Composition and Activity of Gut Microbiota in Inflammatory Bowel Disease

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Composition and Activity of Gut Microbiota in Inflammatory Bowel Disease

Samenstelling en activiteit van de darmflora in chronische darmontstkingen

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General introduction
Chapter 1

Inflammatory bowel disease

Ulcerative colitis (UC) and Crohn’s disease (CD) are inflammatory bowel diseases (IBD). Complex environmental, immunoregulatory, and genetic factors interact in this pathologic process, resulting in chronic, relapsing intestinal inflammation. UC is characterized by a diffuse mucosal inflammation limited to the colon. It involves the rectum in nearly all cases and may extend proximally to the rest of the large intestine. The most specific clinical symptom is bloody diarrhea. CD is characterized by a more discontinuous inflammatory pattern, potentially affecting the whole gastrointestinal tract. The inflammation in CD is transmural with large ulcerations and occasional granuloma. Symptoms of CD are abdominal pain and diarrhea, but patients may also suffer from rectal bleeding, weight loss, and fever. Worldwide, up to two million people suffer from IBD (1-4).
Both UC and CD are mainly located in areas with high bacterial concentrations. There is evidence that IBD is caused by a (genetically determined) altered immune response to antigens of the enteric bacterial microbiota in a genetically susceptible host (5-8). In this hypothesis the gut microbiota, host defense, and genetics of the host are inextricably linked and can be displayed by a vicious circle, where the one process, inflammatory response, may lead to the other process, a change in microbiota composition or activity, or vice versa (Figure 1.1).

**Figure 1.1** Possible interaction and cause- and effect- relations between inflammatory reactions and intestinal microbiota in IBD.

**The role of the microbiota in IBD**

Interactions between the intestinal microbiota and the host have been suggested to play a role in the pathogenesis of CD and UC. First of all, the distal ileum and the colon are the areas of the gut with the highest bacterial concentrations and are the more frequent sites of inflammation in IBD patients as well (9). Furthermore, decreasing bacterial numbers in the intestine, e.g. by using antibiotics, can lead to clinical improvement and decreased inflammation in both humans (10) and animal models of IBD (11,12). Diversion of the faecal stream was studied in patients after curative resection of the distal ileum. A diverting terminal ileostomy was constructed. These patients only had a recurrence of disease after reanastomosis, but not during the six months in which the neoterminal ileum and colon were excluded from intestinal transit (13). This finding further points to the involvement of the microbiota in the etiology of mucosal inflammation in IBD.

A large variety of observations, often using transgenic animals, support the hypothesis that IBD is due to T cell hyper responsiveness towards otherwise harmless components of the indigenous microbiota (14). HLA-B27/ß-microglobulin transgenic rats and IL-10 knock-out mice, develop IBD in the presence of a normal colonic microbiota, but not under germfree conditions (11,15,16).
Moreover, CD4+ T cells (T-helper cells) reactive towards antigens of the enteric bacteria produce colitis after adoptive transfer to immunodeficient hosts (17). It is not so much the presence of a specific microorganism, but the immune response to it that appears critical in the pathogenesis of IBD.

Circumstantial evidence for the role of the microbiota in IBD includes responses to antimicrobial therapy, infiltration of inflammatory bacterial products across the intestinal mucosal barrier, host immunity against (or rather loss of tolerance to) bacteria indigenous to the large intestine, and the role of bacteria in disease development in animal models of IBD. Currently, two hypotheses regarding the role of microorganisms in IBD are under investigation. The first suggests that specific microorganisms play a role in the induction of inflammation (2,18-21). The second suggests that metabolites derived from the microbiota, without regard to species, can be mediators of injury and inflammation (22,23) hereby driving a perpetuous circle as depicted in Figure 1.1.

**Composition of the microbiota**

A wide spectrum of organisms, including *Escherichia coli*, *Bacteroides vulgatus*, *Clostridium difficile*, *Campylobacter jejuni*, *Mycobacterium paratuberculosis*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, is thought to be linked to IBD (7,19,24). If specific microorganisms cause mucosal inflammation, the composition of the microbiota from IBD patients may be different from that of healthy individuals. Various investigators compared the microbiota of IBD patients to that of healthy individuals, but results on this are conflicting (5,7,25). However, in severe, extensive IBD an abnormal composition of the gut microbiota may be different, rather than a cause, of the disease. For instance, diarrhea can lead to secondary, non-specific increases in faecal aerobes and coli forms and thus in a reduced number of anaerobic bacteria and lactobacilli. This is a common feature in active colitis, regardless of the cause (6). Not only a pathological symptom such as diarrhea, but also the treatment of IBD patients with antimicrobials or other drugs can result in a microbiota that is different in composition from that of healthy individuals (5,25).

Also, most data have been obtained using classical plating and speciation techniques. These observations are limited by the fact that only approximately 40% of the microbiota can be cultivated. By taking more modern molecular approaches, such as Denaturing Gradient Gel Electrophoresis (DGGE), fluorescent *in situ* hybridization (FISH), or DNA-arrays (DNA-chips), one may come to well-founded conclusions about microbial compositions, which are more specific and non-conflicting.

**Metabolic activity of the microbiota**

The second hypothesis explaining the role of the microbiota in IBD focuses on its metabolic activity. Published data suggest that the metabolic activity of the large intestinal microbiota of patients suffering from UC and CD is different compared to that of healthy individuals (7,23,26-28). This difference in activity, as evidenced by the production of certain microbial metabolites, may be a mediator of mucosal injury.
The metabolic activity of the colonic microbiota is an anaerobic fermentation process in which substrates are metabolized by the large intestinal microbiota to salvage energy (29). This process is influenced by the composition of the colonic microbiota as well as by the amount and type of substrate that enters the colon, consisting of dietary residues (mainly carbohydrates and proteins) and host-produced substances (e.g. mucin, sloughed-off epithelial cells) (3,22,29,30).

The presence of carbohydrates influences proteolytic fermentation, and vice versa. (2,22,31). Protein fermentation, in particular, gives rise to a variety of metabolites that are potentially toxic, including phenolic and indolic compounds, branched-chain fatty acids (BCFA), sulphur-containing metabolites, and ammonia. In absolute amounts the turnover of nitrogenous substances is less than that of carbohydrates and organic acids (29,32). However, little is known about the production and biological role in vivo of these potentially toxic metabolites derived from proteolytic fermentation.

The within-subject variation turned out to be high when faeces was used to determine concentrations of phenolic and indolic compounds, BCFA, and ammonia (30). Studies focusing on these metabolites in different regions of the colon showed that the proteolytic activity of gut bacteria is higher in the distal parts of the large intestine, where pH is increasing amongst others because of decreased carbohydrate fermentation (2,22,31). The protein fermentative metabolites have been associated with a variety of disease states in humans, including cancer. They seem to have several cytopathic effects on colonic epithelial cells, for instance reduction of their lifespan (2). Protein fermentation potentially plays a role in IBD by causing damage to epithelial cells in genetically susceptible hosts, thereby resulting in inflammatory reactions.

Besides a high proteolytic activity, a low saccharolytic activity can possibly also influence the pathogenesis of IBD. Bacterially-produced short-chain fatty acids (SCFA), especially butyrate, are important energy sources for colonic epithelial cells; the colonocytes acquire approximately 70% of their energy from butyrate (33). Depletion of butyrate or prevention of the oxidative use of butyrate by epithelial cells leads to loss of integrity of these cells and, eventually, results in death and epithelial desquamation (34) (35-38). This is presumably the case in UC (35,39). Renewal of the lost cells increases proliferation and may eventually influence the chance of cancer development (40). In addition, the increased desquamation due to a lack of SCFA leads to a decreased barrier function of the gut wall and makes the colonic epithelium more susceptible to bacterial translocation and inflammation (Figure 1.1)(25). In some initial studies, enemas containing SCFA decreased disease activity in left-sided colitis. Additional studies are needed to evaluate this therapy. Presently, no link has been found between CD and SCFA. Both the reduction in butyrate or other health promoting metabolites and the increase in toxic compounds seem to influence the health status of the epithelial layer lining the colon.

Also the elevation of other bacterial enzymatic activities can contribute to the clinical picture. Patients with active UC have higher mucin sulfatase activity than those with inactive UC. Furthermore Rafii et al. (6) found significantly higher total glycosidase activity in fecal samples of patients with CD than in healthy subjects. Both sulfatase and glycosidase have been implicated in the impairment of the protective function of the mucus barrier. Other enzymes, such as azoreductase, nitroreductase, oxidoreductase, and glucuronidase activities can potentially lead to toxic metabolites (6,41).
Chapter 1

Role of nutrition in IBD

A high prevalence of nutritional disturbances and deficiencies occur in IBD. Nutritional therapies can be used in control of the disease activity in CD. Elemental diets, which are mainly used in enteral nutrition therapy, seem to reduce mucosal inflammations by the elimination of dietary antigens and by reductions in fat. Parenteral nutrition is utilized as complete bowel rest supporting nutrition (42).

In this thesis another role of nutrition in IBD is discussed, based on the knowledge that the microbial activity in the colon may play a role in IBD. This activity is in part defined by the components of the food that reach this part of the digestive tract. Especially the nutritional approach in UC is evident, focusing on the metabolic activity in the colon (43,44). Instead of elimination of dietary compounds and establishing bowel rest, dietary compounds can be added to the normal diet to control disease activity. These dietary compounds are called prebiotics, defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth or activity of one or a limited number of bacterial species already resident in the colon (45). Lactulose, oligofructose, inulin, bran, psyllium, and germinated barley all have been proposed as prebiotics (46-49). Fermentation of prebiotics can for instance result in high amounts of butyrate and they are able to manipulate the microbiota towards a more remedial community (10,50). Also, prebiotics often exhibit waterholding capacity and bile-acid binding capacity. This high water-holding capacity results in a positive effect on stool formation. Furthermore, such prebiotics seem to be more readily utilized by microorganisms. A high capacity for binding bile-acids results in a higher excretion of these compounds, which are a causal factor in colorectal cancer (48,51). However, more detailed studies are needed to clearly establish the relevance of prebiotics in IBD. A short-term synbiotic therapy, in which Bifidobacterium longum was combined with inulin, resulted in improvement of the clinical appearance of chronic inflammation in patients with active UC (52).

Defense

An intact gastrointestinal mucosa, with a normal intestinal permeability and barrier function, is essential for fluid-absorption and -secretion and transport of oxygen, ions, and nutrients. Intestinal epithelial cells that line the gastrointestinal mucosa can be regarded as the most outer defense system preventing microorganisms, including commensals, and endotoxins from reaching systemic organs and tissues (34,53-57).

It has been suggested that both luminal microorganisms and their metabolites, as well as dietary antigens, are capable of initiating and sustaining intestinal mucosal inflammation. An intriguing hypothesis regarding the pathogenesis of CD speculates that a defect in epithelial barrier function initiates the disease (58-60). By studying IL-10 deficient mice, Madsen et al. (1999) found that increased intestinal permeability existed prior to the development of mucosal inflammation, indicating that increased intestinal permeability is thus not secondary to mucosal inflammation (61). When the mucosal barrier function is impaired, bacterial translocation may increase as a result of paracellular- or transcellular migration. Paracellular migration involves the opening up of tight
junctions and subsequent penetration of the bacteria between the enterocytes; in transcellular migration bacteria adhere to the cells, are taken up by endocytosis, and transported within vesicles to the basolateral membrane (62). Besides bacteria, also microbial metabolites can be transported. Both can be regarded as biological active substances that may activate or influence the mucosal immune system leading to an inflammatory reaction (17,25,55,61,63,64).

Failure to terminate or down regulate an immune response results in persistent and chronic inflammation. Many chronic inflammatory disorders show a pattern of relapse and remission. This is best explained by variation in exposure of immunocompetent cells to the antigenic stimulus, but may also reflect phased changes in immune responsiveness caused by nonspecific ‘priming’ of the immune system, as may occur with exposure to antigenically unrelated microorganisms (7).

Finally, the failure to prohibit or down regulate an immune response can be the result of inherited predisposition. Among the candidate genes for IBD investigated to date, only the NOD2/CARD15 gene on chromosome 16 has been definitively associated with CD (65) (66). Nucleotide-binding oligomerisation domain (NOD) molecules detect, together with Toll-like receptors (TLR), microbial ligands in order to initiate a defense response to fight infectious disease. These microbial ligands are structural components of the microorganism that are not subject to much variation, like lipopolysaccharide (LPS) and peptidoglycan from the cell walls of bacteria. NOD2/CARD15 act as an activator of nuclear factor (NF)-κB and is thus involved in innate immunity (65,67-69). Variants of proteins critically involved in bacterial antigen recognition, such as the products of CD-associated NOD2/CARD15 mutations seem to contribute to the loss of tolerance to commensal bacteria in CD (66). Currently, the NOD2-dependent signaling cascade is poorly understood (70).

**Gut Immunology in IBD**

Irrespective of whether or not the primary disorder in IBD is immunological in nature, the immune system is heavily involved in the inflammatory processes that cause this condition. In the healthy intestine, immune systems are self-tolerant and controlled by powerful, self-regulating mechanisms. But when IBD ensues, the inflammatory processes turn into confounding factors that camouflage what could otherwise be an explicit inflammatory response.

**Mucosal B cells**

Activated B cells are called plasma cells. They produce immunoglobulins (Ig), of which there are five classes (IgA, IgG, IgM, IgD and IgE), which bind to invading organisms, eventually leading to their destruction. The IBD-involved intestine shows a massive increase in the number of plasma cells and this increase is not uniform; IgA, IgM, and IgG producing cells increasing 2-, 5-, and 30-fold, respectively (71,72). Furthermore, the two forms of IBD can be distinguished based on IgG subclass: IgG1 increasing more in UC and IgG2 in CD. The reason for this difference is unclear, but distinct immune regulatory mechanisms or genetically conditioned differences may be responsible (73,74) (25). Alternatively, the same antigens driving the disease into a T_{H}-1 (CD) or a T_{H}-2 (UC) response (see below) can also cause the difference in production of IgG subclass.
**Chapter 1**

**Mucosal T cells**

T-helper (T\textsubscript{H}) cells are the T cell type that is involved in IBD. T\textsubscript{H} cells produce cytokines and chemokines and are classified in T\textsubscript{H}-1 cells and T\textsubscript{H}-2 cells. A T\textsubscript{H}-1 response results in an early increase in the expression of interleukin (IL)-2, interferon (INF)-\(\gamma\), and IL-12, followed by a subsequent increase in tumour necrosis factor (TNF)-\(\alpha\) and IL-18, and a compensatory increase in IL-10 and tumour growth factor (TGF)-\(\beta\). T\textsubscript{H}-1 cells mediate cellular immunity and macrophage activation. T\textsubscript{H}-2 cells mediate humoral immunity and produce IL-4, IL-5, IL-6, IL-10 and IL-13. Production of IFN-\(\gamma\) inhibits differentiation of T\textsubscript{H}-2 cells, and IL-10 can diminish T\textsubscript{H}-1 responses (9,75). This latter depends on the local concentrations achieved; high systemic doses seem to be correlated with an immunostimulatory effect of IL-10 (76).

The dysbalance between the humoral and cell-mediated immune responses may play an important role in the pathogenesis of UC and CD. The two major IBD diseases may be different in their etiology as evidenced by the fact that the immunological responses of both CD and UC is of opposite nature. An increase in T\textsubscript{H}-1 cytokines is detected in CD patients, whereas in patients affected by UC, T\textsubscript{H}-2 cell activation was demonstrated (25,59,77,78).

**Macrophages**

Also nonspecific cellular immunity is altered in IBD. In active IBD lesions a large number of blood monocytes can be detected, probably because of an increased demand of macrophages in the inflamed gut (79). This macrophage population is very heterogeneous and they are evident in early IBD lesions (25,80) Intestinal activated macrophages are distinct in phenotype and function from blood monocytes: they retain phagocytic and bacteriocidal activity. Furthermore, they do not produce proinflammatory cytokines, except small amounts of IL-8, upon stimulation by an array of proinflammatory agents (e.g. LPS) (81). Whether intestinal activated macrophages in IBD patients behave like intestinal activated macrophages in healthy individuals is poorly understood.

**Surface boundaries and -excretions**

The structure of both the small and large intestine is made up of four layers: mucosa, submucosa, muscularis, and serosa. The mucosal epithelium consists of a heterogenous population of cells that change in morphology with progression from the crypt base to the villus (82). There are four major intestinal cell types: simple columnar cells (almost 90% of the villus population), large numbers of mucus-secreted goblet cells, enteroendocrine cells and Paneth cells (at the base of the crypt). Lymphatic nodules are present in the mucosa, and the larger aggregates of lymphatic tissue are known as Peyer’s patches (83).
The gut mucosa plays a central role in the exclusion and elimination of potentially harmful antigens and microorganisms, while providing selective absorption (Figure 1.2). Antigen exclusion has been associated with factors such as the capacity of the gut mucosa to produce secretory IgA and mucus. Secretory IgA seems to prevent the adherence of enteral antigens to the mucosal surface, and mucus protects against microbial infestation (84).

**Epithelial cells**

Intestinal immunity consists of a functional integration of local immune cells with cells of nonimmune origin. Among these nonimmune cells, epithelial cells have been studied the most extensively. Epithelial cells are one of many kinds of cells that form the epithelium and they absorb nutrients. Their reciprocal interaction with the adjacent immune cells is well recognized. Human enterocytes and colonocytes express human leukocyte antigen (HLA) class II proteins and can function as antigen-presenting cells (APC), with CD4+ TH cells responding to the antigens displayed on their surfaces (85). Furthermore, the epithelial cells play an important role in the prevention of TLR activation by commensal microorganisms. This family of receptor molecules, of which some are expressed on the epithelial cells, function as sensors of microbial infection and initiate immune defense responses. A normal epithelial surface prevents the translocation of commensals. However, pathogenic bacteria that are translocated can subsequently be detected by the TLR that are expressed on macrophages and dendritic cells (86,87).
Evidence of epithelial cell involvement in IBD is derived from various studies. Epithelial cells respond to IL-1, IL-2, IL-15, and TNF-α and secrete IL-7, which activates lamina propria mononuclear cells. Furthermore, epithelial cells can produce IL-8 and TNF-α; and they express leukocyte adhesion molecules (25,56,88). Abnormalities of epithelial cell phenotype and function are found in IBD, of which a defective capacity to induce CD8+ T-suppressor cells (T_S) is potentially important. Whereas normal epithelial cells preferentially activate T_S cells, a function that might contribute to induction of local tolerance, IBD epithelial cells preferentially activate CD4+ TH cells, perhaps leading to an amplification of local immune reactivity and inflammation. Finally, there is the intriguing suggestion that the colonic epithelium in UC fails to adequately oxidize butyrate. The result can be an energy-deficient condition of the mucosa, and thus the gut barrier function may be impaired (35,89). Irrigation of diversion colitis with SCFA and use of butyrate enemas in distal UC result in marked clinical improvements (25,90).

Endothelial cells

The microvascular endothelial cell is another important nonimmune cell that regulates mucosal homeostasis. Recruitment of activated leucocytes, one of the most important functions of innate immunity, is mediated by cell-adhesion molecules that are induced on the surface of endothelial cells. First, the P- or E-selectins initiate endothelial-leucocyte interactions by binding to fucosylated oligosaccharide ligands on passing leucocytes. The next step depends on a tighter adhesion, which is due to intercellular adhesion molecules (ICAMs) on the endothelium binding to heterodimer proteins of the integrin family on leucocytes. MadCAM-1 is an intercellular adhesion molecule that is specific for the gut epithelium. P- and E-selectins, ICAMs, and MadCAM-1 are expressed on the surface of endothelial cells following production of TNF-α by macrophages, and the adhesive properties of integrins like LFA-1 and MAC-1 on leucocytes are increased in response to IL-8 or other chemokines (85).

Markers of prothrombotic state and endothelial lesion and physiologic inhibitors of coagulation in plasma support the hypothesis that IBD patients harbor endothelial lesions with sustained coagulation activation. This endothelial damage is probably secondary to intestinal injury (25,91). Furthermore, the features of endothelial cells seem to be altered in IBD. Isolated intestinal mucosal endothelial cells of IBD patients show a markedly enhanced capacity to bind leucocytes compared with cells from normal mucosa. This enhanced binding by IBD endothelial cells persists regardless of in vitro culture time, suggesting that persistence of inflammation may also result from a hyperadhesive intestinal microvasculature. Similar functional modifications may very well occur for other cell types, ultimately creating a self-perpetuating proinflammatory loop sustained by the activity of nonimmune cells, a mechanism also underlying other types of chronic inflammation (17,25). Biological therapies against adhesion molecules that are being used in clinical practice at present or investigated for the treatment of IBD are anti-ICAM-1 and the anti-α-4 integrin antibodies natalizumab (Antegren®) and LDP-02 (92-94).
Colonic mucus

The mucosal epithelial cells are protected from mechanical injury, the action of antigens and toxins, and the invasion of enteric bacteria by a layer of mucus. Mucus is secreted by goblet cells; the main constituent is a glycoprotein (mucin) that contains up to 85% carbohydrates. Common constituents of the carbohydrate are fucose, galactose, N-acetyl (Nac)-glucosamine, NAc-neuraminic acid, and mannose in an α- or β-glycoside linkage. Mucus serves as an important energy source for the bacterial microbiota in the intestine, and it acts as a lubricant to ease the passage of stool through the colon (2,95-97).

A breakdown of the mucus barrier will increase the exposure of the epithelium to the full impact of luminal aggressors and may thereby contribute to the pathophysiology of IBD (25,98). Mucus thickness was significantly reduced and the layer became discontinuous in active UC (98). Bacterial enzymes, such as glycosidases and sulfatases, together with host-derived proteases, have been implicated in the impairment of the protective function of the mucus barrier. Together they are responsible for the degradation of the glycoproteins (6,99,100). There is experimental evidence that colonic mucin-glycoconjugates are altered in UC due to bacterial glycosidases, which are able to release monosaccharides from the polysaccharide chains that surround the protein core of the glycoprotein (6). However, studies on mucin composition in UC patients are conflicting. But even if colonic mucin alterations would be present, they alone would probably be insufficient to trigger inflammation (25).

Microbiota

Early exposure at the neonatal stage of the gut mucosa to live microorganisms and bacterial colonization, together with the introduction of dietary antigens, play an important role in the development of gut barrier functions and unresponsiveness to ingested antigens (oral tolerance). The microbiota enhances the development of the barrier by increasing the population of IgA-plasmocytes and the number of enteroendocrine cells in the jejunal and colonic epithelium, enhancing the production of secretory IgA and mucus. The microbiota have also been shown to stimulate proliferation of epithelial cells and increase the total intestinal surface. Demonstration that the intestinal microbiota constitutes an important part of the exclusive components of the mucosal barrier has led to the introduction of novel modes of therapeutic intervention, using specific strains of microorganisms as probiotics (84).

Treatment of IBD

Medical therapy for IBD

Treatment of IBD varies according to the type, distribution and severity of disease. Although surgery is indicated in certain situations, drug therapy is the approach of choice to induce remission and then prevent relapse. Currently no medical or surgical cure exists for CD, for UC cure can be obtained by proctocolectomy, but such extensive surgery is usually only offered when drug therapy has failed or when dysplasia occurs. Thus the goals of medical treatment are generally restricted to
suppress the inflammatory response, to relieve symptoms and to induce disease remission. Drugs in the treatment of IBD can be used topically, orally or intravenously. Commonly used drugs include aminosalicylates, corticosteroids, antibiotics, and immunomodulatory agents (8,101).

**Aminosalicylates**
Sulfasalazine consists of the active component mesalazine (5-aminosalicylic acid; 5-ASA) and sulfapyridine joined by an azo-bond. Sulfasalazine remains inactive in the proximal gastrointestinal tract, but in the colon bacterial cleavage of the azo-bond occurs, making 5-ASA available only to the large intestinal mucosa. There are a number of drugs that contain only 5-ASA, consisting of a single molecule (Pentasa®, Asacol®, Salofalk® and Claversal®/Mesasal®) or 2 molecules joined by an azo-bond (Dipentum®). Various mechanisms to deliver the active drug to its site of action are used; most often the disintegration of the formula is based on pH. Aminosalicylates are used in the treatment of mild to moderately active UC and mildly active CD, and for maintenance of remission (101-103).

**Corticosteroids**
Corticosteroids block the production and effects of cytokines and additional inflammatory mediators, and therefore have effects on many cellular and humoral immune functions. Examples of steroids in the treatment of IBD are prednisolone, budesonide (Entocort®, Budenofalk®), beclomethasone dipropionate, and fluticasone propionate. Corticosteroids are proven efficacious in acute UC and CD, but not as maintenance therapy because of lack of effect and their association with side effects (101,104,105). Especially budesonide is of scientific and clinical interest because of its topical delivery. Therefore, budesonide is less likely to cause serious side effects compared to other corticosteroids (105).

**Antibiotics**
Antibiotics have been used by clinicians for many years. They can selectively decrease tissue invasion by microorganisms and eliminate aggressive bacterial species or globally decrease luminal and mucosal bacterial concentrations, depending on their spectrum of activity. Examples used in both active CD and UC are clarithromycin, ciprofloxin, metronidazole, vancomycin, and imipenem. They sometimes are also used to maintain remission but then appear to have a less significant impact on clinical course and parameters of inflammatory activity in patients with UC (10,12,101,106,107).

**Immunomodulatory agents**
Azathioprine (AZA), its active metabolite 6-mercaptopurine (6-MP), methotrexate (MTX), and cyclosporine are the four primary immunomodulatory agents used in IBD patients. These drugs alter the immune response via inhibition of T-cell mediated responses, natural killer cell activity, or cytokine production. These immunosuppressants are valuable drugs for those forms of IBD that are unresponsive to other forms of treatment. Azathioprine and 6-MP are mainly used for maintenance therapy in both UC and CD. Methotrexate is also used for maintenance therapy in CD.
Cyclosporine is only used in active UC (8,101). Newer immunomodulatory agents that are emerging are mentioned in the next paragraph.

**Biological therapy for IBD**

Novel therapeutic approaches for the treatment of IBD are currently emerging. These often use biologic agents that mechanistically target individual inflammatory pathways. Biological agents that have already been proven to be effective for CD and recently for UC include monoclonal antibodies directed against tumour necrosis factor alpha (anti-TNF-α; infliximab and CDP571) and to the leukocyte adhesion molecule α-4 integrin (natalizumab)(92,93,108). Still under investigation are among others interferon-α, anti interleukin-12 (IL-12) antibody, human growth hormone, IL-10, IL-11, and antisense-oligonucleotides which either block the genetic read out of adhesion molecule genes such as ICAM-1 or of the intracellular regulatory factor NF-κB (77,101,106,109). Besides immunological strategies, also the targeting of therapeutics to improve topical delivery is a strategy for IBD that can be realized using a biological approach (105,106). In microbiological strategies, microorganisms are used to modify the gut microbiota. The used microorganisms are called probiotics. Probiotics are defined as living organisms, which beneficially affect the host. The probiotic organisms influence the diverse functions of the colonic microbiota, which contains more than 500 different bacterial species. It is thought that probiotic organisms can correct any imbalance between the beneficial and harmful activities. Probiotics could also increase the defense of the body against pathogens through immunomodulatory activities (110,111). More recently also genetically engineered bacteria can function as probiotic, such as lactococci that secrete immunosuppressive IL-10 (10).

**Studying microbiota in IBD**

**Faeces**

Faeces can be used to study the role of specific microorganisms in the induction of IBD. Molecular approaches have recently shown that the microbial composition of faeces is comparable to the microbial composition of the luminal contents in the proximal colon, as well as that of the colonic mucosa (112). Zoetendal *et al.* (113), on the other hand, used a 16S rRNA approach to demonstrate that the predominant mucosa associated bacterial community was significantly different from the faecal community. But the community of *Lactobacillus*-like bacteria in biopsy samples was similar to that in faeces for 6 out of 10 individuals.

Faeces does not seem a good indicator of the fermentative activity that occurs in the proximal colon (114). For IBD patients, investigating faecal material is even more complex. Damage to the mucosa of IBD patients, caused by inflammation, can result in inadequate absorption and utilization of metabolites and therefore may result in higher concentrations of metabolites in their faeces than in that of healthy individuals (7). With faeces, no distinction can be made between an inadequate absorption and utilization and an actual altered metabolic functioning by luminal bacteria from IBD.
patients. This latter can be studied in an *in vitro* model of the proximal colon, using faeces as inoculum.

**In vitro model of the large intestine (TIM-2)**

To study microbial activity, a dynamic model of the large intestine was developed by TNO (115). The model (nick-named TIM-2 for TNO’s *in vitro* model of the large intestine) consists of a number of linked glass units with flexible walls inside (Figure 1.3). Peristaltic movements are achieved by pumping water of body temperature (37°C) into the space between the glass jacket and the flexible wall at regular intervals. The computer controls the sequential squeezing of the walls, causing the chyme to be mixed and moved.

The model is equipped with hollow fibre membranes, inside the lumen of the model, to remove water and microbial fermentation products, such as SCFA. Thereby, they maintain the physiological concentrations of small molecules, such as electrolytes, and prevent product inhibition (and eventually microbial death) due to built-up of microbial metabolites (46,116). The model further contains an inlet system for delivery of the artificial ileal delivery medium. The system is kept anaerobic by flushing with gaseous nitrogen, to allow for the growth of a dense, complex microbiota, comparable in composition and activity to that found in humans in the proximal colon (117).

![Figure 1.3](image-url)

Dynamic *in vitro* model of the large intestine (TIM-2).
Cell cultures
Absorption and local and systemic biotransformation of microbial metabolites, as well as tolerance to microbes and microbial metabolites of epithelial cells can be assessed using *in vitro* and *ex vivo* approaches. These assays are especially suitable for screening of microorganisms or microbial metabolites separately and in certain combinations. It has been suggested that both luminal microorganisms and their metabolites are capable of initiating and sustaining intestinal mucosal damage and/or inflammation by negatively affecting defense systems. Among others, the following two cell cultures have been used to study this hypothesis.

*Caco-2*
The human colon carcinoma cell line Caco-2 has been widely used as an *in vitro* model for human intestinal absorption as these cells display, after differentiation, both biochemical and morphological characteristics of intestinal enterocytes (118, 119). Differentiation of Caco-2 cells can proceed along multiple pathways and does not necessarily lead to apoptosis. The phenotypic changes during spontaneous differentiation (17-21 days) mimic those that occur in normal colonic epithelial cells *in vivo* during their migration from the crypt base to the neck. A butyrate-induced differentiation (2-3 days) more closely follows that occurring when normal colonic epithelial cells migrate from the crypt neck to the surface compartment (120).

In this thesis, Caco-2 cells grown on semi-permeable inserts in a two-compartment transport system (Figure 1.4) were used to study the effect of microbial metabolites on the barrier function. The permeability characteristics of compounds across Caco-2 monolayers were found to correlate well with *in vivo* absorption data in humans after an oral intake (121, 122). We also used the model to study immunologic effects of microbial compounds.

Figure 1.4
Monolayer of Caco-2 cells grown on semi-permeable inserts in a two-compartment cell culture system (SEM).
Chapter 1

Macrophages
During a local infection or inflammation, the affected tissue is rapidly infiltrated by large numbers of exudate (inflammatory) macrophages. These inflammatory macrophages have distinct characteristics compared to the resident macrophages that are normally present in various organs (123). Several cell lines are available that are widely used as model systems for monocytes and macrophages. The human monocytic cell line U937, used in this thesis, is a tumor cell line that originates from immature cells of the monocytic differentiation lineage corresponding to monoblasts (124).

By stimulating the cells with phorbol 12-myristate 13-acetate (PMA), one of the most potent tumor promoting agents (125), cells are induced to undergo monocytic differentiation. In this way the cells acquire the typical monocyte/macrophage morphology, become adhesive, express differentiation related antigens and are no longer able to proliferate. Furthermore, the cells become functionally similar to monocyte/macrophage-like cells that can perform phagocytosis, cytokine production, antibody dependent cellular cytotoxicity, antigen presentation and chemotaxis (Figure 1.5)(126).

Figure 1.5

Macrophage with projectile-looking surface is interacting with lymphocytes which are rounded.

A macrophage extends a semiliquid projection, a so-called pseudopodium, towards some bacteria in the foreground.
Aim and outline of this thesis

The experiments described in this thesis envisaged determining whether patients with IBD show evidence of an altered metabolic activity of the gut microbiota and whether this is related to the initiation or exacerbation of the disease. The TNO dynamic in vitro models of the upper gastrointestinal tract and the large intestine, and defense systems such as Caco-2 and macrophage cultures can be used to study the mechanisms that underlie the onset, or the progression, of UC and CD. The provided biomarkers can possibly be used in prevention and treatment of the disease in humans.

In chapter 2 we studied which metabolites are produced during the normal colonic fermentation process by a healthy microbiota and which metabolites are produced if this microbiota is out-of-balance. TNO's dynamic in vitro model of the large intestine (TIM-2) was used to investigate the production of microbial metabolites by a standardized microbiota, representative for the healthy human proximal colon. The effect of (potential) prebiotics on saccharolytic- and proteolytic activity of the colonic microbiota was investigated. The emphasis was on toxic metabolites, such as ammonia, phenols and indoles, and branched chain fatty acids. The proteolytic pathogen Clostridium difficile, prevalent after broad-spectrum antibiotic treatment, was introduced into TIM-2 to study the potential for toxic metabolite production by a microbiota that is out-of-balance.

In the third chapter we studied whether IBD patients show an altered metabolic activity of the microbiota in the proximal colon. It is unknown whether patients with IBD have an altered metabolic function of the intestinal microbiota and whether this is related to the severity of the disease. This study described experiments using TIM-2 inoculated with fresh faeces of 6 healthy individuals and 8 IBD patients. Metabolites from both saccharolytic- and proteolytic fermentation were analyzed to determine whether patients with IBD show evidence of an altered metabolic activity of the microbiota in the proximal colon.

The production of microbial metabolites may play a role in IBD by affecting the gut barrier function. An increase in gut permeability may subsequently trigger the intestinal immune system, leading to unwanted inflammatory reactions. The effects of metabolites produced in the in vitro colon model, TIM-2, on epithelial and immunocompetent cells could give important insight into the mechanisms that underlie the onset of IBD. In chapter 4, we aimed to investigate this by studying which metabolites, and in which concentrations, can cause injury to epithelial cells and subsequently stimulate unwanted immune reactions.
Human interleukin-10 (hIL-10) can be used in the treatment of CD since it diminishes Th-1 responses. A new development is the targeted delivery of hIL-10 to the site of inflammation by bacteria. For this therapy to be efficacious, an important criterion is the survival of these microorganisms in the gastrointestinal tract of the host. \textbf{In chapter 5 we aimed to determine whether targeted delivery of hIL-10 producing} \textit{L. lactis} \textbf{could be obtained by incorporating the strain in an enteric-coated capsule formulation}. The effect of the formulation on survival and metabolic activity of the recombinant \textit{L. lactis} was studied in the TNO \textit{in vitro} model of the stomach and small intestine (TIM-1), which simulates to a high degree the successive dynamic conditions in the upper gastrointestinal tract.

Anti-TNF-\(\alpha\) monoclonal antibodies block TNF-\(\alpha\), a powerful immune stimulator. A single intravenous dose of this biological therapy results in reduced CD activity scores and increased remission. \textbf{In chapter 6 we aimed to investigate whether anti-TNF-\(\alpha\) monoclonal antibody therapy for Crohn’s disease has an effect on the faecal microbiota.} Microbial composition of faecal samples of four CD patients before and after anti-TNF-\(\alpha\) monoclonal antibody treatment were investigated and compared with the microbial composition of faeces of IBD patients in remission and of healthy individuals. Concentrations of microbial metabolites in the faecal samples were determined as well. It was hypothesized that if the microbial composition correlates with clinical outcome, microbial metabolites possibly do as well. Therefore, the second aim of this study was to investigate the possible use of microbial metabolites as disease activity markers in IBD, given its relationship with the microbial composition.

\textbf{Chapter 7 concludes the thesis with a general discussion.} This discussion is focussed on the role of microbial metabolites in IBD and is followed by a summary of the main conclusions derived from the research described in this thesis.
References


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The effect of various inulins and *Clostridium difficile* on the metabolic activity of the human colonic microbiota *in vitro*

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Abstract

The influence of inulins with different average degree of polymerisation (ranging from 3 to 25) on the metabolic activity of the human colonic microbiota with or without the addition of *Clostridium difficile* was investigated *in vitro*. The *in vitro* system used was a dynamic, computer-controlled model that simulates the conditions of the proximal part of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. The addition of inulin stimulated the formation of the total amount of short-chain fatty acids acetate, propionate and butyrate up to 50%, and that of lactate more than 10-fold, while the formation of ammonia and the branched chain fatty acids *iso*-butyrate and *iso*-valerate was suppressed. Ammonia formation was suppressed by about 30% and that of *iso*-butyrate and *iso*-valerate almost completely. These effects became much more pronounced when *Clostridium difficile* was present in the system. The introduction of *Clostridium difficile* caused a stimulation of the production of the protein fermentative metabolites ammonia, branched chain fatty acids and of the phenolic compounds indole, phenol and *p*-cresol. This stimulatory effect of *Clostridium difficile* was almost completely prevented by the addition of inulins. Thus, these results indicate a potential of inulins to shift the metabolic activity of the human colonic microbiota towards the production of less potentially toxic metabolites, both under normal conditions and under conditions with a disturbed microbiota (with a high level of *Clostridium difficile*).
**Introduction**

Inulin is a reserve carbohydrate found in many plants and vegetables (1). It occurs for instance in wheat, onions, garlic and chicory. The inulin content ranges from less than 1% in banana, 1-4% in wheat, 1-7% in onion and leek to 15-20% in chicory (2). The carbohydrate polymer is composed of β-2,1-linked fructosyl moieties mostly with a terminal glucose. The number of fructosyl residues (degree of polymerisation, DP) extends from 3 to 250, mainly depending on the plant species. For about 15 years inulin is also available as an ingredient for foods. It is extracted from chicory roots and purified (see for instance reference 3). These types of inulin have a DP of 2–60, with an average ranging from 9 to 25. Short-chain inulins can be prepared enzymatically from sucrose with fructosyltransferases, or with an endo-inulinase from long-chain inulin (4). All types of inulin are used in a large variety of foodstuffs, both for their technological and nutritional benefits. The technological benefits include fat and sugar replacement (in combination with high intensity sweeteners), low caloric bulking agent, texturing and water binding agent, and filler/binder in tablets. Based on these properties it finds applications in, for instance, dairy products, in bread and other bakery products, in confectionery and ice, and in low or zero fat spreads. The nutritional benefits arise from the fact that inulin resists digestion in the human small intestine. Therefore it reaches the colon intact. In the first part of the colon, the caecum or proximal colon, it is fermented by the microbiota to short-chain fatty acids (SCFA; acetate, propionate and butyrate), lactate and gas. The absorption of the SCFA, and subsequent metabolism by the host, salvages some of the original energy in inulin. However, only about 1.5 kcal/g is delivered from inulin (compared to 3.9 kcal/g from fructose) which explains the low caloric value (5).

Consumption of inulins leads to an increase of *Bifidobacterium* and *Lactobacillus* species in the human faecal colonic microbiota (6,7). This is the so-called prebiotic activity of inulin: it increases the number and/or activity of specific microorganisms in the gastrointestinal tract presumed to be health promoting (8). Apparently the growth of these bacteria in the colon is stimulated by inulins as has also been found in vitro (9). This increase has been implicated to cause an inhibition of growth of pathogens, and other physiological effects both locally and systemically. Among these effects is the increased synthesis of vitamins, an increase in mineral absorption from the colon, a lowering of serum lipids, or the stimulation of immune functions (3,10).

SCFA can be used by the cells of the body. Butyrate is considered a health-promoting metabolite, which functions as the major energy source for epithelial cells of the colon (11,12). Butyrate may play a role in the inhibition of colon carcinogenesis and in the regression of colitis (12,13). Acetate and propionate are used systemically in the body, especially in the liver (12). Lactate is metabolized by the muscle tissue (and will also be used to a large extent by the colonic microbiota). The branched chain fatty acids (BCFA) *iso*-butyrate and *iso*-valerate are produced by fermentation of the amino acids valine and leucine, respectively (14). The latter products may have a negative impact on health and can cause liver problems (12). Proteolytic fermentation can also lead to other (potentially) toxic components, such as ammonia and phenolic compounds (14). Ammonia is toxic to the colonic epithelium and promotes colon cancer in rats (15). In addition, ammonia is a (potential) liver toxin and has been implicated in the onset of neoplastic growth (14,16,17). The
production of phenolic compounds by intestinal bacteria has been associated with a variety of
disease states in humans, including schizophrenia (14). While the role of phenols in cancer is
unclear, nitrosation of dimethylamine, by nitrite, is stimulated by phenol, and the interaction of
phenol with nitrite forms diazoquinone, which is mutagenic in the Ames test. Other potential
(pro)carcinogens can also arise from the metabolic conversions of hepatically detoxified
compounds that are secreted into the gastrointestinal tract by the liver via bile. For instance,
bacterial β-glucuronidase can release carcinogens from hepatically derived glucuronic acid
conjugates (13). Deconjugated bile acids, products of bacterial bile acid metabolism, have been
implicated in the events that lead to colon cancer (18). Although a healthy person can handle a
limited amount of these toxic metabolites, the balance of health promoting and toxic metabolites
produced by the colonic microbiota is thought to be important for a healthy colon (19). In a
disturbed microbiota, e.g. as the result of antibiotic treatment, this balance can be shifted towards
more toxic metabolites.

The objective of this study was to examine the effect of the addition of inulins of different DP on
the activity of the human colonic microbiota in vitro. To introduce a disturbance of the microbiota
we used Clostridium (C.) difficile and we investigated whether the addition of the various inulins
was able to suppress the effects of the introduction of C. difficile. This microorganism is the
causative agent of antibiotic associated diarrhoea (20) and pseudomembranous colitis (21), both
known to be linked to disturbances of the colonic microbiota. Infection with C. difficile is a major
problem in hospitals and leads to significant morbidity and mortality (21).

**Materials and Methods**

**Materials**

Inulins were provided by Sensus Operations (Roosendaal, The Netherlands). Frutafit® IQ with an
average DP of 9 (DP9-inulin; 84.9% > DP5) and Frutafit® TEX! with an average DP of 25 (DP25-
inulin; 99.6% > DP5) are chicory-based materials. Short-chain inulin with an average DP of 3
(DP3-inulin; Actilight®; 5% = monomers, DP2; 38.5% = DP3; 46% = DP4; 7.7% = DP5; 2.1% >
DP5), produced by enzymatic synthesis from sucrose, was obtained from Eridania Béghin-Say
( Neuilly sur Seine, France). All chemicals used were of the highest analytical grade.

**Strains and faecal flora**

Clostridium difficile ATCC 17857, a human isolate, was used in the experiments. It was cultivated
anaerobically in Schaedler bouillon at 37°C and added to the in vitro large intestinal model to a
final number of 5 x 10^8–1 x 10^9 cells per experiment.

Faecal microbiota samples were donated by 10 healthy, adult volunteers (employees of TNO
Nutrition and Food Research; average age 27 yrs). They were non-smokers and had not used
antibiotics or laxatives at least three months prior to the donation, nor had they used probiotic
bacteria 3 weeks prior to donation. Faecal samples were collected into a gastight bag, in a plastic
container containing an Anaerocult® strip to create anaerobic conditions. Within 10 minutes the
material was transferred into an anaerobic cabin. For the production of a standardized, cultivated faecal microbiota, which was used for the in vitro experiments, a mixture was made using about 2 g faeces from each individual. This sample was cultivated in a 5 L fermentor at 37°C and pH 5.8. Using fed batch fermentation a final volume of 5 L was obtained in approximately 40 h. The fermentation medium was the artificial ileal delivery medium as described by Gibson et al. (22), with some modifications (see below). Anaerobic conditions were maintained by flushing the fermentor with gaseous nitrogen. Adequately sized samples were snap-frozen in liquid nitrogen (with glycerol 20% v/v as cryoprotective agent) and stored at –80°C until further use.

Experiments in the in vitro model of the proximal colon (TIM-2)

Details of the in vitro model (TNO Intestinal Model, TIM-2, Figure 2.1) can be found in Minekus et al. (23) and Venema et al. (24). In short, the proximal colon model consists of four glass units with a flexible wall inside (peristaltic compartments). Water of body temperature (37°C) was pumped into the space between the glass jacket and the flexible wall, causing the microbiota to be mixed and moved. The sequential squeezing of the walls, controlled by a computer, caused a peristaltic wave forcing the material to circulate through the loop-shaped system. Appropriate electrolyte and metabolite concentrations in the lumen were maintained with a dialysis system consisting of hollow fibres, running through the lumen of the reactor, through which dialysis liquid was pumped.

The model further contained an inlet system for delivery of the artificial ileal delivery medium, and a level-sensor to maintain the luminal content at a set level. The system was kept anaerobic by flushing with gaseous nitrogen. At the start of each experiment the model was inoculated with approximately 30 mL of the standard, cultivated faecal microbiota. The microbiota was allowed to adapt to the model conditions for 16 h, after which the experiments started.
All experiments were performed in duplicate. Addition of the various inulins started after the adaptation period. The inulins were added to the standard ileal delivery medium (see below) and fed to the system at a rate of 10 g of inulin per day. In the experiments with *C. difficile*, the strain was introduced after the adaptation period in a single dosage of $5 \times 10^8$–$1 \times 10^9$ cells. In all experiments the pH was maintained at 5.8 by titration with 2 M NaOH and the temperature was kept at 37°C. Every 24 h a 45 mL sample was removed from the model to simulate passage of material from the proximal part to the distal colon.

In these luminal samples and samples of the dialysate taken at the same moment in time the concentrations of the various compounds as described below were determined. With these data and the known volume of the dialysate, the cumulative production of SCFA, BCFA, lactate, and ammonia in time was calculated. The presented data are the average of duplicate experiments, and were all within 10% of each average.

**Ileal delivery medium**

Gibson *et al.* (22) described a medium, which simulates the material passing the ileo-caecal valve in humans (ileal delivery). This medium was modified for the experiments in TIM-2 concerning the following components (g/L): 4.7 pectin, 4.7 xylan, 4.7 arabinogalactan, 4.7 amylopectin, 23.5 casein (all from Sigma-Aldrich, Zwijndrecht, the Netherlands), 39.2 starch (BDH, Amsterdam, The Netherlands), 17 Tween 80 (Merck, Amsterdam, the Netherlands), 23.5 bactopeptone (Oxoid, Haarlem, the Netherlands), 0.4 bile (Oxoid).

**Dialysate**

Dialysis liquid contained (per litre): 2.5 g K$_2$HPO$_4$.3H$_2$O, 4.5 g NaCl, 0.005 g FeSO$_4$.7H$_2$O, 0.5 g MgSO$_4$.7H$_2$O, 0.45 g CaCl$_2$.2H$_2$O (all from Merck), 0.05 g bile (Oxoid), and 0.4 g cysteine.HCl (BDH), plus 1 mL of a vitamin mixture containing (per litre): 1 mg menadione, 2 mg D-biotin, 0.5 mg vitamin B12, 10 mg pantothenate, 5 mg nicotinamide, 5 mg *p*-aminobenzoic acid, and 4 mg thiamine (all from Sigma-Aldrich).

**Analytical methods**

**SCFA/BCFA**

Samples were centrifuged (12,000 rpm, 5 min) and a mixture of formic acid (20%), methanol, and 2-ethyl butyric acid (internal standard, 2 mg/mL in methanol) was added to the clear supernatant. According to the method described by Jouany (25), a 0.5 µL sample was injected on a GC-column (Stabilwax-DA, length 15 m, ID 0.53 mm, film thickness 0.1 µm; Varian Chrompack, Bergen op Zoom, the Netherlands) in a Chrompack CP9001 gas chromatograph using an automatic sampler (Chrompack liquid sampler CP9050; Varian Chrompack).

**Lactate**

Samples for lactate analysis were centrifuged as described above. In the clear supernatant both L- and D-lactate were determined enzymatically (based on Boehringer, UV-method, Cat. No. 1112821) by a Cobas Mira plus autoanalyzer (Roche, Almere, the Netherlands).
Ammonia
Ammonia was measured with an ammonia selective electrode (Orion, Beverly, USA), after adjusting the sample with an ionic strength adjustor to bring the pH above 12. The high pH turned all NH$_4^+$-ions into NH$_3$. The electrode measured the released gas, and concentrations in the samples were determined via comparison with a series of standard solutions with known concentrations.

Phenolic compounds
Phenolic compound analyses were performed on a gas chromatograph (GC) according to methods described previously (26,27), with minor modifications. Chloroform was used for extraction of the phenolic compounds. After mixing well, samples were centrifuged (12,000 x g for 5 min), and the chloroform phase was diluted with methanol (1:10). To the diluted sample, 100 µL of the internal standard (p-nitrophenol, 10 mM) was added. Of the obtained mixture 0.5 µL was loaded onto a WCOT Fused Silica GC column (Varian Chrompack) using the automatic sampler.

Determination of C. difficile
All handlings were carried out in an anaerobic cabinet. Serial 10-fold dilutions were made in peptone/physiological saline (Oxoid) and were spread on CD agar (CM0601; Oxoid) with a C. difficile selective supplement (SR096; Oxoid) for enumeration of C. difficile. Plates were incubated anaerobically for 3 days at 37°C. The C. difficile strain used here (ATCC 17857) could be discriminated from other bacteria growing on CD agar due to its large colony size.

Results
SCFA production
Both total SCFA production and SCFA ratios were used for the description of the metabolic activity of a microbiota; straight lines of SCFA production means a continuous production (Figure 2.2).

Figure 2.2 Cumulative SCFA production after the addition of DP9-inulin and in the absence (A) and presence of C. difficile (B). The cumulative production of acetate (◊), propionate (○), n-butyrate (Δ) and of the sum of these (□) in time is shown. The additions (inulin and/or C. difficile) were made to the system at 16 h. The inulins were added at a rate of 10 g/day.
The main SCFA produced were acetate, propionate and butyrate (Figure 2.2 and Table 2.1). Valerate could hardly be detected in the experiments without *C. difficile*; under these circumstances the cumulative production of this compound remained below 1 mmol in 112 h. The total cumulative production as well as the SCFA ratios at the end of the experiments are represented in Table 2.1. Compared to the control experiment, the addition of inulins increased the total amount of SCFA produced and also the relative amount of propionate (Table 2.1). The percentage of butyrate decreased to about 15% with DP3-inulin. The total amount of SCFA increased with increasing chain length of the inulins (Table 2.1).

Table 2.1 The effect of inulins of various DP on the total cumulative SCFA production and SCFA ratios after 112 h of fermentation in TIM-2, in the absence (-C) or presence (+C) of *C. difficile*.

<table>
<thead>
<tr>
<th>SCFA</th>
<th>Control</th>
<th>DP3-inulin</th>
<th>DP9-inulin</th>
<th>DP25-inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total production (mmol)</td>
<td>-C 230</td>
<td>+C 210</td>
<td>-C 271</td>
<td>+C 144</td>
</tr>
<tr>
<td>Acetate (%)</td>
<td>45.6</td>
<td>61.8</td>
<td>51.0</td>
<td>47.9</td>
</tr>
<tr>
<td>Propionate (%)</td>
<td>18.7</td>
<td>0.6</td>
<td>34.3</td>
<td>20.8</td>
</tr>
<tr>
<td>Butyrate (%)</td>
<td>35.7</td>
<td>37.6</td>
<td>14.7</td>
<td>31.3</td>
</tr>
</tbody>
</table>

At 16 h *C. difficile* (single dosage) was added to the system and the addition of inulins was started at 10 g/day. After 112 h samples were taken, analysed for SCFA content as described, and the cumulative SCFA production and percentage of individual SCFA at 112 h were calculated.

The main effect of the addition of *C. difficile* to the model was a suppression of the propionate production (Table 2.1; Figure 2.2B), which also resulted in the total production of SCFA being less. This latter effect could be reversed by the addition of DP9- or DP25-inulin (210 mmol versus 244 respectively 250 mmol; see Table 2.1). With DP3-inulin and *C. difficile* the lowest level of SCFA production was found (Table 2.1). Higher levels of valerate were detected under the conditions with *C. difficile*, but they remained very low compared to the other SCFA (maximally about 4 mmol in 112 h was produced; data not shown).

**Lactate production**

Without inulins added there was almost no lactate produced, but it is clear from Table 2.2 that the addition of any type of inulin stimulated lactate production. The production was slightly larger with shorter chain length of the inulins. There was almost no difference in lactate production with or without the addition of *C. difficile*.
Table 2.2 The effect of inulins with various DP on the cumulative production of lactate after 112 h of fermentation in TIM-2 in the absence (-C) or presence (+C) of *C. difficile*.

<table>
<thead>
<tr>
<th>Lactate</th>
<th>Control</th>
<th>DP3-inulin</th>
<th>DP9-inulin</th>
<th>DP25-inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total production (mmol)</td>
<td>-C 6</td>
<td>+C 7</td>
<td>-C 165</td>
<td>+C 183</td>
</tr>
</tbody>
</table>

At 16 h *C. difficile* (single dosage) was added to the system and the addition of inulins was started at 10 g/day.

**BCFA production**

The BCFA iso-butyrate and iso-valerate arise from deamination of valine and leucine, respectively. Their production by the human colonic microbiota in these experiments was enhanced following the addition of *C. difficile* (Figure 2.3B) compared to the control (Figure 2.3A): it resulted in a two-fold increase of iso-valerate production. Both without and with the pathogen added, the production of BCFA was strongly reduced by the addition of inulins (Figure 2.3C and 2.3D, respectively; only shown for DP9-inulin). Similar effects were observed with DP3- and DP25-inulin (data not shown).

![Figure 2.3](image_url)

**Figure 2.3** Cumulative production of iso-butyrate (◊) and iso-valerate (□) in time during control experiments (A), with the addition of *C. difficile* (B), DP9-inulin (C), or both DP9-inulin and *C. difficile* (D). The additions as described were made to the system at 16 h. DP9-inulin was added at a rate of 10 g/day.
Ammonia production

As with the BCFA production, the ammonia production in the experiments without C. difficile (35 mmol) was suppressed by the addition of inulins by about 30% after 112 h. Introduction of C. difficile resulted in a strong stimulation of ammonia formation (Figure 2.4). This stimulation was suppressed completely by the addition of DP25-inulin, as shown in Figure 2.4. The described effects were identical for DP3- and DP9-inulin (data not shown), indicating that the effect was independent of the average chain length of the fructan.

![Figure 2.4] Figure 2.4 effect of the addition of DP25-inulin (□, ■) compared to the control (◊, ♦) on the cumulative production of ammonia in the absence (open symbols) or presence (closed symbols) of C. difficile. At 16 h C. difficile was introduced to the system and the addition of DP25-inulin was started at a rate of 10 g/day.

Phenolic compounds

The production of phenolic compounds (indole, p-cresol and phenol; skatole could not be detected) was low under all conditions tested. Their presence could only be shown in the lumen samples and not in the dialysis fluid. Hence cumulative production could not be calculated for these components. After the introduction of C. difficile the concentration of indole increased from 612 to 620 µmol/L and only under this condition were p-cresol (143 µmol/L) and phenol (17 µmol/L) detected. In none of the experiments in which inulins were added, the phenolic compounds were present in detectable amounts (data not shown). These results indicated that the various inulins are able to diminish the production of these compounds.

C. difficile content

In all the experiments where C. difficile was added to the in vitro system, their number remained at the starting value (data not shown). Also the content of other microbiological groups did not change (data not shown).
Discussion

The TNO in vitro model has been developed and validated using data from sudden death individuals (11,23). Both with respect to composition and with respect to metabolic activity, a faecal inoculum in the model has been shown to simulate the data from these sudden death individuals very well. In addition, we have performed experiments in the model using different microbiotas from dogs. The microbiotas were originating from the caecum and from faecal material. The microbiotas maintained in TIM-2 originating from either the caecum or faeces were shown to have the same composition and microbial activity as those of the microbiota when freshly obtained from the caecum, supporting the hypothesis that the microbiota in TIM-2 does develop to resemble that of the caecum (unpublished data). We hypothesize that this is also the case for a human microbiota. A human microbiota could be maintained stable in TIM-2 for up to three weeks on the standard pre-digested food, without changes in microbial composition or activity (23).

In the experiments without C. difficile the addition of inulins suppressed the formation of BCFA (Fig. 2.3), ammonia (Fig. 2.4), and phenolic compounds (data not shown) while at the same time SCFA and lactate production was stimulated (Fig. 2.2 and Table 2.2, respectively). Since BCFA, ammonia, and phenolic compounds are products typical of breakdown and fermentation of proteins it can be concluded that inulins inhibit the proteolytic activity of the colonic microbiota and stimulate the saccharolytic activity, as indicated by the increased SCFA and lactate production. With C. difficile introduced into the model, the effects of inulin became even more pronounced. The increase in proteolytic activity caused by C. difficile was suppressed to a large extent by the addition of the inulins, irrespective of the chain length. These results corroborate the hypothesis that fermentable fibres, such as inulin, decrease the amount of protein fermentation products by repressing protein fermentation itself. In an in vitro batch incubation, a reduction of phenol and p-cresol production by 63% and 78%, respectively, was observed during fermentation of a readily fermentable source of carbohydrate (starch) by human faecal bacteria (26). Instead of being fermented, the amino acids and ammonia are incorporated into the bacterial biomass; Levrat et al. (28) observed an increase in the use of ammonia by the microbiota of rats after the addition of inulin to their diet.

Our results demonstrate clearly the potential of inulins to shift the metabolic activity of the human colonic microbiota into a direction resulting in the production of potentially less toxic products, without major changes in the composition of this microbiota. For the inulins with varying DP grossly similar effects were found for the potential to induce changes in the metabolism of the colonic microbiota in this system. So the prebiotic activity (i.e. the ability to bring about a metabolic shift) of the various inulins seems to be relatively independent of the chain length. This may seem surprising in view of the differences reported for the bifidogenic effects of inulins in relation to chain length, with DP3-inulin suggested to be more effective than DP25-inulin (29). However, in the present study we could not find a major difference for the changes in metabolic activity brought about by the different inulins. Our hypothesis is that the complex microbiota present in TIM-2 is equally able to ferment long-chain inulin as short-chain inulin. We propose that endo-inulinase activity quickly reduces long-chain inulin into short-chain molecules. It may well be
that different species are responsible for the fermentation of different inulins, but that the overall metabolic effect is the same.

The introduction of only a small number of *C. difficile* bacteria (only about 1% or less of the total bacterial population was added) caused a major shift in the overall metabolism of the colonic microbiota towards proteolysis. The fact that the number of *C. difficile* did not change after inulin addition is probably due to the constant pH of the system, as May *et al.* (30) suggested that a decrease in pH is necessary to inhibit *C. difficile* growth. Apparently, the metabolic balance, disturbed by the introduction of *C. difficile*, cannot be restored by the microbiota alone, since there was no normalisation of the metabolism during the time course of these experiments without addition of inulins. For instance, the cumulative production of ammonia in time remained virtually unchanged (Fig. 4). Apparently, the addition of e.g. inulins is required to shift the metabolism from proteolysis to saccharolysis. Similar data were found by Terada *et al.* (31) following lactulose consumption by human volunteers. Lactulose consumption suppressed the formation of proteolytic products in faeces (31). In addition, Hidaka *et al.* (32) found similar results in a human study: consumption of short-chain inulins (DP3-inulin) led to a decrease in the faecal concentrations of BCFA and phenolic compounds.

Since we used samples of a standardized faecal microbiota (grown in a fed-batch fermentor) as inoculum, the results of the different experiments can be properly compared. We have previously shown that the microbial composition and activity of the inoculum did not differ substantially from that of the fresh faecal microbiota ((23,24) and data not shown). Therefore we presume that the results as presented reflect the possible *in vivo* effects of inulins or a *C. difficile* infection.

The mechanism causing the differences in metabolic activity between the *C. difficile* containing microbiota with and without the addition of inulin is postulated to be that the inulins also provide a suitable carbon source for the *C. difficile* bacteria, thus preventing protein fermentation. The *C. difficile* strain used in this study was able to ferment the inulins used in this study (unpublished observations) and it is known that *C. difficile* VPI 10463 can grow on short-chain inulins (31). Since clostridia are involved in butyrate production, the observation of a higher relative amount of butyrate with DP3- and DP-25 inulin in combination with *C. difficile* is in accordance with the proposed mechanism described above.

The cause of the decreased propionate production after addition of *C. difficile* or its restoration after inulin addition remains obscure. It could well be that the increased lactate formation following the addition of inulins (Table 2.2) leads to an increased propionate formation (33), but the reduction of propionate production remains unexplained.

The results show that even in a microbiota originating from healthy humans, a low intake of proteins (approximately 5 g/day in the present study) can result in the production of potentially toxic or harmful metabolites. Since the model represents the proximal colon, this indicates that even in a region of the gut where protein fermentation is of minor importance these substances can be produced. Inulins can reduce this production, whereas addition of the *C. difficile* can stimulate it. It is of general interest to determine whether individuals with chronic bowel problems, such as inflammatory bowel disease patients, show evidence of an altered metabolic function of the microbiota in the large intestine and whether this is related to the onset or progression of the disease. Dietary interventions in these patients may have the same effect on the metabolic activity of the microbiota as that shown for healthy individuals. We are currently investigating this.
The relevance of these findings is that the prebiotic action of inulin clearly is not limited to a mere (bifidogenic) change in the composition of the colonic microbiota, but includes a shift in metabolic activity; from proteolysis to saccharolysis. It is certainly a feature of the in vitro system employed in this study that such an effect can be found. In vivo, adequate sampling is impossible, since one can only take faecal samples from which most of the fermentation products (SCFA, ammonia) will already have been absorbed by the body (34,35). It is therefore not surprising that others could not find a change in faecal SCFA after consumption of inulin by human volunteers despite a significant bifidogenic change in bacterial composition of the faecal microbiota (6,34). Similarly, we have found no changes in faecal SCFA after lactulose treatment, whereas the SCFA production in TIM-2, simulating the proximal colon, did change (36).

The effect on metabolic activity seems much more relevant for any potential health implication of the use of inulins in food than a mere shift in bacterial composition. Model studies such as those reported here may prove to be an important tool for establishing the effects of (potential) prebiotic ingredients on the metabolic activity of the colonic microbiota. The model has already been used with cultivated human microbiota from healthy individuals to simulate conditions in the proximal colon (24). From the present study it can be concluded that this in vitro model of the large intestine can also be used to study the effects of prebiotics in the gastrointestinal tract of humans infected with a pathogen or with a disturbed microbiota.
Chapter 2

References


The metabolic activity of faecal microbiota from healthy individuals and patients with inflammatory bowel disease

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Digestive Diseases and Sciences 2004;49:485-91
Abstract

The hypothesis was studied that intestinal microbial metabolites play a role in the pathogenesis of inflammatory bowel disease. For that purpose, an *in vitro* model of the colon was inoculated with fresh feces of 6 healthy individuals and 8 inflammatory bowel disease patients. Samples were taken from the model over time to analyze metabolites from both saccharolytic- and proteolytic fermentation. Microbiotas from inflammatory bowel disease patients produced significantly more short-chain fatty acids and ammonia than microbiotas from healthy individuals. Furthermore, the branched-chain fatty acid production was 25% higher after inoculation with microbiotas from patients than after inoculation with microbiotas from healthy individuals. Phenolic compounds were produced by all microbiotas with large interindividual variation. The production of (potentially toxic) metabolites may play a role in the onset or chronicity of inflammatory bowel disease, because they were produced in higher amounts by microbiotas from these patients than by microbiotas from healthy individuals.
Introduction

Ulcerative colitis (UC) and Crohn’s disease (CD) are intestinal inflammatory disorders with unknown etiology. Inflammatory bowel disease (IBD), the collective term for UC and CD, is possibly caused by a disregulated immune response to antigens of the enteric bacterial microbiota in a genetically susceptible host (1,2). In this hypothesis, host genetics, immune response, and bacterial microbiota are inextricably linked. It is unknown whether patients with IBD have an altered metabolic function of the intestinal microbiota and whether this is related to the severity of the disease. Currently, two hypotheses regarding the role of microorganisms in IBD are under investigation. The first suggests that specific microorganisms play a role in the induction of inflammation (3-7). The second suggests that metabolites derived from the microbiota, without regard to species, can be mediators of injury and inflammation (8,9).

Colonic fermentation is an anaerobic process in which carbohydrates and proteins are metabolized by the large intestinal microbiota to salvage energy (10). This process is regulated by the composition of the colonic microbiota as well as by the amount and type of substrate that enters the colon, consisting of dietary residues and host-produced substances (8,10-12). Protein fermentation, in particular, gives rise to a variety of metabolites that are potentially toxic, including phenolic compounds, branched-chain fatty acids (BCFA), and ammonia (10,13). However, little is known about their production and their biological role in vivo. The within-subject variation turned out to be high in studies using colonic contents (8) or faeces (12) to determine concentrations of phenol, p-cresol, BCFA, and ammonia, respectively indole and skatol. Studies focusing on metabolites in different regions of the colon showed that the proteolytic activity of gut bacteria is higher in the distal parts, where pH is increasing (4,8,14). The protein fermentative metabolites have been associated with a variety of disease states in humans, including schizophrenia and cancer. They seem to have several cytopathic effects on colonic epithelial cells, including reduction of their lifespan (4). Protein fermentation potentially plays a role in IBD by causing damage to epithelial cells in genetically susceptible hosts, resulting in inflammatory reactions. Besides a higher proteolytic activity, a lower saccharolytic activity can possibly also influence the pathogenesis of IBD. Especially depletion of butyrate or prevention of the oxidative use of butyrate by epithelial cells (e.g. by sulphur compounds) leads to loss of integrity of these cells and, eventually, results in death and epithelial desquamation. The latter is presumably the case in UC patients (15,16). Renewal of the lost cells increases proliferation and may eventually influence the chance of cancer development (17). In addition, the increased desquamation and a lack of short-chain fatty acids (SCFA) lead to a decreased barrier function and make the colonic epithelium more susceptible to inflammation (18). Furthermore, the presence of carbohydrates influences proteolytic fermentation. Fermentation of carbohydrates, resulting in a pH decline, reduced production of phenol and p-cresol in vitro, by 63% and 78%, respectively (14), and led to an increased use of ammonia for use in biosynthetic reactions of the microbiota (4,8). We recently showed that inulins can reduce the production of protein fermentative metabolites (19).

Faeces is not a good indicator of the fermentative activity that occurs in the proximal colon (20). For IBD patients, investigating faecal material is even more complex. Damage to the mucosa of
IBD patients caused by inflammations can result in inadequate absorption and utilization of metabolites and therefore results in higher concentrations of metabolites in their feaces than in that of healthy individuals (21). With feaces, no distinction can be made between the above-mentioned theory and an actual altered metabolic functioning by luminal bacteria from IBD patients. The goal of this study was to determine whether patients with IBD show evidence of an altered metabolic function of the microbiota in the proximal colon. This was studied in an in vitro model of the proximal colon.

Materials and Methods

Faecal microbiota

Six healthy individuals (aged 23-31 years) and eight IBD patients with colitis in remission, five with CD affecting the colon and three with UC, aged 28-53 years, were studied. The six healthy volunteers (H1–H6) and two of the CD patients (IBD1 and IBD2) had not taken any medication or probiotics in the 6 months prior to study. The other IBD patients (IBD3–IBD8) had not taken probiotics, but were treated with mesalazine (n=4) and corticosteroids (budenoside, n=4; prednison, n=3).

An early-morning faecal sample was collected from each subject at home and immediately placed into gastight bags containing an Anaerocult® strip to create anaerobic conditions during transport to the anaerobic cabinet. This transport never lasted longer than 3 h. A faecal sample of approximately 30 g was mixed with artificial ileal delivery medium (80 mL; see Gibson et al. (22) and van Nuenen et al. (19)) in an anaerobic cabinet to get a solution resembling the content of the human, proximal colon. These mixtures were used as inocula for TNO’s in vitro model of the large intestine (TIM-2).

Experiments in TNO’s in vitro model of the large intestine (TIM-2)

Details of the in vitro model (TIM-2, Figure 3.1) are given by Minekus et al. (23) and Venema et al. (24). In short, TIM-2 is a computer controlled in vitro model that simulates the human proximal colon (23). It consists of a number of linked glass units with inner flexible walls. Water at 37°C can be pumped into the space between the glass jacket and the flexible wall, yielding peristaltic movements and causing the content to be mixed and moved. Appropriate electrolyte and metabolite concentrations in the lumen were maintained with a dialysis system consisting of hollow-fibres, running through the lumen, through which dialysis liquid (22,19), was pumped. The model further contained an inlet system for delivery of the artificial ileal delivery medium, and a level-sensor to maintain the luminal content at a set level. The system was kept anaerobic by flushing with gaseous nitrogen, to allow for the growth of a dense, complex microbiota, comparable in composition and activity to that found in humans in the proximal colon (24). The model enabled the study of metabolic activity of the colonic microbiota under physiological circumstances.
During each experiment, the TIM-2 system was inoculated with the mix of fresh feces and ileal delivery medium, from a single study subject. The microbiota was allowed to adapt to the model conditions for 16 h, after which the actual experimental period (48 h) started. In all experiments the pH was maintained at 5.8 by titration with 2 M NaOH, the temperature was kept at 37°C, and the microbiota was fed semi-continuously (4.6 mL/h) with the ileal delivery medium. To simulate bowel movements, 30 mL of contents was removed from the model twice daily. In the luminal samples and samples of the dialysis liquid, taken at 16, 40, and 64 h after inoculation, the concentrations of the various metabolites were determined as described below. With these data and the known volume of the dialysis liquid, cumulative productions over time were calculated (mmol or µmol).

**Analyses**

**SCFA, BCFA and lactate**

SCFA (acetate, propionate, butyrate, valerate) and BCFA (iso-butyrate and iso-valerate) analysis were performed on a gas chromatograph (Chrompack CP9001; Varian Chrompack, Bergen op Zoom, the Netherlands) essentially according to the method described by Jouany (25). After centrifugation of the samples (12,000 rpm, 5 min), the clear supernatant was diluted (7%, by volume) with a mixture of methanol, internal standard (2 mg/mL 2-ethyl butyric acid) and formic acid (20%). Of this mixture 0.5 µL was loaded onto a “wide-bore” GC column (Stabilwax-DA; length, 15 m; ID, 0.53 mm; film thickness, 0.1 µm; Varian Chrompack) using an automatic sampler (Chrompack CP9050; Varian Chrompack). Samples for lactate analysis were centrifuged as described for SCFA analysis. In the clear supernatant both L- and D-lactate were determined enzymatically (based on Boehringer, UV-
method, Cat. No. 1112821) by a Cobas Mira plus autoanalyzer (Roche, Almere, the Netherlands). The analysis is based on the conversion of NAD into NADH.

**Phenolic compounds**

Phenolic compound analyses (phenol, \( p \)-cresol, indole, skatol) were performed on a gas chromatograph based on methods described previously (14,26-29). For extraction of the phenolic compounds, 0.5 mL of chloroform was added to the samples. After mixing well, samples were centrifuged (4,500 rpm, 5 minutes). Fifty \( \mu \)L of the chloroform phase was diluted with methanol (1:10). To the diluted sample, 100 \( \mu \)L internal standard (\( p \)-nitrophenol; 1.5 mg/mL in methanol) was added. Of the mixture obtained, 0.5 \( \mu \)L was loaded onto a WCOT Fused Silica GC column (length, 25 m; ID, 0.53 mm; film thickness, 1 \( \mu \)m; Varian Chrompack) using the automatic sampler.

**Ammonia**

Ammonia was measured with an ammonia selective electrode (Orion, Beverly, CA, USA), after the addition of an ionic strength adjustor (ISA) to raise the pH above pH 12. This turned the \( \text{NH}_4^+ \) ions into gaseous \( \text{NH}_3 \). The electrode measured the released gas, and concentrations in the samples were determined via comparison with a series of standard solutions with known concentrations.

**Statistical analyses**

Statistical analyses were performed using the Student’s \( t \)-test. A \( p \leq 0.05 \) was considered significant.

**Results**

**SCFA production**

The total cumulative SCFA production ranged from 98 to 137 mmol in the eight IBD patients (mean, 115 mmol; SD, 11.2 mmol; Table 3.1), whereas production ranged from 63 to 102 mmol for microbiotas from healthy individuals (mean, 89 mmol; SD, 16 mmol). IBD patients produced a significantly higher amount of SCFA than healthy individuals (\( p=0.0036 \)). In four controls and all eight IBD patients acetate production was higher than that of other SCFA (Table 3.1); two healthy individuals (H3 and H6) produced more butyrate.
Table 3.1 SCFA ratios (%) and total cumulative production (mmol) by different types of fresh faeces after 64 h of fermentation in TIM-2.

<table>
<thead>
<tr>
<th>Individual</th>
<th>C2 (%)</th>
<th>C3 (%)</th>
<th>C4 (%)</th>
<th>Total (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBD1</td>
<td>53</td>
<td>24</td>
<td>23</td>
<td>137</td>
</tr>
<tr>
<td>IBD2</td>
<td>64</td>
<td>12</td>
<td>25</td>
<td>107</td>
</tr>
<tr>
<td>IBD3</td>
<td>46</td>
<td>26</td>
<td>28</td>
<td>119</td>
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<tr>
<td>IBD4</td>
<td>54</td>
<td>21</td>
<td>25</td>
<td>98</td>
</tr>
<tr>
<td>IBD5</td>
<td>46</td>
<td>17</td>
<td>37</td>
<td>116</td>
</tr>
<tr>
<td>IBD6</td>
<td>57</td>
<td>17</td>
<td>26</td>
<td>112</td>
</tr>
<tr>
<td>IBD7</td>
<td>55</td>
<td>24</td>
<td>22</td>
<td>118</td>
</tr>
<tr>
<td>IBD8</td>
<td>44</td>
<td>32</td>
<td>25</td>
<td>116</td>
</tr>
<tr>
<td>H1</td>
<td>52</td>
<td>16</td>
<td>32</td>
<td>99</td>
</tr>
<tr>
<td>H2</td>
<td>50</td>
<td>21</td>
<td>29</td>
<td>98</td>
</tr>
<tr>
<td>H3</td>
<td>37</td>
<td>23</td>
<td>40</td>
<td>98</td>
</tr>
<tr>
<td>H4</td>
<td>38</td>
<td>29</td>
<td>33</td>
<td>63</td>
</tr>
<tr>
<td>H5</td>
<td>49</td>
<td>21</td>
<td>30</td>
<td>102</td>
</tr>
<tr>
<td>H6</td>
<td>20</td>
<td>22</td>
<td>58</td>
<td>76</td>
</tr>
</tbody>
</table>

Lactate production

Lactate production did not differ between IBD patients (mean, 2.1 mmol; SD, 1.5 mmol) and healthy individuals (mean, 1.9 mmol; SD, 0.9 mmol; Table 3.2). Total lactate production after 64 h ranged from 0.01 to 3.97 mmol and the amount of D-lactate was always almost twice as high as that of L-lactate (except for individual IBD6; Table 3.2).

Table 3.2. Ratios (%) and total production (mmol) of L- and D-lactate by different types of fresh faeces after 64 h of fermentation in TIM-2.

<table>
<thead>
<tr>
<th>Individual</th>
<th>L (%)</th>
<th>D (%)</th>
<th>Total (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBD1</td>
<td>33</td>
<td>67</td>
<td>3.70</td>
</tr>
<tr>
<td>IBD2</td>
<td>33</td>
<td>67</td>
<td>3.97</td>
</tr>
<tr>
<td>IBD3</td>
<td>27</td>
<td>73</td>
<td>1.84</td>
</tr>
<tr>
<td>IBD4</td>
<td>28</td>
<td>72</td>
<td>1.19</td>
</tr>
<tr>
<td>IBD5</td>
<td>31</td>
<td>69</td>
<td>1.87</td>
</tr>
<tr>
<td>IBD6</td>
<td>0</td>
<td>100</td>
<td>0.01</td>
</tr>
<tr>
<td>IBD7</td>
<td>48</td>
<td>52</td>
<td>3.90</td>
</tr>
<tr>
<td>IBD8</td>
<td>44</td>
<td>56</td>
<td>0.50</td>
</tr>
<tr>
<td>H1</td>
<td>36</td>
<td>64</td>
<td>1.37</td>
</tr>
<tr>
<td>H2</td>
<td>34</td>
<td>66</td>
<td>1.08</td>
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<tr>
<td>H3</td>
<td>35</td>
<td>65</td>
<td>2.78</td>
</tr>
<tr>
<td>H4</td>
<td>38</td>
<td>62</td>
<td>2.64</td>
</tr>
<tr>
<td>H5</td>
<td>34</td>
<td>66</td>
<td>2.62</td>
</tr>
<tr>
<td>H6</td>
<td>28</td>
<td>72</td>
<td>0.76</td>
</tr>
</tbody>
</table>
Chapter 3

BCFA production

The cumulative production of BCFA is shown in Figure 3.2. As of the start of all experiments, BCFA production was rather stable. Overall, the healthy individuals (mean, 3.80 mmol; SD, 1.45 mmol) had an *in vitro* BCFA production that was 25% lower (p=0.053) than that of the IBD patients (mean, 5.10 mmol; SD, 0.81 mmol). Cumulative production was highest for patient IBD3 (5.88 mmol) and lowest for control H6 (1.62 mmol; Figure 3.2). The ratio for *iso*-butyrate versus *iso*-valerate in TIM-2 was 40:60 in all experiments (data not shown).

![Graph showing BCFA production](image1)

**Figure 3.2** Total cumulative BCFA production (mmol; sum of *iso*-butyrate and *iso*-valerate) by different types of fresh faeces, during 64 h in TIM-2.

Ammonia production

Ammonia production was stable during the course of the experiments (Figure 3.3). On average, the healthy individuals produced significantly less ammonia than the IBD patients (28 mmol; SD 8.3 mmol versus 42 mmol; SD 6.3 mmol; p=0.0034). Individual IBD6 produced the highest cumulative ammonia amount (47 mmol). The lowest production was 16 mmol, by individual H6.
**Microbial activity in IBD**

**Figure 3.3** Total cumulative ammonia production (mmol) by different types of fresh feces, during 64 h in TIM-2.

**Phenolic compounds production**

Only indole and $p$-cresol were produced in all TIM-2 experiments. Phenol and skatol production was rare. Overall, production of phenolic compounds was not stable during the two experimental days (Figure 3.4). Furthermore, both cumulative production and the ratios of the compounds differed widely between subjects within one group (IBD, SD = 62% of mean; healthy, SD = 81% of mean). Cumulative production after inoculation of TIM-2 reached various levels, from 21 to 203 µmol.

**Figure 3.4** Total cumulative phenolic compounds production (µmol; sum of phenol, $p$-cresol, indole, and skatol) by different types of fresh feces, during 64 h in TIM-2.
Discussion

Microbes and their metabolic products very likely play a role in IBD. This hypothesis is based on various arguments, obtained both in humans and in animals, including the effect of deviation of the faecal stream on disease activity in CD. Unfortunately, there is a lack of solid data to substantiate the hypothesis. This is primarily due to the fact that the study of microbial metabolic activity is difficult, for among others, reasons of sampling and difficulty mimicking physiological circumstances *ex vivo*. Our TIM-2 system overcomes these difficulties and is therefore relevant for the study of microbial metabolic activities (19,23,24,30). The goal of this study was to determine whether patients with IBD show a different metabolic activity of the microbiota in the proximal colon in comparison with healthy controls. We observed higher production of short- and branched-chain fatty acids, as well as ammonia, in the experiments using faeces of IBD patients compared to that of controls. These differences were not observed for the production of phenolic compounds and lactate, which varied greatly. Furthermore, standard deviations for all determined metabolites (except lactate) were smaller in the IBD group than in the healthy control group, which is surprising, considering the differences in disease course and use of medication in the IBD patients. The higher metabolite production in IBD patients is in agreement with literature data obtained with other methods (16,21,31).

These data suggest that increased metabolite concentrations may affect IBD. The cause of the higher metabolic activity in these patients is unknown. It seems unlikely that only damage to the mucosa, resulting in inadequate absorption of these metabolites, is the cause of the increased metabolite concentrations.

Our results may have been influenced by a number of potential confounders. During the 64 h of fermentation in TIM-2, the pH was set at 5.8. This is the pH in the proximal colon of healthy individuals (32). No extensive research is done in IBD patients concerning the pH in (this part of) the colon. Another debatable point is the digestion of food in the stomach and small intestine; if this function of the gastrointestinal tract in IBD patients also deviates from healthy individuals, the IBD microbiota in TIM-2 are fed other substrates than *in vivo*, which will result in different metabolite profiles. Without extensive knowledge of the environmental circumstances in the proximal IBD colon, our results here may not totally reflect the events *in vivo*.

The proteolytic activity of the gut microbiota can increase remarkably, even by a factor of 5, in the distal colon compared to the proximal colon (4,14,33). Therefore fermentation in the transverse and descending parts of the colon will give more insight into the colonic health status. An *in vitro* system simulating this distal part, with physiological parameters such as lower water content and a higher pH, is in development at our institute and will be used in the future to study the metabolic activity of microbiotas obtained from IBD patients under relevant physiological conditions.

Our results show also that feeding microbiotas of healthy humans approximately 5 g of protein per day, which is only one third of the estimated amount that becomes available in the proximal colon *in vivo* (34,35), results in production of detectable amounts of potentially toxic or harmful metabolites, even when the environmental conditions mimic those of the healthy proximal colon. The ability of the colonic mucosa to detoxify these substances seems to be impaired in UC (14),
causing the colonic tissue of patients to be more susceptible to the toxicity of these compounds than colonic tissue of healthy individuals. If so, the microbial activity in the colon of IBD patients can certainly serve as an indicator of disease severity. However, without ex vivo or in vivo tests on the toxicity of these compounds, we cannot conclude which metabolites (and at which concentrations) cause injury to the epithelial barrier of IBD patients.

Once the role of the metabolic activity of IBD microbiotas has become more clear, dietary interventions of IBD may become feasible. The effect of pre- and probiotics and other (functional) food components on the composition and metabolic activity of different microbiotas are currently being studied in our institute.

In conclusion, we demonstrated that the colonic microbiota from IBD patients produced significantly more short- and branched-chain fatty acids, as well as ammonia, than microbiotas from healthy individuals. The production of (potentially toxic) metabolites may play a role in the onset or chronicity of IBD because they are produced in higher amounts by microbiotas from these patients than by microbiotas from healthy individuals.
References


The influence of microbial metabolites on human intestinal epithelial cells and macrophages *in vitro*

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Koen Venema

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Abstract

Microbial metabolites may influence the metabolic integrity of intestinal epithelial cells and induce mucosal immune responses. Therefore we investigated the effects of the microbial metabolites butyrate, iso-valerate, and ammonium on Caco-2 cells and macrophages. Barrier functioning was determined by measuring transepithelial electrical resistance and basolateral recoveries of metabolites. Tumour necrosis factor-α and interleukin-10 were measured to determine immune reactions. The barrier function of Caco-2 cells remained intact after exposures. Basolateral recoveries ranged from 6.2% to 15.2%. The Caco-2 cells did not secrete both cytokines. Physiological concentrations of butyrate and iso-valerate stimulated the secretion of tumour necrosis factor-α and suppressed the secretion of interleukin-10 by macrophages that are not protected by an epithelial barrier. In contrast, ammonium concentrations as high as those produced by microbiotas of IBD patients suppressed the release of both cytokines when the barrier function is impaired.
**Introduction**

An intact gastrointestinal mucosa, with a normal intestinal permeability and barrier function, is essential for absorption, secretion and transport. Intestinal epithelial cells (IEC) that line the gastrointestinal mucosa can be regarded as the most outer defence barrier preventing microorganisms and endotoxins from reaching systemic organs and tissues (1-4).

The relation between IEC, luminal microbiota, and inflammatory bowel disease (IBD) appears evident. Intestinal epithelial cells play an important role in the interaction between luminal content and the host’s immune system (2,3,5), while a role for microbiota in triggering the inflammatory process has been proposed (6-8). Both luminal microorganisms and their metabolites can possibly influence the metabolic integrity of the IEC, resulting in presentation of these antigens to the mucosal immune system (9-11).

The mucosal immune system controls the production of cytokines. CD4+ T-helper (T\text{H})-1 cells mediate cellular immunity and macrophage activation. Activated macrophages produce proinflammatory cytokines, including TNF-\text{\textalpha}. T\text{H}-2 cells mediate humoral immunity and produce anti-inflammatory cytokines, including IL-10. In IBD, the T\text{H}-1/ T\text{H}-2 balance is disturbed. An increase in T\text{H}-1 cytokines is detected in Crohn’s disease (CD) patients, whereas in patients affected by Ulcerative colitis (UC) T\text{H}-2-like immune activation has been demonstrated (10,12).

In a previous study an in vitro colon model was used to study the role of microbial metabolites in the pathogenesis of IBD. Microbiotas from IBD patients produced significantly more short-chain fatty acids (SCFA, including butyrate) and ammonium than microbiotas from healthy individuals. Furthermore, the branched-chain fatty acid (BCFA, \textit{iso}-butyrate and –valerate) production was 25% higher after inoculation with microbiotas from patients than after inoculation with microbiotas from healthy individuals (13). Butyrate is a major source of energy for colonic epithelial cells. Depletion of butyrate leads to a decreased barrier function and results in a higher susceptibility of the colonic epithelium to inflammation (10,12). UC may be associated with a mucosal defect in the metabolism of butyrate (3). BCFA and ammonium are potentially toxic (on colonic epithelial cells), although little is known about their biological role \textit{in vivo} (14).

We hypothesized that the increase in production of microbial metabolites contributes to IEC injury and/or loss of the integrity of the barrier function in IBD. Enhanced presentation of these microbial metabolites to the mucosal immune system may then play a role in the maintenance of IBD activity. We investigated which metabolites, and at which concentrations, cause injury to epithelial cells and thereby might modulate unwanted immune reactions. This was studied by co-culturing enterocyte-like Caco-2 cells with macrophages in separate compartments of a Transwell\textsuperscript{TM} system. In addition, the direct effect of metabolites on Caco-2 cells and macrophages was tested.
Chapter 4

Materials and Methods

Culture systems

Caco-2 mono-culture
Human colon carcinoma Caco-2 cells (ATCC; Rockville, MD, USA) were cultured (37°C; 5% CO₂) in medium A: Dulbecco’s Modified Eagle Medium, 4.5 g/L glucose, 1% (v/v) MEM non-essential amino acids (100x), 25 mM HEPES, 2 mM L-glutamine, 0.5% 50 mg/mL gentamycin (all from Life Technologies BV, Breda, The Netherlands), 10% (v/v) fetal calf serum (Cambrex Bio Science, Verviers, Belgium). This medium was changed every 2-3 days. Caco-2 cells were then seeded (10⁵ cells/cm²) in Transwell™ polycarbonate cell culture inserts (12 well) with a mean pore size of 0.4 µm (Corning Inc, Schiphol, The Netherlands) and cultured for 15 to 21 days to obtain differentiated monolayers (15).

U937 mono-culture
Human monocyte-like histiocytic lymphoma U937 cells (ATCC; CRL-1593.2, Manassas, VA, USA), were grown (37°C; 5% CO₂) in medium B: RPMI-1640 medium (Life Technologies BV), 10% (v/v) fetal calf serum (Cambrex Bio Science), and 2 mM L-glutamine (Life Technologies BV). The U937 cells were seeded in 12-wells plates (2*10⁶ cells/well) and differentiated overnight into macrophages using phorbol 12-myristate 13-acetate (PMA, 10 ng/mL; Omnilabo, Breda, The Netherlands). Before exposure to the microbial metabolites (see below), the macrophages were allowed to recover from PMA treatment for 48 h, during which culture medium was replaced every day (16).

Co-culture model
Transwell™ inserts covered with differentiated Caco-2 cells were transferred to 12-well culture plates seeded with macrophages. Before transfer, transepithelial electrical resistance (TEER) was measured (see below) and medium A was removed from the inserts (apically). Medium B, used in the basolateral compartment of the co-culture model, was refreshed before the Caco-2 inserts were placed into the compartments.

Exposure to microbial metabolites
Sterile stock solutions of single sodium salts of butyrate (C4) and iso-valerate (i-C5), and ammonium (NH₄⁺)-chloride were prepared in medium A (Caco-2 mono-cultures and co-cultures) or medium B (U937 mono-cultures). Concentrations of the metabolites tested were based on experiments in TNO’s in vitro model of the colon in which faeces from IBD patients or healthy volunteers was used as inoculum (13). Cell viability was determined by cytotoxicity tests (MTT) prior to the actual exposures (data not shown).
Caco-2 mono-culture
Caco-2 mono-cultures were exposed to microbial metabolites to determine 1) injury or loss of barrier function and 2) cytokine production, possibly interfering with the cytokine production by macrophages.

Injury or loss of barrier function were determined by measuring transepithelial electrical resistance (TEER) and basolateral recoveries of the used metabolites. Concentrations of C4, i-C5, and NH₄⁺ were tested in triplicate (pH 6.3-7.3; 10-100 mM) to determine dose-response curves. After the addition of metabolite solutions to the apical compartment of Caco-2 monolayers (TEER>500 Ω*cm²), TEER was measured for a 24 h period (at 0, 0.5, 1, 2, 4, 6, and 24 h) using a Millicel-ERS epithelial voltohmmeter with Ag/AgCl electrodes (Millipore, Bedford, USA) in iso-osmotic (approximately 300 mOsm) media. Metabolites were measured before and after 6 h of exposure of the Caco-2 mono-cultures to calculate mass-balances.

To determine TNF-α and IL-10 secretion by Caco-2 cells upon exposure to microbial metabolites, the following solutions were tested: 50, 100, and 100 mM + lipopolysaccharide (LPS, 1 µg/mL; Sigma-Aldrich, Zwijndrecht, The Netherlands) C4 and i-C5 and 40, 60, 20 mM + LPS, and 40 mM + LPS NH₄⁺. Samples were taken after 6 h of incubation (37°C; 5% CO₂) to determine TNF-α and IL-10 concentrations by ELISA using CytoSet™ antibody pairs (Biosource, Nivelles, Belgium), according to the instructions provided by the manufacturer.

U937 mono-culture
U937 mono-cultures were directly exposed to C4, i-C5, and NH₄⁺ to determine the effect on cytokine production. Concentrations varied from 2 to 100 mM (see Figures 4.1 and 4.2) to mimic 1) a situation in which the macrophages are protected by a barrier (the results of the Caco-2 mono-culture experiments revealed that 6.2-15.2% of the initial amounts of microbial metabolites (20–100 mM) appeared on the basolateral side) and 2) a situation in which the barrier function of the epithelium was abolished. Metabolites were measured before and after 6 h of exposure of the U937 mono-cultures to calculate mass-balances.

Samples of culture medium were collected after 6 h of incubation (37°C; 5% CO₂) to determine TNF-α and IL-10 secretion. U937 macrophages, exposed to LPS, have an optimal secretion of TNF-α and IL-10 at t=6 h, thereafter secretion diminishes (data not shown).

Co-culture model
In the co-culture model experiments, the combined effect of microbial metabolites and a Caco-2 barrier on macrophages was investigated. Butyrate, i-C5, or NH₄⁺ were added for 6 h (t0-t6) to the apical compartments of the co-culture model (C4 and i-C5: 50 and 100 mM; NH₄⁺: 20, 40, and 60 mM). Subsequently, the macrophages were incubated for another 6 h (t6-t12) in the presence of LPS (1 µg/mL). Samples of the culture medium were collected after 6 and 12 h to determine the TNF-α and IL-10 secretion. The TEER of the Caco-2 inserts was measured at t0 and t6. Test solutions were analysed for concentrations of metabolites before exposure of the co-cultures, after 6 h of co-incubation (apical and basolateral), and after another 6 h (t6-t12) of incubation of the macrophages with LPS to calculate mass-balances.
All microbial metabolites were measured as described before (17).

**Statistical Analysis**

Results are expressed as means (+ SD when n>2 or range when n=2). Comparison of TEER and release of cytokines between (positive) control samples and samples exposed to microbial metabolites were performed using ANOVA and Student’s t-test. A p≤0.05 was considered significant.

**Results**

**Integrity of the barrier function**

**TEER**

The TEER was expressed as % of the value of the same well prior to exposure (Table 4.1). In the Caco-2 mono-culture experiments, exposure to C4 or i-C5 did not induce a functional loss of, or have a dose-dependent effect on the barrier function, except at the highest concentration tested (100 mM). In the latter cases, the average TEER dropped significantly compared to the other concentrations tested, as well as compared to the control (p<0.001 in all cases). After exposure to ammonium, the average TEER value declined to 75% (± 11%), which was significantly lower than the control (p=0.010). No concentration-effect could be detected. The addition of LPS to a microbial metabolite solution had no effect on TEER (data not shown).

No functional loss of the barrier function was found with any of the concentrations of C4, i-C5, and \( \text{NH}_4^+ \) tested in the co-culture model experiments. The average TEER value of the Caco-2 cells in the co-culture model was significantly higher (p=0.016) than that of Caco-2 cells in a mono-culture model after 6 h of incubation with medium A (Table 4.1). Furthermore, TEER values were significantly higher when Caco-2 cells were exposed to C4-100 and i-C5-100 in a co-culture model compared to exposure of these cells in a mono-culture model (p=0.004 and p=0.037, respectively). In addition, the effect of \( \text{NH}_4^+ \) was less pronounced in co-culture incubations compared to mono-culture incubations.

**Mass-balances of metabolites**

In the Caco-2 mono-culture experiments, 7.4% (+ 1.1%), 10.8% (+ 2.1%), and 11.2% (+ 3.1%) of the initial amounts of C4, i-C5, and \( \text{NH}_4^+ \), respectively, were recovered from the basolateral side. With macrophages underneath the Caco-2 cells, the average basolateral recovery after 6 h was 6.4% (+ 0.8%) for C4, 6.2% (+ 0.6%) for i-C5 and 15.2% (+ 6.8%) for \( \text{NH}_4^+ \). The sum of the apical and basolateral recovery was in both types of experiments less than 100% of the initial amounts. The initial amounts of all metabolites tested on U937 cells of both the mono- and co-culture models were fully recovered after 6 h of incubation.
Table 4.1. Transepithelial electrical resistance of Caco-2 cells.

<table>
<thead>
<tr>
<th>C4-25</th>
<th>98</th>
<th>7.3</th>
<th>C4-50</th>
<th>94</th>
<th>17.7</th>
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<tr>
<td>C4-65</td>
<td>103</td>
<td>19.6</td>
<td>C4-100</td>
<td>88</td>
<td>7.7</td>
</tr>
<tr>
<td>C4-85</td>
<td>99</td>
<td>6.8</td>
<td>i-C5-50</td>
<td>105</td>
<td>11.0</td>
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<tr>
<td>C4-100</td>
<td>68</td>
<td>1.1</td>
<td>i-C5-100</td>
<td>84</td>
<td>14.7</td>
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<td>i-C5-30</td>
<td>95</td>
<td>2.1</td>
<td>NH₄⁺-20</td>
<td>94</td>
<td>12.7</td>
</tr>
<tr>
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<td>101</td>
<td>5.9</td>
<td>NH₄⁺-40</td>
<td>83</td>
<td>20.1</td>
</tr>
<tr>
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<td>2.7</td>
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<td>106</td>
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<tr>
<td>i-C5-100</td>
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<tr>
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<tr>
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<td>76</td>
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<tr>
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<tr>
<td>Medium A</td>
<td>88</td>
<td>6.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* TEER values measured after 6 h of exposure, expressed as % of values prior to exposure. Presented are the average and standard deviation (SD) of at least three measurements.

**Immunne modulations**

Caco-2 mono-culture cells did not secrete detectable levels of TNF-α or IL-10 after metabolite exposure (with or without LPS).

In the U937 mono-culture experiments, addition of 50 and 100 mM C4, or i-C5, or of 20 and 40 mM NH₄⁺ resulted in dose-dependent inhibition of TNF-α secretion compared to LPS (positive control), while lower concentrations (C4, i-C5: 4-20 mM; NH₄⁺: 2 and 4 mM) stimulated TNF-α secretion (dose-independent; Figures 4.1A, B, and C for C4, i-C5 and NH₄⁺, respectively). IL-10 secretion by these macrophages was suppressed by all metabolites in all concentrations compared to LPS (dose-dependent; Figures 4.2A, B, and C for C4, i-C5 and NH₄⁺, respectively). The lowest concentration of NH₄⁺ (2 mM) was an exception: IL-10 secretion by macrophages exposed to this solution was almost twice as high as with LPS.
Figure 4.1 TNF-α levels, produced by macrophages during 6 h of exposure to concentrations of A) butyrate (C4-mM) and LPS (1 µg/mL), B) iso-valerate (i-C5-mM) and LPS (1 µg/mL), and C) ammonium (NH₄⁺-mM) and LPS (1 µg/mL). * C4-100 versus LPS and all other concentrations tested: p=0.0125. † i-C5-50 versus: i-C5-4 - i-C5-20 and i-C5-100, p<0.001. †† i-C5-100 versus: i-C5-4 – i-C5-20, p=0.001; LPS, p = 0.0289. ††† LPS versus: i-C5-4 - i-C5-20, p<0.001. ‡ NH₄⁺-2 versus: NH₄⁺-40, p=0.0329; LPS, p=0.02549.

Figure 4.2 IL-10 levels, produced by macrophages during 6 h of exposure to concentrations of A) butyrate (C4-mM) and LPS (1 µg/mL), B) iso-valerate (i-C5-mM) and LPS (1 µg/mL), and C) ammonium (NH₄⁺-mM) and LPS (1 µg/mL). * LPS versus all C4 concentrations tested: p<0.001. † i-C5-4 and i-C5-6 versus: all other concentrations tested, p=0.0034; LPS, p= 0.0134. †† LPS versus: i-C5-10 - i-C5-100, p<0.001. ‡ NH₄⁺-2 versus: NH₄⁺-4, p=0.0047; NH₄⁺-20 and NH₄⁺-40, p<0.001. ‡‡ NH₄⁺-4 versus: NH₄⁺-20, p=0.0088; NH₄⁺-40, p=0.0085. ‡‡‡ LPS versus: NH₄⁺-20, p=0.0353; NH₄⁺-40, p=0.0347.
Exposure of the Caco-2 cells and the macrophages of the co-culture model for 6 h (t0-t6) to medium A without LPS (negative control) resulted in a basolateral secretion of 71.5 pg/mL (± 3.8 pg/mL) TNF-α. The average TNF-α secretion after 6 h of exposure to NH₄⁺ was comparable: 73 pg/mL (± 5 pg/mL). Both C4 and i-C5 on the other hand were able to increase TNF-α secretion in the co-culture model significantly (dose-independent; C4: 144.3 pg/mL (± 18.4 pg/mL), p<0.001 and i-C5: 136.2 pg/mL (± 41.2 pg/mL), p=0.015). Interleukin-10 secretion by the cells of the co-culture model could not be detected after 6 h of incubation with the microbial metabolites.

After removal of the Caco-2 inserts, the macrophages of the co-culture model were incubated for another 6 h (t6-t12) with addition of LPS. Compared to LPS, no effects of NH₄⁺ on secretion of TNF-α or IL-10 were observed (Figures 4.3A and B). However, C4 and i-C5 both stimulated TNF-α secretion significantly (dose-dependent; Figure 4.3A). For IL-10 secretion the opposite effect was observed, except for the concentration of 50 mM i-C5. This concentration of iso-valerate stimulated IL-10 secretion significantly as compared to LPS (p=0.0053), while C4-50 mM, C4-100 mM and i-C5-100 mM all suppressed IL-10 secretion by the macrophages significantly (Figure 4.3B).

![Figure 4.3](image-url)  
**Figure 4.3** TNF-α (A) and IL-10 (B) levels, produced by macrophages from the co-culture model during 6 h (t6-t12) of exposure. Concentrations of metabolites (mM) given are the concentrations used at the start of the co-culture experiments and thus applied at the apical site of the Caco-2 cells. In all cases LPS (1 µg/mL) was added at t6. Comparison versus LPS: * C4-50, C4-100, and i-C5-100, p<0.001; i-C5-50, p=0.02.; †C4-50, p=0.0039; C4-100, p<0.001; i-C5-50, p=0.0053; i-C5-100, p=0.012.

**Discussion**

The colon of patients with active IBD contains increased concentrations of microbial metabolites (13). These metabolites may contribute to the chronic inflammatory process by affecting mucosal integrity and by stimulation of macrophages inducing production of pro-inflammatory cytokines.

In vitro exposure experiments showed that the barrier function of IEC is not easily disturbed by microbial metabolites. Exposure to ammonia, or to high concentrations of C4 or i-C5 lowered TEER values, but the absolute TEER levels remained higher than 500 Ω·cm², a level which is compatible with an intact barrier function (15). As a result, basolateral recovery of metabolites was
in the same order of magnitude in all experiments, regardless of type of experiment, metabolite, or concentration of metabolite tested. However, it can not be concluded that intraluminal concentrations of C4, i-C5 and NH4+ in IBD patients do not lead to increased permeability of the barrier function in vivo because we do not know how sensitive intestinal epithelial cells of IBD patients are.

Information about luminal concentrations of C4, i-C5 and NH4+ in vivo is scarce. Most data are traced back from faecal measurements. Especially with IBD patients, faeces is not a good indicator of the fermentative activity that occurs in the proximal colon. Damage to the mucosa of IBD patients can result in inadequate absorption and utilization of metabolites, resulting in higher concentrations of metabolites in their faeces than in that of healthy individuals (18). Two studies give some more information of what can be produced in vivo. Cummings et al. (19) and MacFarlane et al. (20) measured SCFA, BCFA, and ammonium in colonic luminal contents of sudden-death individuals. The intraluminal concentrations of C4, i-C5, and NH4+ in the proximal colon were 38, 2, and 20 mmol/kg wet weight contents, respectively (20). The observed maximum intraluminal metabolite concentrations produced by IBD microbiotas in an in vitro model were approximately 30 mM for C4, 5 mM for i-C5 and 65 mM for ammonium (13).

In case of an impaired epithelial barrier, such as in patients with an inflamed ulcerated colon, colonic macrophages can be exposed to luminal concentrations of microbial metabolites. Butyrate and i-C5 in concentrations that may occur in the colon of IBD patients had a pro-inflammatory effect by stimulating the secretion of TNF-α and suppressing the release of IL-10 by macrophages that are not protected by an epithelial barrier (U937 mono-culture experiments). In contrast, NH4+ in a concentration that may occur in the colon of IBD patients would have an inhibitory effect on release of both cytokines. This is speculative, based on Figures 4.1C and 4.2C, because the highest concentration tested was only 40 mM.

Due to the (intact) barrier function of the Caco-2 cells, the TNF-α and IL-10 secretion levels in the co-culture experiments reflect the effect of exposure of macrophages to approximately 10% of concentrations of initial amounts added to the luminal side. When macrophages are protected by an epithelial barrier, ammonium no longer has an inhibitory effect on secretion of TNF-α and IL-10. The concentrations of C4 and i-C5 tested in the co-culture experiments are higher than the concentrations found in vivo (sudden death individuals) and in vitro (IBD faeces in in vitro model). However, butyrate is likely to reach concentrations of 50 or 100 mM, for instance upon consumption of prebiotics. Then the effect of this metabolite is pro-inflammatory, even when the macrophages are protected by an epithelial barrier (Figures 4.3A and 4.3B).

Both the supposed positive effect of C4 and the supposed negative effect of NH4+ are not confirmed by the present experiments. Our results are contrasting with the limited data available concerning butyrate and the production of cytokines: studies with whole-blood models and isolated mononuclear cells showed that butyrate (to a maximum of 2 mM) reduced LPS induced TNF-α and/or IL-10 production (12,21,22). A complex mixture of microbial metabolites, obtained from TNO’s in vitro model of the large intestine, reduced TNF-α and INF-γ, while inducing IL-10 (van Nuenen et al., manuscript in preparation). This might indicate that metabolites in combinations
might have opposing or synergistic effects. We are currently studying this in more detail, including the effect of the cytokines on the barrier function.

The increased production of C4 and i-C5 by microbiotas of IBD patients, together with an increased exposure of the mucosal immune system to these metabolites, due to loss of integrity of the epithelial barrier function, contribute to an unwanted immune response. On the other hand, ammonium concentrations as high those produced by microbiotas of IBD patients seem to have an anti-inflammatory role by suppressing the release of both TNF-α and IL-10 when the barrier function is impaired.

In conclusion, the barrier function of intestinal epithelial cells in vitro is not disturbed by the microbial metabolites tested in this study, but these metabolites are able to modulate the secretion of both TNF-α and IL-10 by macrophages. The enhanced production of C4 and i-C5 may thus play a role in the maintenance of IBD activity.
References


Ileal delivery of hIL-10 producing *Lactococcus lactis* as therapy for Crohn’s disease; proof of principle using an *in vitro* small bowel model

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Submitted
Abstract

Human interleukin-10 (hIL-10) can be used in the treatment of Crohn’s disease. A new development is the targeted delivery of hIL-10 to the site of inflammation by bacteria. We aimed to determine whether targeted delivery of hIL-10 producing Lactococcus lactis (L. lactis) could be obtained by incorporating the strain in an enteric-coated formulation. Survival and metabolic activity of L. lactis was studied in TNO’s in vitro gastrointestinal model, simulating the successive dynamic conditions in the stomach and the small intestine, as stationary-phase grown cells or after incorporation in two types of enteric-coated formulation (one disintegrating at a pH of 6.0 and higher; another disintegrating at a pH equal or higher than 7.2). Only 0.04% of the stationary-phase grown cells was recovered after oral intake. Incorporating L. lactis in enteric-coated capsules increased the viable cells to 0.28% or 1.21%, respectively, depending on the capsule used. Human IL-10 could not be measured in any of the samples. The results show that the enteric-coated capsules protected lactococci during passage through the upper gastro-intestinal tract. Therefore, enteric-coated capsules for ileal delivery of hIL-10 producing L. lactis can be a potential therapy for Crohn’s disease.
Introduction

Ulcerative colitis and Crohn’s disease (CD) are inflammatory bowel diseases (IBD) caused by a disregulated immune response to antigens of the enteric bacterial microbiota in a genetically susceptible host (1-3). Treatment of IBD varies according to the type, distribution and severity of the disease. Although surgery is indicated in certain situations, drug therapy is the approach of choice to induce remission and thereafter prevent relapses. Drugs in the treatment of IBD can be used topically, orally or intravenously. Currently the drugs used are mesalazine-containing compounds, corticosteroids, immunomodulatory agents, antibiotics, and biological therapies (4,5).

A new therapeutic approach under investigation is the inhibition of TH-1 polarization of the immune system by using the immunomodulatory recombinant human interleukin-10 (hIL-10)(5). IL-10 controls and suppresses inflammation essentially by down regulating pro-inflammatory cytokine production. A number of studies with animal models of IBD have shown the efficacy of IL-10 for the regulation of mucosal inflammation (6,7). However, studies evaluating the therapeutic use of IL-10 in humans have yielded contradictory results.

This may at least in part have resulted from insufficient delivery of the anti-inflammatory cytokine to the site of inflammation (8,9) and to the induction of interferon-γ following systemic administration (10). To overcome these obstacles, a new development is the targeted delivery of IL-10 to the site of inflammation by means of in situ synthesis through recombinant vectors. This new approach may be safety and effective, as it avoids the complications and side effects associated with conventional therapies, resulting in an overall improvement of the quality of life of those affected by IBD. Steidler et al. (2000) tested this new approach of mucosal delivery of IL-10 using a recombinant Lactococcus lactis (L. lactis). Mouse models were used to show that the therapeutic dose of IL-10 can be reduced by localized delivery. In addition, daily ingestion of IL-10 secreting L. lactis caused a 50% reduction in colitis in mice treated with dextran sulphate sodium and prevented the onset of colitis in IL-10 knock-out mice. The therapeutic effect was due to mIL-10, synthesized de novo by IL-10-secreting L. lactis (7).

For this therapy to be efficacious, an important criterion is the survival of the modified L. lactis strain in the gastrointestinal tract of the host (11,12). The environment in the gastrointestinal tract negatively influences the survival of lactic acid bacteria (13,14). This may be overcome by an enteric-coated formulation that protects the bacteria from gastric fluid and bile salts. Because of their active ileal reabsorption, the bile salt concentration is lower in the distal part of the small intestine (15). Therefore L. lactis should be targeted to the ileum in order to protect them from the detrimental bile salts. Besides, for the treatment of patients with CD localization in the terminal ileum, L. lactis should be targeted specifically to the site of inflammation (16). Based on the increasing pH along the GI tract, a pH dependent approach was used in the development of an enteric-coated capsule formulation containing freeze-dried, viable recombinant L. lactis for the ileal delivery of human IL-10 (17).

The aim of the current study was to determine whether targeted delivery of hIL-10 producing L. lactis could be obtained by incorporating the strain in an enteric-coated capsule formulation. The effect of the formulation on survival and metabolic activity of the recombinant L. lactis was studied.
in the TNO *in vitro* gastrointestinal model, which simulates to a high degree the successive dynamic conditions of the human stomach and small intestine (TIM-1)(13,18,19).

**Materials & Methods**

**Strains**

The strains used were *Lactococcus lactis* subsp. *cremoris* MG1363 (20) and *Lactococcus lactis* Thy12. For the latter, the thymidilate synthase gene thyA of *L. lactis* MG1363 was replaced by the expression cassette for hIL-10. This thyA gene is essential for the growth of *L. lactis* as it plays an essential role in its DNA synthesis. The thyA⁻ hIL-10⁺ *L. lactis* strain (*L. lactis* Thy12) produces hIL-10 (21) and when deprived of thymidine or thymine, its viability drops by several orders of magnitude, essentially preventing its accumulation in the environment (21). The strains were tested in the *in vitro* model as stationary phase grown cells or after incorporation in an enteric-coated formulation.

**Glycerol-stocks**

The strains were cultured in M17 (+0.5% glucose; Difco, Becton Dickinson, MD, USA) for 16 h at 30°C. Thymidine (50 µg/mL; Sigma-Aldrich, Milwaukee, USA) was added to the broth for culturing *L. lactis* Thy12. After centrifugation of 10 mL of these cultures (~10⁹ bacteria/mL), the pellets were resuspended into 10 mL BM9 (50 mM CO₃-buffer at pH 8.5, supplemented with the M9 salts (7)). These bacterial suspensions were introduced in TIM-1.

**L. lactis formulation**

For the incorporation in an enteric-coated formulation, the *L. lactis* cultures were grown, harvested (centrifugation), and resuspended in 10% skim milk (Difco, Becton Dickinson, MD, USA) in order to obtain a cell concentration of ±10¹⁰ CFU/mL. This suspension was subsequently freeze-dried. In order to perform visual analysis of disintegration of the enteric-coated capsules, carmin red (rubrum coccineum, Federa, Braine-l’Alleud, Belgium) was added to the freeze-dried powder before filling in ready-to-use enteric-coated HPMC capsules 00. The HPMC capsules (Capsugel, Bornem, Belgium) were coated with 7 mg/cm² (~30µm) of either Eudragit® L30D-55 coating (Röhm, Darmstadt, Germany) dissolving at a pH 6.0 and higher, or Eudragit® FS 30 D (Röhm) dissolving at a pH equal or higher than pH 7.2 (22). The coated bodies were filled with 300 mg of freeze-dried powder (5*10¹⁰ CFU/capsule) and closed manually with the coated caps. After filling, the capsules were packed in aluminum sachets, sealed at 20% relative humidity and stored at -20°C until use in TIM-1.

**Survival and metabolic activity of *L. lactis***

**TIM-1**

Survival of the strains was studied in TIM-1 (Figure 5.1). Details of this computer-controlled system are given in Minekus *et al.* (23) and Marteau *et al.* (13). In short, the model consists of a
number of linked glass units with flexible walls inside. The intestinal content is mixed and moved by peristaltic movements, achieved by pumping water of 37°C around the flexible walls. During passage through the gastrointestinal tract, parameters such as temperature, pH, gastric and intestinal mixing, peristaltic movements, and gastrointestinal secretions are continuously monitored and controlled. The digested and dissolved low-molecular compounds are dialyzed continuously from the jejunal and ileal compartments of the model via hollow fiber membrane systems. Several studies have demonstrated the predictive value of this model in relation to survival of microorganisms (13,24) and oral drug delivery systems (19).

Strain introduction and passage
In duplicate experiments the bacterial suspension or one capsule was mixed with 80 mL stomach water, 20 mL citrate buffer, and 100 mL water (23), and was introduced into the gastric compartment containing 10 mL gastric residue. The bacterial suspensions contained approximately 1.0*10^{10} colony forming units (cfu) and the capsules approximately 5.0*10^{10} cfu of either *L. lactis* MG1363 or *L. lactis* Thy12. TIM-1 was programmed to simulate the adult gastrointestinal conditions after the intake of water (*i.e.*, with a fast gastrointestinal passage)(19). The starting pH was 4.5, decreasing to 1.5 in 90 min. The gastric content was gradually delivered to the duodenum over a period of 3 h, according to data obtained from clinical studies (23). In the duodenal compartment the gastric content was neutralized to pH 6.5 ± 0.2, and bile and pancreatin were secreted continuously. The content of the duodenum (residence time approx. 10-15 minutes) was
delivered into the jejunal compartment (pH 6.8) and after that into the ileal compartment (pH 7.2), according to the physiological intestinal transit after the intake of water by a human adult. It was assumed that the enteric-coated capsules would not disintegrate in the stomach. As the enteric-coated capsules were not able to pass the pyloric valve in the model (because of their size), they were transferred manually after 1 h from the stomach to the duodenal compartment in a simulated housekeeper wave. This had the additional advantage that passage from the stomach to the small intestine was standardized. If an enteric-coated capsule did not disintegrate in the duodenum, or the jejunum, it was again transferred manually after 1 h to the jejunum or the ileum, respectively. The survival experiments lasted 5 h, only for the Eudragit® FS 30 D coated capsules filled with *L. lactis* Thy12 also a 6 h experiment was performed.

**Sampling**

At the end of the ileal compartment, the simulated ‘ileo-caecal valve’ (Figure 5.1H) delivered the intestinal contents into a sampling bottle at 4-8°C (ileal effluent). Every 60 minutes during 5 h (or 6 h) the total collected volume was measured and analyzed for viable microorganisms per mL. Also the cfu per mL at time zero was determined in the ‘meal’ before introduction of the test-product in the gastric compartment. This meant for the capsules, that the initial numbers of bacteria had to be determined in separate capsules. Furthermore, all three residues of the model from respectively stomach and duodenum, jejunum, and ileum were collected at the end of an experiment and analyzed for viable counts. Results were expressed as percentage of the initial intake.

The same ileal effluent samples as mentioned above were also used for analyzing the amount of hIL-10. The Eudragit® L30D-55 coated capsules disintegrated in the jejunum (see results). In this case additional luminal samples from the jejunum (t=3, 4, and 5 h) were also analyzed for the amount of hIL-10.

**Determination of viable counts and hIL-10**

For viable cell count determinations, the samples were diluted (10-fold serial dilution) in pepton-physiological salt and plated on M17 agar (+0.5% glucose; Difco and +50 µg/mL thymidine in case of *L. lactis* Thy12; Sigma-Aldrich).

The concentration hIL-10 in each sample was determined in a sandwich ELISA. Maxisorp F96 plates (Nunc, Roskilde, Denmark) were coated with 2 µg/mL rat anti-human IL-10 (BD Biosciences Pharmingen, San Diego, CA, USA). The plates were blocked with a 0.1% casein solution. A 2-fold dilution series of hIL-10, starting from 1 ng/mL and appropriate dilutions of the samples were loaded on the plates. Between each step the plates were washed with PBS + 0.05% Tween-20. The bound hIL-10 was detected with 1/1000 diluted biotinylated rat anti-human IL-10 combined with horseradish peroxidase coupled streptavidine. The plates were developed with TMB substrate (all from BD Biosciences Pharmingen). The reaction was stopped after 30 min with 1M H$_2$SO$_4$. The absorbance was measured at 450 nm.
Results

Survival

For the survival no distinction was seen between *L. lactis* MG1363 and *L. lactis* Thy12. Therefore, the average cumulative survival out of 4 TIM-1 experiments with stationary phase grown cells is shown in Figure 5.2A. Especially in the first 120 minutes after the intake, bacteria survived the passage through the gastro-small intestinal tract. More than half of the bacteria that survived passage were still in the model as residue after 300 minutes of running. Viable cells could only be detected in the residue of the jejunum and not in the residue of the stomach and duodenum. At the end of the experiment, 0.04% of the ingested stationary phase grown cells (~8.5*10^6 cells) had passed the gastric and small intestinal compartments alive and was recovered in the ileal effluent and residue.

**Figure 5.2** Cumulative survival of lactococci in time (min) as % of the intake (left axis, ileal delivery = white bars and residue = black bars) and absolute (right axis, line). A) stationary phase grown cells; B) Eudragit® L30D-55 coated capsules. For comparison the scales of the y-axes has been kept the same in A) and B).
Incorporation of *L. lactis* MG1363 and *L. lactis* Thy12 in Eudragit® L30D-55 coated capsules, designed to disintegrate in the proximal small bowel, increased survival (Figure 5.2B). The Eudragit® L30D-55 coated capsules showed no disintegration in the stomach or duodenal compartment. The disintegration started immediately in the jejunum compartment (visual analysis; release of carmin red from the coated capsules). About 50% of the volume of the capsule had disappeared at 150 min after intake and the capsules showed complete disintegration at 175 min after intake. With a cumulative survival of 0.28% of the intake (~2.4*10^8 cells), the increase in survival for these encapsulated strains compared to stationary phase grown cells was almost 8-fold. The bacteria were also more gradually delivered to the colon by incorporating them in an enteric-coated capsule before ingestion. Also here, more than half of the cells that survived passage still resided in the model after termination of the experiment, and viable cells were only detected in the residue of the jejunum and ileum.

The Eudragit® FS 30 D coated capsules, designed to disintegrate in the distal small bowel, passed the stomach and small intestine compartments intact. It was observed that the coated capsules became swollen and sticky, and that the pink color (carmin red) of the capsule content became more intense towards the distal region, probably indicating an increased permeability. Between 240 and 300 minutes after intake, the release of *L. lactis* started. At this time, the capsule had been in the ileal compartment for one hour (180-240 minutes; pH 7.2). The final cumulative survival of the released lactococci was 0.17% of the intake (sum of ileal effluent and ileal residue; average for both *L. lactis* MG1363 and *L. lactis* Thy12). The remaining capsules still contained on average 8.44% of the initial amount of lactococci in these capsules (see Table 5.1).

**Table 5.1.** Cumulative survival of lactococci in a Eudragit® FS 30 D capsule, in % of the intake and in absolute numbers (cells), during a 5 h experiments and a 6 h experiment.

<table>
<thead>
<tr>
<th>Eudragit® FS 30 D</th>
<th>Survival during a 5 h experiment</th>
<th>Survival during a 6 h experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of intake</td>
<td>absolute numbers (cells)</td>
</tr>
<tr>
<td>Ileal delivery</td>
<td>0.17</td>
<td>4.6*10^7</td>
</tr>
<tr>
<td>Residue</td>
<td>0.0001</td>
<td>1.3*10^5</td>
</tr>
<tr>
<td>Capsule</td>
<td>8.44</td>
<td>6.4*10^9</td>
</tr>
<tr>
<td>Total</td>
<td>8.61</td>
<td>6.4*10^9</td>
</tr>
</tbody>
</table>

The TIM-1 experiments were repeated (n=2; Eudragit® FS 30 D capsules, only with *L. lactis* Thy12) to study the effect of one extra hour residence time in the ileal compartment (time of experiment=6 h). In this experiment, the capsules totally disintegrated between 300 and 360 minutes, as a result of the combination of pH and peristaltic forces. The total cumulative survival of the lactococci was 1.21% of the intake. Also in this case, after total disintegration of the coated capsules, more than half of the cells that survived passage were still in the model as ileal residue after the 6 h (see Table 5.1).
Finally, a static incubation was performed to study the effect of the ileal contents (e.g. bile) on survival of *L. lactis* Thy12. One Eudragit® FS 30 D coated capsule was incubated for 5 h in a mixture of bile, pancreatic juice, and ileal dialysate, resembling the ileal lumen in TIM-1. The pH was set at 7.2 and the incubation occurred in a shaking water-bath of 37°C. Another Eudragit® FS 30 D capsule was first opened and the content was then mixed with the ileal fluid. From Figure 5.3 it can be concluded that after 300 minutes the presence of viable bacteria in both static incubations was more or less the same. After 60 minutes the release of *L. lactis* Thy12 from the coated capsule started, although visual analysis indicated that the disintegration occurred between 120 and 180 minutes. At 180 minutes, the survival (and/or releases in case of the coated capsule incubation) stabilized in both types of static incubations. These experiments corroborate that survival of *L. lactis* in intestinal fluids is very low. Under these conditions survival as percentage of the initial amount of cells varied from 0.06% (deliberately opened capsule) to 0.25% (intact capsule).

**Figure 5.3** Release and/or survival of *L. lactis* Thy12 cells out of Eudragit® FS 30 D capsules during a static incubation of 5 h. ■ intact capsule • deliberately opened capsule

**hIL-10 analysis**

Human IL-10 was only detected in samples of the log-phase grown cells of *L. lactis* Thy12, taken from the mixture with artificial saliva and water before introduction in the gastric compartment. One mL of bacterial suspension of *L. lactis* Thy12 contained 641 pg hIL-10. Human IL-10 could not be measured in any of the ileal effluent or jejunum samples.

**Discussion**

A new therapeutic approach for CD is the targeted delivery of hIL-10 to the ileum by a recombinant *L. lactis* strain. For this therapy to be efficacious, an important criterion is the survival of *L. lactis* in the gastrointestinal tract. This implies the development of an enteric-coated formulation that
protects the bacteria from gastric fluid and, amongst others, bile salts and releases the bacteria at the site of inflammation. In this study two types of enteric-coated capsules were evaluated for ileal delivery of hIL-10 producing *L. lactis*. This was studied in TIM-1, an *in vitro* gastrointestinal model simulating to a high degree the successive dynamic processes in the stomach and the small intestine (13,23). The study clearly indicates that both types of capsules are effective in protecting lactococci during passage through the stomach and (parts of) the small intestine. Only 0.04% (yet still representing 8.5*10^6 cells) of the stationary-phase grown lactococci cells was recovered after oral intake. This has been observed previously for passage of unprotected lactocci and other lactic acid bacteria through the small intestine (14,19,24,25). Vesa et al. (2000) reported an ileal survival for *L. lactis* MG 1363 of 1.0 % of the intake. This higher survival as compared to our findings with the lactococci as stationary phase grown cells is most likely the result of the use of milk instead of water as meal matrix in the human volunteer study; whereby milk acts as a protecting (e.g. buffering) matrix.

Incorporating *L. lactis* MG1363 and *L. lactis* Thy12 in Eudragit® L30D-55 coated capsules increased the viable bacteria to 0.28%. Eudragit® FS 30 D coated capsules improved this even further, up to 1.21% and 8.61% of the total intake could be recovered, depending on the incubation time in the ileal fluid (6 h, leading to complete disintegration of the capsules, and 5 h, where the capsules were still largely intact, respectively).

This general protecting property is underscored also in studies in pigs, albeit with different efficacy, where the *in vivo* situation shows even higher, nearly complete recovery of the inoculum (21) and unpublished data). A major difference in the both study designs was the site of ‘ingestion’. In our *in vitro* study the capsules were introduced in the stomach. In the pig study the capsules were inserted through a duodenal fistula (pH duodenum 6.2). In addition, the higher recovery in the pigs can be due to the intrinsic differences between the *in vivo* and *in vitro* situation, where the latter for instance does not possess of a mucus layer that may protect the bacteria.

An advantage of the use of *in vitro* systems such as TIM-1 is the fact that time-samples can be taken, combined with the ability to visualize disintegration. Disintegration of the Eudragit® L30D-55 coated capsules started immediately in the jejunum (pH 6.8), and disintegration was completed in about 55 minutes. Therefore, the cells were in contact with the jejunal and/or ileal contents for a minimum period of 125 minutes and a maximum period of 180 minutes, and in this situation the average total cumulative survival was 0.28% (representing 2.4*10^8 cells). From the static incubations with the unprotected cells (deliberately opened capsules) it can be concluded that the survival in ileal contents decreased from 0.31% (120 min of incubation) to 0.06% of the initial intake (180 min of incubation). By comparing the percentages of survival from both type of experiments, it can be concluded that the Eudragit® L30D-55 coated capsules seems to protect the lactococci for a period of approximately 175 minutes, thereby increasing survival 8-fold.

In the TIM-1 experiments that lasted 5 h no visual disintegration of the Eudragit® FS 30 D coated capsules was observed, and viable count analysis showed a release of *L. lactis* in the ileal fluid of 0.17% of the intake (4.6*10^7 cells). The capsules had been in the ileal compartment for 120 minutes and still contained 8.44% of the initial amount of viable cells (6.4*10^9 cells). In the TIM-1
experiments that lasted 6 h, the capsules had been in the ileal compartment for 180 minutes, and were completely disintegrated. Total cumulative survival was 1.21% (1.2*10^9 cells), which is 34-fold higher than the stationary phase, unprotected cells. Furthermore, a static incubation was done with this type of capsule. After 60 minutes, the release of *L. lactis* Thy12 started (Figure 5.3), although visual analysis indicated that the disintegration occurred between 120 and 180 minutes. At 180 minutes, the cumulative survival was 0.09% of the initial amount of cells. For the Eudragit® FS 30 D coated capsules a retention time of at least 60 minutes in an environment resembling the human ileum is necessary for the release of the lactococci. Compared to the Eudragit® L30D-55 coated capsules, the Eudragit® FS 30 D coated capsules are more effective in protecting lactococci during passage through the stomach and the small intestine (0.28% versus 1.21% survival).

Depending of the site of action, the proper capsule can be chosen. If the site of action should be the jejunum, a Eudragit® L30D-55 coated capsule is the preferred choice of drug dosage form; if the site of action is the ileum or proximal colon, a Eudragit® FS 30 D coated capsule should be chosen.

In conclusion, our results show that the enteric-coated capsules protect lactococci during passage through the upper gastrointestinal tract. The choice for one type of formulation, Eudragit® L30D-55 or Eudragit® FS 30 D, depends on the patients's CD localization and ileal pH. The bacterium’s capacity to secrete hIL-10 was validated *in vivo* in pigs (21), but was not seen in our *in vitro* systems. The absolute amount of viable Thy12 bacteria that would have been able to produce hIL-10 *in vitro* varied from 8.5*10^6 to 1.2*10^9 cells. *In vivo* 4.2*10^10 and 4.4*10^11 Thy12 cells were detected in the lumen and in intestinal tissue, respectively. These lower amounts of viable cells *in vitro* go together with hIL-10 concentrations (~0.5 – 5.0 pg/mL) that are below the detection limit of the ELISA assay used.

Even though survival of *L. lactis* is low when expressed in percentages, total viable cell numbers at the end of the small intestine reach 2.4*10^8 – 1.2*10^9. Enteric-coated capsules for ileal delivery of hIL-10 producing *L. lactis* can be a potential therapy for Crohn’s disease. Combining these results with the outcome of a preclinical study in which the same two formulations with hIL-10 producing *L. lactis* has been administered to 10 patients suffering from CD (Amsterdam Medical Centrum, The Netherlands) will provide valuable insight into safety, biological containment and clinical effectiveness of this novel medical strategy.
Chapter 5

References

hIL-10 producing L. lactis as therapy for CD


The effect of anti-TNF-α antibody therapy in Crohn’s disease on composition and activity of faecal microbiota: a pilot study

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Gjalt Welling
Hermie Harmsen
Saskia van der Schaaf
Ernst Kuipers

Submitted
Abstract

We aimed to determine whether anti-TNF-α monoclonal antibody therapy for Crohn’s disease (CD) has an effect on the composition of the faecal microbiota in these patients. It was hypothesized that if the microbial composition correlates with clinical disease activity, microbial metabolites possibly do as well, given their relationship with the microbial composition. Therefore, the second aim of this study was to investigate the possible use of microbial metabolites as markers for disease activity in inflammatory bowel disease (IBD).

The study population consisted of 23 subjects divided over three groups: healthy individuals, IBD patients in remission and patients with active CD. The latter patients were studied twice: immediately before and two weeks after a single, first infusion of infliximab (the anti-TNF-α antibody). Microbial composition was determined using the classical plating technique and FISH, and the following potential disease activity markers were determined in the faecal samples: dry matter, pH, short-chain fatty acid, branched-chain fatty acids, and lactate.

Infliximab therapy led to an increase of the density of the bacterial population, to a decrease of the faecal L- versus D-lactate ratio, and in three of the four patients this therapy led to an increase of faecal dry matter. By comparing the patients with active CD with the healthy controls and the IBD patients in remission, an increase in total number of faecal bacteria and in faecal dry matter, and a decrease in the faecal L- versus D-lactate ratio seem to correlate overall with a lower severity of disease activity, and thus with health status. These three parameters can possibly be used as clinical disease activity markers, as opposed to the absolute excretions of microbial metabolites, given the complex interaction between production, absorption/utilization and excretion.
Introduction

The gastrointestinal microbiota plays a role in Crohn’s disease (CD) and ulcerative colitis (UC). Evidence for this role includes responses to antimicrobial therapy, infiltration of inflammatory bacterial products across the intestinal mucosal barrier, host immunity against (or rather loss of tolerance to) bacteria indigenous to the large intestine, and the role of bacteria in disease development in animal models of inflammatory bowel disease (IBD). Specific microorganisms (1-5) and their metabolites (6,7) have both been hypothesized to be mediators of injury and inflammation.

If (specific) microorganisms cause or maintain inflammation, the composition of the microbiota from IBD patients may be different from that of healthy individuals. However, reports on this are conflicting (3,8-10). Most data on the involvement of microbiota in IBD have been obtained using classical plating and speciation techniques. These techniques are limited by the fact that a major proportion of the microbiota, estimates range between 12 and 60%, can not be cultured (11,12). A molecular approach towards microbiota analysis by means of denaturing gradient gel electrophoresis (DGGE), fluorescent in situ hybridisation (FISH), or DNA-arrays (DNA-chips), may provide better insight in the composition of the faecal microbiota in IBD patients.

The large interindividual variation in the microbial composition in human subjects makes it difficult to conclude if a microbiota is ‘normal’ or ‘abnormal’. Furthermore, in severe extensive IBD an abnormal composition of the gut microbiota may be a result, rather than a cause of the disease. For instance, abnormal gut transit times, the presence of blood, mucus, and shedded epithelial cells, as well as the treatment of IBD patients with antimicrobials or other drugs can result in a microbiota that is different in composition from that from healthy individuals (8,13,14). The determination whether patients with IBD show evidence of an ‘abnormal’ composition of the microbiota in their large intestine and whether this is related to the initiation or exacerbation of the disease is thus a difficult, but important issue to solve. An improvement in the composition of the microbial community may relate to an improved clinical outcome.

The proinflammatory cytokine tumor necrosis factor (TNF)-α has been demonstrated to play a crucial role in the pathogenesis of CD. An increased TH-1 response is detected in CD; these cells mediate cellular immunity and macrophage activation. Activated macrophages produce proinflammatory cytokines, including TNF-α (15-17). Genetically engineered monoclonal antibodies, specifically directed against TNF-α and given intravenously, have been shown to have a dramatic and sustained effect in inducing remission in IBD patients (18-20). The exact mechanism of anti-TNF-α monoclonal antibodies, apart from direct activity that neutralizes both soluble and transmembrane TNF-α, is not fully understood. The proposed mechanisms include inhibition of binding of TNF-α with its receptors, as well as lysis of TNF producing cells via complement fixation, antibody dependent cytotoxicity, and an increased susceptibility to apoptosis of intestinal mucosal T cells (21,22).

Our aim was to determine whether anti-TNF-α monoclonal antibody therapy for CD has an effect on the composition of the faecal microbiota in these patients. It was hypothesized that if the microbial composition correlates with clinical disease activity, microbial metabolites possibly do as
well, given their relationship with the microbial composition. Therefore, the second aim was to investigate the possible use of microbial metabolites as markers for disease activity in IBD.

Materials and Methods

Study population
The study population consisted of 23 subjects divided over three groups: (1) the CD-active group consisting of four patients (CD1-4) with mild-to-moderately active CD with a Clinical Disease Activity Index (CDAI) ranging between 107 and 265, (2) the IBD-remission group consisting of 12 IBD patients in remission, and (3) the control group consisting of 7 healthy subjects. The patients in the CD-active group were studied twice, immediately before and two weeks after a single, first infusion of infliximab (5 mg/kg; Remicade®, Centocor Inc, Horsham, USA). The subjects in the other two groups were studied only once. The IBD-remission group consisted of 7 patients with CD affecting the colon and 5 patients with UC. Two CD patients from the IBD-remission group and all seven healthy subjects had not taken any medication in the 6 months prior to the study. The other patients were treated with mesalazine (n=5), corticosteroids (n=3), or immunomodulatory agents (n=3). Clinical response of the CD-active patients was defined as a reduction of 70 or more points in the score on CDAI or a CDAI score below 150 (=remission). C-reactive protein (CRP) values were also determined to score disease activity.

Faecal Analysis
Fresh faecal material from each subject was collected and frozen at -80°C until analysis.

Microbial composition
Microbial composition was determined using the classical plating technique and FISH. Plating was performed as described before (23). For FISH, weighed portions (about 0.5 g) of each stool were fixed with 3% paraformaldehyde in PBS (per liter 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4, and 0.24 g of KH2PO4) as described previously (24). Samples were applied to glass slides following a protocol described previously (25), except that the dilution of the paraformaldehyde fixed fecal samples was made in PBS and not in 5% Tween solution. The slides were hybridized with the probes listed in Table 6.1 or stained with 4′, 6′-diamidino-2-phenylindole (DAPI) as described (24,25). Fluorescent cells in the samples were counted automatically (25) with a Leica DMRXA epifluorescence microscope (Leica, Wetzlar, Germany) or visually with an Olympus BH2 epifluorescence microscope (in case of numbers below 4 * 10^8 cells/g or inhomogeneous spreading of the cells).

Potential faecal disease activity markers
The following potential disease activity markers were determined in the faecal samples: dry matter, pH, short-chain fatty acid (SCFA), branched-chain fatty acids (BCFA), and lactate. The dry matter (expressed as percentage of a wet weight sample) was determined by drying approximately 1 g of a
Effects of anti-TNF-α antibodies on faecal microbiota

faecal sample in a pre-weighed tube in an incubator of 100°C until no loss of weight could be detected. Both SCFA, BCFA, and lactate concentrations were measured as described before (26). Potential disease activity markers were expressed as unit per gram faeces (wet weight).

Table 6.1 Stain or probes used to determine the microbial composition of faeces by FISH.

<table>
<thead>
<tr>
<th>Target group</th>
<th>Stain or probe</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells</td>
<td>DAPI</td>
<td>(24)</td>
</tr>
<tr>
<td>Bacteroides/ Prevotella</td>
<td>Bac303</td>
<td>(27)</td>
</tr>
<tr>
<td>Eubacterium rectale/ Clostridium coccoides-group (Clostridium group XIVa)</td>
<td>Erec482</td>
<td>(24)</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>Bif164</td>
<td>(28)</td>
</tr>
<tr>
<td>Eubacterium lowG+C #2 (Faecalibacterium sp.)</td>
<td>Elgc01</td>
<td>(24,29)</td>
</tr>
<tr>
<td>Atopobium group</td>
<td>Ato291</td>
<td>(30)</td>
</tr>
<tr>
<td>Clostridium ramosum/ Clostridium spiroforme/ Clostridium cocleatum group</td>
<td>Cspiro222, differentiated on bases of morphology: C. ramosum straight rod, others curved or coiled up rods</td>
<td>(31)</td>
</tr>
<tr>
<td>Clostridium histolyticum/ Clostridium lituseburensense group (Clostridium group I,II and XI)</td>
<td>Chis150/Clit135</td>
<td>(24)</td>
</tr>
<tr>
<td>Lactobacillus/ Enterococcus</td>
<td>Lab158</td>
<td>(32)</td>
</tr>
<tr>
<td>Streptococcus/ Lactococcus</td>
<td>Strc493</td>
<td>(24)</td>
</tr>
</tbody>
</table>

Statistical Analysis

Results for each group are expressed as means ± SEM, except for FISH. There, the median and the range for the groups are shown. All statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) for Windows version 12.0. Descriptive statistics were used to analyse and report the data. Differences in outcome between groups were calculated by means of the Mann-Whitney test. Differences within the CD-active group over time (before versus after treatment) were performed using Wilcoxon’s signed ranks test. A $p \leq 0.05$ was considered significant.

For FISH, results of one IBD-remission subject were not included because of poor hybridization.
Results

Clinical disease activity markers

The patients on anti-TNF-α therapy all showed a reduction in the CDAI score two weeks after the single infliximab infusion (Figure 6.1). Infliximab treatment in patient CD1 caused serious side effects, resulting in an increase in CRP from 59 to 223 mg/dL.

![Image](image_url)

**Figure 6.1** CRP (closed symbols) and CDAI (open symbols) scores of patients CD1 (■, □), CD2 (♦, ◊), CD3 (▲, △) and CD4 (●, ○) before and after anti-TNF-α treatment.

Microbial composition

*Colony forming units (CFU) by plating*

Total bacterial counts as determined by plating were significantly lower in the CD-active patients than in the healthy controls and IBD-remission group (p=0.01 and 0.05, respectively; Table 6.2). There was no difference in total bacterial counts between the healthy controls and IBD patients in remission, but that of the CD-active patients after treatment was still significant lower than that of the healthy volunteers (p=0.02). Infliximab therapy led to an increase in total bacterial counts in the CD-active group. This increase was of borderline significance (p=0.07) and observed in each individual in this group. Bifidobacteria, bacteroides, lactobacilli, and sulphite reducing clostridia were the bacterial genera that all showed increases in counts, except in patient CD4 where the bifidobacteria and bacteroides remained stable upon treatment. The colonization density of enterobacteriaceae and enterococci was already before infliximab therapy similar in the CD-active group to both other groups. The bacterial composition of faeces did not differ between the IBD-
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remission and healthy control group, except for a significantly higher number of lactobacilli in faeces of healthy subjects (p=0.01).

Table 6.2 Microbial composition as determined by plating; log10 CFU/gram faeces (mean ± SEM for healthy controls, IBD-remission, CD-before and CD-after (sub)groups; individual data for the CD-active group).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Healthy controls (n=7)</th>
<th>IBD-remission (n=12)</th>
<th>CD-active (n=4)</th>
<th>CD1</th>
<th>CD2</th>
<th>CD3</th>
<th>CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before</td>
<td>after</td>
<td>before</td>
<td>after</td>
<td>before</td>
<td>after</td>
<td>before</td>
</tr>
<tr>
<td>bifidobacteria</td>
<td>8.7 (0.2)</td>
<td>8.8** (0.2)</td>
<td>7.2* (0.5)</td>
<td>7.7</td>
<td>6.9</td>
<td>7.8</td>
<td>5.6</td>
</tr>
<tr>
<td>bacteroides</td>
<td>9.3 (0.3)</td>
<td>9.3** (0.3)</td>
<td>7.9* (0.4)</td>
<td>8.2*</td>
<td>8.0</td>
<td>8.2</td>
<td>6.5</td>
</tr>
<tr>
<td>lactobacilli</td>
<td>8.7† (0.6)</td>
<td>4.8 (0.4)</td>
<td>3.9* (0.4)</td>
<td>4.9*</td>
<td>1.5</td>
<td>5.0</td>
<td>2.6</td>
</tr>
<tr>
<td>enterococci</td>
<td>6.2 (0.2)</td>
<td>6.5 (0.4)</td>
<td>6.9 (0.8)</td>
<td>6.4</td>
<td>5.0</td>
<td>5.2</td>
<td>3.9</td>
</tr>
<tr>
<td>entero-bacteriaceae</td>
<td>6.7 (0.4)</td>
<td>7.5 (0.5)</td>
<td>7.1 (0.6)</td>
<td>7.3</td>
<td>7.6</td>
<td>7.4</td>
<td>5.1</td>
</tr>
<tr>
<td>sulphite reducing clostridia</td>
<td>7.2 (0.4)</td>
<td>6.7* (0.3)</td>
<td>4.3* (0.7)</td>
<td>6.5</td>
<td>3.9</td>
<td>7.1</td>
<td>1.6</td>
</tr>
<tr>
<td>total counts</td>
<td>9.5 (0.2)</td>
<td>9.4† (0.3)</td>
<td>8.0* (0.4)</td>
<td>8.4*</td>
<td>8.2</td>
<td>8.4</td>
<td>6.6</td>
</tr>
</tbody>
</table>

† p≤0.05 healthy controls versus IBD-remission  
‡ p≤0.05 healthy controls versus CD-before  
# p≤0.05 healthy controls versus CD-after  
** p≤0.05 IBD-remission versus CD-before  
before/after=before/after infliximab treatment

FISH

The relative proportions of the various bacterial target groups were determined by correlating the respective cell counts with the total bacterial counts detected with DAPI staining. Bacteria detected with DAPI staining, but not with any of the specific probes, are hereafter referred to as unidentified bacteria.

The DAPI stained cell counts of the four CD-active patients before treatment (median: log 9.5, range: log 9.3-10.1 CFU/gram) increased after infliximab treatment to log 10.5 (median; range: log 10.0-10.6 CFU/gram; p=0.07). This increase was seen in all CD-active patients and thus matched the plating results. Before treatment, the total counts for the CD-active patients were also significantly lower than those of both the healthy controls and the IBD-remission groups (p=0.01 for both). There were no significant differences in total counts between the CD-active patients after treatment, the healthy controls (median: log 10.9, range: log 10.2-11.0 CFU/gram), and the IBD patients in remission (median: log 10.4, range: log 10.0-10.9 CFU/gram). That between the latter two groups was of borderline significance (p=0.09).

No significant effects of anti-TNF-α antibody treatment on the relative composition of faecal microbiota of the CD-active patients were observed (Table 6.3, results are expressed as % of total
bacteria). Furthermore, no significant compositional differences between the healthy controls and the IBD patients in remission did appear. The composition of the CD-active patients differed from both other groups with respect to a (significant) higher proportion of the Bacteroides/Prevotella group (for significance: see Table 6.3).

**Table 6.3** Composition of the faecal microbiota of healthy controls, IBD patients in remission, and patients with active CD before and after infliximab treatment detected with FISH. Counts are expressed as % of total bacteria detected with DAPI staining (median and range for healthy controls, IBD-remission, CD-before, and CD-after (sub)groups; individual data for the CD-active group).

<table>
<thead>
<tr>
<th>group</th>
<th>Healthy controls (n=7)</th>
<th>IBD remission (n=11)</th>
<th>CD-active (n=4)</th>
<th>CD1</th>
<th>CD2</th>
<th>CD3</th>
<th>CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before</td>
<td>after</td>
<td>before</td>
<td>after</td>
<td>before</td>
<td>after</td>
<td>before</td>
</tr>
<tr>
<td>Bacteroides/Prevotella group</td>
<td>6.7 (3.4-26.1)</td>
<td>6.9† (0.4-21.7)</td>
<td>17.5** (12.3-53.2)</td>
<td>29.9† (12.7-34.6)</td>
<td>16.1</td>
<td>28.0</td>
<td>18.9</td>
</tr>
<tr>
<td>Eubacterium rectale/C. coccoides group</td>
<td>14.6 (6.0-45.8)</td>
<td>22.6 (1.7-50.2)</td>
<td>24.2 (6.7-45.5)</td>
<td>18.9 (15.6-35.2)</td>
<td>6.7</td>
<td>15.6</td>
<td>22.0</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>4.2 (0.7-6.3)</td>
<td>1.8 (0.4-11.1)</td>
<td>3.7 (1.4-7.9)</td>
<td>1.7 (0.7-12.4)</td>
<td>1.4</td>
<td>1.3</td>
<td>2.3</td>
</tr>
<tr>
<td>low G+C Gram-positive bacteria</td>
<td>8.8 (0.3-12.6)</td>
<td>5.3 (0.4-12.2)</td>
<td>2.6 (0.1-23.9)</td>
<td>3.8 (0.8-11.9)</td>
<td>4.9</td>
<td>6.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Atopobium group</td>
<td>2.9 (0.1-7.5)</td>
<td>3.5 (1.4-7.2)</td>
<td>0.9 (&lt;0.1-4.3)</td>
<td>0.4 (&lt;0.1-4.9)</td>
<td>1.1</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>C. ramosum</td>
<td>0.6 (0.2-1.8)</td>
<td>0.6 (0.4-4.2)</td>
<td>0.9 (&lt;0.1-2.9)</td>
<td>0.8 (0.2-1.5)</td>
<td>&lt;0.5</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>C. spiroforme/C. cocleatum</td>
<td>0.1 (0.0-0.2)</td>
<td>0.2 (0.1-0.4)</td>
<td>0.1 (&lt;0.1-0.3)</td>
<td>0.1 (&lt;0.1-0.3)</td>
<td>&lt;0.5</td>
<td>0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>C. histolyticum/C. lituseburense group</td>
<td>0.2 (0.1-0.2)</td>
<td>0.2 (0.0-0.1)</td>
<td>0.2 (0.2-0.6)</td>
<td>0.1 (&lt;0.1-0.6)</td>
<td>&lt;0.5</td>
<td>&lt;0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Lactobacillus/Enterococcus</td>
<td>0.0 (0.0-0.1)</td>
<td>0.1 (0.0-0.1)</td>
<td>0.02 (&lt;0.1-0.5)</td>
<td>0.05 (&lt;0.1-0.1)</td>
<td>&lt;0.5</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Streptococcus/Lactococcus</td>
<td>1.1 (0.3-1.8)</td>
<td>0.3 (0.1-0.4)</td>
<td>0.5 (0.2-3.1)</td>
<td>1.1 (0.2-2.5)</td>
<td>0.4</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>unidentified bacteria</td>
<td>57.6 (23.9-79.0)</td>
<td>50.8 (30.7-87.6)</td>
<td>39.8 (0.69-4.1)</td>
<td>41.2 (21.6-48.1)</td>
<td>69.4</td>
<td>48.1</td>
<td>50.9</td>
</tr>
</tbody>
</table>

† p≤0.05 healthy controls versus CD-after
‡ p≤0.05 IBD-remission versus CD-after
** p≤0.05 IBD-remission versus CD-before
* p≤0.05 IBD-remission versus CD-before
before/after=before/after infliximab treatment

Two other trends could be detected. The lower severity of disease, the higher the percentages of both low G+ C Gram-positive bacteria and unidentified bacteria. So, percentages were on average increasing in the CD-active patients upon infliximab therapy, were even higher for the IBD-remission group, and were highest for the healthy controls. But these trends were not detected for all individual CD-active patients.
Potential faecal disease activity markers

Dry matter and pH
The average faecal dry matter in the CD-active group was 11.0 ± 2.3% before and 17.3 ± 2.1% after treatment. This increase was not significant but was seen in three out of the four CD-active patients. Patient CD3 had the lowest improvement in CDAI score, but showed a remarkable increase in faecal dry matter: from 4% before treatment to 23% after treatment (Table 6.4). Faecal dry matter was significantly higher in healthy subjects than in IBD patients, both those in remission and those with active disease before and after anti-TNF-α treatment (p≤0.02 for all). Also the difference between the IBD-remission group and the CD-active patient before treatment was significant (p=0.04). Faecal pH did not vary significantly between groups; overall faecal pH ranged from 5.3 to 7.2.

Metabolites
The total lactate and SCFA concentrations in faeces showed large interindividual differences. The total lactate concentrations did not differ between groups. In the CD-active group, infliximab treatment had no significant effect on lactate concentrations, but it did change the ratio of L- versus D-lactate (L:D lactate ratio) from 1:0.5 to 1:1.3 (p=0.07). This shift in favor of D-lactate was seen in every individual CD-active patient. The L:D ratio was 1:1.6 for the IBD-remission group and 1:2.9 for the healthy subjects (p≤ 0.04 for healthy controls versus IBD-remission, healthy controls versus CD-active both before and after treatment, and for IBD-remission versus the CD-active patients before and after treatment).

Although no significant differences concerning faecal SCFA or BCFA excretion were observed when comparing the CD-active patients before and after treatment, all excretions (except for iso-butyrate) were higher after the infliximab infusion. On the other hand, the excretions of all individual SCFA and BCFA were lower in faeces of the healthy control group compared to the IBD-remission group (Table 6.4). For acetate and the total SCFA plus BCFA excretion these differences were significant (p=0.02 and 0.04, respectively). All other significant differences between the different study (sub)groups did not correlate with health status (see Table 6.4).
Table 6.4 Potential faecal disease activity markers per gram faeces (wet weight). Dry matter: %; lactate and SCFA: µmol (mean ± SEM for healthy controls, IBD-remission, CD-before and CD-active (sub)groups; individual data for the CD-active group).

<table>
<thead>
<tr>
<th>potential faecal disease marker</th>
<th>Healthy controls</th>
<th>IBD-remission</th>
<th>CD-active</th>
<th>CD1</th>
<th>CD2</th>
<th>CD3</th>
<th>CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=7)</td>
<td>(n=12)</td>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.2 (0.1)</td>
<td>6.3 (0.1)</td>
<td>6.1 (0.4)</td>
<td>5.9</td>
<td>5.8</td>
<td>5.9</td>
<td>7.2</td>
</tr>
<tr>
<td>dry matter</td>
<td>30.2 †(2.0)</td>
<td>21.2** (2.3)</td>
<td>11.0 (2.3)</td>
<td>17.3 †(2.1)</td>
<td>12.0</td>
<td>14.7</td>
<td>12.9</td>
</tr>
<tr>
<td>L-lactate</td>
<td>4.0 (1.3)</td>
<td>9.6 (3.7)</td>
<td>13.6 (10.2)</td>
<td>9.1</td>
<td>43.9</td>
<td>13.9</td>
<td>1.4</td>
</tr>
<tr>
<td>D-lactate</td>
<td>10.1 (2.6)</td>
<td>16.7 (5.6)</td>
<td>4.0 (2.4)</td>
<td>11.8</td>
<td>11.1</td>
<td>17.4</td>
<td>0.8</td>
</tr>
<tr>
<td>total lactate</td>
<td>14.1 (3.6)</td>
<td>26.3 (9.3)</td>
<td>17.6 (12.5)</td>
<td>21.0</td>
<td>54.9</td>
<td>31.3</td>
<td>2.2</td>
</tr>
<tr>
<td>acetate</td>
<td>21.3 †(5.0)</td>
<td>55.0 (9.9)</td>
<td>34.5 (18.9)</td>
<td>59.7 †(8.7)</td>
<td>14.8</td>
<td>83.7</td>
<td>89.1</td>
</tr>
<tr>
<td>propionate</td>
<td>9.3 (1.6)</td>
<td>16.7 (3.4)</td>
<td>7.0 (3.6)</td>
<td>18.9 †(3.3)</td>
<td>5.1</td>
<td>27.0</td>
<td>17.6</td>
</tr>
<tr>
<td>iso-butyrate</td>
<td>1.4 (0.2)</td>
<td>2.0** (0.5)</td>
<td>0.2 (0.2)</td>
<td>0.7 †(0.2)</td>
<td>0.0</td>
<td>0.6</td>
<td>0.0</td>
</tr>
<tr>
<td>butyrate</td>
<td>9.0 (1.8)</td>
<td>14.9 (3.1)</td>
<td>8.7 (4.1)</td>
<td>13.2</td>
<td>2.1</td>
<td>16.5</td>
<td>14.9</td>
</tr>
<tr>
<td>iso-valerate</td>
<td>1.7 (0.3)</td>
<td>3.0** (0.7)</td>
<td>0.3 (0.1)</td>
<td>1.2</td>
<td>0.4</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>valerate</td>
<td>1.5 (0.3)</td>
<td>1.9** (0.6)</td>
<td>0.1 (0.1)</td>
<td>0.6</td>
<td>0.2</td>
<td>1.9</td>
<td>0.0</td>
</tr>
<tr>
<td>total SCFA +BCFA</td>
<td>45.3 †(8.2)</td>
<td>93.4 (17.4)</td>
<td>50.8 (25.1)</td>
<td>94.2 †(13.4)</td>
<td>22.6</td>
<td>130.6</td>
<td>121.8</td>
</tr>
</tbody>
</table>

† p≤0.05 healthy controls versus IBD-remission  
‡ p≤0.05 healthy controls versus CD-before  
¬ p≤0.05 healthy controls versus CD-after  
** p≤0.05 IBD-remission versus CD-before  
SCFA=short-chain fatty acids  
BCFA=branched-chain fatty acids  
before/after=before/after infliximab treatment

Discussion

We aimed to investigate the composition and activity of the faecal microbiota in patients with active Crohn’s disease, to study the effect of anti-TNF-α monoclonal antibody therapy on these microbiotas, and to compare them with faecal parameters in Crohn’s patients in remission and healthy controls.

The first conclusion, found with both the classical plating technique and FISH, was that induction of disease remission in CD-active patients by infliximab therapy led to an increase in bacterial
counts in the faeces. Secondly, a reduced total number of faecal bacteria correlated with a more severe disease activity, but was not inherent to the disease itself since the total bacterial number of the IBD-remission patients resembled that of the healthy subjects. Significant compositional changes that correlated with the physical condition of all studied subjects were only found with the plating technique. The detected increases in the number of bifidobacteria, bacteroides, lactobacilli, and sulphite reducing clostridia occurred also in all individual CD-active patients upon infliximab infusion. Only lactobacilli were present in lower amounts in IBD patients in remission than in healthy controls. The trends detected by FISH, that is higher percentages of both low G+C Gram-positive bacteria and unidentified bacteria in less severe disease activity, needs further study in more detail. In general, molecular approaches provide better insights in the composition of the faecal microbiota than the plating technique; media are often not truly specific or are too selective for certain bacteria (24). But the number of lactobacilli in faeces are often below the detection limit in FISH (<10^7 CFU/gram; see also Table 6.2), so for this latter group one has to rely on the plating technique. Microbial compositions in faeces as well as on the mucosal surface of biopsy specimens have been used to study the role of (specific) microorganisms in IBD. The relevance of these studies was supported by the findings of the CARD15 gene as the first susceptibility gene for CD (33,34). The CARD15 protein is mainly expressed in phagocytic cells. Its encoded protein is able to induce NFκB after recognition of intracellular bacterial peptidoglycan (35,36). NFκB is an important regulator of genes involved in a pro-inflammatory response (37).

A study in which IBD tissue was investigated with molecular techniques reported that the mucosal surface of IBD patients with active disease contained more bacteria than on those of non-IBD controls (38). Others were unable to confirm these findings and reported similar bacterial colonization densities on the colonic mucosal surface of IBD patients and controls (39,40). Apart from this discrepancy with respect to bacterial counts, discrepant results were also observed for the diversity of the microbiota on the colonic mucosal surface in patients with CD. Some reported that this diversity was reduced up to 50% in comparison with controls (38,39). One study group found that CD tissue samples mainly harboured bacteria belonging to Proteobacteria, the Enterobacteriaceae, and the Bacteroides/Prevotella cluster (38), while others found that the reduction in diversity of the microbiota on the colonic mucosa in CD was due to loss of normal anaerobic bacteria such as Bacteroides species, Eubacterium species, and Lactobacillus species (39). In contrast, another group, when comparing tissue samples from the descending colon of patients with UC (two inactive, one active) and healthy individuals, found no significant differences with respect to the composition of the predominant bacterial community, and that of the Lactobacillus group community (40). Investigators, which like us used faeces to detect if the microbiota of IBD patients may be different from that of healthy individuals, also reported varying results. Both an increase (41) and a decrease (42) in faecal Bacteroides have been reported in patients with CD compared to controls. Two study groups showed a reduced score of bifidobacteria in these patients compared to controls, and reported an increased number of enterobacteria in (active and inactive) CD (42) and Escherichia coli in active CD (43). But the significantly reduced Lactobacillus score in patients with CD compared to controls was only found by Giaffer et al. (43). Van der Waaij (44) has recently shown that the microbial composition of (solid) faeces was
comparable to the microbial composition of the luminal contents in the proximal colon, as well as that of the colonic mucosa. But comparing the results of our study with results from IBD tissue investigations requires caution. Zoetendal et al. (40) used a 16S rRNA approach to demonstrate that the predominant mucosa-associated bacterial community was significantly different from the faecal community. Furthermore, Marteau et al., (45) found that the human caecal microbiota differs quantitatively and qualitatively from the faecal microbiota. In conclusion, at this moment results with respect to bacterial counts and the diversity of the microbiota are still sometimes conflicting despite the use of accurate modern molecular techniques. Furthermore, no real consensus exists which material (faeces, colonic contents, biopsy specimens) has to be studied and how to compare the results when different techniques (plating, DGGE, FISH or dot blot) have been used. To overcome these problems, the intestinal microbiota in IBD should be further studied, using different techniques, materials, and patients simultaneously.

A shift in favor of D-lactate and an increase in faecal dry matter was seen for every individual CD-active patient as a result of a first, single infliximab infusion. These two parameters overall correlated with the physical condition of all studied subjects; the less severe disease activity, the lower the L-lactate excretion compared to the D-lactate excretion and the higher the faecal dry matter. Furthermore, given the significant differences concerning these two parameters between the healthy individuals and the IBD patients in remission, lower faecal dry matter and a higher L:D lactate ratio seem inherent to IBD. These two parameters can possibly be used as faecal disease activity marker.

The shift in favor of D-lactate is compatible with reduced host L-lactate production and/or increased bacterial D-lactate formation (46). The increase in total number of faecal bacteria when disease activity became less severe could correspond to an increased bacterial lactate formation. Concerning faecal dry matter we may also conclude that, given the significant difference between the IBD-remission group and the healthy controls, patients suffering from quiescent IBD still show a fast transit time and/or reduced net fluid absorption, resulting in a high water content of their faeces. Furthermore, the differences seen in total number of bacterial counts between the groups with different disease activities was not the result of differences in faecal dry matter. When expressed as CFU/gram dry matter total bacterial counts still were significantly higher in healthy controls compared to CD-active patients before infliximab infusion (plating: p=0.02; FISH: p=0.04).

Faecal SCFA and BCFA excretion did not correlate with anti-TNF-α treatment or with health status of the tested (sub)groups. It is remarkable that for the metabolites most abundantly present in faeces (i.e. acetate, propionate, and butyrate) excretions by the healthy individuals resembled that of the CD-active patients before infliximab treatment, and excretions by the IBD patients in remission were similar to that by the CD-active patients after the treatment. The difficulty with faecal metabolites is that elevated concentrations may reflect impaired absorption and/or utilization as well as increased production. Which of these two aspects applies, or to what extent each of these aspects contributes to increased levels of microbial metabolites in these faecal samples remains to be studied. The phenomenon seen for excretions of acetate, propionate, butyrate, and also total SCFA in faeces can be explained as follows. A higher bacterial colonization density may be
associated with a higher total microbial activity. Excretion of these microbial metabolites in faeces is the result of microbial activity minus mucosal absorption and/or utilization. Therefore, we may conclude that a higher faecal excretion of microbial metabolites then that found for healthy individuals, combined with an equal total faecal number of bacteria to that of healthy individuals mean that the mucosal absorption and/or utilization is impaired. This seems to be the case for the IBD remission group, as well as for the CD-active patients after infliximab treatment (based on faecal number of bacteria determined by FISH and acetate, propionate, butyrate and total SCFA excretion). Furthermore, faecal excretions of microbial metabolites equal to that found for healthy individuals, combined with a lower total faecal number of bacteria to that of healthy individuals can also mean that the mucosal absorption and/or utilization is impaired. This seems to be the case for the CD-active patients before infliximab treatment. To confirm these conclusions, more (infliximab treated) patients should be investigated. Concentrations of SCFA and lactate in faeces of IBD patients have been determined before. The reported results are conflicting, pointing out the difficulty of using faeces. Children with UC and ileocolonic CD had a significant decrease in the faecal concentration of acetate, a significant increase in butyrate, while no changes in fecal lactate were seen compared with age-matched controls (47). Especially the faecal samples of inactive- or mild-UC children showed increased butyrate levels, whereas the faecal samples of moderate- and severe-UC children were mainly responsible for the decreased total SCFA and acetate levels. Others however found increased lactate (mainly L-lactate) levels in adult ulcerative colitis patients with either pancolitis or proctitis compared with quiescent disease. In this study, lactate concentrations correlated with the disease activity index of Truelove. Lactate was also increased in Crohn's colitis compared with quiescent CD, but lactate did not correlate with a disease activity index. This study did not find a correlation of SCFA (including butyrate) with inflammatory activity or localization in either ulcerative colitis or Crohn’s disease (48). Our results correspond partly with these latter observations.

In conclusion, in this pilot-study active IBD is associated with a significant reduced total number of faecal bacteria and a significant lower faecal dry matter than found in healthy controls. Induction of disease remission leads to an increase of both parameters. These changes are associated with a decrease in the L- versus D-lactate ratio. These three parameters can possibly be used as clinical disease activity markers. For this, larger studies need to be undertaken.
References


Effects of anti-TNF-α antibodies on faecal microbiota


Discussion and conclusions
The role of the microbiota in IBD

Intestinal bacteria drive the inflammatory process in IBD. Loss of tolerance to bacteria indigenous to the large intestine, the role of bacteria in disease development in animal models of IBD, responses of IBD patients to antimicrobial therapy, and infiltration of inflammatory bacterial products across the intestinal mucosal barrier, altogether reveal the possible role of microbes in IBD. Some suggest that specific microorganisms play a role in the induction of inflammation, while others think that metabolites derived from the microbiota, without regard to species, can be mediators of injury and inflammation. Unfortunately, there is a lack of solid data to substantiate this latter hypothesis. This is primarily due to the fact that the study of microbial metabolic activity is difficult, among others for reasons of sampling and difficulties in mimicking physiological circumstances *ex vivo*. Our TIM-2 system overcomes a lot of these difficulties and is therefore relevant for the study of microbial metabolic activities (1-4). In this thesis the effect of the microbiota on the onset and exacerbation of IBD was investigated, with a focus on microbial activity.

In the current chapter the results from the preceding chapters are discussed. The discussion focuses on the role of microbial metabolites in IBD and is followed by a summary of the main conclusions derived from the research described in this thesis.
Composition of the microbiota

The first aspect in the study of the role of the microbiota in IBD is determining the composition of faecal microbiota with regard to bacterial counts and species. In chapter 6 we investigated the faecal microbial composition of healthy adults and IBD patients both in remission and during disease exacerbation using classical plating and FISH. The patients with active disease were studied both before and after treatment with a biological therapy (anti-TNF-α antibodies; see also below). With both plating and FISH techniques, we found an overall higher number of bacteria in faeces of Crohn’s patients after treatment than before. These numbers were also compared with outcomes in faecal samples of IBD patients in remission and healthy controls. From these comparisons it may be concluded that disease remission correlates with an increase in total faecal bacterial counts, which approach levels observed in healthy controls.

Metabolic activity of the microbiota

Microbiota originating from healthy individuals versus out-of-balance microbiota

Before investigating the role of microbial metabolites in IBD, we first aimed to understand which metabolites are produced during the normal colonic fermentation process by a microbiota originating from healthy human individuals and which metabolites are produced when this microbiota is out-of-balance. In chapter 2 TNO's dynamic in vitro model of the large intestine (TIM-2) was used to investigate the production of microbial metabolites by a standardized microbiota, representative for the healthy human proximal colon. The emphasis was on toxic metabolites, such as ammonium, phenols and indoles, and BCFA. Clostridium difficile was introduced into TIM-2 to study the potential for toxic metabolite production by a microbiota that is out-of-balance. Furthermore, the effect of inulins with different degree of polymerisation (ranging from 3 to 25) on the saccharolytic- and proteolytic activity of both types of colonic microbiota was investigated. Inulin is regarded a prebiotic; it increases the number and/or activity of specific microorganisms in the gastrointestinal tract presumed to be health promoting (5).

The addition of inulin to the standardized, healthy microbiota suppressed the formation of BCFA, ammonium, and phenolic compounds while at the same time SCFA and lactate production was stimulated. Since BCFA, ammonium, and phenolic compounds are products typical of breakdown and fermentation of proteins it can be concluded that inulins inhibit the proteolytic activity of the colonic microbiota and stimulate the saccharolytic activity, as indicated by the increased SCFA and lactate production. The introduction of C. difficile caused an expected stimulation of the production of the protein fermentative metabolites. This negative effect of C. difficile was almost completely prevented by the addition of inulins, irrespective of the chain length.

In conclusion, the results of the study described in chapter 2 indicate a potential for inulins to shift the metabolic activity of the human colonic microbiota towards the production of less potentially toxic metabolites, both under normal conditions and under conditions with a microbiota that is out-of-balance. The introduction of only a small number of C. difficile bacteria (only about 1% or less
of the total bacterial population was added) caused a major shift in the overall metabolism of the colonic microbiota towards proteolysis.

**Metabolic activity of the microbiota of IBD patients**

Changes in the microbiota may also have an effect on metabolite production, which in theory may lead to increased levels of toxic metabolites in patients with active inflammatory bowel disease. It is therefore of great interest to determine whether individuals with IBD show evidence of an altered metabolic function of the microbiota in the large intestine and whether this relates to the onset or progression of the disease. Dietary interventions in these patients, with for instance prebiotics, may have the same effect on the metabolic activity of the microbiota as that shown for healthy individuals in chapter 2. Some published data suggest that the metabolic activity of the large intestinal microbiota of patients suffering from UC and CD is different compared to that of healthy individuals. If this difference in activity resembles an out-of-balance situation, resulting in a higher level of potentially toxic metabolites, this activity can be a mediator of injury and inflammation.

In the third chapter we therefore investigated whether the microbiota of IBD patients indeed show such an altered metabolic activity in the proximal colon in comparison with healthy controls and whether this is related to the severity of the disease. For that purpose, TIM-2 was inoculated with fresh faeces of 6 healthy individuals and 8 IBD patients. Samples were taken from the model over time to analyze metabolites from both saccharolytic- and proteolytic fermentation. We observed higher production of SCFA and BCFA, as well as ammonia, in the experiments using faeces of IBD patients compared to that of controls. These differences were not observed for the production of phenolic compounds and lactate, which varied greatly.

The higher metabolite production in IBD patients is in agreement with literature data obtained with other methods (6-8). The cause of the higher metabolic activity in these patients is unknown. Using TIM-2, we showed that damage to the mucosa, resulting in inadequate absorption of these metabolites, is not the cause of the increased metabolite concentrations, although it may contribute to a higher excretion of these metabolites in faeces of IBD patients (see chapter 6). The higher production of (potentially toxic) metabolites in IBD patients may play a role in the onset or chronicity of IBD.

The results described in chapter 3 may have been influenced by a number of potential confounders. During the experiments in TIM-2, the pH was set at 5.8. This is the pH in the proximal colon of healthy individuals (9). No extensive research is done in IBD patients concerning the pH in (this part of) the colon. Another debatable point is the digestion of food in the upper gastrointestinal tract in these individuals; if this function of the gastrointestinal tract in IBD patients also deviates from healthy individuals, the IBD microbiota in TIM-2 are fed other substrates than in vivo, which will result in different metabolite profiles. Without extensive knowledge of the environmental circumstances in the proximal colon of IBD patients, the results described in chapter 3 should be interpreted with caution.

The proteolytic activity of the gut microbiota can increase remarkably, in the distal colon compared to the proximal colon even by a factor of 5 (10-12). Therefore, fermentation in the transverse and descending parts of the colon will give more insight into the total colonic health status. An in vitro
system simulating this distal part, with physiological parameters, such as lower water content and a higher pH, is in development at our institute and will be used in the future to study the metabolic activity of microbiotas obtained from healthy persons and IBD patients under even more relevant physiological conditions.

Role of nutrition in IBD

The microbial activity in the colon is in part defined by the components of the food that reach this part of the digestive tract. The results of both the first (chapter 2) and the second (chapter 3) study showed that feeding microbiotas of healthy humans approximately 5 g of protein per day, which is about one third of the estimated amount that becomes available in the proximal colon in vivo (13,14), results in the production of detectable amounts of potentially toxic or harmful metabolites. Since the model mimics the environmental conditions of the healthy proximal colon, this indicates that even in a region of the gut where protein fermentation is of minor importance these substances can still be produced.

To control disease activity, some dietary compounds, such as proteins, can be removed from the diet of IBD patients whereas others, such as prebiotics, can be added to the normal diet. In chapter 2 we showed that inulin indeed acted as a prebiotic; it reduced the production of proteolytic fermentation products, both under normal conditions and under conditions with a microbiota that is out-of-balance. We observed higher production of SCFA and BCFA, as well as ammonia, in the experiments using faeces of IBD patients compared to that of controls (chapter 3); whether the addition of inulins to these microbiotas of IBD patients also results in suppression of the formation of BCFA and ammonia, and in even a further increase in SCFA production needs to be studied. Also more detailed studies are needed to clearly establish the relevance of even small amounts of protein in IBD.

Defense

Intestinal epithelial cells (IEC) that line the gastrointestinal mucosa can be regarded as the most outer defence barrier preventing microorganisms and endotoxins from reaching systemic organs and tissues (15-18). The relation between IEC, luminal microbiota, and IBD appears evident. IEC play an important role in the interaction between luminal content and the host’s immune system (16,17,19), while a role for the microbiota in triggering the inflammatory process has been proposed (20-22). Both luminal microorganisms and their metabolites can possibly influence the metabolic integrity of the IEC, resulting in presentation of these antigens to the mucosal immune system (23-25).

The ability of the colonic mucosa to detoxify metabolites derived from proteolytic fermentation seems to be impaired in UC (11), causing the colonic tissue of patients to be more susceptible to the toxicity of these compounds than colonic tissue of healthy individuals. If so, the microbial activity in the colon of IBD patients can certainly serve as an indicator of disease severity. However,
without *ex vivo* or *in vivo* tests on the toxicity of these compounds, it cannot be concluded which metabolites (and at which concentrations) cause injury to the epithelial barrier of IBD patients.

**Studying defense by using cell cultures**

We hypothesized that the increase in production of microbial metabolites (chapter 3) contributes to IEC injury and/or loss of the integrity of the barrier function in IBD. Enhanced presentation of these microbial metabolites to the mucosal immune system may then play a role in the maintenance of IBD activity. In chapter 4 we aimed to investigate which metabolites, and at which concentrations, cause injury to epithelial cells and thereby might stimulate unwanted immune reactions. This was studied by co-culturing enterocyte-like Caco-2 cells with macrophages in separate compartments of a Transwell™ system. In addition, the direct effect of butyrate (C4), *iso*-valerate (i-C5), and ammonium (NH₄⁺) on Caco-2 cells and macrophages separately was tested. Barrier function of the Caco-2 monolayer (mono-culture and co-culture) was determined by measuring transepithelial electrical resistance and basolateral recoveries of the microbial metabolite. Also the contribution of microbial metabolites to the chronic inflammatory process by stimulating induction of pro- and anti-inflammatory cytokines (TNF-α and IL-10, respectively) by Caco-2 cells and macrophages was investigated. The following concentrations were tested: 5-100 mM (C4), 4-100 mM (i-C5), and 2-40 mM (NH₄⁺).

Information about luminal concentrations of C4, i-C5 and NH₄⁺ *in vivo* is scarce. Two studies give some more information of what can be produced *in vivo*. Cummings *et al.* (26) and MacFarlane *et al.* (27) measured SCFA, BCFA, and ammonium in colonic luminal contents of sudden-death individuals. The intraluminal concentrations of C4, i-C5, and NH₄⁺ in the proximal colon were 38, 2, and 20 mmol/kg wet weight contents, respectively (27). The observed maximum intraluminal metabolite concentrations produced by IBD microbiotas in an *in vitro* model were approximately 30 mM for C4, 5 mM for i-C5 and 65 mM for ammonium (28). However, both these *in vivo* and *in vitro* concentrations were totally dependent on the moment of sampling; it cannot be concluded that intraluminal concentrations of C4, i-C5 and NH₄⁺ in healthy persons or in IBD patients do not reach higher levels, for instance simply because of diet. In case of an impaired epithelial barrier, such as in patients with an inflamed ulcerated colon, colonic macrophages can be exposed to luminal concentrations of microbial metabolites.

Our *in vitro* exposure experiments in chapter 4 showed that the barrier function of IEC is not easily disturbed by microbial metabolites. Exposure to ammonia (10-40 mM), or to high concentrations of C4 or i-C5 (100 mM) lowered TEER values of Caco-2 mono-cultures, but the absolute TEER levels remained higher than 500 Ω*cm², a level which is compatible with an intact barrier function (29). As a result, basolateral recovery of metabolites was in the same order of magnitude in all experiments, regardless of type of experiment (mono- or co-culture), metabolite, or concentration of metabolite tested. Basolateral recoveries ranged from 6.2% to 15.2%. However, it cannot be concluded that intraluminal concentrations of C4, i-C5 and NH₄⁺ in IBD patients do not lead to increased permeability of the barrier function *in vivo* because we do not know how sensitive intestinal epithelial cells of IBD patients are. The Caco-2 cells did not secrete TNF-α or IL-10. Concentrations of butyrate and *iso*-valerate as high as those produced by IBD patients stimulated
the secretion of TNF-α and suppressed the secretion of IL-10 by macrophages that are not protected by an epithelial barrier. In contrast, ammonium concentrations close to those produced by microbiotas of IBD patients suppressed the release of both cytokines when the barrier function is impaired.

Due to the (intact) barrier function of the Caco-2 cells, the TNF-α and IL-10 secretion levels in the co-culture experiments reflect the effect of exposure of macrophages to approximately 10% of concentrations of initial amounts of metabolites added to the luminal side in the Transwell™. In this co-culture set-up, ammonium no longer had an inhibitory effect on secretion of TNF-α and IL-10 by macrophages (levels of both cytokines were as high as with LPS). The effect of the other metabolites remained pro-inflammatory, even when the macrophages were protected by an epithelial barrier. The concentrations of C4 and i-C5 tested in the co-culture experiments are higher than the concentrations found in vivo (sudden death individuals) and in vitro (IBD faeces in in vitro model). However, butyrate is likely to reach concentrations of 50 or 100 mM, for instance upon consumption of prebiotics.

Both the supposed positive effect of C4 and the supposed negative effect of NH₄⁺ are not confirmed by the present experiments. Our results are contrasting with the limited data available concerning butyrate and the production of cytokines: studies with whole-blood models and isolated mononuclear cells showed that butyrate (to a maximum of 2 mM) reduced LPS induced TNF-α and/or IL-10 production (30-32). A complex mixture of microbial metabolites, obtained from TNO’s in vitro model of the large intestine, reduced TNF-α and INF-γ, while inducing IL-10 (van Nuenen et al., manuscript in preparation). This might indicate that metabolites in combinations might have opposing or synergistic effects. We are currently studying this in more detail, including the effect of the cytokines on the barrier function.

In conclusion, the results of the study described in chapter 4 indicate that the barrier function of intestinal epithelial cells in vitro is not disturbed by the microbial metabolites when tested separately, but these metabolites are able to modulate the secretion of both TNF-α and IL-10 by macrophages underlying the epithelium. The enhanced production of C4 and i-C5 by the microbiotas observed in chapter 3 may thus play a role in the maintenance of IBD activity.

### Biological therapy for IBD

Novel therapeutic approaches for the treatment of IBD often use biologic agents that mechanistically target individual inflammatory pathways. Some biological agents have already been proven to be effective for CD and UC (33-35), others are still under investigation (36-39). Besides immunological strategies, also the targeting of therapeutics to improve topical delivery is a strategy for IBD that can be realized using a biological approach (38,40). In microbiological strategies, microorganisms are used to modify the gut microbiota and they are called probiotics. More recently genetically engineered bacteria have been constructed that can function as a probiotic, such as lactococci that secrete immunosuppressive IL-10 (41).
**Interleukin-10 secreting lactococci**

Irrespective of whether or not the primary disorder in IBD is immunological in nature, a good therapeutic approach in CD is the inhibition of \( \text{T}_{\text{H}}-1 \) polarization of the immune system by using the immunomodulatory recombinant human IL-10. This cytokine controls and suppresses inflammation essentially by down regulating pro-inflammatory cytokine production. A number of studies with animal models of IBD have shown the efficacy of IL-10 for the regulation of mucosal inflammation. A new development in IBD-therapeutics is the targeted delivery of IL-10 to the site of inflammation by means of *in situ* synthesis using a recombinant *L. lactis*. For this therapy to be efficacious, an important criterion is the survival of the *L. lactis* strain in the upper gastrointestinal tract of the host (42,43).

In chapter 5 we aimed to determine whether targeted delivery of hIL-10 producing *L. lactis* could be obtained by incorporating the strain in an enteric-coated capsule formulation. Survival and metabolic activity of the recombinant *L. lactis* was studied in TIM-1, simulating the successive dynamic processes in the stomach and the small intestine, as stationary-phase grown cells or after incorporation in two types of enteric-coated formulation (Eudragit® L30D-55, disintegrating at a pH of 6.0 and higher; and Eudragit® FS 30 D, disintegrating at a pH equal to or higher than 7.2). Only 0.04% of the oral intake of viable cells was recovered for the lactococci taken as stationary-phase grown cells. Incorporating *L. lactis* in Eudragit® L30D-55 coated capsules increased the viable cells to 0.28% and Eudragit® FS 30 D coated capsules improved this further to 1.21%. The capacity of the cells to secrete hIL-10 was validated *in vivo* in pigs (44), but was not observed in our *in vitro* systems. The reason for this was that, contrary to the pig experiment, the absolute amount of viable bacteria that would have been able to produce hIL-10 *in vitro* was to low to produce hIL-10 concentrations that are above the detection limit of an ELISA assay. Nevertheless, the study clearly indicated that both types of capsules are effective in protecting lactococci during passage through the stomach and (parts of) the small intestine. Depending of the desired site of action of the cells, the proper capsule can be chosen. If the site of action should be the jejunum, a Eudragit® L30D-55 coated capsule is the preferred choice of dosage form; if the site of action is the ileum or proximal colon, a Eudragit® FS 30 D coated capsule should be chosen.

From chapter 5 we can conclude that, even though survival of *L. lactis* is low when expressed in percentages, total viable cell numbers of *L. lactis* at the end of the small intestine reach 2.4 * 10^8–1.2 * 10^9. In our *in vitro* model we have shown proof-of-principle for the delivery of these cells in the gastrointestinal tract. Enteric-coated capsules for ileal delivery of hIL-10 producing *L. lactis* can thus be a potential therapy for CD. Combining these results with the outcome of a preclinical study in which the same two formulations with hIL-10 producing *L. lactis* has been administered to 10 patients suffering from CD (Amsterdam Medical Centre, The Netherlands) will provide valuable insight into safety, biological containment and clinical effectiveness of this novel medical strategy.

**Monoclonal antibodies against TNF-α**

Another therapy currently in practice in IBD is the use of anti-TNF-α monoclonal antibodies. These antibodies block TNF-α, a powerful immune stimulator. A single intravenous dose of this biological
therapy results in reduced CD activity scores and increased remission. In chapter 6 we aimed to investigate the composition and activity of the faecal microbiota in patients with active CD, to study the effect of anti-TNF-α monoclonal antibody (infliximab) therapy on these microbiotas, and to compare them with faecal parameters in Crohn’s patients in remission and healthy controls. Microbial composition was determined using the classical plating technique and FISH, and the potential disease activity markers determined in the faecal samples were dry matter, pH, SCFA, BCFA, and lactate.

The first conclusion, found with both the classical plating technique and FISH, was that induction of disease remission in CD-active patients by infliximab therapy led to an increase in bacterial counts in the faeces. Secondly, a reduced total number of faecal bacteria correlated with a more severe disease activity, but was not inherent to the disease itself since the total bacterial number of the IBD-remission patients resembled that of the healthy subjects. Increases in the number of bifidobacteria, bacteroides, lactobacilli, and sulphite reducing clostridia correlated with the physical condition of all studied subjects and occurred also in all individual CD-active patients upon infliximab infusion. Only lactobacilli were present in lower amounts in IBD patients in remission than in healthy controls. The trends detected by FISH, that is the lower severity of disease, the higher the percentages of both low G+C Gram-positive bacteria and unidentified bacteria, can be interesting to study in more detail. Microbial compositions in faeces as well as on the mucosal surface of biopsy specimens have been used to study the role of (specific) microorganisms in IBD. In conclusion, at this moment results with respect to bacterial counts and the diversity of the microbiota are still sometimes conflicting despite the use of accurate modern molecular techniques. Furthermore, no real consensus exists which material (faeces, colonic contents, biopsy specimens) has to be studied and how to compare the results when different techniques (plating, DGGE, FISH or dot blot) have been used (45-52). To overcome these problems, the intestinal microbiota in IBD should be further studied, using different techniques, materials, and patients simultaneously.

Concerning the potential disease activity markers determined in the faecal samples, a shift in favor of D-lactate and an increase in faecal dry matter was seen for every individual CD-active patient as a result of a first, single infliximab infusion. These two parameters overall correlated with the physical condition of all studied subjects; the less severe disease activity, the lower the L-lactate excretion compared to the D-lactate excretion and the higher the faecal dry matter. Furthermore, given the significant differences concerning these two parameters between the healthy individuals and the IBD patients in remission, lower faecal dry matter and a higher L:D lactate ratio seem inherent to IBD.

In conclusion, in the pilot-study described in chapter 6 active IBD is associated with a significant reduced total number of faecal bacteria and a significant lower faecal dry matter than found in healthy controls. Induction of disease remission leads to an increase of both parameters. These changes are associated with a decrease in the L- versus D-lactate ratio. These three parameters can possibly be used as clinical disease activity markers. For this larger studies need to be undertaken.
Main conclusions

- When studying the effect of the microbiota on the onset and exacerbation of IBD both the composition of the intestinal microbiota as well as the metabolites derived from this microbiota should be analysed.

- Results concerning the microbial composition of IBD patients are still conflicting although nowadays modern molecular techniques are used. No consensus exists in which material (faeces, colonic contents, biopsy specimens) has to be studied and how to compare the results when different techniques (plating, DGGE, FISH or dot blot) have been used. To overcome these problems, the intestinal microbiota in IBD should be further studied, using different techniques, materials, and patients simultaneously.

- Active inflammatory bowel disease is associated with a significant reduced total number of faecal bacteria and a significant lower faecal dry matter than found in healthy controls. Induction of disease remission leads to an increase of both parameters. These changes are associated with a decrease in the L- versus D-lactate ratio. These three parameters can possibly be used as clinical disease activity markers. For this larger studies need to be undertaken.

- The study of microbial metabolic activity is difficult, among others for reasons of sampling and difficulty mimicking physiological circumstances \textit{ex vivo}. TNO’s \textit{in vitro} system of the proximal colon turned out to be relevant for studying microbial metabolic activity although one should keep in mind the disadvantages of using such a system.

- Inulins inhibit the proteolytic activity of the colonic microbiota and stimulate the saccharolytic activity, both under normal conditions and under conditions with a microbiota that is out-of-balance.

- The introduction of only a small number of \textit{C. difficile} bacteria (\(\leq 1\%\) of the total bacterial population was added) caused a major shift in the overall metabolism of the colonic microbiota towards proteolysis.

- The production of (potentially toxic) metabolites may play a role in the onset or chronicity of inflammatory bowel disease, because they were produced in higher amounts by microbiotas from these patients than by microbiotas from healthy individuals.

- The barrier function of intestinal epithelial cells \textit{in vitro} is not disturbed by the microbial metabolites butyrate (\(\leq 100\) mM), \textit{iso}-valerate (\(\leq 100\) mM), or ammonium (\(\leq 40\) mM). But it cannot be concluded that intraluminal concentrations of butyrate, \textit{iso}-valerate and ammonium in IBD patients do not lead to increased permeability of the barrier function \textit{in vivo} because we do not know how sensitive intestinal epithelial cells of IBD patients are.

- In case of an impaired epithelial barrier, such as in patients with an inflamed ulcerated colon, colonic macrophages can be exposed to luminal concentrations of microbial metabolites. Physiological concentrations of butyrate and \textit{iso}-valerate both stimulated the secretion of TNF-\(\alpha\) and suppressed the secretion of IL-10 by macrophages that are not protected by an epithelial barrier. In contrast, ammonium concentrations close to those produced by microbiotas of IBD patients suppressed the release of both cytokines when the
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barrier function is impaired. The enhanced production of butyrate and iso-valerate by the microbiotas of IBD patients may thus play a role in the maintenance of IBD activity.

• More detailed studies are needed to establish the relevance of a lower proteolytic activity of the colonic microbiota (inulins), a higher saccharolytic activity (inulins, microbial activity of IBD patients in remission), or a higher proteolytic activity (C. difficile, microbial activity of IBD patients in remission) on the gastrointestinal health status.

• Damage to the mucosa, resulting in inadequate absorption of microbial metabolites, contributes to a higher excretion of these metabolites in faeces of IBD patients. We did not only show a higher faecal excretion of microbial metabolites for some IBD patients compared to healthy individuals, but also a higher production of metabolites in IBD patients. To what extent each of these aspects contributes to the onset or chronicity of IBD remains to be studied.

• The cause of the higher metabolic activity in IBD patients is unknown. The role of the total microbial number and the environmental circumstances (pH, amount of food) in the proximal, transverse, and distal colon of IBD patients should all be investigated to determine the effects on the level of microbial activity.

• Novel therapeutic approaches for the treatment of IBD can be investigated using the TNO in vitro models of the upper gastrointestinal tract and the proximal colon. Experiments in TIM-1 showed that the hIL-10 producing L. lactis can indeed be a potential therapy for CD because the enteric-coated capsules protected the lactococci and delivered them to the ileum. The effect of for instance increased TNF-α concentrations at the luminal site of the colon, or a decrease of this cytokine as a result of infliximab treatment on metabolic activity and/or microbial compositions should be studied in TIM-2 to make up the results out of chapter 6. Especially when investigating drugs in the treatment of IBD wherein the microbial activity is involved, as is the case for sulfasalazine, a TIM-2 system simulating the dynamics of the colon of an IBD patient would be an excellent screening tool.

• Once the role of the metabolic activity of the microbiota will become clearer, dietary interventions of IBD may become feasible by the possibility to modulate this metabolic activity. The provided biomarkers can possibly be used in prevention and treatment of the disease in humans.
References


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