fermentation and colonic carcinogenesis

EFFECTS OF RESISTANT STARCH ON LUMINAL CONTENTS AND MUCOSAL PROLIFERATION

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FERMENTATION

AND

COLONIC CARCINOGENESIS

Effects of resistant starch on
luminal contents and mucosal proliferation.

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Chapter 1

Introduction and outline of the study

Large bowel cancer is one of the leading causes of cancer death in the western world. Marked international differences in incidence rates suggests that environmental factors and especially dietary factors play an important role in the etiology of colonic cancer. It has been estimated that 90% of the geographic differences are based on dietary differences, with an increased risk for a high fat intake and a protective effect for dietary fiber. This protection of dietary fiber is based on several mechanisms, fecal bulking and colonic fermentation being the most important factors. Fecal bulking is related to water-absorption of non-digested fiber constituents and results in an dilution of the fecal content and may contribute to a shortening of the colonic transit time. Due to both factors, the contact-time of potential hazardous substances in the colonic lumen will diminish. Part of fiber constituents can be degraded by colonic bacteria. This fermentation process results in the production of short chain fatty acids, which play an important role in intraluminal metabolism and in nutrition of colonocytes. The decrease in intracolonic pH, which is the result of short chain fatty acid production, may influence -amongst others- the transformation of primary into secondary bile acids. These acids can damage the mucosal cells and are thought to be co-mutagenic. Furthermore, the acid environment may result in precipitation of the bile acids.

Studies in which a high-fiber diet was given to healthy volunteers, have been shown conflicting results. Partly, this is related to differences in physico-chemical properties between various fibers, resulting in important differences in water-holding capacity and fermentability. Furthermore, high-fiber diets have been shown less attractive to most western people.

In contrast to the assumption that dietary starch is completely hydrolysed and absorbed in the small bowel, recent studies have been shown that a certain amount of starch, depending on processing, is resistant against small bowel digestion and may be fermented in the colon. In fact, it seems a more important source of fermentable substrate than fiber. As it is possible to incorporate this resistant starch in our normal diet, it seems an attractive alternative for fiber-preparations in stimulating colonic fermentation and hopefully in the prevention of large bowel cancer.

In the next chapter of this thesis, we will give an overview of the effects of fermentation on colonic metabolism, with respect to factors

involved in carcinogenesis. The influence of fiber on bile acid metabolism is described in chapter 3. To study the membrane-damaging potential of bile acids to colonic mucosal cells, we developed a new cytotoxicity assay (chapter 4). An increased prolifer-ation of the colonic mucosal cells, probably mediated by the cytotoxic effects of bile acids, has been shown to be a biomarker for large bowel carcinogenesis. We adapted a Proliferating Cell Nuclear Antigen-immunostaining technic to estimate proliferation in colonic mucosa (chapter 5). In chapter 6, we studied the in vitro fermenta bility of amylomaize, which is a maize-starch containing 62% resistant starch. In chapter 7 is demonstrated that consumption of 45 g/d amylomaize results in a significant stimulation of colonic fermentation, and in chapter 8 we determinated the effects of amylomaize on cytotoxicity of feces, on bile acid metabolism and on colonic mucosal proliferation.

Chapter 2

The role of carbohydrate fermentation in colon cancer prevention

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Abstract.

Diet is an important factor in the development of colonic cancer. Fiber
has been shown to decrease this risk. Part of this protective effect is
probaly mediated by colonic fermentation. About 10% of starch in
the normal diet escapes digestion and absorption in the small bowel,
and is therefore called resistant starch. This is a considerably larger
source of fermentable substrate than fiber in the diet and could thus
contribute significantly to the prevention of this malignancy. Short
chain fatty acids, produced during fermentation, reduce colonic pH,
affecting the intraluminal concentration of the putative co-carcin-
ogenic secondary bile acids by precipitation, and by inhibition of their
enzymatic formation from primary bile acids. The role of secondary
bile acids in promoting colonic carcinogenesis is probably mediated by
their cytotoxic effect on colonic mucosa, leading to a compensatory
increase in proliferation. A hyperproliferative mucosa, having an
enhanced sensitivity to mutagenic substances, is associated with an
increased risk of colorectal cancer. Butyrate, one of the short chain
fatty acids, could be significant as it has anti-neoplastic properties in
vitro and in vivo.
We conclude that fermentation is probably the key factor in the
protective effect of fiber on colon carcinogenesis. Furthermore,
consumption of resistant starch seems to be another way of stimula-
ting fermentation.

Introduction.

The incidence of colon cancer varies largely between different popula-
tions. In western countries it is one of the most frequent cancers, but
low incidence rates are observed in parts of South-America, Africa
and Asia. Genetic factors may play a role in these differences, but it
is widely accepted that environmental factors are the most important.
It has been estimated that differences in diet may account for 90% of
the variation in the incidence of colorectal cancer[1]. Epidemiological
studies in Japan, a country with a traditionally low incidence of colon
cancer, have shown that individuals, migrating to high risk countries,
incurred the same high risk [2]. This increased risk of colon cancer is
supposedly mediated by dietary changes, from the low risk japanese
diet to a high risk western diet. Other immigrant studies showed
comparable results [3]. The incidence of colon cancer also varies
amongst groups with different life styles within one population. For
example there is a much lower incidence amoung Seventh Day
Adventists, who consume a strict vegetarian diet, compared to the
rest of the American population [4]. The observation that Africans, con-
suming a high amount of dietary fiber, have a low large bowel
incidence rate, caused Burkitt to speculate that fiber could have a
protective effect on colon cancer [5]. Since then, several epidemiologi-
cal and case-control studies have been carried out, most of them
supporting the fiber theory [6,7]. Controversy exists about different
protective effects of fiber from vegetables or from cereals. In a large
meta-analysis of 13 case-control studies, an odds ratio of 0.58 was
calculated for high versus low fiber intake and of 0.48 for high versus
low vegetable consumption [8]. In this meta-analysis, seven out of 13
studies strongly supported for the fiber hypothesis, two gave
moderate and two gave an equivocal support. There are few large
scale prospective studies examing the relationship between dietary
fiber consumption and large bowel cancer risk. The Nurses' Health
Study, a prospective study of more than 80.000 American nurses,
has shown that the group with the highest animal fat and the lowest
crude fiber intake had the highest risk for large bowel cancer.
However, the effect of a high fat consumption was more significant
than the low fiber intake [9]. Recently, another prospective study has
found a strong negative correlation between fiber consumption and
colonic adenomas with a relative risk of 0.36 in the high fiber group[10].

Interestingly in familial adenomatous polyposis patients, who have a high risk of developing colonic cancer, supplementation of the diet with wheat bran significantly reduced the number of polyps in the remaining rectum after ileorectal anastomosis [11].
Finally, the protective effect of dietary fiber was recently supported by Cummings et al, who demonstrated a significant correlation between dietary fiber intake and stool weight, and an inverse relationship between stool weight and colorectal cancer incidence in 20 different populations [12].
In conclusion, there is a growing body of support for the protective effect of dietary fiber against the risk of colonic cancer.

Mechanisms for the protection of fiber on colonic carcinogenesis.

Several mechanisms have been proposed to explain the protective effect of dietary fiber. As it is not digested in the small intestine, it will enter the caecum and be partly fermented by colonic anaerobic bacteria. This results in the production of gas (hydrogen, methane, and CO_2) and short chain fatty acids (SCFA's), notably acetate, propionate, and butyrate. SCFA's are an important source of energy for these bacteria and thus the bacterial mass increases [13,14]. Only a small proportion of fiber from cereals is fermented, but the remaining carbohydrates can absorb water and contribute to fecal bulking. The increase in fecal mass due to bacterial growth and residual components of fiber results in a dilution of the contents of the large bowel, and therefore lowers concentrations of putative (co-)carcinogens like faecopentaenes, heterocyclic amines and secondary bile acids. Furthermore, fecal bulking results in a decreased colonic transit, reducing the exposure time to irritants and (co-)car-cinogens.
Secondly, SCFA production will lower the colonic pH. A more acid colonic environment might be associated with a lower colon cancer risk [15,16]. Some studies have shown that decreasing the colonic pH has a protective effect against experimentally induced colon cancer [17], although others have reported conflicting results [18]. The means by which an acid colonic environment protects against carcinogenesis is not fully understood, but it is probable that precipitation of bile acids and inhibition of the formation of secondary bile acids play an

important part in this process.

Finally, it has been suggested that butyrate, one of the SCFA's produced by fermentation, can exert a direct protective effect on the colonic mucosa [19].

The role of bile acids and SCFA's will be discussed extensively.

Fermentable substrate.

The relation between fiber, fermentation, and the prevention of colon cancer has been the subject of extensive research. One of the problems in interpreting the results is, that fiber is the collective term for a number of food components, all having individual physical and chemical properties [20-23]. Fiber is defined as that part of the plant wall matrix, which cannot be broken down by human enzymes. It includes all non-α-glucan polymers. The most important components of dietary fiber are cellulose, hemicellulose, pectin and lignin. They are fermentable to differing degrees, pectin being an example of a readily fermentable substrate and lignin an inert material [24-26]. Furthermore, dietary fiber is not normally consumed alone but as part of grains, vegetables and fruits. The ways in which these food products are processed, and the particle size of fiber also affect fermentability. The different properties of fiber components probably contribute largely to the controversy to its role in colonic cancer prevention.

Besides fiber, several other sources of fermentable substrate have been identified; the most important being the starch (and its degradation products) that escapes small bowel digestion. This so called 'resistant starch' has recently become an interesting area of research. Other substrates for cecal fermentation are proteins, glycoprotein and mucopolysaccharides [27], but these are quantatively less relevant. The daily amount of protein that reaches the large bowel is equivalent to only 1-2 g nitrogen [28]. During rapid fermentation, branched SCFA's are also produced (iso-butyrate and iso-valerate) [29,30]. In subjects with carbohydrate malabsorption (e.g. subjects with lactase deficiency), fermentation is further stimulated by the additional load of unabsorbed carbohydrates [31].

In conclusion, the diet contains a wide variety of potential substrates for fermentation but current findings would suggest the most important are resistant starch, fiber, and to a lesser extent protein.

Resistant Starch.

During the last decade, it has been observed that not all ingested starch is absorbed in the small bowel [27,32-35]. This fraction of non-absorbed starch is called resistant starch. The name was originally introduced by Englyst and Cummings [36] for retrograded amylose, but now the term has expanded and the definition for resistant starch is the fraction of starch and starch degradation products that are not absorbed in the small bowel of healthy subjects. Starch can be classified as readily digestible starch, slowly (but completely) digestible starch and resistant starch, depending on the physical form and the susceptibility to human α-amylase. Three different subgroups of resistant starch can be distinguished: type I or physically inaccessible starch (e.g. partly milled grains); type II or starch in granules (e.g. raw potato and bananas); type III or retrograded starch (e.g. cooked and cooled potatoes, old bread).

In a normal western diet, about 10% of ingested starch escapes small bowel absorption and will enter the colon [35]. The amount of resistant starch in food products depends on cooking and processing. Raw potato starch consists of 75% resistant starch [37]. In freshly cooked and warm potatoes, only 3% of starch is still resistant but after cooling the resistant starch fraction increases to 12% [38]. This is probably related to changes in the three-dimensional molecular structure of the starch chains; starch in highly crystallized form being generally resistant to mammalian α-amylase. Colonic bacterial enzymes however, can degrade this resistant starch completely [39]. In contrast to most fiber components, starch is completely fermented in the large bowel and that is why starch is probably the primary of fermentable substrate [27,35]. The hypothesis that starch can have a protective effect on colonic carcinogenesis is supported by a study of Thornton et al, in which it has been demonstrated that starch fermentation in colonic adenoma-patients is relatively low compared to controls [40].

It is concluded that a significant amount of starch is fermented in the large bowel, and that it is an important source for short chain fatty acid production.

The relation between colonic pH and large bowel cancer.

The production of short chain fatty acids during the fermentation process results in a decrease of the intraluminal pH [41]. This could have implications for colonic carcinogenesis, as several epidemiological studies have supported a lower colon cancer risk with lower fecal pH. In India, railway workers with a fecal pH below 6 had a lower colon cancer risk, while a high incidence was found in persons with a fecal pH of 8 or more [42]. In African ethnic populations, a low colon cancer risk has been associated with a low fecal pH [16], and in American Seventh Day Adventists, who are known to have a low colon cancer risk, the fecal pH is significantly reduced compared to controls and colon cancer patients [43]. Although not supported by all case control studies [44], most authors agree on the protective effect of a low colonic pH on large bowel carcinogenesis. It is possible to decrease the colonic pH to a variable extent by dietary manipulation, by using fibers such as oat bran, wheat bran and cellulose [18,20,45-47] or by supplementation of lactulose [48-51]; although not all studies demonstrate a change of fecal pH after fiber or lactulose supplementation. We speculate that this controversy is related to the fact that fecal pH is a poor indicator of acidity in the proximal colon. Cummings et al demonstrated that in humans the mean caecal pH of 5.6 gradually rose to 6.6 in the descending colon [41], these results were confirmed using a radiotelemetry pH-device [52]. The observed change in pH along the colon is due to the buffering capacity of the luminal contents, and to the rapid absorption of SCFA's during their transport from caecum to descending colon [20,53,54]. Thus fecal pH is not necessarily a good indicator of fermentation and acidity in the proximal colon.

The influence of colonic pH on bile acid metabolism.

In 1981 it was proposed by Thornton that a high colonic pH promotes co-carcinogen formation from bile acids, and that acidification of the colonic contents by SCFA formation may prevent this [15]. Bile acids, especially secondary bile acids, are irritating to the colonic epithelium and several experiments have shown a co-carcinogenic effect (see below). Conjugated primary bile acids, produced in the liver and excreted in bile, are largely (90%) absorbed in the small bowel, the

remaining 10% being transported into the colon. Bacterial enzymes deconjugate these bile acids very rapidly, and anaerobic bacteria, producing 7α-dehydroxylase, convert primary bile acids into the secondary bile acids deoxycholic acid and lithocholic acid [55,56]. The 7α-dehydroxylase-enzymes are pH dependent, with an optimum at pH 7-8, and are inactivated at pH below 5.5 [57]. In a fecal incubation system, we have demonstrated a complete conversion of the primary bile acids, cholic acid and chenodeoxycholic acid (1 mmol/l), into deoxycholic acid and lithocholic acid respectively, during an 24 hour incubation at ph = 6.0 (fig.1A). Decreasing the pH to 5.0, resulted in complete inhibition of the bacterial conversion (fig 1B).

Precipitation of bile acids is the alternative mechanism by which their interaction with colonic mucosa may be prevented. Soluble unconjugated bile acids have pK_a's of around 5.0 [58] and if the pH decreases below this as a result of fermentation, precipitation is possible. As only the soluble bile acids are able to affect the mucosal cells, a shift from the aqueous phase of stool to the insoluble compartment will result in a decrease in their putative damaging effect [59]. Indeed, in a rat model, the toxic effect of deoxycholic acid to the colonic mucosa was prevented by decreasing the pH from 7.9 to 5.5 [60].
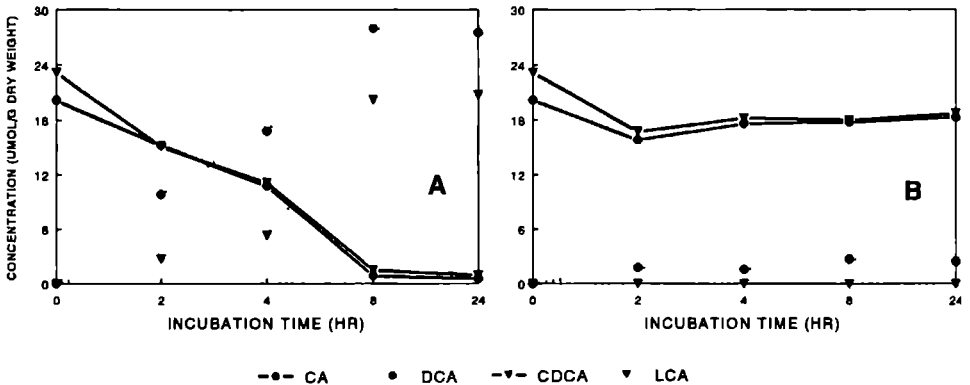

Figure 1: Conversion of primary bile acids in a fecal incubation system at pH = 6.0 (A) and at pH = 5.0 (B). Bile acids were determined by gas chromatography in freeze-dried material (CA: Cholic acid; DCA: Deoxycholic acid; CDCA: Chenodeoxycholic acid; LCA: Lithocholic acid).

It can be concluded that the protective effect of decreased luminal pH on colon cancer incidence is at least partly mediated by the inhibition of formation of bile acids or by their precipitation.

The role of bile acids in colonic carcinogenesis.

In 1969, Aries and Hill proposed that colon cancer was caused by bacterial conversion of bile acids into a carcinogenic metabolite in the gut [61], but to date these specific metabolites have not been found. However, several animal experiments have demonstrated that secondary bile acids have a promoting effect on large bowel carcinogenesis. In experiments with rats, rectal infusion of the secondary bile acids deoxycholic acid and lithocholic acid in rats has been shown to stimulate experimentally-induced carcinogenesis [62]. Similarly, increasing the fecal bile acid concentration by feeding bile acids or cholestyramine, or by diversion of the bile duct to open into the caecum, has also demonstrated an enhancement of mucosal damage and the number of experimentally-induced colorectal tumours in rats [63,64]. Bile acids given intrarectally without chemical carcinogens did not result in tumor formation, suggesting bile acids themselves act as promoter or co-carcinogen but not as real carcinogen. Co-mutagenicity has also been demonstrated in vitro for secondary bile acids using the Ames Salmonella mutation test [65].
Although there is much evidence from animal experiments to support the influence of bile acids on colonic carcinogenesis, their effect in humans is still not proven. Several epidemiological studies have been carried out, comparing the fecal bile acid concentration in high and low risk populations. Most of these population studies have demonstrated a positive correlation between fecal bile acid concentration and colon cancer risk, however case control studies have provided conflicting results [66,67]. We suggest that the discrepancy between these studies is partly related to the fact that only total bile acid concentrations were measured. It should be stressed that most of the fecal bile acids are precipitated or bound to luminal residues, leaving less than 1% in solution, and thus available for interaction with mucosal cells (unpublished results). Only recently has emphasis been placed on the bile acid concentration in the aqueous phase of stool, rather than the total concentration [59,60].

Serum bile acid concentrations are a reflection of the soluble fraction in the gut lumen and are therefore perhaps a better indicator of risk than total fecal concentrations. Van der Werf et al have demonstrated an increased deoxycholic acid absorption in patients with colonic adenomas [68], and very recently a consistently elevated level of serum deoxycholic acid has been found in patients with colonic adenomas [69]. We conclude that, altogether there is substantial evidence supporting the promoting effect of secondary bile acids on colonic carcinogenesis.

Cytotoxicity, hyperproliferation and tumor promotion of bile acids.

Damaging effects of various bile acids on the colonic mucosa have been described at the concentrations present in the aqueous phase of stool. Bile acids can disrupt the integrity of the cell membrane of colonic mucosal cells [60,64,70,71]. The increased cell loss will stimulates a compensatory cell renewal by increased mucosal proliferation. Thus, dietary manipulation resulting in a rise in colonic bile acid concentration causes increased mucosal proliferation [71,72]. Surgical diversion of bile, eliminating bile acids in the colon has also been shown to reduce colonic proliferation [73]. In addition to the attractive hypothesis that hyperproliferation is induced by the cytotoxic potential of bile acids, there is also evidence of a direct stimulatory effect of several bile acids on proliferation, probably mediated by Protein Kinase C activation in the colonic cell [74,75]. Protein Kinase C appears to play a critical role in tumor promotion and in the action of growth factors [76].

The bile acid induced increase in mucosal proliferation may be the key step in the association between bile acids and colon carcinogenesis. It has been demonstrated that a hyperproliferative colonic mucosa is more susceptible to carcinogens than a quiescent mucosa [77-79]. When proliferation is increased, the fraction of cells in S-phase ('target-cells') is relatively high, possibly resulting in an increased potency of intraluminal mutagenic substances.

Large bowel neoplasms are associated with changes in proliferative characteristics and in patients with colonic carcinomas, an overall increased colonic mucosal proliferation has been demonstrated [80].

Furthermore, the proliferative compartment expands from the basal part of the crypts to the luminal surface [81]. Similar changes in proliferative activity can be seen in patients with familial adenomatous polyposis, who are at high risk of developing colonic cancer [82]. The relationship between hyperproliferation and colorectal cancer has led to the use of mucosal proliferation as an established intermediate biomarker in assessing colon cancer risk. In this respect, it is interesting that dietary wheat bran supplementation has been demonstrated to decrease rectal proliferation in high risk patients with previously resected large bowel cancer [83].

In conclusion, the tumor promoting effects of bile acids may be mediated by a direct effect or by their cytotoxic potential and the compensatory epithelial hyperproliferation, leading to a increased effect of mutagenic substances.

The effect of butyrate on cellular differentiation and proliferation.

One of the SCFA's produced by fermentation is butyrate. It is an important factor in the metabolic welfare of the colonic epithelium, and it is the primary energy source for the mucosal cells [84,85]. Besides influencing intraluminal pH (as do other SCFA's), butyrate is purported to have typical anti-neoplastic effects. Several experiments have demonstrated that butyrate can induce differentiation in cultured human colonic adenocarcinoma cells [86-88]. Furthermore, butyrate can reduce the growth characteristics of human colonic cancer cell-lines [86,89] and human colonic mucosal cells in vitro [19] and in vivo [90]. Butyrate derivatives are currently being studied for anti-neoplastic activity [91]. It has been speculated that this regulatory effect is mediated by an inhibition of histone deacetylase and phosphorylation [87], but the exact mechanism is still unclear. In contrast, an increase in mucosal proliferation has also been demonstrated by butyrate [92]. But as it is a major energy-source for the large bowel mucosa, proliferation may be stimulated in certain circumstances by this trophic potential.

The potential beneficial effect of butyrate has been supported by data from case control studies, in which a low fecal butyrate concentration has been found in patients with colonic adenomas, compared to

controls [47,93]. Another study demonstrated a decreased production of butyrate during fiber fermentation in patients with colonic cancer or adenomas [94].

If butyrate does have anti-neoplastic potential, consumption of resistant starch is of particular interest, as fermentation of starch yields twice the amount of butyrate in the colonic SCFA-pool [26,27].

Conclusion.

There is a growing body of evidence that fermentation of fiber and especially resistant starch, has a protective effect on large bowel cancer. In figure 2, we describe the possible mechanisms of this protection. The production of short chain fatty acids decreases the colonic pH, which is probably followed by precipitation of primary and secondary bile acids.
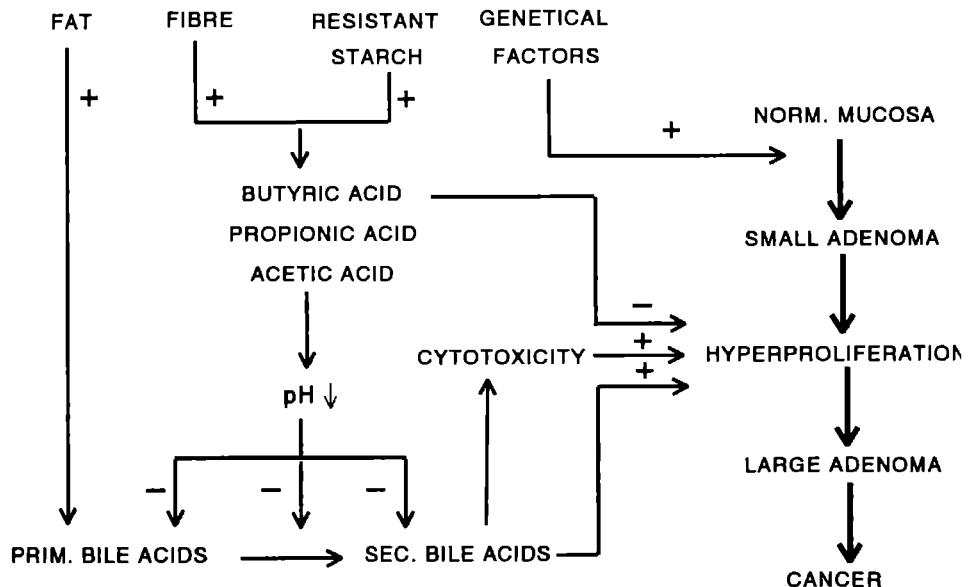
Figure 2: Hypothesis of the influence of fermentation on large bowel carcinogenesis.

When bile acids are precipitated, their interaction with the colonic mucosa is inhibited. Furthermore, the activity of bacterial 7α-dehydroxylase (needed for the conversion of primary into secondary bile acids) is inhibited by an acid environment. Bile acids, especially the secondary bile acid deoxycholic acid, can stimulate colonic mucosal proliferation either directly or mediated by membrane damage. Precipitation of bile acids, or inhibition of the formation of secondary bile acids can inhibit this effect. It's relevance in colonic carcinogenesis is linked to hyperproliferation being a proposed initial stage in the transformation of normal mucosa into cancerous tissue. The short chain fatty acid, butyrate, also appears to play a role in the regulation of the mucosal proliferation, by a direct inhibitory effect.

Although some of these mechanisms are still the subject of debate, we believe that potent stimulation of colonic fermentation, using a substrate such as resistant starch, may play an important role in the prevention of large bowel cancer.

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Chapter 3

The influence of dietary fiber
on bile acid metabolism

Ivo P. van Munster and Fokko M. Nagengast

Introduction.

A great deal of the interest of the effects of dietary fiber on bile acid metabolism is derived from the interrelationship between diet and cholesterol metabolism, and from the role of nutrition in the development of colorectal cancer. In this review the relation between dietary fiber and bile acid metabolism is focused around their role in the development of colorectal cancer.

Bile acids are the major end products of cholesterol metabolism and synthesized in the liver. The primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) are derived via several intermediate steps from cholesterol and secreted in bile as glycine or taurine conjugates. They serve as cholesterol solubilizing agents by the formation of micelles and play an important role in the digestion and absorption of lipids in the small intestine. More than 95 % of the bile acids passing the ileum are reabsorbed and return to the liver through the portal vein. An efficient conservation in the so- called enterohepatic circulation is thus achieved. The proportion of bile acids not absorbed in the terminal ileum is 2-5% per cycle and amounts to an average loss of 20% of the bile acid pool with 6-12 enterohepatic circulations per day. Bile acids that escape absorption in the ileum, are metabolized in the large bowel by the anaerobic bacterial flora. First, deconjugation takes place and the amino acid molecule on the carboxyl group is removed. Secondly, the primary bile acids CA and CDCA are dehydroxylated and converted into the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA) respectively. Further bacterial degradation in the large bowel and alterations in the liver produce the tertiary bile acids (figure 1). DCA is partly absorbed in the colon and enters the enterohepatic circulation, where it is conjugated in the liver and secreted in bile; LCA is almost insoluble and very little is reabsorbed. Both secondary bile acids are excreted in the stool and make up to 95 % of the total amount of excreted bile acids. In the stool the major part of the bile acids are bound to dietary and bacterial residues. In the circulating bile acid pool, CA and CDCA each comprise about 30-40%, DCA about 20-30% and LCA for less than 5% of the total amount [1,2].

It is postulated that secondary bile acids play a promoting role in colonic carcinogenesis [3-6]. According to the hypothesis, these bile acids damage the colonic epithelium, causing hyperproliferation in the
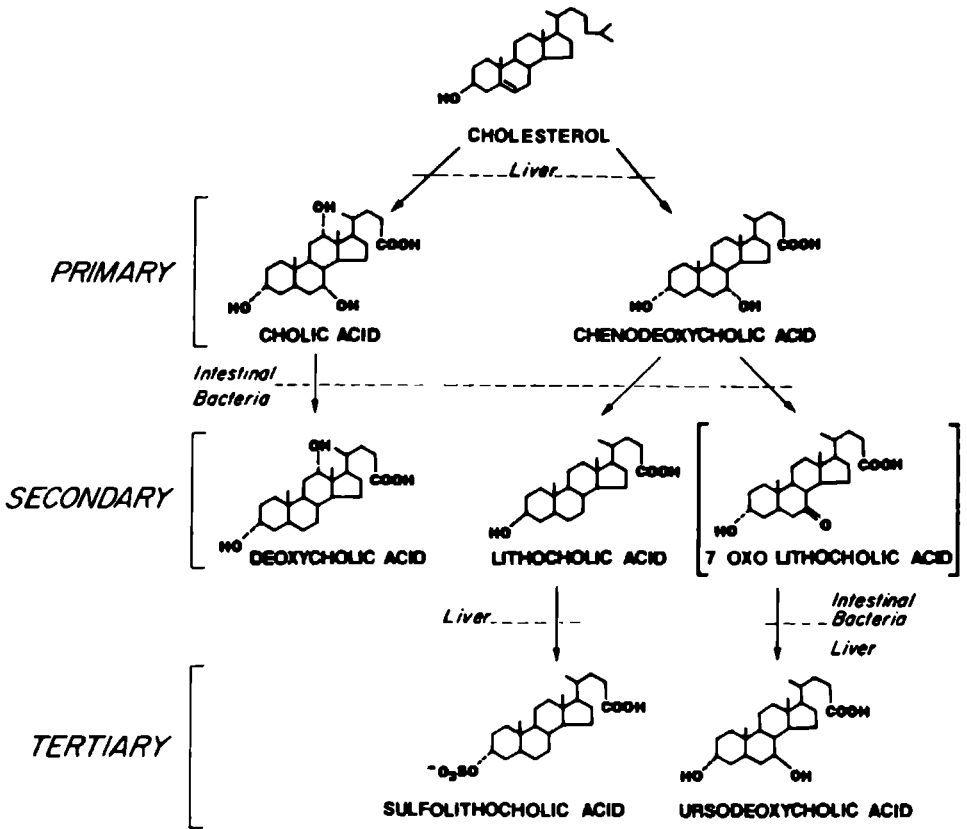
Figure 1: Pathway of primary, secondary and tertiary bile acid synthesis.

colonic crypts, which than leads to an increased risk of malignant degeneration. Of course this is an simplification of reality, since it is well known that many other factors are involved, especially genetic, hormonal and growth factors.

The influence of dietary factors on colonic carcinogenesis.

The incidence of colorectal cancer is high in Western countries and is related to dietary habits. Currently it is assumed that dietary factors modulate a genetic susceptibility. In epidemiological observations the consumption of animal fat is positively related to the incidence of

colon cancer. The intake of fiber is possibly negatively related to this incidence, however, many inconsistencies exist. In a recently published meta-analysis [7] of observational epidemiological and of case-control studies, a protective effect of dietary fiber on the occurrence of colonic cancer was found in most, but not all studies. The overall odds ratio for the high fiber groups in the case-control studies was 0.57 (CI 0.50-0.67). One thing that has to be kept in mind is the often reciprocal relation between fat and fiber intake. An increase in fiber intake will easily lead to a decrease in fat intake or to a change in the total energy intake. An interesting substance that recently has drawn more attention is "resistant starch", defined as starch that can not be absorbed in the small bowel. This resistant starch is supposed to have effects on the large bowel, comparable with fiber. Besides the influence of fiber (resistant starch), fat and total energy intake other dietary constituents like trace elements and vitamins have been suggested to play a more or less important role.

There are several possible explanations for the protective effect of fiber. The most consistent are:

The *stool-bulking effect*, leading to a decrease in concentration of potentially carcinogenic substances, the *acceleration* of gut transit time, the *binding* of bile acids, and the *fermentation* of parts of fiber and probably most of the starch to short chain fatty acids (especially butyrate, which has antineoplastic properties in vitro). The short chain fatty acids will *lower the pH* and this causes a decrease of activity of the enzyme 7α-dehydroxylase, leading to a reduction of the formation of secondary bile acids. Moreover, because of the more acidic environment, bile acids may be precipitated. Fiber and resistant starch are supposed to *alter the bacterial flora* in the colon, leading to a decrease in -possible carcinogenic- nitrogen-containing substances.

The hypothesis postulates that a high fat diet enhances the formation and degradation of bile acids and neutral sterols and that these compounds exert a promoting effect in colonic carcinogenesis [5]. Indeed, it has been found that dietary fat increases the output and fecal concentration of bile acids [10-12] and epidemiological evidence has shown that populations with a high incidence of colorectal cancer, consuming a high fat and animal protein diet, excrete about twice the amount of secondary bile acids [13]. The concentration of these bile acids was also increased. However, other studies in the United

States, Great Britain and New Zealand have failed to demonstrate a correlation between a high fat intake and colorectal cancer incidence [14,15]. Case-control studies have shown conflicting results in this respect [16,17].

Studies about dietary fiber and bile acid metabolism.

Dietary fiber is that part of the plant wall matrix which can not be broken down by human enzymes. The most important resources are wheat, vegetables and fruit.
The most important components of fiber are: cellulose, hemicellulose, pectin and lignin. Different components can exert several physiological effects, which depend on processing, particle size, the source of fiber, and the individual characteristics of the consumer, like transit time and colonic microflora. Since animals often have a very different bile acid pattern, only human studies will be reviewed in this manuscript.
In many of the published studies the main goal was not the effect of fiber or parts of fiber on bile acid metabolism, but to investigate the role of fiber on cholesterol metabolism. This was based on the hypothesis that by increasing the excretion of acidic (bile acids) sterols and neutral sterols through the consumption of dietary fiber, the serum cholesterol level could be lowered. Other studies with regard to the effect of dietary fiber on biliary bile acids were initiated, because of the hypothesis that fiber may decrease the cholesterol saturation index (CSI) of bile, which is a major factor in the development of gallbladder bile stones. Since deoxycholic acid may increase the CSI, many investigations concentrated on the effect of fiber (or fiber components) on individual biliary bile acids.

Pitfalls in the interpretation of dietary fiber studies.

In studies concerning the effect of dietary fiber on bile acid metabolism, *different types or components of fiber* were used. As mentioned above, this can lead to different and sometimes conflicting results.
The *age and gender* of the subjects in the different studies were often

not comparable. It is well known that DCA metabolism is age-dependent [18]. Elderly persons have a higher DCA content in bile than younger persons [6].

In several of the studies, the subjects had *gallstones* or a high cholesterol saturation index. These patients mostly have a higher DCA content, and differences caused by dietary intervention are more likely to occur. Many studies were carried out in hyperlipidemic patients; the close interrelationship between cholesterol and bile acids could have biased the data.

In the dietary intervention studies, the *relation with other dietary factors* like fat and total energy intake is important when looking at the changes in bile acid metabolism. In most of the studies the intake of other food items (like fat) was not controlled for. It is conceivable that even motivated volunteers who have to consume 30 gram bran per day will lower their fat intake. This can have profound consequences on bile acid metabolism.

The *duration of the dietary intervention* may influence the effect on bile acid metabolism. The length of the different investigations varied from 4 days to one year, but in most studies the effect of fiber was investigated for 3-6 weeks. From the results in several investigations it is clear that the effect of fiber on colonic function and bile acid metabolism takes some time to occur. Otherwise, some experimental data suggest an adaptation after a longer period of fiber administration, leading to a reversion of the initial changes. We studied the influence of a low fiber diet, followed by a 10 week diet with a high content of mixed natural fiber. We found a decrease in biliary DCA of 15% and a increase of CA of 24% after 6 weeks high fiber diet, but a return to basal values after 10 weeks [19].

No clear understanding exists at this moment whether long-term administration of a natural high fiber diet can induce changes in bile acid metabolism that would benefit patients at risk of developing colon cancer.

Different endpoints in dietary intervention studies.

Changes in serum bile acid concentrations or biliary bile acid composition are of less interest concerning the potentially protective effect of fiber on colonic cancer because they only reflect indirect

changes in the colonic environment.
The total fecal output of bile acids, mostly secondary bile acids, has been used as an endpoint in several fiber studies. This is probably for the most part correlated to changes in colonic bile acid metabolism, but most of the bile acids are bound to fiber and bacteria and thus not really in interaction with the colonic mucosa.
Perhaps kinetic studies involving the DCA pool size and especially the DCA input rate are a more useful endpoint. The DCA input rate and the unconjugated DCA fraction in serum tells us something about the newly formed and absorbed DCA. This is, in fact, the DCA fraction that is in close contact with the mucosal cells. Unfortunately, only very few studies concerning this matter have been carried out.
Probably the most useful endpoint in fiber studies is the DCA concentration in the aqueous phase of stool. This can give us the most direct information about the micro-environment of the colonic mucosa, that is the place of interaction between secondary bile acids and colonocytes. We will discuss the different fiber studies, looking at the different endpoints described above.

Dietary fiber and biliary bile acids.

In figure 2, a number of studies is shown, in which the effect of wheat bran or natural fiber on the biliary bile acid pattern has been examined.
In most studies 30-60 gram of bran per day was used as a fiber source. These amounts are equivalent to 11-22 gram of dietary fiber. The overall effect was a moderate (13-49%) decline in DCA content. Sometimes a concomitant rise in CDCA content in bile was found.
Tarpila et al [20] and Marcus and Heaton [21], however, detected that a fall in DCA was accompanied by a rise in the CA percentage in bile, while the CDCA fraction remained constant. The same holds true for the study of Thornton [22], which was the only one that used dietary fiber from several sources like cereals, vegetables and fruit. We have recently completed a control-led study with a natural high fiber diet and came to the same results [19].
A hypothesis concerning the reciprocal relationship between DCA and CDCA has been put forward by Pomare et al [23], assuming that DCA inhibits the synthesis of CDCA in the liver.
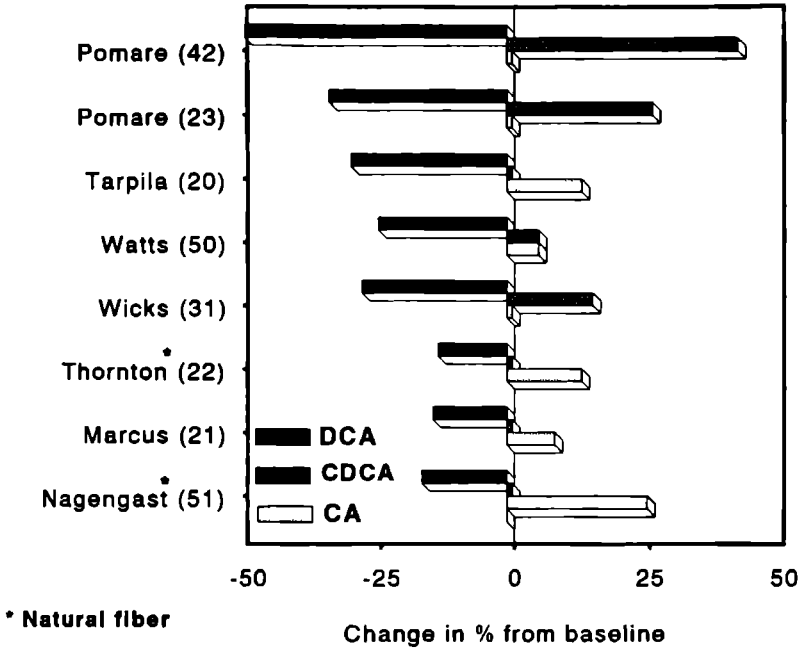
Figure 2: Changes in biliary bile acid composition in studies using wheat bran or a natural high fiber diet.

Some controversy about this hypothesis however, still exists. Of course, the decrease in the percentage of biliary DCA will lead to an obligatory increase in the percentage of the other bile acids.
In figure 3, studies are shown, in which the effect of different components of fiber and of lactulose, a non-absorbable, fermentable disaccharide, was examined. It is striking that in 2 studies in which pectin was used, an increase in biliary DCA was found [24,25]. The explanation is not clear, but perhaps this controversial result is caused by one of the physico-chemical properties of pectin. Pectin is a strong gelformer in the jejunum, which can bind primary bile acids and thus prevent their absorption. In the colon, pectin will be fermented and the liberated primary bile acid will be dehydroxylated to secondary bile acids, which can explain the increase in DCA content. The effects of cellulose and lactulose are comparable to the effects of wheat bran and natural fiber.
The reduction of the DCA content in bile could be explained by several mechanisms, which have been reviewed by Heaton [26].
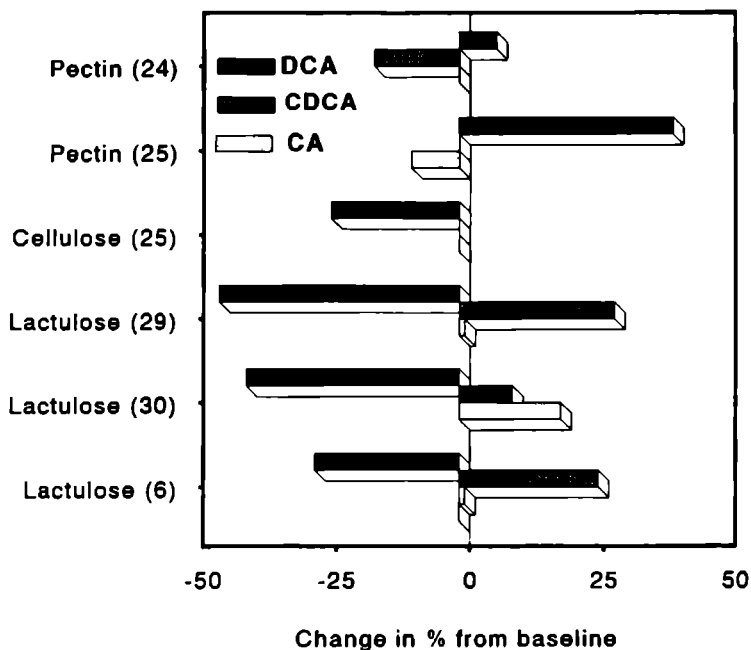
Figure 3: Changes in biliary bile acid composition in studies using pectin, cellulose or lactulose.

Theoretically, the most likely explanations are:

1. By the adsorption of newly formed DCA in the large bowel to dietary fiber, which has been shown in vitro to occur more strongly for DCA than for the other bile acids [27]. Also bacteria that feed and multiply on bran are capable of binding bile acids.

2. By the acceleration of the transit of colonic contents. This would decrease the absorption of DCA. This effect has also been shown to occur with laxatives. However, Marcus and Heaton [28] found no correlation between changes in transit time and alterations in DCA pool sizes or percentages.

3. By the bacterial fermentation of fiber components, which can give rise to the formation of short chain fatty acids and results in a reduction of the intraluminal pH. Such an effect has been found after administration of lactulose, a non-absorbable disaccharide, which is completely fermented in the large bowel and can decrease the DCA content in bile as has been shown by several authors [6,29,30]. Lowering the pH would inhibit bacterial

7α-dehydroxylase and thus reduce the formation of the secondary bile acids DCA and LCA. Acidification of colonic contents might also limit the absorption of DCA by precipitation, because DCA is poorly soluble at low pH.

In summary, dietary fiber can exert effects on secondary bile acid metabolism, which seem largely dependent on the type and source of fiber. Whether in the long run, a natural high fiber diet really can lower the level of circulating secondary bile acids remains to be seen.

Dietary fiber and fecal bile acids.

In figure 4 a review of a number of studies is shown concerning the effect of wheat bran on the concentration and excretion of fecal bile acids. In figure 5 the overall results of the studies using other types of fiber are depicted.
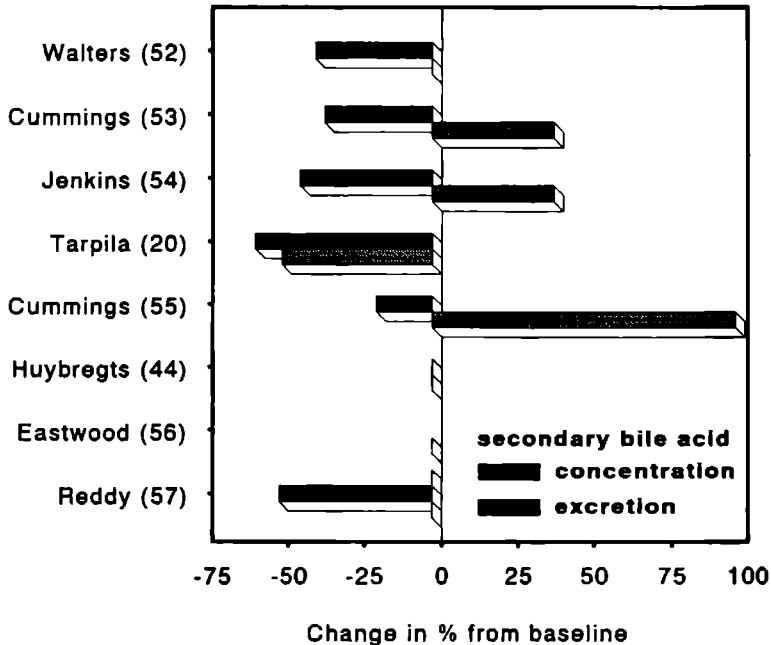
Figure 4: Changes in fecal bile acid excretion and concentration in studies using wheat bran.

It is hypothysed that secondary bile acids will damage the colonic epithelium, causing a hyperproliferation in the colonic crypts. Assuming the hypothesis is correct, than only soluble bile acids should exert the irritative effect on the colonic mucosa. However, in all studies concerning the effect of fiber on fecal bile acids, the total concentration based on stool dry weight or total excretion is given. The concentration of bile acids in fecal water is normally relatively low. From figure 4 it can be seen that consumption of wheat bran generally increases the daily output (excretion) and decreases the concentration of fecal bile acids. In almost all studies fecal weight increased, giving the most likely explanation of the result of decreased concentration and increased excretion.

As shown in figure 5, the results of studies using other types of fiber are less consistent. The suppletion with natural fiber has the same effect as administering wheat bran. The importance of the source of fiber is illustrated in two studies with oat bran. Oat bran has almost no effect on the concentration of fecal bile acids, while the excretion increased [32]. An explanation could be the fact that oat bran contains much less cellulose than wheat bran. Cellulose has been shown to decrease the concentration of fecal bile acids [33]. Pectin in rather small quantities (10-15 gram) can increase bile acid excretion [24,34] and concentration. The source of pectin is important in this respect, as is shown in the study of Stasse-Wolthuis[35]. Ten gram of pure pectin increased the excretion, but the same dose derived from vegetables and fruit had no effect.

Type of fiber	Fecal secondary bile acids	
	concentration	excretion
Wheat bran	≈ or ⇓	≈ or ⇑
Oat bran	≈ or ⇑	⇑
Lactulose	⇓	⇓
Pectin	⇑	⇑
Cellulose	⇓	≈
Lignin	≈	≈ or ⇑
Natural fiber	≈ or ⇓	≈ or ⇑

Figure 5. Effect of different fiber components on fecal bile acids.

It is remarkable that in three studies with doses of 33-60 gram fiber from different natural sources (corn, vegetables and fruit) no effect could be detected [36-38] while we found a decrease in the concentration after suppletion with 15 gram natural fiber [19]. It is conceivable that various components of dietary fiber can have opposite effects, resulting in a net neutral effect. Another reason for differences between the results obtained by several studies are age and gender of the subjects. It has been shown that age dependent differences exist in the concentration of fecal bile acids [2]. In the reported studies age varied from 20-80 years. Gender could also influence results. In many studies only men or women were investigated. Stasse-Wolthuis [37] found a sex difference in secondary bile acid excretion (increase in women, decrease in man), but had no valid explanation for this observation.

A factor that was not accounted for in many studies was the previous nutritional status and consumption pattern of the volunteers. Especially the intake of fiber, fat and cholesterol is important, because these nutrients have profound implications on the baseline concentration and excretion of bile acids.

In subjects consuming lactulose it has been shown that the dehydroxylation of primary bile acids can be inhibited, thereby increasing the concentration of these compounds and decreasing the level of secondary fecal bile acids [6,30]. Fermentation of lactulose in short chain fatty acids leading to a decline in colonic pH, is largely responsible for this effect. Despite the fact that parts of dietary fiber can also be fermented into short chain fatty acids, studies that have looked at individual bile acids did not find a significant increase in primary bile acid concentration. In fact more than 95% of the fecal bile acids on a high fiber diet consisted of the secondary bile acids DCA and LCA. This difference is probably caused by a slow and incomplete fermentation process of fiber.

Dietary fiber and DCA kinetics.

Surprisingly few studies have looked upon the effect of fiber or its components on the kinetic parameters of bile acid metabolism. Hoffmann[39] and Stellaard [40] have demonstrated the complexity of the formation, absorption en enterohepatic circulation of secondary bile

acids. The only way to get a reliable insight into the dynamics of bile acid metabolism is to perform kinetic experiments using radio-active or stable isotopes. However, the availability of this technics is limited and radio-active isotopes can not be used in experiments with healthy volunteers in most countries. Previously, we demonstrated an increased level of DCA input in patients with colonic adenomas, compared to healthy controls [41].

It must be kept in mind that 7α-dehydroxylation is an age-dependent process. Elderly persons have a higher DCA input rate than younger people [18].

Data about the influence of transit time on DCA pool size and DCA input are rather conflicting. Marcus and Heaton found an increased DCA pool size in persons with prolonged transit time in one study [28], but in contrast, three other studies did not show any relation [18,21,30].

In fiber supplementation studies, using kinetic experiments, different results were found. Pomare [23,42] demonstrated that bran reduces the circulating DCA pool and another study showed the same for cereals [43]. After lactulose ingestion, both the DCA pool size and DCA input decreased, although only the DCA pool size changed significant [30].

In contrast, three other studies [21,22,44] with bran and natural fiber did not show any changes in DCA pool size.

Huybregts [44] found a small increase in the DCA input in the circulating pool after wheat bran supplementation. It must be remembered that the 4 healthy subjects in the last study were already consuming a high fiber diet and had small DCA pools to begin with. DCA input increased significantly after 8 weeks, but not after 4 weeks.

In two other studies no significant changes in DCA input where detected [21,22].

Dietary fiber and DCA in the aqueous phase of stool.

Several reports have indicated that the DCA concentration in fecal water is of more relevance concerning the risk of colonic cancer than is the total fecal bile acid concentration [45-49]. This can be explained in theory by the fact that the DCA concentration in fecal water expresses the DCA fraction that is in contact with the mucosal cells. Stadler demonstrated [49] a significant higher DCA concentration in fecal water in patients with adenomatous polyps or colon cancer,

compared to patients without colonic pathology. Geltner [47] showed that a shift from a mixed to a lactovegetarian diet, leading to a decrease in fat intake and an increase in fiber intake, resulted in a significant decrease of the DCA concentration in fecal water from 125 to 75 μmol/l, but not in a change in total bile acid concentration in feces. Also, the cellular toxicity -measured by incubating fecal water with erythrocytes and detecting the lysis of these cells- of the aqueous phase of stool decreased. Although data about bile acid concentrations in fecal water are rather scarce, it is a promising parameter in studying bile acid metabolism in relation to colonic cancer.

Conclusions.

From the reviewed literature it is clear that dietary fiber has an influence upon bile acid metabolism. There is also evidence that a high fiber intake is related with a lower incidence of colonic cancer. However, it is not yet prooven wether this decline in risk is caused by changes in the bile acid metabolism, due to the high fiber diet. In this respect the lower fat intake that goes along with an increase in fiber consumption could have biased the results. One of the problems in determining the exact influence of fiber on bile acid metabolism is that different components of fiber or fiber degradation products have all their own different physiochemical properties, that can even act controversial. Dietary intervention studies in patients at high risk for colonic cancer (patients with adenomas, longstanding ulcerative colitis, familial colon cancer syndromes) could give more insight in the relation between fiber consumption, bile acid metabolism and colonic cancer. Obvious is that we know too little about the mechanism whereby bile acids manipulate colonic carcinogenesis. More research is needed to clarify the interaction and possible beneficial effects of dietary fiber on the metabolism of secondary bile acids.

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Chapter 4

A new method for the determination of the cytotoxicity of bile acids and aqueous phase of stool; the effect of calcium

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Abstract.

Soluble secondary bile acids in the colon are supposed to be cytotoxic for normal colonic cells, resulting in an increased compensatory proliferation of colonic crypt cells, which is associated with an increased risk for colonic cancer. We developed a sensitive method to determine cytotoxicity of bile acids in the HT-29 colon cancer cell line, using a tetrazolium-based colorimetric assay. Only in vital cells, tetrazolium-salts are converted into formazan, which can be measured easily. Chenodeoxycholic acid and deoxycholic acid (DCA) were cytotoxic in concentrations above 100 μM, which is in the physiological range for soluble DCA in feces. Conjugation of bile acids diminished cytotoxicity 7-10 fold. In this concentration range, no effect of calcium or calciumphosphate was demonstrated, suggesting that the effect of calcium on colonic proliferation is not mediated by a precipitation of soluble bile acids in the large bowel. Finally, we could demonstrate a significant correlation between the cytotoxicity of the aqueous phase of feces and the soluble DCA concentration.

Introduction.

Secondary bile acids have been implicated in colonic carcinogen-esis[1-3]. The exact mode of action has not yet been clarified, but is supposed to be mediated by a cytotoxic effect on the colonic mucosa. Most bile acids are cytotoxic to cells, probably by co-micellisation with phospholipids in the cell membrane [4,5]. Therefore it has been postulated that bile acids are only cytotoxic above the critical micellar concentration [5,6]. Another mechanism could be the formation of soaps with calcium in the cell membrane, resulting in the formation of micro-channels [7,8]. This cytotoxicity can cause epithelial cell loss in the large bowel leading to a compensatory crypt cell proliferation [9]. It has been shown that an increased cell proliferation has been linked to a higher risk for the development of colonic cancer [10,11]. One of the proposed mechanisms is an increased susceptibility of the colonic mucosa for mutagenic substances, because of the higher fraction of S-phase cells.

Most bile acids in the lumen of the large bowel are bound to food residue and bacterial mass. This leaves only a minor fraction in the soluble form and thus in contact with colonic mucosal cells. In man more than 95% of the secondary bile acids consist of deoxycholic acid (DCA) and the -almost insoluble- lithocholic acid (LCA). The total concentration of bile acids in the aqueous phase of stool ranges from 50 to 300 μM. DCA is the main soluble bile acid ranging from 10 to 200 μM [12].

Cytotoxicity has been measured by an erythrocyte lysis assay [5,12-14] In this assay erythrocytes are incubated with bile acid solutions and lysis of the erythrocytes is measured by counting intact cells before and after incubation [12] or by the release of iron from destroyed cells [5]. In this assay cytotoxicity of secondary bile acids has been demon-strated in micellar concentrations above 1 to 2 mM. These concentrations are much higher than have been found in the aqueous phase of stool. If cytotoxicity of bile acids is indeed the result of co-micellisation with phospholipids in the cell membrane, for which a micellar concentration is needed, the concentration of the soluble bile acids in the large bowel is far to low to cause this cytotoxic effect. We therefore developed a sensitive and accurate method to determine the cytotoxicity of bile acids in low concentrations, as has been found in the fecal water, on cultured HT-29 colon cancer cells. With this

assay we could also measure the cytotoxicity of fecal water.
Furthermore we used this cytotoxicity assay to study the effect of calcium and calciumphosphate (CaP$_i$) on solutions with bile acids in low concentration. Controversy exists on the effects of calcium or CaP$_i$ on precipitation and cytotoxicity of bile acids and again, studies are done with bile acids in micellar concentrations. To gain insight in the interaction of bile acids and calcium in large bowel, we investigated the effect of calcium and CaP$_i$ on submicellar bile acid concentrations, present in the aqueous phase of stool.

Material and methods.

HT-29 lysis assay.
Cholic acid (CA), DCA, chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), and glyco- and tauro- conjugates were purchased from Sigma (St Louis, USA) and Calbiochem (San Diego, USA). As previously described [15], the colonic cancer cell-line HT-29 was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Flow Laboratories Irvine, Scotland) until 90% confluence. After trypsinization, cells were resuspended and plated in 96 multi-well plates (Costar, Cambridge, Mass, USA) with a concentration of 15.000 cells/well. After 48 hours culture (37°C, 5% CO_2) cells were washed and incubated during 1 hour with 100 μl bile acids solutions in phosphate buffered saline (PBS) or with 100 μl of the aqueous phase of stool. The concentrations of bile acids ranged from 0-3200 μmol/l. Every experiment with a single concentration was performed in octuple. In a number of experiments $CaCl_2$ and/or sodiumphosphate was added to the incubation mixture to a final concentration of 10 mM. After 1 hour incubation, cells were washed and cultured for another 48 hours with DMEM. Then 20 μl of the tetrazolium salt 3-(4,5,-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 1 mg/ml) was added to every well. In the viable cells, MTT will be converted by mitochondrial activity to formazan, a blue dye [16-18]. After four hours cells were washed and lysis was carried out with 100 μl dimethylsulfoxide to free the formed formazan. Absorption was read at 545 nm using a Multiscan (Flow, Helsinki, Finland). The cell survival was expressed as percentage absorption of the maximal absorption, read from the wells incubated with PBS without bile acids.

Erythrocyte lysis assay.
Blood was collected from healthy donors by venipuncture with syringes containing EDTA. Erythrocytes were washed three times with 5 vol of buffer (150 mM NaCl, 25 mM glucose, 10 mM Tris-HCl at Ph 7.4), as described by Rafter et all [12]. Cells were incubated in triplo with bile acid solutions at a final haematocrit of 10% in a total volume of 500 μl. After 6 hours incubation, erythrocytes were counted using a Coulter Counter ZM (Coulter Electronics, England).
The cell survival was expressed as percentage cells, compared with a control incubation with PBS.

Preparation of the aqueous fraction of stool.
Stool samples were collected from healthy volunteers on dry ice and in the laboratory stored at -20 °C until processing. Samples were homogenized with a blender and ultracentrifugated (2 hr, 4°C, 30.000g). The supernatant was carefully removed and filtered (0.2μ filter, Schleicher & Schuell, Dassel, Germany). This fecal water fraction (FW) was stored at -20°C until analysis.

Bile acid analysis.
Bile acids were determined as published before [19], with slight modifications. After enzymatic hydrolysis the bile acids were extracted from fecal water (0.5 ml) by SepPak C18 chromatography. The saponifiable conjugates were then hydrolyzed at 60°C for 2 h in 1 ml of 1 M KOH in methanol. The unconjugated bile acids were separated from the neutral sterols by Lipidex DEAP chromatography and measured after methylation and silylation by capillary gas chromatography (column CP Sil 5CB) on a Packard 430 gas chromatograph.

Statistics.
For every cytotoxicity-experiment, a survival curve was calculated according to the formula: $P = 1/1 + \exp[b(\ln(concentration)-a)]$, in which P is the fraction surviving cells, b is the slope of the curve and a is the concentration resulting in 50% cell-lysis (LC50). Using non linear regression, for every experiment those curves were fitted simultaneously assuming parameter b was the same. The LC50 values were compared using Wald statistics. Bonferroni corrections were applied because of multiple testing.
A Spearman Rank Correlation Coefficient (r_s) was calculated to test

the relation between cytotoxicity and bile acid concentration in fecal water.
Statistic calculations were carried out with the SAS analysis system.

Results.

Validation of the HT-29 assay.
We measured the cytotoxicity of unconjugated DCA in both the HT-29 assay and the erythrocyte lysis assay. Visual inspection with a microscope shortly after the incubation showed a complete lysis of the cells in the cytotoxic concentrations. As demonstrated in figure 1, the HT-29 assay is about 10 times more sensitive than the erythrocyte assay and the cytotoxic potential of DCA is quantifiable in concentrations above 100 μmol/l. This concentration correlates well with the range found in the aqueous phase of feces.
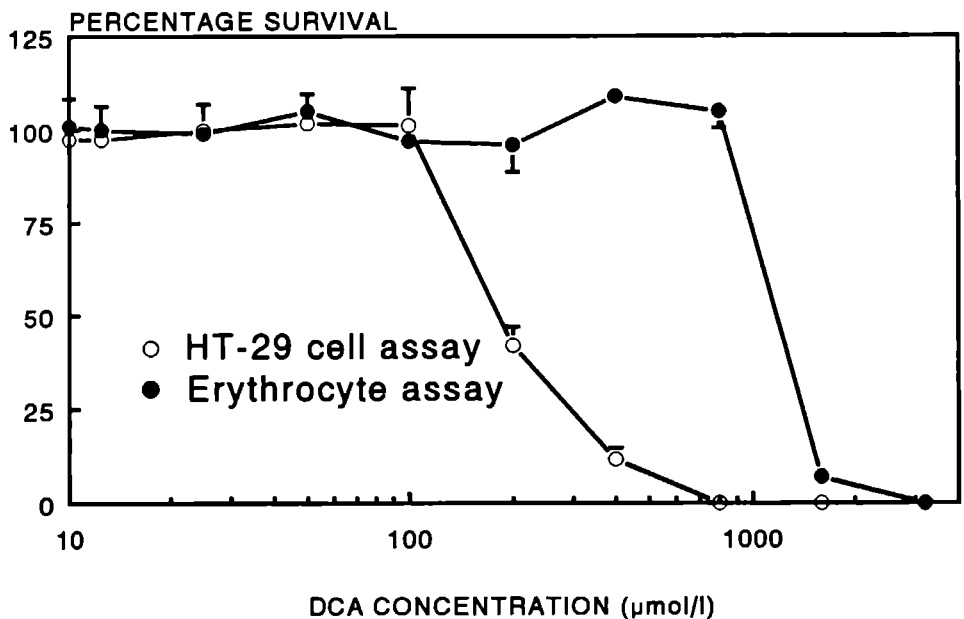
Figure 1. Cytotoxicity of unconjugated DCA in the erythrocyte lysis assay compared to the HT-29 cell assay. Cytotoxicity is expressed as the percentage of maximal cell survival (mean ± S.E.M.).
LC50 of HT-29 vs erythrocyte assay: p< 0.0001)
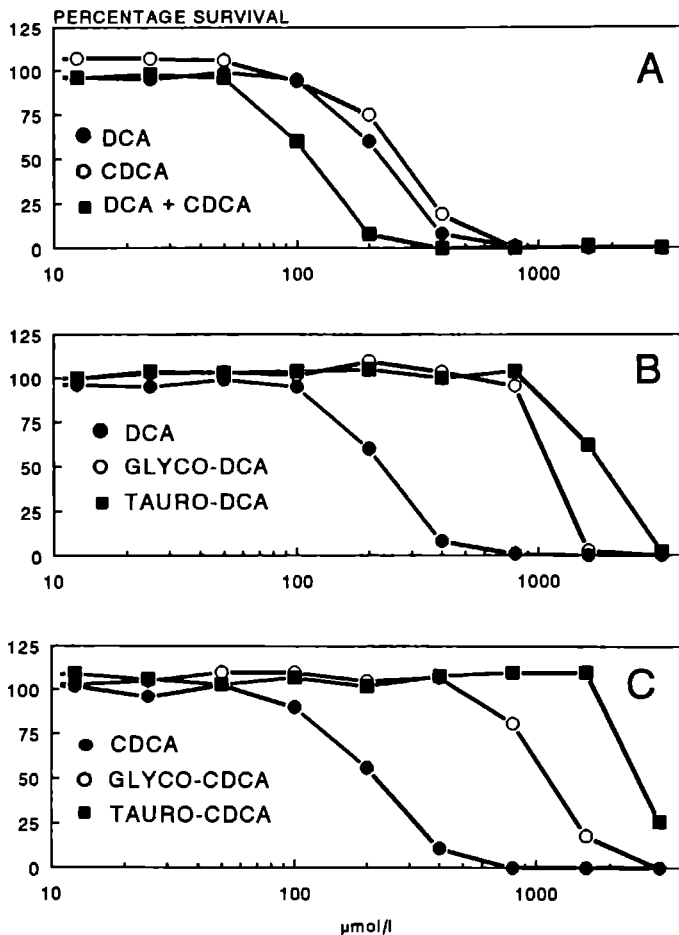
Figure 2. Cytotoxicity of the most important unconjugated (A) bile acids. In the mixture of DCA and CDCA, concentrations of individual bile acids are given. The LC50 of DCA vs DCA+CDCA and of CDCA vs DCA+CDCA: p<0.0001. Conjugation of DCA (B) and CDCA (C) results in a significant decrease in cytotoxicity. LC50 of glyco-DCA vs DCA, tauro-DCA vs DCA, glyco-CDCA vs CDCA and of tauro-CDCA vs CDCA: p<0.0001.

Cytotoxicity of bile acids.

The cytotoxic potential of other bile acids has been displayed in figure 2. For optical reasons only mean values are given, the standard error is like in figure 1 around 5 %. The cytotoxicity of both unconjugated dihydroxy bile acids DCA and CDCA is comparable and a 1:1 mixture of both bile acids resulted in a cumulated cytotoxicity.

Table 1. Cytotoxicity of conjugated and unconjugated bile acids.

BILE ACID	LC50 (μM)	95% Confidence Interval
DCA	192	177- 205
Glyco-DCA	1111	1015 - 1219
Tauro-DCA	1745	1626 - 1855
CDCA	210	191- 230
Glyco-CDCA	1067	975 - 1169
Tauro-CDCA	2715	2607 - 2833
CA	>3200	
UDCA	>3200	

The LC50 (μM) is the concentration resulting in 50% of the maximal cell survival. CA and UDCA were not cytotoxic up to concentrations of 3200 μM. All conjugated bile acids vs unconjugated forms: $p < 0.0001$; glyco vs tauro conjugated bile acids: $p < 0.0001$; DCA vs CDCA: n.s.

The cytotoxicity decreased 7-10 fold for taurine or glycine conjugated DCA and CDCA (figure 2). In contrast, no cytotoxicity was found for CA and UDCA in concentrations up to 3200 μmol/l. The LC50 values of all tested bile acids are given in table 1.

Effect of Calcium and Calciumphosphate (CaP$_i$).
We studied the effect of calcium and CaP$_i$ on the cytotoxicity of DCA and CDCA in concentrations present in the aqueous phase of stool. The cytotoxicity of the bile acids increased after addition of $CaCl_2$ in a final concentration of 10 mM ($p < 0.0001$).
No protective effect of 10 mM CaP$_i$ on bile acid cytotoxicity was demonstrated when equimolair quantities of $CaCl_2$ and sodiumphosphate (Ph = 7.40, final concentration 10 nM) were added to form CaP$_i$. This resulted only in a partial inhibition of the increase in cytotoxicity which was seen after addition of $CaCl_2$. For Sodiumphosphate itself no protective effect on bile acid cytotoxicity was seen (Figure 3).

Cytotoxicity of fecal water.
To study the cytotoxic effect of soluble bile acids in stool, fecal water was prepared from 22 stool samples from healthy volunteers. The cytotoxicity of those samples was determined with the HT-29 assay,

PERCENTAGE SURVIVAL

DCA CONCENTRATION (μmol/l)
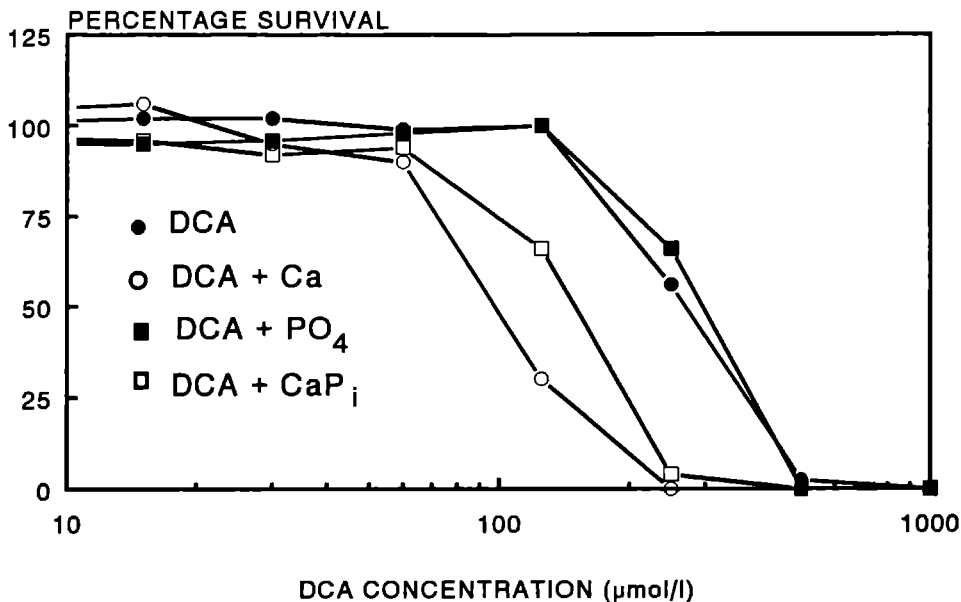
Figure 3. The effect of calcium, phosphate and CaP, on cytotoxicity of DCA.
LC50 of DCA vs DCA + Ca: p < 0.0001; DCA vs DCA + CaP; p < 0.0001; DCA vs
DCA + PO₄: n.s.

and bile acids were measured by gaschromatography. A significant
correlation was found between the cytotoxicity and the concentration
of the most important soluble bile acid DCA, as represented in figure
4 (r_s = -0.44, p < 0.05). It is important to mention that several FW
samples with low DCA concentration showed a marked cytotoxicity.
Other bile acids (CDCA, CA, LCA) were present in very low
concentrations and did not show any correlation with cytotoxicity.

Discussion.

The determination of the cytotoxicity of the major bile acids as well
as of the aqueous phase of feces using this HT-29 assay gives
reproducible results. With this method it is possible to perform
cytotoxicity experiments on large scale. Compared to the erythrocyte
lysis assay, the HT-29 assay is more sensitive for the cytotoxicity of
bile acids. Bile acids, in concentrations physiologically present in fecal
water, are cytotoxic in this assay.

SURVIVAL (%)

r_s = -0.44
p<0.05

DCA (μmol/l)
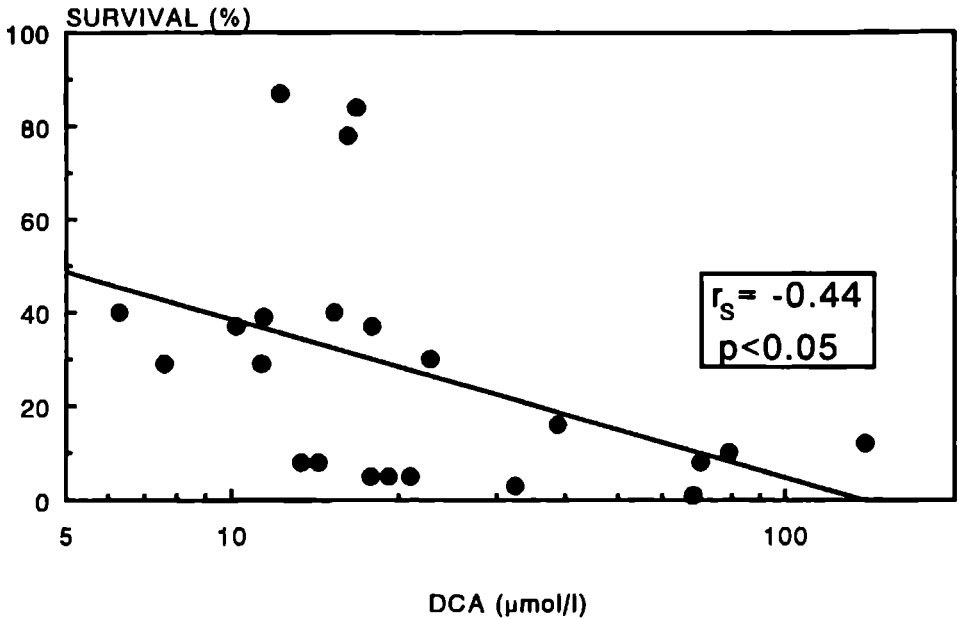
Figure 4. Relation between cytotoxicity and DCA concentration in the aqueous phase of feces. (r_s = -0.44, p < 0.05).

We can only speculate about the role of action of cytotoxicity of bile acids. If indeed the effect on the cell membrane is the most important effect [14,20] then the difference in cell membrane composition between erythrocytes and HT-29 cells could be the explanation for the observed difference in sensitivity between both assay's. The particular structure of the intestinal cell membrane [21] is quite different from erythrocytes. In contrast to erythrocytes, HT-29 cells are polarized cells [22,23], with the apical side exposed to the bile acid solutions. This mimics the normal physiological situation in the large bowel. Furthermore it is conceivable that the unconjugated hydrophobic bile acids DCA and CDCA are rapidly transported into the cell. These bile acids could then exert a cytotoxic effect on the nucleus or in the cytoplasm. Especially mitochondria are sensitive to bile acids, and changes in mitochondrial morphology and function occur during the early phase of bile acid induced cytotoxicity [24-26]. This could be another explanation for the difference in sensitivity between the two assay's, since the metabolic activity in erythrocytes

is relative low.

It seems possible that bile acids can induce mitochondrial dysfunction without immediate cell death [25]. This may interfere with the presented cytotoxicity assay, as basicly mitochondrial function is measured. However, the absence of a gradual decline of tetrazolium conversion before cell lysis occurs, suggests that this is not the case. Furthermore, clear cell lysis was observed by microscopic inspection of the cultured cells after incubation with bile acids in concentrations that were cytotoxic in this assay.

Only few data are available about the cytotoxicity of bile acids on other celltypes than erythrocytes. Two studies about the effect of DCA and CDCA on isolated rat hepatocytes demonstrated a cytotoxic effect at concentrations above 400 μmol/l [27,28]. In one study, the membrane-damaging effect of bile acids was measured on CaCo-2 cells, a cancer cell line with small bowel-characteristics by quantifing the leakage of a fluorescent probe. Only micellar concentrations of bile acids were toxic [6].

The relative high cytotoxicity of the dihydroxy bile acids DCA and CDCA, compared to the more hydrophylic bile acids CA and UDCA, is in agreement with previous studies using other models [5,6,28]. The decrease of cytotoxicity, for both glycine and taurine conjugated DCA and CDCA has been demonstrated before in the erythrocyte model [5].

Several hypotheses are suggested for the proposed protective effect of calcium on the colonic mucosa: a direct effect of Ca^{2+} on the mucosa [29], precipitation of bile acids with calcium in the small bowel or in the colon [30] or precipitation of bile acids with CaP_i [5,31]. Results about the in vitro precipitation of bile acids in micellar concentrations with calcium have been controversial. [5,31-35]. Calcium alone [32-34] or CaP_i [5,31] have been found to precipitate bile acids or to decrease cytotoxicity. In contrast, other studies showed a marked increase in cytotoxicity in the presence of calcium [5,8,35]. It has been demonstrated that the cytotoxicity of bile acids in sublytic concentrations is calcium dependent. Perhaps bile acids in low concentrations can promotion of the calcium uptake [8,35] which could be the factor leading to cellysis. Our data, showing a increased cytotoxicity after calcium addition, support this last theory.

In studies by vd Meer [5,31] addition of CaP_i but not $CaCl_2$ to micellar bile acid solutions caused a decrease of soluble DCA from 2.0 mM to 1.3 mM resulting in a disappearance of cytotoxicity in the erythrocyte

assay. In contrast to their data we found no protective effect of CaP$_i$. This is probably caused by the difference in sensitivity between both assays, as the remaining DCA concentration after a possible precipitation with CaP$_i$ is still about 1.3 mM which is cytotoxic in the HT-29 assay but not in the less sensitive erythrocyte assay.

It is unclear wether calcium or CaP$_i$ can precipitate bile acids in concentrations present in the soluble phase of stool. We hypothesize that if this is true, the remaining concentration of soluble bile acids will still be cytotoxic. Van der Meer demonstrated after calcium suppletion a change in biliary bile composition, but not in the soluble bile acids in the large bowel [31].

Finally for the first time a correlation was demonstrated between cytotoxicity and DCA concentration in fecal water. This supports the theory that DCA is a significant contributor to the cytotoxic potential of fecal water. The DCA-concentrations at which cytotoxicity was found are much lower compared to the in vitro experiments. The observation that even fecal water samples with a low DCA concentration showed a marked cytotoxicity, suggests that other factors play an important role in this interaction. For example, it has been demonstrated that fatty acids are cytotoxic to colonocytes [32,36], and that the cytotoxicity of fecal water was consistently associated with those fractions that contained free bile and fatty acids [37]. Furthermore, recently it has been shown that bile acids and fatty acids have a synergetic effect on cytotoxicity in the erythrocyte model [38]. This increase in cytotoxicity was most marked for the secondary bile acids deoxycholic and lithocholic acid. Unfortunately, it was not possible to measure fatty acids in fecal water in the present study. Further studies are necessary to elucidate other factors that are responsible for the cytotoxicity of colonic contents and to investigate its clinical relevance in carcinogenesis.

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Concordance between Proliferating Cell Nuclear Antigen immunostaining and ³H-Thymidine autoradiography in human colonic mucosa

Ivo P. van Munster and Fokko M. Nagengast.

Abstract.

Proliferating Cell Nuclear Antigen (PCNA) is a cell cycle related nuclear antigen, expressed in late G1-, S-, and early G2- phase. Recently, it has been used in assessing cellular proliferation. In contrast to the more traditional methods to determine cellular proliferation like [3H]-Thymidine or Bromodeoxyuridine, no incubation of fresh tissue is necessary when using PCNA immunohistochemistry. In this study, the concordance between PCNA immunostaining with a PC-10 antibody and [3H]-Thymidine autoradiography as an established S-phase marker, was investigated in human colonic mucosa using a double labelling technique. The intensity of PCNA immunostaining was classified as strong positive, weak positive or negative. Eighty-three percent of the PCNA positive cells was strong PCNA-positive and 17% weak PCNA-positive. When only strong PCNA-positive cells were classified as PCNA positive, sensitivity and specificity of PCNA as a S-phase marker were 80 and 99.8% respectively. When both weak and strong PCNA-positive cells were classified as PCNA positive, sensitivity and specificity were 96 and 98%.
We conclude that PC-10 immunostaining of human colonic mucosa after ethanol fixation is a useful S-phase marker in concordance with [3H]-Thymidine autoradiography. We suggest that besides strong PCNA-positive cells, also the less intense stained cells should be classified as PCNA positive.

Introduction.

Assessment of colonic cell proliferation is of interest in clinical and experimental studies, because a sustained increased proliferation is supposed to play a role in the development of colonic neoplasia [1-3]. Several methods have been used so far. In animals, proliferation can be measured after injection of [³H]-Thymidine by autoradiography of sections with incorporated [³H]-Thymidine or after injection of Bromodesoxyuridine (BrdU) by visualisation of the incorporated BrdU with a monoclonal antibody. In human studies only in vitro-incubation of tissue, after resection or biopsy, with [³H]-Thymidine or BrdU is possible. One of the disadvantages of these methods is, that incubation has to start immediately, with fresh tissue and under standardized conditions. This can be difficult, especially in larger scale multicenter studies. Another problem is the variability in tissue penetration of [³H]-Thymidine or BrdU, resulting in focal differences of the expression of the proliferation markers in the same tissue. Recently, cellular proliferation can be assessed using antibodies against Proliferating Cell Nuclear Antigen (PCNA) [4-8]. PCNA is an evolutionary highly conserved 35 kD nuclear protein, also known as Cyclin or DNA Polymerase δ-associated antigen [9]. One of the major advantages of assessing proliferation with PCNA antibodies is, that no incorporation is necessary because PCNA is a natural occurring antigen. Originally, autoantibodies against PCNA were detected in serum of Systemic Lupus Erythematosus patients [10], later eleven different monoclonal antibodies were raised by Waseem and Lane [11]. One of the antibodies, PC-10, has been used in assessing cell proliferation of colonic mucosa. The expression of the antigen starts late in G1, is maximally in S-Phase and decreases in G2-phase [12]. Compared to [³H]-Thymidine or BrdU, which are specific S-phase markers, PCNA is present during a longer period of the cell cycle. A methodological problem of the PCNA technique is the gradual increase and decrease of the antigen, resulting in a gradual scale of immunohistological staining.

The present study was undertaken to correlate in a double labelling technique [³H]-Thymidine autoradiography with PCNA staining in human colonic tissue using a PC-10 monoclonal antibody. The first aim was to validate PC-10 immunostaining as a S-phase marker for human colonic mucosa. The second goal was to find out whether the

intensity of staining was related to the cell cycle. In other words: are only strong positive cells in S-phase or should strong and weak positive cells be counted to measure cellular proliferation?

Material and Methods.

Biopsies and sections:
At least three colonic biopsies were taken by sigmoidoscopy from 10 patients without colonic abnormalities. Biopsies were cut into small pieces of about 3 mm and stretched under a binocular microscope on a piece of filter paper with the mucosal surface side upside. Immediately afterwards, the material was incubated for 1 hour at 30 psi and 37°C with DMEM medium (Flow laboratories, Irvine, Scotland) containing 4 μCi/ml [3H]-Thymidine (Amersham International Plc, Buckinghamshire, UK) and fixed in 70% ethanol. The tissue was processed and 4 μm sections were cut on poly-L-Lysine coated slides (Sigma, St Louis Mo USA).

PCNA Immunostaining.
After deparaffinization and blocking of endogenous peroxidase activity, sections were incubated with a PC-10 antibody (Dako, Glostrup, Denmark) for 1 hour in a 1:400 dilution in PBS at 24°C. After washing with PBS, a streptavidine-biotine system with AEC chromogen (Histostain-SP, Zymed Laboratories, San Francisco, CA, USA) was used to visualize the antibody. Counterstaining was performed with Mayer's haematoxyline during 10 seconds.

Autoradiography of [3H]-Thymidine.
After PCNA staining, slides were air dried and coated with NTB-2 emulsion (Eastman Kodak, Rochester, USA). The exposure time was 14 days. The autoradiographs were developed in Dektol (Kodak) and fixed.

Scoring method.
Sections were microscopically examined with a magnification of x 400. For PCNA, all homogeneous stained, distinct red cells (estimated as 75-100% of maximal staining intensity) were classified as strong PCNA-positive, less intens stained nuclei (estimated as 25-75% of

maximal staining intensity) were called weak PCNA-positive. For autoradiography all nuclei with at least 5 grains were assessed as [^{3}H]-Thymidine-positive.

Results.

The immunostaining for PCNA showed a low background staining and although most of the positive cells were strong positive, 17% showed a weak positive staining pattern. In the autoradiography, some parts of the sections showed none or very few grains, especially in the central parts of the tissue. Only those parts with a normal and homo-geneous distribution of [^{3}H]-Thymidine labelled cells were used for scoring.
A total number of 10.000 cells were evaluated, approximately 1000 for every patient. Every single cell was scored in PCNA staining as negative, weak positive or strong positive and in [^{3}H]-Thymidine labelling as negative or positive. In most of the cells [^{3}H]-Thymidine labelling and PCNA-staining were in accordance, although some of the cells showed only PCNA-staining or [^{3}H]-Thymidine positivity (figure 1). In table 1, the distribution of cells is given when only strong PCNA-positive cells were classified as positive.

*Table 1: Distribution of [^{3}H]-Thymidine and PCNA labelled
nuclei in human colonic mucosa, considering only
PCNA strong positive cells as PCNA positive.*

	PCNA +	PCNA -
[^{3}H]-Thymidine +	1048	266
[^{3}H]-Thymidine -	14	8545

In table 2, both strong and weak PCNA-positive cells are classified as PCNA positive.
Assuming [^{3}H]-Thymidine as the golden standard for S-phase determination in colonic mucosa, sensitivity, specificity and positive and negative predictive value of PCNA immunostaining compared to

Table 2: Distribution of [³H]-Thymidine and PCNA labelled nuclei in human colonic mucosa, considering PCNA weak and strong positive cells as PCNA positive.

	PCNA +	PCNA -
[³H]-Thymidine +	1265	49
[³H]-Thymidine -	161	8398

[³H]-Thymidine are given in table 3. When only strong PCNA-positive cells are assessed as positive, this method gives an sensitivity of 80% and a specificity of more than 99% for determination of cells in S-phase. From the 364 weak PCNA-positive cells 217 cells (60%) are also [³H]-Thymidine positive, while 147 (40%) were [³H]-Thymidine negative.

Table 3: Sensitivity, specificity and predictive values for PCNA as S-phase marker, compared to [³H]-Thymidine labelling.

	strong PCNA-positive cells	all PCNA-positive cells
sensitivity	0.80	0.96
specificity	0.998	0.98
positive predictive value	0.99	0.88
negative predictive value	0.97	0.99

In the left column data are given when only strong PCNA positive cells were assessed positive, in the right column data are given when strong and weak positive cells are classified as PCNA positive.

When both weak and strong PCNA-positive cells are assessed as PCNA-positive, the sensitivity of PCNA staining as S-phase marker increased to 96%, specificity was 98%. The positive predictive value decreased slightly to 88%.
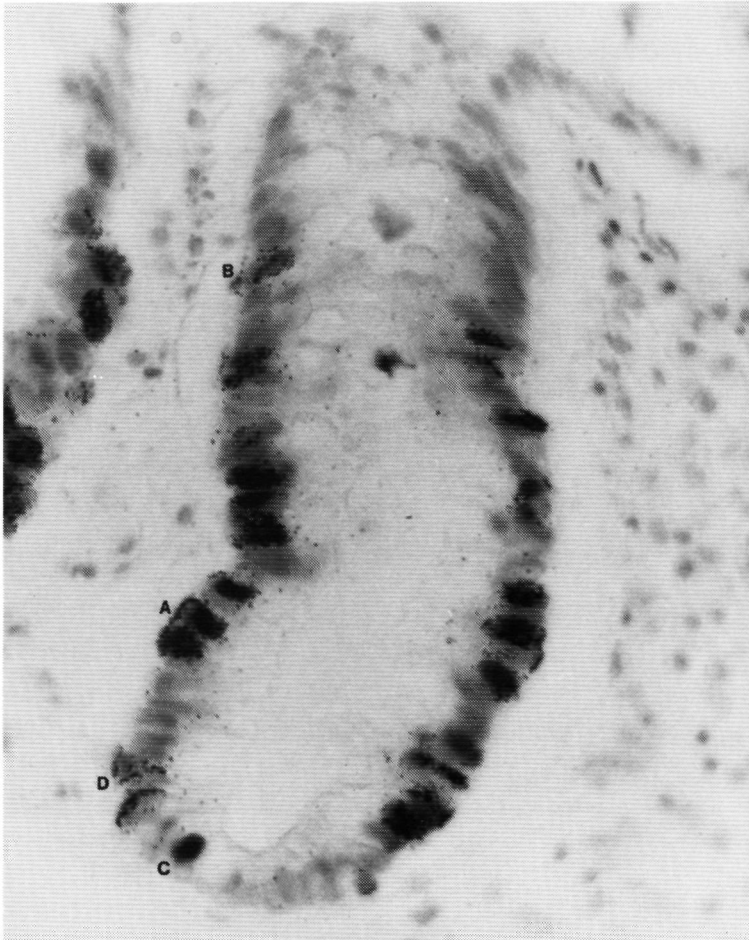
Figure 1: Double labelled section of human colonic tissue, original magnification x 400. A: [³H]-Thymidine positive and strong PCNA-positive cell, B: [³H]-Thymidine positive and weak PCNA-positive cell, C: [³H]-Thymidine negative and PCNA-positive cell, D: [³H]-Thymidine positive and PCNA-negative cell.

Discussion.

The measurement of colonic cell proliferation is currently used as an intermediated endpoint in intervention studies in patients at risk for developing colonic cancer [13,14]. These studies are often multicentric. PCNA immunostaining has the major advantage of a simple handling of mucosal biopsies without incubation. Because PCNA immunostaining is a relatively new technique, only few data are

available about validation of PCNA as S-phase or proliferation marker, especially in human tissue.

Data about the correlation between PCNA and BrdU or [^{3}H]-Thymidine in cancer cell cultures are conflicting, depending on the type of cell line, the antibody used and the method of fixation [7,8,15-17]. The lack of a relationship between PCNA and cell cycle in some of the cell lines is probably caused by the high proliferation rate and the long half life of PCNA. Presumably, this is also the explanation of the observed discrepancy between PCNA immunostaining and flow cytometric data in several [7,18], but not all tumours [4,19].

In rat, in vivo incorporation of [^{3}H]-Thymidine [5,17,20] or BrdU [8,17] correlates well with PCNA labelling in different tissues, although in most studies after formaldehyde fixation, PCNA showed in general a 2 times higher labelling index [5,8,20]. Galand et al performed a double labelling study of PCNA and [^{3}H]-Thymidine in rats [5]. They found that in methanol fixed intestinal tissue, 13% of PCNA positive cells were negative for [^{3}H]-Thymidine and 14% of [^{3}H]-Thymidine positive cells were negative for PCNA. In contrast, after formalin fixation, 41% of PCNA positive cells were [^{3}H]-Thymidine negative.

This difference in PCNA immunostaining after alcohol or formaldehyde fixation has been confirmed by others [16,21] and is important in interpretating results of different studies. This might be explained by the observation that two different populations of PCNA exist during the cell cycle [22]. One is a nucleoplasmic form of PCNA, present during the whole cell cycle and easily extracted, depending on the method of fixation. The other form is strictly nuclear, and present during the S-phase. If human colonic tissue is incubated with highly diluted PC-10 antibody after ethanol fixation, this results, in our experience, in an intens nuclear staining of positive cells and a low nonspecific cyto-plasmatic staining pattern. In contrast, after methanol fixation a high non-specific cyto-plasmatic staining was obtained (data not presented). In human tonsillar tissue, PC-10 immunostaining was optimal after alcohol fixation [21].

At this moment, data about PCNA immunostaining of human colonic mucosa are scarce. After formalin fixation, a 50% labelling index has been found [6], which is much higher than BrdU or [^{3}H]-Thymidine labelling. Again this is probably related to the fixation procedure. We found in this study, using the combination of ethanol fixation and PC-10 immunostaining, a labelling index of about 10% which correlates

well with the [^{3}H]-Thymidine labelling. To our knowledge, this is the first study about the concordance between PCNA immunostaining and [^{3}H]-Thymidine labelling in human colonic mucosa.

In the present study we also investigated whether the intensity of PCNA-immunostaining is of importance in relation to the S-phase determination.

The cells with a strong positive PCNA immunostaining pattern will be in S-phase, but when we consider only those cells as PCNA positive, a 20 percent underestimation of S-phase fraction as determined with [^{3}H]-Thymidine labelling was found. On the other hand, 40 % of the weak PCNA-positive cells was [^{3}H]-Thymidine negative. We assume that these cells have just passed the S-phase and PCNA is - in smaller quantities - still present because of its relative long half life.

In human studies, cell kinetic parameters are frequently used as intermediate risk markers. In this context, the question arises if measuring only S-phase cells (in particular only strong PCNA-positive cells) will give more information than determination of the total PCNA positive cell fraction. Most of these cells are in S-phase (89%) and the remaining fraction is supposed to be in the late G1 or early G2. To our opinion, these cells should be taken into account in the determination of the labelling index, as they all belong to the proliferating fraction of cells.

In summary, we demonstrated that PC-10 immunostaining after ethanol fixation is a good S-phase marker, in concordance with [^{3}H]-Thymidine autoradiography. We would stress the importance of agreement between different laboratories in fixation procedures and in classifying PCNA cells as positive or negative. We suggest to consider both strong and weak PCNA-positive cells as positive. Studies are in progress to assess the clini-cal relevance of PCNA as a proliferation marker in human colonic mucosa.

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Chapter 6

Fermentation of lactulose and resistant starch by high- and low- methane producing fecal inocula

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Abstract.

Fecal inocula from 17 healthy volunteers were incubated under anaerobic conditions with the rapidly fermentable substrate lactulose and with the resistant starch compound native amylomaize. Seven mixtures produced high amounts of methane, 10 mixtures low amounts. In the mixtures with a high methane production, fermentation of lactulose resulted in hydrogen production, which disappeared at the end of the incubation period. The methane production steadily increased. In these inocula, amylomaize fermentation resulted only in methane production. In the mixtures with a low methane production, only hydrogen was released both after lactulose and amylomaize fermentation. Although large interindividual differences existed, fermentation of both substrates seemed to elapse more rapidly in low compared with high methane producing inocula. Fermentation of lactulose and amylomaize starch resulted in an increase of the total short chain fatty acid concentration and in a relative enrichment of the butyrate fraction, from 10 to 17% ($p < 0.001$).

We conclude that fermentation of lactulose and of the resistant starch amylomaize yielded comparable amounts of short chain fatty acids, and that both substrates gave an enrichment of the butyrate-fraction. No significant differences could be demonstrated between low and high methane producing fecal inocula.

Introduction.

Fermentation is the degradation of carbohydrates by anaerobic bacteria. Amongst the several substrates available, fibre, non-digested starch and protein are quantitatively the most important [1]. Until recently, starch was believed to be completely digested in the small bowel. However, depending on the physical form and the way of food-processing, a substantial part of ingested starch will escape small bowel digestion and reach the colon [2-5] to provide substrate for the bacterial flora. Fermentation will result in the production of short chain fatty acids (SCFA) and the gasses hydrogen (H_2), methane (CH_4) and carbon-dioxide (CO_2). Methane is formed out of carbon-dioxide and hydrogen in approximately half of the Western population by the bacterial species Methanobrevibacter Smithii [6]. Hydrogen can also be metabolised to acetate [7] or it can reduce sulphate into hydrogensulfide (H_2S) [8]. Hydrogen and methane are excreted by breath or by flatus [9]. It has been suggested that subjects, excreting large amounts of methane are at an increased risk for colon cancer [10,11]. The most important SCFA's are acetic acid, propionic acid and butyric acid, which are supposed to play an important role in large bowel homeostasis and metabolism [12]. These acids are the most important energy source for colonocytes [13,14], and a deficiency of SCFA's may be the causative factor in the pathogenesis of ulcerative colitis [15]. Furthermore, they might be protective in large bowel carcinogenesis. The SCFA related decrease in intraluminal pH is supposed to diminish the formation of carcinogens [16,17]. Moreover, butyrate is of special interest because it has a differentiating and growth-regulating potential [18-21]. Depending on the substrate for fermentation, differences in rate and proportional production of SCFA have been observed [22-24]. A relatively high yield of butyrate has been demonstrated when starch is fermented.

To gain insight into fermentation of resistant starch and into the possible differences between low and high methane producing fecal flora, fecal samples from methane and non-methane excreting subjects were incubated with lactulose and with the resistant starch compound amylomaize. Hydrogen and methane formation, pH, and short chain fatty acid production were measured as a gauge for fermentation.

Materials and Methods.

Study protocol. Fresh fecal samples were obtained from 17 healthy volunteers. Within 10 min, 50 g of stool was diluted with Sodium Phosphate buffer (0,1M, pH = 6.5) to a final concentration of 16 % (w/v) and blended under oxygen free nitrogen to maintain anaerobiosis. The substrates used were lactulose (Inpharzam, Almere, The Netherlands) and native amylomaize starch (Hylon VII, National Starch & Chemical Company, Zutphen, The Netherlands). Five ml of this fecal slurry was mixed in 20 ml vials with 50 mg lactulose, 50 mg native amylomaize, or without extra substrate, flushed with oxygen free nitrogen and sealed. The total of 30 vials was incubated in a shaking waterbath at 37 °C. Two lactulose vials, two amylomaize vials, and two control vials were removed at the start and after 2 hr, 4 hr, 8 hr and 24 hr. Samples were taken from the gas-phase for measurement of hydrogen and methane, pH was measured and the slurry was centrifugated with 10.000g, during 5 min at 4°C. The supernatant was removed, snap frozen in liquid nitrogen, and stored at -20°C until analysis.

Hydrogen measurement.
A 0.5 ml sample of the head space was injected in triplo into a Packard gas-chromatograph with a Porapak Q 80-100 mesh column and a temperature of 110°C. Calibration was done with a 50.000 ppm and a 100.000 ppm standard gas.

Methane measurement.
A 0.4 ml gas sample was injected in triplo into a Pye gas-chromatograph, with a Porapak Q 100-120 mesh column and a column temperature of 50°C. Calibration was done with a 30 ppm standard gas. In methane concentrations above 60 ppm, samples were diluted and injected again.

Short chain fatty acid analysis.
Ten μl of a solution of 0.17 M 2-ethylbutyric acid (internal standard) in 100% formic acid was added to 100 μl of the supernatant. The mixture was centrifugated and 0.7 μl of the resulting clear supernatant was directly injected in a Packard gas chromatograph (column: 10% SP1200 / 1% H_3PO_4 on 80/100 Chromosorb WAW). The

injection port of the chromatograph was installed with an 8 cm long glass liner with an internal diameter of 3 mm, stoppered with a glass wool plug. Injection was performed in the space inside the liner above the glass wool plug. Details of the gas-chromatographic separation of SCFA's have been described elsewhere [25].

Statistical analysis.
All values are given as mean ± sem, unless specified.
Short chain fatty acid production, pH and gas formation were used as gauge for fermentation. Differences in these parameters between lactulose-, amylomaize-, or control fermentation were tested with the Friedman test. Differences between low- and high-methane producing inocula were tested with a Wilcoxon rank test, the difference in fermentation rate was estimated according to Koziol [26]

Table 1. Production of hydrogen and methane (mean ± sem) of 7 high-methane and 10 low-methane producing human fecal inocula after 24 hour with or without supplementation with 50 mg native amylomaize or lactulose.

	hydrogen μl	methane μl	H_2-equivalent μl
high methane producing inocula			
control	2.3 ± 1.2	298 ± 81^b	1148 ± 212^b
amylomaize	0.2 ± 0.1^b	$793 \pm 167^{a,b}$	$3559 \pm 598^{a,b}$
lactulose	$40 \pm 34^{a,b}$	$679 \pm 168^{a,b}$	$3068 \pm 629^{a,b}$
low-methane producing inocula			
control	7.1 ± 1.8	5.8 ± 3.1	32 ± 13
amylomaize	148 ± 51^a	5.8 ± 3.1	173 ± 58^a
lactulose	464 ± 135^a	0.6 ± 0.2	467 ± 135^a

Not all hydrogen measurements were availible from one high methane producing inoculum. Hydrogen production and H_2-equivalents were calculated from the remaining 6 HMP inocula.
[a] $p < 0.05$ compared with control incubations, [b] $p < 0.01$, compared with low-methane producing inocula.

Results.

Gas-production.

The methane production of incubation mixtures showed a bimodal distribution. In 10 mixtures less than 3 μl methane was produced (range 0.1-2 μl) after 24 hour fermentation of lactulose, in the other 7 samples at least 100 μl was produced (range 100-1060 μl). Methane production after amylomaize starch supplementation showed a comparable distribution, with low production in the same fecal samples. An arbitrary limit of 100 $\mu l/24hr$ methane in the lactulose incubations was taken to classify fecal inocula as high-methane producing mixtures (HMP) or low-methane producing mixtures (LMP). The gas production after 24 hr incubation for HMP and LMP mixtures is given in table 1.
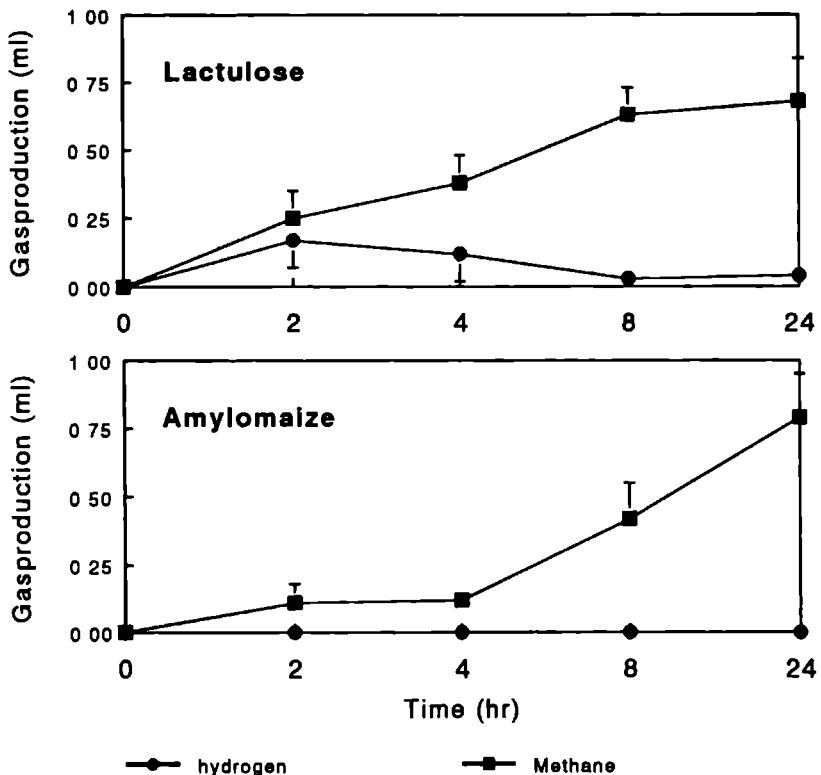

Figure 1: Mean hydrogen and methane production during fermentation of 50 mg lactulose (A) or native amylomaize (B) by seven high-methane producing fecal inocula. Bars indicate one standard error of the mean.

The CH_4-production significantly increased in HMP mixtures during lactulose and amylomaize fermentation, compared to control incubations ($p < 0.05$). The hydrogen production in the control-incubations was minimal, in HMP 2.3 ± 1.2 μl and in LMP 7.1 ± 1.8 μl after 24 hours. The HMP mixtures produced only a small amount of hydrogen after 24 hr incubation with either substrate, in contrast to LMP mixtures, in which a considerable amount of hydrogen was produced (table 1).

The mean hydrogen and methane production of the HMP inocula during fermentation of lactulose and amylomaize is given in figure 1.

Amylomaize fermentation resulted in a steadily increasing methane production. During the first 4 hours of lactulose fermentation, both hydrogen and methane were released, but hydrogen disappeared thereafter from the headspace. In LMP mixtures, the hydrogen production also increased faster during lactulose compared with amylomaize fermentation.

Table 2. Decrease in pH (mean ± sem) of 7 high-methane and 10 low-methane producing fecal inocula after 24 hr with or without supplementation with 50 mg native amylomaize or lactulose.

substrate	high-methane inocula	low-methane inocula
control	0.30 ± 0.06	0.40 ± 0.08
amylomaize	1.33 ± 0.13*	1.28 ± 0.16*
lactulose	1.75 ± 0.06*	1.83 ± 0.04*

* p < 0.05, compared with decrease of pH in control incubation.
The difference between high- and low-methane producing inocula was not significant.

Acidity of the mixture.
At the start of the incubations, the mean pH was 6.59 and this pH decreased significantly in the mixtures with amylomaize and lactulose ($p < 0.05$), but not in the control incubations (table 2). The pH decreased faster and more marked in the lactulose incubation compared to the amylomaize incubation ($p < 0.01$). No differences were observed between HMP and LMP mixtures.

Short chain fatty acid production.
The SCFA production rose gradually during the 24 hr incubation and reached twice the amount of SCFA in the mixtures with lactulose and starch compared to the control incubations (table 3). Lactulose was more rapidly fermented than amylomaize, and the former process was already completed at 8 hr.

Table 3. Total short chain fatty acid concentration (mean ± sem) in mmol/l after 4, 8 and 24 hr incubation of 7 high-methane and 10 low-methane producing fecal inocula without supplementation or with 50 mg native amylomaize or lactulose.

substrate	4 hr	8 hr	24 hr
high-methane producing inocula			
control	18.4 ± 3.4	19.9 ± 2.1	30.7 ± 5.3
amylomaize	23.9 ± 3.9	35.1 ± 4.8*	65.3 ± 8.7*
lactulose	56.7 ± 10.5*	76.9 ± 8.5*	88.6 ± 7.9*
low-methane producing inocula			
control	23.0 ± 2.7	24.9 ± 3.2	41.5 ± 6.9
amylomaize	32.8 ± 5.0	51.0 ± 10.9*	72.4 ± 10.0*
lactulose	75.6 ± 7.6*	90.8 ± 6.8*	87.5 ± 4.8*

* $P < 0.02$, compared with control incubations.
Differences between high- and low- methane producing inocula were not significant.

The total 24 hr SCFA-production was not significantly different between lactulose and amylomaize mixtures. During the first eight hours of incubation, SCFA production tended to be higher in the LMP compared with the HMP mixtures, but the difference was not statistically significant.
The proportional distribution of the individual SCFA's changed during fermentation. The fraction butyrate increased from 10% to 17% after 24 hr fermentation in the mixtures with lactulose or starch ($p < 0.001$), but not in the control mixtures (table 4). No significant differences were found between LMP and HMP mixtures.
At the start of the incubations, the molar ratio of acetate, propionate,

and butyrate was 65:17:10. After 24 hr fermentation of lactulose, the fraction of butyrate increased ($p < 0.001$) and the ratio was 67:10:17. Comparable changes after 24 hours were seen in the amylomaize mixtures (ratio 62:12:17).

Table 4. Concentration and molar fraction of butyrate (mean ± sem) in fecal inocula during fermentation with or without supplementation with 50 mg native amylomaize or lactulose.

time fraction	control Butyrate	fraction	amylomaize Butyrate	fraction	lactulose Butyrate	
	mmol/l	%	mmol/l	%	mmol/l	%
0 hr	1.0 ± 0.1	10.1	1.1 ± 0.1	10.2	1.1 ± 0.2	9.2
2 hr	1.7 ± 0.2	9.9	2.4 ± 0.4	10.9	7.0 ± 0.7	13.1
4 hr	2.2 ± 0.3	10.4	3.5 ± 0.6	11.2	10.0 ± 1.0	14.8
8 hr	2.4 ± 0.3	10.0	6.3 ± 1.3	13.2	13.2 ± 0.9	15.8
24 hr	4.7 ± 1.0	11.7	11.8 ± 1.2	17.1	15.0 ± 0.9	17.3

Discussion.

This study demonstrates that the in vitro fermentation of lactulose and amylomaize results in an increased production of hydrogen, methane, and short chain fatty acids. Furthermore, the fraction of butyrate increased. The total SCFA production is comparable between HMP and LMP incubations, suggesting that the overall fermentation is comparable. The much lower hydrogen production in the LMP mixtures, compared to the calculated H_2-equivalent in HMP mixtures is suggestive for a different pathway for hydrogen disposal in the low methane producing flora. Indeed, it has been demonstrated that hydrogen could be used by sulphate reducing bacteria to form H_2S [8], and by acetogenic bacteria to produce acetate [7]. Unfortunately, it was not possible to measure sulfide-compounds in this study.

Lactulose is rapidly fermented, as can be seen from the increase in SCFA concentration. In HMP inocula, this resulted in the production and release of both hydrogen and methane during the first hours of incubation. After 2 hours, the hydrogen concentration decreased to

almost zero. We suppose that the formation of methane out of hydrogen is the rate limiting step in methanogenesis, and that the excess hydrogen is temporarily released into the headspace and utilized afterwards. In contrast, during the more slowly fermentation of amylomaize, HMP inocula released only methane. To our opinion, all hydrogen produced in these inocula was directly converted into methane.

Both lactulose and amylomaize addition resulted in an increased SCFA production and in a decrease of the pH in the fecal samples. The production of SCFA's elapsed faster for lactulose compared to amylomaize. We speculate this is due to the more complex molecular structure of amylomaize compared to the disaccharide lactulose. It is obvious that the resistant starch must be broken into smaller fragments before final fermentation occurs.

Differences in individual SCFA profiles have been previously described, depending on the substrate supplied. Emphasis has been put on the concentration and the proportional fraction of butyrate, because of its putative anti-neoplastic effects. However, data are conflicting. In some studies, fermentation of lactulose resulted in a decrease in the butyrate fraction [22,28], whilst other authors have found an unchanged fraction [29]. Starch fermentation has been shown to increase the butyrate fraction [24,30]. In the present study we found an increased butyrate fraction both after lactulose and starch fermentation, suggesting that the kind of substrate is not of major importance.

The higher levels of SCFA's in the LMP mixtures during the first 8 hours of incubation suggests a more rapid fermentation in these inocula. This finding is supported by a recent study which showed the same difference [24]. However, the differences between LMP and HMP mixtures were not statistically significant in the present study. We assume that this is due to the large interindividual variation in the samples tested.

In conclusion, this study shows that a resistant starch compound (amylomaize) produced equivalent amounts of hydrogen, methane and short chain fatty acids compared to lactulose in a fecal incubation system. However, the fermentation elapsed more slowly. The proportion of butyrate increased similarly for both substrates. No significant differences were found between low- and high- methane producing inocula.

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Chapter 7

The effect of resistant starch on breath hydrogen and methane excretion in healthy volunteers

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Abstract.

Colonic fermentation of dietary carbohydrates and fiber might produce a protective effect against the development of large bowel cancer. Resistant starch, i.e. starch that escapes small bowel digestion, is a candidate fermentable substrate that has hitherto been little studied. We supplemented 19 healthy volunteers with 3x15 gram per day of native amylomaize (Hylon-VII), containing 28 gram of type II resistant starch, or with dextrins as a placebo for seven days in a cross-over design. Pre-experimentally, 11 subjects regularly produced breath methane an 8 did not. Resistant starch increased 24-hour integrated excretion of breath hydrogen. The mean rise relative to placebo was 35% (P = 0.03) for all subjects, and 60% for 8 subjects not producing methane (P = 0.02). The 11 methane producers showed a 93% increase in breath methane excretion on resistant starch (P = 0.03).
Continued consumption of 25 gram per day of type II resistant starch is well tolerated, and increases colonic fermentation in healthy volunteers.

Introduction.

Starchy foods such as wheat, potatoes, and cassava are the main source of energy in most human diets. Cooking serves to release starch from the granules in which it is stored, and makes it available for digestion. Dietary starch is largely hydrolyzed and absorbed in the small bowel, but a part is resistant to digestion, depending on processing and type of starch [1-3]. This resistant starch fraction enters the cecum and is a potential substrate for fermentation [1,4]. Fiber is another important source of fermentable carbohydrate, but high-fiber diets have proved less than attractive to most western people, and adherence to such diets is poor. Resistant starch might conceivably provide a useful alternative to fiber. However, very little is known about the actual fate of resistant starch in the colon.

Dietary fiber can offer protection against the development of colonic cancer [5-8]. Fermentation of fiber into short chain fatty acids is one of the proposed mechanisms for this effect. Thornton et al demonstrated that in patients with colonic neoplasms the fraction of dietary starch reaching the colon was smaller than in healthy controls [9].

Resistant starch has been classified into 3 groups: type I represents physically inaccessible starch such as in intact or partly milled grains; type II consists of starch enclosed in granules, such as in raw potato or unripe bananas; and type III represents retrograded amylose [1].

The proportion of resistant starch in several foods has been calculated by feeding them to colectomized patients and determining the residual starch in the ileostomy output. The proportion of starch that was resistant to small bowel digestion and absorption varied from 1% in potatoes [10] to 89% in unripe bananas [11]. Molis et al intubated the cecum of healthy volunteers and found that 49% of ingested retrograded high amylose maize starch was recovered from the cecum [12]. In common foods, only a relatively small proportion of dietary starch appears to be resistant starch [1,13]. Using the intubation technique, Flourie et al [14] have demonstrated that 4% of starch in a average meal reaches the colon. Stephen et al used the same intubation technique and recovered 10% of starch in meals from the terminal ileum; In some subjects the figure was as high as 20% [15]. In general, such studies show 4-10% of dietary starch to be resistant to digestion.

One endproduct of cecal fermentation of starch is hydrogen, which

can be measured in the expired breath. If the colonic bacterial flora contains a sufficient amount of methanogenic bacteria, hydrogen will in its turn be used to synthesize methane [16]. This can be found in the expired air and flatus of approximately fifty percent of the western population [17]. Therefore, the excretion of these two gasses can be used as a gauge for colonic fermentation [18].

If indeed fermentation is the mechanism through which fiber has a protective effect on colon cancer development, we speculate that resistant starch could have the same effect. As far as we know, only a few single dose experiments have been done with resistant starch in human subjects, but no data are available of the effect of continued supplementation with resistant starch. Here we report the effects of one week feeding of 45 gram native amylomaize, containing 28 gram resistant starch per day on colonic fermentation in healthy volunteers.

Subjects and Methods.

Hypothesis.
The study was set up to test the following hypotheses:
1. Resistant starch supplementation will cause a higher breath hydrogen excretion in both methane producers and non-methane producers than maltodextrin supplementation.
2. Resistant starch supplementation will increase breath methane excretion in methane producers.

Subjects and design.
Twenty-two healthy male volunteers (mean age 46 yr, range 21-76) entered the study. Women were excluded because of the influence of the menstrual cycle on starch absorption and fermentation [19]. Other exclusion criteria were: smoking, recent use of laxatives or antibiotics and previous or current bowel disease. Four breath samples were collected on two separated days from each volunteer before the study, so as to classify him as a methane excreter or non-excreter. A subject was classified as methane excreter when the methane concentration exceeded 3 ppm in 2 or more of the 4 breath-samples[20]. Ten volunteers were non-excreters, and 12 were methane excreters. One non-methane excreter dropped out because of

influenza, and one excreter and one non-excreter provided insufficient breath samples, leaving a total of 19 who completed the trial successfully.

The design was a two period, placebo-controlled cross-over study with periods of 7 days and a 7-day wash-out period between treatments. During the last 3 days of each supplementation period, volunteers recor-ded their diet and any symptoms of abdominal discomfort in a special diary. The purpose and design of the study and the possible consequences of resistant starch consumption were thoroughly explained to the sub-jects, who then gave their informed consent in writing. The study was approved by the Human Experimentation Committee of the Academic Hospital Nijmegen.

Diets and food intake.
Participants consumed their regular diets during the study, but they were asked to refrain from consuming beans and peas. During the supplementation periods, subjects consumed 3x15 gram per day of native amylomaize starch (Hylon-VII, National Starch & Chemical Company, Zutphen, The Netherlands) or 3x15 gram maltodextrin (Cerestar SF 01904, Cerestar Benelux BV, Sas van Gent, the Netherlands) with their meals. Native Hylon-VII contains a high proportion of amylose; about 62% on gross weight basis is type II resistant starch as determined by the in vitro method of Englyst et al [21]. Consequently, 45 gram native amylomaize contains 28 gr resistant starch. Maltodextrin is a rapidly absorbable, partially hydrolyzed maize-starch.

Diet composition was calculated from the dietary records using the 1987 release of the Netherlands nutrient data bank.

Breath hydrogen and methane.
The subjects collected breath samples in 60 mL syringes at 2 hr intervals from 8.00 am until 24.00 hr and again at 8.00 pm the next morning during the last 24 hours of each supplementation period. Immediately after breath collection another syringe was filled with ambient air, and both syringes were sealed.

Hydrogen was measured within 24 hours after collection using a standard electrochemical cell. Calibration was performed with a standard gas (AGA-gas, Amsterdam, The Netherlands) with a hydrogen concentration of 95.7 ± 2.5 ppm. For the determination of

methane concentration, 0.4 mL breath samples were injected in triplicate into a Packard gas-chromatograph, equipped with a Porapak Q 100-120 mesh column at an oven temperature of 50°C. Calibration was done with a standard gas (AGA-gas, Amsterdam, The Netherlands, methane concentration 28.4 ± 0.4 ppm). The methane concentration in each breath sample was corrected for the concentration of the matching ambient air sample.

Symptom score.
On the final 3 days of each supplementation period, the volunteers scored complaints of bloating, flatulence, abdominal cramps, belching and diarrhea in a diary on a scale running from 0 (none) to 4 (severe). The mean value of the median symptom score was calculated for each complaint during both periods.

Data analysis and statistics.
All values are given as mean ± sem. The effect of resistant starch supplementation and maltodextrin supplementation on hydrogen and methane excretion , calculated as the area under the 24 hour time-concentration curve, was tested one-tailed using the method of Pocock [22]. With a confidence-level of $a = 0.10$, no interaction could be demonstrated between period and supplementation. Under the assumption of equal period effects, differences between methane and non-methane producing subjects were tested with a two-way ANOVA without interaction (classifications: type of supplement, methane producer/non-producer). Calculations were done with the SAS analysis system.

Results.

As shown in table 1, values for carbohydrate and energy intake were those typically found in adult Dutch men. No significant differences in food intake were seen between treatments for either methane or non-methane excreters. The 45 g/d of native amylomaize was well tolerated (table 2); only flatulence was somewhat increased.

Table 1. Intake of energy and carbohydrates from regular foods in volunteers participating in a controlled crossover trial on the fermentation of resistant starch. In addition, subjects received 45 g/d of native amylomaize (Hylon-VII) or of rapidly digestible starch (maltodextrin) for 7 days each.

	Subjects	N	amylomaize	maltodextrin
Energy	All	21	11.4 ± 2.4	12.0 ± 2.7
(MJ/24 hr)	Methane excreters	12	11.3 ± 2.4	12.5 ± 3.2
	Non-methane excreters	9	11.6 ± 2.5	11.2 ± 1.7
Mono and	All	21	153 ± 43	156 ± 30
disaccharides	Methane excreters	12	153 ± 51	156 ± 33
(g/24hr)	Non-methane excreters	9	153 ± 32	156 ± 29
Starch	All	21	165 ± 41	166 ± 59
(g/24hr)	Methane excreters	12	168 ± 43	182 ± 65
	Non-methane excreters	9	162 ± 40	146 ± 45
Dietary fiber	All	21	29 ± 11	31 ± 14
(g/24hr)	Methane excreters	12	32 ± 13	34 ± 18
	Non-methane excreters	9	26 ± 7	26 ± 7

Table 2. Mean and range of symptom-scores as recorded by 21 subjects during supplementation with 45 g/d of native amylomaize (Hylon-VII) and of maltodextrin. Subjects scored symptoms on a scale from 0 (none) to 4 (severe).

Symptom	amylomaize		maltodextrin	
	Mean	range	Mean	Range
Bloating	0.4	0-2	0.3	0-3
Flatulence	1.4	0-3	0.7	0-2
Cramps	0.1	0-1	0.2	0-3
Belching	0.2	0-1	0.2	0-1
Diarrhoea	0.0	0-1	0.0	0-1

Values are means ± SD for days 5-7 of the combined supplementation periods and include those for two subjects who provided insufficient breath samples for H_2 and CH_4 analysis. Differences between periods were not significant either biologically or statistically for any nutrient.

Hydrogen excretion.

The hydrogen concentration in expired air ranged from 1.2 to 91.5 ppm during consumption of resistant starch and from 0 to 57.6 ppm during the maltodextrin period. The mean hydrogen excretion rose during the day, and peaked late at night, several hours after the last dose of resistant starch (figure 1).

A significant rise in integrated 24-hour hydrogen excretion during amylomaize supplementation relative to control treatment was found in both the whole group and in the subjects not excreting methane (table 3). The effect was more modest in methane excreters.

Table 3. Mean 24-hour integrated breath hydrogen excretion (ppm x hr) ± SEM of healthy subjects after seven days supplementation with 45 g/d of native amylomaize (Hylon-VII) or maltodextrin.

	N	amylomaize	maltodextrin	difference[+]
All subjects	19[++]	601 ± 61	443 ± 46	155 ± 75[*]
Methane excreters	11	592 ± 97	488 ± 62	105 ± 110
Non-methane excreters	8	612 ± 64	383 ± 65	230 ± 90[**]

[+] *The difference is corrected for the period effect.*
[++] *Another 2 subjects had provided insufficient sample for hydrogen analysis.*
[*] *p = 0.03* [**] *p = 0.02*

Methane excretion.

The mean concentration of methane in ambient air was 3.9 ± 1.2 ppm. During amylomaize supplementation, the mean methane concentration in breath, corrected for ambient air concentration, ranged from 17 to 26 ppm in methane excreters (figure 2). The area under the curve of concentration versus time was significantly higher during amylomaize supplementation than during control treatment (table 4).

In subjects initially classified as 'non-methane producers", methane excretion was indeed negligible on either treatment (table 4).
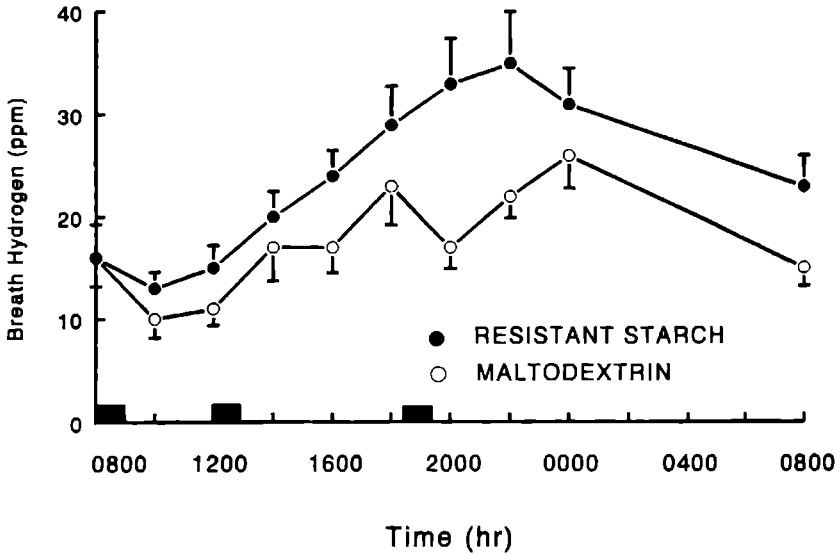
Figure 1. Mean breath hydrogen concentration on the seventh day of supplementation of a normal Dutch diet with 3 x 15 g per day of maltodextrin or of native amylomaize (Hylon-VII), providing 28 g/d of type II resistant starch. Bars indicate one standard error of the mean. (■■■ : time of ingestion of supplement).
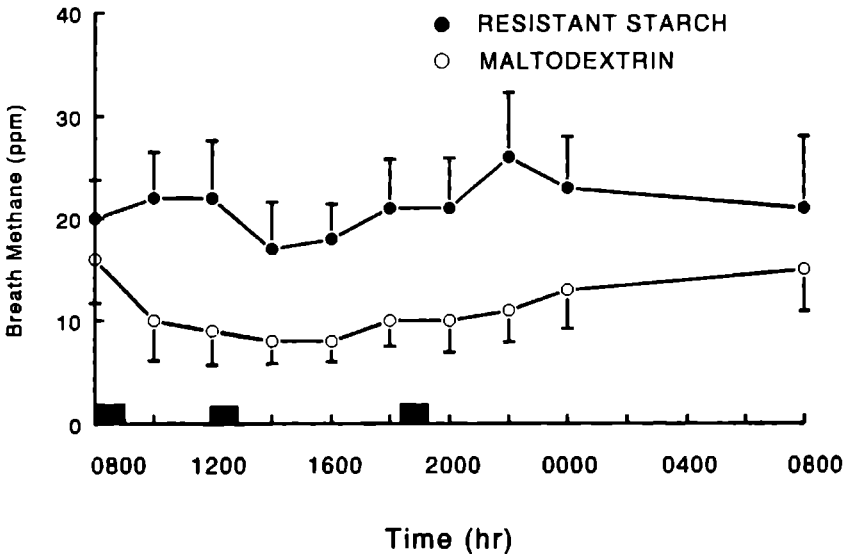

Figure 2. Mean breath methane concentration of 11 methane producing subjects on the seventh day of supplementation of a normal Dutch diet with 3 x 15 g per day of maltodextrin or native amylomaize (Hylon-VII). Bars indicate one standard error of the mean. (■■■:time of ingestion of supplement).

Table 4. Mean 24-hour integrated breath methane excretion (ppm x hr) ± SEM of healthy subjects on the final of 7 days of supplementation with 45 g/d of native amylomaize (Hylon-VII) or maltodextrin.

	N	amylomaize	maltodextrin	difference [+]
Methane excreters	11	506 ± 109	280 ± 73	247 ± 119[*]
Non-methane excreters	8	1 ± 1	4 ± 3	-3 ± 3

[+] *The difference is corrected for the period effect.*
[*] *p = 0.03.*

H₂-equivalents in methane producing subjects.
In subjects who produce methane, hydrogen formed by fermentation is converted into methane according to the equation: $4H_2 + CO_2 \dashrightarrow CH_4 + 2H_2O$ [23]. The primary 24 hour integrated hydrogen production in such subjects (H_2-equivalent) was estimated according the formula: area under the curve (H_2) + 4x area under the curve (CH_4). This sum rose significantly in methane producing subjects from 1607 ± 283 ppm x hr during placebo supplementation to 2617 ± 419 ppm x hr during resistant starch supplementation. After correction for a period effect, the difference between the two supplementations was 1094 ± 447 (p = 0.015).

Discussion.

Data about hydrogen excretion after resistant starch ingestion are scarce. A positive response has been demonstrated after single doses of raw potato starch [24] and raw banana starch [25], and consumption of a type III resistant starch as cornflakes resulted in an elevation of breath hydrogen on the first day of consumption [26]. However, several studies have demonstrated an adaptation of the colonic flora after chronic consumption of fermentable substrate [27,28]. Therefore, the increased fermentation after continued resistant starch consumption found in this study is more relevant to real-life situations than the effect of a single dose. Studies concerning hydrogen excretion after fiber ingestion have shown conflicting results [4,29,30], which could partly be explained by differences in fermentability of different fibers.

As hydrogen is produced by bacterial fermentation in the colon, the rise of hydrogen excretion is probably the result of fermentation of amylomaize escaping digestion and absorption in the small bowel. Hydrogen excreted during the maltodextrin period is the result of background fermentation of other food constituents in the normal diet. This integrated hydrogen excretion during the placebo supplementation was similar to that reported by other authors [4]. Hydrogen produced in the large bowel may be further metabolized to acetate by acetogenic bacteria, to H_2S by sulphate reducing bacteria, or to methane by methanogenic bacteria [16]. Competition between these metabolic pathways exists, and only half of the western population shows significant excretion of methane [17]. In the present study, methane excreting subjects showed a significant increase of methane excretion after resistant starch supplementation. Flourie et al found that a large increase in the intake of digestible starch also resulted in an increased excretion of methane, but not of hydrogen [14]. Presumably, an overload of digestible starch results in a partial escape from small bowel digestion and to colonic fermentation. Studies about the effect of fiber consumption on methane excretion have shown conflicting results [31-34], although in general the effect is minimal. This suggests that resistant starch may more readily increase colonic fermentation than does dietary fiber.

Those of our subjects who did not excrete methane when on their regular diets also did not do so after resistant starch supplementation. A lack of excretion of methane thus appears to be due to a difference in colonic bacterial composition rather than to a lack of fermentable substrate in the habitual diet.

Hydrogen excretion did not increase significantly in the methane excreting subjects after supplementation of resistant starch. We speculate that this is due to excess hydrogen being channelled into methane production. Formation of one molecule of methane requires four molecules of hydrogen. The equivalent H_2 excretion, calculated as $H_2 + 4CH_4$, rose significantly in methane producers after resistant starch consumption. This suggests that the difference in response of breath hydrogen to resistant starch between methane and non-methane-excreting subjects was due to a difference in utilization of the hydrogen produced, rather than to a difference in colonic production of hydrogen from resistant starch. In a previous study using whole body calorimetry, lactulose consumption caused a lower

hydrogen excretion but a higher excretion of total H_2-equivalents in methane producers than in non-methane-excreting subjects [25]. This is in line with our results.

The relatively large amount of resistant starch (28 g/d) given by us was well tolerated; the subjects reported an increase in flatulence but no other discomfort or symptoms.

We conclude that a supplementation of the diet of healthy volunteers with 28 g/d of type II resistant starch in the form of 45 g/d of native amylomaize is well tolerated, and results in an increased colonic fermentation. Epidemiological studies are warranted to elucidate a possible protective effect of resistant starch on the development of colon cancer.

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Chapter 8

The effect of resistant starch on colonic fermentation, bile acid metabolism and mucosal proliferation

Ivo P. van Munster, Albert Tangerman and Fokko M. Nagengast.

Abstract.

Background: Resistant starch is by definition that part of starch that escapes digestion in the small bowel. Cecal fermentation of resistant starch into short chain fatty acids will result subsequently in a decrease in pH. Thus, resistant starch may have the same effect on colonic luminal contents and mucosa as some fiber components.

Methods: We studied the effects of adding 45 g native amylomaize (Hylon-VII) to a standardized diet in 14 healthy volunteers on fermentation and colonic mucosal proliferation. Hylon-VII is a high amylose maize starch, containing 62% resistant starch.

Results: During amylomaize consumption, breath hydrogen excretion raised with 85% and fecal short chain fatty acid output increased with 35% ($p < 0.01$). Excretion of primary bile acids increased and the soluble deoxycholic acid concentration decreased by 50% ($p = 0.002$). Subsequently, cytotoxicity of the aqueous phase of feces -as measured on a colon cancer cell line- decreased ($p = 0.007$). Colonic mucosal proliferation in rectal biopsies (Proliferating Cell Nuclear Antigen immunostaining) decreased from 6.7 to 5.4% ($p = 0.05$). We speculate that resistant starch consumption decreases the colonic mucosal proliferation as a result of the decreased formation of cytotoxic secondary bile acids, which is possibly mediated through acidification of the large bowel by production of short chain fatty acids.

Introduction

Fermentation of fiber to short chain fatty acids (SCFA) in the colon has been postulated to be one of the factors that protect the mucosa against malignant transformation. In man, low fecal butyrate concentrations have been associated with an increased risk for the development of colonic neoplasms [1-3]. Cancer cell experiments have shown that butyrate has antineoplastic characteristics. It promotes cell differentiation and reduces the growth rate of cultured cells by decreasing the doubling time [4,5]. Besides a direct effect of butyrate on cellular proliferation and differentiation, production of SCFA also lowers the colonic pH. This results in inhibition of bacterial transformation of primary into secondary bile acids [6,7] and in a decreased solubility of these acids [8]. Secondary bile acids are cytotoxic to colonic cells, subsequently resulting in an increased cell proliferation[9]. An increased cell proliferation is associated with a higher susceptibility for the development of colonic cancer [10-12]. The fraction of S-phase cells is increased in a hyperproliferative mucosa. Subsequently, mutagenic substances that are present in the colon, can exert more effect because the number of "target cells" (S-phase cells) is increased. This mechanism could explain the demonstrated (co-) carcinogenicity of bile acids [13-15].
Several epidemiological studies have shown a protective effect of dietary fiber on the development of colonic cancer, although this is not a consistent finding [16-20]. The interpretation of the relation between fiber and colonic cancer risk is complicated by the fact that different types of fiber can have different effects [21-23]. Fermentation of components of fiber could contribute to a diminished risk, as could accelaration of transit time and dilution. On the other hand, gelforming types of fiber could bind bile acids and increase the colonic bile acid load. In a recent meta-analysis, it has been shown that fiber derived from vegetables, fruit or cereals can have a different protective effect on colon cancer formation [24]. It is yet unclear to what extent the difference in fermentability of types of fiber contributes to these differences.
Resistant starch (RS) is also a potential source of fermentable substrate, resulting in SCFA production. RS is defined as starch escaping digestion in the small bowel [25-27] and can be classified in 3 groups [25]: type I or RS_1, representing physically inaccessible starch

like whole or partly milled grains; type II or RS_2, representing starch in granules, like raw potato, unripe bananas; and type III or RS_3, representing retrograded amylose starch. Native Hylon-VII, a high amylose maize starch, is an example of a type II resistant starch.

Assuming that fermentation contributes to a lower colon cancer risk, resistant starch might be an attractive substance in dietary intervention studies. However, only few data on fermentation of RS in the human large intestine are available. In two previous studies about the relation between starch fermentation and colonic cancer risk, conflicting results have been found [28,29]. The effect of RS on bile acid metabolism and colonic proliferation in humans has not been investigated to our knowledge. We therefore have studied in a pilot experiment the effects of supplementation of native amylomaize to a standardized diet of healthy volunteers on colonic fermentation, bile acid metabolism, cytotoxicity of fecal water and colonic mucosal proliferation.

Materials and methods

Outline of the study.
Fourteen healthy volunteers (9M, 5F, age 28-73 years) participated in a 3 week study. The average weight of the volunteers was 77.7 ± 2.0 kg and did not change during the experiment. A short outline of the study is given in figure 1. A dietary history was taken by a dietician, and the volunteers were instructed to take an average Dutch diet containing approximately 20 g natural fiber and 120 g fat of which 60% was unsaturated. Only minor modifications of the habitual diets of the volunteers were necessary. In the second and third week of the study, a daily dose of 3x15 gr native, uncooked amylomaize (Hylon-VII) was added to the diet. Hylon-VII (National Starch & Chemical Company, Zutphen, The Netherlands) is a high amylose maize starch, containing about 62% of type II resistant starch, as determined by the in vitro method of Englyst et al. [30]. At the end of the first week of the study, during which the volunteers were on the standardized diet (control period), and at the end of the third week, while the diets were supplemented with amylomaize for two weeks (experimental period), rectal biopsies were taken, without previous laxation. In these biopsies, colonic cell proliferation was

determined. At the end of the control and experimental periods, the 72 hour fecal output was collected on dry ice for determination of starch content, short chain fatty acids, total and aqueous phase bile acid concentrations and cytotoxicity of fecal water. Furthermore, breath samples were taken at the end of the first week, 2 days after the start of amylomaize supplementation and at the end of the third week for determination of hydrogen concentrations.

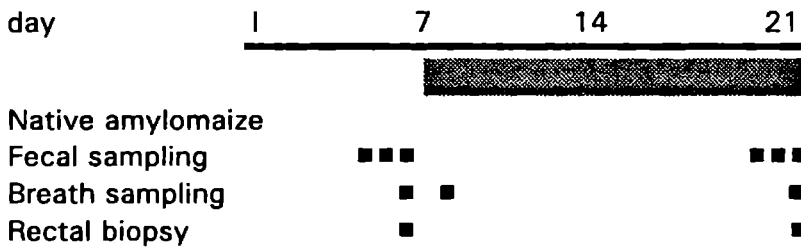The study was approved by the Ethical Committee of the University Hospital Nijmegen.

day	I	7	14	21

Native amylomaize
Fecal sampling
Breath sampling
Rectal biopsy

Figure 1. Design of the study. During week 2 and 3, subjects received 45 g/d of amylomaize.

Dietary diary for food composition.
To check for dietary compliance, during the last 4 days of the control period and of the experimental period, all participants recorded the total amount of food intake in a dietary diary. From these diaries, diet composition was calculated using the 1987 release of the Netherlands nutrient data bank.

Breath hydrogen measurement.
Breath samples were collected in 60 ml syringes at 2 hr intervals from 8.00 am until 24.00 hr and at 8.00 pm the next morning. Within 24 hour, hydrogen concentrations were measured employing a standard electrochemical cell (Lactoscreen, Hoekloos, Amsterdam, The Netherlands). Previously, we found that the hydrogen concentration did not decrease during 48 hr storage (unpublished results). The hydrogen excretion was expressed as area under the concentration-time curve (AUC). The percentual increase in hydrogen excretion in

the experimental period compared to the control period was calculated.

Preparation of the aqueous fraction of stool.
Stool samples were collected on dry ice and subsequently stored at -20°C until processing. Samples were homogenized with a blender and ultracentrifugated at 30.000 g for 2 hr at 4°C. Fecal water (FW) was carefully removed and filtered employing a 0.2μ filter, (Schleicher & Schuell, Dassel, Germany) and stored at -20 °C until analysis.

Short chain fatty acid analysis.
Ten μl of a solution of 0.17 M 2-ethylbutyric acid (internal standard) in 100% formic acid was added to 100 μl of fecal water. The mixture was centrifugated and 0.7 μl of the resulting clear supernatant was directly injected in a Packard gas chromatograph (column: 10% SP1200 / 1% H_3PO_4 on 80/100 Chromosorb WAW). The injection port of the chromatograph was installed with an 8 cm long glass liner with an internal diameter of 3 mm, stoppered with a glass wool plug. Injection was performed in the space inside the liner above the glass wool plug. Details of the gas-chromatographic separation of SCFA have been described elsewhere [31].

Bile acid analysis.
Bile acids were determined as published before [32,33], with slight modifications. After enzymatic hydrolysis the bile acids were extracted from fecal water (0.5 ml) by SepPak C18 chromatography. The saponifiable conjugates were then hydrolyzed at 60°C for 2 h in 1 ml of 1 M KOH in methanol. The unconjugated bile acids were separated from the neutral sterols by Lipidex DEAP chromatography and measured after methylation and silylation by capillary gas chromatography (column CP Sil 5CB) on a Packard 430 gas chromatograph.

Residual starch in stool.
The amount of residual starch present in the feces was determined according Björck et al [34]. Shortly, 50 mgr freeze dried feces was suspended in 2 ml water and free glucose was measured after heating (5min, 100 °C). To measure the total glucose, which is the sum of

starch and free glucose, 50 mgr freeze dried feces were suspended in 1 ml water and 1 ml 4 M KOH and incubated during 30 min at RT. The suspension was neutralized with HCL and 1 + 1 diluted with Na-acetate buffer (0.4 M, pH 4.75). The mixture was incubated with 20 μl amyloglucosidase (1gr/100ml, Boehringer) during 30 min, at 60°C. Total glucose was determined in the supernatant. The amount of starch is the difference between total glucose and free glucose.

Cytotoxicity assay with HT-29 cells.
We used a colonic cancer cell-line, HT-29, to measure cytotoxicity [35]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Flow Laboratories Irvine, Scotland) until 90% confluence. After trypsinization, cells were resuspended and plated in 96 multi well plates (Costar, Cambridge, Mass, USA) with a concentration of 15.000 cells/well. After 48 hours culture at 37°C under 5% CO2, cells were washed and incubated for 1 hour with 100 μl fecal water. Every experiment with a single fecal water sample was performed in octuple. After 1 hour incubation, cells were washed and cultured for another 48 hours with DMEM. Then 20 μl of a 1 mg/ml 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (Sigma St. Louis, MO, USA) was added to every well. In the viable cells, the tetrazolium salt will be converted by mitochondrial activity to formazan, a blue dye [36-38]. After four hours cells were washed and lysis was carried out with 100 μl dimethylsulfoxide to free the formed formazan. Absorption was read at 545 nm using a Multiscan (Titertek Multiskan, Flow, Helsinki, Finland). The cell survival was expressed as percentage absorption of the maximal absorption, read from the wells incubated with PBS. Cytotoxicity is inverse correlated with the percentage survival (100 - survival).

PCNA proliferation in rectal biopsies.
Three rectal biopsies were fixed in 70% ethanol for Proliferating Cell Nuclear Antigen immunostaining. Standard histological sections were prepared on poly-L-Lysine (Sigma) coated slides. After deparaffinization and blocking of endogeneous peroxidase, sections were incubated with PC10 antibodies in a 1:400 dilution for 1 hr at 25°C (Dako, Glostrup, Denmark). After washing, a streptavidine-biotine system (Histostain-SP, Zymed Laboratories, San Francisco, USA) was used to visualize the anti-body. Sections were microscopically

examined under code with a 40x objective, at least 10 completely longitudinally sectioned crypts were scored and the mean value was calculated. All cells with a homogeneous and distinct red nucleus were counted as positive. The fraction positive cells in the whole crypt was calculated, as well as in 5 crypt-segments, from the basal to the luminal side of the crypt.

Statistics.
Results are expressed as mean ± SEM. The averages of each test during the control and experimental period were compared, using a Wilcoxon signed rank test, the two tailed P-value for normal approximation is given. For Hydrogen and SCFA changes, the one tailed P-value is given.

Results.

One volunteer dropped out of the study because of abdominal cramps and discomfort after starting with the amylomaize. The other 13 participants tolerated the amylomaize well. The relevant part of food composition data is given in table 1.

Table 1. Food composition data of 13 subjects during the control period and during supplementation of 45 g/d Amylomaize.

	Control period	Native amylomaize supplementation
Energy (Kcal/day)	2427 ± 116	2462 ± 128
Fat (g/day)	123 ± 6	125 ± 8
Fat (energy %)	45 ± 1	46 ± 1
Sat. Fat (%)	39 ± 2	40 ± 4
Starch (g/day)	124 ± 7	121 ± 9
Fiber (g/day)	17 ± 1	16 ± 1
Calcium (mg/day)	953 ± 94	1024 ± 142

Values are given as mean ± SEM. In addition, subjects received 45 g/d of amylo-maize during the experimental period. None of the differences is significant.

No significant changes were found between the control period (week 1) and the experimental period (week 3), so we assume the food intake did not influence other parameters studied.

Fermentation.
The stool consistency and bowel frequency did not change significantly. The mean wet weight of the daily stool increased by 23% (p = 0.04), the mean percentage dry matter did not change (25 vs 24%), nor did the fecal pH (table 2).
The integrated hydrogen excretion increased from 101 ± 19 ppm.hr during the control period to 186 ± 38 ppm.hr on the second day of the experimental period (85%, p = 0.002) and was still increased at the end of the experimental pariod (44%, p = 0.03). The fecal SCFA excretion increased from 7.1 to 9.6 mmol/day (fig. 2). This increase was mainly caused by a 35% rise in acetate and a 47% rise in butyrate excretion. Excretion of total SCFA's and of acetate and butyrate were significantly higher during amylomaize suppletion (p = 0.005).
No significant changes were found in the fecal concentrations of SCFA's. The total SCFA concentration varied from 87.8 ± 10.5 mM in the control period to 89.6 ± 10.5 mM in the experimental period, acetate from 55.3 ± 3.6 to 56.6 ± 3.6 mM, propionate from 15.5 ± 0.9 to 15.2 ± 0.9 mM and butyrate from 9.1 ± 1.1 to 10.4 ± 1.5 mM.

Table 2. Fermentation data of 13 subjects during the control period and during supplementation of 45 g/d native amylomaize.

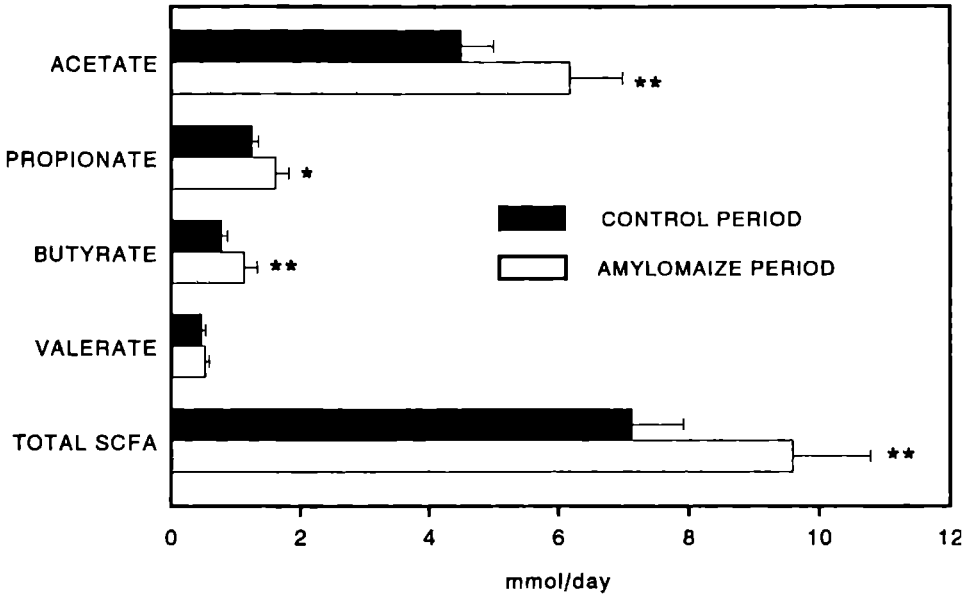|	Control period	Native amylomaize supplementation	P
Stool weight (gr/day)	119 ± 9	147 ± 10	0.04
Stool pH	6.6	6.7	n.s.
Hydrogen excretion (ppm.hr)			
day 2 supplementation	101 ± 19	186 ± 38	0.002
day 14 supplementation		144 ± 19	0.03
SCFA excretion (mmol/day)	7.1 ± 0.8	9.6 ± 1.4	0.005
Residual starch (gr/day)	0.10 ± 0.03	0.26 ± 0.08	n.s.

Values are given as mean ± SEM.

Figure 2. Fecal excretion (24 hour) of acetic, propionic, butyric and valeric acid (mean ± SEM) during control and experimental period.
`* p<0.05 and ** p<0.005, compared with control period.*

In the control period, 0.1 ± 0.03 g/day of residual starch was found in the feces. During the experimental period, with a daily suppletion of 45 gr amylomaize, the residual starch content rose to 0.26 ± 0.08 mg/day. This points to an almost complete fermentation of amylomaize.

Fecal bile acids.
The concentrations of total bile acids (in freeze dried feces) are given in table 3. The primary bile acid concentration rose from 1.5 to 3.9 μmol/gr dry weight (p=0.03). The concentration of secondary bile acids tended to decrease from 25.3 to 20.4 μmol/gr dry weight (p = 0.08), and the fraction secondary bile acids decreased from 93% to 82% (p = 0.02).
In the aqueous phase of stool the concentration of total soluble bile acids decreased from 177.0 to 99.8 μmol/l (p = 0.004). The deoxycholic acid concentration decreased by 49.8% (from 78.0 to 39.2 μmol/l, p = 0.002) (Figure 3).
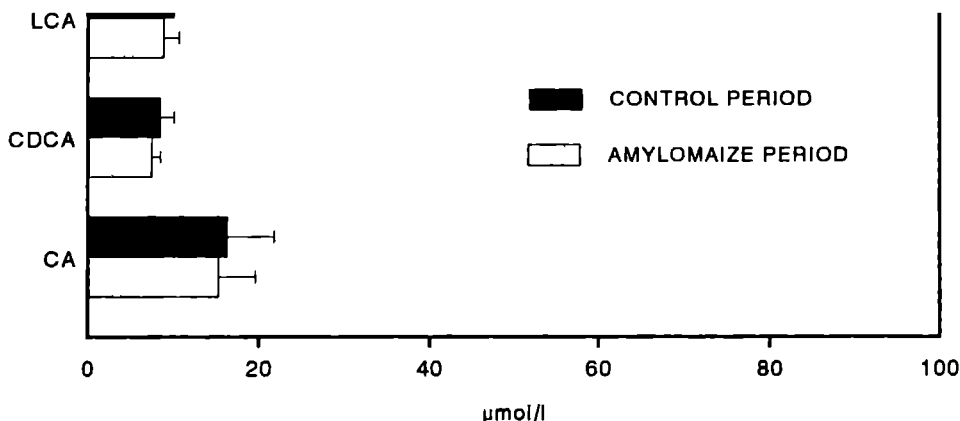
Figure 3. Concentration of bile acids in the aqueous phase of stool during control and experimental period (mean ± SEM). The soluble DCA concentration decreased with 49% (p = 0.002). DCA: deoxycholic acid; LCA: lithocholic acid; CDCA chenodeoxycholic acid; CA: cholic acid.

Cytotoxicity of the aqueous phase of stool.

In the cytotoxicity assay, all but one of the volunteers demonstrated a decrease in the cytotoxic potential of the FW (fig 4). The mean cytotoxicity decreased from 84 ± 6% to 72 ± 9% (p = 0.007).

Colonic mucosal proliferation.

Biopsies from one volunteer did not show sufficient intact crypts to score. Proliferation of colonic crypt cells in the other 12 subjects was determined in the total crypt and in 5 different crypt segments. An example of PCNA labelled crypts is given in figure 6. A decrease in proliferation was found (fig. 5), the total labelling index decreased from 6.7 ± 1.7 to 5.4 ± 1.1 % (p = 0.05). Since in those healthy volunteers almost no labelling was observed in the upper compartments, the decrease in proliferative activity could mainly be attributed to the 3 basal compartments.
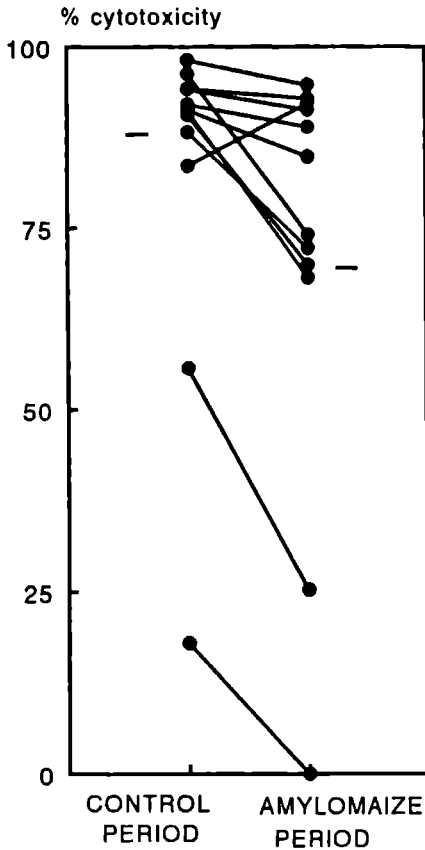
Figure 4. Cytotoxicity of individual fecal water samples as determined with the HT-29 assay. During the native amylomaize supplementation the mean cytotoxicity decreased from 84 to 71 % (p = 0.007).

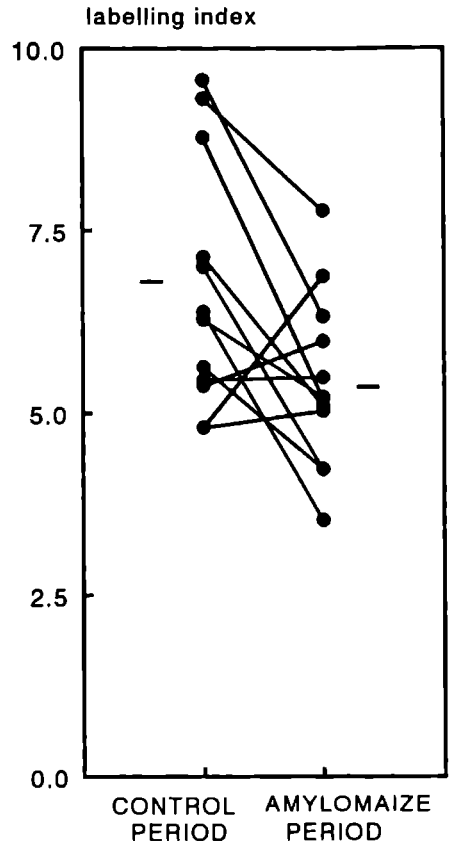Figure 5. PCNA labelling index of colonic mucosa in 12 subjects before and during the amylomaize supplementation. The mean labelling index decreased from 6.7 ± 1.7 to 5.4 ± 1.1 % (p = 0.05).

Discussion.

This study shows important changes in fecal bile acid composition and colonic cell proliferation during the supplementation of resistant starch to a normal Dutch diet. Because no changes were observed in food composition during the study, we assume that the changes were caused by the resistant starch supplementation. The almost complete absence of residual starch in the feces points to a complete utilisation of the starch in the large bowel.
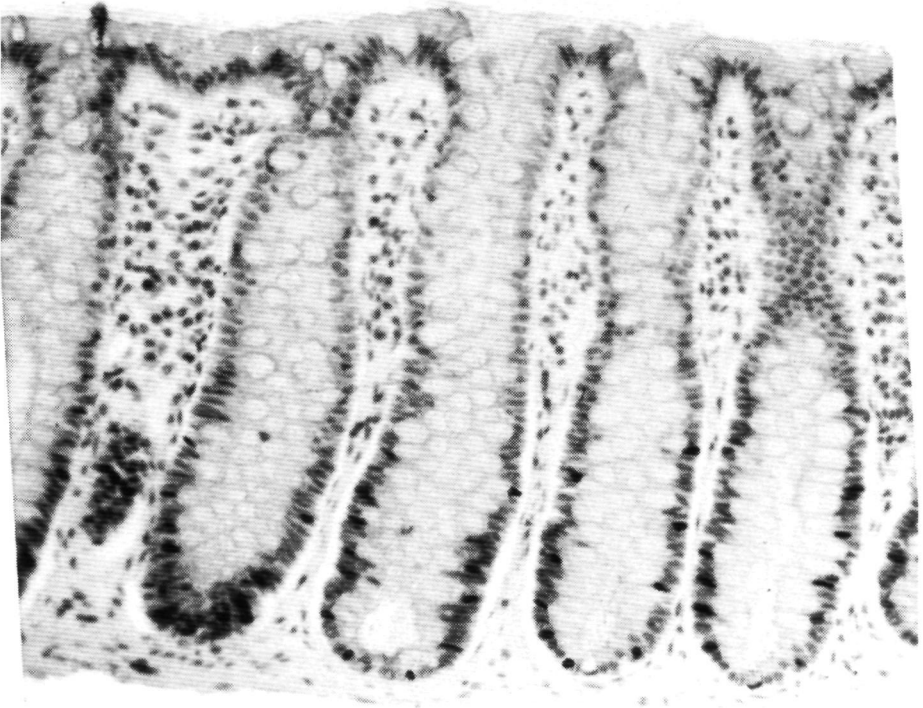
Figure 6. Immunostaining of colonic mucosal crypts with a PCNA-antibody.

The increased hydrogen excretion was the result of fermentation of resistant starch. The more marked hydrogen excretion in the first days after amylomaize supplementation compared to the end of the study period, is suggestive of a certain degree of adaptation which has been described previously [39,40].

The rise in SCFA excretion was relatively minor, but we assume that this is the result of the efficient absorption and utilisation of SCFA's in the large bowel [41-43]. The increase of the butyrate excretion during RS is of special interest, because differentiating and anti-neoplastic effects have been demonstrated in vitro [5,44-46]. The relative enrichment of butyrate in the SCFA pool after starch or RS fermentation has been demonstrated recently [47,48].

Non-digested starch will be fermented after entering the cecum [49], resulting in a lower pH in this part of the large bowel. It has been demonstrated that the pH of the colonic contents gradually rises during the transport from cecum to sigmoid [42,50]. This is probably due to the absorption of SCFA's. Thus fecal pH is not necessarily a good

indicator for fermentation and acidity in the proximal colon, and this may be the explanation for the absence of change of the fecal pH.

We have studied the effect of RS fermentation on soluble and total fecal bile acids. Most important, to our opinion, is the bile acid composition of the aqueous phase of stool, because only the soluble bile acids are in contact with the colonic mucosa and can cause mucosal damage [8]. Precipitation or binding of bile acids, resulting in a shift from the "soluble" compartment to the inert, "solid" compartment is reflected in the bile acid concentrations in the total freeze dried feces [51]. Therefore, changes in total and soluble bile acid composition have to be considered separately, and to our opinion the 50% reduction of the soluble DCA concentration is the most important change.

A decrease in the pH in the proximal colon can have two separete effects on bile acid metabolism. Firstly, the bacterial conversion of primary bile acids into secondary bile acids is inhibited at a pH value below 6 [8]. Formation of secondary bile acids primarily occurs in the cecum. In animal studies it has been shown that cecectomy abolishes secondary bile acid formation [52,53]. The inhibition of bacterial conversion may explain the decrease in the total secondary bile acid concentration and in soluble DCA. It does not explain why the amount of soluble primary bile acids did not increase. Secondly, it seems likely that acidification of the colonic contents by RS fermentation leads to precipitation of unconjugated bile acids (pKa's around 5.0) [54]. This results in a shift of soluble bile acids to the insoluble fraction. Bacterial conversion of primary bile acids is thus inhibited, resulting in an important decrease in soluble DCA and to an increase of the total primary bile acid concentration. Precipitation of bile acids has been demonstrated in rats in whom an important decrease of cytotoxicity and mucosal damage has been shown after lowering the pH in the large bowel from 7.9 to 5.5 [8].

Previously, a shift towards a more fermentation-prone diet resulted in a 41% decrease of soluble DCA [55], which is in line with the data we present in our study. Studies about the effect of fiber on bile acid metabolism have shown controversial results [22,23]. In general, wheat bran causes a decrease of the biliary DCA fraction and total fecal DCA concentration, but pectin showed opposite results.

One should suspect that the effects of amylomaize on bile acid metabolism show a certain similarity with the effects of lactulose, a

non-absorbable disaccharide, which is rapidly fermented in the cecum. Indeed, several studies have shown a decrease in DCA formation and poolsize after lactulose consumption [7,56,57]. To our knowledge only one animal study about the effect of RS on bile acid metabolism has been published [56]. In this rat model, a decrease in cecal pH, an increase in SCFA and a decrease in the formation of secondary bile acids was demonstrated. The use of Acarbose (an *α*-glucosidase inhibitor) causes starch malabsorption followed by fermentation in the large bowel, like RS. In a human study with Acarbose, stool weight increased and the excretion and concentration of both secondary bile acids decreased by approximately 50% [59].

We speculate that the decrease in soluble DCA contributes to the reduction in fecal water cytotoxicity. We have found a significant correlation between soluble DCA concentration in FW and cytotoxicity[35]. Cytotoxicity of FW has been found to influence colonic proliferation [9]. We speculate that the lower cytotoxicity of FW decreases colonic cell proliferation after amylomaize supplementation. This is of particular interest, as a low colonic proliferation is supposed to be associated with a reduced colon cancer risk. Whether the decreased colonic mucosal proliferation is caused by the increased SCFA formation due to the starch fermentation or by the decreased secondary bile acid concentration is not clear at this moment. Other hypotheses are possible to explain this effect of fermentation on proliferation. It has been speculated that an increased carbohydrate fermentation can reduce protein fermentation, resulting in a decreased formation of fecal carcinogens [60]. Only few data are available regarding the effect of starch or fiber on colorectal cell proliferation. In humans, a decrease of rectal proliferation has been demonstrated after wheat bran suppletion [20]. In animals, conflicting effects of starch or fiber suppletion on the colonic cell proliferation have been published [46,61,62]. Long term, controlled dietary intervention studies are necessary to elucidate the possible protective effect of resistant starch on colonic cancer formation.

In conclusion we have demonstrated that amylomaize is fermented in the colon, resulting in a decrease of soluble secondary bile acids, a decrease in the cytotoxicity of fecal water and a decrease of colonic mucosal proliferation. We assume that this study points towards a beneficial effect of resistant starch on several parameters, involved in colonic cancer risk.

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Chapter 9

Summary and Conclusions

In chapter I, the term resistant starch is introduced. It is defined as that part of starch and starch degradation products that escapes small bowel digestion in healthy individuals. It is speculated that resistant starch could be an attractive alternative for fiber in the prevention of colon cancer.

In chapter II, the role of fermentation in the prevention of cancer of the large bowel is described. Resistant starch is probably a more important source of fermentable substrate than fiber. Short chain fatty acids are produced by fermentation of starch or fiber, resulting in a decrease in intraluminal pH. This acidic environment may decrease the concentration of soluble secondary bile acids by precipitation of bile acids and by diminishing bacterial transformation of primary bile acids. The promotion of colon cancer is probably mediated by the cytotoxic effect of secondary bile acids on mucosal cells, which results in increased cell proliferation. A hyperproliferative colonic mucosa has been demonstrated to be associated with an enhanced risk of colon cancer.
In theory, stimulation of colonic fermentation will decrease the intracolonic pH, the soluble secondary bile acids and mucosal proliferation, thus possibly garding against colonic carcinogenesis.
Studies and arguments that support this hypothesis are discussed.

In chapter III, an overview of the literature is given concerning the effect of consumption of fiber on bile acid metabolism. It has been concluded that changes in deoxycholic acid kinetics and changes in deoxycholic acid concentration in the aqueous phase of feces are probably the most relevant parameters, however data on these topics are scarce. Bile acids are for 99% precipitated or bound to fecal bulk, and thus not in interaction with the colonic mucosa. Therefor, the total bile acid concentration in feces seems to be less relevant. Furthermore, the literature clearly illustrates that various fiber constituents can have different, and sometimes even counteracting, effects.

In chapter IV, we describe a new method for measuring the cytotoxi-city of bile acids. Cells from a colonic cancer cell-line (HT-29) were incubated with bile acid solutions and aqueous phase of stool, and the fraction of surviving cells was determined. This assay proved to

be more sensitive than the previously used erythrocyte-cytotoxicity assay. Chenodeoxycholic acid and deoxycholic acid were cytotoxic in concentrations above 100 μmol/l, which is in the physiological concentration range of soluble bile acids in feces.

Using this assay, the effects of calcium and calciumphosphate on bile acid cytotoxicity were studied. Previous investigations, using the erythrocyte model and bile acids in unphysiologically high concentrations, have demonstrated a decrease of cytotoxicity when calcium or calciumphosphate was added. At physiological concentrations, we could not find any evidence demonstrating that calcium or calciumphosphate offers protection against cytotoxicity caused by bile acids. Furthermore, the cytotoxicity of the aqueous phase of feces could be measured and, in fact, is significantly correlated to the concentration of deoxycholic acid. This finding suggests that soluble deoxycholic acid is indeed an important factor in mucosal injury.

Since mucosal proliferation seems to be a biomarker to assess the risk of colon cancer, we have adapted an immunohistochemical method which stains proliferating cells with a antibody against Proliferating Cell Nuclear Antigen. This method is described in chapter V. Proliferating Cell Nuclear Antigen is a nuclear protein that is present in low concentrations during the entire cell cycle, and in high concentrations during late G2 and S-phases. Using a commercially available monoclonal antibody, it is possible to distingish proliferating cells.

In a double labelling technique, we validated the immunostaining with an already established proliferation marker, ^{3}H-Thymidine autoradiography. We found a sensitivity of 96% and a specificity of 98% for PCNA, and concluded that Proliferating Cell Nuclear Antigen immunostaining is a reliable method to assess colonic mucosal proliferation. The main advantage is that no special tissue preparation or incubation is necessary.

In chapter VI, we studied the in vitro fermentation of amylomaize, a maize starch that contains 62% resistant starch. Amylomaize fermented more slowly than the disaccharide lactulose, but after 24 hours, the total production of short chain fatty acids was the same. In methane producing fecal inocula, all hydrogen was converted to methane during the regular amylomaize fermentation. In contrast

during the more rapid lactulose fermentation, the production rate of hydrogen exceeded the rate of which methane forming bacteria could consume it, thus both hydrogen and methane were released in the first 4 hours of incubation.

Fermentation in low methane producing inocula elapsed faster than in in high methane producers. Furthermore we found an increase of the butyrate fraction from 10 to 17% during fermentation of both, lactulose and amylomaize, compared to control incubations.

In chapter VII, the results of a cross-over study are described, in which the normal diet of healthy volunteers was supplemented with 45 g/d of amylomaize or placebo (maltodextrin) for 1 week, with a wash out period of 1 week between treatments. This dose of resistant starch was well tolerated; only a minor increase in flatulence was observed. The 24 hour integrated hydrogen excretion increased significantly in all subjects. In the methane excreting subjects, we found a significant increase in integrated methane excretion.

We believe this increased hydrogen and methane excretion to be the result of stimulation of fermentation by amylomaize.

In chapter VIII, we studied the effects of amylomaize supplementation on fecal weight, fermentation, bile acid metabolism, cytotoxicity of the aqueous phase of stool, and colonic mucosal proliferation. We therefore supplemented the standardized, normal Dutch diet from healthy volunteers with 45 g amylomaize per day. Breath samples, 3 day fecal output, and rectal biopsies were collected before and at the end of the supplementation period. Fecal weight, 24 hour integrated hydrogen excretion, and fecal short chain fatty acid excretion increased, suggesting that amylomaize stimulated colonic fermentation. The soluble deoxycholic acid concentration decreased by 50 % during amylomaize supplementation while the total primary bile acid concentration (in freeze dried feces) increased. This suggests inhibition of the conversion of primary into secondary bile acids, which is probably mediated by precipitation of primary bile acids.

Furthermore, the cytotoxicity of the aqueous phase of stool as well as the colonic proliferation decreased significantly.

In conlusion, we suggest that supplementation of the normal diet with resistant starch may have a positive effect on intermediate factors

that increase the risk of colon cancer.

In summary we have demonstrated that amylomaize-resistant starch is well tolerated by healthy subjects and that in vitro as well in vivo fermentation is stimulated by this supplementation. Several factors, which are thought to be involved in colonic carcinogenesis, changed in a favourable way during amylomaize supplementation. Further long term studies are warranted to elucidate the possible protection that resistant starch can offer against colonic carcinogenesis.

Hoofdstuk 9

Samenvatting en Conclusies

Voeding en dikke darmkanker.

Dikke darmkanker is de op één na meest voorkomende oorzaak van sterfte door kanker in de Westerse wereld. Over de oorzaken van dikke darmkanker is nog onvoldoende bekend. Het is wel duidelijk geworden dat bij een deel van de personen die dikke darmkanker krijgen erfelijke factoren een rol spelen, maar dat omgevingsfactoren meestal van veel meer invloed zijn. Dit is onder andere gebleken uit studies, waarbij mensen gevolgd werden nadat ze geëmigreerd waren van een land met een laag risico op dikke darmkanker naar een hoog-risico gebied. Na verloop van tijd blijken deze emigranten hetzelfde risico te krijgen als de autochtone bevolking in hun nieuwe thuisland.

Aangezien het milieu van de dikke darm bepaald wordt door wat we eten, wordt voeding beschouwd als de belangrijkste factor in het ontstaan van kanker in de dikke darm. Er zijn vele epidemiologische studies gedaan om te onderzoeken welke voedingsmiddelen nu daadwerkelijk schadelijk zijn en welke juist beschermend werken. Uit de meeste van deze studies blijkt dat de consumptie van veel vet het ontstaan van dikke darmkanker bevordert, terwijl het eten van veel vezels juist een beschermend effect heeft. Mogelijk heeft ook de consumptie van calcium een gunstige invloed.

Voedingsvezels zijn bestanddelen afkomstig van planten, die niet door menselijke enzymen afgebroken kunnen worden. Deze vezels komen dus onverteerd in de dikke darm terecht, waar ze voor een deel door de daar aanwezige bacteriën wel afgebroken kunnen worden. Dit proces heet fermentatie. Voor een groot deel echter worden voedings-vezels onveranderd met de ontlasting uitgescheiden.

Het beschermend effect van vezels wordt grofweg aan twee mogelijke factoren toegeschreven. Op de eerste plaats is er een 'mechanisch' effect. De in de dikke darm aanwezige vezels fungeren als bindmiddel of 'bulkvormer', waardoor vele schadelijke en kanker-verwekkende stoffen gebonden kunnen worden. Zij kunnen dan dus niet meer in contact komen met het dikke darmslijmvlies. Verder worden deze schadelijke stoffen door het grotere faecesvolume verdund.

Op de tweede plaats is het mogelijk dat juist de fermentatie van vezels in de dikke darm verantwoordelijk is voor het beschermend effect. Bij die fermentatie ontstaan zogenaamde korte keten vetzuren, zuren die bestaan uit een keten van 2 tot 6 koolstofatomen. Deze

korte keten vetzuren zorgen voor een verlaging van de zuurgraad in de dikke darm, waardoor verschillende omzettingsprocessen anders gaan verlopen. Verder is van één van deze stoffen, namelijk boterzuur of butyraat, aangetoond dat het groeiremmende eigenschappen heeft en mogelijk ook eigenschappen, die de celrijping bevorderen (differentiatie). Ook hierdoor zou het ontaarden van gezonde cellen in kankercellen tegengegaan kunnen worden.

Of nu het 'bulkvormend effect' of juist de fermentatie van vezels de oorzaak is van het beschermend effect op het ontstaan van dikke darmkanker, is niet helemaal duidelijk. Onder andere vanwege de beschreven effecten van butyraat denken we dat de fermentatiefactor wellicht het belangrijkst is.

Om die reden hebben we een aantal studies gedaan met 'resistant starch', een voedingsmiddel dat, veel meer dan vezels, leidt tot toename van de fermentatie. Normaal wordt zetmeel in de dunne darm geheel afgebroken tot suikers, die daar opgenomen worden. Resistant starch is een speciaal soort zetmeel dat zodanig is opgebouwd dat het niet of slechts ten dele afgebroken kan worden in de dunne darm. Het grootste deel ervan komt dus in de dikke darm terecht, waar het - net als componenten van vezels - afgebroken wordt door bacteriële enzymen. Op deze manier kan de fermentatie sterk toenemen, mogelijk resulterend in een bescherming tegen dikke darmkanker. Aangezien dit resistant starch veel gemakkelijker in ons voedingspatroon is te integreren, zou het een veelbelovend alternatief kunnen zijn voor vezels in de preventie van dikke darmkanker.

HOE ONTSTAAT DIKKE DARMKANKER ?

Er is geen zekerheid over het exacte ontstaan van dikke darmkanker. Wel zijn er sterke aanwijzingen dat het proces begint met veranderingen in de snelheid en in de plaats van celdeling in het dikke darmslijmvlies. Sterk uitvergroot ziet het slijmvlies er uit als een vlak oppervlak met talloze openingen, die leiden naar korte buisjes, loodrecht op het oppervlak. In een dwarsdoorsnede ziet het slijmvlies er dus uit als een aaneenschakeling van buisjes, de cryptes. Op de bodem van deze cryptes vindt een continue celdeling plaats, waarna de nieuwe cellen als in een file geleidelijkaan naar boven toe verhuizen. Boven aangekomen worden ze losgelaten en verlaten ze het lichaam.

Onder invloed van genetische en omgevingsfactoren kan de celdeling versneld gaan verlopen en ook hogerop in de crypte plaats gaan vinden. Als men een dergelijke crypte in de lengte zou doorsnijden en bekijken, dan is het percentage delende cellen (de labellingsindex) toegenomen. Tevens zullen ook hogerop in de crypte nog delende cellen worden aangetroffen, in tegenstelling tot de normale situatie.

De constante vernieuwing van crypte-cellen is noodzakelijk omdat deze aan vele beschadigende stoffen, die in de darm aanwezig zijn, blootstaan. Hoe sterker het celbeschadigend effect van de darminhoud (de cytotoxiciteit), des te groter zal het aantal kapotte cellen zijn en des te sneller zal de celdeling moeten zijn om deze te vervangen.

Van galzouten is bekend dat ze cellen sterk beschadigen (cytotoxisch zijn) en verschillende studies hebben dan ook een relatie laten zien tussen galzouten en het ontstaan van dikke darmkanker. Galzouten worden in de lever gemaakt als de zogenaamde primaire galzouten. In de darm werken ze als zeep: ze verdelen het geconsumeerde vet in kleine partikeltjes, waardoor deze door enzymen beter en sneller afgebroken kunnen worden. Aan het einde van de dunne darm worden de meeste galzouten weer opgenomen in het bloed en naar de lever getransporteerd. Een deel van de galzouten ontsnapt echter aan deze opname en komt in de dikke darm terecht, waar ze door bacteriën worden omgezet in de secundaire galzouten lithocholzuur en deoxycholzuur. Vooral van deoxycholzuur wordt aangenomen dat het het ontstaan van dikke darmkanker kan stimuleren. Deze bacteriële omzetting is afhankelijk van de zuurgraad in de dikke darm en is geblokkeerd bij een lage zuurgraad. Verder is de oplosbaarheid van deze secundaire galzuren matig en eveneens afhankelijk van de zuurgraad: hoe zuurder hoe slechter oplosbaar.

Als fermentatie een beschermend effect heeft op het ontstaan van dikke darmkanker, dan zou dat verklaard kunnen worden doordat de omzetting van primaire in -schadelijke- secundaire galzouten geremd wordt door de verlaging van de zuurgraad in de dikke darm als gevolg van de fermentatie. Tevens worden de gevormde galzouten in het zuurdere milieu neergeslagen, waardoor ze niet meer in contact kunnen komen met de crypte-cellen. Daarmee is ook hun schadelijke invloed afgenomen en zal de celdeling in de cryptes minder gestimuleerd worden. Dit alles zou kunnen resulteren in een kleinere kans op kwaadaardige ontaarding van het slijmvlies.

DE OPZET VAN DIT PROEFSCHRIFT.

De hypothese van dit proefschrift is dat een toename van de fermentatie door consumptie van resistant starch zal leiden tot een verminderde concentratie van opgeloste secundaire galzouten in de dikke darm en daardoor tot een vermindering van de cytotoxiciteit en tot een verlaging van snelheid van celdeling in de cryptes van de dikke darm. In hoofdstuk 2 en 3 wordt een uitgebreid overzicht gegeven van de literatuurgegevens die deze hypothese ondersteunen.

Eerst werd een methode ontwikkeld om snel en reproduceerbaar de cytotoxiciteit van darminhoud en galzouten te kunnen meten (hoofdstuk 4). Vervolgens wordt een methode beschreven en getoetst om delende cellen in een darmcrypte aan te kleuren met behulp van antistoffen tegen het PCNA, een eiwit dat in delende cellen in grote mate aanwezig is (hoofdstuk 5).

Vervolgens wordt in hoofdstuk 6 beschreven hoe het door ons gebruikte resistant starch (amylomaize) door dikke darmbacteriën afgebroken wordt.

In het volgende hoofdstuk wordt het effect beschreven van consumptie van 45 gr resistant starch per dag op de fermentatie bij 19 gezonde vrijwilligers.

In de laatste studie (hoofdstuk 8) wordt wederom 45 gr resistant starch gegeven aan 13 gezonde vrijwilligers. Daarbij werd niet alleen naar het effect op fermentatie gekeken, maar ook naar het effect op secundaire galzouten, op cytotoxiciteit van de faeces en op de delingssnelheid in de darmcryptes.

METING VAN DE CYTOTOXICITEIT.

In hoofdstuk 4 wordt een nieuwe methode beschreven die ontwikkeld is om het cytotoxisch effect van galzuren te meten. Hierbij werden aan een kweek van colonkankercellen verschillende galzuuroplossingen toegevoegd, met een oplopende concentratie, waarna het percentage overlevende cellen werd gemeten. Dezelfde experimenten werden uitgevoerd met de waterige fractie van menselijke faeces, waarin onder meer de opgeloste galzouten uit de ontlasting aanwezig zijn. Deze methode blijkt veel gevoeliger om de cytotoxiciteit te meten dan een door andere onderzoekers gebruikte methode met rode bloed-

cellen. De galzouten Na-Chenodeoxycholzuur en Na-deoxycholzuur zijn cytotoxisch in concentraties vanaf 100 μmol/l, hetgeen in de orde van grootte ligt van de deoxycholzuur-concentratie die in opgeloste vorm in menselijke ontlasting gevonden wordt.

Tevens werd met deze methode het effect van calcium en calciumfosfaat op de cytotoxiciteit van galzuren onderzocht. In vroegere studies vond men dat toevoegen van calcium of calciumfosfaat aan (onfysiologisch) hoge concentraties galzuren een daling van de cytotoxiciteit liet zien. Bij gebruik van lage concentraties galzuren, zoals die voorkomen in opgeloste vorm in faeces, konden wij geen beschermend effect van deze stoffen vaststellen.

Tenslotte werd met deze methode de cytotoxiciteit gemeten van faecaal water. Dat is het water in de ontlasting, waarin alle opgeloste stoffen aanwezig zijn; dit in tegenstelling tot de vaste massa, waarin alle gebonden en neergeslagen bestanddelen aanwezig zijn. Men neemt aan dat het faecaal water erg belangrijk is, omdat daarin de stoffen aanwezig zijn, die in contact komen met de slijmvliescellen. De cytotoxiciteit van faecaal water bleek significant gecorreleerd met de concentratie opgelost deoxycholzuur. Dit geeft steun aan de hypothese dat deoxycholzuur een belangrijke rol speelt bij de beschadiging van het dikke darmslijmvlies.

METING VAN CELDELING MET BEHULP VAN PCNA.

In hoofdstuk 5 wordt een methode beschreven die gebruikt kan worden om de celdeling in het dikke darmslijmvlies te meten. Hierbij werd met een commercieel verkrijgbare antistof een eiwit aangetoond, dat aanwezig is in delende (prolifererende) cellen. Dit eiwit heet 'Proliferating Cell Nuclear Antigen', afgekort tot PCNA. PCNA is met name aantoonbaar in de late G2-fase en in de S-fase van de celcyclus, dat is de periode dat de cel in deling is. Deze methode werd tegelijkertijd gebruikt met de ^{3}H-Thymidine methode. Dat is de 'gouden standaard' methode om delende cellen aan te tonen, door aan weefsel radioactieve DNA-bouwstenen toe te voegen, die in de delende cellen ingebouwd worden en later weer zichtbaar te maken zijn. In dit experiment hebben we beide methoden toegepast op dezelfde stukjes dikke darmslijmvlies en gekeken in hoeverre met de PCNA-methode dezelfde cellen aangetoond werden als met de ^{3}H-Thymidine methode.

Hierbij vonden wij een sensitiviteit van 96% en een specificiteit van 98%, waaruit geconcludeerd mag worden dat PCNA-kleuring een goede methode is om de delingsactiviteit van het dikke darmslijmvlies vast te stellen. Het belangrijkste voordeel van de PCNA-methode is dat de cellen direct gekleurd kunnen worden, zonder voorbehandeling met b.v. radioactieve bestanddelen.

FERMENTATIE VAN AMYLOMAIZE.

Vervolgens wordt in hoofdstuk 6 onderzocht hoe amylomaize gefermenteerd kan worden. Amylomaize is een speciaal soort zetmeel dat voor 62% bestaat uit resistant starch, zetmeel dat niet in de dunne darm verteerd en opgenomen kan worden. Amylomaize werd in een proefopstelling samen met bacteriën uit de dikke darm in een kweekflesje gemengd, vervolgens werd gekeken hoe snel de afbraak verliep en wat voor stoffen geproduceerd werden. Dit werd vergeleken met een andere fermenteerbare stof namelijk lactulose.

Daarbij bleek dat de afbraak van amylomaize langzamer verloopt, maar dat de uiteindelijke opbrengst aan korte keten vetzuren vergelijkbaar is. Bij fermentatie van amylomaize en lactulose werd niet alleen een grotere hoeveelheid korte keten vetzuren geproduceerd, ook het relatieve aandeel van butyraat hierin bleek duidelijk toe te nemen. Dit lijkt van belang omdat aan butyraat een belangrijke regulerende functie bij de celdeling en mogelijk ook beschermende eigenschappen tegen de ontwikkeling van dikke darmkanker toegekend worden.

CONSUMPTIE VAN RESISTANT STARCH DOOR PROEFPERSONEN.

Daarna wordt in hoofdstuk 7 onderzocht of amylomaize ook in werkelijkheid leidt tot een toename van de fermentatie. Tijdens fermentatie ontstaat waterstof en bij een deel van de mensen ook methaan. Beide gassen worden in het bloed opgenomen en vervolgens via de longen uitgeademd. De hoeveelheid in de uitademingslucht is dus een maat voor de fermentatie.

In deze studie werd aan de normale voeding van 19 gezonde vrijwilligers gedurende een week dagelijks 45 g amylomaize (62% resistant starch) of 45 g maltodextrine (0% resistant starch) toegevoegd, zonder dat de proefpersonen of de onderzoekers wisten in welke volgorde dat gebeurde.

Deze hoeveelheid resistant starch werd goed verdragen en veroorzaakte uitsluitend enige toename van de winderigheid. De uitademing van waterstof gedurende 24 uur nam bij alle vrijwilligers significant toe tijdens resistant starch toevoeging en bij de methaan producerende proefpersonen nam ook de methaan uitscheiding significant toe ten opzichte van de maltodextrine periode. Deze stijging van de uitscheiding van waterstof en methaan betekent dat de fermentatie in de dikke darm inderdaad toeneemt na consumptie van resistant starch.

HET EFFECT VAN RESISTANT STARCH OP DE DIKKE DARM.

Tenslotte werd bij gezonde vrijwilligers een onderzoek gedaan naar de veranderingen die door de toegenomen fermentatie tijdens amylomaize consumptie in de dikke darm veroorzaakt worden. Hierbij waren wij geïnteresseerd in veranderingen in faeces gewicht, in de uitscheiding van galzuren in de faeces, in de cytotoxiciteit van het faecaal water en in de delingssnelheid van het dikke darmslijmvlies. Deze proliferatie-activiteit is een maat voor de celdeling in de dikke darmcryptes. De resultaten van deze studie zijn beschreven in hoofdstuk 8.

Aan de normale voeding van 14 gezonde vrijwilligers werd gedurende 2 weken dagelijks 45 g amylomaize (62% resistant starch) toegevoegd. Voorafgaand aan en op het einde van de periode van deze toevoeging werden ademmonsters en faeces van 3 dagen verzameld; tevens werden kleine weefsel-biopsiën uit de endeldarm genomen om de proliferatie-activiteit te meten.

Net als in de voorgaande studie werd gevonden dat de uitademing van waterstof significant toenam gedurende resistant starch consumptie. Ook de uitscheiding van korte keten vetzuren in de ontlasting nam tijdens resistant starch gebruik toe, hetgeen eveneens wijst op een toegenomen fermentatie. De concentratie deoxycholzuur in het faecaal water verminderde met 50% tijdens de consumptie van resistant starch en de totale concentratie primaire galzuren in de ontlasting nam toe. Dit wijst op een remming van de omzetting van primaire in secundaire galzouten. Wij denken dat dit vooral veroorzaakt wordt doordat primaire galzuren neerslaan als gevolg de lagere pH door de toegenomen fermentatie in de dikke darm. Tenslotte werd ook een significant lagere cytotoxiciteit van het faecaal water vastgesteld, alsmede een verminderde proliferatie-activiteit van het slijmvlies.

Samenvattend toont deze studie aan dat toevoeging van resistant starch in de vorm van amylomaize een gunstig effect heeft op verschillende parameters die samenhangen met het ontstaan van dikke darmkanker.

SAMENVATTING EN PERSPECTIEF.

Concluderend blijkt uit deze studies dat resistant starch in de vorm van amylomaize goed wordt verdragen door gezonde vrijwilligers en dat consumptie ervan leidt tot een toegenomen fermentatie. De concentratie van opgeloste galzouten in de ontlasting neemt sterk af tijdens resistant starch gebruik en -mogelijk- hierdoor ook het cel-beschadigend (cytotoxisch) effect van de ontlasting. Ook de delings-snelheid van het dikke darmslijmvlies neemt af, naar we aannemen als gevolg van de verminderde cytotoxiciteit.

Van deze factoren wordt aangenomen dat zij betrokken zijn bij het ontstaan van dikke darmkanker. De bevinding, dat deze factoren in gunstige zin beïnvloed worden tijdens amylomaize consumptie, maakt het aannemelijk dat ook de kans op dikke darmkanker hiermee kan worden beïnvloed.

Een deel van de effecten van resistant starch is vergelijkbaar met die van vezels. Een belangrijk voordeel van resistant starch is dat het gemakkelijker in onze dagelijkse voeding kan worden geïntegreerd dan een grote hoeveelheid vezels.

Een volgende stap in het onderzoek is het nagaan van de lange termijn-effecten van resistant starch toediening. Bij gezonde vrijwilligers zal gekeken worden of de beschreven effecten blijvend aanwezig zijn. Verder zal bij mensen met een verhoogd risico op dikke darmge-zwellen onderzocht worden of het ontstaan van poliepen en kanker inderdaad geremd kan worden door langdurige consumptie van resistant starch.

Dankwoord

Het doen van een promotieonderzoek moet men zien als een hobby: het kost tijd, maar het is ook leuk om te doen. Voor velen is werk tevens hun hobby; een proefschrift is dus eigenlijk ook gewoon een stukje werk. Het leuke ervan is dat het je verder leert kijken dan je eigen vak breed is. Daarbij zijn een aantal mensen in het bijzonder behulpzaam geweest, die ik expliciet wil bedanken:

Fokko Nagengast, onze samenwerking begon als assistent en chef de clinique op B5 en bereikte een voorlopig hoogtepunt in dit proefschrift. Op de meest ongelegen en ongeregelde momenten, vaak op de fiets naar huis, hadden we overleg en dat leidde tot prachtige ideeën en soms zelfs tot fraaie onderzoeken. Ik leerde van jou scopieren, en de gastroenterologie à la van Tongeren. En daardoor was het moeilijk kiezen tussen blijven of naar den Bosch gaan!

Jan Jansen, je komst naar Nijmegen gaf nieuwe impulsen aan het onderzoek binnen de afdeling. Hoewel deze studie wat buiten jouw directe gebied lag, werkte je elan aanstekelijk. Je 'laagdrempelige' manier van leidinggeven vormde mede de prima sfeer op de afdeling.

Martijn Katan, je inbreng bij vooral hoofdstuk 7 heb ik zeer gewaardeerd. Het is even wennen als een goed artikel in zwart bij je ingeleverd wordt en in rood terugkomt, maar het wordt er veel beter van!

Prof Wargovich, dear Mike, thank you for giving me the opportunity to stay at the MD Anderson Institute in Houston in your lab, to learn the mysteries of proliferation markers.

De vele proefpersonen die meewerkten aan het onderzoek ben ik zeer erkentelijk. Consumptie van 45 gram Hylon mag dan goed verdragen worden, het dagenlang verzamelen van ontlasting op droogijs moet beschouwd worden als een hele opgaaf.

Keuzevakstudenten spelen een ondergewaardeerde rol bij het hedendaagse promotieonderzoek. In het bijzonder wil ik Pieter Friederichs, Hilde de Boer, Margje Jansen, Natale Adamo en Marcel Twickler bedanken voor hun gemotiveerde inzet.

De bepaling van galzouten en korte keten vetzuren middels een zogenaamde gaschromatograaf is voor een clinicus toch ingewikkeld. Ik ben blij dat Annie van Schaik en Albert Tangerman dat voor mij deden.

Op Koos Diepenbroek en de overige medewerkers van het laboratorium van de afdeling Pathologische Anatomie kon ik altijd een beroep doen om steeds veel te kleine rectumbiopten zo aan te snijden dat coupes met fraaie, longitudinaal getroffen cryptes verkregen werden,

die gebruikt konden worden voor de PCNA-kleuring.

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Louwrens Davids bewees het bestaansrecht van de Apple, door daarmee de fraaie voorkant te ontwerpen.

Aan Henk Festen laat ik de eer dat hij mij erop gewezen heeft dat werken in een groot perifeer ziekenhuis ook voordelen heeft, vooral ben ik hem dankbaar dat ik -altijd laat op de avond- welkom was om onder het genot van een glas wijn gebruik te maken van zijn laserprinter.

Verder ben ik allen die zich voornamen om dit boekje nu eens te lezen dankbaar voor deze intentie. Mijn eigen ervaring heeft mij geleerd dat het dankwoord en de nederlandse samenvatting al een hele opgave is. Mijn ouders ben ik werkelijk dankbaar dat zij mij leerden leren en leerden relativeren. Marie-José, Kim, Sanne en Marit leerden mij dat een proefschrift inderdaad een hobby is, vaak leuk, soms tijdrovend, maar dat er ook nog andere hobby's zijn.

Curriculum vitae

Ivo van Munster werd geboren op 23 oktober 1956 te Nijmegen. Hij behaalde zijn VWO diploma aan het Canisius College - Mater Deï te Nijmegen in 1975. Vanaf hetzelfde jaar studeerde hij geneeskunde aan de Katholieke Universiteit te Nijmegen. In 1982 behaalde hij zijn artsexamen, aansluitend daaraan werd hij tot 1987 opgeleid tot internist in het Academisch Ziekenhuis te Nijmegen (hoofd: prof. dr. A. van 't Laar). Vanaf 1 maart 1987 tot 1 mei 1993 was hij werkzaam op de afdeling Gastroenterologie van het Academisch Ziekenhuis te Nijmegen (hoofd tot 1 januari 1991 dr. JHM van Tongeren, vanaf 1 juni 1991 prof. dr. JBMJ Jansen). Op deze afdeling verrichtte hij onder leiding van prof. dr. SH Yap gedurende 1½ jaar onderzoek naar Hepatitis C, tot diens vertrek naar Leuven. Daarna werd het in deze dissertatie beschreven onderzoek gedaan. Tevens werd de auteur in deze periode opgeleid tot gastroenteroloog.

Vanaf 1 mei 1993 is hij gevestigd in het Bosch Medicentrum, locatie Groot Ziekengasthuis te 's-Hertogenbosch, waar hij samenwerkt met dr. JThM Burghouts, dr. JLJ Jansen, dr. MI Koolen, dr. RMA Kurstjens, FJHM van Liebergen, PM Netten en dr. HPM Festen. Met laatstgenoemde beoefent hij speciaal de gastroenterologie.

Hij is getrouwd met Marie-José van Herpen en vader van Kim, Sanne en Marit.