Formation of carboxylic acids in rats and humans given indigestible carbohydrates Effects of monomeric composition, solubility, molecular weight and probiotics

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Formation of carboxylic acids in rats and humans given indigestible carbohydrates

Effects of monomeric composition, solubility, molecular weight and probiotics

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Abstract

There is increasing evidence that fermentation in the large intestine is important for health, and that health-promoting effects are mediated by fermentation products such as butyric and propionic acid. As the formation of carboxylic acids (CAs) from various carbohydrates reaching the colon has been shown to vary, it is of great interest to identify food factors that could be of importance to this process. Few studies have concentrated on the effects of the physico-chemical properties of the carbohydrates and the effects of probiotics. The present work was an attempt to increase our knowledge in this area. For this purpose, a rat model was set up. A human model was also used to investigate how the colonic concentration of carboxylic acids in healthy subjects would be influenced by dietary supplementation with a β-glucan-enriched oat bran, and how the concentration varied over three consecutive days.

The degree of polymerization (DP) and the solubility of the fructo-oligosaccharides were of great importance for CA formation, while the monomeric composition was of less importance. Fructo-oligosaccharides with a low DP generated high levels of butyric acid throughout the colon, whereas those with a high DP gave high levels of propionic acid. A low solubility of the fructo-oligosaccharides was related to a lower degree of caecal fermentation, and more extensive formation of butyric acid in the distal part of colon. Lactitol and lactulose yielded high proportions of acetic acid and low proportions of butyric acid.

The probiotic bacteria affected both the pattern of CAs and the site of release in the hindgut of rats. However, the type of indigestible carbohydrate had more pronounced effects on the profiles and concentrations of CAs in the caecum than the probiotic added. In the distal part of the colon, on the other hand, the combination of pre- and probiotics was of great importance for the results. Bb-12

in combination with inulin of low solubility specifically increased the caecal pool of propionic acid in the rats. The amount of lactic acid, however, increased in the distal colon, which may have been due to the limited amount of substrate in relation to the number of probiotics in this part. Together with lactitol, Bb-12 reduced the concentration of CAs in the distal colon, indicating an accelerated absorption of CAs. The concentration of CAs was higher throughout the hindgut of rats fed pectin and Bb-12. With UCC500 and pectin, the formation of CAs shifted from the caecum to the distal part of the colon, while the combination with lactitol stimulated formation of CAs in the caecum.

Together with inulin of high solubility, none of the probiotics investigated increased the proportion of butyric acid and propionic acid at any place in the hindgut of rats. The proportion of lactic acid was generally higher, however, and similarly for succinic acid in rats fed UCC500. Rats fed GG had the lowest body weight gain and the highest caecal tissue weight. The caecal pH in rats fed GG and Bb-12 was lower than expected from the concentration of CAs, indicating that these strains reduce the formation of alkaline components e.g. ammonia.

β-glucan-enriched oat bran increased the faecal concentration of CAs in humans after 8 weeks, indicating an increased concentration in the distal colon also. No significant differences in CA concentrations could be seen for different days, which supports the adequacy of the experimental periods. An experimental design, with 20 subjects and a dietary supplementation with the test food product for 8 weeks, thus appears suitable for screening of potential differences in faecal carboxylic acid patterns.

List of Papers

Paper I **Short-chain fatty acid formation in the hindgut of rats fed oligosaccharides varying in monomeric composition, degree of polymerisation and solubility.**
Ulf Nilsson and Margareta Nyman
British Journal of Nutrition (2005), 94, 705–713

Paper II ***Bifidobacterium lactis*** **Bb-12 and** ***Lactobacillus salivarius*** **UCC500 Modify Carboxylic Acid Formation in the Hindgut of Rats Given Pectin, Inulin, and Lactitol.**
Ulf Nilsson, Margareta Nyman, Siv Ahrné, Eilbhis O Sullivan, and Gerald Fitzgerald
The Journal of Nutrition (2006), 136, 2175–2180

Paper III **Carboxylic acids in the hindgut of rats fed highly soluble inulin and** ***Bifidobacterium lactis*** **(Bb-12),** ***Lactobacillus salivarius*** **(UCC500) or** ***Lactobacillus rhamnosus*** **(GG).**
Nilsson Ulf and Nyman Margareta
Scandinavian Journal of Food and Nutrition (submitted).

Paper IV **β-glucan-enriched oat bran increases faecal concentration of carboxylic acids in healthy subjects.**
Nilsson U, Johansson M, Nilsson Å, Björck I and Nyman M
Manuscript.

The present author's contribution to the papers

Paper 1: The author, U. Nilsson, took part in the design of the study, performed the experimental work, evaluated the results and was responsible for writing the manuscript.

Paper II: The author, U. Nilsson, took part in the design of the study, performed the experimental work (except for some microbial analysis), evaluated the results and was responsible for writing the manuscript.

Paper III: The author, U. Nilsson, took part in the design of the study, performed the experimental work, evaluated the results and was responsible for writing the manuscript.

Paper IV: The author, U. Nilsson, supervised all experimental work except the dietary calculations, evaluated the results and was responsible for writing the manuscript.

Abbreviations

AACC	American Association of Cereal Chemists
AOAC	Association of Official Analytical Chemists
CA	Carboxylic acid
DP	Degree of polymerization
DW	Dry weight
GLC	Gas-liquid chromatography
SCFA	Short-chain fatty acid
UC	Ulcerative colitis
IG	Immunoglobulin
GALT	Gut-associated lymphoid tissue
NFκB	Nuclear factor-κB
NSP	Non-starch polysaccharides
OF	Fructo-oligosaccharides, DP 2–8
IN	Fructo-oligosaccharides, DP 10–60
IN-ls	Fructo-oligosaccharides with low solubility, DP 10–60
Mix OF-IN	Fructo-oligosaccharides, DP 2–8 and 10–60
Bb-12	*Bifidobacterium lactis*
UCC500	*Lactobacillus salivarius*
GG	*Lactobacillus rhamnosus*
GLP–1	Glucagon-like peptide-1
PYY	Peptide YY
IC	Indigestible carbohydrate
DSS	Dextran sulphate sodium

Contents

1. Introduction

There is mounting evidence that inflammation is a common denominator of many diseases such as cardiovascular disease, diabetes and cancer and that this may be mediated via the colon. In this respect, the composition of the colonic microbiota and the types of substrates present in the colon may be of great importance. Originally, interest was focused to a great extent on dietary fibre, i.e. indigestible plant cell wall material, but in recent years the effects of probiotics, prebiotics, and other indigestible carbohydrates have been attracting increasing attention. Also, the immune system, with its strong presence in the colon, may be affected and controlled by the colonic microbiota and the substrates available.

Food components not digested by the enzymes in the upper part of the gastro-intestinal tract will enter the colon and can act as substrates for the microbiota. Quantitatively, the most important sources of components reaching the colon are the carbohydrates, although some proteins and fats are also delivered. The microbial activity in the colon is considerable and the carbohydrates are mainly fermented to short-chain fatty acids: acetic, propionic and butyric acid. Different short-chain fatty acids have been associated with different physiological effects. Propionic acid has, for example, been suggested to play a role in lipid and glucose metabolism, and butyric acid as a moderator of colonic health. Interestingly, different types of carbohydrates give rise to different amounts and patterns of short-chain fatty acids, and in principle it would be possible to control the formation of short-chain fatty acids through the diet. A number of factors are of importance for the formation of short-chain fatty acids, such as the types of linkages between the carbohydrate monomers and also their physico-chemical properties. Another factor of importance is the composition of the microbiota. Very few studies, however, have focused on the possibility of changing the formation of short-chain fatty acids by adding probiotics to the diet. This prompted us to study the effects of some probiotics (*Bifidobacterium animalis* (Bb-

12), *Lactobacillus salivarius* (UCC500) and *Lactobacillus rhamnosus* (GG)) on carboxylic acid formation with different types of indigestible carbohydrates (pectin, lactitol, inulin of low and high solubility). Other factors studied in this thesis were the effects of monomeric composition, molecular weight and solubility of the carbohydrates. The rat was used as a model for these experiments. A human model was also evaluated. The concentrations of carboxylic acids were then determined in healthy subjects given β-glucan-enriched oat bran, a fibre source that has been shown previously to increase the faecal butyric acid concentration specifically in patients with ulcerative colitis.

2. Background

2.1 Colon function

The function of the human colon was previously thought to involve mainly absorption of water and minerals, and storage and excretion of faeces. However, in the past few decades it has become clear that the colonic microbiota make use of food components that are not digested in the upper intestinal tract, yielding energy and giving rise to metabolically active compounds such as short-chain fatty acids, which are of importance for human nutrition and health. Today, there is also increasing evidence that the colon plays a role in the prevention of many diseases (Björksten, 2006) such as ulcerative colitis, Crohn's disease, cancer and cardiovascular disease.

The colon is 1–2 m in length and contains 60–900 g of material, as shown in a study of sudden death victims from Africa and the UK (Cummings, 1990). The moisture content is higher in the caecum and proximal colon (~85%) than in the distal part (~77%). Fermentation dominates large bowel function and this affects processes associated with mineral and water absorption, pH, epithelial cell metabolism, motility, bowel habit and colonisation. Furthermore, the degradation products that are formed can be absorbed and reach the liver and peripheral tissues (Cummings, 1995). Quantitatively, the most important substrate entering the colon is the carbohydrate. When carbohydrates are degraded by the saccharolytic species in the colon, bacterial growth is stimulated (biomass), and short-chain fatty acids and the gases H_2, CO_2 and CH_4 are mainly produced.

2.2 Carboxylic acids

2.2.1 Formation

Acetic, propionic and butyric acid are the main acids formed during colonic fermentation, and these are usually referred to as short-chain fatty acids (SCFAs). They are formed in molar ratios of approximately 60:20:20 (Cummings *et al.*, 1979). Other acids and components that can be used as energy sources are ethanol and lactic, succinic, valeric and caproic acids, which are formed in small amounts (Jorgensen *et al.*, 1997), as are branched SCFAs (iso-butyric and iso-valeric acid, which amount to 2–10% of all SCFAs formed). Lactic and acid succinic acid are also formed during fermentation, and when these are included the term carboxylic acid (CA) is normally used. This will be the case throughout this thesis. Both the total amount and the pattern of CAs produced are highly dependent on the substrate that reaches the colon. However, the kinds of bacteria present in the colon may also be important for the amount and the type of CA present, and it has been shown that each genus has different fermentation end-products. This is readily seen when bacteria are grown as pure cultures (Macfarlane & Gibson, 1995). For example, *Fusobacterium* and *Clostridium* are known to produce butyric acid, while *Bacteroides* and *Propionibacterium* produce propionic acid (Hill, 1995). Acetic acid is produced by many different genera, for example *Bifidobacterium* and *Lactobacillus*. These genera also produce lactic acid (Hill, 1995). In the conditions of the colon, however, where many species co-exist, these individual differences do not always show up (Cummings, 1995). Due to the complexity of the microbiota, it has also been suggested that it is better to treat the microbiota as a single unit and to study changes in pH and CA formation instead (Rowland, 1992).

The complexity of the microbiota is also shown by the fact that lactic and succinic acid can be fermented further. Succinic acid, for example, can be converted to

propionic acid by some species of *Bacteroides* fragilis, whereas the propionibacteria can utilize lactic acid, producing propionic acid and acetic acid (Macfarlane & Cummings, 1991). Also, butyric acid may be produced from lactic acid. Thus, human faecal microbiota taken from three healthy individuals have been shown to produce butyric acid from D- and L-lactate during *in vitro* fermentation (acetyl-CoA pathway), which was not the case for ruminal microbiota (Bourriaud *et al.*, 2005). Other species isolated from a healthy infant (*Faecalibacterium pransnitzii* and *Roseburia* spp) have been shown to produce butyric acid from acetic acid (Duncan *et al.*, 2004).

The branched CAs, isobutyric and isovaleric acid, are not formed from carbohydrates but from protein and amino acids such as valine and leucine (Smith & Macfarlane, 1997). Other end-products from bacterial degradation of proteins include NH_3, phenols, indoles and amines, some of which have toxic properties (Salminen *et al.*, 1998).

The production of CAs is influenced by a number of factors, including the numbers and types of the microbiota present in the colon, the source of substrate and gut transit time (Wong *et al.*, 2006). Different types of substrates give rise to different amounts and patterns of CAs, and the monosaccharide composition and the types of linkages between the carbohydrate monomers appear to have a major influence (Mortensen *et al.*, 1988; Salvador *et al.*, 1993), (Berggren *et al.*, 1993). Pectin, containing high proportions of uronic acids, seems especially prone to give high yields of acetic acid, while guar gum (galactomannan) gives high amounts of propionic acid and β-glucans, and inulin and some types of resistant starches high amounts of butyric acid — as shown both in *in vitro* and *in vivo* studies (Englyst *et al.*, 1987; Berggren *et al.*, 1993; Roland *et al.*, 1995; Casterline *et al.*, 1997; Djouzi & Andrieux, 1997; Karppinen *et al.*, 2000). The amounts of substrate available for fermentation and the bacterial activity decrease through the colon, which is also reflected by the

decline in CA production (Savage, 1986; Macfarlane *et al.*, 1992). The total concentration of CAs in the proximal colon of humans is estimated to be between 70 and 140 mM, and in the distal part of colon between 20 and 70 mM (Topping & Clifton, 2001; Wong *et al.*, 2006). Neither total CAs nor the individual ones in the distal colon are predictive of those found proximally (Wong *et al.*, 2006).

The gut transit time is related to total faecal concentration and individual concentrations of CAs (especially butyrate). With a gut transit time of more than 50 hours, butyrate cannot be detected, probably because of colonic uptake (Topping & Clifton, 2001).

The total amounts and the profile of CAs formed as well as the place of fermentation may be important for diseases of the colon, especially in cancer and ulcerative colitis where disease often occurs distally. Thus, it is important to increase the CA production and the delivery of CAs distally, especially butyrate (Morita *et al.*, 1999; Wong *et al.*, 2006).

2.2.2 Absorption and metabolism

The primary effects of CAs are on colon function, although they are also metabolic substrates for other host tissues. The absorption of CAs in the caecum and the colon is a very efficient process, with only 5–10% being excreted in the faeces (McNeil *et al.*, 1978). The CAs formed can be used as an energy source by the host, and it has been estimated that one gram of fermented carbohydrate provides about 7 kJ (Livesey *et al.*, 1995). Two proposed mechanisms of absorption are non-ionic diffusion of protonated CAs and anionic exchange with HCO_3^- (Cook & Sellin, 1998). At least 60% of the uptake is thought to be by simple diffusion of protonated CAs (Cook & Sellin, 1998). Acetic, propionic and butyric acids are absorbed by the colonic mucosa at similar rates in different regions of the colon (Engelhardt, 1995; Fleming *et al.*, 1991) in a concentration-dependent manner (Ruppin *et al.*, 1980). The transport of

water appears to be greater in the distal than in the proximal colon (Bowling *et al.*, 1993; Wong *et al.*, 2006). Under the pH conditions normally found in the human colon, 5.5–7.5, more than 50% of CAs are present in the dissociated form. The rate of absorption within this range is not affected by pH to any great extent (Fleming *et al.*, 1991), suggesting that any regional differences in absorption are due to colonocyte metabolism and not to the local luminal environment (Topping & Clifton, 2001).

The CAs are metabolised at three major sites in the body: (1) in the colonic mucosa (mainly butyric acid), (2) in the liver cells (residual butyric acid, propionic acid and acetic acid, and (3) muscle cells (acetic acid). Valeric, hexanoic and octanoic acids have also been reported to be capable of being utilized as energy by the colonic epithelium (Jorgensen *et al.*, 1997). The CAs are metabolised to CO_2 and ketone bodies in the colonic mucosa, and their oxidation supplies 60–70% of the energy needs of isolated colonocytes (Scheppach, 1994). Butyric acid is the preferred substrate, and it is metabolised in preference to glucose and glutamine (Roediger, 1982). It has been suggested that butyric acid is oxidised more in the proximal colon than in the distal colon (Wong *et al.*, 2006).

Until recently, it was generally believed that most of the butyric acid was efficiently metabolised by the colonic epithelium. However, results from an experiment with catheterised pigs showed that increased formation of butyric acid in the colon increased the circulation level of butyric acid (Bach Knudsen *et al.*, 2003). In this connection, it should be noted that butyric acid not only affects gut cells, but has effects on a number of other cell types — including breast cells, where it influences the expression of members of the IgF-binding proteins. Thus, at high production rates of butyric acid the molecule may be distributed to cells not in direct proximity to the gut.

2.2.3 Models for studying CA formation

Studies of CA formation are mostly conducted *in vitro* using human faecal inocula, or *in vivo* using animal models. One reason for this is that the human proximal colon, where most of the fermentation takes place, is inaccessible. *In vitro* studies are cheap and easy to perform but comparisons are difficult because of the lack of methodological standardisation (Barry *et al.*, 1995). The disadvantages of such techniques are the difficulty in regulating pH and in avoiding accumulation of fermentation products. Furthermore, the presence of digestible components during fermentation in an *in vitro* system may also interfere with and modify the formation of CAs.

The rat is the most common *in vivo* model when studying CA formation, although there have also been some studies in pigs (Berggren *et al.*, 1993; Henningsson *et al.*, 2002; Henningsson *et al.*, 2003; Roland *et al.*, 1995; Brown *et al.*, 1997; Djouzi & Andrieux, 1997; Bird *et al.*, 2000; Nilsson & Nyman, 2005). All models have advantages and disadvantages. In an *in vitro* study, it was shown that rat faecal inocula gave a pattern of CAs similar to that of human inocula, while faecal inocula from pigs did not (Lupton & Villalba, 1988). This suggests that the rat is a good model, although the rat is a caecum fermenter and may practice coprophagy. Rats and man have also been shown to correlate well concerning total fermentability and bulking capacity (Nyman *et al.*, 1986). Thus, the use of a rat model is well motivated when testing different experimental parameters that may influence CA formation, such as the effects of processing conditions and microstructures. Moreover, the primary interest is not to determine the CA formation in absolute figures but rather to rank and compare substrates. This might have been difficult or even impossible in human studies. However, quantitative faecal data in humans are also needed, to characterize products intended for use in therapeutic or prophylactic trials. Studies in man have mainly been performed by measurement of CAs in faeces. They have therefore been suggested to be inadequate, since such analyses do not give any indication of the

formation of CAs in the proximal colon where most of the fermentation actually takes place. However, the fact that ulcerative colitis always affects the rectum with a variable extension in the proximal direction, and that colon cancer appears most commonly in the distal colon, supports the relevance of faecal CA measurements.

2.3 Physiological effects of CAs

2.3.1 Colonic health

Trophic effects. One of the most important properties of CAs is their trophic effect on the colonic mucosa in humans (Figure 1). Thus, studies on mucosal biopsies have shown morphometric changes (Mortensen *et al.*, 1991), and the mucosal weight of the rat hindgut increases (Tulung *et al.*, 1987) while parenteral nutrition induces atrophy of the colonic mucosa. Of the major acids, butyric acid seems to be the most effective and acetic acid the least (Clausen & Mortensen, 1994). Butyric acid is metabolised by the colonocytes to a great extent (70–90%) and is the preferred energy substrate over glucose or glutamine transported by the blood (Fleming & Floch, 1986, Salminen, 1998 #110). A high production of butyric acid has been suggested to be associated with a decreased risk of colonic diseases, suggesting that the mucosal defence barrier is maintained to a greater extent, preventing influx of pathological organisms, toxic components and proinflammatory factors (Bengmark & Jeppsson, 1995; Salminen *et al.*, 1998). The trophic properties of CAs may also be of interest for patients receiving enteral or parental nutrition.

Another interesting finding is that infusion of CAs into the hindgut of rats leads to trophic effects in the small intestine (Sakata, 1987; Frankel *et al.*, 1994). Thus, when CAs were infused into the caecum of innervated rats, there was an increase in jejunal DNA, villous height, surface area, crypt depth and gastrin without any change in colonic variables, whereas this could not be seen in denervated rats. The mechanisms

behind this are not fully understood. The motility of the proximal parts of the small intestine has also been shown to be affected by CAs (Cherbut *et al.*, 1997).

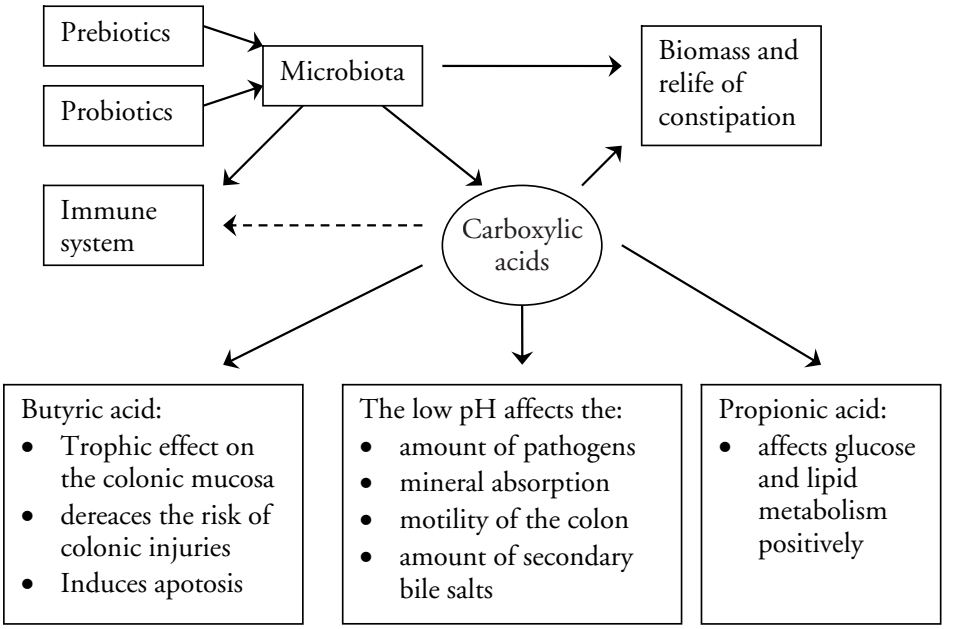

Figure 1. Physiological effects of CAs.

Colon cancer. Human colorectal cancer is unevenly distributed throughout the world (Burkitt, 1971). It is amongst the three most common malignancies in industrialised countries, and the survival rate has improved little during the past few decades, being in the order of 40% at 5 years. In high-risk countries, the most common locations of colorectal cancer are the left colon and the rectum, whereas right colon cancers are proportionally more common in low-risk areas such as Japan. About 5% of colorectal cancers are truly genetic diseases, with autosomal dominant transmission, but the majority of colorectal cancers are sporadic and are mostly influenced by environmental factors, particularly diet, with a possible interaction between genetic background and diet (Boutron *et al.*, 1996; Salminen *et al.*, 1998).

Although butyric acid is the preferred energy substrate and stimulates cell proliferation in normal colonocytes in a dose-dependent way (Sakata, 1987), it suppresses proliferation of colon adenocarcinoma cells. This apparent inconsistency has been termed the "butyrate paradox" (Lupton, 2004; Scheppach & Weiler, 2004). Sodium butyrate has been shown to have an anti-proliferative activity against many cell types, and studies on animals and in cell lines have shown that butyrate has preventative effects on colon cancer and adenoma development (Bornet *et al.*, 2002). A possible mechanism behind this phenomenon may be that butyric acid affects histone acetylase, an enzyme that is involved in the DNA repair system (Cummings & Macfarlane, 1991). Growth and doubling time of cancer cells isolated from the colon is suppressed by butyrate, and to some extent also by propionate (Whitehead *et al.*, 1986; Gamet *et al.*, 1992). Moreover, butyrate at physiological concentrations (2–4 mmol/l) may induce apoptosis (programmed cell death) in colon cancer cell lines (Hague *et al.*, 1993). Acetic acid and propionic acid have also been shown to induce apoptosis in colorectal tumour cell lines, but to a much lesser extent than butyric acid (Hague *et al.*, 1995; Scheppach *et al.*, 1995). Butyric acid may also stimulate immunogenicity of cancer cells (Perrin *et al.*, 1994). All this is interesting, as carbohydrates giving rise to high amounts of CAs — especially butyric acid — may contribute to a reduced risk of cancer.

From animal studies, there are evidence that fructo-oligosaccharides, which yields high proportions of butyric acid, may reduce the risk of colon cancer (Pool-Zobel *et al.*, 2002). An increased number of Bifidobacteria, or increased formation of CAs — especially butyric acid — have been suggested to be protective. In one study on transgenic mice, inulin was shown to stimulate intestinal tumour formation (Pajari *et al.*, 2003), but this has not been verified by others (Pierre *et al.*, 1997; Kaur & Gupta, 2002).

Inflammatory bowel diseases. Two main types of inflammatory bowel disease (IBD) exist: Crohn's disease and ulcerative colitis (UC). Patients having these diseases suffer from chronic diarrhoea and weight loss, abdominal pain, fever and fatigue. Extra-intestinal manifestation can also occur, including skin ulcers, arthritis and bile-duct inflammation, the latter being especially common in UC. Both UC and Crohn's disease are characterised by mucosal ulceration, which is patchy in Crohn's disease but continuous in UC. Crohn's disease may affect any part of the gut from mouth to anus, while UC appears only in the colon, preferentially in the distal parts. Inflammatory bowel diseases involve genetic predisposition; however, other factors such as the composition of the microbiota and the diet may also have an effect (Macdonald & Monteleone, 2005).

CAs have also been considered in relation to inflammatory bowel diseases, especially UC. A defect in butyrate metabolism has been identified in UC patients, and may be induced by sulphuric compounds generated in the colon (Pitcher & Cummings, 1996). Roediger demonstrated that colonocytes of individuals with active and quiescent ulcerative colitis have less butyrate oxidation than controls (Roediger, 1980). Enemas with CAs, especially butyrate, have also been used as a possible treatment for diversion and ulcerative colitis, but the results were not consistent (Wong *et al.*, 2006). Furthermore, patients with UC have been shown to have low faecal concentrations of acetic, propionic and butyric acids and very high concentrations of lactic acid (Vernia *et al.*, 1988). In a recent study in humans, it was shown that patients with ulcerative colitis eating a diet containing high amounts of a β-glucan-enriched oat bran underwent a specific increase in the concentration of butyric acid in the faeces. Unlike the controls, the patients showed no increase in complaints during the trial. Ingestion of suitable fermentable carbohydrates may thus be another way to increase the colonic concentration of butyric acid, and possibly prevent UC. Interestingly, germinated foodstuffs — also containing high amounts of β-glucans and known to give rise to high amounts of butyric acid in rats (Berggren *et*

al., 1993) — have been shown to reduce the epithelial inflammatory response, both in humans and in a mouse model (Kanauchi *et al.*, 2001; Kanauchi *et al.*, 2003a; Kanauchi *et al.*, 2003b). The beneficial effects of butyric acid-producing substrates seen in UC patients with a very sensitive mucosa indicate that these substrates may have preventive effects. Oral butyrate administered in enteric-coated tablets has been shown to be effective in the treatment of patients with mildly and moderately active Crohn's disease (Di Sabatino *et al.*, 2005).

Diarrhoea. Globally, acute diarrhoea is responsible for 3–4 million deaths annually, many of which are in children, and accounts for 20–30% of all childhood mortality (Salminen *et al.*, 1998).The principal pathogens are *Escherichia coli*, *Campylobacter* spp and *Salmonella* spp. A high production of CAs may also protect against different types of diarrhoea. Ramakrishna & Mathan (1993) showed that the sodium and water absorption was restored in patients with acute diarrhoea when CAs were administrated rectally. These data indicate that a reduction in colonic CAs may be a factor contributing to abnormal fluid absorption and colonic dysfunction.

Constipation. Constipation is a colonic disorder defined in terms of defaecation problems, and relates to bowel frequency. There are many reasons for constipation, particularly diets with low dietary fibre. The principal treatment is to increase the amount of dietary fibre in the diet, carbohydrates that are resistant to fermentation such as those from whole-grain cereals. CAs may also contribute to the bulking capacity through stimulating an increase in bacterial mass during fermentation.

Other effects in colon. A high production of CAs *per se* may also be of nutritional interest, as these acids affect colonic and intracellular pH, cell volume and functions associated with ion transport (Cook & Sellin, 1998). The lower pH induced by an increase in CAs also indirectly influences the composition of the colonic microbiota, for example by reducing potentially pathogenic clostridia. Furthermore, absorption of

ammonia is reduced by the protonic dissociation of ammonia and other amines (Vince *et al.*, 1978; Jenkins *et al.*, 1987; Wong *et al.*, 2006). As a result, there will be less ammonia in the circulation, which may be important for patients who have problems with proper detoxification of ammonia in the liver (hepatic encephalopathy). These types of patients have also been shown to be successfully treated with lactulose (Dhiman *et al.*, 2000) and lactitol (Clausen & Mortensen, 1997), carbohydrates that are highly fermented in the colon and which give rise to high amounts of CAs. A low pH also reduces the solubility of free bile acids (Grubben *et al.*, 2001). This is an important factor, as only the soluble bile acids come into contact with the colonic mucosa, causing mucosal damage (van Munster *et al.*, 1994). Also, the activity of a bacterial enzyme that degrades primary bile acids to secondary bile acids is inhibited by low pH (Thornton, 1981). Secondary bile acids have been suggested to promote tumour activity, as co-carcinogens. A low pH in itself has also been suggested to be protective against colon cancer (Cummings & Branch, 1992), and a high production of CAs has been shown to increase colonic blood flow and oxygen uptake in dogs (Kvietys & Granger, 1981).

2.3.2 Lipid and glucose metabolism

Lipid metabolism. Of the CAs formed, it is mainly propionic acid that has been proposed to have positive effects on lipid metabolism, while acetic acid — being the primary substrate for cholesterol synthesis via acetyl-CoA in the liver — may have hypercholesterolaemic effects. A number of studies have indicated that propionic acid may have an inhibitory effect on hepatic cholesterol synthesis (Bush & Milligan, 1971; Chen *et al.*, 1984; Wright *et al.*, 1990; Hara *et al.*, 1998; Hara *et al.*, 1999). There is also evidence that it would be advantageous to have a high propionic acid to acetic acid ratio (Wolever *et al.*, 1991; Luo *et al.*, 1996). Studies in humans, however, have not been conclusive. Subjects given rectal infusions of acetate and propionate separately or in combination showed a dose-dependent increase in total serum cholesterol levels with acetate, which was reduced by propionate (Wolever *et al.*,

1989; Wolever *et al.*, 1991). It has, however, been claimed that non-physiological levels of acetyl-CoA were formed in these studies, which may have diverted CAs to lipid synthesis rather than oxidation (Royall *et al.*, 1990). In another study, propionic acid was shown to reduce serum lipid levels by the inhibition of acetic acid incorporation into triacylglycerols (Wolever *et al.*, 1995). Rectally administered propionate reduced the total amount of cholesterol in the liver (Berggren, 1996) in rats, but not in pigs (Bach Knudsen & Canibe, 1993). An inhibition of cholesterol synthesis from acetic acid has also been seen when liver cells were cultured with propionic acid (Demigne *et al.*, 1995). Interestingly, recent studies have also shown that butyric acid may have an effect on lipid metabolism (Marcil *et al.*, 2002). Thus, in the presence of oleic acid, butyrate reduced the production of triglycerides and phospholipids in Caco-2 cells. These results suggest that substrates that lead to high amounts of propionic acid and butyric acid by fermentation may inhibit cholesterol synthesis.

Several mechanisms behind the positive effects of propionic acid on lipid metabolism have been suggested. Observations in animals have shown that propionic acid inhibits cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA synthase and 3-hydroxy-3- methylglutaryl-CoA reductase (Bush & Milligan, 1971; Rodwell *et al.*, 1976). Other possibilities are increased activity of cholesterol 7α-hydrolase in the presence of propionic acid, which may result in increased synthesis and excretion of bile acids (Imaizumi *et al.*, 1992). Similar effects have been seen with butyric acid (Marcil *et al.*, 2003).

Fructo-oligosaccharides, which are known to give rise to high amounts of CAs during colonic fermentation, have been shown to reduce cholesterol levels in humans and rats in several studies, reviewed by Delzenne & Williams (2002). Furthermore, in other studies it has been shown that triacylglycerol, total and LDL-cholesterol in serum were lowered by fructo-oligosaccharides, reviewed by Williams & Jackson

(2002). Lactulose, another oligosaccharide that gives rise to high amounts of CAs by fermentation, has been shown to cause increased cholesterol levels (Jenkins *et al.*, 1991). Interestingly, fructo-oligosaccharides yield high amounts of propionic and/or butyric acid, while lactulose forms high amounts of acetic acid (Femia *et al.*, 2002; Nilsson & Nyman, 2005; Pool-Zobel, 2005). Easily-fermentable types of dietary fibre also have an effect on lipid metabolism. Oat fibre, for example, was found to reduce total and LDL-cholesterol in several studies (Anderson *et al.*, 1991; Braaten *et al.*, 1994a; Braaten *et al.*, 1994b; Biörklund *et al.*, 2005). The mechanism behind these effects may, however, be related to the high intake of soluble and viscous fibre, and not to the formation of propionic and butyric acid during fermentation (Anderson & Hanna, 1999; Kerckhoffs *et al.*, 2002).

Glucose metabolism. Propionic acid and acetic acid have also been suggested to have effects on glucose metabolism. Acetic acid stimulates gluconeogenesis, but the effects of propionic acid are difficult to interpret since this acid both inhibits and stimulates gluconeogenesis (Venter *et al.*, 1990). Thus, propionic acid is metabolised to succinyl-CoA, which can be converted to oxaloacetate, and in this way stimulate the gluconeogenesis. However, propionic acid may also reduce the citrate concentration — stimulating glycolysis and inhibiting gluconeogenesis. Propionic acid has also been suggested to affect plasma fatty acid concentrations, which may in turn affect hepatic glucose metabolism (Wong *et al.*, 2006). There have been very few studies on acetic/propionic acid formed by colonic fermentation in relation to glucose metabolism. However, in a study by Thornburn *et al.* (1993) the hepatic glucose release was shown to be reduced by the CAs formed during carbohydrate fermentation, while the glucose tolerance increased in healthy subjects after an overnight fast. Furthermore, barley products served as an evening meal and capable of promoting colonic fermentation, improved glucose tolerance the following morning (Nilsson *et al.*, 2006a).

Glucoincretins. Several hormones formed in the endocrine system of the gut have been identified to regulate appetite, which may lead to new approaches in the treatment of obesity and the metabolic syndrome. Ghrelin stimulates appetite, while the hormones glucagon-like peptide-1 (GLP-1), oxyntomodulin, peptide YY (PYY), cholecystokinin and pancreatic polypeptide inhibit appetite (Small & Bloom, 2004). GLP-1 is also involved in insulin secretion, and is therefore associated with type-2 diabetes (Delzenne *et al.*, 2005). Also, GLP-1 and PYY are involved in the control of gastric emptying. The effect on gastric emptying can be interpreted as being part of the "ileal break mechanism", an endocrine feedback loop that is activated by nutrients in the ileum (Beglinger & Degen, 2006). CAs formed in the colon may be a part of this phenomenon, since rectal infusion of CAs has been shown to delay gastric emptying (Ropert *et al.*, 1996). It has also been shown *in vitro* that butyric acid increases PYY and a proglucagon to GLP-1 (Drucker, 2003; Zhou *et al.*, 2006). Several studies have demonstrated that fructo-oligosaccharides increase the production of GLP-1, PPY and ghrelin, and therefore may have effects on satiety and glucose metabolism (Kok *et al.*, 1998; Cani *et al.*, 2004; Cani *et al.*, 2005; Gee & Johnson, 2005). One possible mechanism behind this could be the CAs formed during fermentation. In a study on rats fed 10% fructo-oligosaccharides, the postprandial insulin levels were reduced by 30%; triglyceride and glucose levels were also decreased, while GLP-1 increased (Kok *et al.*, 1998). Lactitol has been reported to significantly increase PYY response and reduce weight gain in growing rats (Gee & Johnson, 2005).

2.3.3 Mineral absorption

As early as the 1970s, an increased absorption of calcium in humans was seen as a result of pectin, which is known to give high amounts of CAs by fermentation (James *et al.*, 1978). Later studies have also demonstrated that other easily-fermentable carbohydrates such as lactulose, fructo-oligosaccharides, galacto-oligosaccharides and sugar alcohols increase mineral absorption in rats (Brommage *et al.*, 1993; Delzenne

et al., 1995; Ohta *et al.*, 1998; Chonan & Watanuki, 1996; Mineo *et al.*, 2002; Zafar *et al.*, 2004). One probable mechanism responsible for this increase is the solubilisation of calcium salts by CAs produced through the microbial fermentation (Levrat *et al.*, 1991; Younes *et al.*, 1996). The reduced colonic pH may also increase the availability of calcium for binding to free bile acids and fatty acids (Wargovich *et al.*, 1984). Another possible mechanism is the enhancement of paracellular calcium transport by direct stimulation of the intestinal epithelium. Fructo-oligosaccharides and raffinose have been shown to increase net calcium absorption in human intestinal Caco-2 cell monolayers by increasing paracellular transport through the physiological control of tight junctions (Suzuki & Hara, 2004; Tokunaga, 2004; Ducros *et al.*, 2005).

2.3.4 Immune system

The largest immune organ in the body is the gastro-intestinal tract, in a system called the gut-associated lymphoid tissue (GALT). It contains 80% of all antibody-producing cells in the body, and produces more antibodies than any other part of the body (Ouwehand *et al.*, 2002a). The major functions of the intestinal immune system are exclusion of antigens and providing tolerance to selected antigens, since all food components and the normal intestinal microbiota are in principle antigens. Many studies have provided evidence that probiotics may modulate the intestinal immune system in some way (Salminen *et al.*, 1998; Gill & Rutherfurd, 2001; Shu & Gill, 2002).The main type of antibody in the intestine is immunoglobulin (Ig) A, which — in contrast to IgG — does not elicit an inflammatory reaction. IgA can thus bind antigens and exclude them from the intestinal mucosa without causing inflammation. The main function of IgA is to prevent the attachment of intestinal pathogens, while IgE is responsible for most allergic reactions (Kagnoff, 1993).

Although less well investigated than probiotics, carbohydrates that are highly fermented by the colonic microbiota have also been shown to affect the immune

system. Thus, pectin, guar gum and lactulose have been found to increase the secretion of IgA into the gut lumen (Lim *et al.*, 1997; Kudoh *et al.*, 1999; Yamada *et al.*, 2003). In addition, polydextrose and lactitol have been shown to increase IgA production. The effect was more pronounced when the two carbohydrates were combined (Peuranen *et al.*, 2004). Other fermentable carbohydrates (pectin and chitosan) seem to reduce IgE production (Lim *et al.*, 1997). The low intake of dietary fibre in western countries might therefore play a role in the high prevalence of hypersensitivity. Fermentable carbohydrates seem to have the opposite effect on IgA and IgE compared to bile acids, suggesting that bile acids are also involved in the mechanism (Lim *et al.*, 1994; Lim *et al.*, 1997).

High levels of glutamine in the circulation have been reported to improve the immune function (Wu *et al.*, 1991; Karinch *et al.*, 2001) and reduce the risk of sepsis (Andrews & Griffiths, 2002). This is interesting, as fermentable carbohydrates giving high amounts of butyric acid reduce the utilization of glutamine by the colonocytes, and in that way the level of glutamine in the circulation may increase (Jenkins *et al.*, 1999). Interestingly, administration of lactulose has been shown to increase serum glutamine levels (Schley & Field, 2002).

It is possible that colonic fermentation of carbohydrates to CAs is an important factor in modification of the immune system. A number of studies support direct or indirect immunomodulatory properties of CAs (Pratt *et al.*, 1996; Bohmig *et al.*, 1997a; Schley & Field, 2002). Thus, supplementing rats with CAs by total parenteral nutrition increased the natural killer cell activity (Pratt *et al.*, 1996) and butyrate was shown to down-regulate the stimulatory function of peripheral blood-derived antigen-presenting cells, which is a potential mechanism for modulation of T-cell responses (Bohmig *et al.*, 1997b). Butyric acid has been reported to suppress both constitutive and cytokine-induced expression of the nuclear factor κB (NFκB) in the colonic cell line HT-29 (Inan *et al.*, 2000). Pharmacological doses of acetic acid administered

intravenously to both healthy subjects and cancer patients increased peripheral blood antibody production, natural killer cell activity and the allogeneic mixed lymphocyte reaction (Ishizaka *et al.*, 1993). Whether these effects occur at concentrations seen after high intake of fermentable fibres is not known.

2.4 Microbiota

In an adult human the gastrointestinal tract is colonised by over 10^{14} microorganisms, weighing over 1 kg and corresponding to more than 10 times the total number of cells in the body (Björksten, 2006). However, at birth the gastrointestinal tract of the newborn is sterile. The development of the microbiota is individual and several factors such as genetic factors, contact with the surrounding environment, diet and disease have a great influence on the outcome. Thus, the microbiota is unique for every individual and even homozygotic twins differ in the composition of their microbiota (Isolauri *et al.*, 2004). Over 400 species have been identified, but some 30–40 species belonging to 5–6 genera account for 99% of the biomass (Cummings, 1995). However, as many as 1,000 species has been suggested to exist, since not all anaerobic organisms can be cultured (Ouwehand *et al.*, 2002a). Most of the species are saccharolytic, i.e. they use carbohydrates as energy source; however, some can also ferment proteins e.g. Clostridia.

2.4.1 Development

The microbial colonisation starts directly after birth, and bacteria start to appear in the faeces within hours after birth. The initial sources of bacteria are the surrounding environment and the mother's birth canal (Ouwehand *et al.*, 2002a). Thus, it has been shown that babies delivered with caesarean section have a different microbiota, and it can take months before the microbiota is similar to the ones delivered vaginally, if ever (Grönlund *et al.*, 1999). How the infants are fed is also under debate—

whether it effects the colonisation or not. Breast-fed infants are traditionally thought to be mainly colonised by bifidobacteria, while formula-fed infants have a more mixed microbiota which is not so high in bifidobacteria. However, by inclusion of fructo- and galacto-oligosaccharides in infant formulae, the number of bifidobacteria has been shown to increase to a similar level to that in breast-fed infants (Rinne *et al.*, 2005b). By the age of two years, the microbiota is very similar to that of an adult (Ouwehand *et al.*, 2002a). The intestinal microbiota has been reported to be fairly stable during adult life, which could be due to similar dietary habits throughout life. However, some major changes can take place again in old age. In most cases, the levels and the number of species of *Bifidobacterium* become reduced (Hopkins & Macfarlane, 2002).

Studies to date suggest that the individual composition of the microbiota is very stable, but great differences occur between individuals. If the microbiota is altered in some way, either through feeding of pro- or prebiotics or through other circumstances such as intake of antibiotics, the microbiota will act to restore the *status quo*. The regulatory mechanisms are not known in detail but probably involve competition, antagonism, parasitism and predation (Lim *et al.*, 2005). Competition is most probably the major regulatory mechanism and organisms that are in competition cannot coexist (Kassen & Rainey, 2004).

2.4.2 Composition

The composition of the microbiota in the human gastro-intestinal tract varies in different parts of it (Table 1). In the oesophagus there is normally no residential microbiota; the bacteria present here originate from the food or from the mouth. The saliva can contain 10^8 colony-forming units (CFU)/ml and dental plaque up to 10^{11}

Table 1. The numerically dominant microbial genera in the adult human gastrointestinal tract (Isolauri *et al.*, 2004)

Oesophagus	Stomach	Duodenum	Jejunum	Ileum	Colon
-	10^4 CFU/g	10^3–10^4 CFU/g	10^5–10^7 CFU/g	10^7–10^8 CFU/g	10^{10}–10^{11} CFU/g
No microbiota of its own	*Candida albicans* *Helicobacter pylori* *Lactobacillus* *Streptococcus*	*Bacteroides* *Candida albicans* *Lactobacillus* *Streptococcus*	*Bacteroides* *Candida albicans* *Lactobacillus* *Streptococcus*	*Bacteroides* *Entero-bacteriaceae* *Clostridium* *Enterococcus* *Lactobacillus* *Veillonella*	*Bacteroides* *Bacillus* *Bifidobacterium* *Clostridium* *Enterococcus* *Eubacteria* *Fusobacterium* *Peptostreptococus* *Ruminococcus* *Streptococcus*

CFU/g. Due to the low pH in the stomach, the CFU/ml in gastric juice is at most 10^4. The microorganisms found are usually lactobacilli and streptococci. Many people are also colonised by *Helicobacter pylori*. The low pH affects the microbiota in the duodenum also; here, the CFU counts are still low and the composition of the microbiota is similar to that in the stomach. In the jejunum, counts of 10^7 CFU/ml have been measured, and lactobacilli, streptococci, *Bacteroides* and *Haemophilus* are part of the normal flora. In the ileum with its slower passage, the microbiota starts to resemble that of the colon and levels of 10^8 CFU/ml can be reached in the distal part. *Enterobacteriaceae, Bacteroides*, *Veillonella*, *Clostridium*, lactobacillus and enterococci are the major organisms found here. Of the different parts of the gastrointestinal

tract, the colon has by far the highest diversity and about 10^{10}–10^{11} microorganisms are present per gram of colonic contents. *Bacteroides*, *Bifidobacterium*, *Clostridium, Eubacterium*, and *Bacillus* are the major genera in the colon (Ouwehand *et al.*, 2002a).

2.5 Colonic substrates

The principal role of the intestinal microbiota is to salvage energy from carbohydrates not digested in the upper gut, through fermentation. Fermentation involves a variety of reactions and metabolic processes in the anaerobic microbial breakdown of organic matter, yielding metabolisable energy for microbial growth and maintenance and other metabolic end-products for use by the host (Macfarlane & Gibson, 1995). Total substrate availability in the human adult colon is 20–60 g carbohydrate and 5–20 g protein per day (Cummings & Englyst, 1987; Cummings *et al.*, 1989; Langkilde *et al.*, 1990). It has been suggested that at least 30 g carbohydrate per day is required to maintain the microbiota (Salyers, 1983). Carbohydrates entering colon are usually referred to as indigestible carbohydrates (IC). The most well-known source of IC is dietary fibre. Dietary fibre was originally defined by Trowell as indigestible plant cell wall material (Trowell, 1972). As this definition also includes indigestible components other than carbohydrates (such as protein, waxes and cutin), dietary fibre was redefined in 1976 to include only indigestible polysaccharides and lignin (Trowell, 1976). In this definition other indigestible polysaccharides than from cell walls are included, such as food additives. Some workers wants to limit the the definition to include only non-starch polysaccharides (NSP) of the cell walls (Englyst *et al.*, 1992b). However, today we know that certain oligosaccharides and some of the starch in our common foods reaches the colon (resistant starch). Consequently the Association of Official Analytical Chemists International (AOAC) and the American Association of Cereal Chemists (AACC) have now defined dietary fibre to include all oligosaccharides, resistant starch and lignin, that have physiological effects (Prosky,

2001). This means that fructo-oligosaccharides, lactitol and lactulose will also be included in this definition. The EU has not adopted any definition yet, although different suggestions are under consideration. In this thesis dietary fibre is defined as it was done by Trowell 1976 — as indigestible polysaccharides and lignin — for analytical reasons. Currently used dietary fibre methodologies use ethanol to precipitate dietary fibre, and in these analyses only carbohydrates with a degree of polymerisation (DP) of around 10 or more are precipitated (Johansson, 1987). Other carbohydrates reaching the colon, such as resistant starch and oligosaccharides, are included in the term IC. Examples of oligosaccharides are lactitol, lactulose and fructo-oligosaccharides.

The indigestible carbohydrates provide the principal substrates for bacterial growth in the colon. The amount of carbohydrates reaching the human colon varies considerably with diet, but is around 20 g per day for a normal western diet, while it increases to about 60 g for a vegetarian diet. The recommended intake is 3 g/MJ, which corresponds to a fibre intake of between 25 and 35 g (NNR, 2004). The estimated intake of resistant starch is 3–8 g per day (Brighenti *et al.*, 1998; Liljeberg Elmstahl, 2002)

Significant regional differences occur in bacterial activity in the colon. The right (proximal) colon is characterised by a high substrate availability (due to dietary input), low pH (due to CAs produced during fermentation) and rapid transit. The left, or distal, colon has a lower concentration of available substrate, the pH is approximately neutral and the bacterial activity lower. The proximal region tends to be a more saccharolytic environment than the distal gut, the latter having higher bacterial proteolysis (Salminen *et al.*, 1998). However, CAs measured in gut contents taken from victims of sudden death have shown that molar ratios of acetic, propionic and butyric acids are very similar in the proximal and distal colon. This does not mean that fermentation reactions are uniform throughout these regions of the gut,

but reflects the relative absorptive capability of the gut mucosa in different segments of the colon (Macfarlane & Gibson, 1995).

2.5.1 Dietary fibre

Dietary fibre covers a large variety of NSP. The classification of NSP was based originally on the methodology used for extraction and isolation of polysaccharides. The complexity of the structure has made it difficult to achieve a clear-cut classification of NSP, but NSP usually falls into three main groups—namely cellulosic, hemicellulosic and pectic components (Cummings *et al.*, 1997).

Cellulose is the most abundant organic compound in nature, comprising over 50% of all the carbon in plants. Cellulose is a linear homopolymer of (1→4)-β-glucose units. It is of high molecular weight, consisting of up to 7,000–10,000 glucose units, and cellulose can be found in all plant materials (Van Soest, 1973). Celluloses in cereals are more crystalline than in vegetables. Cellulose has very low solubility.

Hemicelluloses consist of a wide variety of polysaccharides with a high variation in water solubility; at least 250 are known. The largest group consists of pentosans such as xylans and arabinoxylans, and another group consists of hexose polymers such as galactans. Xyloglucans are the predominant hemicelluloses in the cell walls of vegetables, while arabinoxylans predominate in wheat and rye (McDougall *et al.*, 1996). β-glucan is found in most cereals, being particularly high in barley and oats (Lambo *et al.*, 2005). The ubiquitous structural features of these polysaccharides are well established. They consist of a linear chain of glucose units joined by both β-(1→3) and β-(1→4) linkages. The molecular weight of β-glucans in barley is higher than in oats, and they are also less soluble (Lambo *et al.*, 2005).

Pectic polysaccharides, which can be found mostly in fruit and vegetables, refer to galacturonans—or more commonly rhamnogalacturonans — in which (1→4)-α-D-

galacturonan chains are interrupted at intervals by (1→2) -α-L-rhamnose residues. Pectic substances are highly soluble in water. The solubility depends on the extent of esterfication of the carboxylic group. Other constituent sugars attached as side chains include D-galactose, L-arabinose, D-xylose and, less frequently, L-fucose and D-glucuronic acid. Most of these sugars occur in short side chains, although D-galactose and L-arabinose are often found in multiple units. Extremely complicated side chains containing neutral polymers such as galactans and arabinans, xyloglucans and galactomannans have been reported (Pilnik & Voragen, 1970). Other polysaccharides included in the dietary fibre concept are gums, mucilages and algal polysaccharides. One of the most frequently used in this group is guar gum, obtained from the leguminous seed *Cyamopsis tetragonoloba.* Guar gum is highly fermented in the colon, giving high amounts of propionic acid, and it has also been proposed to have prebiotic properties (Ishihara *et al.*, 2000; Maisonnier *et al.*, 2003). Polydextrose, produced from glucose and maltose under high pressure and temperature, is another polysaccharide that is increasingly used to reduce sugar content and increase fibre content in different food products.

Lignin is an aromatic polymer with a molecular weight of about 10,000, is based on coniferyl and sinapyl alcohols, and can be found in woody plant tissues. Since it is virtually indigestible and structurally related to NSP, lignin is usually classified as part of dietary fibre. Lignin is often analysed as the residue insoluble in concentrated (12M) sulphuric acid (Klason lignin). Most lignin in food is found in brans and whole-grain cereals, while vegetables and fruits only contain minor amounts. Lignin is usually completely resistant to fermentation (Crawford & Crawford, 1980).

2.5.2 Resistant starch

Some starches in commonly used food are resistant to enzymatic hydrolysis and thus reach the colon. This type of starch is usually referred to as resistant starch, and is another major source of fermentable substrate for the bacteria in the colon

(Cummings *et al.*, 1997). Resistant starch cannot be regarded as a homogenous material. The degree to which it remains unhydrolysed in the small intestine and its relative availability for bacterial fermentation in the colon is dependent on its source and structure (Englyst *et al.*, 1992a). Resistant starches can be classified into four categories (Englyst *et al.*, 1992a). Type I is physically inaccessible starch granules such as whole or partially-milled grains. Type II is native starch granules found in, for example, raw potato, green bananas and high amylose maize. Retrograded starch formed after heating and cooling, makes up type III, and type IV are chemically modified starches altered by cross-linking, esterfication, or etherfication (Brown, 1996).

2.5.3 Oligosaccharides

Oligosaccharides are usually defined as glycosides that contain between three and ten sugar moieties. The most common types in food are the galactosides raffinose, stacchyose and verbascose, which are found in lentils, peas and beans, and the fructo-oligosaccharides, mostly found in onions, leeks, artichokes and chicory, and in small amounts in cereals. Oligosaccharides can also be produced commercially using enzymatic processes involving either the hydrolysis of polysaccharides or synthesis from smaller sugars using transglycosylases. Over the last two decades, a number of indigestible oligosaccharides have been developed, initially as low-calorie, low-cryogenic sucrose substitutes for use as bulking agents in foods. The one exception is soybean oligosaccharides, which are directly extracted. Currently, nine different types of indigestible oligosaccharides are produced commercially, mainly in Japan and Europe (Crittenden & Playne, 1996; Crittenden, 1999). Almost all of these are claimed by their manufacturers to be bifidogenic. The ability to act as a prebiotic has become a marketing edge for these products, and has promoted research into the ability of oligosaccharides to induce beneficial changes in the composition and metabolism of the colonic microbiota. The fructo-oligosaccharides, transgactosyl oligosaccharides and soybean oligosaccharides have been more thoroughly studied

than other oligosaccharides, and so far these have the best-documented evidence of prebiotic effects in humans (Crittenden, 1999).

Fructo-oligosaccharides are produced commercially either by synthesis from sucrose or by enzymatic hydrolysis of inulin extracted from chicory. Although produced in different ways, they have been reported to be structurally similar (Crittenden, 1999). Fructo-oligosaccharides manufactured using both techniques have been shown to increase faecal bifidobacteria levels in humans (Gibson *et al.*, 1995; Roberfroid *et al.*, 1998).

Inulin used commercially is usually extracted from chicory, and is used as a food ingredient with application as a fat substitute. Like its hydrolysis product, oligofructose, inulin is not hydrolysed or absorbed in the small intestine, and has been demonstrated to act as a prebiotic. *In vitro* studies indicate that the larger components of inulin (DP >10) are fermented more slowly by the faecal flora (Roberfroid *et al.*, 1998). Inulin and other fructo-oligosaccharides have been shown to increase the number of bifidobacteria (Gibson & Wang, 1994; Kleessen *et al.*, 1997b). It is therefore possible that they are fermented slightly more distally in the colon, although this has not been confirmed in humans (Crittenden, 1999). Today, different types of inulin can be found on the market with different physico-chemical properties (ORAFTI, Tienen, Belgium).

2.5.4 Disaccharides and sugar alcohols

Other carbohydrates entering the colon are sugar alcohols. The synthetic lactose derivatives lactulose and lactitol are mainly used as bulk laxatives for the treatment of constipation and hepatic encephalopathy. Lactitol is produced industrially by reducing lactose and lactulose by isomerisation of lactose; it can also be made through heat-treatment of milk (Olano *et al.*, 1989). These sugars are readily fermented by the colonic microbiota. Both sugars have also been shown to increase the number of

bifidobacteria and reduce the number of pathogenic organisms in the human colon, and therefore support the claim of being prebiotic (Ballongue *et al.*, 1997; Crittenden, 1999). Lactitol and lactulose also give an increase in faecal CAs. Sorbitol and xylitol may be only partially absorbed in the small intestine and therefore reach the colon (Saunders & Wiggins, 1981). In some individuals, lactose and fructose, when ingested in high amounts, may enter the colon and act as a substrate for the colonic microbiota (Riby *et al.*, 1993).

2.5.5 Protein

Carbohydrates are the preferred energy source of the intestinal microbes. Proteins and amino acids can also be effective as growth substrates for colonic bacteria, however, and some protein is also needed to ignite the fermentation, but it is enough with the proteins obtained from the colonic mucosa. In the absence of carbohydrates, certain groups of microbes such as Clostridia turn to protein fermentation, which produces potentially harmful nitrogenous metabolites in the lumen (e.g. biogenic amines, indoles and ammonia). Chronic putrefaction can increase the risk of colon cancer through the production of both ammonia — which can lead to neoplastic growth of the colon epithelium—and phenols, which can act as co-carcinogens (Ouwehand *et al.*, 2005). These include degraded products from elastin, collagen and albumin, as well as bacterial protein released after cell lysis.

2.5.6 Other substrates

In addition to components from food, the body itself produces a large quantity of endogenous compounds, such as mucins (which are glycoproteins) (Cummings & Macfarlane, 1997). These are rich in carbohydrates and are degraded by highly specialised members of the colonic microbiota (Ouwehand *et al.*, 2005). Pancreatic enzymes represent another source of nitrogen. Bacterial secretions, lysis products and sloughed-off epithelial cells also contribute as sources of substrate for the colonic microbiota.

2.5.7 Prebiotics

A prebiotic is an indigestible food ingredient that affects the host in a beneficial way by selectively stimulating the growth of one or a limited number of bacteria in the colon, which can improve the health of the host (Gibson & Roberfroid, 1995). In order for a food ingredient to be classified as a prebiotic, it must not be either hydrolysed or absorbed in the upper part of the gastro-intestinal tract, it must be able to alter the colonic microbiota in favour of a healthier composition, and induce luminal or systemic effects that are beneficial to the health of the host. In the past, proliferation of bifidobacteria was considered a proof efficacy in itself (Ouwehand *et al.*, 2005). However, according to Ouwehand *et al.* (2005), it is important to prove that a high level of bifidobacteria does indeed cure, prevent or reduce the risk of disease. *Bifidobacterium* counts are therefore not a sufficient biomarker, and other biomarkers such as lactobacilli, clostridia and *Bacteroides* are needed (Palframan *et al.*, 2003). Others suggest that microbial metabolites should also be included among the biomarkers, e.g. the CAs (Ouwehand *et al.*, 2005). Oligosaccharides have been the primary focus of the research on prebiotics, since many of them have been shown to selectively stimulate bifidobacterial populations resident in the human gut. These oligosaccharides include lactulose, raffinose, stachyose, fructo-oligosaccharides, gluco-oligosaccharides, xylo-oligosaccharides, galacto-oligosaccharides and isomalto-oligosaccharides. However, all components reaching the colon and utilized by the microbiota are in fact potential prebiotics. A more flexible definition of prebiotics has been suggested to be "potential substrates for bacterial inhabitants of the intestine", encompassing dietary fibre, oligosaccharides, resistant starch and lignin (Lim *et al.*, 2005).

Of the fructo-oligosaccharides, inulin and oligofructose have had the most widespread appeal as prebiotics, and have been shown to selectively stimulate the growth of bifidobacteria (Lim *et al.*, 2005). Also, lactitol has been shown to have prebiotic

properties (Ballongue *et al.*, 1997) and probably also some dietary fibre. Dietary fibre and prebiotics have many properties in common, and in some cases these terms are used interchangeably — but they are not the same. Prebiotics are fermented selectively in the colon, whereas dietary fibre is fermented by a wide range of colonic microbes or may not be fermented at all (Ouwehand *et al.*, 2005). However, it cannot be excluded that stimulation of a diverse microbiota is preferable to a selective stimulation.

Prebiotics are generally considered to be safe for human consumption, as many of them are constituents of the normal diet, albeit at lower concentrations. The main side effects of prebiotic over-consumption are bloating and diarrhoea. Diarrhoea is often claimed to be caused by an increase in osmotic load from the prebiotics or their fermentation products. However, since CAs have been shown to be rapidly absorbed and are also concentration dependent — i.e. the more CAs formed the more CAs and water are absorbed — this seems unlikely to be a probable explanation. Another likely explanation might be that the prebiotic *per se* has osmotic effects before it is fermented. Another side effect is flatulence, but such symptoms usually disappear with time.

2.6 Probiotics

Probiotics are live microorganisms that confer a health benefit on the host if administered in adequate amounts (Reid *et al.*, 2003). The most commonly used strains are some of the lactic acid bacteria: lactobacilli, enterocoocci and bifidobacteria. In recent years, many other microbes and even yeast have been developed as potential probiotics (Ouwehand *et al.*, 2002b). Already in the beginning of the last century, it was proposed by Metchnikoff that the lactobacilli present in yoghurt would have a health-promoting effect. Lactobacilli are still the most

commonly used bacteria, perhaps due to the fact that main food vehicle is fermented dairy products (Ouwehand *et al.*, 2002b).

2.6.1 Important probiotic properties

Many factors influence the choice of a probiotic. The microbe must be resistant to low pH, bile and pancreatic enzymes in order to survive the passage through the gastrointestinal tract. Adhesion to the intestinal mucosa is another factor considered to be important for immune modulation, pathogen exclusion, healing of damaged mucosa and prolonged transient colonisation. Probiotics should also be able to be cultured in a large scale, have an acceptable shelf life, and, in the case of fermented products, also contribute to a good taste (Ouwehand *et al.*, 2002b). Several clinical studies have investigated the use of probiotics, mainly lactobacilli and bifidobacteria, and there are lines of evidence suggesting that specific strains selected from a healthy microbiota may be anti-pathogenic and anti-inflammatory (Isolauri *et al.*, 2004). Not all studies have shown an effect of probiotics. The absence of effects might be due to differences in the amount of probiotics administered.

2.6.2 Colonisation

Whether probiotics can alter the composition of the bowel microbiota is hard to say, since most measurements are done in faeces. Also, neither lactobacilli nor bifidobacteria, on which most studies have been done, are major components of the microbiota of adult humans (Lim *et al.*, 2005). With the exception of *Lactobacillus salivarius* and *Lactobacillus ruminis*, the lactobacilli detected in human faeces originate from food and oral cavity sources rather than being established inhabitants of the colon (Tannock, 2003; Lim *et al.*, 2005). Even long-term feeding of probiotics does not necessarily change this situation, as seen in a feeding study of *Lactobacillus rhamnosus* (DR20) in milk given to human subjects (Tannock *et al.*, 2000). In most of the subjects, the probiotic strain could only be detected in faeces during the 6 months the probiotic was given. Administration of bifidobacteria and other

lactobacillus species showed similar results (Lim *et al.*, 2005). Although probiotics do not have the capacity to colonise the colon, they may have an effect on the host as long as he/she is are exposed to them. Furthermore, they may be effective in the small intestine. Consumption of a probiotic product usually delivers about 10^9 bacterial cells to this site with every dose. A realistic target might be the stimulation of the mucosal immune system of the small intestine (Lim *et al.*, 2005).

2.6.3. Synbiotics

Modification of the microbiota by dietary means seems to be one important target for development of functional foods. The microbiota is dependent on a continuous supply of substrate to survive, and an important factor for the ecosystem of the colon is the type of substrate reaching it. In recent years, a new term — synbiotics — has been introduced, for combinations of pro- and prebiotics. Synbiotics have been suggested to have greater nutritional implications than pro- or prebiotics alone. Synbiotics are defined as a mixture of probiotics and prebiotics that improves the survival and implantation of live microbial dietary supplements in the gastro-intestinal tract, either by stimulating growth or by metabolically activating the health-promoting bacteria (Kaur *et al.*, 2002).

2.6.4 Physiological effects related to probiotics

Diarrhoea. The most extensively documented probiotic intervention is the treatment and prevention of acute rotavirus diarrhoea (Szajewska & Mrukowicz, 2001; Isolauri *et al.*, 2002). The beneficial clinical effect of probiotic therapy in infantile diarrhoea has been explained by stabilisation of the indigenous microbiota, reduction of the duration of rotavirus shedding, and reduction of the increased gut permeability caused by rotavirus infection, together with a significant increase in cells secreting IgA against rotavirus (Isolauri *et al.*, 2004).

L. rhamnosus (GG) is one of the most extensively studied probiotic organisms, and there is evidence that this strain reduces the severity and duration of diarrhoea. When consumed as a dairy product or as a lyophilised powder, GG colonises the gastrointestinal tract for 1–3 days in most individuals and up to 7 days in about 30% of subjects (Kaur *et al.*, 2002). Other strains of lactobacilli have also been shown to reduce the mean duration of diarrhoea by an average of 30.5 hours (Allen *et al.*, 2004). However, no clear-cut conclusions can be drawn at present regarding the prevention of diarrhoea by probiotics (Isolauri *et al.*, 2004).

Inflammatory bowel diseases. Probiotics have been suggested to affect inflammatory bowel disease (IBD). Selected probiotics, such as *L. salivarius* and *L. rhamnosus*, have been observed to reduce the number of relapses and to prolong the period of remission in these types of patients. Interestingly, not only the lactic acid bacteria, *L. salivarius* (UCC118) and *L. rhamnosus* (GG), but also *S. cerevisiae* (*boulardii*) and a strain of *E. coli* (Nissle) have been observed to be effective in alleviating the symptoms of IBD (Gupta *et al.*, 2000; Guslandi *et al.*, 2000; Ouwehand *et al.*, 2002b).

Fat storage. In recent years, probiotics have also been suggested to have an effect on fat storage. Germ-free mice induced with microbiota from the caecum of conventional mice showed an increase in body fat content of 60% despite reduction in food intake (Bäckhed *et al.*, 2004). In contrast, in another study on mice — a comparison of total body fat in germ-free mice and conventional mice with a normal microbiota — it was found that the conventional mice had 40% more total body fat than the germ-free mice, even though these mice consumed less food per day . This was explained by the fact that the conventional mice can salvage energy from otherwise indigestible dietary polysaccharides (Bäckhed *et al.*, 2005). Fermentable carbohydrates are known to provide energy to the host (6.3–7.1 kJ/g) (Livesey *et al.*, 1995; Roberfroid, 1999).

The immune system. By modulating the composition and/or activity of the intestinal microbiota through the diet, the response to dietary antigens can be changed. Selected lactobacilli and bifidobacteria have been shown to be capable of enhancing the production of IgA (Ouwehand *et al.*, 2002a). It has also been shown in germ-free mice that the number of IgA-secreting cells were reduced tenfold as compared to controls containing a normal microbiota (Salminen *et al.*, 1998). The precise mechanism behind the immune modulation by these bacteria is still largely unknown, but adhesion to the intestinal mucosa is thought to be of importance (Morata de Ambrosini *et al.*, 1998). Both lactobacilli and bifidobacteria have been related to an increased immune response (Matsuzaki *et al.*, 1998; Arunachalam *et al.*, 2000; Chiang *et al.*, 2000). Consumption of *Bifidobacterium lactis* has, for example, been shown to increase the natural immune function (Arunachalam *et al.*, 2000).

Allergy. Numerous studies have indicated that the composition of the colon microbiota is different in atopic and non-atopic individuals, and between individuals in industrialised and developing countries (Björksten *et al.*, 1999; Björksten *et al.*, 2001; Kirjavainen *et al.*, 2001). The allergic children had increased levels of aerobic microbes and reduced levels of anaerobic microbes, particularly lactobacilli. In addition, infants that have developed allergies have been shown to harbour lower levels of bifidobacteria and enterococci but increased levels of clostridia and *S. aureus,* further indicating that the composition of the microbiota may be an underlying factor in allergic diseases regardless of other environmental differences that exist in different countries (Noverr & Huffnagle, 2005).

Several studies have shown that probiotics can reduce the frequency of atopic diseases. It has, for example, been demonstrated that mothers given *Lactobacillus* GG before the birth of the child, and continuing during breast feeding, reduce the frequency of atopic eczema by 50% (Kalliomäki *et al.*, 2001). In a four-year follow-up of the study, there was still an effect (Kalliomäki *et al.*, 2003). *Lactobacillus* GG has also been

shown to increase the number of Ig-secreting cells in infants during breastfeeding, indicating that probiotics influence gut immunity positively (Rinne *et al.*, 2005a). Furthermore, *Lactobacillus* GG stimulates the local release of interferons, proteins produced by the cells of the immune system that act against viruses, bacteria, parasites and tumor cells, assisting antigen transport to underlying lymphoid cells (Kaur *et al.*, 2002). In addition, gut bacteria are involved in vitamin synthesis, especially of vitamins B and K (Salminen *et al.*, 1998), and increased microbial activity may increase the availability of these vitamins.

3 Objectives of the present work

The overall aim of the present work was to study the content and pattern of carboxylic acids formed upon colonic fermentation of indigestible carbohydrates, using the rat as a model. The effects of physico-chemical properties such as the monomeric composition, the degree of polymerization and solubility was evaluated, as well as the impact of probiotics — factors that have not been studied to any great extent yet. In addition, a human model was set up to study the individual variation and the day-to-day variation, in order to be able to test various food products in the future.

In paper I, indigestible carbohydrates with different monomeric composition, molecular weight and solubility were studied in the rat model. For this purpose, lactitol, lactulose and four types of fructo-oligosaccharides were chosen.

In papers II and III, indigestible carbohydrates were combined with probiotic bacteria and given to the rats. Pectin, inulin with low and high solubility, and lactitol were supplemented with *Bifidobacterium lactis* and *Lactobacillus salivarius* (papers II and III), and inulin with a high solubility was also supplemented with *Lactobacillus rhamnosus* or a mixture of the three bacterial strains (paper III).

In paper IV, healthy subjects were given β-glucan-enriched oat bran for 12 weeks.

4 Materials & Methods

The materials and methods used in papers I to IV are summarised below. The original papers contain more detailed information.

4.1 Carbohydrates

In paper I, the effect of monomeric composition, degree of polymerisation and solubility on CA formation was investigated. For this purpose, the following ICs were selected: Raftilose®P95 (oligofructose, OF), Raftiline®HP (long-chain inulin, IN), Raftiline®HPX (long-chain inulin with a low solubility, IN-ls), Raftilose®SYNERGY1 (mixture of oligofructose and inulin, a 1:1 mix OF-IN) delivered by ORAFTI (Tienen, Belgium), lactulose, a disaccharide consisting of fructose and galactose (Calbiochem, Darmstadt, Germany) and lactitol, a sugar alcohol consisting of galactose and glucitol (Danisco, Copenhagen, Denmark). OF had a low DP, of between 2 and 8, while those of IN and IN-ls were higher, between 10 and 60 (average 23) (Franck, 2002). OF is produced by partial enzymatic hydrolysis of naturally occurring inulin extracted from chicory root, while IN is obtained by removing fructo-oligosaccharides of low DP from natural inulin (Franck, 2002). IN-ls has a lower solubility (1 g/l at 25°C) than IN (20 g/l at 25°C) and higher gel strength in fat substitution applications, according to the product sheets (ORAFTI). This is obtained by modifying powder characteristics in the spray-drying process.

In the second and third papers, where pre- and probiotic interactions were investigated, pectin, IN, IN-ls and lactitol were included (Table 2). The pectin

obtained from Danisco (Copenhagen, Denmark), was derived from citrus fruit (lime), and had a degree of esterification of 70.7%.

In paper IV, in which the CA formation in humans was investigated, a β-glucan-enriched oat bran was used (Swedish Oat Fiber, Väröbacka, Sweden). This specific oat bran has been shown to give high amounts of butyric acid in subjects with ulcerative colitis.

Table 2. Outline of the papers, number of groups and the carbohydrates and bacterial strains that were used

Paper	Number of groups	Carbohydrates/main monomers/DP	Bacterial strains
I	7 (n = 7 rats)	-	
		Lactitol/ galactose, glucitol/ 2	
		Lactulose/ galactose, fructose/ 2	
		OF/ fructose/ 2–8	
		IN/ fructose/ 10–60	
		IN-ls/ fructose/ 10–60	
		OF-IN/ fructose/ 2–8 + 10–60	
II	9 (n = 7 rats)	Lactitol	-, Bb-12, UCC500
		IN-ls	-, Bb-12, UCC500
		Pectin (citrus, degree of esterfication 70.7%)	-, Bb-12, UCC500
III	5 (n = 7 rats)	IN	-, Bb-12, UCC500, GG, mixture
IV	1 (n = 20 humans)	β-glucan-enriched oat bran/ glucose, arabinose, xylose	

4.2 Bacterial strains

Three bacterial strains were selected to investigate pre- and probiotic interactions in paper II and III: *Bifidobacterium lactis* (Bb-12, Chr. Hansen, Hørsholm, Denmark), *Lactobacillus salivarius* (UCC500, University College Cork, Ireland) and *Lactobacillus rhamnosus* (GG; Valio, Helsinki, Finland) (paper III)(Table 2). All three strains were delivered freeze-dried and they were included in the rat diets (10^9–10^{10} CFU/day) as single strains or as a combination of the three (paper III).

4.3 Animal studies (Papers I–III)

Diets. A basal diet containing no dietary fibre was prepared. This contained casein (Sigma Chemical Co., St. Louis, MO) as protein source, sucrose (Danisco Sugar, Malmö, Sweden), maize oil (Mazola, Bestfoods Nordic A/S, Copenhagen, Denmark), DL-methionine (Sigma), choline chloride (Aldrich Chemie, Steinheim, Germany), mineral mixture and vitamin mixture (Apoteket, Malmö, Sweden) and wheat starch (Lundbergs, Malmö, Sweden). Wheat starch is completely digested and absorbed, and does not contribute to any hindgut fermentation (Björck *et al.*, 1987). In the test diets, the different ICs and bacterial strains were substituted for wheat starch. The ICs were added at a level of 80 g/kg diet and amounts of the probiotic strains were adjusted to give each rat an amount of Bb-12, GG and UCC500 corresponding to 1×10^{10} CFU/day, 1×10^{10} CFU/day and 1×10^9 CFU/day, respectively. In the diet containing a mixture of the probiotic strains, one-third of each strain was added. The diets containing the probiotics were kept refrigerated until they were fed to the rats.

Animals. Male Wistar rats (average weight 84.5 ± 0.9 g), three to four weeks old, were used, with seven in each group. The intake of food was restricted to 12 g dry weight/day and water was given *ad libitum*. The rats were allowed 7 days to adapt to

the diet, which was followed by a 5-day-long experimental period, during which faeces and food residues were collected daily. The faeces were stored at –20°C and then freeze-dried and milled before being analysed for remaining ICs. During the following 24 h of the experiment, fresh faeces were collected on dry ice for CA determination and bacterial analysis (6-d faeces) (paper II). The 6-d faeces from rats fed UCC500 were frozen and sent for microbial analysis (UCC, Cork, Ireland), while faeces from rats fed Bb-12 were collected in transport medium consisting of 0.9% NaCl, 0.1% peptone, 0.1% Tween 80 and 0.02% cysteine, and analysed directly. The animals were sacrificed using carbon dioxide narcosis, and the caecum and proximal and distal colon were removed. Caecal tissue weight, content and pH were measured directly and the different parts of the hindgut were frozen and stored at –40°C until analysis. The Ethics Committee for Animal Studies at Lund University approved the animal experiments.

Experimental design. In paper I, the CA formation in rats fed lactulose, lactitol and fructo-oligosaccharides of different degrees of polymerisation was investigated. In paper II and III, pre- and probiotic interactions were studied. Thus, the formation of CAs was studied in the hindgut of rats fed pectin, IN-ls and lactitol together with Bb-12 and UCC500 (paper II), and in rats fed IN in combination with Bb-12, UCC500 or GG as single strains or as a mixture of all three strains (paper III).

4.4 Human study (Paper IV)

Subjects. Twenty-five healthy young volunteers aged 20–47 years with a mean age of 24.0 ± 1.3 years (10 men and 15 women) participated in the study. The subjects were recruited by announcing in a local paper. All subjects fulfilled the inclusion criteria: they were over 20 years old, had no known gastrointestinal or metabolic diseases, had not used antibiotics for in the previous 6 months, and had not had any episodes of

severe diarrhoea in the 6 months prior to the study. During the trial, 40 g of a β-glucan-enriched oat bran, corresponding to 20 g fibre (10 g β-glucans) was added to the daily diet in the form of bread (4 slices) without changing the normal diet to any greater extent. The subjects had regular contact with a dietician, and every fourth week they came to the department to receive a new package of deep-frozen bread and for reporting of compliance. All subjects carried out two dietary registrations over 4 days, one before the study and one after 8 weeks.

Twenty subjects completed the study. One subject dropped out because of gastrointestinal symptoms (diarrhoea), and three for personal reasons (lack of time). Due to sampling error, the entry sample was lost from one subject. All subjects gave their informed consent and were aware that they could withdraw from the study at any time they desired. They were also informed that the high amount of dietary fibre could cause gastrointestinal problems, such as gas. The study was approved by the Ethics Committee for human studies at Lund University.

Oat bread. The oat bread was baked from 23.7 kg water, 10.8 kg oat bran (Swedish Oat Fiber, Väröbacka, Sweden), 5.6 kg white wheat flour (Nord Mills, Malmö, Sweden), 1.1 kg yeast, 1.8 kg gluten, 1.4 kg sugar, 0.3 kg E 472 and 0.3 kg salt. The dough was proofed at room temperature for 60 min and was then divided into pieces and baked at 240°C for 40 min at a commercial bakery (Skogaholm, Lund, Sweden). After baking, the loaves were sliced and stored frozen until use. Each slice of bread contained 5 g of oat fibre.

Sample collection. Faecal samples from three consecutive days were collected at entry (time-point 0), and then after 4, 8 and 12 weeks during the intervention period with oat bran. The samples were frozen immediately, delivered to the Bacteriology Laboratory at Lund University Hospital, and stored at –40°C until analysed for CA content.

4.5 Analysis

Indigestible carbohydrates. The amount of fructo-oligosaccharides, lactitol and lactulose in the raw material was suggested to be as determined by the manufacturers. Dietary fibre in pectin and in the β-glucan-enriched oat bran was isolated using the method developed by Asp and colleagues (Asp *et al.*, 1983). The composition of dietary fibre in the isolated fibre residues and in faeces was analysed using GLC for the neutral sugars (as their alditol acetates), and using a spectrophotometric method for the uronic acids (Theander *et al.*, 1995). The β-glucan content was quantified using an enzyme kit (Megazyme International, Wicklow, Ireland), based on the procedure developed by McCleary and Codd for mixed-linkage β-glucans (Mccleary & Codd, 1991), which has been approved by the AACC (method 32-23) and the AOAC (method 995.16).

The amount of fructo-oligosaccharides in faeces was first estimated enzymatically (Boehringer, Mannheim, Germany; number 139106) after hydrolysis in 0.6 M perchloric acid (for 15 min, at 80°C) (Nilsson & Bjorck, 1988). Only rats fed IN and IN-ls had detectable amounts of fructose in the faeces. The amounts of fructo-oligosaccharides in faeces from these rats were therefore also quantified with a more reliable method, AOAC method 999.03 (McCleary *et al.*, 2000). With that method, fructo-oligosaccharides are treated with fructanase (exo-inulinase) and the amount of fructose is quantified by the PAHBAH reducing-sugar method. Lactitol and lactulose in faeces were extracted in 50% ethanol (v/v) for 30 min at room temperature according to a previously developed method (Ekvall *et al.*, 2005). Arabinose was used as internal standard. The sugars were then analysed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Dionex 500, Sunnyvale, CA) and NaOH (15 mmol–200 mmol) was used as the

eluent with a flow rate of 1.0 ml/min. In paper III, only small amounts of inulin were detected in the faeces of these rats (0.070 g in 5 days), and as the faecal weights were similar in rats fed the diets containing probiotics, it was assumed that inulin had been highly fermented in these rats also.

Carboxylic acids. A gas-liquid chromatographic method was used to determine the amounts of SCFAs (formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic and heptanoic acids). Other CAs quantified with that method were lactic acid and succinic acid (Richardson *et al.*, 1989). The intestinal content and the faecal samples were homogenised (using a Polytron®; Kinematica, Switzerland) together with an internal standard (2-ethylbutyric acid; Sigma). Hydrochloric acid was added to protonise the CAs, and in order to be able to extract them in diethyl ether. After being silylated with n-(tert-butyldimethylsilyl)-n-methyltrifluoroacetamide (MTBSTFA; Sigma) the samples were allowed to stand for 48 h to complete derivatisation. Samples were then injected onto an HP-5 column (GLC, HP 6890; Hewlett-Packard, Wilmington, DE). Chem Station software (Hewlett Packard) was used for analysis.

Microbial analysis. Analysis of bacteria was done in paper II only. Bifidobacteria and lactobacilli were grown at 37°C for 3 days on Wilkins Chalgren agar (Oxoid) with mupirocin or on LBS agar (lactobacillus selective agar), respectively (Rogosa *et al.*, 1951; Rada & Petr, 2000). Identification of bifidobacteria was done by genus-specific PCR and randomly amplified polymorphic DNA (RAPD) (Roy & Sirois, 2000). Typing of lactobacilli was performed by RAPD (Coakley *et al.*, 1996) on colonies that were randomly picked from medium selective for lactobacilli (Rogosa *et al.*, 1951).

4.6 Calculations and statistical evaluation

All analyses were performed at least in duplicate. The dry matter digestibility was calculated as:

$$1 - \frac{\text{g dry matter in faeces}}{\text{g dry matter ingested}}.$$

The caecal pools of CAs were calculated as the levels of each acid (μmol/g) multiplied by the weight of the caecal content. The values were extrapolated to complete intake of indigestible carbohydrates (4.8 g) and thus corrected for the small amount of feed residues. Bulking capacity was calculated as faecal dry weight in g/g of IC eaten. The gain in body weight was calculated in g/g feed eaten, while the caecal content and faecal dry weights were calculated in g/g IC eaten.

All statistical analyses were performed with Minitab® statistical software (Release 13.32/14.00). The significance of differences between means was determined by General Linear Model (GLM, ANOVA) followed by either Tukey's test for multiple comparisons (papers I and II) or by Dunnett's procedure (papers II–IV). To determine the effects of ICs, probiotics and their interactions, two-way ANOVA was used (paper II). Correlations between CA concentrations in the distal colon and faeces were calculated with linear regression (papers II and III). When CA values in the caecum and distal colon were compared, the two-sample Student t-test was used (paper I). In the human study the coefficient of variation (CV), i.e. the standard deviation divided by the mean value (%), was used to give a measure of the variation in faecal concentrations over the three consecutive days. When comparing CA values for different weeks, the mean value of the three consecutive days was used. The level of significance used was $P < 0.05$.

5 Results & Discussion

5.1 Carboxylic acid formation in rats

There is evidence that butyric and propionic acid may have health-promoting effects, and as the formation of CAs from various carbohydrates in the colon has been shown to vary, it is of great interest to identify food factors that may be of importance for this. Studies in the literature have identified a number of factors, such as monomeric composition, type of linkage, branching and the composition of the microbiota. However, few studies have concentrated on the effects of the physico-chemical properties of the carbohydrates and of probiotics on CA formation. The present work was an attempt to increase our knowledge in this area. For this purpose a rat model was used, which had been shown earlier to correlate well with humans concerning total fermentability and bulking capacity. The rats were given the diets for 13 days, which has been shown to be sufficient for optimal fermentation of dietary fibre components and for a stable pattern of CAs (Tulung *et al.*, 1987; Brunsgaard *et al.*, 1995).

In the first study, four types of fructo-oligosaccharides, lactulose and lactitol were used to study whether the monomeric composition, molecular weight and the solubility of the carbohydrates had any effect on CA formation. The choice of fructo-oligosaccharides was motivated by the possibility of obtaining well-defined preparations from the manufacturer. Thus, oligofructose (OF) had a DP of between 2 and 8, inulin (IN) a DP of between 10 and 60, IN also exist in a variant with lower solubility (IN-ls), and finally a mixture of oligofructose and inulin (1:1, Mix OF-IN). These fructo-oligosaccharides are widely used as prebiotic food ingredients, especially oligofructose. Lactulose was chosen because it also contains fructose. Lactitol, due to its similar composition of that of lactulose.

In the second and third study, carbohydrates with different fermentation rates (pectin, lactitol, and inulin of high and low solubility) and known to give specific CAs were combined with probiotic strains, in order to ascertain whether it was possible to increase the amount of CAs formed, as well as to study whether the CA profile and the site of release of CAs could be modified. The probiotic bacteria *Bifidobacterium animalis* (Bb-12), *Lactobacillus salivarius* (UCC500), *Lactobacillus rhamnosus* (GG), or a mixture of all three was investigated.

The total amount of CAs in the caecum of rats fed a control diet without any indigestible carbohydrates was 42 µmol. When rats were fed test diets containing indigestible carbohydrates, the caecal pool of CAs increased 2 to 5-fold (97–226 µmol), and when probiotics were added the caecal pools were even higher with some combinations (up to 322 µmol). The major acids formed were acetic acid (41–64%), propionic acid (14–31%) and butyric acid (7–22%), and these acids accounted for 81–93% of the total amount of the CAs analysed. The remaining part could be attributed to lactic acid (1–7%), succinic acid (2–10%) and other minor acids (1–6%).

5.1.1 Effects of molecular weight, solubility and composition

The degree of polymerization and the solubility of the fructo-oligosaccharides had pronounced effects on CA formation, while the fructose content *per se* was of less importance. Thus, the highest caecal concentrations and pools of butyric acid were found in rats fed diets containing OF (18 µmol/g versus 5-13 µmol/g for the other test diets, $P < 0.05$), while the highest caecal concentrations of propionic acid were found in rats fed the IN diet (22 µmol/g versus 13–16 µmol/g for the other test diets, $P < 0.05$). Similar tendencies could generally be seen in the proximal and distal colon. These differences were also reflected in the proportions of CAs. This is interesting, as

these carbohydrates are both composed of fructose with β–2, 1 linkages and it therefore seems that other factors, such as the DP, are of importance. OF consists of fructo-oligosaccharides with a DP between 2 and 8, while IN contains fructo-oligosaccharides with a DP between 10 and 60.

From studies in the literature, it is difficult to draw any clear conclusions about the DPs of the fructo-oligosaccharides used, but most of the studies seem to have investigated fructo-oligosacharides with a low DP. These studies also show that fructo-oligosaccharides give high yields of butyric acid, at least compared to other oligosaccharides (Roland *et al.*, 1995; Campbell *et al.*, 1997; Poulsen *et al.*, 2002). However, one study employing different doses of inulin reported high proportions of propionic acid (Levrat *et al.*, 1991), which could be due to the use of fructo-oligosaccharides with a high DP. Also, high amounts of propionic acid were seen in rats fed fibre diets when these were partially replaced with long-chain inulin but not oligofructose (Kleessen *et al.*, 2001). However, in that study no differences could be seen in the concentrations of butyric acid despite the different DPs. The concentrations of butyric acid, on the other hand, were higher than before when the fibre had been partially replaced with the fructo-oligosaccharides. This may be explained by the fact that the diet contained other indigestible carbohydrates. Thus, it has been shown that certain combinations of indigestible carbohydrates yield a higher proportion of butyric acid than the single substrates (Henningsson *et al.*, 2002). Similarly, Topping and collaborators (1985) reported that a mixture of gum arabic and cellulose gave more butyric acid when combined than when used as individual substrates. Rats fed a diet containing OF also gained most in weight (26.6 g in 5 days as compared to 18.7 ± 0.8 g for rats fed other fibre diets). Fermentable carbohydrates are known to provide energy to the host (6.3–7.1 kJ/g) (Livesey *et al.*, 1995; Roberfroid, 1999). However, in this study all carbohydrates tested were more or less fermented. It may therefore be questioned whether the CA profile would be of any importance in this context.

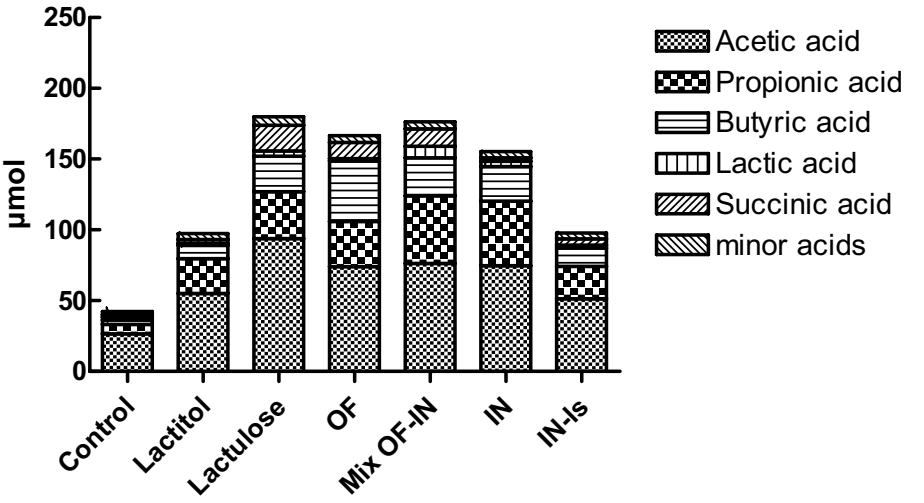

Figure 2. Caecal pools (µmol) of carboxylic acids in rats fed a control diet and test diets containing lactulose, lactitol and fructo-oligosaccharides with various degrees of polymerisation (DP) and solubility

Although the fructo-oligosaccharides, lactulose and lactitol were more or less readily fermented (86–100%), the caecal pools varied (Figure 2). Lactitol and IN-ls gave a lower caecal pool of CAs (mean 97 and 98 µmol, respectively), than the other test diets (173 ± 9 µmol, $P < 0.5$). Concerning IN-ls, this may be due to this substrate being fermented to a greater extent in the distal part of the hindgut than the other substrates, due to its lower solubility. A lower degree of caecal fermentation was also suggested by the lower caecal pH with IN-ls (6.9 versus mean 6.6 ± 0.1 for the other oligosaccharides) as well as the lower caecal pool of CAs (98 µmol versus 168 ± 8 µmol for the other fructo-oligosaccharides). IN-ls was also shown to be somewhat more resistant to fermentation, as evidenced by the higher faecal excretion of fructo-oligosaccharides (0.44g in 5 days), than of IN (0.07 g in 5 days). Similar results have

been seen in *in vitro* studies. Thus, fructo-oligosaccharides with a DP of > 10 were fermented more slowly than those with a DP of < 10 (Roberfroid *et al.*, 1998).

The low caecal pools of CAs using lactitol as substrate (97 µmol) also indicate a low degree of fermentation. However, this substrate gave the highest caecal content (2.8 g as opposed to 1.8–2.3 g). One explanation of these contradictory results would be that unfermented lactitol contributes to the osmosis in the caecum to a greater extent than formation of CAs. Water would then rather be retained than absorbed from the caecum, resulting in an enlarged, distended organ. Feeding rats with lactulose, on the other hand, resulted in the highest caecal concentrations (90.5 µmol versus mean 66.4 µmol with the other oligosaccharides) and pools of CAs (187.7 µmol as opposed to 139.7 µmol with the other oligosaccharides), indicating that this disaccharide is rapidly fermented. Interestingly, in studies in humans, lactulose resulted in more pronounced changes in e.g. CA production and activity of pro-carcinogenic enzymes than with lactitol (Ballongue *et al.*, 1997), as well as in a lower colonic pH (5.1 as opposed to 5.6 with lactitol) (Patil *et al.*, 1987). Effects on microbial composition also seem to be different between these two disaccharides. Lactulose has been shown to selectively stimulate the number of bifidobacteria in humans (Tuohy *et al.*, 2002), whereas lactitol has been reported to reduce the bifidobacterial and bacteroides populations in an *in vitro* culture system when added to a growth medium containing low amounts of dietary fibre (Probert *et al.*, 2004). In the same in vitro model, oligofructose was shown to increase the number of bifidobacteria (Gibson & Wang, 1994). However, in contrast to the present study, lactitol was more prone to produce butyric acid than oligofructose (Probert *et al.*, 2004), which may be due to the different methodologies used (an *in vitro* model versus an *in vivo* model).

The butyric acid concentration increased along the hindgut of rats fed OF, IN and IN-ls. Similar results were observed for propionic acid in rats fed IN and IN-ls. The transit time through the gastrointestinal tract has been reported to influence bacterial

activities and pathways, and as a result, the proportion of individual CAs may change (Oufir *et al.*, 2000). It has been reported that the shorter the caecal transit times, the higher the proportion of butyric acid (Mathers & Dawson, 1991). Since the transit time was not measured in the present study, it remains to be seen if this is the case also for OF, IN and IN-ls.

The increased concentration of butyric acid along the hindgut in rats fed IN-ls may also be due to the slower fermentation of this substrate. Wheat bran, another substrate that is slowly fermented, has been shown to give high amounts of butyric acid in the distal part of the hindgut of rats (Henningsson *et al.*, 2002). Furthermore, in another study on rats the site of fermentation of easily fermentable RS was shifted to the distal part of the colon when RS was combined with psyllium fibre. A concurrent increase in faecal butyrate concentration could also be seen. Psyllium fibre is another example of a comparatively resistant type of fibre (Morita *et al.*, 1999). This is interesting and may be important from a nutritional point of view, as most colonic diseases also occur in the distal part of the colon.

Lactulose generally gave low concentrations and proportions of propionic acid throughout the hindgut and high concentrations and proportions of acetic acid, compared with the other substrates. This may have metabolic consequences, as propionic acid has been suggested to lower serum lipids and cholesterol, and the higher the propionic:acetic ratio the greater the effects. Thus, when humans were given rectal infusions of propionate and acetate, it was shown that propionate repressed the utilization of acetate for the synthesis of cholesterol (Wolever *et al.*, 1991). Similar results were obtained in obese rats (Berggren *et al.*, 1996). Interestingly, it has been shown in a human study on healthy subjects that lactulose increases serum lipids and LDL-cholesterol (Jenkins *et al.*, 1991), and it may be speculated whether this is due to the comparatively high amounts of acetic acid formed. In a human study on non-insulin-dependent diabetic patients, total and LDL

cholesterol in serum were found to be lowered by fructo-oligosaccharides (Yamashita *et al.*, 1984). The discrepancies in results between fructo-oligosaccharides and lactulose were suggested to be due to a lower acetate:propionate ratio with fructo-oligosaccharides than with lactulose (Luo *et al.*, 1996). Another explanation might be differences in metabolic status between healthy and non-insulin-dependent subjects.

Small caecal concentrations of lactic acid (1–4 µmol/g) and succinic acid (2-9 µmol/g) were also detected, and lactulose, OF and Mix OF-IN produced the highest amounts of succinic acid (6-9 µmol/g). Similarly, an increase in the formation of succinic acid has been seen in other studies on rats with dextran sulphate sodium (DSS)-induced acute colitis that were fed fructo-oligosaccharides (Mix OF-IN). Thus, compared to rats given a control diet without any fibre and rats treated with probiotics (bifidobacteria), those given fructo-oligosaccharides had higher faecal amounts of succinic acid (Osman, 2006). It may be questioned why there is an increased formation of succinic acid, propionic acid can be formed from succinic acid, but not at a low bacterial activity. One could speculate that a specific increase in bifidobacteria reduces the number of bacteria which degrades succinic acid to propionic acid.

5.1.2 Pre- and probiotic interactions

In paper II, the effect of *Bifidobacterium lactis* (Bb-12) and *Lactobacillus Salivarius* (UCC500) on the formation of carboxylic acids was studied in rats fed pectin, inulin with low solubility, and lactitol. Since all these carbohydrates are readily fermented, they have prebiotic potential. The carbohydrates used were selected because of their different rates of fermentation and capacities to form different amounts and types of CAs. Thus, pectin was chosen because this polysaccharide has been reported to give high amounts of acetic acid during fermentation, whereas the inulin used has a low solubility and has been shown to give high amounts of propionic and butyric acid in the distal part of colon (Paper I). Inulin has also been shown to stimulate the growth

of bifidobacteria specifically. Lactitol was chosen because of its unexpectedly low amounts of CAs in the caecum during fermentation, and it has also been proposed to be rapidly fermented.

Addition of probiotics to the diet was shown to have an influence on both the degree of fermentation and CA formation. However, different effects were seen with the various combinations of probiotics and fermentable carbohydrates (Figure 3). This contrasts with *in vitro* studies with pig caecal bacteria, which have shown that the same amounts of CAs are produced but at a faster rate when probiotics are added (Sakata *et al.*, 2003).

IN-ls and Bb-12. When Bb-12 was added to the IN-ls diet, the caecal concentration of CAs increased (from 59.6 to 70.0 µmol/g), while it decreased in the distal part of the colon (from 88.4 to 63.2 µmol/g). Thus, it seems that Bb-12 stimulates the caecal fermentation of IN-ls, resulting in lower amounts of substrate reaching the distal part of the colon, despite a similar net production of CAs — as verified by the similar amounts (~5%) of fructo-oligosaccharides excreted in faeces. The increased concentration of CAs in the caecum was the result of a specific increase in propionic acid. Interestingly, the proportion reached a level (31%, Figure 3) that was higher than for inulin of high solubility (27%) — the type of fructo-oligosaccharide with a high DP that has previously been shown to be especially prone to generate propionic acid (paper I). In the distal part of the colon, on the other hand, the proportions of propionic and butyric acid decreased, while that of lactic acid increased. Carbohydrates fermented by bifidobacteria have been reported to use another metabolic pathway, the "bifid shunt", producing high amounts of acetic acid and lactic acid (in the molar ratio 3:2) (Scardovi, 1986), while propionic and butyric acids are not produced through this pathway. It may therefore be speculated whether the number of bifidobacteria in relation to the amount of fermentable substrate is of importance for the profile of CAs formed. Thus, it appears that high amounts of

lactic acid are formed when the amount of substrate is limiting, as found in the distal part of the colon in rats fed Bb-12 and inulin. On the other hand, propionic acid seems to be formed when there is a surplus of substrate, as found in the caecum of these rats. Interestingly, Bb-12 combined with inulin (Raftiline and Raftilose) has been shown to raise the integrity of tight junctions in an *in vitro* model, a phenomenon that has been suggested to protect the mucosal barrier from disruption (Commane *et al.*, 2005).

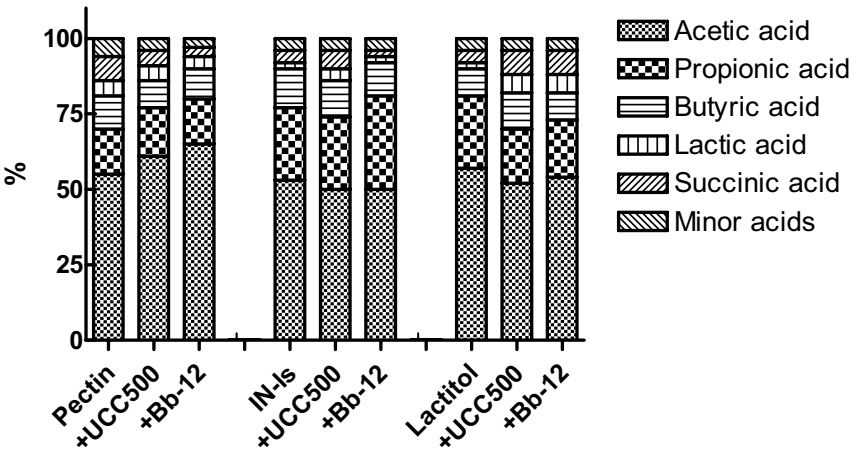

Figure 3. Proportions (%) of carboxylic acids in the hindgut of rats fed *Lactobacillus salivarius* (UCC500) and *Bifidobacterium lactis* (Bb-12) in combination with pectin, fructans or lactitol

Pectin and Bb12. When rats were fed pectin and Bb-12, the concentrations of CAs were greater throughout the hindgut — especially in the distal part of the colon — as compared to rats fed only pectin, leading to a higher net production of CAs ($P < 0.05–0.001$). As the faecal excretion of pectin was very similar regardless of whether Bb-12 was added to the diet or not, it appears that pectin was more effective in generating CAs in the presence of Bb-12. The mechanisms behind this can only be speculated upon, but evidently the colonic microbiota metabolises pectin through

other pathways when Bb-12 is added, as indicated by the different profiles of CAs. In the distal part of the colon, for example, the proportion of lactic acid decreased, while that of propionic, butyric and acetic acid increased. Since pectin stimulates the growth of both bacteroides and eubacteria (Salyers *et al.*, 1977a; Salyers *et al.*, 1977b), supplementation with Bb-12 leads to a more complex situation in the hindgut with more metabolic interactions between the microbial species than with IN-ls. The bacteria *per se* may also contribute to the amount of substrate available in the colon by upregulating mucus production. It has been shown that probiotics increase the production of mucins from HT-29 cells (Mack *et al.*, 2003) as well as from the jejunum and ileum of the rat (Ahrné, S., unpublished results).

Lactitol and Bb-12. The low amounts of CAs in the caecum of rats fed lactitol (97 µmol as compared to 226 µmol, for example, with pectin) indicate a low degree of caecal fermentation. This has also been seen by others (Ballongue *et al.*, 1997). However, lactitol was readily fermented during passage through the hindgut, as evidenced by the finding that no lactitol was excreted in faeces. This was the case regardless of whether or not probiotics were added. In rats fed lactitol and Bb-12, the concentration in the distal part of the colon was even lower than without this strain (57.2 versus 86.3 µmol/g, $P < 0.001$). The reason for this is not known, but may be ascribed to an accelerated formation and absorption of CAs when this strain is included, leading to less substrate being delivered to the distal part. Others have also found that the absorption of CAs in rats may increase when probiotics are added to the diet (Berggren, 1996). For example, when *Lactobacillus reuteri* was added to a rat diet containing a mixture of easily fermentable fibres (oat fibre, potato fibre, pectin and pea fibre), there was a general decrease in the faecal concentrations of CAs (Berggren, 1996).

Pectin and UCC500. UCC500 added to the pectin diet reduced the caecal pool of CAs in the rats (from 226 to 129 µmol, $P < 0.05$), but increased the concentration of

CAs in the distal colon (49.3 vs. 75.3 µmol/g, $P < 0.01$), indicating that the site of fermentation had been changed to the distal part of the colon. This could be due to this strain inhibiting the activity of pectin-degrading enzymes in the caecum, resulting in reduced formation of CAs. As a consequence, more substrate would be available distally, which was also seen in the higher faecal excretion of pectin when UCC500 was added (12% versus 4%, $P < 0.05$). Interestingly, there was an increase in the concentration of propionic and butyric acids in the distal part of the colon when UCC500 was added. This may be interesting from a nutritional point of view, as most colonic diseases occur in the distal part.

IN-ls, lactitol and UCC500. The concentrations of CAs in rats fed IN-ls or lactitol were not affected by UCC500. However, the caecal pool of CAs in rats fed lactitol increased from 97 to 195 µmol, indicating an overall increase in bacterial activity or a change in the composition of the caecal microbiota. This was further evidenced by the fact that the proportion of lactic and succinic acid in the caecum increased at the expense of propionic acid, compared with lactitol alone. Concerning IN-ls, there was a lower proportion of acetic acid in the distal part of colon in rats fed UCC500. No other effects could be seen when UCC500 was added to the IN-ls diet.

5.1.3 Effects of probiotics with IN as colonic substrate

In paper III, a long-chain fructo-oligosaccharide (DP 10-60) with high solubility (IN) was combined with three probiotic strains, Bb-12, UCC500 and GG, in order to investigate whether the CA formation could be modified. GG has been found to have positive effects when treating diarrhoea (Isolauri *et al.*, 1991) and atopic eczema (Isolauri *et al.*, 1999) and also to survive passage through the gut (Goldin *et al.*, 1992; Millar *et al.*, 1993; Saxelin *et al.*, 1995). Bb-12, the most thoroughly studied probiotic *Bifidobacterium* strain currently on the market, has been shown to be effective in preventing traveller's diarrhoea, in reducing the risk of constipation, and

in modification of the immune response (Alander *et al.*, 2001). *Lactobacillus salivarius* has not been studied to any great extent yet, but some strains have been shown to have anti-inflammatory properties (Peran *et al.*, 2005). As it has been suggested that a combination of probiotics may have more pronounced effects in the colon than a single strain, a mixture of the three strains was also investigated.

+Bb-12. The caecal pool of CAs increased when rats were fed IN and Bb-12 (from 155 µmol to 356 µmol, $P < 0.001$). There was a similar increase in acetic, propionic and butyric acid, and only the proportion of lactic acid increased from 3% to 7%. Fructo-oligosaccharides, such as inulin and oligofructose, have been reported to be preferred substrates for bifidobacteria and the number of these bacteria increases in the faeces when fed to humans (Gibson & Roberfroid, 1995; Kleessen *et al.*, 1997b) and rats (Campbell *et al.*, 1997). This may explain the considerably higher caecal formation of CAs in rats fed Bb-12 than with IN only, which gave low amounts of CAs compared to many other substrates (Berggren *et al.*, 1993). In a previous study on IN-ls, the caecal formation of CAs also increased when rats were fed Bb-12, but in that study there was a specific increase in propionic acid (paper II). One reason for the difference could be that IN-ls gave considerably lower caecal amounts of CAs, including propionic acid. In the present study the proportion of propionic acid was already very high without the addition of Bb-12, and it is questionable whether it is possible to increase the proportion further, or if a maximum has already been reached. Thus, the substrate seems to be of crucial importance for the formation of CAs, as was also demonstrated in paper II.

+UCC500. UCC500 also gave a higher caecal pool of CAs together with IN when fed to rats. To some extent, this could be ascribed to succinic acid. This is interesting, as this acid has been associated with reduced or changed bacterial activity, such as after some types of antibiotic treatment (Berggren, 1996). Low numbers of certain bacteria, such as bacteroides and propionibacteria, have been reported to reduce the

formation of propionic acid whereas the formation of succinic acid was found to increase (Macfarlane & Gibson, 1995). One could speculate whether UCC500 together with IN modifies the composition/activity of certain organisms, resulting in an increase in the amount of succinic acid formed. The production of propionic acid from succinic acid by certain microorganisms has been shown to be dependent on vitamin B12 (Strobel, 1992), and if the B12-producing components of the microbiota are reduced by, for example, antibiotic treatment, the synthesis of this vitamin will decline. In paper II, no increase in the CA formation and only minor effects on the proportions of CAs could be seen in the hindgut of rats when UCC500 was added to a diet containing IN-ls. These discrepancies, with different types of inulin, show that the outcome of the CA formation depends to a great extent on physico-chemical properties, such as solubility of the substrate and also on the addition of probiotic, at least for inulin.

GG and the mixture of probiotics. The caecal concentrations of CAs were lower with GG (52.7 µmol/g, $P < 0.05$) and the mixture of probiotic strains (41.5 µmol/g, $P < 0.01$) in the diet than with the diet witout any probiotics (79.1 µmol/g). This was partly due to the higher weight of the caecal content. Another explanation could be increased adhesion of GG to the mucosa, causing increased absorption of CAs, or that less bifidobacteria can utilize the bifidogenic inulin.

Colonic pH. Caecal pH decreased when probiotics were added to the diets, from 6.8 to 6.1 with Bb-12 and 6.2 with GG ($P < 0.05$). The change in caecal pH in rats fed IN, in relation to its concentration, was in accordance with findings in previous studies (Berggren *et al.*, 1993). However, when Bb-12 and GG were included in the diet, the pH was significantly lower than expected from the CA concentration. Bb-12 did not have the same effect in paper II. The reason for this is not known, but it might be ascribed to components other than CAs. Ammonia, for example, is formed during colonic fermentation of protein (Macfarlane & Cummings, 1991) and it may

be speculated, whether less amounts are formed in the presence of GG and Bb-12. Interestingly, bifidobacteria have been shown to decrease the number of ammonia-producing bacteria such as Clostridia, resulting in reduced production of ammonia and possibly explaining the low pH (Zhao *et al.*, 2004). GG and Bb-12 may be especially prone to inhibit the growth of pathogens. Subjects with impaired liver function have an increased production of ammonia (Zhao *et al.*, 2004), and it has been shown that probiotics can reduce this production (Sakata *et al.*, 1999).

Lactic acid. When the rats were fed probiotics, the proportion of butyric acid was lower and that of lactic acid generally higher, both in the caecum and distal colon. Furthermore, the proportion of propionic acid was lower in the distal colon than in rats fed IN without probiotics. Both *Lactobacillus* and *Bifidobacteria* have been reported to form high amounts of lactic acid (Hill, 1995) and when the number of these bacteria increases, others are suppressed, leading to a modified profile of CAs and increased proportions of lactic acid. These probiotic effects were seen especially in the distal part of the colon. Similar results were seen in paper II, where the property of a probiotic strain to form a specific CA seemed to depend, to a great extent, on the amount of substrate available (Nilsson *et al.*, 2006b). Thus, with limited amounts of substrate in relation to the number of bifidobacteria there was an increased formation of lactic acid, while abundant amounts of substrate seem to regulate the CA formation.

Caecal tissue weight. Rats fed GG had the highest caecal content (0.73 g versus mean 0.53 ± 0.05 g per gram ingested IC for the other groups) and tissue weight (1.02 g versus mean 0.82 ± 0.03 g for the other groups). The increased weight of the caecal content in rats fed GG may be due to an increased formation of CAs, increased water/dry matter content, or the addition of bacteria as such. However, the caecal concentrations were lower and the caecal pools of CAs were similar to those in rats fed IN only, and could therefore not be an explanation. Another reason could be that

the bacteria added inhibit the enzymes responsible for the degradation of IN, resulting in a lower degree of caecal fermentation, as indicated by the lower caecal concentrations of CAs. However, this suggestion is contradictory to the similar concentrations of CAs in the proximal and distal colon of rats with GG in the diet, as compared to IN only. Also, the faecal dry weights were similar in rats fed the two diets. The amount of GG added was only 4 g/kg diet (corresponding to 0.05 g per day per rat) and could thus not contribute to the increased caecal content *per se*. A more likely explanation would therefore be that GG increased the bacterial activity/metabolism, leading to an increase in bacterial mass and thus also higher amounts of water in the caecum (Montesi *et al.*, 2005), which in turn may also explain the higher caecal tissue weight. This seems to be a probable explanation, as the correlation between caecal content and tissue weight was found to be high ($r = 0.78$, $P < 0.05$), whereas there was no correlation between the formation of CAs and caecal tissue weight ($r = 0.40$, $P < 0.05$), indicating that the hypertrophic effect observed in rats fed oligosaccharides was mainly due to increased caecal content, rather than the formation of CAs. Similar observations have been made by others (Wyatt *et al.*, 1988; Kishida *et al.*, 2001) and explained by an increase in mechanical stress (Calvert *et al.*, 1989). The correlation between caecal tissue weight and content was also higher than the correlation between CAs and caecal tissue weight in paper II ($r = 0.90$, $P < 0.05$). It has also been suggested that the caecal enlargement is associated with an increase in mucosal cell proliferation, which is stimulated by CAs mediated by a systemic mechanism *in vivo* (Sakata, 1987). Furthermore, an increased production of CAs may increase the proglucagon mRNA expression and the secretion of glucagon-like peptide-2, which are other factors that affect mucosal cell proliferation (Massimino *et al.*, 1998). CAs enemas have also been shown to have a transmural trophic effect and to preserve the mucosal surface area of dysfunctional and atrophic colon in rats (Kissmeyer-Nielsen *et al.*, 1995).

Interestingly, rats fed GG had significantly higher caecal tissue weights than rats fed the diet wiout any probiotics, although they had a similar caecal pool size. One explanation for this finding could be that this strain increases the absorption of CAs in some way. In this respect, it is noteworthy that some types of probiotic bacteria (*Lactobacillus salivarius*, UCC 118) have been shown to increase calcium uptake in human intestinal-like Caco-2 cells, and were therefore suggested to have the ability to increase intestinal calcium uptake (Gilman & Cashman, 2006). The caecal tissue weight and content was only significantly higher for rats fed GG, although the same tendencies were seen in rats fed the other probiotic strains. A longer duration of the study might have given more pronounced differences. However, the rat model used has been shown to yield stable fermentation of different types of dietary fibre after an adaptation time of 5–7 days (Nyman & Asp, 1985; Brunsgaard *et al.*, 1995; Kleessen *et al.*, 1997a). Whether or not this is valid when probiotics are included in the diet remains to be investigated.

5.1.4 Correlation of CAs in the distal colon and faeces

The concentrations of CAs were generally higher in faeces than in the distal colon, but a strong correlation was seen regarding the concentrations of acetic, propionic and butyric acid ($r = 0.96–1.0$, $P < 0.05$). However, when lactic acid was included there was no longer any correlation ($r = 0.80$, $P = 0.19$). Furthermore, different substrates gave different slopes and this depended on the probiotic added. This is interesting, as most colonic diseases such as ulcerative colitis and cancer mainly appear in the distal part of the colon and measurements of CAs in humans are mostly done in faeces.

5.2 Effects of probiotics on body weight increment

The body weight gain was significantly lower in rats fed IN-ls and UCC500 (0.15 g/g, $P < 0.01$) (paper II) than in rats fed only IN-ls. The mechanism behind this is not known. It could not be due to an increased formation of CAs, as caecal pools and concentrations throughout the hindgut were very similar irrespective of whether probiotics were added or not. No effects could be seen with any other combinations in paper II. In paper III, the weight gain was lower in rats fed GG and IN (0.21 g/g, $P < 0.05$) than rats fed IN only (0.29 g/g). In this case, the lower weight might be explained by the CA concentrations, as these were lower in rats fed GG and IN than with IN only. In paper I, the highest weight gain was demonstrated in rats fed OF. These rats had a high concentration of butyric acid, and maybe this could be a reason. Concerning IN-ls and UCC500, no differences in the proportion of butyric acid could be seen irrespective of whether or not UCC500 was added. However, the proportion of acetic acid was lower in the distal part of the colon in rats fed UCC500 (34% as opposed to 46%). There have been suggestions that the microbiota may affect lipid metabolism, and that some probiotic strains may offer protection against obesity if included in the diet (Bäckhed *et al.*, 2004; Ley *et al.*, 2005). Ley *et al.* (2005) found that there were considerable differences in the composition of the gut microbiota in obese mice as compared with lean mice, suggesting that intentional manipulation of the gut microbiota may be useful for regulating energy balance in obese individuals.

5.3 CA formation in humans

The use of a rat model is justifiable for mechanistic work and testing of different experimental parameters, such as the effects of molecular weight and process

conditions. However, CA patterns following ingestion of food products containing various prebiotic carbohydrates need to be evaluated in man for selection of relevant foods for interventions in target groups.

Most of the CAs formed in the human colon are absorbed, and it can be argued that it is difficult to see any effects of diet by studying faecal concentrations of CAs. However, as most colonic diseases occur in the distal part of the colon, faecal concentrations of CAs may reflect the distal concentration and therefore be justified. Studies on rats have also shown that there is a good correlation between the distal and faecal concentrations of acetic, propionic and butyric acids, although the faecal concentrations were generally somewhat higher (paper II) (Nilsson *et al.*, 2006b). Another important issue is that faecal concentrations reflect the exposure and not the utilization of CAs in the distal part of the large intestine. Thus, if the proportion that is absorbed in the rectum and distal colon remains unchanged but the faecal volume increases after indigestible carbohydrate intake, faecal data will give an underestimate of any increase in rectal utilization of CAs.

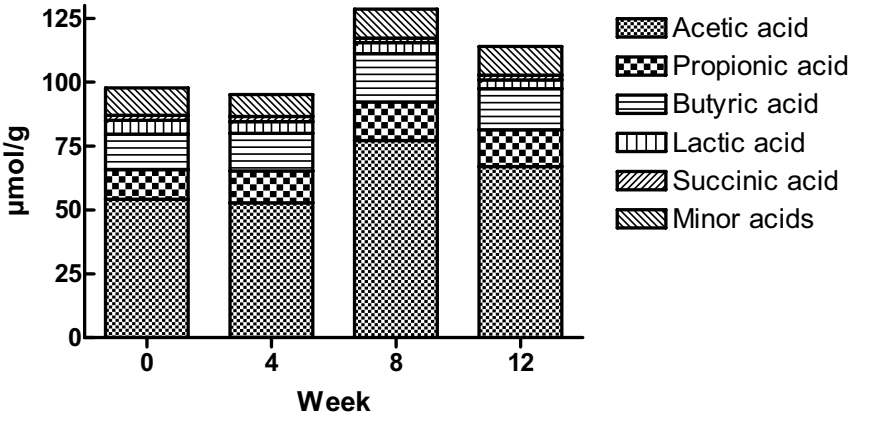

Figure 4. Concentrations (µmol/g wet content) of carboxylic acids in faeces of humans given β-glucan enriched oat bran bread

The aim of this study was to elucidate how the faecal concentrations of CAs vary in healthy individuals following dietary supplementation with β-glucan enriched oat bran bread, and whether the concentration varies over time during consecutive days. For this purpose, 25 healthy subjects were recruited for the study. The subjects were given 40 g β-glucan-enriched oat bran as four slices of bread per day. CAs were analysed in faeces collected over three days at entry and after 4, 8 and 12 weeks on this diet.

No effects on CA concentration were seen after 4 weeks (Figure 4), except for a reduced formation of formic acid. However, after 8 weeks the mean concentration of acetic, propionic and butyric acids had increased ($p < 0.001$). Thus, the mean concentration of acetic acid increased from 54.2 μmol/g to 77.2 μmol/g, that of propionic acid from 11.6 to 15.0 μmol/g and that of butyric acid from 13.9 to 19.0 μmol/g. Furthermore, in 19 of the 20 subjects who completed the study, the total concentration of CAs was higher than ($n = 17$) or similar to ($n = 2$) values at entry. Similar profiles were seen with acetic, propionic and butyric acid. The concentrations of isobutyric acid ($P < 0.001$) and isovaleric acid ($P < 0.05$) were also higher after 8 weeks on the oat bran diet, while that of lactic acid was lower ($P < 0.05$). After 12 weeks, the faecal concentrations were still higher and that of lactic acid lower; the exceptions were butyric and isovaleric acid.

The present study shows that it is possible to increase the CA concentration by adding β-glucans to the diet. The increase in butyric acid concentration must be considered positive, as butyric acid has been suggested to protect against colonic diseases. However, the concentration of CAs increased first after 8 weeks. The reason for this is not known, but it may be due to a quite stable and balanced composition of the colonic microbiota in healthy subjects, and therefore a comparatively longer time may be needed to increase the number of bacteria that can ferment the increased amount of available substrate. This may also explain the smaller range in the

concentration of CAs (data not shown) as compared to a previous study on patients with ulcerative colitis who ate the same type of oat bran (Hallert *et al.*, 2003). An increase in the faecal excretion of CAs has also been seen in other studies on humans when dietary fibre was added to the diet (Jenkins *et al.*, 2001).

The individual range of faecal concentrations of CAs was considerable. However, at entry the concentrations of acetic, propionic and butyric acid were comparatively assembled, and there were only a few outliers (two to three of twenty subjects), while the individual variation was higher with lactic acid. After 8 weeks on the oat bran, the individual variation was considerably higher.

The faecal concentration of CAs was also determined over three consecutive days. No significant differences in the concentration of CAs could be seen in any of the subjects over these days, which may justify the use of this human model for evaluating food products especially prone to generate butyric acid.

The total faecal concentrations of CAs at the start of the study were similar to those in previous studies on subjects with ulcerative colitis (Hallert *et al.*, 2003) and irritable bowel syndrome (Molin G, Noeback S, Johansson M-J, Berggren A, Nyman M, Björck I, Jeppsson B, unpublished results). However, the concentration of butyric acid was higher in healthy subjects than in patients with ulcerative colitis (13.9 µmol/g versus 11.1 µmol/g), while the concentration of lactic acid was lower (5.4 versus 15.9 µmol/g) (Hallert *et al.*, 2003). Thus, the average proportion of butyric acid at the entry of the study was high (14%) compared to studies on patients with ulcerative colitis (11%) having the same type of oat bran. Differences in the concentrations of butyric acid and lactic acid between healthy individuals and patients with ulcerative colitis have also been shown by others. Thus, Vernia and colleagues (Vernia *et al.*, 1988) found that the concentrations of butyric acid were particularly low in patients with severe ulcerative colitis, and those of lactic acid high.

Interestingly, high colonic concentrations of lactic acid have been associated with increased risk of diarrhoea and mucosal inflammation (Cummings, 1995).

The increase in the concentration of CAs seen after 8 weeks was due to all the main acids, i.e. acetic, propionic and butyric acid. Similar results, with an increase in acetic, propionic and butyric acids have been obtained in rats with β-glucans from barley (Berggren *et al.*, 1993). However, in a study on humans with ulcerative colitis where the same type of β-glucan enriched oat bran was used as in the present study, there was a specific increase in butyric acid already after 4 weeks (Hallert *et al.*, 2003). The differences could be due to the fact that patients with ulcerative colitis have a diminished capacity to utilize butyric acid and thus the excretion of this specific acid increases. Another difference may be that patients with ulcerative colitis have another composition of the microbiota than healthy people, leading to the use of different metabolic pathways by the microbiota, and different CAs. Thus, subjects with ulcerative colitis have been shown to have a higher number of sulphate-reducing bacteria (Cummings, 1995) and in another study it was shown that in the active phase of the disease, the number of lactobacilli was significantly lower than in remission (Bullock *et al.*, 2004). The differences may also be due to the oat bran, since the bread was much denser in the present study — indicating a different structure of the oat β-glucans and differences in baking properties — which might lead to another profile of CAs. In a study on rats, fructo-oligosaccharides of different molecular weight were shown to give different patterns of SCFAs during fermentation (Nilsson & Nyman, 2005). Thus, fructo-oligosaccharides with a low molecular weight gave high proportions of butyric acid, and those with high molecular weight were especially prone to yield propionic acid. Moreover, Wood (2004) found that β-glucans with different physico-chemical properties behave differently when frozen.

6 Conclusions

- The DP and the solubility of the fructo-oligosaccharides were of great importance for CA formation, while the monomeric composition seemed to be of less importance. OF with a low DP generated high levels of butyric acid, whereas IN with a high DP gave high levels of propionic acid. IN-ls with a comparatively low solubility gave a higher formation of butyric acid in the distal part of the colon than IN. Lactulose and lactitol both yielded high proportions of acetic acid and low proportions of butyric acid.

- Probiotic bacteria affected both the CA pattern and the site of CA release in the hindgut of rats. The combination of pre- and probiotics was of great importance for the outcome of the results. The effect of probiotics appears to be more pronounced in the distal part of the colon, where the amount of substrate is probably limited in relation to the number of probiotics.

- Bb-12 in combination with the slowly fermentable IN-ls specifically increased the caecal concentration of propionic acid in the rats, but decreased it in the distal part of the colon. An increase in lactic acid could be seen instead. When Bb-12 was combined with lactitol, the concentration in the distal colon decreased, indicating an accelerated absorption of CAs. The concentration of CAs was higher throughout the hindgut with pectin, because of increased formation of acetic acid, and in the distal part of the colon also because of increased amounts of propionic and butyric acid.

- When rats were fed UCC500 together with pectin, there was a shift in the amount of CAs from the caecum to the distal part of the colon. The formation of CAs in combination with lactitol seemed to be stimulated in the caecum of rats. UCC500

had no effect on CA formation when given together with IN-ls, except for a lower proportion of acetic acid in the distal part of the colon.

- When diets containing IN were supplemented with Bb-12 and UCC500, the caecal pool of CAs increased compared with IN only, while the concentrations of CAs decreased after supplementation with GG and a mixture of the strains. None of the probiotics investigated increased the proportion of butyric acid and propionic acid anywhere in the hindgut of rats. The proportion of lactic acid was generally higher, however, and in rats fed UCC500, also that of succinic acid. Rats fed GG had the lowest weight gain and the highest caecal tissue weight. The caecal pH in rats fed GG and Bb-12 was lower than expected from the concentration of CAs, indicating that these strains reduce the formation of alkaline components, e.g. ammonia.

- β-glucan enriched oat bran increased the faecal concentration of CAs in healthy subjects after 8 weeks. There was no further increase after 12 weeks. No significant differences in CA concentrations could be seen between three consecutive days, supporting the stability in faecal levels of CAs after these experimental periods. An experimental design, with 20 subjects and dietary supplementation with the test food product for 8 weeks, thus appears suitable for screening of potential differences in faecal CA patterns achievable by dietary means.

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