaken for 30 minutes and subsequently centrifuged for 20 minutes (10000 g, 4°C). The supernatant was used for analysis of calprotectin using a standard ELISA kit (HBT) according to the manufacturer's instructions.

Statistical analysis

The non-parametric Wilcoxon signed-rank two-tailed test was used to identify significant differences over time within the butyrate or placebo treated group. Mann-Whitney U two-tailed test for non-parametric data was used to identify significant differences between the changes in the butyrate compared to the placebo group. Spearman's rank correlation coefficient was determined to identify significant correlations between parameters. Differences were considered statistically significant if $p<0.05$. Statistical analyses were performed using SPSS 11.0 software.
RT-PCR data were analysed using a Gaussian linear regression as described previously.[9] Finally, data were presented as fold change and the confidence interval. The gene under consideration was denoted to be differentially expressed if the confidence interval did not include 1.

Results

Patient characteristics

Forty UC patients agreed to participate in the study. After initial screening, four UC patients appeared to have a relapse before the start of the study and were therefore excluded. Due to antibiotic use one other patient in the butyrate group was excluded during the study period. Thirty-five UC patients completed the study: 18 in the placebo group (50% male, median age: 59 (38-64) years) and 17 in the butyrate group (65% male, median age: 54 (31-65) years), respectively.
In the placebo group, 7 patients (39%) were on maintenance therapy for UC using mesalazine (n=4), azathioprine (n=1), azathioprine in combination with mesalazine (n=1) or sulfalazine (n=1). In the butyrate group, 13 patients (76%) were on maintenance therapy for UC using mesalazine (n=12) or sulfalazine (n=1). In all subjects, the dose and type of medication did not change during the study period.
The compliance based on returned empty enema bottles and questionnaires was 99% in both the placebo and the butyrate group.

Faeces consistency and frequency and the mean daily symptom score did not differ as a result of the enemas during the intervention period within each group, nor between the butyrate and the placebo group (Table 5.2).

Table 5.2 Daily symptom score, defaecation frequency and consistency scores according to the Bristol scale (from 1: hard lumps, to 7: watery diarrhoea) before and during the intervention period with a daily enema containing 100 mmol/L butyrate or placebo for 20 days (median (range))*.

	Butyrate group		Placebo group	
	Before n=17	During n=17	Before n=18	During n=18
Daily symptom score	0.6 (0-7.4)	0.4 (0-5.6)	0.4 (0-13.2)	0.4 (0-5.4)
Stool consistency score	4.7 (3.1-6.0)	4.5 (3.8-6.0)	4.1 (3.0-5.7)	4.2 (2.1-5.4)
Stool frequency	1.6 (1.0-3.6)	1.6 (0.9-3.2)	1.4 (0.9-6.9)	1.6 (0.7-3.9)

* None of the differences between and within the two groups is statistically significant.

Inflammatory parameters

Table 5.3 shows the effects of the butyrate and placebo intervention on the different parameters of inflammation determined in colonic mucosal biopsies (i.e. IL-1β, IL-5, IL-6, IL-10, IL-12, IL-10/IL-12 ratio, IFN-γ, IL-8, CCL5, MCP-1, MPO), in faeces (i.e. calprotectin), in plasma (i.e. CRP) as well as by using general scoring systems (i.e. CAI, EGS and histology score). A significant increase in the mucosal IL-10/ IL-12 ratio was found within the butyrate group (p=0.022), but not if comparing the changes between the butyrate and placebo group. Within the placebo group only a significant decrease in the concentrations of CCL5 was observed (p=0.012) and this was also significant if the changes between the butyrate and placebo group were compared (p=0.029). All other parameters of inflammation did not differ significantly within or between placebo and butyrate treatment (Table 5.3).

Table 5.3 Parameters of inflammation and clinical symptoms before and after the intervention period with a daily enema containing 100 mmol/L butyrate or placebo for 20 days (median (range)).

	Butyrate group		Placebo group	
	Before n=17	After n=17	Before n=18	After n=18
IL-1β (ng/g protein)	1.96 (0.42-242.33)	1.48 (0.59-39.37)	1.14 (0.45-79.43)	1.64 (0.42-18.42)
IL-5 (ng/g protein)	0.005 (0.002-0.054)	0.005 (0.002-1.118)	0.004 (0.002-0.059)	0.009 (0.002-0.060)
IL-6 (ng/g protein)	0.19 (0.09-0.93)	0.16 (0.08-4.89)	0.21 (0.07-7.20)	0.17 (0.01-3.43)
IL-10 (ng/g protein)	0.076 (0.004-0.437)	0.107 (0.020-0.993)	0.084 (0.011-4.913)	0.095 (0.011-0.863)
IL-12 (ng/g protein)	0.073 (0.002-0.293)	0.033 (0.002-0.261)	0.061 (0.002-0.383)	0.032 (0.003-0.379)
IL-10/IL-12 ratio	1.40 (0.03-18.00)	5.10 * (0.33-86.00)	3.46 (0.16-28.00)	5.04 (0.23-74.00)
IFN-γ (ng/g protein)	5.81 (0.55-48.01)	4.77 (0.72-16.72)	6.57 (3.82-22.42)	6.23 (3.59-16.36)
IL-8 (ng/g protein)	2.89 (0.66-40.65)	4.62 (1.33-138.79)	5.65 (0.63-119.72)	3.79 (1.46-32.27)
CCL5 (ng/g protein)	485.4 (247.2-1413.6)	550.7 ** (269.7-1455.6)	621.4 (392.1-8784.4)	579.1 * (322.9-1512.9)
MCP-1 (ng/g protein)	21.64 (8.79-51.02)	28.19 (10.68-95.61)	22.40 (13.39-262.87)	26.21 (16.26-56.70)
MPO (μg/g protein)	82.7 (24.3-1931.3)	90.3 (23.9-1792.1)	64.8 (20.1-2395.9)	55.0 (23.7-717.9)
Calprotectin (μg/g faeces)	54.1 (3.2-496.6)	88.8 (6.7-486.5)	62.0 (6.7-359.7)	39.5 (3.9-403.4)
CRP (mg/l)	2.8 (1.0-14.9)	3.4 (1.0-14.0)	1.7 (1.0-12.4)	1.0 (1.0-35.0)
Histology score	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-1)
CAI	2 (0-10)	2 (0-9)	2 (1-7)	2.5 (1-5)
EGS	0 (0-5)	1 (0-8)	0.5 (0-3)	0 (0-3)

* P<0.05 compared to pre-treatment values of the same group (Wilcoxon signed-rank test); ** P<0.05 for comparison of the change between the placebo and the butyrate group (Mann-Whitney U test); IL: interleukin; IFN-γ: interferon-γ; CCL5: chemokine (C-C motif) ligand 5; MCP-1: monocyte chemotactic protein-1; MPO: myeloperoxidase; CRP: C-ractive protein; CAI: colitis activity index; EGS: endoscopic grading system. Cytokines and MPO were determined in colonic mucosal biopsies and CRP was determined in plasma.

Oxidative stress parameters

Table 5.4 shows the effects of the butyrate and placebo intervention on the different parameters of oxidative stress measured in colonic mucosal biopsies (i.e. total GSH, GSH, GSSG, GSH/GSSG ratio, UA, TEAC, GST, MDA). These

parameters did not differ significantly within or between placebo and butyrate treatment.

Table 5.4 Parameters of oxidative stress determined in colonic mucosal biopsies before and after the intervention period with a daily enema containing 100 mmol/L butyrate or placebo for 20 days (median (range))*.

	Butyrate group		Placebo group	
	Before n=17	After n=17	Before n=18	After n=18
tGSH (nmol/mg protein)	20.8 (16.1-25.1)	20.7 (17.1-27.0)	22.6 (17.9-27.6)	21.8 (15.2-27.8)
GSH (nmol/mg protein)	16.0 (11.9-24.7)	17.7 (14.5-23.6)	16.9 (10.8-24.7)	18.0 (10.1-25.3)
GSSG (nmol/mg protein)	1.0 (0.1-4.5)	1.6 (0.1-4.3)	2.2 (0.2-4.6)	2.0 (0.1-5.3)
GSH/GSSG ratio	17.9 (2.7-247.5)	13.3 (3.5-213.7)	7.5 (2.5-102.7)	9.0 (2.7-153.5)
UA (nmol/mg protein)	2.9 (1.8-4.1)	2.6 (1.5-3.7)	2.5 (1.7-4.1)	2.8 (1.7-3.6)
GST (U/mg protein)	0.12 (0.08-0.17)	0.14 (0.09-0.16)	0.13 (0.08-0.19)	0.14 (0.09-0.18)
TEAC nmol trolox eq/mg protein	118.2 (82.1-171.6)	111.1 (82.8-191.5)	121.9 (94.6-163.6)	110.6 (87.2-163.7)
MDA (nmol/mg protein)	0.62 (0.26-2.16)	0.92 (0.08-2.7)	0.91 (0.34-1.75)	0.93 (0.18-2.43)

* None of the differences between and within the two groups is statistically significant. tGSH: total glutathione; GSH: glutathione; GSSG: glutathione disulfide; UA: uric acid; GST: glutathione-S-transferase; TEAC: trolox equivalent antioxidant capacity; MDA: malondialdehyde.

However, a significant negative correlation ($p = 0.018$) was found between the level of inflammation measured by calprotectin at the start of the study and the change in tGSH due to the butyrate intervention (Figure 5.1).

Expression of GCLC and XDH

mRNA expression of the genes coding for the rate limiting enzymes for GSH (GCLC) and uric acid (XDH) synthesis in the mucosal biopsies were determined as a previous study in healthy volunteers showed that these expression levels were regulated by butyrate.[9] Compared to placebo, the butyrate intervention did not significantly affect the expression of GCLC (fold change (confidence interval): 0.94 (0.89-1.01)), while the expression of XDH was significantly downregulated after the butyrate intervention compared to placebo (fold change: 0.89 (0.81-0.97)).

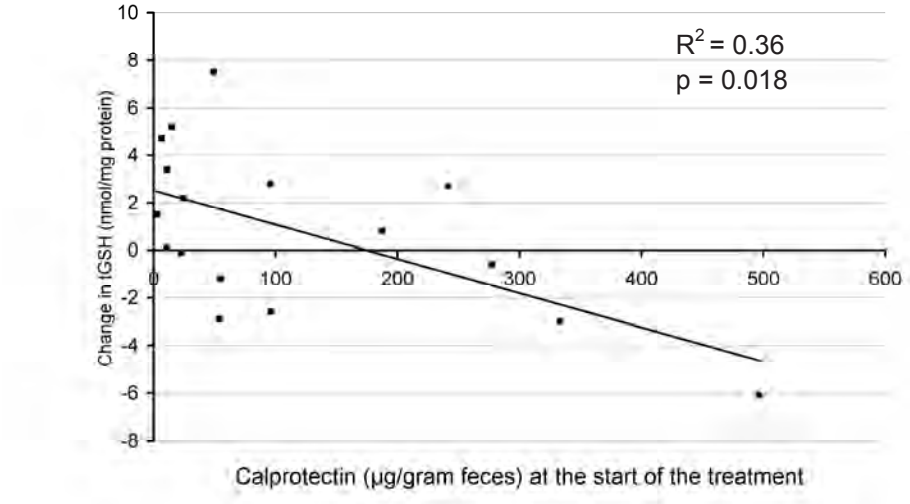

Figure 5.1 Correlation between the change in the concentrations of total GSH (tGSH) in the colonic biopsies (concentration tGSH in the biopsy taken after the treatment with butyrate or placebo minus the concentration in the biopsy taken at the start of the study) and the concentrations of calprotectin determined in faeces obtained at the start of the study.

Discussion

In the present study UC patients in remission were included to evaluate the effects of butyrate supplementation in a situation of chronically mildly elevated levels of inflammation and oxidative stress.[16-18] The results of the present study showed minor effects of butyrate on colonic inflammation. Only a significant increase of the colonic IL-10/IL-12 ratio was found within butyrate treated patients, and colonic concentrations of CCL5 were increased after butyrate compared to placebo treatment. In addition, in contrast to previous results in healthy volunteers no significant effects of butyrate administration on parameters of oxidative stress were found. Although in general butyrate did not affect colonic GSH levels, the effects of butyrate enemas on total colonic GSH correlated negatively with the level of inflammation at the start of the study.

A comparison of pre-treatment values of the included UC patients with values from healthy volunteers[9], confirmed the presence of a low-grade inflammation and oxidative stress in these patients as indicated by significantly increased levels of IL-1β, IL-12, IFN-γ, calprotectin and GSSG, and significantly decreased levels of GSH, the GSH/GSSG ratio, GST activity and TEAC.[9] Rectal enemas were chosen as route of butyrate administration in order to standardise the location and concentration of butyrate in the distal colon. An additional advantage of this specific region is that mucosal biopsies could be obtained from this distal part of colon without the need for prior bowel cleansing, as this could affect the physiological situation. The actual concentrations of butyrate would be unknown when using a dietary fibre intervention to increase local concentrations of butyrate and this would also have led to other changes that could have affected colonic health, such as the composition of the colonic microbiota. The concentration of butyrate given was based on expected local concentrations after consumption of a high fibre diet.[9] To minimise confounding effects of diet and medication, patients were asked to adhere to their habitual diet including the use of pro- and prebiotics and all patients were on stable medication throughout the entire study period. However, the use of medication was not equally distributed between the butyrate and the placebo group: 76% of the butyrate-treated and 39% of the placebo-treated patients used maintenance therapy for UC. The most frequently used drug was 5-aminosalicylate, which uses the same transporter for absorption from the intestinal lumen as butyrate. Although we acknowledge that this might have affected the bioactivity of butyrate, it was ethically not acceptable to stop or interfere with this maintenance therapy. However, a previous study has shown that butyrate enemas in combination with 5-aminosalicylate was significantly more effective than 5-aminosalicylate alone in the management of refractory distal colitis.[29]

The results of this double blind, placebo controlled trial demonstrated minor effects on inflammation: only a significant increase of the colonic IL-10/IL-12 ratio was found within butyrate treated patients, and colonic concentrations of the chemokine CCL5 were increased after butyrate compared to placebo intervention. Previously, using human monocytes, Seaman *et al.*[30] have also demonstrated that butyrate inhibits the pro-inflammatory cytokine IL-12, while it increased the production of the anti-inflammatory cytokine IL-10. With regard to chemokine production, controversial effects of butyrate have been reported using different conditions and intestinal epithelial cell lines.[31-34] It has been reported that butyrate was able to either increase or decrease IL-8 production depending on the duration of the incubation[32] and the concentration of butyrate applied.[31] In the present study, enemas containing 100 mmol/L butyrate once daily for three weeks resulted in increased concentrations of CCL5, while other chemokines (MCT-1 and IL-8) were unaffected. *In vitro,* CCL5 is a potent chemotactic agent for eosinophils, basophils,

monocytes, NK cells, and CD4 memory T lymphocytes.[35] By affecting chemokine concentrations different immune cell types are activated for host defence against luminal antigens[36], although the relevance of this increase in only CCL5 remains to be determined. Other determined cytokines were not significantly affected by the butyrate intervention, while previous *in vitro* studies have reported reduced concentrations of pro-inflammatory cytokines after incubation with butyrate[7](submitted for publication). However, most effects were observed after the addition of a pro-inflammatory stimulus and can therefore not be compared to the present study.

To our knowledge this is the first study reporting the effect of butyrate in a situation with low-grade inflammation and oxidative stress. Apart from the findings mentioned above, no changes in other cytokines and overall inflammatory parameters were observed. This is in contrast to what was expected as various studies in UC patients with active disease revealed a significant improvement of clinical and inflammatory parameters due to butyrate administration.[14,15,29,37,38] Some other clinical trials, however, did not find beneficial effects[39] or observed only trends towards clinical improvement.[40,42] The studies that reported beneficial effects on clinical scores used enemas containing SCFA mixtures[15,38] or similar concentrations of butyrate[14,29,37] as used in the present study, but those enemas were administered twice daily compared to the once daily administration in the present study. A single rectal nightly enema was given, as this is less cumbersome to the patient and thereby is expected to increase their compliance. However, more prolonged exposure and/or higher dosages might be required for stronger anti-inflammatory effects.

To protect against oxidative stress, the intestinal epithelium contains multiple enzymatic and non-enzymatic antioxidants including GSH, which is considered an essential antioxidant in the colon.[43] When GSH is used to reduce hydrogen peroxide and lipidhydroperoxides, GSSG is formed that can be reconverted to GSH[44]. GSH deficiency has been proposed to be a therapeutic target in UC.[45,46] As expected GSH was found to be decreased and GSSG was found to be increased compared to previously reported data in healthy volunteers.[9] However, the butyrate intervention did not change these concentrations nor the expression of GCLC, one of the rate-limiting enzymes for GSH synthesis, in UC patients in remission. It was expected that butyrate would increase the concentrations of GSH since a previous study by our group demonstrated that an identical once daily rectal administration of 100 mmol/L butyrate for 14 days significantly increased colonic GSH concentrations and GCLC expression in healthy subjects.[9] In addition, a fibre-supplemented diet two weeks prior to and during the induction of colitis in rats led to an increased production of butyrate and a restoration of the colonic GSH levels.[11] In agreement with the previous study in healthy volunteers, the butyrate intervention resulted in a significant decrease in XDH, the enzyme responsible for

the formation of uric acid. During uric acid production reactive oxygen species are produced, and therefore a decrease in its activity could decrease oxidative stress.[9]
In the present study, significant negative correlations were found between the level of inflammation at the start of the study and the change in tGSH, indicating that the effects of butyrate on GSH may depend on the level of inflammation. Therefore, we hypothesise that butyrate at the concentration used in the present study may only be able to increase GSH if inflammation is minimal or absent. In active UC patients butyrate oxidation is decreased[47,48] and furthermore, butyrate oxidation could be inhibited by hydrogen sulphide, which is increased in UC patients compared to healthy controls.[49] In addition, in UC patients, colonic inflammation has also been shown to cause downregulation of the H^+-coupled butyrate transporter.[50] Therefore, as the uptake and/or oxidation of butyrate may differ in UC patients with varying degrees of inflammation, the dose of butyrate needed to achieve a beneficial effect could be different depending on the level of inflammation. It seems that in UC patients either the butyrate concentrations should be increased or the exposure time extended.
The exposure time to butyrate can be increased if colonic levels of butyrate are increased by dietary means, for example through the addition of fermentable dietary fibres to the diet. It remains to be determined whether the concentration needed for beneficial effects of butyrate during low-grade inflammation and oxidative stress can be achieved, especially in the distal colon, via these dietary interventions. However, beneficial effects on inflammation and oxidative stress may not be entirely due to the increased butyrate production, but may rather by the result of a broad range of effects of fermentable fibre supplementation, such as the production of other SCFA, a decreased intraluminal pH and changes in microbial composition.

In conclusion, this intervention study with nightly butyrate enemas for three weeks in patients with UC, characterised by low-grade inflammation and oxidative stress, showed minor effects on inflammation and no significant effect on oxidative stress parameters. On the other hand, the effect of butyrate on tGSH appeared to be inversely related to the level of inflammation. Future studies should focus on the level of butyrate needed to result in beneficial effects during low-grade as well as active inflammation and whether these could be achieved via fermentable fibres as a dietary component.

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Chapter 6

Analyses of human colonic mucus obtained by an *in vivo* sampling technique

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Abstract

Background

The mucus layer is an important dynamic component of the epithelial barrier. It contains mucin glycoproteins and other compounds secreted by the intestinal epithelium, such as secretory IgA. However, a standardised in vivo sampling technique of mucus in humans is not yet available. Therefore, the aim was to assess the validity and feasibility of mucin and protein determinations in human colonic mucus collected under physiological conditions.

Methods

Triplicate colonic mucus samples were collected in 11 healthy volunteers using cytology brushes during sigmoidoscopy. As an indication of the quantity of collected mucus, total protein and mucin concentrations were determined by measuring oligosaccharide equivalents and monosaccharides. Also secretory IgA and sialic acid concentrations were determined and proteomic analysis was performed using SELDI-TOF-MS.

Results

Mean values of secretory IgA and sialic acid corrected for the amount of mucus ranged from 0.16 to 1.81 gram secretory IgA /mmol oligosaccharide equivalents and from 12.6 to 48.6 gram sialic acid /mmol oligosaccharide equivalents. Proteomic analysis of mucus is feasible and cluster analysis showed subject specific profiles.

Conclusion

Using cytology brushes, human colonic mucus can be sampled and under physiological conditions. These samples could give information on the composition and quality of the mucus layer.

Introduction

The intestinal mucus layer is the first line of defence to the external environment and protects the mucosa against mechanical, chemical or microbiological aggressions.[1] It is composed of a loosely adherent layer and a layer firmly attached to the mucosa. The mucus layer mainly contains mucus glycoproteins (mucins), which are secreted by goblet cells and consist of a central protein backbone with high numbers of oligosaccharides attached through O-linkage with N-acetyl-galactosamine to serine or threonine in the protein core. The oligosaccharide side chains of colonic mucins mainly comprise galactosamine, glucosamine, fucose, galactose and can be either acidic containing sialic acid or sulphate groups, or neutral.[2]

In addition to mucins, a large number of other secreted proteins can be found in the mucus layer, such as trefoil factor family proteins and secretory IgA (sIgA), which are able to interact with mucins or benefit from the viscoelastic, aqueous environment provided by the mucus gel.[1] The main functions of sIgA are to neutralise toxins and prevent epithelial adherence and penetration of invasive pathogenic micro-organisms by direct non-specific binding.[3] Trefoil factors are mucin-associated proteins that are involved in the protection of gastrointestinal epithelium and mucosal healing, and contribute to the viscoelastic properties of the mucus layer.[4,5]

Alterations in the intestinal mucus layer, which may lead to diminished protection of the colonic mucosa, have been reported in various intestinal disorders.[2,6,7] For example in ulcerative colitis, a reduced mucus thickness, depleted goblet cells, altered glycosylation, reduced sulphation and a reduced presence of sIgA have been reported.[7,8] Other factors such as diet[9], stress[10,11] and age[12,13] can also affect the colonic mucus layer. Studies with rats have shown that dietary fibre deficiency leads to a decreased mucus thickness[9], while acute exposure to stress decreased colonic IgA levels.[10] Although studies on the effect of age on the colonic mucus layer are hardly available, changes have been demonstrated to occur in the gastric mucus layer with advancing age[12,13]. Farinati *et al.*[12] showed that the number of human gastric mucus-secreting cells decreases with increasing age and Corfield *et al.*[13] showed a decrease with advancing age in total sialic acid concentration in human gastric juice, suggesting that there is also a qualitative change in gastric mucus with increasing age.

Because of its importance, techniques to sample intestinal mucus are of interest. Sampling under physiological conditions is important when subtle changes are expected, for example in case of dietary interventions. Currently, the human mucus layer can be studied using mucosal biopsy specimens or resection material. However, analyses in the latter can be affected by preoperative antibiotic treatment and operation associated stress.[6] In addition, standard histological techniques dehydrate the mucus layer, hampering its visualisation.[14] Analyses of components

of the mucus layer can also be performed in faeces, but this does not provide site-specific information.[15] Previously, rectal colonic mucus has been studied by using the mucosal patch technique[16] or a filter paper inserted through a rigid sigmoidoscope.[17,18] Although gastric mucus has been sampled *in vivo* before[19], there are no reports on the collection of mucus samples from the large intestine *in vivo*.

In this study, we used a cytology brush to collect mucus under physiological conditions in order to evaluate the composition of the mucus layer. In the collected colonic mucus samples both mucin components and proteins were determined using biochemical assays as well as surface enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS).

Methods

Subjects

Twelve healthy non-smoking volunteers participated in the study (33% male, median age 23 (18-62) years). Subjects were on a stable Western diet, had no history of gastrointestinal diseases and did not use immunosuppressive drugs, antibiotics, anti-diarrhoeal drugs or laxatives in the three months prior to the study. All subjects gave written informed consent. The medical ethics committee of the University Hospital Maastricht approved the study.

Collection of colonic mucus

Triplicate colonic mucus samples were obtained during sigmoidoscopy over the last 30 cm of the colon without prior bowel cleansing using cytology brushes (Boston Scientific, Natick, USA; Figure 6.1).

Sterile cytology brushes (protected by a sleeve and a cap) were inserted through the working channel of the flexible endoscope to collect mucus from a standardised location of the sigmoid (approximately 20 cm from the anal verge at the location of the arteria iliaca communis). On the site of sampling, the protecting cap was removed and the brush was placed on the colonic wall for 30 seconds while gently moving over the epithelium. Subsequently, it was retracted in its sleeve and removed from the endoscope. The brush was cut off, placed immediately in cold 600 μL buffer (0.1 M TrisHCl, in 154 mM NaCl, 0.01% azide, 0.02% Tween-20, pH 7.4) and rocked (5 min, 150 rpm, 4°C). From each sample, 50 μL was immediately frozen at -80°C for SELDI-TOF-MS. The remaining part was sonicated for 45 min on ice to retrieve all the mucus from the brushes. Mucus samples were then centrifuged (5 min, 350 g, 4°C) to remove bacteria, cells and large faecal particles. The supernatant was frozen at -80°C until further analyses.

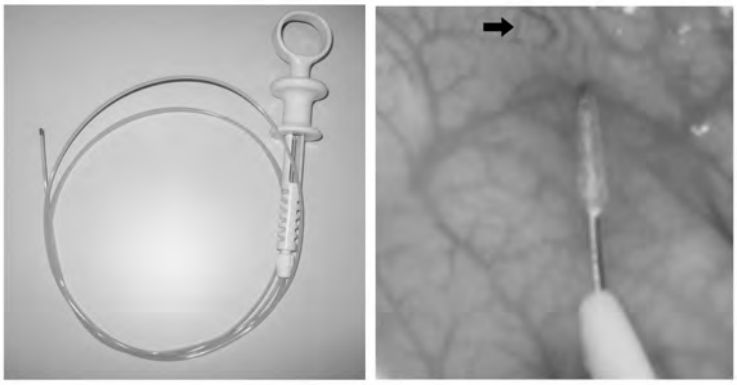

Figure 6.1 Cytology brush for the collection of intestinal mucus during a sigmoidoscopy. The grey protection cap, preventing contamination during insertion of the brush, is pushed off and is indicated by an arrow in the right picture.

Analyses of colonic mucus samples

Oligosaccaride equivalents

Mucins were extracted and determined as described previously.[20,21] First, glycosidases were denatured and mucins were solubilised[20]. Samples were filtered (ultrafree MC 30000 NMWL, Millipore, Bedford, USA) during centrifugation (3000 g, until the complete sample passed the membrane). Subsequently, 200 μL methanol was added and centrifuged (3000 g) to wash the membrane. After drying by air, the mucin retentate was dissolved in PBS to the original volume and the amount of oligosaccharide side chains liberated from mucins were quantified using a fluorimetric assay.[22] The total amount of mucins was expressed as μmol oligosaccharide equivalents.[21]

Monosaccharide analysis

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) was performed using a Dionex ICS 3000 HPLC system equipped with a Dionex CarboPac PA-1 (4 mm ID x 250 mm) column and an amino trap column (4 mm ID x 50 mm) (Dionex, Amsterdam, the Netherlands). Monosaccharides were separated using an isocratic elution with 20 mM NaOH for 25 min at 30°C at a flow-rate of 0.5 mL/min and detected with a pulsed amperometric detector.[23] Filtered mucus samples were hydrolysed in 2 M trifluoroacetic acid for 1 hour at 121°C.[24] Glucose, fucose, galactose,

galactosamine and glucosamine were used as a monosaccharide standard and fetuin as a glycoprotein standard.[24]

Total protein concentration, sialic acids and sIgA

Total protein concentrations in the unfiltered mucus samples were quantified using the BCA^{tm} protein assay kit (Pierce, Rockford IL, USA).
Bound sialic acids in sialoglycoproteins were determined by the acidic ninhydrin reaction.[25] N-acetylneuraminic acid (Sigma, Zwijndrecht, the Netherlands) was used for a standard curve. Briefly, 50μL glacial acetic acid and 50 μl acidic ninhydrin reagens (0.5 g ninhydrin (VWR, Amsterdam, The Netherlands) in 12 mL glacial acetic acid and 8 mL 37% HCL) was added to 50 μL of the standards and unfiltered mucus samples, heated for 10 min at 100°C and after cooling, the absorbance of the samples was measured at 470 nm.
SIgA concentrations in the unfiltered mucus samples were determined using an enzyme-linked immunosorbent assay (ELISA) as described previously.[15] Briefly, plates were coated with monoclonal mouse α human anti-secretory component (IgA) antibody (1:10000; Sigma). Purified human IgA isolated from colostrum (Sigma), was used for the standard curve. SIgA was detected using a monoclonal biotinylated mouse anti-human IgA1/2 monoclonal antibody (1:500; BD Pharmingen, Breda, the Netherlands), followed by the addition of streptavidin-conjugated horseradish peroxidase (1:10000; Sanquin CLB, Amsterdam, the Netherlands) and enzymatic colour development with tetramethylbenzidine (Sigma).

Proteomics

To study a wide range of (unknown) proteins present in the mucus layer, SELDI-TOF-MS was applied. The SELDI-TOF-MS technology (Ciphergen Biosystems, Fremont, USA) consists of the protein chip array, mass spectrometer and data analysis software. Optimal conditions for mucus profiling were determined by testing three protein chip array surfaces (Q10, CM10 and IMAC-Cu) with different binding/washing conditions and dilutions of mucus. The binding/washing buffer for the anionic exchange arrays (Q10) contained 0.1% Triton X-100 with 0.1M Tris–HCl (pH 8) or 0.1M sodium acetate (pH 6) and that for the cationic exchange arrays (CM10) contained 0.1% Triton-X100 with 0.1 mM ammonium acetate (pH 4.5 or pH 6). Both Q10 and CM10 chips were pre-incubated with binding buffer. The metal affinity-binding IMAC-Cu chips were loaded with 50 μL 0.1 M copper sulphate by vigorous shaking for 10 min. After washing with water, the chip surface was neutralised using 150μL sodium acetate buffer pH 4 followed by a short wash with water and pre-incubation with binding buffer (0.1 M Tris-HCl, pH 7.4) containing 0.1% Triton X-100 and 100 mM or 500 mM NaCl. Mucus samples were diluted 1:10 or 1:100 in binding buffer (total volume of 100 μL) and applied at random and in

duplicate to the chip and incubated for 1 h shaking on a mixer. Afterwards, spots were washed six times with 150 μL binding buffer for 10 min (three times with and three times without 0.1% Triton X-100). Before application of the matrix (Sinapinic acid (SPA; Ciphergen Biosystems) in 50% acetonitrile/0.1% trifluoroacetic acid), another short wash with HPLC-gradewater was performed and the chips were airdried for 10 min. The matrix was applied twice (0.8 μL each time, 1 min apart) and the chips were airdried prior to reading by a ProteinChip Reader IIC instrument (Ciphergen Biosystems), using the following settings: detector sensitivity 9; detector voltage 2900; positions 20-80 were read with an increment of 10; 50 laser shots were collected on each position; two warming shots were fired at each position; lag time focus of 241 ns; laser intensity 180. Calibration was done with a 7–30 kDa proteins mixture. After baseline subtraction, peak labelling was performed with CiphergenExpress Software (version 3.0) for peaks with a signal-to-noise (S/N) ratio of ≥3 in the m/z range from 1.5–30 kDa, and then normalised by total ion current.

Statistics

Results are expressed as median and range. A two-sided p-value ≤0.05 was considered to be statistically significant. Pearson correlations coefficients were calculated between mucin and protein determinations. The mean variation coefficient and the interclass correlation coefficient (ICC) including the 95% confidence interval were calculated to evaluate the reliability of triplicate samples. The ICC was interpreted as follows: ICC ≤0.4 = poor reproducibility; 0.4-0.75 = fair to good reproducibility; ≥0.75 excellent reproducibility of the variance within persons (i.e. triplicate samples) compared to the variance between persons.[26] Statistical analyses were performed using SPSS 11.0 software. For analyses of proteomic profiles including all peaks, hierarchical clustering was used based on similarity as described by Eisen *et al.*[27]

Results

Eleven healthy volunteers completed the study. One subject was excluded because no brushes could be taken due to severe faecal contamination in the colonic lumen.

Mucins and total protein

To evaluate the quantity of mucus in the collected samples, the concentrations of mucins and total protein were determined in the samples. As indicators for mucin concentrations, the amounts of oligosaccharide equivalents and the individual and

sum of the monosaccharides (fucose, galactosamine, glucosamine and galactose) were determined. In Table 6.1, the Pearson correlation coefficients between total protein and indicators of mucin concentrations are shown. The amount of oligosaccharide equivalents correlated significantly with the sum of monosaccharides and with all individual monosaccharides. Total protein concentrations did not correlate with the sum of monosaccharides, but correlated significantly with the amount of oligosaccharide equivalents as well as with the concentrations of galactose.

Table 6.1 Pearson correlation coefficients between individual and total monosacchacharides, total oligosaccharide equivalents and total protein concentrations measured in mucus samples from all subjects.

	Sum of Monosaccharides	Oligosaccharide equivalents	Total protein
Fucose	0.987**	0.959**	0.299
Galactosamine	0.976**	0.951**	0.326
Glucosamine	0.995**	0.981**	0.291
Galactose	0.996**	0.978**	0.359*
Sum of monosaccharides	1	0.983**	0.326
Oligosaccharide equivalents	0.983**	1	0.348*

* $p<0.05$; ** $p<0.01$

Sialic acid and sIgA

To correct for the amount of mucus collected, concentrations of sialic acid and sIgA were expressed per mmol oligosaccharide equivalents. Mean triplicate values of sialic acids ranged from 12.6 to 48.6 g/mmol oligosaccharide equivalents (Figure 6.2A). The median variation coefficient of triplicate samples per subject was 33.3% (6.5-75.1) and the ICC was 0.19 (-0.14-0.61). Mean triplicate values of sIgA concentrations ranged from 0.16 to 1.81 g/mmol oligosaccharide equivalents (Figure 6.2B). The median variation coefficient of triplicate samples per subject was 37.6% (14.0-80.6) and the ICC was 0.59 (0.24-0.85).

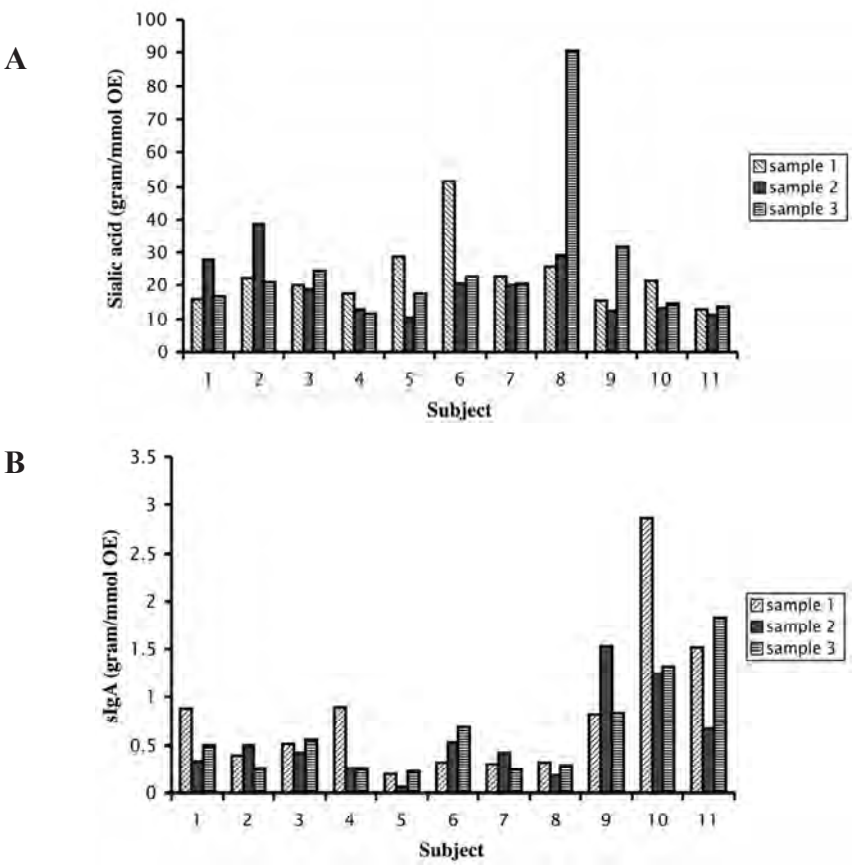

Figure 6.2 Concentrations of sialic acid (A) and sIgA (B) expressed as g/mmol oligosaccharide equivalents (OE) in triplicate colonic mucus samples collected from 11 healthy volunteers using cytology brushes.

SELDI-TOF-MS analysis

Using all three protein chip array surfaces (Q10, IMAC-Cu, CM10) only small differences were found comparing 1:10 with 1:100 dilutions of mucus samples. The 1:100 dilution was chosen to reduce the risk of ion suppression due to the presence of abundant proteins in the mucus samples. On CM10 chips, best results were achieved with binding buffer of pH 4.5 showing 61 different peaks (30 in the range 1–10 kDa and 31 from 10-100 kDa). Q10 chips displayed a total of 28 peaks with binding buffer pH 8, of which most peaks were observed in the range from 10–100 kDa. Lowering the pH from 8 to 6 decreased the intensity of peaks in the whole spectrum. Using IMAC-Cu chips, an almost equal number of peaks was detected as with the CM10 chips. An increase in salt concentration to 500 mM had very little effect on the spectra. The CM10 chip was chosen for further analysis of all samples, because of the high number of peaks with a good intensity. Duplicate

proteomic analyses of one mucus sample resulted in highly reproducible profiles (data not shown).
Representative protein profiles in the range of 1–25 kDa from triplicates of two donors on a CM10 chip are shown in Figure 6.3.

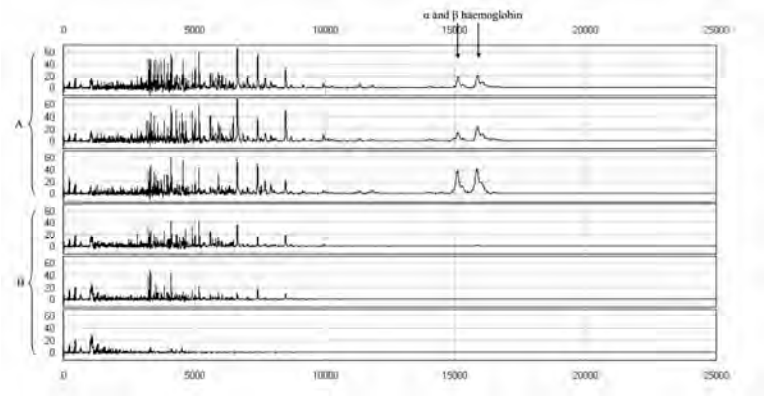

Figure 6.3 Representative triplicate proteomic profiles from colonic mucus samples collected from two volunteers (A and B), measured using SELDI-TOF-MS. Arrows indicate α- and β-haemoglobin.

Using a hierarchical cluster analysis, it was shown that all three replicate mucus samples clustered in 5 out of 11 subjects. Two out of 3 replicate samples clustered in another 5 subjects (Figure 6.4).

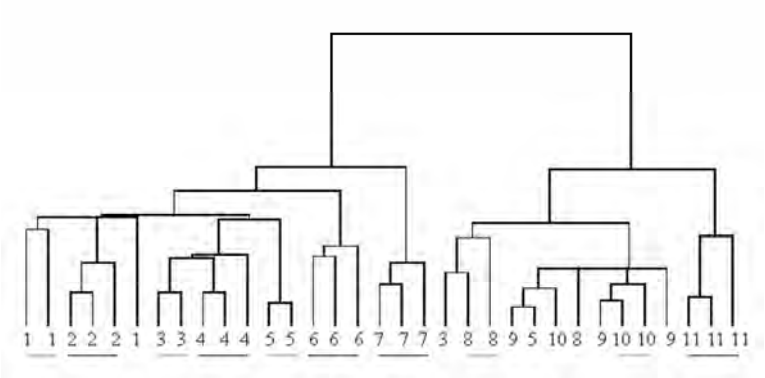

Figure 6.4 Cluster analysis of the proteomic analysis by SELDI-TOF MS. Numbers refer to the 11 volunteers. Mucus samples that cluster together are underlined with grey and black lines, indicating clustering of 2 out of 3 or clustering of all 3 samples, respectively.

Discussion

In this study, we showed that colonic mucus could be sampled *in vivo* in humans using a cytology brush during an endoscopic procedure and mucins, sIgA and sialic acids could be determined. In addition, proteomic analysis of colonic mucus samples is feasible and subject specific profiles can be obtained.

To our knowledge this was the first time that cytology brushes were used successfully to collect colonic mucus samples under physiologic conditions *in vivo*. However, although handling of the brushes was standardised, the amount of mucus collected cannot be adequately controlled. Therefore, this technique is not suitable for quantification of the mucus layer. For quantification of the intestinal MUC2 synthesis, infusion of ^{13}C labelled threonine can be considered.[28] The reported technique is, however, suitable for the evaluation of the composition of the mucus layer, including mucins and other proteins secreted by the epithelium. To compare differences in components between distinct mucus samples, correction for the amount of mucus sampled is warranted. As specific markers for the mucus layer are lacking, total protein, oligosaccharide equivalents and monosaccharides were determined as indicators for the quantity of mucus in the collected sample. As to be expected, concentrations of oligosaccharide equivalents showed a good correlation with the sum of monosaccharides, but the correlation between total protein concentrations and both indicators of mucin concentrations was rather poor. The total protein concentration was determined in the unfiltered sample using the BCA assay. This gives a global indication of the total amount of the collected sample. However, heavily glycosylated mucins that form the dominant macromolecular component of mucus are known to have poor reactivity with the BCA assay, which could explain the poor correlation. Therefore, in the present study the indicators of mucin concentrations were used for the standardization of the quantity of the samples. An alternative way to standardise the mucus samples could be by analyzing the amount of MUC2 using Western blotting, which is the predominant mucin in the human colon.

We demonstrated that sIgA and sialic acids could be measured in the collected mucus samples. Although their concentrations were corrected for the total mucin concentrations, there still appeared to be a large variation between triplicate samples. Similar variations were observed when correction for total protein concentration or the sum of monosaccharides was applied (data not shown). The ICC gave a fair to good reproducibility for sIgA. This indicates that the variability between triplicates was relatively small compared to the variation between subjects. The ICC was low for sialic acid, indicating a poor reproducibility of the triplicates compared to the intersubject variance. This could probably be explained by the lack of difference in concentrations of sialic acid between subjects. Larger differences in sialic acid concentrations are expected when samples are taken from different locations of the large intestine or from different patient groups.

To minimise the variation between triplicates, the location and procedure of sampling were standardised. Because we aimed to sample mucus under physiological conditions, mucus was collected without bowel cleansing, which could provoke stress to the colonic mucosa and may alter the composition of the mucus layer. However, as a consequence, faecal contamination cannot be excluded. Possibly better results regarding the reproducibility could be obtained by using prior bowel cleansing or mildly rinsing the sampling location during the sigmoidoscopy. On the other hand, due to the viscous properties of the mucus layer it remains possible that proteins secreted in the mucus layer are not evenly distributed.

Besides measuring sIgA and sialic acid concentrations, other proteins present in the mucus layer can be determined, such as cytokines, defensins or trefoil factors. This can be analysed by biochemical techniques that focus on individual molecules or by global protein analysis using SELDI-TOF-MS. This provides a complementary visualization technique to 1D and 2D gel electrophoresis that is more sensitive for the characterization of low molecular weight proteins and requires smaller amounts of sample. The value of the SELDI-TOF-MS lies in the ability to obtain and compare sprectra from a significant number of samples in a relatively short time with very little sample preparation. By comparing protein profiles from a larger set of samples from different patient groups during different physiological and pathological conditions, (unknown) proteins can be identified that characterise these groups.[29,30] Good protein profiles were obtained from mucus samples collected in the present study. However, these profiles only provide information on the low molecular weight proteins up to 25 kDa using the cationic CM10 chip and is not suitable for the determination of larger proteins such as sIgA (385 kDa)[31] and mucins (550 kDa (MUC2)).[32] Although cluster analysis clearly pointed towards subject specific profiles, not all triplicates clustered together. Deviating samples might be caused by contamination with faecal matter or blood, the latter was visualised with clear peaks of α- and β-haemoglobin (Figure 6.3).

In this methodological study we focussed on the feasibility of sampling and analysis of the colonic mucus obtained under physiological conditions. In this first study only triplicate samples of one anatomic location in healthy subjects were analysed. Future research could focus on changes in composition of the mucus layer in subjects with advancing age, between different patient groups, between different stages of the disease or after dietary interventions with for example dietary fibres. In addition, attention should be paid to the reproducibility of the colonic mucus sampling of one person over time and the variation over different locations in the intestine under pathological and non-pathological conditions.

In conclusion, mucus samples collected with cytology brushes during endoscopy can provide useful information on the composition of this layer *in vivo*. However and importantly, some deviant triplicates indicate that analyzing only one mucus

sample may result in an observation bias and, therefore, at least three triplicate samples should be analysed independently.

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Chapter 7

Butyrate enemas do not affect human colonic mucosal MUC2 and TFF3 expression

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Submitted

Abstract

Introduction

The colonic mucus layer plays an important role in the protection of the intestinal epithelium and mainly consists of mucin glycoproteins (primarily MUC2 in the colon) but also contains trefoil factor 3 (TFF3) and secretory IgA. Butyrate is a major end-product of fermentation of dietary fibres and is associated with beneficial effects on colonic health. Previous *in vitro* and animal studies showed that butyrate modulates MUC2 and TFF3 expression and mucin secretion, although data from human studies are not yet available.

Methods

16 healthy volunteers and 35 UC patients in clinical remission administered daily a 60 mL rectal enema containing 100 mmol/L butyrate or placebo for two and three weeks, respectively. After each treatment, biopsies were taken from the distal sigmoid for quantitative RT-PCR and immunohistochemical analysis of MUC2 and TFF3. In addition, mucosal sections were stained with high iron diamine-alcian blue to distinguish between sialomucins and sulphomucins. To analyse total mucin secretion and secretory IgA concentrations, faeces was collected over a period of 24h during the day before endoscopic examination,

Results

The butyrate intervention did not significantly modulate the expression of MUC2 (fold change: 1.04 and 1.05 in healthy volunteers and UC patients, respectively) or TFF3 (fold change: 0.91 and 0.94 in healthy volunteers and UC patients, respectively). Furthermore, the percentage of sialomucins, mucus secretion and secretory IgA concentrations were not affected by the butyrate intervention in both groups.

Conclusion

Butyrate exposure in healthy volunteers and UC patients in clinical remission did not affect quality parameters of the colonic mucus layer.

Introduction

The intestinal mucus layer forms a dynamic defensive barrier between the luminal contents and the epithelial lining, which prevents invasion of intestinal bacteria, protects the epithelial lining against toxic substances and is a medium for lubrication and transport. Therefore, the mucus layer forms an important part of the colonic barrier[1], which can be affected by environmental factors such as diet[2] and has been shown to be impaired in various intestinal disorders, either as a cause or a consequence of the disease.[3] The mucus layer mainly contains mucus glycoproteins (mucins), which are secreted by goblet cells and consist of a central protein backbone with large numbers of attached oligosaccharides containing sialic acid or sulphate residues. The mucins are classified into neutral and acidic subtypes and the latter category includes sialomucins and sulphomucins. Mucin subtypes vary spatially throughout the gastrointestinal tract and the acidic subtypes are found to dominate in the large intestine.[4] Until now seventeen mucin-genes (MUC) genes have been identified in humans, of which MUC2 is predominantly expressed in the human colon.[3]

In addition to mucins, a large number of other secreted proteins can be found in the mucus layer, such as trefoil factor family proteins (TFF) and secretory IgA (sIgA).[1] The main function of sIgA is to neutralise toxins and to prevent epithelial adherence and penetration of pathogens.[5] TFFs are mucin-associated proteins involved in protection of the gastrointestinal epithelium and mucosal healing, and contribute to the viscoelastic properties of the mucus layer.[6,7] TFF1, TFF2 and TFF3 have been identified and are expressed in a site-specific pattern along the gastrointestinal tract. Trefoil factor 3 (TFF3) is predominantly produced by the intestinal goblet cells along the small and large intestine and has shown to be important for maintenance and repair of the intestinal mucosa.[8]

An impaired mucus layer has been reported in active ulcerative colitis (UC), as shown by a reduced mucus thickness, sulphation, MUC2 synthesis and MUC2 secretion. During clinical remission these parameters of the quality of the mucus layer did not significantly differ from the normal situation.[3,9] It has been proposed that dietary factors are able to affect the mucus layer.[10] For example, studies with rats have shown that dietary fibre deficiency leads to a decreased mucus thickness.[2] Butyrate, a short chain fatty acid, is one of the main end-products of intestinal microbial fermentation of mainly dietary fibre. Its production has shown to be related with several beneficial effects on colonic health such as a reduction of inflammation, carcinogenesis and oxidative stress.[11] A number of studies also indicate that butyrate affects the composition and the thickness of the colonic mucus layer.[12-22] Several *in vitro* studies demonstrated that butyrate increased the *MUC2* gene expression in different cell lines[15,17,22], but a decreased *MUC2* gene expression has been reported by others.[12] In addition, butyrate stimulated mucin synthesis in human colonic biopsies *ex vivo*[14] and increased mucus secretion in

isolated perfused rat colon.[13] Other *in vitro* and animal studies demonstrate that butyrate affects TFF3 expression[18,19,21], although not all study results point into the same direction.
Although several *in vitro*, *ex vivo* and animal studies show that butyrate affects the mucus layer, to our knowledge these effects have not yet been evaluated in the healthy and/or mildly inflamed human situation. Therefore, the present study aims to determine the effects of butyrate administration on MUC2 and TFF3 expression, the proportion of sialomucins and sulphomucins, total mucin secretion, and sIgA concentrations in both healthy volunteers as well as in UC patients in clinical remission.

Methods

Patients

Sixteen healthy subjects and thirty-five UC patients in clinical remission participated in this study. Patients had a well-defined diagnosis of UC established by clinical, endoscopical, histological and/or radiological criteria. Patients were in clinical remission and were excluded from participation if the Endoscopic Grading System (EGS)[22] score was higher than 5 at the first endoscopy. The healthy subjects and patients were between 18-65 years of age and consumed a stable Western diet. Exclusion criteria comprised: proctitis only, pregnancy, lactation, changes in medication and/or pre- and probiotic intake 2 weeks prior to and during the study, the use of corticosteroids, enemas or suppositories two weeks prior to or during the study, the use of antibiotics 3 months prior to or during the study, other clinically significant systemic diseases and previous radiotherapy or chemotherapy. All healthy subjects and patients gave their written informed consent before participation. The study was performed in accordance with the principles of the declaration of Helsinki and was approved by the Medical Ethics Committee of the University Hospital Maastricht, the Netherlands.

Study design

The design of study with healthy volunteers was according to a randomised, double blind, placebo controlled, crossover design with a wash-out period of two weeks[23], whereas, the study with UC patients was according to a randomised, double blind, placebo controlled parallel design. In short, during each intervention period, patients self-administered a 60 mL enema containing either a sodium butyrate solution (100 mmol/L) or a placebo solution (140 mmol/L NaCl) with a neutral pH. The butyrate enemas were made isotonic by the addition of NaCl (40 mmol/L). Patients were instructed to self-administer the enema once daily prior to sleeping,

and to remain in a left-lateral supine position for at least 15 min. The administration of the enemas continued for 14 and 20 days in healthy subjects and UC patients, respectively. Compliance was evaluated on the basis of a questionnaire and the returned enema-bottles.
In the study with the healthy subjects a sigmoidoscopy was performed after each intervention period and in the study with UC patients a sigmoidoscopy was performed before and after the intervention period. Each sigmoidoscopy was performed in the morning after an overnight fast and without prior bowel cleansing. During all sigmoidoscopies, mucosal biopsies were obtained from a standardised location of the sigmoid (approximately 20-25 cm from the anal verge at the location of the arteria iliaca communis). Two mucosal biopsies for RT-PCR analysis were immediately frozen in liquid nitrogen and two other mucosal biopsies were fixed in formalin and embedded separately in paraffin for (immuno)histochemistry.
The day before each sigmoidoscopy, participants collected 24-hour faeces. Part of the collected faeces was freeze-dried for the determination of mucin secretion and another aliquot was stored at -80°C for determination of faecal sIgA and calprotectin.

RT-PCR for MUC2 and TFF3

The expression of MUC2 and TFF3 was determined using RT-PCR. RNA was isolated from a frozen biopsy using TRIzol reagent (Invitrogen, Carlsbad, USA). RNA was purified with an RNeasy mini kit (Qiagen, Venlo, The Netherlands) combined with a DNase treatment using the RNase-Free DNase Set (Qiagen). 500 ng total RNA was used as a template for the cDNA reaction, which was synthesised using the iScript cDNA Synthese kit (Bio-Rad, Veenendaal, The Netherlands). The cDNA was diluted to a concentration of 0.32 ng/μL. Each reaction contained 12.5 μL iQ Sybr Green Supermix (Bio-Rad), 1 μL each of 10 μmol/L gene-specific forward and reverse primers, 4 μL cDNA template solution and 6.5 μL sterile H_2O. Primer sequences are listed in Table 7.1. Housekeeping genes included were 18SrRNA, GAPDH and CANX. Reactions were run on the My IQ Single Colour RT-PCR Detection System (Bio-Rad). PCR conditions used were 3 min at 95°C, followed by 40 amplification cycles of 10 sec at 95°C and 45 sec at 60°C.

Table 7.1 Primer information of MUC2, TFF 3 and housekeeping genes.

	Sequence ID	Forward primer (5' → 3')	Reverse primer (5' → 3')
MUC2	NM_002457	GTCAACCCTGCCGACACCTG	ACTCACACCAGTAGAAAGGACAGC
TFF3	NM_003226	CTTGCTGTCCTCCAGCTCT	CCGGTTGTTGCACTCCTT
18SrRNA	M10098	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
CANX	NM_001024649	CCACTGCTCCTCCTTCATCTCC	CGGTATCGTCTTTCTTGGCTTTGG
GAPDH	NM_002046	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

MUC2: mucin2; TFF3: trefoil factor 3; 18SrRNA: 18S ribosomal RNA; CANX: calnexin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase;

(Immuno)histochemistry

Serial tissue sections (2 μm) were stained with high iron diamine-alcian blue (HID-AB) at pH 2.5 to distinguish non-sulphated (sialomucins) from sulphated (sulphomucins) acidic mucins[24] and with immunohistochemical stainings for MUC2[25] and TFF3.[26] Paraffin sections for immunohistochemistry were deparaffised and endogenous peroxidase was blocked with 3% (vol/vol) hydrogen peroxide in PBS for 30 minutes. Antigen retrieval was performed by boiling the sections in 10 mmol/L citrate buffer (pH 6.0), After cooling down to room temperature, sections were incubated with 1% (wt/vol) blocking agent (Boehringer, Mannheim, Germany) in PBS for 30 minutes. Primary antibodies, monoclonal anti-MUC2 raised in mouse[25] (We9, 1:200) and polyclonal anti-TFF3 raised in rabbit[26] (1:2000) diluted in PBS were incubated overnight. Subsequently, slides were incubated with biotinylated secondary antibody and avidin-biotin peroxidase complex (Vectastain ABCkit, Vector laboratories, Burlingamer, UK) and binding was visualised in 0.5 mg/mL 3,3'-diaminobenzidine, 0.02% (vol/vol) hydrogen peroxide in 30 mmol/L imidazole and 1 mmol/L EDTA (pH 7.0).
Two blinded observers analysed the slides semi-quantitatively by scoring the percentage of non-sulphated sialomucins, MUC2 and TFF3 of at least three entire crypts from each biopsy specimen.

Total mucin determination in faeces

Mucins were extracted from freeze-dried faeces and quantified fluorimetrically as described previously.[27] First, freeze-dried faeces was reconstituted in PBS and glycosidases were denatured and mucins were solubilised.[28] Samples were filtrated (ultrafree MC 30000 NMWL, Millipore, Bedford, USA) during centrifugation (3000 g, until the complete sample passed the membrane). Subsequently, 200 μL methanol was added and centrifuged (3000 g) to wash the membrane. After drying by air, the mucin retentate was dissolved in PBS to the original volume and the amount of oligosaccharide side chains liberated from mucins were quantified using

a fluorimetric assay.[29] The total amount of mucins was expressed as μmol oligosaccharide equivalents.[27]

sIgA in faeces

Secretory IgA concentrations in supernatants of 10% (w/v) faecal homogenates in PBS were determined using an enzyme-linked immunosorbent assay (ELISA) as described previously.[30] Briefly, plates were coated with monoclonal mouse α human anti-secretory component (IgA) antibody (1:10000; Sigma). Purified human IgA isolated from colostrum (Sigma), was used for the standard curve. sIgA was detected using a monoclonal biotinylated mouse anti-human IgA1/2 monoclonal antibody (1:500; BD Pharmingen, Breda, the Netherlands), followed by the addition of streptavidin-conjugated horseradish peroxidase (1:10000; Sanquin CLB, Amsterdam, the Netherlands) and enzymatic colour development with tetramethylbenzidine (Sigma).

Statistical analysis

The non-parametric Wilcoxon signed-rank test was used to identify significant differences between dependent samples. Mann-Whitney U test for non-parametric data were used to identify significant differences between independent samples. Spearman's rank correlation coefficient was determined to identify significant correlations between parameters. Differences were considered statistically significant if $p<0.05$. Statistical analyses were performed using SPSS 11.0 software.

RT-PCR data were analysed using a Gaussian linear regression as described previously.[23] Finally, data were presented as fold change with the confidence interval. The gene under consideration was denoted to be differentially expressed if the confidence interval (CI) did not include 1.

Results

Patient characteristics

All 16 healthy volunteers (25% male, median age: 23 (18-62) years) completed both study periods with butyrate and placebo enemas. Thirty-five UC patients completed the study: 18 placebo-treated patients (50% male, median age: 59 (38-64) years) and 17 butyrate-treated patients (65% male, median age: 54 (31-65) years). The compliance based on returned empty enema bottles and questionnaires was 99% both in the placebo and the butyrate group of the UC patients and was 100% for the healthy volunteers.

MUC2 and TFF3 mRNA expression levels

Using RT-PCR the mRNA levels of both MUC2 and TFF3 were analysed in the mucosal biopsies. Both MUC2 and TFF3 mRNA was not significantly affected by the butyrate intervention in healthy volunteers nor in UC patients (Table 7.2). There was a trend, although not significant, towards a decrease in the expression of TFF3 due to the butyrate intervention in both groups as indicated by the confidence interval that just included “1”.

Table 7.2 Fold changes and confidence interval of the MUC2 and TFF3 as a result of the butyrate intervention in healthy subjects and UC patients in remission.

Gene		Fold change
MUC-2	Healthy subjects	1.04 (0.90-1.19)
	UC patients	1.04 (0.94-1.14)
TFF3	Healthy subjects	0.91 (0.82-1.01)
	UC patients	0.92 (0.83-1.03)

MUC2 and TFF3 protein expression

The protein expression of MUC2 and TFF3 was analysed by immunohistochemistry. In both healthy volunteers and UC patients in remission, MUC2 and TFF3 was expressed throughout the entire crypt. No differences in MUC2 and TFF3 protein expression were seen after the butyrate intervention nor between both intervention groups.

HID-AB

To distinguish between sulphomucins and sialomucins, the HID-AB staining was applied on sections from the colonic mucosal biopsies obtained before and after the intervention with butyrate. The percentage of non-sulphated sialomucins did not differ significantly between both groups. Nor was there change in the percentage of sialomucins after the intervention with butyrate treatment compared to placebo in the healthy volunteers and UC patients in remission (Table 7.3).

Table 7.3 The percentage of sialomucins in colonic mucosal biopsy specimens of healthy volunteers (cross-over design) and UC patients in remission (parallel design) after placebo or butyrate treatment.

	Treatment		% sialomucins
Healthy subjects	Placebo (n=16)		31 (20-65)
	Butyrate (n=16)		39 (10-63)
UC patients	Placebo (n=18)	Before	23 (5-60)
		After	18 (8-43)
	Butyrate (n=17)	Before	28 (8-48)
		After	26 (5-55)

No significant differences between groups and treatment.
The HID-AB staining was used to distinguish between sialomucins and sulphomucins.

Mucin determination in faeces

As an indicator of the mucin secretion, the amount of mucins in 24 hour faecal collections were determined and expressed as μmol oligosaccharide equivalents per gram dry faeces. Mucin secretion was not different after butyrate treatment compared to placebo treatment in the healthy subjects and in UC patients in remission, nor were there any changes between both intervention groups (Table 7.4).

sIgA in faeces

Faecal sIgA concentrations are shown in Table 7.4. A significant decrease in sIgA was observed ($p = 0.028$) in the UC patients after the placebo intervention, which was not seen after the butyrate intervention. In healthy volunteers and UC patients in remission there was no significant effect of the butyrate intervention on the sIgA concentrations. In addition, there were no differences in faecal sIgA concentrations between healthy subjects and UC patients in remission.

Table 7.4 Mucin secretion expressed as μmol oligosaccharide equivalents (OE) /g dry weight, measured in faecal samples of healthy volunteers (cross-over design) and UC patients in remission (parallel design) after placebo or butyrate treatment.

	Treatment		Mucins (μmol OE/g dry weight)	sIgA (μg/mg protein)
Healthy subjects	Placebo (n=16)		2.2 (1.2-4.1)	5.8 (0.1-63.4)
	Butyrate (n=16)		2.2 (1.3-8.7)	5.0 (0.1-48.5)
UC patients	Placebo (n=18)	Before	2.9 (0.7-6.7)	8.6 (<0.1-51.2)
		After	2.6 (0.6-6.2)	2.8 (<0.1-21.2)*
	Butyrate (n=17)	Before	3.3 (0.2-5.6)	3.6 (<0.1-78.5)
		After	3.4 (0.1-7.8)	7.3 (0.2-67.4)

* Significant decrease compared to the value before the placebo treatment ($p<0.05$).

Discussion

The aim of the present study was to evaluate the *in vivo* effect of butyrate on the human colonic mucus layer in two groups, healthy volunteers and subjects with a minor degree of mucosal inflammation, i.e. UC patients in clinical remission. Our results show that butyrate does not affect the expression of MUC2 and TFF3 in colonic mucosal biopsies of both groups. Furthermore, the butyrate intervention did not change the percentage of sialomucins, nor the total mucin secretion and sIgA concentrations determined in faeces.

The lack of an effect of butyrate on MUC2 and TFF3 in the present study contradicts to the findings of most of the *in vitro* and animal studies. The production and secretion of MUC2 are known to be important factors in the protection against colorectal diseases, since *Muc2* gene targeted mice have been shown to develop colitis spontaneously.[31] A recent study in mice by Gaudier *et al*. demonstrated that daily rectal enemas containing 1 mL 100 mmol/L butyrate for 7 days induced a 6-fold increase in *Muc2* gene expression in both the proximal and the distal colon.[16] Furthermore, the *ex vivo* addition of 0.1-1 mmol/L butyrate to human colonic biopsies stimulated mucin synthesis[14] and an increase of MUC2 after butyrate supplementation up to 2 mmol/L has been reported in several colonic epithelial cell lines[15,17,22], especially if a glucose-deprived medium was used.[15] However, one other study reported that 5 mmol/L butyrate repressed MUC2 expression in HT-29 cells.[12] In the light of these previous findings it is rather surprising that no effect of butyrate on MUC2 was found in the present study. The discrepancy between the present study and former mice and *in vitro* studies could be due to the concentration of the butyrate supplementation. A previous *in vitro* study by Burger-van Paasen *et al*. showed dose dependent effects of butyrate on MUC2 expression[40]. Low concentrations (up to 5 mmol/L) increased MUC2 expression in LS174T cells, while higher concentrations of butyrate decreased its expression to control values[40]. In the present study, the nightly rectal enemas containing 100 mmol/L butyrate were self-administered for 14 and 20 days in healthy volunteers and UC patients, respectively. This dose was chosen since such a local concentration could be reached when given a high fibre diet[23] and furthermore this concentration showed to be beneficial in the treatment of active UC.[32] However, it is possible that the dose, frequency and/or length of the intervention period was either not sufficient or even too high to achieve the same effect on MUC2 as previously has been reported in cell cultures and animal studies.

Besides MUC2, TFF3 is another essential protein present in the colonic mucus layer. TFF3 knock-out mice are proven to be very sensitive to developing colitis[33], while mice overexpressing TFF3 displayed increased resistance to intestinal damage and ulceration.[34] In the present study no effect of butyrate on TFF3 could

be demonstrated, while previous *in vitro* and rat studies have shown equivocal effects of butyrate on TFF3. In a rat TNBS model of colitis, TFF3 expression was decreased during active disease, and intra-colonic daily administration of 80 mmol/L butyrate for 7 days increased TFF3 expression.[19] However, butyrate in concentrations of 1-5 mmol/L inhibited the expression of TFF3 in various cell lines.[18,21] Although in the present study the effect of butyrate on TFF3 was not significant in the present study, both intervention groups showed a decrease of TFF3 expression. Further studies are needed to determine whether the effect of butyrate on TFF3 is also related to the administered dose and whether the effect depends on the presence and degree of inflammation.
The most frequently reported mechanism by which butyrate could induce the changes of MUC2 and TFF3 is by inhibition of histone deacetylase.[35,36] This results in hyperacetylation of histones and enhancement of the accessibility of transcription factors to nucleosomal DNA. However, it has also been reported that butyrate is able to induce changes in DNA methylation and acetylation of transcription factors.[35] Therefore, it is possible that butyrate is able to increase the expression of one gene, while it decreases the expression of another gene.

Other parameters of the mucus layer that were evaluated in the present study were distribution of sialomucins and sulphomucins, mucin secretion, and faecal sIgA concentrations. We evaluated these parameters because several animal studies have previously demonstrated that dietary fibre interventions, associated with a fermentation-mediated increase of butyrate, resulted in increased numbers of sulphomucin positive goblet cells[37], increased sIgA production[30,38], increased mucus secretion[13] and increased thickness of the mucus layer.[14-16,20,39] In the present study no significant increase in these parameters was observed due to the butyrate intervention. However, the significant decrease of sIgA in the placebo group was not found in the butyrate treated UC patients. Possibly, this difference between the butyrate and placebo group could be significant when larger numbers of patients would have been included. As determinations in faeces reflect changes in the entire gastrointestinal tract, it is possible that small changes restricted to the distal colon remained to be undetected. On the other hand, changes might also depend on the dose of the administrated butyrate, and therefore it cannot be excluded that other concentrations do show an effect.

In contrast to the cross-over design that was applied to the study with healthy volunteers, a parallel design was chosen for the study with UC patients in order to limit the study duration because medical interventions apart from maintenance therapy are not allowed and the course of the disease may fluctuate over time. As a consequence, more UC patients than healthy subjects were included and pre-treatment as well as post-treatment values were determined in the group of UC patients.

Although the primary aim of the present study was not to evaluate changes between healthy volunteers and UC patients in clinical remission, no significant differences between these groups were observed, despite the clear presence of low-grade inflammation and oxidative stress in these patients (data not shown).

In contrast to results from previous *in vitro* and animal studies, butyrate administered in healthy volunteers and UC patients in clinical remission using nightly rectal enemas containing 100 mmol/L butyrate for 14 and 20 days, respectively, did not show a beneficial effect on various protein constituents of the colonic mucus layer. Most likely the concentration and duration of exposure are pivotal factors in achieving a beneficial effect of butyrate supplementation on the colonic mucus layer. It has to be determined whether this concentration can also be achieved by addition of fermentable dietary fibres to the diet.

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Chapter 8

General discussion

General discussion

Protective effects of dietary fibre intake on colorectal cancer[1], cardiovascular disease[2,3] and intestinal inflammation[4] have been shown in several epidemiological studies. Nevertheless, not all studies were able to find such effects.[5] One of the proposed mechanisms for these health-promoting effects of high dietary fibre intake is the increased fermentation-mediated production of short chain fatty acids (SCFA) in the colonic lumen. The most frequently studied SCFA is butyrate, which, besides being an energy source for the epithelial cells, influences a wide array of cellular functions affecting colonic health mainly by acting as an inhibitor of histone deacetylase (**chapter 2**). Generally, intestinal bacteria favour fermentation of carbohydrates (fibres) over proteins. Therefore, saccharolytic fermentation, which leads to the production of SCFA, is characteristic for the proximal colon that contains non-absorbed and non-fermented carbohydrates available as substrates. Proteolytic fermentation, yielding potentially harmful metabolites, mainly occurs in the distal colon where these substrates are depleted. In contrast to our remote ancestors who consumed a high fibre plant-based diet, today's Western diet is low in fibre and it could be speculated that this plays a role in the increasing incidence of colonic pathology such as colorectal cancer[6] and inflammatory bowel disease.[7] Therefore nowadays, functional food manufacturers are interested in adding fermentable fibres to dietary products. Their main challenge is to design and select the fibres that increase microbial SCFA production along the entire length of the colon.

Evidence for the health promoting characteristics of SCFA originates from epidemiological studies. Beneficial effects of SCFA have, however, mainly been described in animal and *in vitro* studies and relatively little studies have evaluated the direct health promoting effects of these SCFA in humans. Human studies with SCFA have all been carried out in patients with active intestinal inflammation and mainly deal with clinical parameters (**chapter 2**). So far no studies have investigated the local effects of SCFA in the healthy or mildly disturbed colon, while healthy subjects or individuals with chronic mild intestinal complaints comprise an important target group for functional foods. Examples of patient groups characterised by such a chronically mildly disturbed colonic health are patients with irritable bowel syndrome, microscopic colitis, Crohn's disease in remission or ulcerative colitis (UC) in remission. In the present thesis the latter patient group was used as a model and indeed, when comparing the parameters of inflammation and oxidative stress between healthy volunteers (**chapter 4**) and these UC patients in clinical remission (**chapter 5**), our data confirmed that these patients had an increased level of inflammation and oxidative stress.

In the performed human intervention studies we were mainly interested in the local colonic effects of topical butyrate administration. When studying systemic effects,

blood can easily be sampled. In contrast, the colon is physically quite inaccessible for the sampling of tissue and luminal contents, in particular the proximal part of the colon. A full colonoscopy is cumbersome for the patient and there is a risk - although small- for colonic perforation.[8] Furthermore, to be able to reach the proximal colon, prior bowel cleansing is needed, which disturbs the normal physiologic environment and results in an unknown bias in the determined parameters. Restricting the endoscopic examination to the distal colon makes it possible to avoid prior bowel cleansing and the endoscopic procedure is shorter and thereby less cumbersome for the participants. Another reason why the human intervention studies described in the present thesis focus on the distal colon is that this part of the colon naturally contains the lowest concentration of SCFA and is often involved in various gastrointestinal disorders.

In the present thesis results of three separate studies have been presented. In the first study an *in vitro* model was applied, which compared the effects of 5 different SCFA with carbon chain lengths varying from 2 to 6 atoms in three different concentrations. Secondly, in healthy volunteers the effects of butyrate, being the most potent SCFA, were investigated using a randomised placebo controlled cross-over design. Finally, the effects of butyrate on colonic health in UC patients in clinical remission were evaluated using a randomised placebo controlled parallel design. To evaluate the effects of the SCFA on colonic health, different parameters of colonic inflammation, oxidative stress and mucus composition were assessed. The results on these three topics will be discussed and integrated in the following sections.

SCFA and colonic inflammation

Different SCFA with varying carbon chain lengths (acetate (C2), propionate (C3), butyrate (C4), valerate (C5) and caproate (C6)) are being produced in the human colon by microbial fermentation. The amount and type of these SCFA depends largely on the structure of substrates available for fermentation and the composition of the gut microbiota. Using an *in vitro* model that combined colonic epithelial (Caco-2) cells with stimulated human whole blood cultures, it was shown that especially butyrate, but also valerate, propionate and caproate, possessed potent anti-inflammatory properties. No such effects on inflammation were observed for acetate (**chapter 3**). Anti-inflammatory effects of C3-C6 SCFA were demonstrated by a decrease in the release of different pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, due to incubation with these SCFA. Besides being the most potent anti-inflammatory compound, butyrate was the only SCFA that was able to significantly improve the TEER, which is an indicator of epithelial integrity.
The human intervention studies in the present thesis focused on the local colonic effects of topically administrated butyrate on inflammatory activity, expressed as

MPO activity and several pro-inflammatory and anti-inflammatory cytokines in colonic mucosal biopsies as well as faecal calprotectin. Despite the clear anti-inflammatory effect observed *in vitro*, no apparent decrease of inflammation was observed in the *in vivo* study with healthy human subjects (**chapter 4**). Even though the healthy colon does not present active inflammation, immune cells are still present and, although small, effects on inflammation could have been expected. To further evaluate the effects of butyrate on inflammation, patients with UC in remission were included in the subsequent study, as they are characterized by low-grade colonic inflammation. In these patients, the butyrate intervention only resulted in a slight increase of the IL-10/IL-12 ratio and the chemokine CCL5, but overall no evident anti-inflammatory effect of butyrate was observed (**chapter 5**).

The fact that the results from the *in vitro* study could not be reproduced in the human intervention studies may have several explanations. First of all, although a complex co-culture model was used, it remains a simplification of the *in vivo* situation that has multiple feedback systems to control inflammation. In addition, even though the Caco-2 cell line is a widely used model for the normal intestinal epithelium, it originates from tumour tissue, which may have an altered cellular metabolism. Furthermore, Caco-2 cells do not produce mucus. In contrast, *in vivo,* intestinal cells are covered by a thick mucus layer, which lowers the concentration of luminal components that are in contact with the colonocytes. This may affect the effective concentration *in vivo*. The dose of butyrate given by the rectal enemas was 100 mmol/L in 60 mL. This dose was chosen as this concentration could be reached locally when giving a high fibre diet and furthermore this concentration showed to be beneficial in the treatment of active UC.[10,11] For practical reasons, once daily rectal enemas were chosen for the administration of butyrate. Although administration just before going to bed maximised the exposure, there was no continuous exposure to butyrate throughout the intervention period. Possibly, in humans, more prolonged exposure and/or higher dosages are required for stronger anti-inflammatory effects as were demonstrated *in vitro*.

An alternative explanation for the fact that the anti-inflammatory effect could not be reproduced in the human intervention studies is the difference in the inflammatory state of the cells. The whole blood cultures in the *in vitro* study were stimulated with a pro-inflammatory stimulus after a short period of co-incubation of the co-culture system with the different SCFA. Such a pro-inflammatory stimulus is often applied to augment the effect of the SCFA.[12] Whereas the human intervention studies were performed in the normal physiologic situation. A low level of inflammation is always present, which is essential for the resistance against infections.[13] On the other hand, exaggerated immune responses are unwanted as they may contribute to a further deterioration of the intestinal barrier function. It could be concluded that butyrate, and to a lesser extent also propionate, valerate and caproate, are able to attenuate exaggerated immune responses as was demonstrated *in vitro*, but

butyrate maintains a low level of controlled inflammation as observed in the described human intervention studies.

SCFA and oxidative stress

Not only inflammatory parameters, but also parameters of oxidative stress were determined in our studies. Levels of GSH were significantly increased in colonic biopsies from healthy volunteers after the intervention with butyrate (**chapter 4**). GSH is an important component of the antioxidant defence network, as also demonstrated by severe degeneration of intestinal epithelial cells in GSH deficient mice.[14] Butyrate increased the concentration of GSH in the *in vitro* study using unstimulated differentiated Caco-2 cells (**chapter 3**). In healthy volunteers, this increase in concentrations of GSH was accompanied by an increase in the expression of GCLC, the rate-limiting enzyme in GSH production. Surprisingly, this increase in GSH levels and GCLC expression was not observed in the study with UC patients in clinical remission (**chapter 5**). However, a significant negative correlation was found between the level of inflammation at the start of the study and the change in total GSH, indicating that the effects of butyrate on GSH may depend on the level of inflammation. Therefore, we hypothesise that butyrate at the concentration used in the present study may only be able to increase GSH when inflammation is minimal or absent. This implies that butyrate used in this concentration does not function as a therapeutic agent, but may be able to improve the protection against toxic stimuli in healthy individuals (i.e. preventive effect). Possibly, higher concentrations are needed for a therapeutic effect of butyrate in UC patients. This may be due to the fact that uptake and/or oxidation of butyrate differs in UC patients with varying degrees of inflammation.[15-17]
The levels of uric acid were significantly affected by the butyrate intervention in healthy volunteers (**chapter 4**). The observed decrease in uric acid was accompanied by a decrease in XDH, the enzyme that converts purines into uric acid and superoxide. Although uric acid is an antioxidant, superoxide is a reactive oxygen species and we suggest that a decrease in its production protects against oxidative stress. In the study with UC patients the same significant decrease in expression of XDH was found, but the decrease in uric acid did not reach significance (**chapter 5**). Further studies are needed to determine whether an increased GSH and a decreased uric acid production leads to an improved barrier function and protection against inflammation in humans.

Several studies have shown that increased inflammation is related to increased levels of oxidative stress. During inflammation, large quantities of reactive oxygen and nitrogen species are released that contribute to oxidative stress, antioxidant depletion and subsequent destruction of cells and disruption of the mucosal barrier function.[18] In the clinical study with UC patients in remission, no significant

correlation was found between oxidative stress parameters (GSH/GSSG ratio and MDA) and inflammation. However, although these patients showed increased inflammatory activity compared to the healthy subjects, this activity was still low compared to patients with active inflammation. It can be expected that excessive inflammation, observed in patients with active UC, does correlate with the oxidative stress parameters.

SCFA and the colonic mucus layer

The colonic mucus layer is an important dynamic component of the epithelial barrier, which can be affected by diet and is impaired in several intestinal disorders.[19] It contains mucin glycoproteins and many other compounds secreted by the intestinal epithelium, such as sIgA. Several studies have proposed a beneficial role for butyrate in the maintenance of the colonic mucus layer. However, the colonic mucus layer is difficult to study. Therefore we developed a standardised *in vivo* sampling technique to obtain human colonic mucus under physiological conditions during an endoscopic procedure using protected cytology brushes (**chapter 6**). The obtained colonic mucus samples were analysed for concentrations of mucins, sialic acid residues and sIgA. Furthermore, using SELDI-TOF-MS, low molecular weight proteins in the mucus samples were profiled to identify (unknown) proteins present in these samples. Sampling and analysis of colonic mucus was feasible using these brushes. However, this sampling technique was not sensitive enough to evaluate the rather subtle changes that occurred after the butyrate intervention, due to a relatively large variance between triplicate samples. Most likely, contamination with traces of faeces or blood was responsible for this variance. Although prior bowel cleansing could reduce this contamination, it was not applied since it could affect protein profiles in a yet unknown way.
Other mucus related parameters were also studied in the mucosal and faecal samples obtained during both butyrate intervention studies, including RT-PCR and immunohistochemical analysis of MUC2 and TFF3, distribution of sialomucins and sulphomucins, total mucin excretion and sIgA concentrations in faeces (**chapter 7**). These analyses revealed no significant effects of butyrate on the determined mucus-related parameters. The lack of an effect of butyrate on colonic mucus contradicts the findings of most of the previous *in vitro* and animal studies, demonstrating that butyrate indeed affected MUC2[20] and TFF3[21] expression levels and increased mucus synthesis[22] and secretion.[23] Possibly the lack of effect in the present study could again be due to the 'physiological' dose applied as well as the once daily administration of butyrate. In addition, the limited number and size of the biopsies that could be obtained during a sigmoidoscopy influenced the choices of the parameters to be analysed. Future studies could also evaluate the thickness of the mucus layer as it has been shown that fibre deficiency leads to a decreased thickness and a decreased protective potential of the mucus layer using a model.[19]

Future perspectives and general conclusions

Several *in vitro* studies, including the *in vitro* study described in the present thesis suggest a beneficial role of SCFA, especially butyrate, on colonic health. However, in the performed human intervention studies the effects on colonic health, measured by assessing parameters of inflammation, oxidative stress and the mucus layer, were not as prominent as expected based on the *in vitro* data that showed a reduction of inflammation, an increase in the antioxidant capacity and an improvement of the barrier function. Nevertheless, increased concentrations of colonic glutathione were conformed in healthy volunteers indicating that butyrate is able to beneficially modulate oxidative stress.

In the intervention studies described in the present thesis a standardised topical administration of butyrate by enemas was applied to assess the contribution of butyrate to the proposed beneficial effects of dietary fibre on colonic health. This enabled us to study the effects of butyrate without changing the microbial composition, intraluminal pH and production of other metabolites. Another possibility to increase the colonic SCFA concentrations is the administration of dietary fermentable fibres or acetylated starches that are not digested and absorbed in the small intestine and rely on the colonic microbiota for the production of SCFA.[24,25] This way there is a more continuous exposure of the colonocytes to SCFA, however the exact local concentrations of SCFA would remain unknown. Furthermore, an intervention with dietary fermentable fibres would also influence other factors that can affect colonic health besides the SCFA production.[26] For example, fermentation of dietary fibres may lead to modification of the intestinal microbial composition, reduction of potentially harmful by-products of protein fermentation, reduction of colonic pH, inhibition of growth of pathogens, increase of intestinal bulk and acceleration of intestinal transit.

Due to the invasiveness of the clinical studies it was chosen to evaluate the effects of only one concentration of butyrate on colonic health. The concentration of 100 mmol/L of butyrate was applied, as this is the expected local concentration after consumption of a high fibre diet. However, although *in vitro* fermentation models can be used to predict the SCFA production, *in vivo* data are hardly available due to the inaccessibility of the human colon.[9] Therefore, future research should also focus on the quantification of the production of SCFA in health and disease and to which extent these concentrations can be increased by fermentation of indigestible carbohydrates. It may be possible that other concentrations or more prolonged exposure to SCFA results in more similar effects as observed *in vitro*.

In general, the results described in this thesis support the rationale for the addition of fermentable dietary fibres that increase colonic concentrations of SCFA to dietary products in order to improve host health. Nevertheless, large prospective human intervention trials with long-term fermentable dietary fibre supplementation are needed to evaluate whether increased colonic SCFA levels are causatively

related to improvements of functional parameters, such as colonic permeability and symptom scores. Because our studies demonstrated that the effects of SCFA on the colonic mucosa could depend on the state of the mucosal cells, future studies should also focus on the effects of SCFA in the healthy versus the (mildly) inflamed colon.

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Summary

Summary

Short chain fatty acids (SCFA) are end-products of luminal microbial fermentation of predominantly non-digestible dietary carbohydrates. SCFA with different carbon chain lengths (acetate (C2), propionate (C3), butyrate (C4), valerate (C5) and caproate (C6)) are produced in varying amounts depending on the diet and the composition of the intestinal microbiota. In contrast to potentially toxic metabolites that are formed through fermentation of proteins, SCFA are proposed to beneficially affect colonic health. Therefore, food manufacturers are interested in the development of non-digestible carbohydrates as ingredients for novel functional foods. Especially butyrate has become of major interest as it is the main energy source for colonocytes. In **chapter 2** the current knowledge on the effects of butyrate on colonic health has been reviewed. From this review it can be concluded that butyrate can affect colonic health by reducing inflammation and carcinogenesis, decreasing oxidative stress, and improving the colonic defence barrier. However, these previous studies have mainly investigated the effects of butyrate in animal and *in vitro* models. Only limited numbers of human studies have been performed, which have all been carried out in patients with active intestinal inflammation and mainly focus on clinical parameters. Overall, these studies suggest that luminal administration of butyrate results in an amelioration of the symptoms and inflammation in active UC patients.

So far no studies have investigated the local effects of SCFA in the healthy or mildly disturbed colon, while healthy subjects or individuals with chronic mild intestinal complaints comprise an important target group for functional foods.

Although several *in vitro* studies have investigated the effects of butyrate on colonic homeostasis, the effects of other SCFA with different chain lengths have been studied less extensively. In addition, effects are hard to compare as they involve separate studies and/or different models. Therefore, the aim of the study described in **chapter 3** was to compare the effects of C2 to C6 SCFA on parameters of inflammation, antioxidant capacity and epithelial integrity in one study using a co-culture model of human epithelial cells (Caco-2) and human whole blood cultures. The results of this *in vitro* study showed that butyrate inhibits the release of several pro-inflammatory cytokines, improves of the trans-epithelial resistance and increases the concentration of the antioxidant glutathione. Remarkably, such effects were also observed for valerate, propionate and caproate with a rank order of potency being butyrate >> valerate > propionate > caproate.

In **chapter 4** the effect of butyrate, being the most potent SCFA, was investigated in 16 healthy volunteers in a randomised placebo controlled cross-over study. The intervention with daily butyrate enemas (60 mL, 100 mM) for 14 days resulted in significantly increased colonic concentrations of the antioxidant glutathione as was also observed in the *in vitro* study. Furthermore, the colonic concentration of uric acid decreased, indicating that the intervention with butyrate decreased oxidative

stress in healthy volunteers. In the following study, the effects of butyrate enemas (60 mL, 100 mM) for 20 days on inflammation and oxidative stress were evaluated in patients with low-grade colonic inflammation using a randomised placebo controlled parallel design. For this study 36 patients with ulcerative colitis (UC) in clinical remission were included. The results, described in **chapter 5**, confirmed the presence of a low-grade inflammation and oxidative stress in these UC patients in clinical remission as indicated by significantly increased levels of pro-inflammatory cytokines (IL-1β, IL-12, IFN-γ), faecal calprotectin and oxidised glutathione, and significantly decreased levels of reduced glutathione, antioxidant enzyme activity and total antioxidant capacity in colonic biopsies compared to healthy controls. However, the butyrate intervention only resulted in a slight increase of the IL-10/IL-12 ratio and the chemokine CCL5. Overall, no evident anti-inflammatory effect of this butyrate treatment was observed in these patients. Also no effects on colonic glutathione levels were found, but the effects of butyrate enemas on total colonic glutathione appeared to be inversely related to the level of inflammation at the start of the study. This may indicate that the effective dose of butyrate to decrease inflammation and oxidative stress depends on the level of colonic inflammation.

Besides inflammation and oxidative stress, several studies have proposed a beneficial role for butyrate in the maintenance of the colonic mucus layer. The colonic mucus layer is the first line of defence to the external environment and protects the mucosa against mechanical stress and chemical or microbiological aggressors. Since the mucus layer is impaired during intestinal disease and it can be affected by the diet, it is interesting to evaluate the composition of the colonic mucus layer in relation to colonic health. However, as mucus samples are difficult to collect, it is complex to study the human colonic mucus layer *in vivo*. In **chapter 6** a standardised *in vivo* sampling technique to obtain human colonic mucus under physiological conditions during an endoscopic procedure using protected cytology brushes is described. The obtained colonic mucus samples were used for analysis of the concentrations of mucins, sialic acid residues and secretory IgA. Furthermore, using SELDI-TOF-MS, low molecular weight proteins in the mucus samples were profiled to identify (unknown) proteins present in these samples. Sampling and analysis of colonic mucus was feasible using these brushes and revealed subject specific protein profiles. However, this sampling technique was not sensitive enough to evaluate the rather subtle changes that can be induced by the butyrate intervention, due to a relatively large variance between triplicate samples of one subject. To evaluate the effects of the butyrate intervention in healthy volunteers and UC patients in clinical remission other mucus related parameters were studied in colonic mucosal biopsies and faecal samples obtained during both intervention studies. These parameters include RT-PCR and immunohistochemical analysis of MUC2 and TFF3, the distribution of sialomucins and sulphomucins, total mucin excretion and secretory IgA concentrations in

faeces. The results that are described in chapter 7 revealed no significant effects of butyrate on the determined mucus-related parameters analysed.
In conclusion, the studies described in this thesis support the rationale for the addition of non-digestible fermentable dietary fibres to dietary products in order to increase colonic SCFA concentrations and thereby improve host health. Especially butyrate has shown to be an important metabolite that can decrease colonic inflammation, improve intestinal barrier function and reduce oxidative stress as observed in an *in vitro* model. Although not as prominent results were found *in vivo*, butyrate can improve the antioxidant defence capacity in healthy humans. However, the optimal SCFA concentration in health and disease remains to be determined. In addition, large prospective human intervention trials with long-term fermentable dietary fibre supplementation are needed to evaluate whether increased colonic SCFA levels are causatively related to improvements of functional parameters, such as colonic permeability and symptom scores.

Samenvatting

Samenvatting

Korte keten vetzuren (KKVZ) zijn eindproducten van bacteriële fermentatie van met name onverteerbare koolhydraten in de dikke darm. Afhankelijk van de samenstelling van het dieet en micro-organismen in de dikke darm worden KKVZ met koolstof (C) ketens van verschillende lengtes geproduceerd (acetaat (C2), propionaat (C3), butyraat (C4), valeraat (C5) en caproaat (C6). In tegenstelling tot de potentieel toxische metabolieten afkomstig van eiwit fermentatie, wordt verondersteld dat KKVZ gunstige effecten hebben op de gezondheid van de dikke darm. Daarom zijn levensmiddelen fabrikanten geïnteresseerd in het ontwikkelen van koolhydraten die niet verteerd worden in de dunne darm, maar wel gefermenteerd worden in het colon als ingrediënten voor nieuwe functionele voeding. Er is met name veel interesse in het verhogen van de concentratie van butyraat in het colon omdat het de belangrijkste energiebron is voor de epitheelcellen. **Hoofdstuk 2** geeft een overzicht van de huidige kennis over de effecten van butyraat op de gezondheid van het colon. Uit dit overzicht kan geconcludeerd worden dat butyraat de gezondheid van het colon bevordert door de ontsteking en oxidatieve stress te verlagen, carcinogenese te remmen en de barrière functie van het colon te versterken. Deze effecten zijn echter met name aangetoond met behulp van diermodellen en *in vitro* onderzoeken. Slechts weinig studies hebben de gezondheidsbevorderende effecten van butyraat en andere KKVZ onderzocht in mensen. De humane studies naar de effecten van KKVZ die uitgevoerd zijn betreffen alleen studies met patiënten met actieve ontsteking van de dikke darm en bekijken met name het effect op klinische parameters. Over het algemeen tonen deze studies aan dat rectale toediening van KKVZ en/of butyraat symptoom scores en ontstekingswaarden kan verlagen in patiënten met actieve colitis ulcerosa. Tot op heden zijn er geen studies waarin de directe effecten van KKVZ zijn onderzocht in gezonde vrijwilligers en patiënten met slechts een milde ontsteking van het colon, terwijl deze mensen een belangrijke doelgroep vormen voor het nuttigen van de nieuwe functionele voedingsproducten.
Diverse *in vitro* onderzoeken hebben de effecten van butyraat op de gezondheid van het colon onderzocht, maar de mogelijke effecten van de andere KKVZ zijn nauwelijks bestudeerd. Daarnaast zijn de effecten moeilijk te vergelijken omdat ze in verschillende studies en met behulp van verschillende modellen zijn onderzocht. Daarom had het onderzoek beschreven in **hoofdstuk 3**, de doelstelling om de effecten van de KKVZ met verschillende lengtes, variërend tussen 2 en 6 koolstof atomen, in één studie te vergelijken met betrekking tot parameters voor ontsteking, de antioxidant capaciteit en epitheliale integriteit. Hierbij werd gebruik gemaakt van een ‘co-culture’ model van een humane epitheliale cel lijn (Caco-2) en humaan volbloed. De resultaten van dit *in vitro* onderzoek laten zien dat butyraat de meest potente effecten heeft op de remming van pro-inflammatoire cytokines, verbetering van de transepitheliale resistentie en de toename van het antioxidant glutation.

Deze effecten werden ook waargenomen voor valeraat, propionaat en caproaat, maar niet voor acetaat. De grootte van de effecten was als volgt: butyraat >> valeraat > propionaat > caproaat.
Omdat butyraat de sterkste effecten had, zijn in **hoofdstuk 4** de effecten van butyraat op de gezondheid van het colon onderzocht in 16 gezonde vrijwilligers in een gerandomiseerde placebo gecontroleerde cross-over studie. De interventie, die bestond uit dagelijks een butyraat klysma (60 mL, 100 mM) gedurende 14 dagen, toonde een significante stijging van de concentratie van het antioxidant glutation in het weefsel van de dikke darm. Deze resultaten zijn in overeenstemming met de resultaten van de *in vitro* studie beschreven in **hoofdstuk 3**. Daarnaast werd ook een daling in de concentratie urinezuur gemeten. Deze resultaten lijken te wijzen op een vermindering van de oxidatieve stress als gevolg van de toediening van butyraat. In een volgende studie werden de effecten van een dagelijks butyraat klysma (60 mL, 100 mM) gedurende 20 dagen op ontsteking en oxidatieve stress geëvalueerd in patiënten met een laaggradige ontsteking van de dikke darm. Voor deze studie met een gerandomiseerd placebo gecontroleerd parallel design, werden 36 patiënten met colitis ulcerosa in klinische remissie geïncludeerd. De resultaten van deze studie die zijn beschreven in **hoofdstuk 5**, bevestigen de aanwezigheid van een laaggradige ontsteking en een lichte verhoging van oxidatieve stress in deze patiënten. Ten opzichte van waardes gemeten in gezonde vrijwilligers werden in biopten van het colon van deze patiënten significant hogere concentraties pro-inflammatoire cytokines (IL-1β, IL-12, IFN-γ), geoxideerd glutation en calprotectine gemeten. Tevens waren de hoeveelheden van het gereduceerd glutation, antioxidant enzymen en de totale antioxidant capaciteit in biopten van het colon juist verlaagd in de patiënten ten opzichte van gezonde controles. De interventie met butyraat resulteerde slechts in een lichte verhoging van de IL-10/IL-12 ratio en het chemokine CCL5. Over het algemeen werden er geen grote effecten van de interventie met butyraat op ontsteking of oxidatieve stress geobserveerd in de patiënten met colitis ulcerosa in remissie. Echter, uit de resultaten kon wel opgemaakt worden dat het effect van butyraat op totaal glutation omgekeerd correleerde met de mate van ontsteking. Hieruit kan geconcludeerd worden dat de effectieve dosis van butyraat mogelijk afhangt van de mate van ontsteking van de darm.
Verschillende studies met cellijnen en diermodellen hebben aangetoond dat butyraat ook een effect kan hebben op de mucuslaag, die op de epitheelcellen van de darmwand ligt. Deze mucuslaag vormt een belangrijke beschermlaag tegen mechanische stress en chemische of microbiologische stressoren die aanwezig zijn in het darmlumen. Het bestuderen van de mucuslaag is zeer interessant, maar het wordt bemoeilijkt door het verkrijgen van monsters van deze humane mucuslaag *in vivo*. In **hoofdstuk 6** wordt een gestandaardiseerde techniek beschreven om met een cytologie borsteltje, gedurende een endoscopie, monsters te nemen van de mucuslaag onder fysiologische omstandigheden. In deze

monsters konden verschillende analyses worden gedaan, zoals het bepalen van de hoeveelheid mucines, siaalzuur residuen en secretoir IgA. Daarnaast konden met behulp van SELDI-TOF-MS, persoon specifieke eiwit profielen van de mucus monsters worden verkregen. Deze techniek om monsters te verkrijgen was echter niet sensitief genoeg om de subtiele effecten van de interventie met butyraat aan te tonen. Om de effecten van butyraat op de mucuslaag in gezonde vrijwilligers en patiënten met colitis ulcerosa in remissie te onderzoeken werden vervolgens andere parameters geanalyseerd: expressie van MUC2 en TFF3 en verdeling van sialo- en sulfomucines in colonbiopten, en totale mucine secretie en secretoir IgA concentraties in faeces. De resultaten die beschreven staan in **hoofdstuk 7**, toonden echter geen significante effecten van butyraat op de gemeten mucus gerelateerde parameters.

Concluderend kan worden vastgesteld dat de verschillende studies beschreven in dit proefschrift de hypothese ondersteunen die stelt dat toevoeging van onverteerbare, fermenteerbare koolhydraten die leiden tot een verhoging van de KKVZ concentratie in de dikke darm aan de voeding kan bijdragen aan een verbetering van de darmgezondheid. Met name butyraat blijkt een belangrijke metaboliet te zijn dat ontsteking en oxidatieve stress in de dikke darm kan verlagen en de barrière functie van de darm kan versterken, zoals geobserveerd werd in een *in vitro* model. Ondanks dat de resultaten van de *in vivo* studies niet even prominent waren, werd een toename van de antioxidant capaciteit geobserveerd na toediening van butyraat in gezonde vrijwilligers. Echter meer onderzoek is nodig om de optimale KKVZ concentratie gedurende gezondheid en ziekte te bepalen. Daarnaast zijn er grotere prospectieve lange termijn humane interventie studies met fermenteerbare koolhydraten nodig om te evalueren of een verhoging van KKVZ causaal gerelateerd is met een verbetering van functionele parameters als permeabiliteit van de darm en symptoom scores.

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Dankwoord

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Publications & curriculum vitae

Publications

Hamer HM, Jonkers DMAE, Venema K, Vanhoutvin SALW, Troost FJ, Brummer RJM. Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther. 2008; 27(2): 104-19

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Curriculum vitae

Henrike Maria Hamer was born on May 13th 1981 in Delft, the Netherlands. After completing secondary school in 1999 at the 'Zandevelt College' in 's Gravenzande, she started the study Health Sciences, at the Maastricht University. As a part of her specialization 'Biological Health Sciences' she performed two research internships. The first internship in 2002 was performed at the University of Kuopio in Finland at the department of Clinical Nutrition where she took part in a twin study to evaluate the heritability of overweight and taste perception. She wrote her final thesis about malnutrition and oxidative stress after her second internship performed at the Institute of Nutrition and Food Technology in Santiago, Chile in 2003. After her graduation in February 2004, she worked as a research assistant for NutriScience, a research and consultancy organization specialised in functional foods. In October 2004 she started working as a PhD fellow at the department of Internal Medicine from the Maastricht University. Her research was part of the TI Food and Nutrition project entitled 'microbe-mediated gut metabolism'. The research performed during this period is described in the present thesis. She presented parts of the research findings at several national and international conferences. In 2007 she received the 'NWO-Foppe ten Hoor Young Investigator Award'. From December 1st 2008, Henrike Hamer is working as a post-doc at the Department of Gastrointestinal Research at the Catholic University of Leuven in Leuven, Belgium.