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# Attenuation of oxidative stress, inflammation and colorectal oncogenesis by food constituents: Ischaemia/reperfusion injury and induced colitis in rodents

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2010





# Attenuation of oxidative stress, inflammation and colorectal oncogenesis by food constituents: Ischaemia/reperfusion injury and induced colitis in rodents

Åsa Håkansson

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**LUNDS UNIVERSITET**

Lunds Tekniska Högskola

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# Abstract

The enteric microbiota is increasingly implicated as a central factor in the development of intestinal inflammation in humans and experimental animals, and the intestinal environment is considered important in both colorectal cancer development and modulation of mucosal immunity. Diet-induced changes in the different populations of the intestinal microbiota can be achieved by use of probiotic bacteria, dietary fibre and berries rich in phenolic compounds. The present thesis aims to find relevant animal models with clinical similarities and use these models to evaluate the anti-inflammatory effects of feed supplements.

Supplementation of *L. plantarum* and rose hip in combination decreases lipid-peroxidation in caecum independently of the vitamin C content and inhibits the growth of *Enterobacteriaceae*, in response to ischaemia/reperfusion injury.

An attempt was made to understand how the immune system is regulated and responds to variations in the composition of the intestinal microflora during DSS-induced acute colitis. An increased immunological response against LPS, increased populations of regulatory T-cells and higher levels of IL-6, IL-17 and KC were found. The amount of lactobacilli decreased, while that of *Akkermansia*, the total amount of bacteria and the incidence of *Enterobacteriaceae* increased. As revealed by T-RFLP patterns, the composition of the bacterial flora changed during induction of colitis and there was a complex close relationship in comparison to multiple immune parameters. Supplementation of probiotics and blueberry husks decreased disease activity, *Enterobacteriaceae* load, bacterial translocation and inflammation, and increased lactobacilli count and affected bacterial fermentation in the gut.

Long-term colonic inflammation promotes carcinogenesis and histological abnormalities of the liver. Two different ways of inducing chronic inflammation and colorectal tumours were investigated and cyclic DSS administration showed similar clinical and histopathological features of both colon and liver. Dysplastic lesions and a continuous mucosal inflammation were observed along with increased *Enterobacteriaceae* load and liver dysfunction. Also, the SCFAs patterns coincide with clinical findings. Supplementation of probiotics and blueberry husks during cyclic DSS administration delayed carcinogenic development as evaluated by lower numbers of dysplastic lesions. The mucosa was less affected after feed supplementation which may be favourable for SCFA absorption. *Enterobacteriaceae* load was decreased, especially by the addition of probiotics, and lactobacilli count was increased. Judged by histopathological evaluation, bacterial transloca-

tion and the concentration of propionic acid in blood, supplementation of probiotics seemed to moderate the negative liver effects and protect against injury, i.e. the findings indicated the potential of probiotics and blueberry husks in prevention of colonic inflammation and tumour development.

## List of papers

### Paper I

Håkansson, Å., Stene, C., Mihaescu, A., Molin, G., Ahrné, S., Thorlaciuss, H., and Jeppsson, B. Rose hip and *Lactobacillus plantarum* DSM 9843 reduce Ischaemia/reperfusion injury in the mouse colon. *Dig Dis Sci* 2006;51:2094-2101.

### Paper II

Håkansson, Å., Bränning, C., Adawi, D., Molin, G., Nyman, M., Jeppsson, B., and Ahrné, S. Blueberry husks, rye bran and multi-strain probiotics affect the severity of colitis induced by dextran sulphate sodium. *Scand J Gastroenterol* 2009;44:1213-1225.

### Paper III

Håkansson, Å., Bränning, C., Molin, G., Adawi, D., Hagslätt, M-L., Nyman, M., Jeppsson, B., and Ahrné, S. Colorectal oncogenesis and inflammation in a rat model based on chronic inflammation due to cycling DSS-treatments. (In manuscript).

### Paper IV

Håkansson, Å., Bränning, C., Molin, G., Adawi, D., Hagslätt, M-L., Jeppsson, B., Nyman, M., and Ahrné, S. Blueberry husks and probiotics attenuate colorectal inflammation and oncogenesis, and liver injuries in rat exposed to cycling DSS-treatment. (In manuscript).

### Paper V

Håkansson, Å., Baridi, A., Tormo-Badia, N., Xu, J., Molin, G., Hagslätt, M-L., Karlsson, C., Jeppsson, B., Cilio, C., and Ahrné, S. Immunological alteration and changes of gut microbiota after dextran sulphate sodium (DSS) administration in mice. (In manuscript).



## The author's contribution to the papers

- Paper I      The author, Å. Håkansson, coordinated and performed the experimental work and the analysis of malondialdehyde levels together with C. Stene and A. Mihaescu. ÅH performed the analysis regarding the intestinal microflora. ÅH evaluated the results and wrote the paper in collaboration with CS and AM.
- Paper II     The author, Å. Håkansson, coordinated and performed the experimental work together with C. Bränning. ÅH performed the analysis regarding disease activity index, myeloperoxidase activity, malondialdehyde levels, interleukin-12 in blood, viable count, randomly amplified polymorphic DNA and 16S rRNA gene sequencing. ÅH evaluated the results and wrote the paper in collaboration with CB.
- Paper III    The author, Å. Håkansson, coordinated and performed the experimental work together with C. Bränning. ÅH performed the analysis regarding disease activity index, haptoglobin concentration in blood, myeloperoxidase activity, viable count, randomly amplified polymorphic DNA and 16S rRNA gene sequencing. Histology samples were analysed in collaboration with M-L. Hagslätt. ÅH evaluated the results and was responsible for writing the manuscript.
- Paper IV    The author, Å. Håkansson, coordinated and performed the experimental work together with C. Bränning, performed the analysis regarding disease activity index, haptoglobin concentration in blood, myeloperoxidase activity, viable count, randomly amplified polymorphic DNA and 16S rRNA gene sequencing. Histology samples were analysed in collaboration with M-L. Hagslätt. ÅH evaluated the results and was responsible for writing the manuscript.
- Paper V     The author, Å. Håkansson, coordinated and performed the experimental work and the analysis of flow cytometry, malondialdehyde levels, myeloperoxidase activity and cytokines/chemokines in blood. ÅH evaluated the results in collaboration with A. Baridi and N. Tormo-Badia. ÅH was responsible for writing the manuscript.

# Abbreviations

AOM	Azoxymethane
BHI	Brain heart infusion
CA	Carboxylic acid
CD	Cluster of differentiation
CFU	Colony-forming units
CRC	Colorectal cancer
CTLA	Cytotoxic T-lymphocyte-associated protein
DAI	Disease activity index
DSS	Dextran sulphate sodium
dwb	Dry weight basis
ELISA	Enzyme-linked immunosorbent assay
ENV	<i>Escherichia coli</i> numbering
FOXP3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GLC	Gas-liquid chromatography
H & E	Haematoxylin and eosin
IEL	Intraepithelial lymphocytes
IFN	Interferon
IL	Interleukin
i.p.	Intraperitoneal
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MLN	Mesenteric lymph nodes
MPO	Myeloperoxidase
MW	Molecular weight
OFR	Oxygen free radicals
PCA	Principal component analysis
PCR	Polymerase chain reaction
PP	Payer's patches
RAPD	Randomly amplified polymorphic DNA

rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acids
TNF	Tumour necrosis factor
TLR	Toll-like receptor
T-RFLP	Terminal restriction length polymorphism
UC	Ulcerative colitis
VRBG	Violet-red bile glucose
w/v	Weight/volume

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# Introduction

The potential role of intestinal microbiota in modulating inflammation has been indicated in several clinical conditions, including ulcerative colitis and colorectal cancer. Given the intense exchange between the intestinal microbiota and the mucosal immune system, it is easily conceivable that perturbation of the intestinal microbiota or of the immune control mechanisms can initiate or contribute to pathological and potentially chronic inflammatory reactions. The indigenous intestinal microbiota and an intact mucosa are vital components of body defences against luminal pathogenic bacteria. In general, intestinal inflammation is accompanied by bacterial imbalance and overrepresentation of harmful species. During inflammation, mucosal permeability is enhanced, allowing indigenous bacteria or endotoxin within the gastrointestinal tract to reach systemic organs and tissues, causing extraintestinal infections with a subsequent systemic inflammatory response and possibly even sepsis with multiple-organ dysfunction.

Normalisation of an unbalanced indigenous microbiota by specific strains of health providing gut bacteria constitutes the principles of probiotic therapy. Specific components of the intestinal microflora, including *Lactobacillus* and *Bifidobacterium*, have been associated with beneficial effects on the host, such as maintaining intestinal barrier function, antagonisms against pathogens and modulation of immune responses. In this regard, the interest for use of probiotic bacteria as a prophylactic or curative treatment against inflammation is receiving a strong scientific support.

When considering optimal nutrition for individuals in inflammatory states, nutrition strategies to protect intestinal damage and/or provoke intestinal restitution should be explored.

The diet may exert a major effect on the composition and activity of the gut microbiota and consumption of dietary fibre and phenolic compounds may further modify the numbers and types of bacteria. Bacterial metabolism will also generate bioactive compounds with anti-inflammatory actions and benefits on the intestinal mucosal architecture.

Relevant animal models of intestinal inflammation are imperative in order to study normal and pathophysiological conditions in the gut and the effect of immunomodulating substances. The work performed and reported in this thesis primarily attempts to evaluate a model that clinically resembles the development of acute and chronic inflammation with subsequent colorectal cancer development and also considers nutritional potential for improving pathophysiology and clinical out-



comes. However, the starting point of the work was an evaluation of the antioxidative protection of dietary supplements in an Ischaemia-reperfusion model.

# Background

## *In vivo* models

### Acute colitis, chronic colitis and colorectal cancer

Experimental acute and chronic colitis and colorectal carcinoma induced in rodents by feeding of DSS has been extensively described, and has clinical and histopathological similarities to human ulcerative colitis (Okayasu *et al.*, 1990 and Dieleman *et al.*, 1998). DSS-induced acute colitis shows marked loss of body weight, rectal bleeding, reduction of colonic length and destruction of the epithelial layer and glandular architecture of the large intestine (Cooper *et al.*, 1993 and Okayasu *et al.*, 1990). The mechanism by which DSS induces both colonic inflammation and cancer is still unknown. A disruption in the balance between epithelial cell apoptosis and proliferation could be implicated and abnormal increase of both proliferation and apoptosis rate of colonic epithelial cells have been demonstrated after repeated cycles of DSS administration.

In long-term DSS administration, dysplasia and/or cancer occur as flat lesions or as a dysplasia-associated lesion or mass (DALM) as observed in humans (Cooper *et al.*, 2000). Because DSS responds negatively in the Ames test for mutagens (Nagoya *et al.*, 1981), the neoplastic changes seem to originate from the mucosal inflammation itself. These changes can be attributed to the recurrence of inflammatory ulceration and regeneration of the colonic mucosa, a recurrence–remission cycle typical of clinical ulcerative colitis (Neuman 2007). Therefore, DSS-induced colitis seems to closely resemble ulcerative colitis not only with respect to the pathology but also to the process of tumour development (Cooper *et al.*, 2000). However, colorectal tumour development in the DSS colitis model typically requires a relatively long exposure period or cycles of DSS administration and the incidence and/or multiplicity of induced tumours is relatively low (Okayasu *et al.*, 2002).

Significant increases in the population of members of *Enterobacteriaceae*, *Bacterodaceae* and of *Clostridium* spp. after DSS administration has been reported (Okayasu *et al.*, 1990) and due to increased colonic mucosal permeability, translocating bacteria and endotoxin may cause destruction of the epithelial cells of basal crypts and induce an inflammatory reaction (Kitajima *et al.*, 1999).

AOM is a mutagen that induces G to A transitions and when used alone, it induces polypoid tumours in rodents with clinical, histological and molecular features that mimic human sporadic colon cancer (Takahashi & Wakabayashi, 2004). However, when DSS is given after AOM administration, tumorigenesis is strongly promoted (Okayasu *et al.*, 1996) and the AOM/DSS model generates neoplasms that develop through dysplastic lesions in the colonic mucosa (Suzuki *et al.*, 2004). AOM is metabolised in the liver into methylazoxymethanol, which is catalysed by the enzyme cytochrome P450 E1. Metabolic activation of methylazoxymethanol to a highly reactive electrophile (methyl diazonium ion) occurs in the liver and colon, which is recognised as inducing oxidative stress (Sohn *et al.*, 1991).

### Ischaemia/reperfusion injury (I/R)

A rodent model of intestinal I/R injury, in which the superior mesenteric artery (SMA) is occluded, resulting in damage to the intestinal epithelium, has been proposed as a clinically relevant animal model mimicking the clinical setting seen in critically ill patients (Turnage *et al.*, 1994 and Haglund & Bergqvist, 1999). This model can be chosen to mimic a crucial period of intestinal hypoperfusion, occurring in most critically ill patients, which results in the loss of mucosal barrier function, increased translocation of enteric bacteria, and elevation of systemic cytokines. The insult in Balb/c mice resulted in reproducible macroscopic and microscopic mucosal injury that correlates with survival. In addition, the histological scoring system developed for this model of intestinal injury accurately reflects the aspects of mucosal injury seen clinically after ischaemic insult, such as inflammation and variable areas of epithelial damage (Haglund & Bergqvist, 1999). SMA occlusion induced the greatest degree of damage in the distal jejunum and proximal ileum with reduced damage to the duodenum and distal ileum and no damage to the colon (Stallion *et al.*, 2005). However, occlusion of superior mesenteric artery has also been shown to cause I/R injury in colon of rats and mice respectively (Leung *et al.*, 1992 and Riaz *et al.*, 2002).

## Mucosal architecture

The intestinal mucosa comprises three main elements: epithelium (of the surface and crypts), lamina propria and muscularis mucosae; the latter separates the mucosa from the deeper submucosa. The intestinal epithelium is composed of a single layer of columnar epithelial cells, which are connected by tight junctions and form an impermeable barrier between the body and the luminal contents. The epithelial cells are responsible for both importing luminal nutrients and releasing IgA into the lumen. In addition, they play an active role in immunity by producing antimicrobial peptides, e.g. defensins and lysozyme, and proinflammatory cytokines in response to microbes (Artis, 2008 and Fahlgren *et al.*, 2003). Epithelial renewal occurs in the crypts through a coordinated series of events such as proliferation,

differentiation and migration toward the intestinal lumen (van de Wetering *et al.*, 2002).

Besides intestinal epithelial cells, the epithelium includes specialised cells such as goblet cells, which secrete the protective mucus layer limiting the contact between bacteria and epithelial cells, and Paneth cells, which reside in the crypts of the small intestine and secrete antimicrobial peptides (Cash *et al.*, 2006). In addition to the immune function provided by the epithelial cells themselves, populations of immune cells can be found within the epithelial cell layer. These include dendritic cells that sample antigens directly from the lumen (Rescigno *et al.*, 2001) and intraepithelial lymphocytes (IEL), which consist mostly of T cells. More immune populations are found in the lamina propria, a layer of loose connective tissue supporting the epithelium.

The intestine is a primary site of foreign antigen encounter and it is associated with several types of lymphoid organs collectively referred to as gut-associated lymphoid tissue (GALT). Some of them, such as Peyer's patches (PP) and the isolated lymphoid follicles, are within the mucosa itself. In addition, intestinal lymph drains into the mesenteric lymph nodes (MLN), which constitute a key checkpoint to determine the anatomical location of tolerogenic or inflammatory responses (Izcue *et al.*, 2009). PP are secondary lymphoid organs that arise in the fetal small intestine independently of the intestinal flora (Mowat, 2003). PP lie at regular intervals along the small bowel and their organisation is comparable to that of lymph nodes, with large B cell follicles and T cell areas. In contrast, isolated lymphoid follicles are distributed along the small intestine and colon and their architecture resembles that of PP, with the exception that they lack a discrete T zone (Platt & Mowat, 2008). Unlike PP, their development seems to be triggered by the intestinal flora (Lorenz *et al.*, 2003) and their number increases during chronic inflammation.

Almost 70% of the immune competent cells are located in the intestinal mucosa, making it the most important immune organ of the body. The intestinal immune system has developed a tightly regulated control system to ascertain optimal protection against pathogens, while at the same time avoiding unnecessary immune activation in response to harmless antigens of the intestinal microflora (MacDonald, 2005).

# The bacterial flora of the gut

## Composition

The gut is a complex anaerobic environment with a diverse microbial community playing a fundamentally important role in health and disease. Culture-independent techniques have allowed insight into gut microbial diversity but the function of most gut bacteria is still unknown, due to an inability to culture many of these micro-organisms (Hooper & Gordon, 2001 and Hooper *et al.*, 2001).

The commensal and mutualistic bacteria of the gut constitute a heterogeneous microbial ecosystem composed of anaerobic, aerobic and facultative aerobic bacteria. The conditions in the various sections of the gastrointestinal tract differ considerably, reflected by the uneven distribution of the bacterial cells. The stomach and duodenum harbour relative low numbers of micro-organisms adhering to the mucosal surface or in transit, typically less than  $10^3$  bacteria cells per gram of intestinal content. The numbers are low because of acid, bile, and pancreatic secretions, and short transit time impedes stable colonisation. Gram-positive bacteria such as *Streptococcus* and *Lactobacillus* dominate in the upper small intestine (Evaldson *et al.*, 1982). There is a progressive increase in both numbers and of different species along the jejunum and ileum, from approximately  $10^4$  in the jejunum to  $10^7$  colony-forming units per gram of contents in distal ileum. The latter part usually contains bacteria like those found in the colon, although in smaller numbers (Evaldson *et al.*, 1982).

The proportion of anaerobic bacteria gradually increases from the proximal to distal regions of the intestine and beyond the ileo-caecal valve, but the strict anaerobes outnumber the facultative anaerobes in the lumen by 100 to 1000 times, and bacterial numbers typically exceed  $10^{11}$  g<sup>-1</sup> (Holzapfel *et al.*, 1998). The environment is favourable for bacterial growth with a slow transit time, the availability of nutrients and higher pH. A comprehensive investigation of gut mucosal and faecal microbial diversity revealed *Firmicutes* and *Bacteroidetes* as the dominating phyla (Eckburg *et al.*, 2005). The majority of *Firmicutes* belonged to the clostridia with 42 phylotypes of this group recognised as butyrate-producing bacteria. On a more refined level the fecal microbiota is a highly complex and diverse bacterial ecosystem dominated by anaerobic bacteria, represented by the genera *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Peptostreptococcus*, *Ruminococcus*, *Clostridium* and *Propionibacterium*, and sub-dominant bacteria of the *Enterobacteriaceae* family and the genera *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Fusobacterium*, *Desulfovibrio* and *Methanobrevibacter* (Harmsen *et al.*, 2002 and Wang *et al.*, 2005). Bifidobacteria, belonging to the phyla *Actinobacteria*, has been shown to comprise about 5% of the colonic microbiota (Flint, 2006). In addition, however, members of some of the less abundant phyla in the human gastrointestinal tract can have a disproportionately large influence on health maintenance and disease progression (Duncan *et al.*, 2007).

Surface-adherent and luminal microbial populations may be distinct and may fulfil different roles. The mucosal microbiota is in close contact with the underlying gut epithelium and facilitates beneficial functions including nutrient exchange and induction of host innate immunity (Sonnenburg *et al.*, 2004). Fecal samples are often used to investigate the intestinal microflora because they are easily collected. However, the degree to which composition and function of the fecal microflora differ from mucosal microflora is not clear, but it has been postulated that the fecal microbiota represents a combination of shed mucosal bacteria and a separate non-adherent luminal population (Eckburg *et al.*, 2005).

At the level of bacterial species and strains, there is a high degree of variability among human individuals (Holzapfel *et al.*, 1998).

### Metabolic functions

The major metabolic function of the microflora is in the fermentation of dietary carbohydrates that have escaped digestion in the small intestine (Cummings & Macfarlane, 1997). These include resistant starches, dietary fibre (cellulose, hemicellulose, pectin, inulin), and unabsorbed sugars and sugar alcohols. However, dietary protein and protein from pancreatic enzymes and gastrointestinal secretions as well as mucus produced by the host and sloughed epithelial cells also contribute to some extent to the supply of substrates to intestinal bacteria (Macfarlane, 1995). The fermentation gives rise to short-chain fatty acids (SCFAs) mainly acetate, propionate, and butyrate as well as to carbon dioxide and molecular hydrogen. Lactic acid, ethanol, succinic acid, and pyruvate are important intermediates that are degraded to SCFAs, CO<sub>2</sub>, and H<sub>2</sub>. Fermentation of peptides and amino acids also results in the formation of SCFAs, CO<sub>2</sub>, and H<sub>2</sub>, but at the same time, it generates a series of potentially toxic substances including ammonia, amines, phenols, thiols and indols (Macfarlane, 1995).

The presence of SCFAs is critical to the normal physiology of colon and SCFAs coupled with sodium absorption is one of the major mechanisms for salt and water uptake (Murray, 1990). With a normal intestinal flora, the presence of their fermentation end products in the colon facilitates salt and water absorption, and thus prevents diarrhoea. SCFAs can be rapidly converted to ketone bodies, which in turn can be used as an energy source. Butyrate is the major energy source for colonocytes and it has been estimated that in humans, up to 70% of the energy used by colonocytes is derived directly from luminal sources and not from the circulation (Kien, 1990). Consequently, SCFAs exert a significant trophic effect on the colonic mucosa (Sakata, 1987). Furthermore, both ketone bodies and SCFAs can enter the circulation and serve as an energy source for other tissues.

All three major SCFAs are present in portal blood at concentrations several times greater than in peripheral venous blood. Hepatic vein SCFA concentrations are only 39% of those in portal blood. As portal blood provides two-thirds to three-quarters of total hepatic blood flow in man (Schenk *et al.*, 1962) there is

clearly significant uptake of these anions by the liver. Further uptake occurs in peripheral tissues because peripheral venous blood levels are again lower; about 53% of hepatic venous blood. Propionate is largely taken up by the liver and acetate enters the peripheral circulation to be metabolised by peripheral tissues (Wong *et al.*, 2006). Arterial blood supplying the gut contains predominantly acetate (Cummings *et al.*, 1987). Acetate and propionate might also have a role as modulators of glucose metabolism. Absorption of these short-chain fatty acids would result in lower glycaemic responses to oral glucose or standard meal, a response consistent with an ameliorated sensitivity to insulin (Venter *et al.*, 1990 and Brighenti *et al.*, 1995).

Metabolic functions of the microflora also include the production of some vitamins (K, B12, biotin, folic acid, pantothenate), synthesis of amino acids from ammonia or urea (Hooper *et al.*, 2002) and activation or destruction of mutagenic metabolites (Rowland, 1988).

### Protective functions

One of the essential functions of the colonic microbiota includes the barrier effect that prevents invasion by pathogens. The resident bacteria represent a crucial line of resistance to colonisation by exogenous microbes or opportunistic bacteria that are present in the gut, but have restricted growth. Several mechanisms appear to be involved and include competition for substrate and for mucosal adhesion receptor sites. Adherent non-pathogenic bacteria can prevent attachment and subsequent entry of pathogen enteroinvasive bacteria into the epithelial cells (Bernet *et al.*, 1994). The production of a physiologically restrictive environment in terms of, for example, pH, redox potential, hydrogen sulphide production, production of metabolites toxic to other bacteria, and production of antibiotic substances such as bacteriocins (van der Waaij, 1988) are also involved in the process. However, most of the bacteriocins are protein compounds degradable by digestive proteases, so the role of bacteriocins is mainly restricted to localised niches (Guarner & Malagelada, 2003).

### Interactions with immune cells

Microbial colonisation of the gastrointestinal tract affects the composition of gut associated lymphoid tissue. Immediately after exposure to luminal microbes, the number of intraepithelial lymphocytes expands greatly (Umesaki *et al.*, 1993 and Helgeland *et al.*, 1996), germinal centres with immunoglobulin producing cells arise rapidly in follicles and in the lamina propria (Cebra *et al.*, 1998), and concentrations of immunoglobulin increase substantially in serum (Butler *et al.*, 2000).

There is a complex relationship between the intestinal immune system and the bacterial flora and it is extremely important for the intestinal epithelial cells and the mucosal immune system to distinguish between pathogenic and non-pathogenic bacteria. The intestinal bacterial flora can significantly alter the re-

sponse and the interaction can occur in different ways. The follicle-associated epithelium, which covers Peyer's patches, is located along the small intestine and is particularly abundant in the ileum. The epithelium harbours shorter villi and contains specialised cells, called M (microfold) cells. M cells have the capacity to capture soluble antigens, apoptotic epithelial cells or bacteria from the luminal compartment, and transport them to Peyer's patches for sampling by dendritic cells (DCs) or destruction by macrophages (Newberry & Lorenz, 2005). DCs may present antigen locally to T cells, migrate to T-cell zones or to mesenteric lymph nodes, or interact with memory B cells (Pickard *et al.*, 2004). Both pathogenic and non-pathogenic bacteria can also enter into the mucosal tissue through lamina propria DCs, which extend their dendrites through epithelial cell tight junctions (Rescigno *et al.*, 2001). Also the intraepithelial lymphocytes located in the epithelium might recognise microbial antigens (Cheroutre, 2004).

Intestinal epithelial cells are capable of detecting bacterial antigens and initiating, participating in and regulating both innate and adaptive immune responses. Through molecules expressed on the epithelial cell surface such as major histocompatibility complex I and II molecules, and Toll-like receptors (TLRs), signals from bacteria can be transduced to adjacent immune cells in lamina propria, such as macrophages, DCs and lymphocytes (Cario *et al.*, 2002 and Hershberg & Mayer, 2000). In healthy adults, TLRs are expressed in most tissues, including myelomonocytic cells, endothelial and epithelial cells. Interaction of TLRs and bacterial molecular patterns results in activation of a complex intracellular signalling cascade, up-regulation of inflammatory genes and production of pro-inflammatory cytokines and interferons and recruitment of myeloid cells. It also stimulates expression, upon antigen-presenting cells, of co-stimulatory molecules required to induce an adaptive immune response (Testro & Visvanathan, 2009). The expression level of TLRs on the gut epithelium is particularly sophisticated to prevent over-stimulation.

Whilst TLR responses are critical for survival and defence against invading pathogens, inappropriate signalling in response to alterations in the local bacterial environment can be detrimental. Human intestinal epithelial cells normally express TLR3 and TLR5, whilst TLR2 and TLR4 are barely detectable (Cario & Podolsky, 2000 and Melmed & Thomas, 2003). The two most thoroughly studied TLRs are TLR4 and TLR2, the receptors for Gram-negative and Gram-positive bacterial products, respectively. TLR4 is best known as the LPS receptor and TLR2 recognises bacterial lipoproteins and peptidoglycans. TLR4 and TLR2 also recognise a range of endogenous ligands that are released into the circulation during periods of cellular stress or necrosis. TLR3 is responsible for recognising double-stranded RNA while TLR5 controls responses to bacterial flagellin (Shi & Walker, 2004). In health, TLR signalling protects the intestinal epithelial barrier and confers commensal tolerance. In disease, aberrant TLR signalling may stimulate diverse inflammatory responses leading to acute and chronic intestinal inflammation with many different clinical phenotypes including UC (Zhang *et al.*, 2006).



## Ulcerative colitis (UC)

UC is an inflammatory disease of the rectal and colonic mucosa. It often arises primarily in the rectum and subsequently extends proximally to a variable extent and it seems to result from a complex series of interactions between environmental, genetic, and immunologic factors in which an uncontrolled immune response within the intestinal lumen leads to inflammation in genetically predisposed individuals (Karlinger *et al.*, 2000). Although the aetiology of the disease remains unknown, the clinical features, histopathological findings and the therapeutic efficacy of immunosuppressive drugs indicate an involvement of the immune system in the pathogenesis of the disease (Deusch & Reich, 1992). The clinical symptoms of UC include weight loss, diarrhoea accompanied by blood, and abdominal pain (Podolsky, 2002). A dysregulation of sodium absorption in the distal colon involving the epithelial sodium channel may be responsible for the observed diarrhoea (Amasheh *et al.*, 2004). UC is characterised by periods of remission marked by episodes of clinical relapse caused by acute intestinal inflammation. Treatment is primarily aimed at reducing inflammation during relapse and secondarily at prolonging the time spent in remission of clinical symptoms (Neuman, 2007). The histopathological features of UC are characterised by architectural distortion of colonic crypts with frequent depletion of goblet cell mucin and diffuse infiltration of lymphocytes and plasma cells.

### Inflammatory response

During the acute phase of inflammation, macrophages, neutrophils and eosinophils infiltrate the lamina propria of the colonic mucosa. Neutrophils especially aggregate near the crypt, leading to the formation of abscesses (Asakura *et al.*, 1999). Activated DCs and macrophages secrete several cytokines that actively regulate the inflammatory response in UC. Once secreted, these cytokines trigger and differentiate many T cells, activating the adaptive immune response. Increased populations of CD4-positive and CD8-positive cells have been found in the colonic lamina propria of patients with active UC, with preference to CD4-positive dominance (Sasakawa *et al.*, 1995). Upon antigenic stimulation, naive CD4+ T cells are activated, expand and differentiate into different effector subsets, Th1, Th2 and Th17 cells, characteristic of the production of distinct cytokines and effector functions (Blumberg, 2009). In both UC and Crohn's disease, polarised immune activity towards Th1 (marked by up-regulation of TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$ , IL-6) and Th17 (marked by IL-17 secretion) response is reported, while UC appears to exhibit an added contribution of Th2 responses (characterised by secretion of IL-4, IL-5, and IL-13) (Dharmani & Chadee, 2008). Cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ , increase the expressions of TLR4 in intestinal epithelial cell and result in increased LPS responsiveness (Abreu *et al.*, 2002). During UC, the expression of TLR4 is increased on mucosal DCs as well as on intestinal epithelial cells in inflamed and

non-inflamed mucosa throughout the colon and terminal ileum (Baumgart *et al.*, 2009 and Cario & Podolsky, 2000).

The CD4+ T cell phenotype expressing CD25<sup>high</sup> and fork-head box protein 3 (FoxP3) has been recognised as the functional representative of regulatory T cells (Treg). The Treg is known to down-regulate immune responses to both foreign and self-antigens (Kanai & Watanabe, 2005) and a significant number of T-regulatory cells can be found in the inflamed intestine. Their ability to overcome the inflammatory response is hypothesised to be a major reason for remission and is therefore a major goal of therapies that aim to enable the regulatory functions of these naturally immunosuppressive cells (Blumberg, 2009).

## Intestinal microbiota

Although the aetiology of UC still is unknown, it seems that the intestinal microbiota plays a crucial role in the pathogenesis of the disease. UC patients have higher numbers of bacteria associated with biopsies than healthy subjects, and the difference may reflect the altered nature of the mucus present on the mucosal surface of the colon of UC patients which is thinner and less sulphated than that of healthy subjects (Pullan *et al.*, 1994 and Corfield *et al.*, 1996). The thinner layer may provide a more secure habitat for bacterial proliferation, provided by decreased mucus flow. The bacteria might also be more numerous because the non-sulphated mucins in the mucus are more easily degraded by bacterial cells and therefore provide an improved nutritional environment (Robertson & Corfield, 1999). A thin mucus layer containing larger than normal numbers of bacteria might facilitate contact between bacterial antigens and the mucosal immune system.

Microbial compositions of active UC patients have also been shown to be significantly less diverse (Nishikawa *et al.*, 2009). A significant decrease in the number of lactobacilli is found in the mucosa of UC patients (Fabia *et al.*, 1993) and predominantly detected in inactive patients, suggesting its role in the induction of remission (Andoh *et al.*, 2007). It has been suggested that the changing condition in the intestine may influence the *Lactobacillus* composition (Zoetendal *et al.*, 2002) and the composition of lactobacilli adhered to the ulcerated and non-ulcerated tissue within the same patient varied greatly and may be caused by differences in the physiological status of the mucosa (Zhang *et al.*, 2007).

It is not clear whether endogenous intestinal bacteria and/or specific bacterial pathogens is directly or indirectly involved in the initiation and/or perpetuation of UC, and which bacterial components or antigens that are responsible for the unrestrained inflammatory response.

The colonic surface area and the infected area of the UC patients have been shown to be colonised by a wide variety of organisms, such as bacteria belonging to the gamma subdivision of *Proteobacteria*, the *Enterobacteriaceae*, the *Clostridium histolyticum/Clostridium lituseburense* group, the *Clostridium coc-*

*coides/Eubacterium rectale* group, the *Bacteroides/Prevotella* cluster, high G+C Gram-positive bacteria, or sulphate-reducing bacteria. The presence of a wide spectrum of organisms suggests that the endogenous intestinal flora plays a role in the pathogenesis of UC (Kleessen *et al.*, 2002). Bacteria of the *Clostridium histolyticum/Clostridium lituseburense* groups made up 21% of the total bacteria in the specimens, while these organisms were not found in controls. These phylogenetic groups contain mainly clostridia belonging to clusters I and II and in part of cluster XI of Gram-positive bacteria (Collins *et al.*, 1994), e.g. species such as *C. histolyticum*, *C. beijerinckii*, *C. perfringens*, *C. botulinum*, *C. intestinalis* or *C. lituseburense*, *C. difficile*, *C. bifermentans* (Franks *et al.*, 1998). A high proportion of these organisms may be pathogenic through their proteolytic capabilities and toxin production (Sartor, 1997). *Enterobacteriaceae* have also been considered as being involved in the pathogenesis of UC, owing to the ability to adhere to the intestinal mucosa and to produce enterotoxins (Kotlowski *et al.*, 2007). A subgroup of the *Enterobacteriaceae* (e.g. *E. coli* and *Klebsiella*) accounted for 25% of the mucosa-associated and 20% of the penetrated bacteria in colonic specimens of UC patients (Kleessen *et al.*, 2002). Sulphate-reducing bacteria have received attention due to their ability to reduce sulphate to sulphide, a by-product of their respiration. Hydrogen sulphide is freely permeable to cell membranes and inhibits butyrate oxidation in colonocytes (Rowan *et al.*, 2009). Hydrogen sulphide has been implicated in the pathogenesis of ulcerative colitis, since increased amounts have been found in faeces (Pitcher *et al.*, 2000).

## Bacterial translocation

The intestinal mucosa functions as a major local defence barrier that helps to prevent the invasion and systemic spread of bacteria and endotoxin normally contained within the intestinal lumen. However, under certain conditions, intestinal mucosal barrier function appears to be impaired or overwhelmed, allowing indigenous bacteria or endotoxin within the gastrointestinal tract to reach systemic organs and tissues, a process termed bacterial translocation (Berg & Garlinton, 1979).

Severe translocation takes place when the intestinal wall is damaged, which can occur as a consequence of a number of events, including inflammation, ischaemia, trauma, hyperthermia, the presence of vasoactive agents or ionising radiation (Van Leeuwen *et al.*, 1994). This process may initiate the development of systemic sepsis and multisystem organ dysfunction syndrome by activating lamina propria macrophages, leading to the release of proinflammatory cytokines (Strober *et al.*, 2002 and Rakoff-Nahoum *et al.*, 2004). During UC, systemic endotoxaemia have been shown to correlate positively with anatomic extent and clinical activity (Gardiner *et al.*, 1995).

The physical barrier that prevents translocation is the monolayer of intestinal epithelial cells with its overlying mucus (Turner, 2009). Except for being thinner

and less sulphated during UC (Pullan *et al.*, 1994 and Corfield *et al.*, 1996), there is evidence that several strains of enteropathogenic *Escherichia coli*, as well as other pathogens, bind to the mucus gel better than nonpathogenic bacteria (Beachey, 1981 and Savage, 1984). While the intestinal epithelial cell membranes are essential to mucosal barrier function, the paracellular space between adjacent cells must be sealed and this function is mediated by the apical junctional complex that is composed of tight junctions, adherens junctions, and desmosomes (Turner, 2009).

There is good evidence that portal vein endotoxaemia of gut origin in minute amounts is a normal physiological phenomenon (Jacob *et al.*, 1977 and Nolan, 1981). During normal conditions, this low-grade endotoxaemia of gut origin is rapidly cleared by the cells of the reticuloendothelial system of the liver (Mathison & Ulevitch, 1979 and Ruiter *et al.*, 1981). The diseased gut, however, can translocate endotoxin in large amounts (Van Leeuwen *et al.*, 1994). Increased paracellular permeability has been documented in the epithelial lining from both the acutely inflamed and chronically damaged areas of the intestine (Schmitz *et al.*, 1999). Histological evaluation reveals local epithelial defects ranging from micro-erosions to gross ulcer-type lesions and neutrophil infiltration of the crypts, which may cause epithelial destruction (Glickman, 1991). Moreover, epithelial cell death by apoptosis is more frequent in UC (Sträter *et al.*, 1997) and may also destroy crypt integrity (Pritchard *et al.*, 1998).

Gram-negative bacteria have been cultured in the portal blood and from liver biopsy samples in patients with UC undergoing colectomy (Eade & Brooke, 1969). In addition, circulating agglutinating antibodies against various enteric bacteria have been identified in active UC (Hedde & Shearman, 1979).

## Hepatobiliary complications

A variety of hepatobiliary abnormalities have been described in patients with UC. These complications include fatty changes in the liver, cholelithiasis, pericholangitis, primary sclerosing cholangitis, cirrhosis, chronic active hepatitis, amyloidosis, and bile duct cancer, with primary sclerosing cholangitis being the most common form (Williams & Harned, 1987). The pathogenetic mechanisms responsible for the hepatobiliary alterations are not known, but several hypotheses have been proposed, including autoimmunity, genetic factors, virus infections, and gut-derived bacterial antigens or toxins (Masubuchi & Horie, 2004). Because gut derived components are easily accessible to the liver via the portal vein, it is suggested that increases in the permeability of the intestinal epithelium during inflammation allow bacterial antigens and toxins to enter the lamina propria and cause an inflammatory reaction when the bacterial products such as endotoxin reach the liver (Masubuchi & Horie, 2004).

Endotoxins are mostly lipopolysaccharide (LPS) constituents of the outer membrane of Gram-negative bacteria. Animal and clinical studies have identified

endotoxins as the main trigger of septic shock in Gram-negative infection. Immunocompetent cells, such as monocytes and macrophages, are the main cellular targets of endotoxins and many of the symptoms associated with septic shock are mediated through endotoxin-induced cytokine production and production of reactive oxygen intermediates by these cells (Van Leeuwen *et al.*, 1994 and Arthur *et al.*, 1988).

Through the portal blood flow draining the GI tract, intestinal bacteria and bacterial products, such as LPS, reach the liver. The Kupffer cells and endothelial cells of the liver serve as a cellular back-up system to prevent entrance into the systemic circulation (Nakao *et al.*, 1994). However, bacterial particles entering the circulation can also be cleared and detoxified to some extent in the serum by serum proteins such as LPS binding protein, bactericidal/permeability increasing protein, and high density lipoprotein (Van Leeuwen *et al.*, 1994). The liver's primary role in clearing LPS can be demonstrated in patients with liver failure and systemic endotoxaemia without evidence of Gram negative infection.

Endotoxaemia is frequently found in patients with cirrhosis, and the degree of endotoxaemia is correlated with the degree of liver failure (Lin *et al.*, 1995). Intestinal endotoxins also played an important part in the pathophysiology of hepatic failure after hepatic resection and in turn, initiated an accelerated catabolic response to the surgical injury. Significant hepatic injury and a high death rate occurred in conjunction with these changes. Changing the gut contents before operation through administration of lactulose reduced the level of endotoxaemia, blocked the catabolic response, and protected the liver with an enhanced survival (Van Leeuwen *et al.*, 1991).

## Colorectal cancer

Patients with UC represent a risk group for developing colorectal cancer (CRC) and the two most important risk factors are the duration and extent of the disease. Other factors suggested as risk factors for development of CRC are primary sclerosing cholangitis, family history of CRC, younger age at diagnosis and severity of inflammation. The severity of inflammation has been shown to correlate with an increased frequency of dysplasia and thus a greater CRC risk (Rutter *et al.*, 2004). Macroscopic features of previous and ongoing inflammation (post-inflammatory polyps, scarring, strictures, backwash ileitis and severe inflammation) signified an increased risk whereas a macroscopically normal-looking colonoscopy returns the cancer risk to that of the general population (Rutter *et al.*, 2004). Correlation between the degree of inflammation and cancer risk was also shown in other studies (Rubin *et al.*, 2006 and Gupta *et al.*, 2007) and these findings suggest that chronic inflammation of the colon is a key predisposing factor to CRC, and so may be an initiator and promoter of colon carcinogenesis.

Clinically, patients with chronic UC typically display a recurrence-remission cycle of mucosal ulceration, and the repetition of the cycles is thought to enhance the transformation of the colon mucosa epithelium to neoplastic cells. The elevated rate of cell turnover associated with the epithelial damage-restitution cycle may increase the occurrence of mitotic aberrations and other genetic and epigenetic changes, as well as take part in the promotion stage of cancer development (Parsonnet, 1997).

UC-associated colorectal cancer is often multicentric in origin and frequently associated with dysplastic lesions. Dysplasia is suggested as a precursor of cancer (Riddell *et al.*, 1983) and is defined as an unequivocal neoplastic change that is intraepithelial and within the confinement of the glandular basement membrane (Warren & Sommers, 1949).

Many of the molecular alterations responsible for sporadic CRC development also play a role in UC-associated colon carcinogenesis. However, there are several differences in the sequence of molecular events leading from dysplasia to invasive adenocarcinoma in UC as compared with sporadic CRC. For example, APC loss of function, considered to be a common early event in sporadic CRC, is much less frequent and usually occurs late in the colitis-associated dysplasia-carcinoma sequence. On the contrary, p53 mutations in sporadic neoplasia usually occur late in the adenoma-carcinoma sequence, whereas in patients with UC, p53 mutations occur early and are often detected in non-dysplastic mucosa. (Xie & Itzkowitz, 2008).

## Injury of ischaemia and reperfusion (I/R)

When the blood supply to a tissue is interrupted, a sequence of chemical events is initiated that leads to cellular dysfunction, oedema and ultimately, cell death (Grace, 1994). Oxygen as basic fuel is crucial to cell function and lack of oxygen results in anaerobic metabolism and an increased local concentration of lactic acid. The resulting acidosis alters normal enzyme kinetics and the cell is deprived of the energy needed to maintain homeostasis. (Rhodes & De Palma, 1980). Revascularisation of ischaemic tissue results in further injury. Leukocyte adhesion to the endothelium is significantly enhanced within minutes after the onset of reperfusion and remains elevated for hours. Soluble mediators released during I/R exert chemotactic actions and direct leukocyte accumulation to the site of injury. These chemotactic substances include several proinflammatory cytokines/chemokines and leukotrienes released by the postischaemic endothelium, but also by resident leukocytes (Massberg & Messmer, 1998).

In addition, oxygen-derived free radicals (OFR) have been identified as playing a significant role as the mediators of the reperfusion component of ischaemia-reperfusion injury (McCord, 1985). OFRs are defined as molecules having an unpaired electron in the outer orbit e.g. superoxide anion ( $O_2^-$ ), hydroxyl radicals

(OH) and the non-radical hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and are constantly generated under normal conditions as a consequence of aerobic metabolism and they are generally unstable and very reactive (Gilbert, 2000). They are particularly transient species due to their high chemical reactivity and can react with DNA, proteins, carbohydrates and lipids in a destructive manner. The cell is endowed with an extensive antioxidant defence system to combat OFRs, either directly by interception or indirectly through reversal of oxidative damage. When OFRs overcome the defence systems of the cells and redox homeostasis is altered, the result is oxidative stress (Curtin *et al.*, 2002). The most damaging effect of free radicals is lipid peroxidation. Cell membranes are composed of polyunsaturated fatty acid and phospholipids. OFRs induce lipid peroxidation, which results in structural and functional cell damage (Grace, 1994).

Colonic ischaemia is a prevalent form of gastrointestinal ischaemia and it is frequently encountered in trauma, strangulated bowel, low flow states and haemorrhagic shock (Collard & Gelman, 2001). Colon has a lower resting blood flow than the small intestine. Previous studies have shown that colon is less susceptible to ischaemia injury compared to the small bowel (Leung *et al.*, 1992), which may be related to protection afforded by the high population of strictly anaerobic bacteria prohibiting the formation of oxygen free radicals or the reduced basal metabolic and cell turnover rate. Consequently, colon has been shown to harbour a reduced amount of protective antioxidant enzymes (such as superoxide dismutase and catalase) relative to the liver and small bowel (Grisham *et al.*, 1986).

UC-patients have already a reduced antioxidant defence in colonic tissue, rendering them more susceptible to oxidative tissue damage, hindering recovery of the mucosa and return of epithelial cell layer integrity (Buffinton & Doe, 1995). Intestinal damage varies depending on the severity of the ischaemic injury to the mucosa and disruption of the protective epithelium is a potential portal for the entry of bacteria and their products into the systemic circulation. This loss of epithelial cell integrity may very well be a source or promoter for the systemic inflammatory process (Stallion *et al.*, 2005).

## Protective effects of feed supplements

### Probiotic bacteria

Probiotics are live organisms that, when ingested in sufficient amounts, have a beneficial effect on the overall health of the host (Agostoni *et al.*, 2004) and the most commonly used probiotic bacterial strains belong to the genera *Lactobacillus* and *Bifidobacterium* (Saxelin *et al.*, 2005). Probiotics elicit beneficial effects through different potential mechanisms of action and not all probiotic strains exert the same mechanisms. Certainly, some probiotic bacteria will have multiple activities and can affect various stages of the defence.

Some probiotics possess the ability to transiently colonise the gastro-intestinal (GI) tract, increase the concentration of beneficial microbes, and thereby create a balance in the gut microbiota to the ultimate benefit of the host. Direct antimicrobial effects through production of bacteriocins have also been shown. These antimicrobial factors inhibit the growth and virulence of enteric bacterial pathogens (Sherman *et al.*, 2009).

Interaction with epithelial cells lining the GI tract to prevent the binding of enteric pathogens has been documented. Bacteria possessing hydrophobic cell surface properties are able to bind non-specifically to host cell surfaces, including the apical microvillus membrane of epithelial cells, thereby preventing pathogens from binding and invading the host (Johnson-Henry *et al.*, 2007). Attachment of bacteria to the mucosal surface is a prerequisite in the pathogenesis of many infections originating from the gut (Le Bouguenec, 2005), and colonisation of gram-negative intestinal bacteria is most likely mediated by mannose-sensitive adhesins (Johnson, 1991). The probiotic strain *Lactobacillus plantarum* 299v (Lp 299v) has excellent adherence characteristics using the mannose binding sites on mucosal cells (Adlerberth *et al.*, 1996), and may therefore be able to compete for receptor binding. The same strain also has the capacity to enhance the production and secretion of mucins from human intestinal (HT-29) epithelial cells (Mack *et al.*, 1999). The enhanced mucus layer overlying the epithelial lining can serve as an antibacterial shield that prevents the binding of enteric pathogens and increase clearance from the GI tract (Linden *et al.*, 2008).

Probiotics have a direct effect on enhancing the integrity of the epithelial barrier by preventing changes in tight junction proteins (such as occludins and claudins) and by enhancing the electrical resistance of tight junctions contained in the apical junction complexes between adjacent polarised epithelia (Johnson-Henry *et al.*, 2008 and Seth *et al.*, 2008). By reducing cytokine-induced epithelial cell apoptosis, the barrier resistance is also enhanced (Yan *et al.*, 2007).

Through bacterial-epithelial cell interactions, probiotics are able to affect both the innate and adaptive immune system. Some strains have the ability to promote the differentiation of B cells into plasma cells and increase the production of secretory immunoglobulin A (Corthésy *et al.*, 2007). Other ways of affecting the immune response involve prevention of activation of the proinflammatory nuclear transcription factor, NF- $\kappa$ B (Riedel *et al.*, 2006) and induction of immunoregulation by increasing the number of regulatory T-cells (Di Giacinto *et al.*, 2005).

## Phenolic compounds

Antioxidants are agents that interrupt peroxidation: they prevent tissue damage and the production of peroxides and further free radicals (Grace, 1994). Many diseased states including cancer are associated with an oxidative and inflammatory stress imbalance (Okada & Fujii, 2006). A regular intake of fruits, vegetables and cereals, rich in antioxidant and anti-inflammatory compounds, is known to mark-



edly decrease the risk of developing certain diseases such as colon cancer (Riboli & Norat, 2003 and Gill & Rowland, 2002). Phenolic acids, flavonoids, and anthocyanins may contribute to this effect (Duthie *et al.*, 2000).

Antimicrobial activities of polyphenols have been demonstrated (Azis *et al.*, 1998) and the level of inhibition varies depending on the bacterial species and the chemical structure of the compound. Growth inhibition of *Enterobacteriaceae*, *Clostridium* and *Bacteroides* has been shown, and interestingly, pathogenic *Clostridium* spp. was generally inhibited, while commensal *Clostridium* spp. was enhanced (Lee *et al.*, 2006). Probiotics such as *Lactobacillus* and *Bifidobacteria* was relatively unaffected, so probiotic colonisation in the intestine should continue in the presence of phenolics to improve the intestinal microbial balance and inhibit pathogen growth (Lee *et al.*, 2006).

Analysis of human fecal water indicates that polyphenols entering the colon are extensively metabolised by intestinal bacteria (Jenner *et al.*, 2005). However, only a limited part of the intestinal bacterial flora can metabolise phenolics. In the genus *Lactobacillus*, strains of the closely related species *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* often possess tannase activity, which enables the bacteria to metabolise polyphenols (Osawa *et al.*, 2000). Furthermore, strains of *L. plantarum* and the close relatives are also able to metabolise phenolic acids, producing substituted phenyl propionic acids (Barthelmebs *et al.*, 2000 and Barthelmebs *et al.*, 2001). Derivates of propionic acid are used commercially as an anti-inflammatory drug Ibuprofen®.

Blueberries are a rich source of a variety of polyphenols, most of which are attached to cell components of plants and have the potential to reach colon before they are released (Kahle *et al.*, 2006). The phenolics from blueberries have also been shown to inhibit colon cancer cell proliferation and induce apoptosis (Yi *et al.*, 2005). It has been proposed that the protective effect of blueberry phenolics as anti-inflammatory agents in the colon is likely to occur as a result of microbial metabolism, and that this depends on the composition of the individual colonic microbiota (Russell *et al.*, 2007).

Rose hips are often used in nutrition because of their ascorbic acid content, well known to have antioxidant properties (Hemilä, 1992). However, the antioxidant properties of rose hips are not due only to ascorbic acid, but also to polyphenols (Daels-Rakotoarison *et al.*, 2002). Rose hips have been shown to inhibit neutrophil oxidative burst and the effect was not because of the vitamin C content of the extract (Kharazmi & Winther, 1999). Also, rose hips lowered the level of the acute phase protein C-reactive protein and the chemotaxis of peripheral blood polymorphonuclear leucocytes, comparable to non-steroid anti-inflammatory drugs such as ibuprofen (Kharazmi & Winther, 1999, Winther *et al.*, 1999). The results have been attributed to the high content of polyphenols, as well as to the presence of galactolipids (Daels-Rakotoarison *et al.*, 2002 and Larsen *et al.*, 2003).

## Dietary fibre

Dietary fibres have been put forward as cancer-protective food components (Hill, 1995) and they may protect against colon cancer through secondary events resulting from the fermentation of carbohydrates by the microbiota. This will lead to fecal bulking, increased speed of colonic transit, increase in nitrogen metabolism, increased bacterial load in the colon, acidification and to the production of short chain fatty acids (Hill, 1995 and Harris & Ferguson, 1993).

The three major SCFAs produced during fermentation are acetate, propionate, and butyrate (Wong *et al.*, 2006) and they are absorbed from the colonic lumen by passive and active transport over the epithelium (Cook & Sellin, 1998). Butyrate is the most extensively studied SCFA, and its anti-inflammatory capacity is well documented during UC (Scheppach *et al.*, 1992 and Steinhart *et al.*, 1996). Butyrate is seen as promoting growth and proliferation of normal colonic mucosa, while suppressing cancer cells (Clausen *et al.*, 1991). The pre-treatment of colon cells with butyrate also resulted in significant protection against oxidative DNA damage induced by H<sub>2</sub>O<sub>2</sub> (Abrahamse *et al.*, 1999). However, anti-inflammatory properties comparable to those of butyrate have also been demonstrated for acetate and propionate.

SCFAs have been shown to inhibit LPS-induced TNF- $\alpha$  release, but not IL-8 secretion from human blood-derived neutrophils. The three acids inhibit TNF- $\alpha$ -mediated activation of the NF- $\kappa$ B pathway in a human colon adenocarcinoma cell line with the rank order of potency being butyrate>propionate>acetate (Tedelind *et al.*, 2007). Bacterial fermentation of dietary fibre in the colon does not necessarily change fecal SCFA concentrations because absorption may be greater than the rate of formation (Rasmussen *et al.*, 1987).



# Aims

The general aim of this thesis was to evaluate appropriate animal models for studying the preventative effects of probiotic bacteria, berries and cereals on colonic inflammation and cancer development. This was done in five papers whose specific aims were to:

- I evaluate the ability of *Lactobacillus plantarum* and rosehips to attenuate lipidperoxidation and *Enterobacteriaceae* load after Ischaemia/reperfusion induced colonic injury.
- II rate the anti-inflammatory properties of a mixture of *Lactobacillus crispatus*, *Lactobacillus gasseri* and *Bifidobacterium infantis*, with and without blueberry husks or rye bran, on induced acute colitis and the associated disease activity, bacterial translocation, inflammatory infiltration, oxidative stress, bacterial flora changes and fermentation.
- III develop, characterise and compare two models of chronic inflammation, colorectal cancer development and liver abnormalities with clinical reference. Focus was placed on disease activity, bacterial translocation, macroscopic and microscopic evaluation, local and systemic effect of inflammation, bacterial flora changes and SCFAs.
- IV use a model of chronic inflammation and colorectal cancer development to determine the preventative effects of *Lactobacillus plantarum*, *Lactobacillus gasseri* and *Bifidobacterium infantis* separately and in combination with blueberry husks in cancer development and liver impact. Focus was put on disease activity, bacterial translocation, macroscopic and microscopic evaluation,

local and systemic effect of inflammation, bacterial flora changes and SCFAs.

- V evaluate a model of acute colitis with regard to small intestinal and colonic affection, bacterial flora changes, histological findings of the small intestine, colon and liver, involvement of various cell populations of the immune system, and the relationship between multiple immune parameters and the composition of the colonic microflora.

# Materials and methods

## Animals and experimental design

All animals were kept at room temperature and maintained under standard laboratory conditions with a controlled 12 h light and 12 h dark cycle. Before experimental inclusion, they were acclimatised for 1 week. All experimental procedures were performed in accordance with legislation on the protection of animals and the protocols were approved by the Animal Ethics Committee at Lund University. Animal species, feed supplements and experimental models are summarised in Table I.

Table I. Animal species, feed supplements and experimental models in paper I-V.

Papers	Animals	Feed supplement	Model
I	Male Balb/c/J mice	Rose hip powder, or vitamin C <i>Lactobacillus plantarum</i> DSM 9843 (= strain 299v)	Ischaemia/ Reperfusion injury
II	Female Sprague-Dawley rats	Rye bran or blueberry husk <i>Lactobacillus crispatus</i> DSM 16743 <i>Lactobacillus gasseri</i> DSM 16737 <i>Bifidobacterium infantis</i> DSM 15158 (=strain CURE 19)	DSS-induced acute colitis
III	Female Sprague-Dawley rats	Oat bran	DSS and/or AOM-induced colorectal cancer
IV	Female Sprague-Dawley rats	Oat bran and/or blueberry husk <i>Bifidobacterium infantis</i> DSM 15159 (=strain CURE 21) <i>Lactobacillus gasseri</i> DSM 16737 <i>Lactobacillus plantarum</i> DSM 15313 (=strain HEAL 19)	DSS-induced colorectal cancer
V	Male C57BL/6 mice	-	DSS-induced acute colitis

## Ischaemia/reperfusion injury (paper I)

Six different treatment groups were selected and the mice were treated for 7 days with either *Lactobacillus plantarum* 299v ( $10^9$  CFU/mouse), rose hip powder (1.6 g/mouse), a combination of the two, or vitamin C (0.014 g/mouse). *L. plantarum* was provided by Probi AB (Ideon, Lund, Sweden) and grown in still culture at 37°C overnight in *Lactobacillus* carrying medium (LCM) (Efthymiou & Hansen, 1962) supplemented with 1% glucose. The cells were harvested by centrifugation and resuspended in freezing media (3.6 mM  $K_2HPO_4$ , 1.3 mM  $KH_2PO_4$ , 2.0 mM Na-citrate, 1.0 mM  $MgSO_4$ , and 12% glycerol) and freeze stored in -80°C until feeding. The feed (nonradiated R3, Lactamin, Sweden) was dissolved in water to soften the consistency prior to addition of supplementation. Supplementary carbohydrates (fructose and glucose, 0.65 g of each/mouse) were used to compensate for carbohydrate supply of rose hips, and freezing media was given to groups without bacteria supplementation.

The injury of the small intestine and colon was induced by occlusion of the superior mesenteric artery for 30 min followed by 240 min of reperfusion.

## Dextran Sulphate Sodium (DSS)-induced acute colitis (paper II and V)

In paper II, animals were divided into 6 groups and the feed (Table II) was supplemented with either a probiotic mixture (*Lactobacillus crispatus* ( $5 \times 10^{10}$ - $1 \times 10^{11}$  CFU), *Lactobacillus gasseri* ( $5 \times 10^{10}$ - $1 \times 10^{11}$  CFU), *Bifidobacterium infantis* ( $2 \times 10^{10}$  CFU), rye bran, a combination of rye bran and probiotics (rye bran+probiotics), blueberry husk, and a combination of blueberry husk and probiotics (blueberry husks+probiotics). Rye bran (Lantmännen (Järna, Sweden) and blueberry husk (Probi AB, Lund, Sweden) were included at a level of 80 g dietary fibre/kg in the diets (dwb) and the dry matter content of the diets was adjusted with wheat starch (Cerestar, Krefeld, Germany). The probiotic bacteria was provided by Probi AB; *B. infantis* as a prepared frozen culture, which after centrifugation was resuspended in bacterial suspension medium (sodium chloride, 8.5 g/l; bacteriological peptone (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England), 1 g/l; Tween 80, 1 g/l; L-cysteine hydrochloride monohydrate (Merck, Darmstadt, Germany) 0.2 g/l) before administration. The preparations of lactobacilli were made from fresh cultures in LCM (Efthymiou & Hansen, 1962), incubated anaerobically at 37°C for 48 h, harvested by centrifugation and resuspended in bacterial suspension medium. As compensation to the groups not given probiotics, bacterial suspension medium was used.

In paper V, mice were fed ordinary food with no supplements.

Acute colitis was induced in rats by 5% (w/v) DSS (molecular weight 36,000-50,000 kDa; ICN Biomedicals, Inc., Aurora, Ohio, USA) dissolved in water and

given by oro-gastric tube for 7 days (paper II), or by 4% (w/v) DSS (molecular weight 35.000-48.000 kDa; TdB Consultancy, Uppsala, Sweden) dissolved in drinking water, for 7 days (paper V).

Table II. Composition of the feed (g/kg dry feed)

Component	
Casein	120
DL-methionine	1.2
Maize oil	50
Mineral mixture <sup>1</sup>	48
Vitamin mixture <sup>2</sup>	8
Choline chloride	2
Sucrose	100

<sup>1</sup>Containing (g kg<sup>-1</sup>) 0.55 CuSO<sub>4</sub>·H<sub>2</sub>O, 2.0 ZnSO<sub>4</sub>·7H<sub>2</sub>O, 498 KH<sub>2</sub>PO<sub>4</sub>, 258 NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 487 CaCO<sub>3</sub>, 0.1 KI, 86 MgSO<sub>4</sub>, 12 FeSO<sub>4</sub>·7H<sub>2</sub>O, 5 MnSO<sub>4</sub>·H<sub>2</sub>O, 0.03 CoCl<sub>2</sub>·6H<sub>2</sub>O, 153 NaCl, 0.02 CrCl<sub>3</sub>·6H<sub>2</sub>O, 0.02 Na<sub>2</sub>Se.

<sup>2</sup>Containing (g kg<sup>-1</sup>) 0.62 menadion, 2.5 thiamin hydrochloride, 2.5 riboflavin, 1.25 pyridoxin hydrochloride, 6.25 calcium pantothenate, 6.25 nicotinic acid, 0.25 folic acid, 12.5 inositol, 1.25 p-aminobenzoic acid, 0.05 biotin, 0.00375 cyanocobalamin, 0.187 retinol palmitate, 0.00613 calciferol, 25 d- $\alpha$ - tocopheryl acetate, 941.25 maize starch.

## DSS and/or azoxymethane (AOM)-induced colorectal cancer (paper III and IV)

In paper III, the animals were divided in three groups and the feed (Table II) was supplemented with oat bran (Lantmännen) as the major dietary fibre source. Oat bran was included at a level of 50 g dietary fibre/kg (dwb), and the dry matter content was adjusted with wheat starch (Cerestar). Colorectal cancer was induced in two ways: either by an intraperitoneal injection (15 mg/kg bodyweight) of AOM (Sigma, St. Louis, USA) followed by 5% DSS (ICN Biomedicals, Inc.) dissolved in drinking water for 7 days, or by cyclic administration of 7 days of 4% DSS administration followed by 10 days of tap water. This cycle was repeated 11 times. The experimental period lasted 5 months (paper III) or 6 months (paper IV). The animals who received cyclic DSS administration, was also applied as non-treated animals in paper IV.

In paper IV, the above mentioned model of cyclic DSS administration was used and the feed of the six groups included was supplemented with either oat bran (as the major dietary fibre source), a probiotic mixture (*Bifidobacterium* (2x10<sup>9</sup> CFU),



*Lactobacillus gasseri* ( $1 \times 10^9$  CFU), *Lactobacillus plantarum* ( $3 \times 10^9$  CFU)), blueberry husk (an extra blueberry shot (2B)), a combination of blueberry husk and probiotics (an extra blueberry shot (2B)+probiotics), blueberry husk and oat bran (blueberry husks (B)), or a combination of blueberry husk, oat bran and the probiotic mixture (blueberry husks (B)+probiotics). When solely comprised, blueberry husk and oat bran were included at a level of 50 g dietary fibre/kg in the diets (dwb), while half of the amount was used when combined. Blueberry husks and oat bran were supplied by Lantmännen and the probiotic bacteria was cultured and freeze dried by Probi AB, who also supplied freezing media as compensation to the appropriate groups.

## Surgical procedures and sample collections

Animals were anaesthetised with 7.5 mg Ketamine (Ketalar 50 mg/ml, (Pfizer, UK)) and 2.5 mg Xylazine (Narcoxy 20 mg/ml, (VeterinariaAG, Schweiz)) per 100 g body weight by intraperitoneal injection (paper I), or by subcutaneous injection of a mixture (1:1:2) of Hypnorm (Division of Janssen-Cilag Ltd., Janssen Pharmaceutica, Beerse, Belgium), Dormicum (F. Hoffmann-La Roche AG, Basel, Switzerland) and water at a dose of 0.15 ml/100 g (papers II-IV). No anaesthetics were given to animals in paper V, since they were immediately sacrificed by cervical spine dislocation. All animals were non-fasting and surgery was performed with careful attention to sterile technique and the laparotomy was performed through a midline incision.

Arterial blood was withdrawn for cytokine assay (papers II-V) and for SCFAs (papers III and IV). The samples were centrifuged for 15 min (2500xg) and stored at  $-40^{\circ}\text{C}$  (papers III and IV) or for 10 min (3000xg) and stored at  $-80^{\circ}\text{C}$  (papers II and V). For analysis of translocating bacteria, the caudate lobe of the livers and mesenteric lymph nodes were isolated (papers III and IV). The luminal content of caecum and colon was removed and caecal tissue weight, content and pH were measured before the luminal content was stored at  $-40^{\circ}\text{C}$  for analysis of CA (papers II-IV). For microflora analysis, the following samples were collected: caecal content (paper I), caecal tissue (paper II), faecal samples (papers III and IV) and colonic tissue (paper V). Samples saved for analysis of translocating bacteria and microflora was collected in sterile tubes containing freezing media (papers I-IV) or in TE-buffer (10mM Tris; 1mM EDTA, pH 8.0) (paper V). Caecal tissue (paper I), small intestinal tissue (paper V) or colonic tissue (papers II and V) were saved in tubes protected from light for analysis of malondialdehyde (MDA) and small intestinal sample (paper V) as well as colonic tissue (papers II-IV) were collected for analysis of myeloperoxidase (MPO) activity. All samples were immediately frozen in liquid nitrogen. For histopathological evaluations, sections from the following organs were included: caecum (paper I), small intestine (paper V), colon (papers III-V), liver (papers III-V).

In papers III and IV, blood samples from the saphenous vein were taken at the beginning of the study, and during cycle 1, 5 and 10 for analysis of haptoglobin and SCFAs in serum. During the same time points, faecal samples for microflora analysis were collected and body temperature was measured.

In paper V, spleen, Payer's patches (PP) and mesenteric lymph nodes (MLN) were collected and saved on ice in HANK's buffer until immediate analysis.

## Analyses

### Disease Activity Index scoring

The Disease Activity Index (DAI) was used to validate severity of illness. The scoring system has been validated (Murthy *et al.*, 1997) and shown to correlate histologically with pathological findings (Cooper *et al.*, 1993). DAI was applied daily from day 0-7 (papers II and V) and during every 7-day cycle of DSS administration (papers III and IV) for assessment of the severity of colitis. The index was evaluated on a scale ranging from 0 to 4 according to the following criteria: stool consistency (normal, loose, diarrhoea), presence or absence of faecal blood (test slides with Hemocult II (SmithKline Diagnostic, USA) and macroscopic evaluation of the anus) and weight loss.

The arithmetical mean of the three scores was calculated and determines the DAI value. The original scoring of DAI by Cooper *et al.*, (1993) was used, but the limits of body-weight change have been modified. Weight changes were based on the starting weight of each rat at initiation of DSS administration (papers II and V) and at the start of the respective DSS cycles (papers III and IV). Weight-loss scores were determined as 0=no weight loss; 1=>0-4.99% weight loss; 2= 5.00-9.99% weight loss; 3=10.00-19.99% weight loss; 4=>20.00% weight loss. Stool scores were determined as 0=normal stool, well-formed pellets; 2=loose stool, pasty stool that does not stick to the anus; 4=diarrhea, liquid stool that sticks to the anus. Bleeding scores were determined as 0=no bleeding; 2=positive Hemocult test, 4=gross bleeding.

To quantify the amount of drinking water and DSS load ingested by the animals, the drinking volumes were recorded and DSS load were calculated as: Total drinking water (ml)x(DSS (g)/100 ml).

### Body temperature measurement

Body temperature of each rat was recorded using a rectal digital thermometer. The animals were placed in a restraining device when the body temperature was measured (papers III and IV).

## Body weight change and organ weights and lengths

Body weights were determined for each animal just before experimental inclusion and before sacrifice (papers I-V) as well as daily during DSS administration (papers II-V). Body weight change was calculated as g/kg feed/animal (paper II) and as g/animal (papers III-IV).

As an indication of inflammation, the weight of caecum (papers II-V), spleen (paper V) and colon (paper V) as well as colon length (paper V) were recorded. In paper V, caecum was emptied, washed, gently dried, weighed and incubated in an 80°C oven for 48 hours. The weight was re-measured, after 24 and 48 hours, and the wet-to-dry weight ratio was determined as a measure of edema (Rachmilewitz *et al.*, 1989). Colon was vertically suspended with a 1.5g weight to provide uniform tension, before measuring of the length.

## Macro and microscopic evaluation

In papers III and IV, the large bowels were initially macroscopically inspected for gross lesions (ulcers, dysplastic lesions and polyps) all of which were recorded.

All specimens (paper I and papers III-V) were fixed in phosphate buffered 4% formaldehyde, dehydrated and paraffin-embedded following standard procedures. After hematoxylin-eosin (H & E) staining, serial sections were cut and evaluated by light microscopy (papers I and III-V).

In papers III and IV, the degree of colonic dysplasia was scored from normal mucosa to mucosa with mild dysplasia (with distorted crypts of increased length and orientation), and severe dysplasia (with severe crypt distortion, atypical epithelial cells, reduction or loss of goblet cells, hyperchromatic cell nuclei and increased numbers of cell mitoses). For statistical evaluation, a numerical scoring system was used (1: normal mucosa, 2: low grade dysplasia, 3: high grade dysplasia).

For the degree of liver steatosis (papers III and IV), specimens were evaluated according to Brunt *et al.*, (1999), and scored as absent (=0), mild when present in <1/3 of the hepatocytes (=1), moderate when present in 1/3 – 2/3 of the hepatocytes (=2), and severe when present in >2/3 of the hepatocytes (=3). The presence and location of infiltrating inflammatory cells and liver injury was also recorded, and a semiquantitatively graded scale of 0 (absent), 1 (mild), 2 (moderate), and 3 (extensive) were used (Deutschman *et al.*, 2006) to grade for the degree of inflammation in steatotic and non-steatotic areas, stasis and loss of parenchyma.

In paper V, one-centimeter long specimens from the most distal part of the colon, and the left lobe of the liver were microscopically evaluated. The evaluation of histopathological changes in the colon was done according to Cooper *et al.*, (1993).

## Lipid peroxidation

The concentration of MDA was determined in colonic segments as an index of lipid peroxidation, using the colorimetric assay MDA 586TM (R&D, Europe Ltd., Abingdon, Oxon, UK).

The segments were collected, rinsed in ice-cold Dulbecco's PBS, weighed, and then frozen immediately at  $-70^{\circ}\text{C}$  for later evaluation. Lipid peroxidation was estimated by adding 1 ml PBS with 5 mM butylated hydroxytoluene to the samples prior to homogenisation. The samples were then centrifuged at 4,000g for 10 min at  $4^{\circ}\text{C}$ . An aliquot (200  $\mu\text{l}$ ) of standard or supernatant was added to a reaction mixture containing 640  $\mu\text{l}$  of N-methyl-2-phenindole, 10  $\mu\text{l}$  probucol, and 150  $\mu\text{l}$  of 12 M hydrochloric acid. The samples were incubated in a water bath for 60 min at  $45^{\circ}\text{C}$  and centrifuged at 10,000g for 10 min at  $4^{\circ}\text{C}$ . The absorbance of standard and supernatant was measured by spectrophotometry at 586 nm. Tetramethoxypropane (TMOP), which is hydrolysed during the acid incubation step at  $45^{\circ}\text{C}$  generating MDA, was used as standard since MDA is unstable. The results were expressed as nmol MDA/g tissue.

## MPO activity

MPO is an enzyme found in cells of myeloid origin, and has been used extensively as a biochemical marker of granulocyte (mainly neutrophil) infiltration into gastrointestinal tissues (Perner *et al.*, 2001).

In papers II-V, MPO activity was estimated in the whole colonic tissue (Khan & Al-Awadi, 1997), containing mucosa and muscle layers. Specimens of distal colon were collected and weighed prior to storage at  $-70^{\circ}\text{C}$  until time of assay. The segments were homogenised in 1 ml potassium phosphate buffer (20 mM, pH 7.4) for 60 s. Subsequently, the homogenates were centrifuged (14000 rpm, 10 min) and the pellets were resuspended in 1 ml potassium phosphate buffer (50 mM, pH 6.0) containing 0.5% hexadecyl-trimethyl-ammonium bromide. The samples were then freeze-thawed once, sonicated (90 s) and kept in water bath for 120 min ( $60^{\circ}\text{C}$ ). Next, the samples were centrifuged (14000 rpm, 10 min) and the MPO activity of the supernatants (20  $\mu\text{l}$ ) assessed in 96-well plates (Nunc, Invitrogen A/S, Taastrup, Denmark). The enzyme activity was determined spectrophotometrically at 450 nm. MPO (Sigma Chemical Co., St. Louis, MO, USA) was used as standard and the activity was expressed as units per gram of wet weight of the tissue.

## Haptoglobin

Haptoglobin is an acute phase reactant that has been implicated as a useful marker of inflammation in rats (Giffen *et al.*, 2003).

The concentration of serum haptoglobin was analysed using a manual microplate (96 microwell plates, Nunc<sup>TM</sup>, Roskilde, Denmark) method at room tem-

perature. In this assay, serum was incubated with haemoglobin (Hb) (0.12 mg/ml bovine haemoglobin (Sigma Aldrich, St Louis, USA) in 0.15 M NaCl (Merck Schuchardt, Hohenbrunn, Germany)) which combined with any haptoglobin present in the samples, will lead to preservation of peroxidase activity of the Hb. The peroxidase activity of free, unbound Hb was inhibited by performance at low pH. The preserved activity, measured by the addition of a peroxidase substrate (chromogenic solution; 0.5 M citrate buffer pH 3.8 (0.5 M sodium citrate dihydrate (J.T Baker B.V., Deventer, Holland), 0.5 M citric acid-1-hydrate (Merck)), 1% Tween 20 (Merck), 20 mM phenol (International Biotechnologies Inc., Eastman Kodak Co. Rochester, NY), 0.39 mM dithioerythritol (Sigma), 1.6 mM 4-aminoantipyrine (Sigma), 1 mM 8-anilino-1-naphthalene sulphonic acid (Sigma) and 1 µl 30% H<sub>2</sub>O<sub>2</sub>/0.7 ml solution (Merck)), is directly proportional to the amount of haptoglobin in the samples. A colorimetric reaction showing the peroxidase activity was compared with a haptoglobin standard (2 mg/ml) (Tridelta Development Ltd, Maynouth County Kildare, Ireland). Absorbance was measured at 600 nm (SpectraMax® M2 Multi-detection Microplate Reader, Molecular Devices, Sunnyvale, California).

## Cytokines

### *Enzyme-Linked ImmunoSorbant Assay (ELISA)*

In paper II, interleukin-12 was analysed in serum by use of solid phase sandwich ELISA.

The active form of IL-12 is a 70 kDa (p70) glycoprotein composed of 40 kDa (p40) and 35 kDa (p35) subunits. The individual p40 and p35 subunits show no IL-12 activity, however, p40 does exist as a dimer that has been shown to bind the IL-12 receptor and act as an IL-12 antagonist (Trinchieri G *et al.*, 1998). The interleukin-12 Rat ELISA Kit (Rt IL-12) (BioSource International, Inc., USA) quantifies natural and recombinant Rt IL-12 heterodimer and the free p40 unit. The analysis of cytokine protein expression was done according to the manufacturer's recommended protocols and all samples were analysed in duplicate. The minimum detectable amount of Rt IL-12 is <3 pg/mL. Samples that are >1000 pg/mL should be diluted with standard diluent buffer for serum/plasma samples. Unfortunately, a replication of the analysis was not feasible, due to freezer failure.

### *Multiplex cytokine profiling*

Multiplex cytokine profiling is a sensitive and quantitative simultaneous measurement of a broad spectrum of cytokines/chemokines.

A multiplex sandwich immunoassay from Millipore Corp. (Billerica, MA, USA), MILLIPLEX™ MAP rat or mouse cytokine kit, was used in paper III and V respectively, and the following cytokines/chemokines were analysed: IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17, IL-18, TNF-α, IFN-γ) and leptin (paper III) and IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, TNF-α, IFN-γ and KC (paper V).

The assay contains fluorescence-labeled microspheres conjugated with monoclonal antibodies specific for the target cytokines/chemokines of interest. Antibody-coupled beads were incubated with the serum sample, after which they were incubated with biotinylated secondary antibodies before finally being incubated with streptavidin-phycoerythrin. Bound molecules were read by a Luminex 200 instrument (paper III) or by Luminex 15 v. 2.3, which use Luminex fluorescent-bead-based technology. For evaluation of the results, Milliplex™ Analyst v. 3.4 (Vigenetech, Millipore) was used. In all steps, the procedures were performed in accordance to the manufacture's instructions and all samples were run in duplicate.

## Viable count

### *Translocation*

The passage of viable indigenous bacteria from the intestinal tract through the epithelial mucosa to the mesenteric lymph nodes, and then to systemic circulation was measured by culturing of the caudate lobe of the liver (papers II-IV) and mesenteric lymph nodes (papers III and IV).

Before culturing, the liver samples in paper II were placed at room temperature for 5 days, to stimulate multiplication of possible existing bacteria. This procedure was not performed in papers III and IV, since the probability of finding living bacteria in these models were regarded as increased due to the condition of the animals.

Samples were placed in an ultrasonic bath (Millipore, Sundbyberg, Sweden) for 5 min and swirled on a Chiltern (Therma-Glas, Gothenburg, Sweden) for 2 min. Viable counts were obtained from Violet-red bile glucose (VRBG) agar (Oxoid), incubated aerobically at 37°C for 24 h (*Enterobacteriaceae* count) from Rogosa agar (Oxoid), incubated anaerobically (Gas Pack System, Gas Pack; Becton-Dickinson Microbiology Systems, Cockeysville, Md., USA) at 37°C for 72 h (lactobacilli count), and from brain heart infusion (BHI) agar (Difco, Detroit, Mich., USA), incubated both aerobically and anaerobically, as described above, at 37°C for 72 h (aerobic and anaerobic count, respectively). Results were expressed as incidences of positive cultures/group. In papers III and IV, colonies were randomly picked and were subcultured on Rogosa or BHI agar plates before subjected to 16S rDNA sequencing.

### *Gut*

Determination of lactobacilli and *Enterobacteriaceae* counts were performed in papers I-IV.

Samples from papers I and II were placed in an ultrasonic bath for 5 min and swirled on Chiltern, while faecal samples from papers III and IV were homogenised in freezing media. All samples were serially diluted in dilution liquid (sodium chloride (Merck), 8.5 g/l; Bacteriological peptone (Oxoid), 1 g/l; Tween 80 (Merck), 1 g/l; L—Cystine hydrochloride monohydrate (Merck), 0.2 g/l) and

spread plated. Viable counts were obtained from Rogosa agar and from VRBG, under the same culturing conditions as mentioned above. The number of colonies formed on each plate was counted and *corrected* for the weight of the original tissue or faeces. In papers II-IV colonies were randomly picked from Rogosa agar, recultured to verify their purity and subsequently subjected to Randomly Amplified Polymorphic DNA (RAPD) analysis and 16S rRNA gene sequencing.

### Randomly Amplified Polymorphic DNA (RAPD)

Amplification of random DNA fragments by the RAPD technique generates a reproducible fingerprint of the PCR products and the method was administered in papers II-IV (identification of lactobacilli (papers II-IV) and translocation (papers III and IV)).

As template for the polymerase chain reaction, crude cell extract was used (Quednau *et al.*, 1998). The polymerase chain reaction of random fragments was run with a 9-mer primer with the sequence 5'-ACG CGC CCT-3' (Scandinavian Gene Synthesis AB, Köping, Sweden) (Quednau *et al.*, 1998). PCR templates (1 µl) were used in a total reaction volume of 50 µl containing PCR reaction buffer with 1.5 mM MgCl<sub>2</sub> (Roche Diagnostics GmbH, Mannheim, Germany), 0.2 mM dNTPs (Roche) and 2.5 units of Taq DNA polymerase (Roche). The PCR amplification was performed in a Perkin-Elmer thermal cycler, using the following temperature profile: 94°C for 45 s, 30°C for 120 s, 72°C for 60 s, for four cycles, followed by 94°C for 5 s, 36°C for 30 s (with an extension of 1 s per cycle), and 72°C for 30 s, for 26 cycles. The PCR reaction was terminated at 72°C for 10 min, followed by cooling to 4°C. Agarose gel electrophoresis was run, the gels were stained with ethidium bromide and photographed under UV illumination.

### 16S rDNA sequencing

For species identification, 16S rRNA gene sequencing was used in papers II-IV (identification of lactobacilli (papers II-IV) and translocation (papers III and IV)) and the two primers used for amplification were ENV1 (5'-AGA GTT TGA TII TGG CTC AG-3', *Escherichia coli* numbering 8-27) and ENV2 (5'-CGG ITA CCT TGT TAC GAC TT-3', *E. coli* numbering 1511-1492) (Brosius *et al.*, 1978). The PCR reaction mixture contained 0.2 µM of both primers, 5 µl of template DNA, 5 µl of 10x PCR reaction buffer with 1.5 mM MgCl<sub>2</sub> (Roche), 200 µM of each deoxyribonucleotide triphosphate, and 2.5 U of Taq DNA polymerase (Roche). Water was added to a final volume of 50 µl. PCR was performed in a PCR Mastercycle 5333 (Eppendorf Hamburg, Germany) with the following profile: 1 cycle at 94°C for 3 min, followed by 30 cycles of 96°C for 15 s, 50°C for 30 s, and 72°C for 90 s, with an additional extension at 72°C for 10 min. The amplification products (5 µl) were checked by running the products on 1.5 % (wt/vol) agarose gel in 1xTBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3), after ethidium bromide staining. Amplicons were sent to MWG (Biotech,

Ebersberg, Germany) for single strand sequencing. 16S rDNA sequences were searched against Genbank (blastn) option at the homepage of the National Centre for Biotechnology (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Altschul *et al.*, 1990) or aligned to 16S rDNA encoding sequences downloaded from the Ribosomal Data Base (RDP-II) (Cole *et al.*, 2003) for an approximate phylogenetic affiliation.

## DNA extraction

DNA extraction from colonic mucosal samples in paper V was done by using BioRobot EZ1 DNA tissue kit (Qiagen) as described elsewhere (Karlsson *et al.*, 2009).

## Terminal restriction fragment length polymorphism (T-RFLP)

To assess bacterial communities and diversity T-RFLP was applied in paper V.

### *PCR amplification for T-RFLP analysis*

The 16S rRNA genes were amplified with primer FAM-ENV1 (5'-AGA GTT TGA TII TGG CTC AG-3') and ENV2 (5'-CGG ITA CCT TGT TAC GAC TT-3') (Wang *et al.*, 2005). The forward primer ENV1 was synthesized and fluorescently labeled with FAM (Applied biosystems, Foster city, CA, USA) at 5' end. The PCR reaction mixture in a total volume of 25 µl, contained 0.4 µM of primer FAM-ENV1 and 0.2 µM of primer ENV2, 2.5 µl of 10 x PCR reaction buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM MgCl<sub>2</sub>, pH 8.3), 0.2 mM of each deoxyribonucleotide triphosphate, 2.5 U of FastStart Taq DNA polymerase (Roche), and 2 µl of template DNA. The PCR was performed in an Eppendorf MasterCycler (Eppendorf) using the following program: 95°C for 3 min, 94 °C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 45 s, and 72°C for 2 min. Finally, an additional extension at 72°C for 7 min was done. To diminish the PCR bias, triplicate reactions were carried out for each sample and a negative control was included in all the PCR runs. After the amplification, the PCR products were verified by agarose gel electrophoresis. PCR products of each sample were then pooled and purified by MiniElute PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was eluted in 30 µl of sterile water and DNA concentration was measured by Nanodrop ND-1000 (Saveen Werner, Limhamn, Sweden).

### *T-RFLP*

200 ng of the purified PCR products were digested separately with 10 U of the restriction endonucleases *Msp*I (Fermentas Life Science, Burlington, Canada) in a total volume of 10µl for 5 h at 37°C. Enzyme was then inactivated by heating at 65°C for 20 min. After digestion, aliquots of the products were diluted 5 times with sterile water in a sterile 96-well plate (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were then sent to DNA-lab at Malmö University Hospital



(UMAS) for the T-RFLP analysis. Analysis was performed on a 3130xl Genetic Analyzer (Applied Biosystems) and in all samples a DNA size marker GeneScan™ LIZ 600 (Applied Biosystem) was included. Fragment sizes, peak height and peak area were analysed with Genemapper® software version 4.0 (Applied biosystems). Local Southern method was chosen for size calling, and the size range was set between 40 bp and 580 bp. The peak amplitude thresholds were set to 50 relative fluorescence units (rfu) for samples, and 10 rfu for standards. The total peak area for each sample was calculated by summarizing the area for all peaks in a sample. The relative peak area of each peak was expressed as percentage of the total area.

### Quantitative Real-Time Polymerase Chain Reaction (qPCR)

To establish the DNA copy number after cycles of amplification, qPCR was used in paper V.

Standards for the qPCR that were used to quantify the *Lactobacillus* and *Enterobacteriaceae* were prepared by cloning of the corresponding 16S RNA fragment using primers listed in Table III in a PCR. The bacterial DNA was extracted from pure cell culture of *Lactobacillus plantarum* 299v CCUG 35035 and *Escherichia coli* CCUG 29300 (Culture collection, University of Gothenburg, Sweden) and used as template.

For total bacteria another set of primers were used with *L. plantarum* 299v DNA as template (Table III).

The purified PCR products were then cloned by using pGEM-T vector system (Promega, Madison, USA) and then transformed into *E. coli* JM109 high efficiency competent cells. Colonies were blue/white screened and cells containing inserts were collected and stored in freezing media at -80°C. The correct size of the insert was checked by PCR using the primers RIT28 (5'-AAA GGG GGA TGT GCT GCA AGG-3') and RIT29 (5'-GCT TCC GGC TCG TAT GTT GTG TG-3') that anneal to the flanking regions of the insert (Hultman *et al.*, 1991).

The four different clones were then cultured in LB-broth with ampicillin at 37°C overnight. The plasmid DNA was extracted by using QIAprep® Miniprep kit (Qiagen). The concentrations of the plasmid DNA were measured by Nanodrop ND-1000 (Saveen Werner, Limhamn, Sweden). For preparation of the standards, tenfold dilution series were made of the extracted plasmid DNA in TE buffer (10 mM Tris, 1mM EDTA, pH 8.0) supplemented with 0.1 ug/µl Herring sperm DNA (VWR International, West Chester, PA, USA) and the copy numbers of each standard were calculated.

The quantitative PCR (qPCR) was run in a Mastercycler® ep realplex 1.5 real-time PCR system (Eppendorf) separately for the different groups of bacteria. The qPCR reaction consisted of 10 µl of 2X Platinum®SYBR® Green qPCR Super-Mix-UDG (Invitrogen A/S, Taastrup, Denmark), 0.5 µM each of the forward and the reverse primer (Table III) and 2 µl of template DNA. Sterile ddH<sub>2</sub>O was added

to the total volume of 20  $\mu$ l. Triplicate of standards and samples as well as triplicate negative controls were prepared in a sterile 96-well polypropylene microplate (Eppendorf). The qPCR was run under the following conditions. Initially the temperature were set to 50°C for 2 min, followed by 95°C for 2 min. 40 cycles were then run with the following parameters: 95°C for 15 s, primer annealing for 30 s and 72°C for 30 s (Table III). For amplification of the total bacteria the elongation time was set for 45s at 72°C. Finally a melting curve analysis was performed by a temperature gradient from 60-95°C for 20 min and a final denaturation at 95°C for 15 s. For data analysis CalQplex algorithm and automatic baseline with drift correction were chosen for all the quantifications.

Table III. Primers used in qPCR to amplify four target regions

Name	Sequence (5'—3')	Target group	Amplicon size (bp)	Annealing term (°C)	Reference
Lact-F	AGCAGTAGGGAATCTCCA (19)	<i>Lactobacillus</i>	327	61	Walter <i>et al.</i> , 2001
Lact-R	CACCGCTACACATGGAG (17)				Heilig <i>et al.</i> , 2002
Uni331-F	TCCTACGGGAGGCAGCAGT (19)	Total bacteria	466	58	Nadkarni <i>et al.</i> , 2002
Uni797-R	GGACTACCAGGGTATCTAATCCTGTT (26)				
Eco1457-F	CATTGACGTTACCCGAGAAGAAGC (25)	<i>Enterobacteriaceae</i>	195	60	Bartosch <i>et al.</i> , 2004
Eco1652-R	CTCTACGAGACTCAAGCTTGC (21)				
AM1-F	CAGCACGTGAAGGTGGGGGACC (22)	<i>Akkermansia</i>	327	60	Collado <i>et al.</i> , 2007
AM2-R	CCTTGCGGTTGGCTTCAGAT (20)		327		

## Flow cytometry

In paper V, FACS analysis was used to evaluate proportions of leukocytes according to expressed surface antigens.

### FACS staining

Spleen, MLN and PP cell suspensions were prepared by pressing the organs between the frosted ends of sterile glass microscope slides and suspending the cells in Hanks-BSS. Isolations of single cell suspensions were obtained by pressing the suspension through a nylon cell strainer (Becton, Dickinson and Company, USA). Red blood cells in spleen were lysed with ddH<sub>2</sub>O for 13 seconds in room temperature. Cells from all tissues were washed twice with Hanks-BSS (Gibco, Invitrogen, Paisley, UK) at 1300 rpm for 10 min. The cells were counted and approximately  $1.5 \times 10^6$  cells were plated for each staining in a 96 well round-bottom plate. After washing the plated cells with 200  $\mu$ L staining buffer (1x PBS (AppliChem GmbH,

Darmstadt, Germany); 0.1% NaAz (Scharlau Chemie S.A., Sentmenat, Spain); 2% Fetal Bovine Serum (VWR International, Sweden)) at 1800 rpm for 1 min, the cells were stained in 25 µL antibody solution for surface markers (CD11b, CD11c, CD8 (BD Pharmingen<sup>TM</sup>, USA); CD3, CD4, CD25, CD49b, B220, CCR9 (BioLegend, San Diego, USA); CD69 (eBioscience, Inc., San Diego, USA)) for 30 min at 4°C in dark. After staining, cells were washed twice with 175 µL FACS buffer (1x PBS (Applichem, Germany); 0.1% NaAz (Scharlau Chemie S.A., Sentmenat, Spain); 2% Fetal Bovine Serum (VWR International, Sweden); 2% Formaldehyde (Apoteket, Sweden)) at 1800 rpm for 1 min and re-suspended in 200 µL FACS buffer. Instead the cells stained for intracellular markers (FoxP3 (eBioscience, Inc., San Diego, USA); CTLA-4 (BioLegend, San Diego, USA)) were fixed and permeabilised according to manufacturer's instructions for FoxP3 (eBioscience, Inc., USA) and then re-suspended in 200 µL FACS buffer. Stained cells were stored at 4°C until FACS analysis, which was performed next morning.

#### *Antibody panel*

Cells were incubated with sets of four monoclonal antibodies and the four-colour panels consisted of FITC, PE, PerCP, APC and reagents in the following antibody combinations:

CD3/B220/CD8/CD4; CD3/CD25/CD8/CD4; CD69/CD25/CD4/FoxP3; CD4/CTLA4/CD25/FoxP3; CD4/CD69/CD8/CCR9; CD3/CD49b/CD8/CD4; DAMP channel\* (CD11c/CD11b); DAMP channel\* (CD11c/TLR4); DAMP channel\* (CD11c/CD11b/TLR4).

\*DAMP channel: CD3, B220, CD4, CD8

#### *Flow cytometry analysis*

The FACS analysis was performed on FACS Calibur (Becton, Dickinson and Company, USA) and 30 000 lymphocytes in live gate were acquired for analysis. The data was analysed in FlowJo software (Treestar, Inc., Ashland, USA).

#### *Dietary fibres*

A gravimetrically method by Asp *et al.*, (1983) was used to determine soluble and insoluble dietary fibre in blueberry husk, rye bran and oat bran (papers II-IV). The composition of the luminal content fibre residues was analysed by gas-liquid chromatography (GLC) for the neutral sugars as their alditol acetates and spectrophotometrically for the uronic acids (Theander *et al.*, 1995).

#### *Carboxylic acids*

##### *In serum*

Acetic, propionic, isobutyric, butyric, isovaleric and valeric acids were analysed in serum in papers III and IV using a GLC method by Zhao *et al.*, (2007). To the serum samples, water and 2-ethylbutyric acid (internal standard), (Sigma Chemical

Company, St Louis, MO, USA) were added and SCFAs were protonised with hydrochloric acid. A hollow fibre was immersed in the serum solutions and SCFAs were extracted into the lumen of the fibres, resulting in an enrichment of acids. After 16 h of extraction, the lumen content was flushed and mixed with hydrochloric acid and the samples were injected onto a fused-silica capillary column (DB-FFAP 125-3237, J&W Scientific, Agilent Technologies Inc., Folsom, CA, USA). For evaluation, GC ChemStation software (Agilent Technologies Inc., Wilmington, DE, USA) was used.

#### *In luminal content*

A GLC method developed by Richardson *et al.*, (1989) was applied in paper II, to assess the amounts of acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic, heptanoic, succinic and lactic acid. An internal standard (2-ethylbutyric acid) was added to caecal and colonic contents and the samples were homogenised using an Ultra Turrax® T25 basic homogeniser (IKA®-Werke, Staufen, Germany). After complete derivatisation by hydrochloric acid and n-(tert-butyltrimethylsilyl)-n-methyl trifluoroacetamide (Sigma Chemical Company, St Louis, MO, USA) the samples were injected onto an HP-5 column (Hewlett Packard, GLC, HP 6890, Wilmington, DE, USA) and for evaluation of the results, Chem Station software (Hewlett Packard) was used.

In papers III and IV, the caecal and colonic amounts of acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic and heptanoic acids were analysed by a GLC method by Zhao *et al.*, (2006). Water mixed with hydrochloric acid and 2-ethylbutyric acid (Sigma) was added to the samples before homogenisation using Ultra Turrax® T25 basic (IKA®-WERKE). After centrifugation (MSE Super Minor, Hugo Tillquist AB, Solna, Sweden) the samples were injection onto a fused-silica capillary column (as mentioned before).

#### Statistical evaluation

If a large number of variables are obtained, principal component analysis (PCA) can be performed to reveal the internal structure and possible correlations of the data, in a way which explains the variance.

A principal component analysis was performed in paper V by using the software Umetrics SIMCA-P+ 12.0.1 (Umetrics, Umeå, Sweden). For the analysis, the relative peak area and/or flow cytometry data was used.

In papers I and III-V, feed intake, DSS consumption, body weight change, DAI scores, caecal weight (only paper V), colon length and weight, spleen weight, MPO activity, MDA levels, cytokines/chemokines, leptin, haptoglobin, body temperature, lactobacilli and *Enterobacteriaceae* counts, number of dysplastic lesions, number of ulcers, and scoring used for histopathologic evaluation of colonic- and liver samples were presented as medians with 25 and 75 percentiles. The statistics were conducted in SigmaStat® version 2.0 (paper I) and 3.0 (papers II-V), (SPSS Inc., Chicago, Ill., USA). Differences between all groups were evaluated by

Kruskal-Wallis test oneway ANOVA on ranks followed by all pairwise multiple comparison procedures (Student-Newman-Keuls method), if appropriate.

The correlation between expectations of benefit was ascertained using Pearson's correlation coefficient (papers I-III) or Spearman Rank Order (paper V).

Calculation of the incidence of steatosis, cellinfiltration, stasis, loss of parenchyma and translocation to the liver (papers II-IV), as well as statistical comparison of the total received score of steatosis compared to maximum score (paper IV) and the incidence of *Enterobacteriaceae* growth (paper V) was conducted in QuickStat version 2.6 and was evaluated by the Fisher exact test.

The Shannon-Wiener diversity index (H') (Krebs, 1998) was calculated by using the relative peak area (paper V). Mann-Whitney Rank Sum Test (SigmaStat) was used to for pairwise comparison.

For evaluation of feed intake (paper II), bodyweight change (paper II), caecal content (papers II-IV), caecal tissue weight (papers II-IV), caecal pH (papers II-IV) and CAs (papers II-IV), one-way ANOVA using the general linear model procedure (GLM, ANOVA) was used. When error variance was found to be heterogeneous, data was transformed by Box-Cox-transformation before ANOVA. To make these evaluations, the Minitab statistical software (Release 14) was used.

Caecal pools of CAs (paper II) and SCFAs (paper III) were calculated as the concentrations of each acid ( $\mu\text{mol/g}$ ) multiplied by the caecal content (g). The values were extrapolated to a complete intake of dietary fibre during the experimental period, to correct for small amounts of feed residues (paper II). Analysis of SCFAs in serum taken from the saphenous vein (paper IV) was performed on pooled samples.

For all analysis,  $p$ -values  $\leq 0.05$  were considered significant.

# Results and Discussion

## Physiological findings and clinical scoring reflecting intestinal inflammation

### Ischaemia/reperfusion – oxidative stress

The human colon is supplied by the superior and inferior mesenteric artery, while the superior mesenteric artery (SMA) is dominant in rodents (Leung *et al.*, 1992). The Ischaemia/reperfusion injury model has been verified in C57BL/6 mice (Riaz *et al.*, 2002), by clamping of the SMA. In paper I, SMA was identified and occluded and after clamp removal, immediate reperfusion was observed (Fig 1).

The mice underwent 30 min of Ischaemia and 240 min of reperfusion. The time-intervals were chosen according to Riaz *et al.*, (2002), showing the highest level of colonic MDA. During the seven day-period of feed supplementation, all animals gained weight and the products were well tolerated.

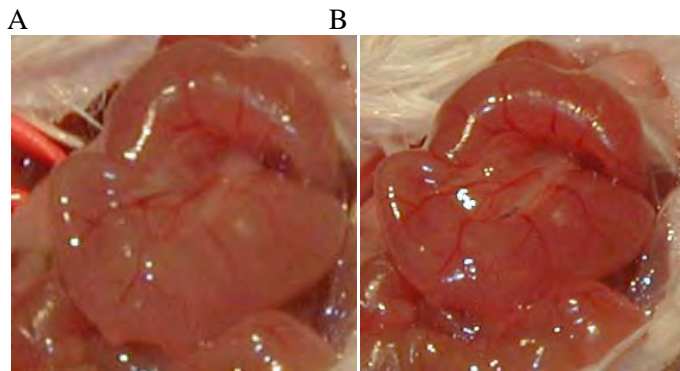


Figure 1A, B. Superior mesenteric artery was identified and occluded, resulting in Ischaemia (A). After clamp removal, immediate reperfusion was observed (B) (paper I).

## DSS-induced colitis

### *Weight loss*

In UC patients, diarrhoea and bleeding as well as inadequate food intake due to abdominal pain and loss of appetite, results in body weight loss during acute flare-ups.

Induced acute colitis resulted in loss of body weight compared to healthy animals (paper V). By feed supplementation, the body weight loss was significantly reduced in all treatment groups, with the exception of rye bran only, which was not significantly differed although a trend towards decreased body weight loss was definitely seen (paper II).

In papers III and IV, the animals were held for 6 months (animals belonging to group AOM were purchased at the same time as the other animals to avoid eventual batch differences) and during this time, the animals continued to grow and develop, so a difference in body weight change was not observed, when comparing start weight with the weight at the end of the experimental period. However, starting from cycle 3, all animals from all groups (paper IV) lost weight during DSS administration, but during the following water periods they were able to gain more weight than they lost.

### *Organ morphology*

Extensive inflammation with accumulation of inflammatory cells and associated edema contribute to increased organ weight (Tracey *et al.*, 1988) and the DSS-induced model of colitis is associated with a significant decrease in colon length (Fiocchi *et al.*, 1998 and Hendrickson *et al.*, 2002) due to intestinal wall-thickening and healing ulcers and fistulas (Meira *et al.*, 2008). The weight of caecum (papers II-V), colon (paper V) and spleen (paper V) as well as colonic length (paper V) were measured as morphological parameters for the degree of inflammation in DSS-induced acute and chronic colitis. During acute colitis, significant differences of caecal weights were found between non-treated animals and animals supplemented with blueberry husks or rye bran (paper II) and between non-treated animals and healthy ones (paper V). However, induction of chronic inflammation and colorectal cancer did not give rise to any differences in caecal tissue weights (papers III and IV). Measurements of caecal tissue weights were in papers II-IV registered as wet weight, while wet/dry weight ratio was used in the last paper (paper V) and may be a more reliable method for claimed edema. Acute colitis also increased colon weight and decreased the length (Fig 2), but the spleen weight was not affected even though an increase is regarded as a colitis-related disease marker (Meira *et al.*, 2008).

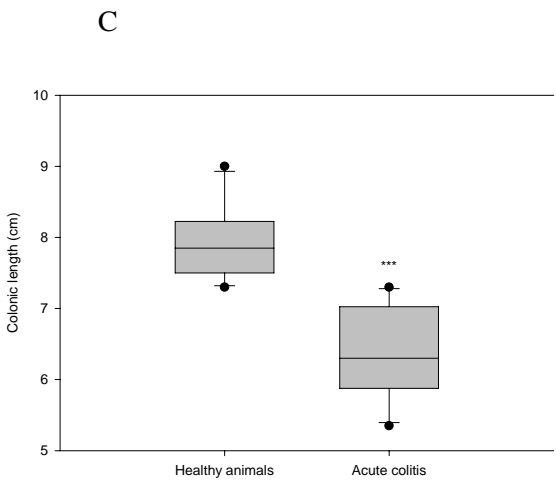
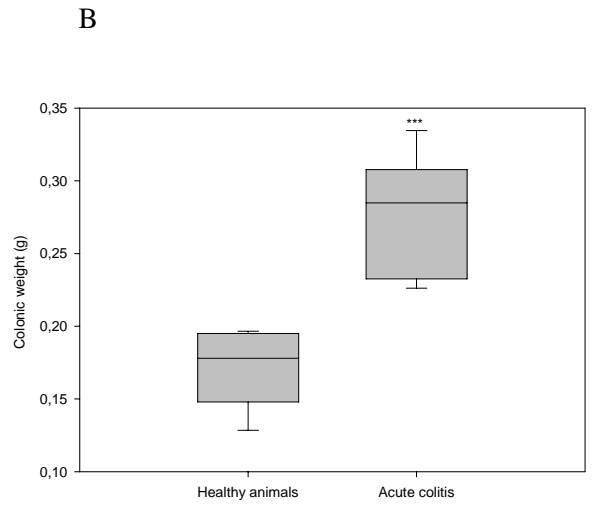
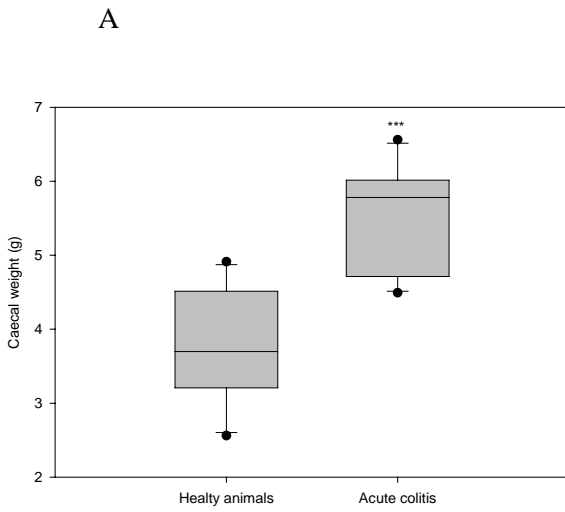


Figure 2A, B, C. Caecal wet/dry weight ratio (A) and colonic wet weight (B) was significantly increased, while the colonic length was significantly decreased during acute colitis (C). \*\*\* denotes  $P < 0.001$  vs healthy animals (paper V).



### *Disease Activity Index*

In all cycles of DSS administration (papers II-V), daily body weights, positive hemocult or presence of gross blood per rectum, and stool consistency were determined. The clinical parameters used are comprehensive functional measures, analogous to subjective clinical symptoms observed in human UC. This method of scoring has been validated by repeated studies (n=600, data from 1997, Murthy *et al.*, 1997) showing a coefficient of variation of 4.8%. The index correlates well with histologic healing measured as crypt scores (Cooper *et al.*, 1993) and significant decreases in DAI are considered an end point of a successful therapy (Murthy *et al.*, 1997). The scoring limits of body weight changes were slightly modified in papers II-V to make the judgements between scores 1 and 2, and 2 and 3 objectively facilitated (Table IV).

Table IV. Difference between scoring limits of Disease Activity Index (DAI).

Score	Weight loss (Cooper <i>et al.</i> , 1993)	Weight loss (paper II-V)	Stool consistency	Rectal bleeding
0	None	None	Normal	Normal
1	1-5%	1-4.99%		
2	5-10%	5-9.99%	Loose stools	Hemocult+
3	10-20%	10-19.99%		
4	>20%	>20%	Diarrhea	Gross bleeding

Disease activity index is the mean of the combined scores of weight loss, stool consistency and rectal bleeding. The stool is judged according as well formed pellets=normal stool, pasty and semiformal stool which do not stick to the anus=loose stool and liquid stool that stick to the anus=diarrhea.

### *Acute colitis*

In paper II, DAI score on day 7 was lower in all treatment groups and a negative correlation was found between DAI and the total concentration of CAs, acetic acid and butyric acid found in luminal content of caecum. This correlates well with the observation of low CA levels and impaired utilisation of butyric acid during severe colitis (Vernia *et al.*, 1995 and Hallert *et al.*, 2003).

DAI between the two models of acute colitis was compared (papers II and V) and no difference was found. C57BL/6 mice are known to be highly sensitive to DSS (te Velde *et al.*, 2006), also verified here, since no difference in disease severity was found compared to rats fed a fibre-free diet that worsened the state. In some degree, the differences may be caused by handling of various animal species.

### *Chronic colitis*

In paper IV, the signs of colitis gradually disappeared between the cycles of DSS administration but over time, DAI gradually increased and did not revert. This may suggest a chronologic sequence, where repeated uninhibited acute inflammatory responses with less extension of mucosal repair develop chronicity. At the 11<sup>th</sup> cycle of DSS administration, decreased scoring values were obtained by supplementation of blueberry husks alone or combined with probiotics.

## Lipid peroxidation attenuated by probiotics, berries and seeds rich in phenolic compounds

### Oxidative stress

Reperfusion injury after ischaemia is a severe complication that is associated with the systemic release of toxic compounds when the blood flow is restored and the injury is considered to be an inflammatory condition (Granger *et al.*, 1994). The local intestinal damage is partly induced by the formation of toxic oxygen free radicals (OFR), (Parks *et al.*, 1982 and Granger *et al.*, 1981) and administration of antioxidant substances with the capacity to scavenge OFR may attenuate the injury. Lipid peroxidation is a well known mechanism of OFR-induced cellular injury and is used as an indicator of oxidative stress in tissues (Esterbauer *et al.*, 1991 and Ohkawa *et al.*, 1979).

In papers I and II, malondialdehyde (MDA) was determined as an index of lipid peroxidation in caecum and colon, respectively. In paper I, lipid peroxidation was a result of induced I/R injury and the antioxidative properties of rose hips, which has been attributed not only to the content of vitamin C, but also to the pharmacological properties of polyphenolics (Daels-Rakotoarison *et al* 2002), as well as *L. plantarum* possessing tannase activity (Osawa *et al.*, 2000), was supplemented to the animals. As evident by significantly decreased caecal levels of MDA, lipid peroxidation was attenuated by the combination of rose hips and *L. plantarum* 299v and a significant lower level was also found in comparison with the groups given vitamin C or bacteria alone. It is hypothesised that the combined effect from rose hips and *L. plantarum* 299v may depend on the antioxidative capacity of rose hips and on the potential of the bacteria to metabolise polyphenoles and as a result, for example, substituted phenyl propionic acids can be produced (Barthelmebs *et al.*, 2001). Propionic acid derivates are commercially used in anti-inflammatory drugs such as Ibuprofen® (Peters *et al.*, 1983).

### Lactobacilli and *Enterobacteriaceae*

In the intestinal microflora of mice, lactobacilli constitute a major group (Savage *et al.*, 1968) and this may be an explanation to the smaller difference between the

group supplemented with rose hip and bacteria in combination, compared to the group supplemented with rose hip alone. However, the tannase activity of the lactobacilli naturally occurring in mice is not clarified, but the actual difference between the two groups is expected to be more pronounced in a clinical situation, where lactobacilli regularly are present but in smaller proportions (Suau *et al.*, 1999 and Wilson *et al.*, 1999). Moreover, a positive correlation between MDA levels and *Enterobacteriaceae* count was found in paper I, and according to Brown *et al.*, (1998), LPS caused an elevated level of lipid peroxidation in colonic tissue.

## MDA levels

Lipid peroxidation causing an increased MDA concentration in the UC mucosa of humans and during DSS-induced acute colitis in rats, have been shown (Kruidenier *et al.*, 2003 and Arafa *et al.*, 2009). In paper II, colonic MDA was significantly reduced through supplementation of blueberry husks, rye bran and probiotics, but to a smaller extent with probiotics alone. A negative correlation was also found between MDA levels of acetic acid and butyric acid concentrations in distal colon, which can be compared to the protective effect of acetic acid and butyric acid against oxidative DNA damage induced by H<sub>2</sub>O<sub>2</sub> (Abrahamse *et al.*, 1999), also known to cause lipid peroxidation (Asghar *et al.*, 2006).

For some reason, the opposite relationship between inflammation and MDA levels was found in paper V. After DSS administration, significantly lower levels were found in both colon and small intestine compared to healthy controls. However, contradictory results have also been demonstrated in humans. Bhaskar *et al.*, (1995) did not find any increased MDA levels or any differences in mucosal activities of antioxidant enzymes between the control group and UC patients. In fact, antioxidant potential was found to be higher, and MDA levels lower in inflamed biopsies compared to normal mucosa from the same subjects (Durak *et al.*, 2000). The differing results may be explained by experimental differences in the severity of the condition and corresponding differences in the residual antioxidant capacity of the mucosa.

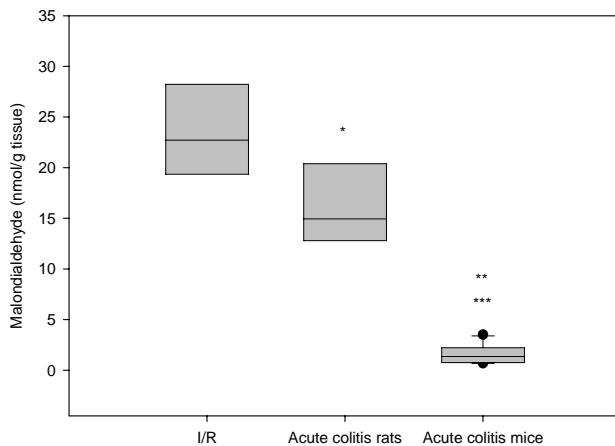


Figure 3. Malondialdehyde (MDA) levels after Ischaemia-reperfusion (I/R) injury (paper I) and during acute colitis (paper II and V). \* denotes  $P < 0.05$  compared to I/R injury, \*\* denotes  $P < 0.01$  compared to acute colitis rats, and \*\*\* denotes  $P < 0.001$  compared to I/R injury.

By comparing non-treated animals from paper I (I/R injury), paper II (acute colitis in rats) and paper V (acute colitis in mice), the difference is obvious. The levels of MDA were significantly higher after Ischaemia/reperfusion injury (Fig 3) compared to both colitis models and there was also a difference between these two. In paper II, the non-treated animals were given a fibre free diet, which probably worsen the condition compared to animals fed ordinary food. Lipidperoxidation also seems to be more pronounced and indicative of I/R injury (Fig 3).

## Infiltration of neutrophils and histological abnormalities influenced by feed supplements

### MPO activity

MPO is an enzyme found in neutrophils and its activity is linearly related to neutrophil infiltration. The assessment of MPO activity is well established for quantifying acute intestinal inflammation (Krawisz *et al.*, 1984). Acute colitis revealed significantly elevated MPO activity in colon but not in the small intestine, compared to the healthy animals (paper V). In paper II, MPO activity was significantly reduced by blueberry husks+probiotics and a decrease was also seen in the group supplemented with rye bran+probiotics compared to rye bran alone.

The chronic colitis and colorectal models (papers III and IV) showed higher levels only during cyclic administration of DSS, indicating a lower level of mucosal inflammation in the AOM group, since neutrophil infiltration reflects the mucosal inflammation state (Boughton-Smith *et al.*, 1988 and Roncucci *et al.*, 2008).

In paper IV, no effect on MPO activity was reached in any of the supplemented groups. Repression of the inflammation to that extent may not be expected after 11 cycles of DSS administration. Neutrophils represents the initial cell infiltration during acute inflammation, while the subsequent on-going reaction may represent a later mononuclear cell infiltration, more characteristic of chronic inflammation (Boughton-Smith *et al.*, 1988). MPO activity is not specific for polymorphonuclear leukocytes and mononuclear cells may also contribute to increased activity (Boughton-Smith *et al.*, 1988). The colonic MPO activity is increased during chronic inflammation, as evaluated by comparison of non-treated animals from paper II and paper III (Fig 4). Local high myeloperoxidase content presented here did not always correlate with histological findings or with the objective macroscopic evaluation.

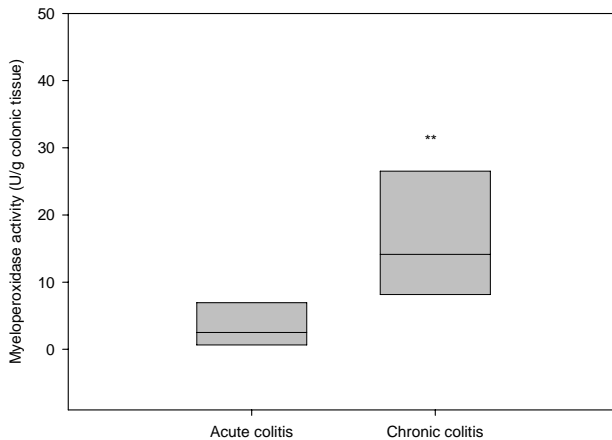


Figure 4. Myeloperoxidase activity was significantly increased during chronic colitis (paper III and IV) \*\* denotes  $P < 0.01$  compared to acute colitis (paper II).

There is an association between the content of MPO and the presence of inflammation in tissues. Notwithstanding, during a chronic condition and the analysis being done in whole colonic tissue, where the extravascular content cannot be

strictly defined, it may also reflect circulating neutrophils (Queiroz-Junior *et al.*, 2009).

No difference in MPO levels were found between non-treated animals in papers II and V (acute colitis).

## Histology

Microscopically, caecal samples after I/R injury (paper I) shows severe diffuse injury indicated by mucosal damage, edema and congestion in non-treated animals. Much milder changes were found in the treatment groups, with less damage found in the group supplemented with rose hip and *L. plantarum* 299v in combination.

In paper V, distal colonic samples were evaluated according to Cooper *et al.*, (1993). No histological abnormalities were found in samples from healthy animals. After DSS administration, 3 out of 5 showed the earliest histological change with shortening and loss of the basal one-third of the actual crypt and dilation and thinning of the remaining epithelium. The separation between the base of the crypt and the muscularis mucosae was described by Cooper *et al* (1993) as a thickening of the pericrypt collagen sheath and to a lesser extent, a mild diffuse increase in intercrypt collagen. The changes probably occurred through a dissolution or disappearance of the basal third of the crypt, but in the absence of any destructive inflammatory infiltrate. Of the remaining preparations, one had grade 2 lesions showing loss of the basal two thirds of the crypt, and in the last preparation, evaluated as grade 3-4, loss of the entire crypt was shown and a mild inflammatory infiltrate, primarily composed of polymorphonuclear cells, was found in the lamina propria and submucosa.

The microscopic evaluation of colonic samples from papers III and IV verified a normal mucosal architecture of the healthy animals (paper III) (Fig 5).

Histological evaluation of samples taken from animals after cyclic DSS administration (papers III and IV) showed colonic inflammation mostly confined to the mucosa and submucosa, with loss of surface epithelium, inflammatory cell infiltrations, loss of goblet cells, crypt distortion and abscesses, mucosal ulceration and erosion, and accompanying submucosal edema and the abnormalities was particularly prominent on the left side of the large intestine and transverse colon. Regenerative and hyperplastic epithelium which morphologically mostly resembled low-grade dysplasia with some sections of high grade dysplasia and polyps diagnosed as adenocarcinomas were observed (Fig 6).

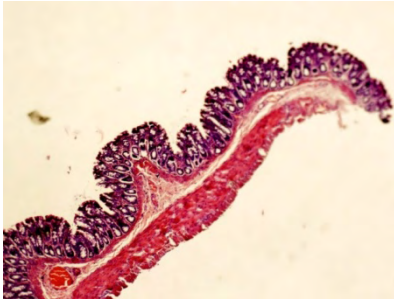


Figure 5. Normal crypt architecture and absence of inflammation seen in colonic mucosa from healthy animals (paper III).

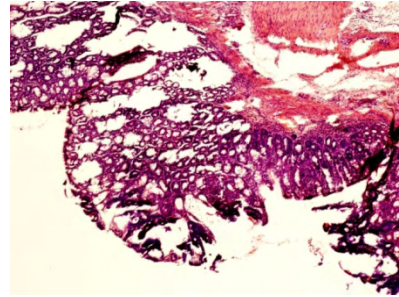


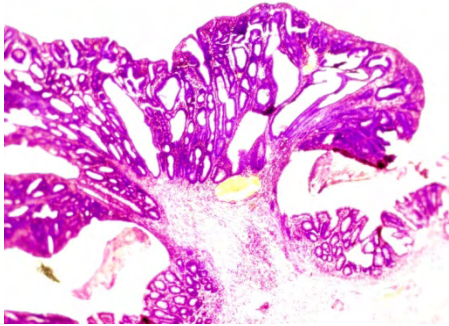
Figure 6. Colonic mucosa showing a dysplastic lesion in association with inflammation. The sample was taken from non-treated animals after cyclic DSS administration (paper III and IV).

## Tumour development

Ulcerative colitis associated dysplasia is a useful prognostic marker for subsequent cancer development (Sjöqvist *et al.*, 2004) and in colitic mucosa, the dysplasia is usually multifocal and widespread, reflecting a broader “field effect” (Kraus *et al.*, 2009 and Morson *et al.*, 1967).

In paper III, the incidence of preparations scored as low grade dysplasia was significantly higher after cyclic DSS administration, than in healthy animals and in the AOM group. Although not significant compared to the other groups, high grade dysplasia with occasional pedunculated adenocarcinomas showing fibrovascular stalks and heads containing abundant dysplastic epithelial glands (Fig 7A), was frequently found in the AOM group. In contrast to cyclic DSS administration, the polyps in the AOM group were segregated by minimal inflammation, erosion and hyperplastic epithelium, indicating that the disease process was not continuous (Fig 7B). However, examination of specific histologic scores showed no significant difference, but it must be noted that sample sites of histological evaluation were chosen and whole mucosal sections between affected sites were not objected to histological evaluation.

A



B

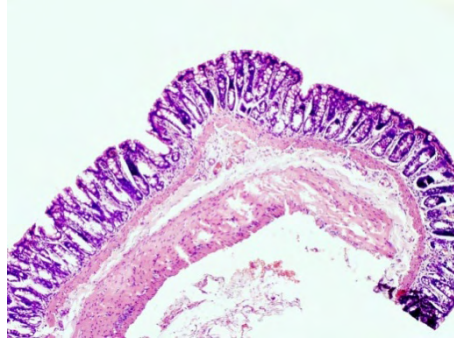


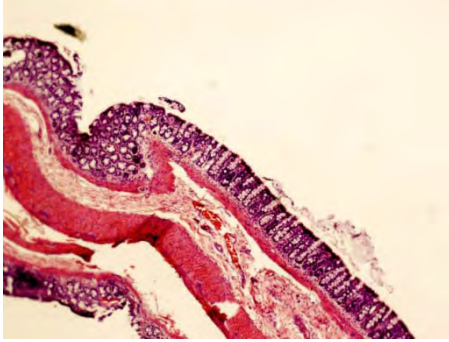
Figure 7A, B. Pedunculated adenocarcinoma with fibrovascular stalk and heads containing dysplastic epithelial glands in colonic mucosa from the AOM group (A). Colonic mucosa free from dysplastic foci in the area between the polyps in the AOM group (B) (paper III).

In paper IV, the histological changes were less severe in most treatment groups. In groups supplemented with a double portion of blueberry husks, i.e. rats getting an extra blueberry shot (representing group 2B in paper IV) alone (Fig 8A) or in combination with probiotics (Fig 8B), no sections of high-grade dysplasia and adenomatous polyps were found. None of the animals in groups supplemented with blueberry husks alone or in combination with probiotics showed gross mucosal ulceration. The bleeding per rectum was caused by small and focal erosions. The scores and the incidence of low and high grade dysplasia in the selected areas chosen for histological evaluation did not show any significant difference between the groups.

The inhibition in DAI in papers III and IV was obviously more effective than histological scores. The evaluation of DAI is more subjective than improvements seen in histology. On the contrary, the improvements in DAI signal the clinical condition without complete regeneration of crypts and complete resolution of the underlying inflammation.



A



B

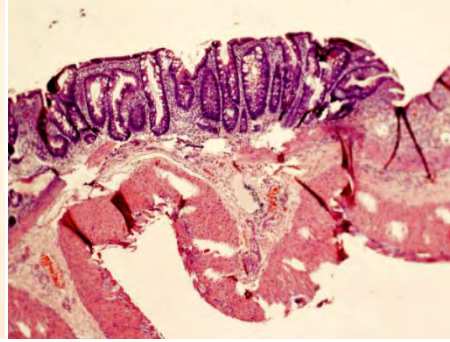


Figure 8A, B. An example of mucosa with essentially intact appearance after supplementation of blueberry husks (2B), (A). A picture chosen to illustrate signs of slight dysplasia in the epithelium after supplementation of blueberry husks (2B) + probiotics, (B) (paper IV).

### Interpretation of luminal SCFAs relative histopathological findings on the mucosa

Butyrate is the most extensively studied SCFA, and its anti-inflammatory capacity is well documented. However, both acetate and propionate have been demonstrated to have these properties, comparable to those of butyrate (Tedelind *et al.*, 2007).

#### *Acute colitis*

During acute colitis (paper II) the proportion of propionic acid in caecum increased by supplementation of blueberry husks + probiotics compared to blueberry husks alone. In a previous study by Bränning *et al.*, (2009), where healthy rats were given blueberry husks (corresponding to 120 g indigestible carbohydrate/kg diet) for 12 days, this caecal increase was also found. However, in healthy rats, an increased proportion of propionic acid and butyric acid was also found in the distal colon and faeces, respectively, compared to non-treated animals, and this pattern was not seen during acute colitis. In both studies, the non-treated animals were fed a fibre free diet.

In colitic animals (paper II), the addition of probiotics to the fiberfree diet resulted in increased proportion of butyric acid and propionic acid in the proximal colon, as well as an increased proportion of butyric acid in distal colon. This may reflect an increased production of fermentable mucus. However, these results were not seen in healthy rats. Unfortunately, no histological evaluation was performed in paper II. On the other hand, DAI have been shown to correlate well with histologic healing (Cooper *et al.*, 1993) and in paper II all treatments decreased DAI.

It may be speculated whether a healing process due to the supplements increase the utilisation of butyrate by colonocytes (Moreau *et al.*, 2004).

### *Chronic colitis and tumour development*

The capacity of stool-derived bacteria from patients with colonic adenomas to produce butyrate was found to be significantly reduced (Clausen *et al.*, 1991) and lower proportions of butyrate and higher proportions of acetate was demonstrated (Weaver *et al.*, 1988). Supplementation of an extra blueberry shot, along with cyclic administration of DSS, decreased the proportion of butyric acid and the proportion of acetic acid increased in caecum, proximal and distal colon compared with blueberry husks and non-treated animals. The same trend was seen when animals were given an extra blueberry shot+probiotics. The higher proportion of acetic acid was seen also during acute colitis (paper II) and can be explained by the comparatively high content of uronic acids in blueberry.

The utilisation of butyrate is significantly impaired during both severe colitis (Vernia *et al.*, 1995) and during hepatic cirrhosis (Onori *et al.*, 2001). In paper IV, the histological examination revealed less epithelial affection after supplementation with an extra blueberry shot alone or combined with probiotics (Fig 8A and 8B), which may explain the superior disposal of butyrate (Moreau *et al.*, 2004). In the same groups, total levels of SCFAs in caecum, proximal and distal colon were lower, which further indicate an advanced absorption by the less affected epithelium.

In non-treated animals (papers III and IV) significantly higher proportions of acetate and lower proportions of butyrate in the content of caecum, proximal and distal colon were found compared to healthy animals. Also, as in the study of Weaver *et al.* (1988), the concentration of acetate and propionate were significantly higher in the distal colon. These results demonstrate to some extent the similarities between the animal model of cyclic DSS administration and the clinical situation.

## Blueberry husks and probiotics repress carcinogenesis development

### Dextran Sulphate Sodium

Unlike colitis-associated neoplasia models using genotoxic colon carcinogenesis as initiators, for example the AOM-model, DSS itself is not a mutagen or genotoxin (Mori *et al.*, 1984). DSS administration has been shown to result in mucosal and systemic activation of macrophages and other inflammatory cells, indicating that carcinogenesis arising in these settings may solely be a manifestation of chronic inflammation (Okayasu *et al.*, 1990, Shintani *et al.*, 1997, Westbrook *et al.*, 2009).

Morphological examination demonstrated visible thickening of the colon wall after cyclic DSS administration (papers III and IV) as well as in all treatment groups, while the interpolyp sections of the AOM group mostly had a macroscopically normal-like appearance (paper III) (Fig 7B). Invaginations and dilated descending colons were seen in the AOM group, after cyclic DSS administration, (papers III and IV, respectively) and in treatment groups supplemented with blueberry husks alone or combined with probiotics (paper IV).

## Blueberry

During quantitative evaluation, the number of lesions classified as low grade dysplasia, was significantly higher after cyclic DSS administration than in healthy animals and in the AOM group (paper III) and when the numbers were matched against the different treatment groups, significant reductions were found after supplementation of an extra blueberry shot (2B) + probiotics, blueberry husks (B) + probiotics and in group 2B (paper IV). These findings may be of clinical interest since it is likely that most (if not all) colorectal carcinomas evolve through stages of increasingly severe epithelial dysplasia before becoming invasive lesions (Tanaka *et al.*, 2009).

Similar patterns as above mentioned, were found for colonic ulcers (papers III and IV, respectively) and no polyps were found after supplementation of an extra blueberry shot alone or combined with probiotics. Even though no difference was found through histological scoring of selected sections, or through analysis of myeloperoxidase activity, we hypothesize that the lower number of dysplastic lesions found in groups supplemented with blueberry and probiotics, is due to decreased mucosal inflammation. This is in accordance with findings from Cooper *et al.*, (2000) showing that after DSS administration, animals with dysplasia/cancer had a significantly higher inflammation score than those without dysplasia and or cancer. Also, Okayasu *et al.*, (1990) demonstrated that repeated administration of DSS in mice induced colorectal tumours predominantly on the left colon, where both pathology scores of DSS-induced colitis and mucosal regeneration activity were the highest. Contingently, similar results would have been achieved by assessment of sections other than the biopsies taken at chosen sampling sites (polyps or dysplastic lesions and surrounding mucosa).

## Probiotics and dietary fibres and phenolics affect the gut microbiota during inflammation

Alterations of the composition of the intestinal bacterial flora can significantly alter host immunity and the course of mucosal inflammation (Hooper *et al.*, 2001 and Madsen *et al.*, 1999). Due to LPS, *Enterobacteriaceae* triggers the release of numerous inflammatory mediators such as reactive oxygen species and is regarded as a potent exogenous inducer of inflammation. The interfering ability of specific

probiotic strains of *Lactobacillus* and *Bifidobacterium*, against common pathogens, has been demonstrated (Falagas *et al.*, 2008).

### Ischaemia/Reperfusion

Only a slight increase in *Enterobacteriaceae* count could be found after I/R injury (paper I) and it can be speculated whether the time period from ischaemia to sacrifice of the animals is not long enough to induce a detectable difference. Nevertheless, compared to animals receiving no feed supplements, only probiotics or vitamin C, a significant decreased *Enterobacteriaceae* count was found by the addition of probiotics and rose hips (paper I). Although not significant, a decrease was also found by supplementation of rose hips alone. No significant difference was found in lactobacilli counts even though probiotics was added. This may also reflect the shorter time period of injury induction, which is probably not sufficient for a detectable decrease caused by the inflammation.

### Colitis

There is considerable evidence that the components of the resident colonic microflora can play an important role in initiation of UC (Bullock *et al.*, 2004). Different members of the *Enterobacteriaceae* family and different *Clostridium* species have been found to increase in accordance with a decrease in bifidobacteria and lactobacilli (Mylonaki *et al.*, 2005 and Bullock *et al.*, 2004), leading to an imbalance between potentially beneficial and adverse bacteria. Therefore, manipulation of the mucosal microbiota to reduce the inflammatory potential of colonising bacteria might be an attractive therapy for UC.

#### *Enterobacteriaceae*

In accordance with Okayasu *et al.*, (1990), an increase in *Enterobacteriaceae* load was observed after 7 days of DSS administration and the increase was significantly higher after AOM injection followed by a single treatment with 5 % DSS compared to 4 % of DSS administration in the cyclic model (Paper III). At the end of the study, *Enterobacteriaceae* load was still increased compared to start values by cyclic DSS administration, which was not the case in the AOM group. At this point, *Enterobacteriaceae* load was significantly higher than those found in both healthy animals and in the AOM group.

From papers III and V the conclusion can be drawn that during intestinal inflammation, viable count of *Enterobacteriaceae* in faecal samples (paper III) as well as the incidence found on colonic mucosa (paper V) are increasing.

In papers II and IV, almost all feed supplements significantly reduced viable count of *Enterobacteriaceae*, except for administration of blueberry husks alone (group B in paper IV). In paper IV, this was also the only treatment group showing an increased *Enterobacteriaceae* count over time, from start to end of the study. In general, the addition of probiotics seems to further suppress *Enterobacteriaceae*.

### *Akkermansia*

In paper V, the amount of the gram-negative, mucin-degrading bacterium *Akkermansia* was increased. Mucus isolated from UC patients is thinner and less sulphated than normal (Pullan *et al.*, 1994, Corfield *et al.*, 1996), which may promote the contact between the microflora and the immune system (Bibiloni *et al.*, 2006). Although higher prevalence of sulphate-reducing bacteria has been associated with UC (Zinkevich *et al.*, 2000), the potential link to pathogenesis is not clear. Sulphate reducing bacteria reduce sulphate to sulphide, which is toxic to colonic epithelial cells (Pitcher *et al.*, 1998) and inhibits their butyrate metabolism (Roediger *et al.*, 1993). However, the sulphate-reducing capacity of *Akkermansia* is currently not known.

### *Lactobacilli*

During acute colitis, the total amount of colonic mucosal bacteria increased, coincidentally as the amount of lactobacilli decreased. Lactobacilli count in faecal samples was also decreased at the end of the study in the AOM group and after 11 cycles of DSS administration (paper III) compared to healthy animals.

When feed supplements were administered during induction of acute colitis, viable count of lactobacilli increased in the groups given rye bran, rye bran+probiotics and blueberry husks+probiotics compared to non-treated animals and the addition of probiotics to blueberry husks verified an increase compared to blueberry husks alone. Induction of chronic colitis and colorectal cancer resulted in increased faecal viable count of lactobacilli after supplementation of an extra blueberry shot (2B) + probiotics, blueberry husks (B) or blueberry husks (B) + probiotics while a decrease was found by supplementation of an extra blueberry shot alone. Increased counts were also found by the addition of probiotics to an extra blueberry shot compared to the same diet without probiotics.

Lactobacilli species were identified from caecal (paper II) and faecal samples (papers III and IV) through RAPD band pattern comparison and 16S rDNA sequencing and the results are shown in Table V and VI.

Table V. *Lactobacillus* species identified from caecal samples (paper II), (NT= non-treatment, P=probiotics, R=rye bran, RP=rye bran+probiotics, B=blueberry husks, BP=blueberry husks+probiotics). (*L.*=*Lactobacillus*).

Group	Active colitis
NT	72% <i>L. reuteri</i> and 22% <i>L. animalis</i>
P	28% <i>L. gasseri</i> and 67% <i>L. reuteri</i>
R	67% <i>L. reuteri</i> and 17% <i>L. animalis</i>
RP	67% <i>L. reuteri</i> and 22% <i>L. animalis</i>
B	91 % <i>L. animalis</i>
BP	69% <i>L. reuteri</i> and 23% <i>L. animalis</i>

Table VI. *Lactobacillus* species identified from faecal samples (paper III and IV), (DSS=cyclic DSS administration, P=probiotics, 2B=an extra blueberry shot, 2B+P=an extra blueberry shot+probiotics, B=blueberry husks, BP=blueberry husks+probiotics).

(*L.* = *Lactobacillus*; *B.* = *Bifidobacterium*).

Group	Base line	Time point after DSS cycle 1	Time point after DSS Cycle 10	Termination
Healthy animals	<i>B. animalis</i> <i>L.reuteri</i> <i>L.murinus</i>		<i>L.murinus</i> <i>L.murinus</i> <i>L.murinus</i>	
AOM	<i>L.reuteri</i> <i>L.intestinallis</i> <i>L.intestinallis</i>	<i>L.gasseri</i> <i>L.reuteri</i> <i>L.gasseri</i>		<i>L.vaginalis</i> <i>L.vaginalis</i> <i>L.vaginalis</i> <i>L.reuteri</i>
DSS	<i>L.murinus</i> <i>L.murinus</i>	<i>L.murinus</i> <i>L.murinus</i>	<i>L.murinus</i> <i>L.murinus</i>	<i>L.murinus</i> <i>L.murinus</i>
P	<i>L.murinus</i> <i>L.murinus</i> <i>L.johnsonii</i> <i>L.johnsonii</i>	<i>L.murinus</i> <i>L.johnsonii</i> <i>L.plantarum</i>	<i>L.murinus</i> <i>L.murinus</i> <i>L.reuteri</i>	<i>L.murinus</i>
2B	<i>L.murinus</i>	<i>L.murinus</i>	<i>L.reuteri</i> <i>L.reuteri</i> <i>L.murinus</i>	<i>L.murinus</i> <i>L.murinus</i> <i>L.murinus</i>
2B+P	<i>L.gasseri</i>	<i>L.plantarum</i> <i>L.plantarum</i>	<i>L.plantarum</i> <i>L.plantarum</i> <i>L.plantarum</i>	<i>L.murinus</i> <i>L.gasseri</i> <i>L.gasseri</i> <i>L.plantarum</i>
B	<i>L.murinus</i>	<i>L.reuteri</i> <i>L.murinus</i>	<i>L.murinus</i> <i>L.murinus</i> <i>L.reuteri</i> <i>L.murinus</i>	<i>L.johnsonii</i> <i>L.reuteri</i> <i>L.johnsonii</i>
B+P	<i>L.vaginalis</i> <i>L.vaginalis</i> <i>L.vaginalis</i>	<i>L.plantarum</i>	<i>L.murinus</i> <i>L.plantarum</i> <i>L.plantarum</i> <i>L.plantarum</i>	<i>L.plantarum</i>

After 7 days of DSS administration (paper II), *L. reuteri* and *L. animalis* constituted the dominant part in 4 out of 6 groups (non-treatment, rye bran, rye bran+probiotics and blueberry husks+probiotics). The composition of the lactobacilli flora was different by supplementation of probiotics only and by supplementation of blueberry husks (Table V). *L. murinus* and *L. reuteri* comprise a considerable part of the intestinal *Lactobacillus* population of rats (Hemme *et al.*, 1980 and

Molin *et al.*, 1992) and from faeces of mice, bacteria with tannase activity have been identified as *L. animalis* or *L. murinus* (i.e. *L. animalis* is relatively closely related to *L. murinus*) (Sasaki *et al.*, 2005). Since no fibre was added together with the probiotics, the fibre-free diet may not provide optimal survival and persistence conditions for *L. animalis*, while blueberry husks improved growth performance and competing ability. In paper III and IV, *L. murinus* was identified at different time points during cyclic DSS administration and in healthy rats, but not in the AOM group (paper III) (Table VI). Supplementation of probiotics gradually changed the dominance of *L. murinus* and isolates identified as *L. plantarum* and isolated from groups supplemented with probiotics, were identified as the administered strain *L. plantarum* HEAL19 (Table VI).

#### *The composition of the bacterial flora*

Rather than the presence of certain pathogens, the global composition of the intestinal microbiota have been suggested to be more relevant to the pathogenesis of intestinal inflammation (Andoh *et al.*, 2007). Faecal microbial communities between active UC patients and healthy individuals have been shown to be different and the differences were thought to be associated with the appearance of unique T-RFs, only detected in UC patients (Andoh *et al.*, 2007).

In paper V, T-RFLP analysis was used to investigate bacterial communities in colonic mucosal samples after induction of acute colitis and the incidence of T-RFs in healthy and colitic mice was calculated. 11 T-RFs of different size were detected with significantly different occurrence between the two group and 8 of them were detected only during active colitis. T-RFLP patterns were also used to calculate the Shannon-Wiener diversity index ( $H'$ ) but in contrast to previous findings (Andoh *et al.*, 2007), no significant difference between the groups was found. By the use of principal component analysis, T-RFLPs obtained from each individual were analysed, showing clearly distinct distribution of the microbial communities in healthy mice compared to the communities found in colitic animals (Fig 9).



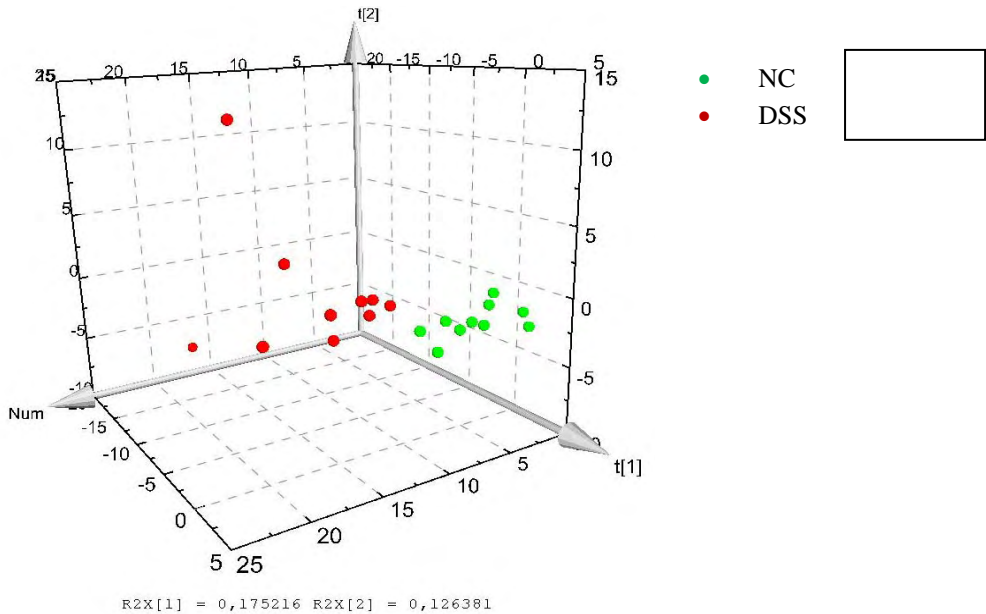


Figure 9. Principal component analysis of T-RFLP data shows distinct distribution of the microbial communities in healthy animals compared to the communities found in colitic animals (paper V).

## Probiotics decrease translocation and liver injury during colitis

Increased epithelial permeability and the inflammatory process are intimately linked and play a central role in perpetuating the chronic mucosal damage during UC (Laukoetter *et al.*, 2008). *Lactobacillus* and *Bifidobacterium* are thought to be important in the maintenance of colonisation resistance and intestinal barrier function, preventing translocation of bacteria and endotoxin from the gut to extraintestinal sites (Tuohy *et al.*, 2003 and Sullivan *et al.*, 2001). Gram-negative bacteria have been found in portal blood and liver biopsies from patients with UC (Eade *et al.*, 1969) and a part of the therapeutic strategy for UC aims at restoring the intestinal integrity (Gardiner *et al.*, 1995). After 7 days of DSS administration, resulting in an acute colitis (paper II), the incidence of translocation to the caudate lobe of the liver was evaluated and found to be significantly decreased by supplementation of probiotics and/or blueberry husks and by rye bran + probiotics.

Chronic colitis as in UC is frequently associated with pathologic findings in the liver and biliary tract, ranging from minor alterations, such as liver fatty changes,

to severe conditions, like primary sclerosing cholangitis (Bargiggia *et al.*, 2003 and Wewer *et al.*, 1991). Gut-derived components, accessible to the liver through the portal vein, are proposed as a possible pathogenic mechanism responsible for the hepatobiliary alterations (Masubuchi *et al.*, 2004). Malignant tissue may disrupt the bowel architecture and increase gut permeability during colonic cancer (Lescut *et al.*, 1990).

In papers III and IV, histological abnormalities and bacterial translocation were evaluated in the AOM group and after cyclic DSS administration to induce chronic colitis and colorectal cancer. After cyclic DSS administration, a mild to moderate degree of steatosis was found. Liver lobules had occasional focal areas with parenchymal loss, haemorrhage, and small inflammatory infiltrations in non-steatotic areas (papers III and IV). The livers were much less affected in the AOM group and only slight hepatic steatosis could be seen and no parenchymal inflammatory infiltration, or areas with parenchymal loss or haemorrhage was found (paper III). The incidence of stasis and the incidence and degree of inflammatory infiltrations in the parenchyma were significantly increased during cyclic DSS administration compared to healthy animals and compare to the AOM group. The occurrences of steatotic areas were more frequently found after cyclic DSS administration but these findings also pass for healthy animals (paper III). During intestinal inflammation, fatty infiltration of hepatocytes has been reported (Bargiggia *et al.*, 2003). The colonic mucosa was inflamed after cyclic DSS administration but no inflammation was evident in the mucosa of healthy animals and the reason for steatosis might be increased body weight.

No potential pathogenic bacteria were found in the livers of the healthy animals, even though no significant difference in the incidence of translocation was found between the groups (paper III). *C. ramosum* and *C. perfringens* were isolated after cyclic DSS administration and from the AOM group, respectively (paper III). Species of *Clostridium* have been implicated in the induction of intestinal inflammation and, according to Okayasu *et al* (1990), increasing numbers of *C. ramosum* were particularly significant after repeated administration of DSS. *C. ramosum* has also been one of the most frequently isolated anaerobes from the inflamed mucosa of UC patients and an enhanced antibody response against the bacteria has been demonstrated (Matsuda *et al.*, 2000). The translocation of *Clostridium* species may be deleterious (García-Lafuente *et al.*, 1998), evidenced by *Clostridium* bacteriemia found in patients with gastroin-testinal or haematologic malignancies (Myers *et al.*, 1992) where a gastrointestinal source of infection, particularly carcinoma of the colon or rectum or enterocolitis, was evident in most cases (Myers *et al.*, 1992).

The overall histological changes after feed supplementation were similar to those found in non-treated animals, but could be more or less severe (paper IV). The degree of parenchymal infiltration in non-steatotic area was significantly reduced by supplementation of blueberry husks and probiotics. The incidence of stasis and translocation to the liver was decreased by supplementation of blueberry

husks (B) together with probiotics. Although the incidence of translocation was not affected, supplementation with an extra blueberry shot together with probiotics resulted in no findings of *Clostridium* in the livers.

### Interpretation of SCFAs in blood relative histopathological findings in the liver

It has been speculated to which extent patients with cirrhosis are still able to metabolise portal derived SCFAs, which would otherwise lead to a rise in systemic concentrations (Bloemen *et al.*, 2009). The concentrations of propionic acid in aortic blood differed between the groups in paper III, and are mentioned in falling order: healthy rats > cyclic DSS administration > the AOM group. Of the groups in paper III, the livers of the AOM group were less affected and these animals also showed the lowest feed intake and body weight gain. Since propionate serves as a substrate for gluconeogenesis (Bloemen *et al.*, 2009), the lower levels of propionic acid may indicate a higher gluconeogenic rate due to starvation caused by occlusive colon cancer.

We hypothesise that the higher concentration of propionic acid in aortic blood, is due to the impaired capability of the liver to perform the metabolism. Our hypothesis is strengthened by a report by Minushkin *et al.*, (2003), who found that an increased concentration of propionic acid in serum of patients with chronic hepatitis of various etiology at the stage of hepatic cirrhosis with hepatic encephalopathy syndrome, correlated with the results of clinical and laboratory methods. Evaluation of propionic acid in aortic blood after feed supplementation (paper IV), revealed significantly lower concentrations by addition of probiotics. When the proportion of propionic acid in aortic blood was taken into account, lower values were found by all supplements, i.e. both probiotics and blueberry. Propionic acid levels in blood taken from the saphenous vein tended to increase in all groups at the end of the study, which strengthen the fact that the livers were affected from the colonic pathogenesis.

By feeding soluble oat fibre, the fatty acid and cholesterol synthesis in the liver are increased (Illman *et al.*, 1985). The increase in fatty acid synthesis is probably due to the increased flow of SCFAs in the liver from portal blood, and a positive correlation between plasma propionate concentrations and hepatic fatty acid synthesis has been found (Illman *et al.*, 1985). Although not previously shown for healthy animals (like in paper III), fat accumulation in the liver of hypercholesterolaemic rats fed oat fibres have been reported (Mälkki *et al.*, 1992). In the study by Illman *et al.*, (1985), no cirrhotic, necrotic or inflammatory changes were observed, so except for the steatosis, the livers were not otherwise affected (Illman *et al.*, 1985).

In paper IV, the livers were affected from chronic intestinal inflammation, probably impairing normal functions. The addition of probiotics caused a significant increase in total scoring of steatosis compared to those animals fed diets

without bacteria, but the concentration of propionic acid in the blood decreased. We speculate that the livers from groups supplemented with probiotics and blueberry have a better preserved capability of fatty acid synthesis, which may reflect the liver capacity. Of course, it is likely that the concentrations of SCFAs in portal blood are lower than normal in all groups, reflecting the affected colonic mucosa.

## Cytokine profiles and decreased systemic inflammatory response by feed supplements

### IL-12

Growing evidence supports a role for various cytokines, released by epithelial and immune cells, in the pathogenesis of colitis. IL-12 is a pleiotropic and proinflammatory cytokine that promotes cell-mediated immunity via different mechanisms (Watford *et al.*, 2004 and Dong *et al.*, 2001) and one of the major functions of IL-12 is to induce proliferation of T cells leading their differentiation to the Th1 subset (Nielsen *et al.*, 2003). The underlying mechanisms for increased expression of IL-12 in UC are unclear, but elevated levels of IL-12 mRNA expression in the mucosal tissues of UC patients have been demonstrated (Nielsen *et al.*, 2003 and Pang *et al.*, 2007).

In paper II it was shown, that serum level of IL-12 was significantly decreased by supplementation of blueberry husks. Even though only one cytokine was measured, the clinical relevance of these findings may be strengthened by the proposal, that inhibition of IL-12 or IL-17 might be future therapeutic targets in UC and by the fact that antibodies to IL-12 can abrogate experimental colitis (Neurath *et al.*, 1995).

### IL-6, IL-17 and chemokine KC

The serum cytokine profile of acute colitis was characterised by significantly increased concentrations of IL-6, IL-17 and the chemokine KC (paper V). Although not significant, increased concentrations of TNF- $\alpha$  was also found. These findings are in accordance with a previous study by Alex *et al.*, (2009). By disruption of the epithelial cell barrier, the normal mucosal microbiota may activate mucosal macrophages, which leads to the production of immunomodulatory cytokines (Kitajima *et al.*, 1999). The macrophage-induced inflammation and tissue damage is accompanied by a cellular cytotoxic-mediated inflammatory response (macrophage/Th1/Th17 chemotactic profile), along with progression of colitis (Strober *et al.*, 2002). KC has been identified as the chemokine that drives the acute DSS Th1 response (Alex *et al.*, 2009) and determines the severity of experimental colitis (Tsuchiya *et al.*, 2003). This is consistent with the findings of increased colonic MPO activity (paper V).

## Haptoglobin

Haptoglobin is an acute phase reactant that has been implicated as a useful marker of inflammation in rats (Giffen *et al.*, 2003) and it is elevated during DSS administration (Li *et al.*, 2008). In paper III, no difference was found between the three groups at the end of the experimental period. We expected a higher haptoglobin level in the heavily inflamed DSS-group and a lower level in healthy rats. From the histopathological evaluations of the livers, an infiltration of fat was obvious in the healthy rats, which may explain higher haptoglobin levels. It is also known that liver injury can result in decreased haptoglobin concentrations, i.e. both inflammation due to colitis and steatosis tended to increase the haptoglobin levels, and probably to the same degree (Gangadharan *et al.*, 2007 and Körmöczi *et al.*, 2006).

High levels of haptoglobin have been implicated in patients with colorectal cancer and development of hepatic metastases (Ward *et al.*, 1977). In paper IV, all groups exhibited a successive rise in the concentration of haptoglobin from the beginning to the end of the study. At the end, the animals given an extra blueberry shot showed significantly lower levels and a similar trend was found when the blueberry was supplemented with probiotics.

## Leptin

Leptin is regarded as a proinflammatory adipocytokine, but based on results from UC patients, it has been suggested that chronic intestinal inflammation may decrease circulating leptin values (Karmiris *et al.*, 2006) and the lowest level of leptin was found after cyclic DSS administrations, coinciding with clinical findings.

The concentration of leptin is directly correlated with body fat stores (Friedman *et al.*, 1998) and increased levels of haptoglobin have been demonstrated in obese animals (Li *et al.*, 2008). Since both leptin and haptoglobin was increased in healthy animals (paper III), this might indicate mild overweight. Visceral adipose tissue is also strongly associated with steatosis (Bahl *et al.*, 2008), which agree to the findings in this group. Low-grade asymptomatic bowel inflammation was shown in obese subjects (Poullis *et al.*, 2004), however, this was not confirmed by histological evaluation and MPO analysis in healthy animals (paper III). If the hypothesis is correct and the healthy animals are afflicted with mild overweight, this is most likely to be due to a match between age and low physical activity, rather than an effect from the feed constituents itself.

## Regulatory functions and bacterial-induced activation in mesenteric lymph nodes

Initiation and perpetuation of the inflammatory intestinal responses in UC may result from an exaggerated host defence reaction of the intestinal epithelium to

endogenous bacterial flora (Cario *et al.*, 2000). In many respects, the immune system resembles an ecosystem. It is remarkably complex and has many overlapping components capable of achieving the same ultimate functions.

In paper V, an attempt was made to evaluate various cell populations in Payer's patches (PP), mesenteric lymph nodes (MLN) and spleen, after 7 days of DSS administration to C57BL/6 mice, which are highly sensitive to DSS (te Velde *et al.*, 2006). Significantly higher numbers of cells were found in MLN after DSS administration, whereas fewer cells were found in PP, all compared to healthy animals. A primary role of macrophages in the pathogenesis of mucosal inflammation has been suggested (Rogler *et al.*, 1997 and Ishikura *et al.*, 2003). The percentage of CD11b and c double positive cells was significantly increased in mesenteric lymph nodes after DSS administration, and higher expressions of CD11b and CD11c also have been found in biopsies of patients with UC (Rogler *et al.*, 1997). This may reflect either a change in phenotype of resident cells or cellular recruitment (Rogler *et al.*, 1997).

TLR4 are expressed on the apical surface of enterocytes (Cetin *et al.*, 2004), and on peripheral blood leukocytes, macrophages, and dendritic cells (Chow *et al.*, 1999). TLR4 are important in the recognition of the Gram-negative cell-wall component LPS (Qureshi *et al.*, 1999 and Poltorak *et al.*, 1998) and LPS-mediated signalling through TLR4 leads to activation of the transcription factor NF- $\kappa$ B and the release of proinflammatory cytokines (Takeda *et al.*, 2004). DSS administration induced an increased proportion of CD11c+TLR4+ cells in MLN, which corresponds to the situation in UC patients, where an increase in TLR4 positive intestinal DCs confined to areas of inflamed tissue was demonstrated (Hart *et al.*, 2005). This may eventually lead to increased recognition of bacterial products and enhanced responses to them. TLR4 signalling may recruit regulatory T-cells to intestinal sites (Heimesaat *et al.*, 2007) and during UC, an increased frequency of CD4+CD25+FoxP3+ T-cells have been found in mucosal lymphoid tissue (Yu *et al.* 2007).

During acute colitis, the percentage of CD4+CD25+FoxP3+ expressing T-cells in MLN was increased and so was also the percentages of CD4+CD25+FoxP3+ cells co-expressing CD69 and CTLA-4. Positive correlations between CD11b+/CD11c+ cells and CD4+CD25+FoxP3+ cells with or without co-expression of CD69 and CTLA-4 indicates a potential interaction of these cells (Yu *et al.*, 2007). It has been speculated by Yu *et al.*, (2007), why the regulatory T-cells, despite increased frequency and potent suppressor activity *in vitro*, fail to control the development of colitis. It is possible that the suppressive activity is abrogated through co-stimulatory molecules or TLR signalling (Baecher-Allan *et al.*, 2004 and Pasare *et al.*, 2003). The microbial induction of the Toll pathway may block the suppressive activity of the regulatory T cells, in a process that is in part dependent on IL-6 (Pasare *et al.*, 2003). Other options are strong T-cell receptor stimuli that abrogate the suppression or render the effector T-cells resistant to suppression (Baecher-Allan *et al.*, 2002) and a high number of regulatory T-cells

could also, to some extent, limit the severity of inflammation, despite their inability to reverse it (Yu *et al.*, 2007). Increased proportions of CD4+CD25+FoxP3+ cells co-expressing CTLA-4 was found in PP and spleen. Although to a smaller extent, the results from PP and spleen confirm some affection, but our observations align with previous data suggesting a regulatory role for MLN during colitis (Spahn *et al.*, 2002).

## Interactions between the bacterial flora and the immune system

Determining whether the global composition of the intestinal microbiota rather than the presence of single pathogens are responsible for the abnormal microbe versus immune system interplay during inflammation, is not a simple process. Associations of the intestinal microbiota with the development of the immune reactions during acute colitis were evaluated by using principal component analysis (paper V). T-RFs were combined with cellular findings from the flow cytometry analysis and distinct differences between the groups appeared, indicating a relationship between the two components (Fig 10).

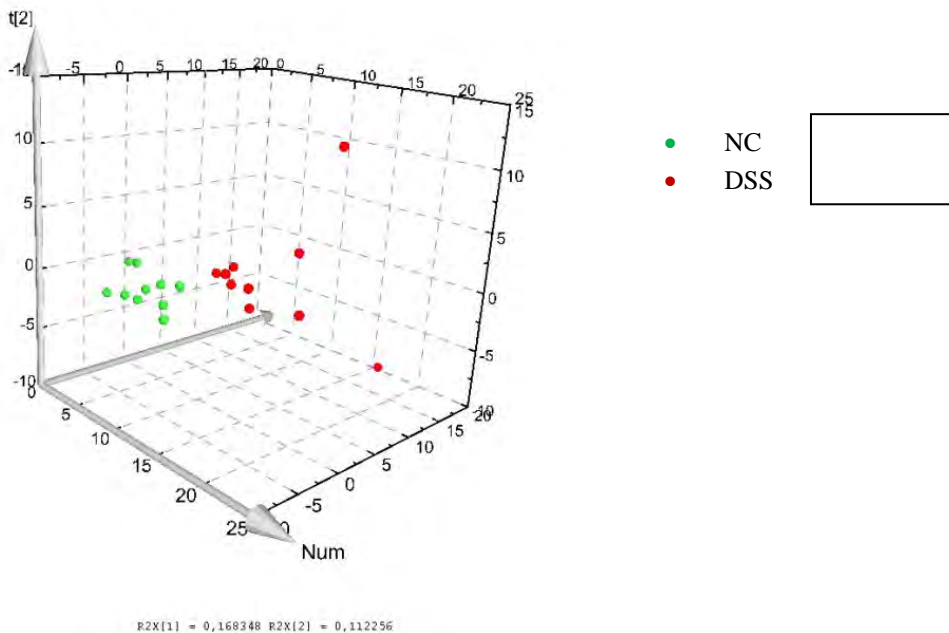


Figure 10. Principal component analysis of T-RFLP data combined with cellular findings from the flow cytometry analysis. Distinct differences between the groups indicate a relationship between the two components (Paper V).

# General conclusions and future considerations

In response to I/R injury in mice, administration of *L. plantarum* and rose hip in combination decreased lipidperoxidation in caecum independently of vitamin C content, and it also inhibited the growth of *Enterobacteriaceae*. The effect of such treatment might be more pronounced in other parts of the intestinal tract e.g. the small intestine or proximal and even distal colon as well as in humans, where lactobacilli comprise smaller proportions of the normal microbiota.

DSS-induced acute colitis primarily affects colon and rectum, which is consistent with UC. A dominant function of MLNs was indicated, but increased populations of regulatory T-cells were found in MLNs, PP and spleen and higher levels of IL-6, IL-17 and KC were found in serum. A significantly lower amount of lactobacilli was found on the colonic mucosa but the amount of *Akkermansia* was increased as was the incidence of *Enterobacteriaceae* (both taxa are gram-negative and, hence has LPS associated to the cell wall). The composition of the microflora as well as the amount of mucosal bacteria was significantly different during colonic inflammation and an increased immunological response against LPS was revealed. The short-term DSS administration did not significantly affect the liver. Unravelling the interaction of intestinal microbiota with the host and particularly the mucosal immune system will help in understanding the disease, as well as in prevention or treatment. A complex close relationship between multiple immune parameters and the composition of the colonic microflora was found at colonic inflammation. However, identification and characterisation of the causative species and a more precise investigation of the relationship need to be further elucidated.

Supplementation of probiotics, blueberry husk and rye bran in a model of acute colitis, significantly improved disease activity, bacterial translocation, inflammatory infiltration, lipid peroxidation, serum concentration of IL-12, *Enterobacteriaceae* load, while lactobacilli counts increased and most improvements were confined to blueberry husks and probiotics. Posi-



tive correlations were also found between disease activity and *Enterobacteriaceae* load and colonic neutrophil infiltration. Bacterial fermentation resulted in different carboxylic acid patterns and negative correlations were found between disease activity and total amounts of carboxylic acids in caecum, and the level of lipidperoxidation seemed to increase with lower concentrations of acetic acid and butyric acid in distal colon. Although the question of whether the alteration of intestinal microbiota in patients with UC is the cause or consequence of the disease still lacks a satisfactory answer, our data suggests the possibility of microbial re-establishment using probiotics.

Different levels of lipidperoxidation were found in acute colitis and the reason for these findings and the validity of this parameter in this model needs to be confirmed.

Two different ways of inducing chronic inflammation and colorectal tumours were investigated and cyclic DSS administration was the one with similar clinical and histopathological features of colon and liver to those found in chronic human UC and UC-associated colorectal carcinoma development. Dysplastic lesions and continuous colonic mucosal inflammation were observed along with increased *Enterobacteriaceae* load and liver dysfunctions. Also, the SCFA patterns coincide with clinical findings. This model may be a valuable tool for further investigations of the promotion of chronic inflammation, microbial imbalance and carcinogenic prevention.

Supplementation of probiotics and blueberry husk during cyclic DSS administration significantly delayed carcinogenic development as evaluated by lower numbers of dysplastic lesions. The mucosa was generally less affected after feed supplementation and this condition may be favourable for SCFA absorption. *Enterobacteriaceae* load was decreased, especially by the supplementation of probiotics, and lactobacilli count was increased. Judged by histopathological evaluation, bacterial translocation and the concentration of propionic acid in blood, supplementation of probiotics seemed to moderate liver affection and protect against damage. However, the common knowledge of correlations between concentrations of propionic acid in blood during liver dysfunctions is limited and may be an attractive field for investigation.

For some of the investigated parameters, a general issue is the similarities of effects obtained from berries alone compared to berries and probiotics in combination. The phenolic compounds in the berries have a strong antimicrobial effect that is strengthened by probiotics. Even the amount of lactobacilli normally existing in the animal seems to be affected. However,

by addition of probiotics, the lactobacilli increased. The microflora of the animals differs in composition from that of humans, and this must be taken into account when interpreting the results. Even though some of the supplemented strains may not be re-isolated, the individual composition of the intestinal microbiota may be changed. Manipulating the intestinal microbiota may be used as prevention for inflammation development or as a useful treatment alone or in combination with current medical therapy. However, further studies on mechanisms underlying the compositional alteration of intestinal microbiota are needed.



# Populärvetenskaplig sammanfattning

I tarmen lever en stor mängd bakterier som bildar ett komplicerat ekosystem och bygger upp det vi kallar den normala tarmfloran. Dessa bofasta bakterier förhindrar på olika sätt tillväxten av andra, skadliga bakterier, som bildar gifter och därmed skadar tarmslemhinnan.

Tarmslemhinnan utsätts konstant för en stor mängd främmande ämnen vilka kan framkalla en immunologisk reaktion. Det är därför inte förvånande att ca 80 procent av immunsystemet finns i anslutning till tarmen. Tarmens immunsystem är uppbyggt av lymfkörtlar, aggregat av vita blodkroppar som kallas Peyerska plack (fläckar), samt enskilda vita blodkroppar som är spridda i slemhinnan. En viktig egenskap hos tarmen är en skyddande barriär mellan tarmen och blodet. Denna barriär vidmakthålls genom att tarmväggens celler håller tätt ihop, så att utrymmet mellan dem blir så litet som möjligt och genom olika skyddsfunktioner, t.ex. genom produktion av antikroppar och ett skyddande slemlager. En annan uppgift för bakterierna i tarmen är att bryta ner de kolhydrater som inte kan absorberas i tunn-tarmen och därmed passerar till tjocktarmen. Då dessa fibrer fermenteras av bakterierna bildas kortkedjiga fettsyror, som har viktiga effekter på slemhinnans celler. Den normala tarmfloran har ytterligare en rad viktiga funktioner såsom aktivering av immunsystemet och produktion av vissa B-vitaminer och K-vitamin.

Ulcerös kolit är en kronisk inflammatorisk tarmsjukdom som varje år drabbar cirka 500 till 1000 personer i Sverige. Sjukdomen orsakar inflammationer och sår i ändtarmen och tjocktarmen. De vanligaste symtomen är diarré, ofta blandat med slem och blod. Vid mera uttalad sjukdom kan feber, illamående, viktnedgång och uttalad orkeslöshet förekomma. Ibland drabbas även andra delar av kroppen av inflammationen, såsom leder, hud och ögon, men ofta påverkas även levern. Ulcerös kolit innebär också en ökad risk för cancer i tjocktarmen och ändtarmen. Den rådande uppfattningen idag är att en samverkan mellan genetiska anlag, miljöfaktorer, den normala floran i tarmen och immunförsvaret leder till utveckling av sjukdomen.

En försämrad barriärfunktion i tarmen kan uppkomma till följd av inflammation. Detta gynnar överväxt av mer aggressiva Gram-negativa bakterier som har komponenter i sina cellväggar som kan orsaka inflammation och som brukar kallas endotoxiner. Vid en försämrad slemhinnebarriär passerar tarmbakterier och endotoxin genom tarmväggen och förs via blodet till levern. Vid högt upptag av bakte-

rier och endotoxin från tarmen överskrids leverns skyddande kapacitet och därmed kan andra organ påverkas.

En störd bakterieflora i tarmen har visat sig kunna ha betydelse för uppkomsten av sjukdomar såväl i tarmen som i övriga kroppen. Det finns nu allt starkare bevis för att skyddande probiotiska bakterier kan förebygga och bota många av dessa tillstånd. Tillförsel av probiotiska bakterier, oftast laktobaciller eller bifidobakterier, kan påtagligt sänka halterna av potentiellt sjukdomsframkallande bakterier, stimulera kroppens immunförsvar, samt stärka tarmens barriärfunktion. Prebiotiska kostfibrer stödjer probiotikans positiva effekt genom att stimulera dess tillväxt. Studier har också visat att kombination av laktobaciller och vissa frukter har en stor anti-inflammatorisk effekt, då laktobacillerna med hjälp av ett enzymssystem kan bryta ner fenolföreningar i frukt och bär och därmed bilda ämnen med dessa egenskaper. Frukter och bär är dessutom rika på antioxidanter som skyddar cellerna mot skador från fria radikaler.

Målsättningen med detta avhandlingsarbete har varit att i olika djurmodeller studera de preventiva effekterna av probiotika och bär på uppkomst av inflammation och tumörutveckling i tarmen. För att de uppnådda resultaten ska kunna värderas och omsättas i en klinisk situation, så är det högst väsentligt att det finns stora likheter mellan den mänskliga situationen och den djurmodell som används. I ett av delarbetena har därför två modeller för tumörutveckling i tjocktarmen inbördes jämförts och utvärderats.

En akut skada på tjocktarmen på grund av syrebrist i tarmväggen orsakas ofta av en störning i blodcirkulationen och den efterföljande reaktionen orsakar en akut inflammation med tillströmning av vita blodkroppar och en ökad produktion av fria radikaler. Vid tillförsel av laktobaciller och nyponpulver till djurens foder, var det möjligt att minska cellskadan och även minska mängden inflammationsdrivande bakterier, d.v.s. *Enterobacteriaceae*, i tarmen.

Ett mycket intressant område är hur sammansättningen av tarmfloran påverkar vårt immunsystem och för att studera hur sammansättningen av bakterier återspeglar den immunologiska reaktionen vid inflammation, så studerades en modell för akut inflammation, vilken har stora likheter med utvecklingen av ulcerös kolit hos människor. Olika typer av vita blodkroppar i mesenteriska lymfkörtlar, Peyerska plack och i mjälten studerades och en viss typ av vita blodkroppar i de mesenteriska lymfkörtlarna (vilka är lokaliserade närmast tjocktarmen) visade ett ökat svar gentemot inflammationsdrivande bakterier i tarmen. På tarmslemhinnan minskade mängden laktobaciller medan mängden Gram-negativa bakterier ökade. Många grupper av bakterier på slemhinnan fanns också endast vid inflammation och då resultaten från den immunologiska analysen kombinerades med analysen av tarmfloran, så återfanns ett starkt samband. Detta styrker uppfattningen om att tarmfloras sammansättning spelar stor roll vid inflammation och den immunologiska reaktionen.

Samma modell användes också för att studera de preventiva effekterna av laktobaciller, bifidobakterier, råg och blåbär vid inflammationsutveckling. Vid tillsats av

dessa supplement minskade sjukdomssymtomen, tarmens barriär förstärktes och därmed återfanns färre bakterier i levern. Koncentrationen av *Enterobacteriaceae* i tarmen minskade medan de hälsofrämjande bakterierna, laktobacillerna, ökade. Det var också möjligt att minska tillströmningen av vita blodkroppar till tarmslemhinnan och minska skadan orsakad av fria radikaler. Den allra bästa effekten återfanns då blåbär kombinerades med probiotika.

Eftersom det finns en ökad risk för utveckling av tjocktarmscancer i samband med ulcerös kolit och kronisk inflammation, så fanns det också ett stort intresse för att utvärdera den preventiva effekten i en för ändamålet lämplig modell. Två ofta förekommande djurmodeller utvärderades och jämfördes med de förändringar som ses hos människor. Ett väl överensstämmande mönster sågs hos en av modellerna beträffande en kraftigt inflammerad tarmslemhinna, många av de cellförändringar som anses vara förstadiet till cancer, de så kallade dysplasier, produktionen av kortkedjiga fettsyror, koncentrationen *Enterobacteriaceae* i tarmen och leverpåverkan. Denna modell användes för vidare studier.

Supplementering med blåbär och probiotika visade sig minska sjukdomssymtomen och den systemiska påverkan. Koncentrationen av *Enterobacteriaceae* minskade, medan mängden laktobaciller ökade. Tarmslemhinnan skyddades och färre sår och sjukliga cellförändringar återfanns. Tillsats av probiotika verkade dessutom ha en skyddande effekt på levern.

Sammanfattningsvis har detta avhandlingsarbete visat en påtagligt skyddande effekt av probiotika och bär, i synnerhet blåbär, vid inflammatoriska tillstånd i tarmen och tumörutveckling hos försöksdjur. Det är en stor förhoppning att dessa resultat ska kunna leda fram till ett komplement för behandling av inflammation i tarmen hos människor.



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