DOI 10.1002/mnfr.200600084 Mol. Nutr. Food Res. 2006, *50*, 1006 – 1012

Human rectal mucosal gene expression after consumption of digestible and non-digestible carbohydrates

Jürgen Schauber, Frank Weiler, Andrea Gostner, Ralph Melcher, Theodor Kudlich, Hardi Lührs and Wolfgang Scheppach

Department of Medicine II, Division of Gastroenterology, University of Würzburg, Würzburg, Germany

The effect of regular consumption of the low-digestible and prebiotic isomalt versus the digestible sucrose on gene expression in rectal mucosa was examined in a randomized double-blind crossover trial. Nineteen healthy volunteers received 30 g isomalt per day or 30 g sucrose as part of a controlled diet over two 4-week test periods with a 4-week washout period in between. At the end of each test phase rectal biopsies were obtained. After RNA extraction mucosal gene expression was assayed using GeneChip microarrays. In addition, expression of cathelicidin hCap18/LL37, cellular detoxification enzymes GSTπ, UGT1A1 and CYP3A4, cyclooxygenase 2 and barrier factors MUC2 and ZO-1 were determined by real-time RT-PCR. Microbiological analyses of fecal samples revealed a shift of the gut flora towards an increase of bifidobacteria following consumption of the diet containing isomalt. Isomalt consumption did not affect rectal mucosal gene expression in microarray analyses as compared to sucrose. In addition, the expression of cathelicidin LL37, GSTπ, UGT1A1, CYP3A4, COX-2, MUC2 and ZO-1 was not changed in rectal biopsies. We conclude that gene expression of the human rectal mucosa can reliably be measured in biopsy material taken at endoscopy. Dietary intervention with the low digestible isomalt compared with the digestible sucrose did not affect gene expression in the lining rectal mucosa.

Keywords: Colon epithelium / Gene expression / Low digestible carbohydrate / Prebiotic / Sucrose

Received: June 15, 2006; revised: August 5, 2006; accepted: August 7, 2006

1 Introduction

The polyol isomalt (Palatinit®) is a well-established sugar substitute with low caloric, non-cariogenic [1] and very low glycemic and very low insulinemic properties [2]. Chemically, isomalt is a mixture of the polyols 1-O-α-D-glucopyranosyl-D-mannitol (1,1-GPM) and 6-O-α-D-glucopyranosyl-D-sorbitol (1,6-GPS). It is derived from sucrose in two major processing steps: (i) the enzymatic rearrangement of sucrose (2-O-α-D-glucopyranosyl-D-fructofuranose) into isomaltulose (6-O-α-D-glucopyranosyl-D-fructofuranose), and (ii) the catalytic hydrogenation of isomaltulose into isomalt. In the upper digestive tract isomalt is only slowly and incompletely hydrolyzed to glucose, sorbitol and mannitol. Released glucose is actively absorbed, whereas sorbitol and mannitol are partially absorbed from the small intestinal lumen. Undigested and/or unabsorbed portions reach the colon and are completely fermented by the gut microflora to gases and short chain fatty acids [3].

Recently, the results of a nutrition intervention trial comparing test diets containing isomalt or sucrose documented the safety and tolerance of isomalt consumption in healthy volunteers [3, 4]. It was shown that, like other low-digestible carbohydrates such as resistant starch, non-starch polysaccharides and non-digestible oligosaccharides, isomalt provides substrates for microbial fermentation and acts as a prebiotic. In addition, isomalt consumption changed the bacterial enzyme patterns in stool samples [4]. It is speculated that long-term consumption of low-digestible carbohydrates may be protective against Western diseases, like cardiovascular disease, type 2 diabetes and related complications and disorders, metabolic syndrome and functional bowel disorders [5]. Thus, due to its prebiotic and non-prebiotic beneficial health effects, isomalt may be a promising "functional food" component.

Besides functioning as a nutrient-transporting and metabolizing organ, the colonic mucosa is challenged with multi-

Correspondence: Dr. Jürgen Schauber, M.D., Dept. of Medicine, Div. of Gastroenterology, University of Würzburg, Josef-Schneider-Str. 2, 97080 Würzburg, Germany
E-mail: jschauber@ucsd.edu
Fax: +49-931-201-36101

Abbreviations: CK18, cytokeratin 18; **Ct**, number of cycles needed to cross the threshold limit; **GAPDH**, glyceraldehyde-3- phosphate dehydrogenase; **SCFA**, short-chain fatty acids; **TPA**, 12-O-tetradecanoylphorbol-13-acetate

ple other tasks. Among those, the colon epithelium serves as an active barrier against pathogens and luminal flora. Physical and chemical barriers such as the formation of tight junctions and the production of mucin have been identified as important factors for host defense. In addition, innate and adaptive immune responses orchestrate appropriate mucosal defense mechanisms [6]. Moreover, potentially harmful luminal contents are being metabolized and detoxified by epithelial enzyme systems [7]. As a result, the intestinal mucosa actively shapes the luminal bacterial flora and microenvironment. In turn, alterations of the resident flora in addition to the generation of fermentation products exert profound effects on colon mucosa metabolism and physiology [8].

Numerous studies have been published investigating the effects of nutritional interventions in humans. The parameters tested often included analyses of serum or stool parameter changes or alterations of the intestinal flora [4, 9]. In animal studies, the influence of nutritional intervention on mucosal gene expression has been investigated [10]. To our knowledge, the influence of nutritional factors on human intestinal gene expression has not been investigated so far.

The objective of this study was to analyze the effect of consumption of the digestible *vs*. a low-digestible prebiotic carbohydrate on the colon mucosa on a molecular level and to study the applicability of modern molecular biological techniques. Gene chip array analyses and real-time RT-PCR were applied to investigate global and specific gene expression in the rectal mucosa of healthy volunteers after nutritional intervention.

2 Materials and methods

2.1 Study participants

Twenty healthy volunteers were recruited for the trial. Nineteen volunteers completed the study (twelve women, seven men) aged 21 – 53 years (median 30.5). One volunteer dropped out due to a febrile tonsillitis that had to be treated with antibiotics. Intake of antibiotics, laxatives, motility affecting or lipid altering medications was not allowed during the study. Intake of antibiotics more than 6 weeks prior to the start of the study was tolerated. Other exclusion criteria were: history of severe chronic medical disease, including gastrointestinal diseases; severe abdominal discomfort; severe constipation; unusual dietary habits; known or suspected lack of compliance with the study protocol; and pathological clinical parameters. All participants signed informed consent forms after written and oral information about aim, course, and potential hazards of the trial. The study was approved by the Ethics Committee of the Faculty of Medicine, University of Würzburg, Germany.

2.2 Study design

During two consecutive study periods, volunteers consumed a controlled diet [3, 4]. The diets were composed as a typical Western diet (low-fiber, high-fat). The diet compositions were identical except that isomalt was added to one diet and sucrose to the other diet. The test products were prepared and supplied by SÜDZUCKER (SÜDZUCKER, Mannheim/Ochsenfurt, Germany). In a randomized, double-blinded fashion using a crossover design the volunteers consumed the diet containing isomalt for a 4-week period followed by a 4-week phase during which isomalt was replaced by sucrose. A 4-week washout period without intervention was interposed in between study periods. Volunteers consuming the sucrose containing diet during the first study period (4 weeks) continued with the isomalt diet for another 4-week period. For better adaptation, the dose was slowly increased from 5 g/d to 30 g/d in the first week of each test phase. Breakfast, morning snack, lunch, afternoon snack and dinner were provided with each diet. A 7-day rotating menu including ready-to-eat foods (Eismann Tiefkühl-Heimservice, Mettmann, Germany) was used. Additional food was not allowed, the volunteers were asked to consume the delivered portions completely. Regular contact with the study nutritionist was maintained to ensure volunteers' compliance. In addition, compliance was monitored by determination of urinary excretion of mannitol, a metabolite of isomalt.

2.3 Rectal biopsy sampling

At the end of each study period two rectal biopsies were taken from each volunteer during rectoscopy using a standard forceps. Biopsies were immediately transferred to an RNA stabilization solution (RNAlater®, Ambion, Austin, TX) and flash frozen in liquid nitrogen. Biopsies were stored at $-80^{\circ}C$ until processing.

2.4 RNA extraction and real-time PCR

Biopsies were homogenized and RNA extracted using Trifast1 (Peqlab, Erlangen, Germany) with an additional clean up protocol followed by treatment with RNase-free DNase (Qiagen, Hilden, Germany). All RNA material was denatured at $94^{\circ}C$ for 5 min and chilled to $4^{\circ}C$. Reverse transcription and real-time PCR were performed as described [11]. In brief, for quantification of the expression of the target genes and the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cytokeratin 18

Table 1. Real-time PCR primers and probes applied to evaluate gene expression levels in rectal biopsies

Gene target	Primer pair 5′ to 3′	Gene probe 5′-FAM to TAMRA-3′
GSTπ	TCATGGATCAGCAGCAAGTCC GGAGGCAAGACC-TTCATTGTGG	ACCAGATCTCCTTCGCTGACTACAACCTGC
COX2	CCTTCCTCCTGTGCCTGATG ACAATCTCATTTGA-ATCAGGAAGC	TGCCCGACTCCCTTGGGTGTCA
CK18	GAGGCATCCAGAACGAGAAGG TGCTCCCGGATTTTGCTCTC	CGGTTCTCGGTCTCCAGGCTCCTCAG
UGTA1	GGTGACTGTCCAGGACCTATTGA TAGTGGATTTT-GGTGAAGGCAGTT	ATTACCCTAGGCCCATCATGC
CYP3A4	CAGGAGGAAATTGATGCAGTTTT GTCAAGATACT-CCATCTGTAGCACAGT	CCCAATAAGGCACCACCCACCTATGA

(CK18) were measured in triplicate using an iCycler real-time PCR Detection System (Bio-Rad, Hercules, CA). GAPDH transcript numbers were measured by PCR reactions using the GAPDH-PDAR-Housekeeping Kit (ABI, Foster City, CA), which does not amplify GAPDH pseudogenes. CK18 is a marker for colon epithelial cells [12]. The expression of CK18 as a second housekeeping gene was determined in each biopsy to acknowledge the inconsistency of epithelial and subepithelial tissue portion in the obtained biopsy material. The sequences of forward and reverse primers as well as for the fluorogenic probes, as designed by Primer Express (ABI) or Beacon Designer 2.1 (Premier Biosoft, Palo Alto CA) for quantification of COX-2, GSTπ, UGTA1, CK18 and CYP3A4 mRNA are listed in Table 1. Primers and FAM-labeled probe for the quantification of MUC2 were obtained as predeveloped assay (ABI). The primers and probe used for analysis of cathelicidin expression have been described elsewhere [13]. Standard curves were constructed from serial dilutions of cDNA synthesized from a known quantity of total RNA from U937 monocytic cells or T84 colon cells (DSMZ Braunschweig, Germany). Expression of the target genes in unknown samples was quantified by measuring Ct and reading the corresponding value off the standard curve. Target gene expression was then normalized to CK18 expression (gene expression strictly restricted to epithelial cells, *e.g.* COX-2, Muc-2, ZO-1) or a correction factor CK18 expression/GAPDH expression (*e.g.* GSTπ, cathelicidin, UGTA1, CYP3A4).

2.5 Gene expression profiling

RNA from 19 biopsies (one biopsy from each participant) obtained after 4-week consumption of the isomalt- and the sucrose-containing diet was pooled. Subsequently, RNA was reverse transcribed and submitted to hybridization on a GeneChip microarray (Affymetrix, Santa Clara CA). The cDNA microarrays were performed in cooperation with the Institute for Clinical Biochemistry and Pathological Biochemistry in Würzburg. Briefly, cDNA was marked with either Cy3 or Cy5 and hybridized to slides preliminary spotted with Affymetrix gene chip 12k according to customer's information. Independent images were obtained for Cy3 and Cy5 fluorescence emitted from hybridized microarrays by a commercial microarray scanner. Raw data from data files were produced using ScanAlyze software (M. Eisen, Stanford University, Palo Alto, CA) and statistical analyses were performed with Microsoft Excel and SigmaStat 2.03 (SPSS, San Rafael, CA). Signal and background intensities of each spotted cDNA element were calibrated by a correction factor ($e^{(\text{mean lnCy3})}/e^{(\text{mean lnCy5})}$). Signal intensities of Cy3 and Cy5 were determined by subtracting the local background from signal intensity values. Transcript abundance is determined by the ratio of Cy3/Cy5. The median values of Cy3/Cy5 ratios of each gene from three independent array analyses were taken as a representative value.

2.6 Statistics

All statistical analyses were performed using SigmaStat 2.03. Values are given as means ± SEM. Nonparametric Wilcoxon rank-sum test for paired data was used for comparisons.

3 Results

To investigate markers in the gene expression pattern of the epithelial cells lining the colon a dual approach was chosen. The mRNA from rectal biopsies from study participants was isolated, mRNA from both groups was pooled and subjected to cDNA microarrays covering 9500 genes. In addition, mRNA from biopsies obtained from individual patients was analyzed for the expression of representative genes involved in mucosa metabolism, barrier function, inflammation and antimicrobial defense. Out of possible genes on the cDNA array used 9000 genes could be evaluated. Alternating fluorescence dye combinations were used in three arrays to minimize sources of error. Gene expres-

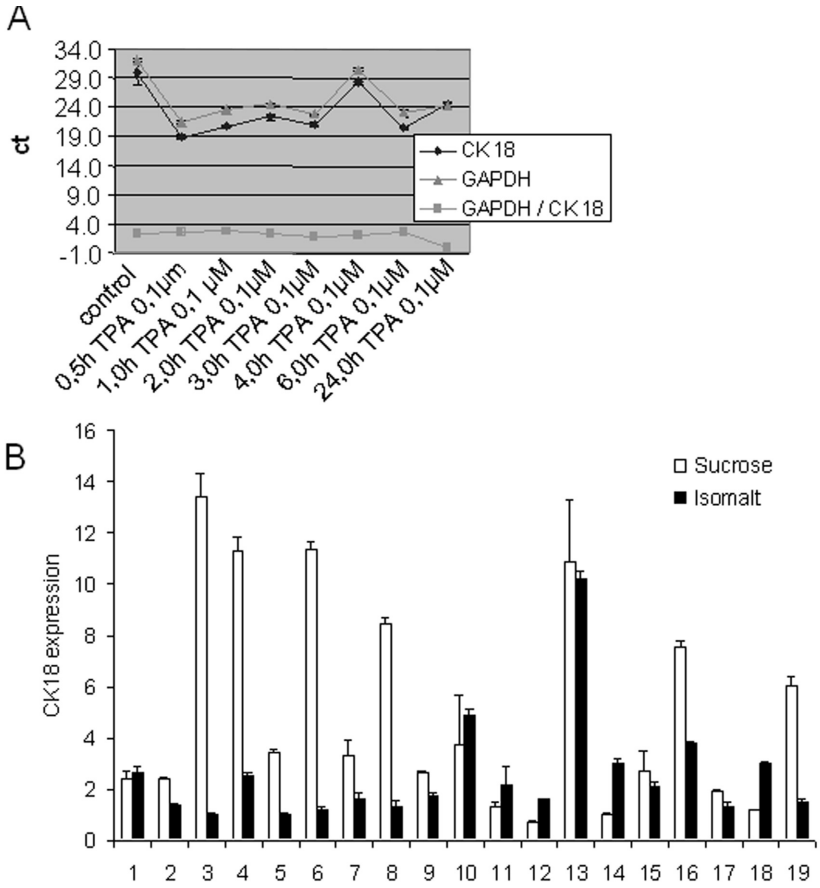

Figure 1. Establishment of CK18 as second housekeeping gene. (A) T84 colon cells grown in monolayers were stimulated with TPA at different concentrations for up to 24 h. Total RNA was isolated and the expression of CK18 and GAPDH determined by real-time RT-PCR using fluorescence labeled probes. Ct values corresponding to a threshold of emitted fluorescence during the respective real-time PCR reaction are displayed. One experiment representing repeated analyses is displayed. (B) Heterogeneous CK18 expression in biopsy material obtained from study patients. Rectal biopsies were obtained after control and isomalt treatment periods, total RNA extracted, and CK18 expression evaluated. CK18 expression per biopsy was normalized to GAPDH expression. Mean ± SD from three independent PCR reactions are displayed.

sion patterns were similar after isomalt or sucrose diet periods (data not shown).

In addition to semi-quantitative cDNA array technology, expression of a subset of specific genes involved in various mucosal functions was determined by real-time RT-PCR using gene probes for target genes and housekeeping genes. GAPDH is a commonly used housekeeping gene and its abundance is utilized to normalize gene expression in various model systems [13]. GAPDH is considered to be constitutively expressed in colorectal mucosa and in colon cell lines. However, in this study, a number of genes expressed solely by epithelial cells were investigated. To exclude that the comparisons of the relative expression of these genes between treatment groups is biased by the differing amounts of adjacent epithelial tissue CK18 expression was established as a second housekeeping gene. CK18 is a type 1 keratin protein that is primarily found in non-squamous epithelia [12]. In T84 colon epithelial cells, CK18 is constitutively expressed and abundance is not influenced by external stimuli like TPA (12-O-tetradecanoylphorbol-13-acetate) a strong inducer of morphological changes (Fig. 1A). In rectal biopsy material CK18 transcript abundance varied considerably between individuals, suggesting heterogeneous epithelial content (Fig. 1B). To address this, a correction factor for each biopsy was established and used for quantification of relative expression of epithelial expressed genes. For this purpose, CK18 expression in each sample was normalized to GAPDH expression and the resulting correction factor used to determine relative target gene expression.

Expression of all specific investigated genes except for cathelicidin was detected in study patients. Among the

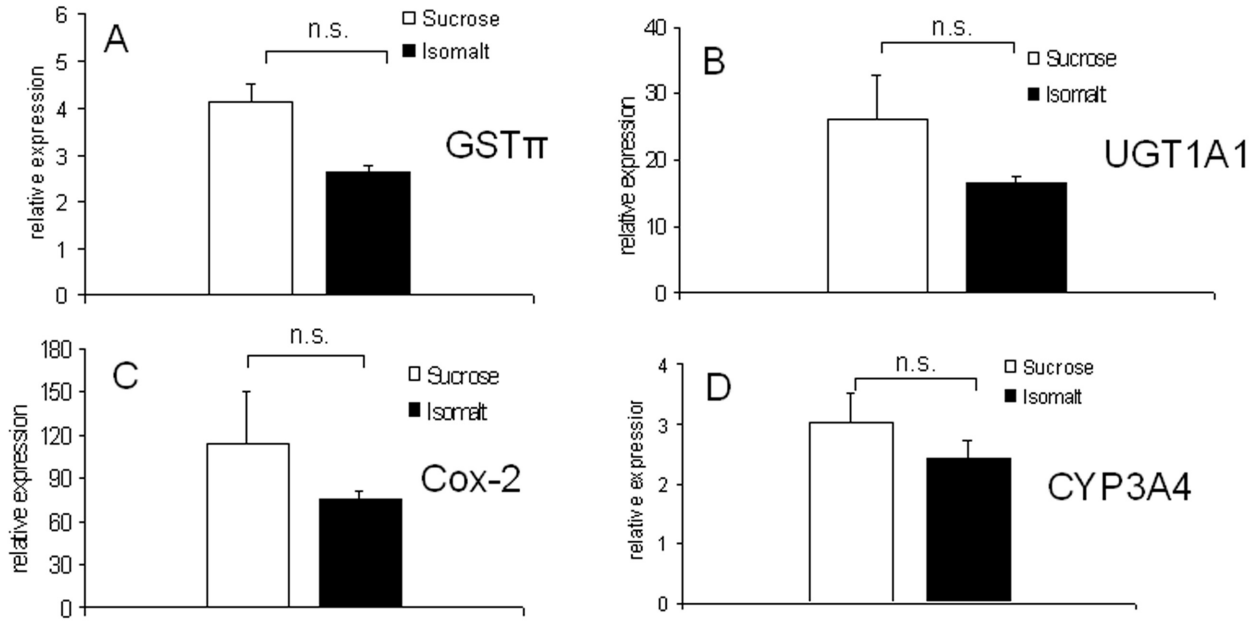

Figure 2. Expression of specific genes in rectal mucosa after isomalt or sucrose consumption (I). Expression of GSTp (A), UGT1A1 (B), Cox-2 (C) and CYP3A4 (D) in rectal biopsies from healthy volunteers after 4 weeks on each test diet. Relative gene expression was determined in rectal biopsy specimens by real-time RT-PCR applying specific gene probes and normalized to CK18 and GAPDH expression. (* $p < 0.05$; n.s.: not significant).

genes studied, GST are detoxifying enzymes expressed by the intestinal mucosa. Fermentation products such as short-chain fatty acids (SCFA) induce GST in colon cells *in vitro* [7]. When GSTπ expression was normalized relatively to CK18 and GAPDH expression no significant difference between study groups was observed (Fig. 2A). In addition, cDNA array results suggest that GSTπ transcript abundance is not significantly affected by the diet regimen as GSTπ expression together with 14 other GST was not changed in these analyses (not shown).

UDP-glucuronyltransferases (UGT) are a protein family of detoxifying enzymes comprising nine members. UGT1A1 is a member of the UGT protein family expressed by colonic epithelium. The dietary interventions used in this study did not influence the expression of UGT1A1 in healthy volunteers (Fig. 2B).

Cyclooxygenase 2 (COX2) is an enzyme involved in prostaglandin metabolism and colonic expression induced during inflammation [14]. Basal COX2 expression was detected in all biopsies obtained from non-inflamed mucosa during this study. No differences in COX2 mRNA in rectal biopsy specimen were observed (Fig. 2C).

Another detoxifying enzyme CYP3A4, a member of the cytochrome p450 family, was detected in all specimens; however, greatly variable expression levels were observed interindividually. In both study periods similar CYP3A4 expression levels were found (Fig. 2D).

Besides analyses of enzymes involved in detoxification, factors important to mucosal barrier function and defense were studied. In analyses of specific gene expression, antimicrobial cathelicidin expression was detected in 9 out of 19 study patients, indicating a low colonic cathelicidin expression under non-inflammatory conditions. In these 9 patients, cathelicidin mRNA abundance in rectal biopsy material of both interventions were comparable (data not shown). In addition to secreted defense factors like cathelicidin, MUC-2 and ZO-1 are factors involved in the formation of a physical barrier at the epithelial surface of the intestine. Expression of both genes was detected in all specimens at similar levels with both dietary regimens (Figs. 3A and B).

4 Discussion

In the present study, changes in rectal mucosal gene expression after nutritional intervention in healthy volunteers were investigated. Study participants received isomalt- or sucrose-containing diets over two 4-week periods in a crossover design. After each study period, changes in stool parameters such as bacterial content were evaluated. It was shown that isomalt favorably alters the colonic microflora

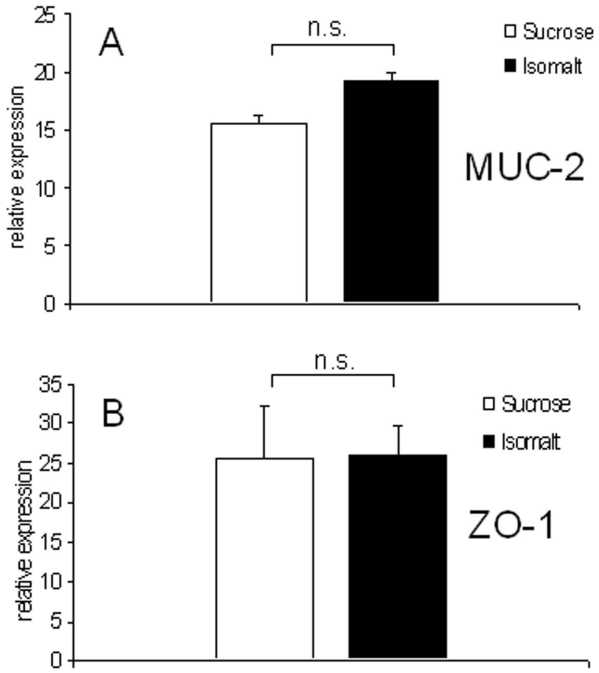

Figure 3. Expression of specific genes in rectal mucosa after isomalt or sucrose consumption (II). Expression of MUC2 (A) and ZO-1 (B) in colon biopsies after 4 weeks on each test diet. RNA was isolated from rectal biopsies of volunteers at the end of each study period. MUC2 and ZO-1 expression was analyzed by real-time RT-PCR and gene expression was normalized to CK18 and GAPDH. (n.s.: not significant).

as well as the activity of luminal enzymes such as bacterial β-glucosidase [3, 4]. However, in gene chip arrays and real-time PCR analyses applying specific gene probes no changes in rectal mucosal gene expression as compared to sucrose were observed. Thus, changes in the colonic microenvironment by nutritional intervention were not reflected by rectal mucosal gene expression in this study.

Biopsy samples from study participants were used to evaluate changes in expression in genes relevant to barrier function, immune defense and detoxification. Despite limited sample material analyses with real-time RT-PCR using Taqman® probes showed expression of all the genes studied. This quantitative technique is therefore considered a promising tool not only in animal but also in human intervention trials. In a second approach, alterations in gene expression were evaluated in gene array experiments. No differences were found in these assays either. These results could be explained by our approach to pool individual biopsy samples. This approach was necessary to obtain enough RNA material for the analyses. However, consequently, differences in heterogeneously expressed genes in individuals might have been harder to identify.

Among the variety of fermentation products in the colon lumen SCFA have been demonstrated to influence colon mucosal physiology and function [15]. SCFA are produced by fermentation of undigested carbohydrates such as isomalt in the colonic lumen. This process mainly occurs in the caecum [16]. Indeed, *in vitro* isomalt is rapidly fermented by different bifidobacteria strains and yields high levels of butyrate [4]. Fermentation products are subsequently absorbed by the colon epithelium and concentrations vary when comparing the different colonic segments [16]. The highest concentrations are observed in the caecum with decreasing contents towards the distal segments. SCFA in concentrations sufficient to alter target gene expression might therefore not reach the rectum unless produced in high concentrations. When measuring SCFA content in feces, most likely reflecting concentrations in the rectum, isomalt did not result in measurable increase in SCFA concentrations. Butyrate, the most important SCFA, exerts a variety of gene expression alterations in colonocytes, but is also readily absorbed by the mucosa. Butyrate has profound effects on the gene expression of factors investigated and found not to be changed in this study such as cathelicidin LL-37 [13]. These findings are most likely explained by absorption of SCFA by gut epithelium lining the colon [4]. Analyses of gene expression patterns in caecal mucosa might thus have revealed different results. However, a much more invasive approach is needed to obtain mucosal samples from the proximal colon to investigate potential effects of undigested carbohydrates. To our knowledge, no such experiments have been conducted so far.

In addition to the possibility that nutritional intervention changes may not be reflected in the gene expression in the rectum, the inherited mucosal metabolic profiles in individual patients might be too large to detect a perturbation by a nutritional agent. Lack of differences in array analyses might also have been due to heterogeneity within each study group. Individual gene array analyses on patient samples, however, were limited by the amount of RNA extracted from single biopsies.

Microbiological analyses of fecal samples indicated a shift of the gut flora towards an increase of bifidobacteria following consumption of isomalt in comparison with the control diet [4]. However, also the modulation of bacterial luminal composition did not result in altered rectal gene expression of the lining mucosa in this study.

In summary, colonic mucosal gene expression can be reliably measured in biopsy material taken at endoscopy. In samples from the human rectum, gene expression did not differ following the consumption of low-digestible and readily digestible carbohydrates. Further studies are needed to evaluate the influence of dietary intervention in other segments of the human large bowel.

5 References

[1] Gehring, F., Karle, E. J., *Z. Ernährungswiss*. 1981, *20*, 96 – 106.

[2] Gee, J. M., Cooke, D., Gorick, S., Wortley, G. M., *et al.*, *Eur. J. Clin. Nutr.* 1991, *45*, 561 – 566.

[3] Gostner, A., Schaffer, V., Theis, S., Menzel, T., *et al.*, *Br. J. Nutr*. 2005, *94*, 575 – 581.

[4] Gostner, A., Blaut, M., Schaffer, V., Kozianowski, G., *et al*., *Br. J. Nutr*. 2006, *95*, 40 – 50.

[5] Scheppach, W., Luehrs, H., Menzel, T., *Br. J. Nutr*. 2001, *85*, S23 – 30.

[6] Hecht, G., *Am. J. Physiol*. 1999, *277*, C351 – 58.

[7] Wollowski, I., Rechkemmer, G., Pool-Zobel, B. L., *Am. J. Clin. Nutr*. 2001, *73*, 451S – 455S.

[8] Kanauchi, O., Matsumoto, Y., Matsumura, M., Fukuoka, M., Bamba, T., *Curr. Pharm. Des*. 2005, *11*, 1047 – 1053.

[9] Abe, S., Yamaguchi, H., *Nippon Ishinkin Gakkai Zasshi* 2000, *41*, 77 – 81.

[10] Yang, K., Yang, W., Mariadason, J., Velcich, A., *et al.*, *J. Nutr*. 2005, *135*, 2710 – 2714.

[11] Schauber, J., Iffland, K., Frisch, S., Kudlich, T., *et al*., *Mol. Immunol*. 2004, *41*, 847 – 854.

[12] Bosch, F. X., Leube, R. E., Achtstatter, T., Moll, R., Franke, W. W., *J. Cell. Biol*. 1988, *106*, 1635 – 1648.

[13] Schauber, J., Svanholm, C., Termén, S., Iffland, K., *et al*., *Gut* 2003, *52*, 743 – 751.

[14] Singer, I. I., Kawka, D. W., Schloemann, S., Tessner, T., *et al*., *Gastroenterology* 1998, *115*, 297 – 306.

[15] Scheppach, W., *Gut* 1994, *35*, S35 – 38.

[16] Cummings, J. H., Pomare, E. W., Branch, W. J., Naylor, C. P., Macfarlane, G. T., *Gut* 1987, *28*, 1221 – 1227.

