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Structural Stability and Prebiotic Properties of Resistant Starch Type 3 Increase Bile Acid Turnover and Lower Secondary Bile Acid Formation

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Microbial metabolism is essential in maintaining a healthy mucosa in the large bowel, preferentially through butyrate specific mechanisms. This system depends on starch supply. Two structurally different resistant starches type 3 (RS3) have been investigated with respect to their resistance to digestion, fermentability, and their effects on the composition and turnover of bile acids in rats. RSA (a mixture of retrograded maltodextrins and branched high molecular weight polymers), which is more resistant than RSB (a retrograded potato starch), increased the rate of fermentation accompanied by a decrease of pH in cecum, colon, and feces. Because they were bound to RS3, less bile acids were reabsorbed, resulting in a higher turnover through the large bowel. Because of the rise of volume, the bile acid level was unchanged and the formation of secondary bile acids was partly suppressed. The results proved a strong relation between RS3, short chain fatty acid production, and microflora. However, butyrate specific benefits are only achieved by an intake of RS3 that result in good fermentation properties, which depend on the kind of the resistant starch structures.

KEYWORDS: Resistant starch type 3; short chain fatty acid; bile acid; rat

INTRODUCTION

Strong interactions exist between intestinal microflora and large bowel mucosa. The predominantly anaerobic bacteria contribute to healthy gut function by protective mechanisms against different pathogens and by producing butyrate as a product of fermentation for the metabolism of epithelial cells of the colonic mucosa. The complex bacterial ecosystem formed postnatally comprises more than 50 genera and over 400 species (*1*, *2*). The stabilization of an apathogenic bacterial community structure depends on diet-derived substrates, including carbohydrates, proteins, and undigested dietary fiber, which escape degradation and absorption in the small intestine. Carbohydrates are the main substrate for bacterial fermentation. As end products of fermentation, short chain fatty acids (SCFA) are essential for maintaining healthy gut tissue and the normal continuous regeneration of the large bowel mucosa. Butyrate plays a particularly crucial role as the most important substrate of the oxidative energy metabolism and as a signal metabolite of proliferation and differentiation of colonocytes (*3*, *4*). Nearly all absorbed butyrate is metabolized in epithelial cells of the colonic mucosa. Furthermore, butyrate also influences the protein and enzyme levels of colonocytes directly through histone hyperacetylation and interaction with butyrate specific response elements upstream of multiple genes, resulting in a higher expression of regulatory proteins such as p21, cyclin D3, the protein kinase C-δ, mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase, glutaminase, etc. (*5*−*7*). It has been proposed that a high luminal level of butyrate is beneficial for the large bowel health, since this SCFA can lower the risk of inflammatory diseases and cancer (*8*, *9*). Starch is well-known to increase prebiotically the growth of butyrate-forming bacteria, most of which belong to Gram-positive bacteria of the XIV_a cluster related to the *Eubacteria rectale* group (*10*, *11*).

Primary bile acids are synthesized from cholesterol in hepatocytes and conjugated in rats with taurine, before being secreted into the bile. After deconjugation by bacteria, most bile acids are absorbed in the distal small intestine. Subsequently, colonic bacteria dehydroxylate unabsorbed, deconjugated bile acids to secondary bile acids such as deoxycholic acid (DCA), lithocholic acid (LCA), 12-ketolitocholic acid (KLCA), and hyodeoxycholic acid (HDCA). Secondary bile acids are well-known tumor promoters (*12*), and a high-fat diet has been linked to an increase in colorectal cancer risk (*13*). Butyrate can hinder the secondary bile acid-mediated toxic effects (*4*, *14*). Prevention with prebiotic high-quality resistant starch type 3 (RS3) would therefore be beneficial (*15*).

Starches are capable of binding bile acids. Investigations with radioactive labeled cholic acid (CA), chenodeoxycholic acid (CDCA), and conjugated bile acid compounds revealed that the dissociation constants of bile salt−starch complexes decrease from CA over DCA to CDCA by nearly 2 orders of magnitude (*16*). It is assumed, therefore, that intake of RS3 influences the

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Table 1. Composition of the Diets Used

ingredients	g/100 g diet: control group	RSA group	RSB group
starch	57[a]	57[b]	57[c]
casein[d]	18	18	18
sunflower oil[e]	13	13	13
cellulose[f]	5	5	5
mineral mixture[g]	5	5	5
vitamin mixture[h]	2	2	2

[a] Starch basis: waxy maize starch (National Starch and Chemical Company, Bridgewater, United States); final RS content, 0 g/100 g diet. [b] Starch basis: mixture of waxy maize starch and partially debranched, recrystallized (retrograded) maltodextrin (Cerestar R&D, Vilvoorde, Belgium) (RS3, RSA); final RS content, 5.7 g/100 g diet. [c] Starch basis: mixture of waxy maize starch and modified, recrystallized (retrograded) potato starch (RS3, RSB); final RS content, 5.7 g/100 g diet. [d] Dauermilchwerk Peiting GmbH (Landshut, Germany). [e] Thomy GmbH (Karlsruhe, Germany). [f] Rettenmeier GmbH (Ellwangen, Germany). [g] Altromin GmbH (Lage, Germany). The composition of the mineral mixture (g/kg): Ca, 185; P, 145; K, 140; Na, 88; Cl, 72; S, 34; Mg, 16; Fe, 4; Mn, 2; Zn, 0.6; Cu, 0.16; F, 0.08; I, 0.008; Se, 0.004; and Co, 0.002. [h] Altromin GmbH. Composition of vitamin mixture (mg/kg): choline, 50000; vitamin E, 8000; *p*-aminobenzoic acid, 5000; myo-inositol, 5000; niacin, 2500; pantothenic acid, 2500; vitamin B_1, 1000; vitamin B_2, 1000; vitamin B_6, 750; folic acid, 500; menadione, 500; vitamin A, 225; biotin, 10; vitamin B_{12}, 1.5; and cholecalciferol, 0.65.

rate of bile acid absorption in the small intestine and bile acid modifications in the large bowel.

Our aim was to identify optimal substrate conditions for colonic fermentation by manipulating the intake of RS3. Two RS3 preparations, named RSA and RSB, were tested with respect to their structural composition, fermentation properties, and effects on composition and turnover of bile acids.

MATERIAL AND METHODS

Study Design. Thirty male rats (Schoe-Wist; Tierzucht Schönwalde GmbH, Schönwalde, Germany) with an initial body mass of 210 ± 5 g were used. During an adaptation period (7 days), the animals were fed a freshly prepared semisynthetic diet free of resistant starch (RS). The rats were randomly assigned into three groups of 10 animals each: a control group fed the RS-free standard diet and two experimental groups fed diets containing RSA or RSB. The RSA group received a diet with partially debranched and retrograded maltodextrin from potato (RS3 content in diet, 5.7 g/100 g). The RSB group received a diet with modified, retrograded potato starch (RS3 content in diet, 5.7 g/100 g). The modification of the potato starch was performed by subjecting the starch to a controlled temperature program in the presence of bacterial α-amylase as previously described (*17*). The rats were placed separately in macrolon cages on wire grates and housed in a room at 22 ± 2 °C and 60 ± 5% relative humidity and with a 12 h light−dark cycle. The animals were allowed to consume their diets and water ad libitum. The composition of the diets is given in **Table 1**.

The effect of the two different RS3s on physiological parameters was studied over a 70 day period after changing the diets. At the end of the experiment, the rats were sacrificed by CO_2 asphyxiation and the entire intestine was removed. The terminal ileum, cecum, and colon were weighted, the contents were removed, and the tissue was reweighted. All treatments and diets were formally approved by the Animal Welfare Committee of Brandenburg, Germany.

Characterization of RS Preparations. The oligomer and polymer composition of the RS3 preparations was analyzed using high-performance anion exchange chromatography (HPAEC) from Kontron (Neufahrn, Germany) with a Carbopak PA1 column (1.9 mm × 250 mm) and precolumn (Dionex, Idstein, Germany), an alkaline gradient, and a Chiralyser detector (IBZ-Messtechnik GmbH, Berlin, Germany) (*18*).

Feces and Urine Collection and Treatment. Feces were collected from each rat on the seventh day of the adaptation period, weighed, and homogenized with water (feces/water = 1:5, w/v); then, the pH was measured straight away before the samples were stored at −20 °C. During the RS3 feeding period, feces were collected daily from each animal and treated in the same manner.

For collection of urine, the animals were placed in individual metabolic cages for 10 days of the experimental period. Daily, urine collections were made from the sixth day. Urine samples were weighed and stored at −20 °C until analysis.

Analytical Methods. Adiabatic bomb calorimetry was used for the determination of the energy content of the diets used and of the feces. The nitrogen content of urine and feces was determined using an automated Kjeldahl procedure (*19*).

The RS content of the diets was determined by a modified method of Berry (*20*). The method is based on in vitro hydrolysis with pancreatic α-amylase (EC 3.2.1.1) for 16 h, degradation of the residue with heat-stable α-amylase (Termamyl) and amyloglucosidase, and determination of total glucose with the GOD-PAP method (Boehringer, Mannheim, Germany) (*21*). Fresh cecal and colonic contents were homogenized with water (material/water = 1:5; w/v) and the pH as for fecal samples was determined using a standard glass electrode.

Gas chromatography was used for determination of SCFA. Suspensions prepared from feces and contents of cecum or colon with water (material/water = 1:5; w/v) were first homogenized, and their dry matter was determined. Approximately 400 mg of the suspension was weighed in a sample tube and centrifuged. *iso*-Butyric acid (internal standard) solution, perchloric acid solution, and sodium hydroxide solution were added to 200 μL of the supernatant. The tube was capped, and the solution was homogenized and frozen at −80 °C overnight. The frozen material was dried under vacuum. The dried sample was dissolved in 200 μL of 5 mol/L formic acid and 800 μL of acetone, homogenized, and centrifuged. One microliter of the organic phase was injected onto a 25 m × 0.32 mm (i.d.) Carbowax 20M column using a temperature program. The GC system from Hewlett-Packard (Waldbronn, Germany) consisted of a HP 5890 Series II gas chromatograph, HP 7673 GC/SFC injector, HP GC Auto Sampler Controller, Detector FID, and Software—HP Chemstation. Helium was used as the carrier gas. All samples were analyzed in duplicate (*18*).

For determination of bile acids using reversed phase high-performance liquid chromatography (RP-HPLC), freeze-dried feces material or contents of cecum (50 mg) were treated with 25 mL of 1 mol/L NaOH in 90% EtOH for 1 h at 80 °C (*22*). After centrifugation (15 min at 4 °C and 5000*g*), the EtOH was removed from the supernatant under vacuum. After the addition of 7 mL of 0.1 mol/L NaOH and 2 mL of MeOH, the nonpolar neutral sterols and lipids were separated by repeated extraction (three times) with 10 mL of hexane. For the removal of residues of organic solvents, the aqueous phase was partly evaporated under vacuum. The pH was adjusted to 8−10 with 0.1 mol/L NaOH and water was added up to a total volume of 50 mL. Samples of 15 mL were used for purification by solid phase extraction on Bakerbond spe C_{18} columns using a BAKER spe-12G system (J. T. Baker, Gross Gerau, Germany). The bile acids were analyzed by HPLC using precolumn derivatization and fluorescence detection as previously described (*23*). Free and glycine-conjugated bile acids were directly derivatized with 4-bromomethyl-7-methoxycoumarin (BMC) in the presence of 18-Crown-6 as a catalyst. Taurine conjugates that cannot react with BMC were enzymatically hydrolyzed with cholylglycine hydrolase before their derivatization and analyzed as free bile acids. The BMC-labeled derivatives were analyzed on a nonpolar stationary phase (Nucleosil 100 Å; C_{18}; 5 μm; 250 mm × 4.6 mm) at 40 °C in HPLC equipment from Gynkotek (Germering, Germany) with online degasser DG 1310, gradient pump M 480, injection automate GINA 160, column oven (Peltier), fluorescence detector RF 1002 (excitation λ, 320 nm; emission λ, 385 nm) (Shimadzu Europe, Duisburg, Germany), and GynkoSoft software. Linear gradients consisting of acetonitrile (30−100%), methanol (40−0%), and water (30−0%) (v/v/v) were applied.

Statistical Analysis. Statistical analysis was conducted using the Microsoft Excel software and the Statistical Package for Social Sciences software SPSS 11.0 (SPSS Inc., Chicago, IL). Data are presented either

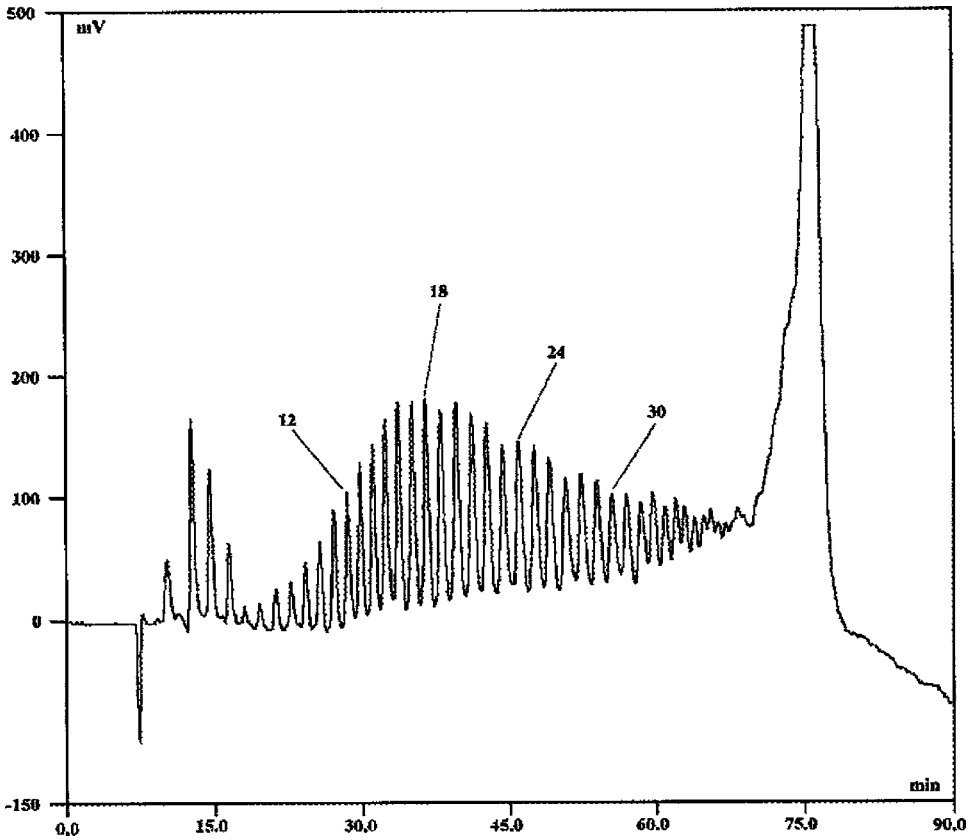

Figure 1. HPAEC chromatogram of RS from maltodextrin (RSA). Numbers above the peaks mark glucose units of the maltooligo- and polysaccharides.

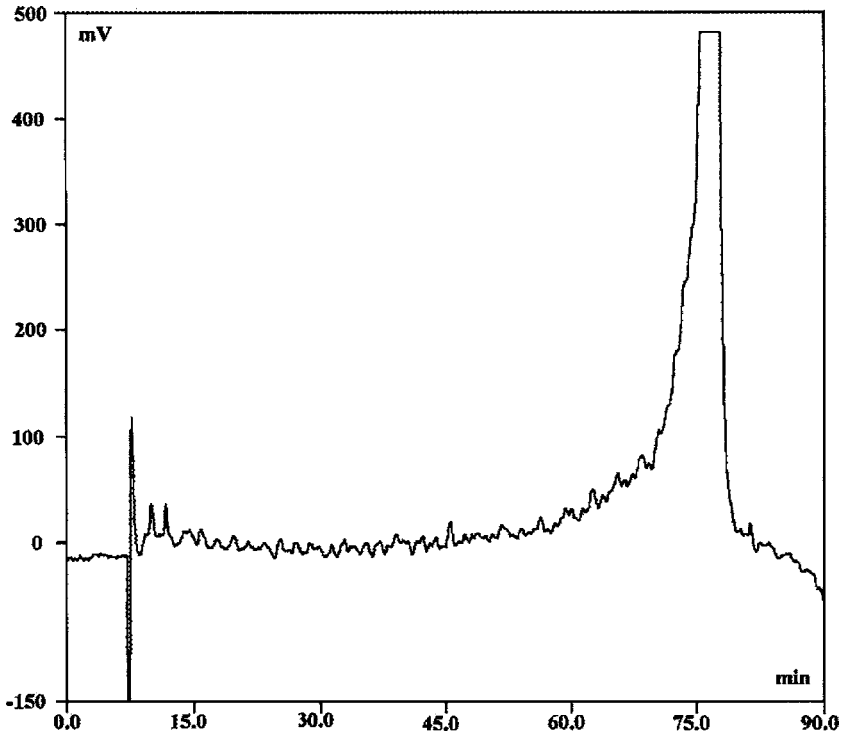

Figure 2. HPAEC chromatogram of RS from modified potato starch (RSB).

as means with standard errors of the mean (SEM) or, in the case of RS effects on energy and nitrogen digestibility, as means with standard deviation (SD). Furthermore, data were analyzed by one-way analysis of variance, and differences between the RS3 groups and the control group were evaluated by Dunnett's *t*-test and Dunnett's T3-test for multiple posthoc comparisons. Differences with $P < 0.05$ were considered significant.

RESULTS

Chromatographic Characterization of RSA and RSB. **Figures 1** and **2** show the HPAEC chromatograms of both RS3 samples. The numbers above the peaks mark glucose and glucose units of α-1,4-D-glucans. RSA consisted of linear low molecular weight polymers and branched high molecular weight

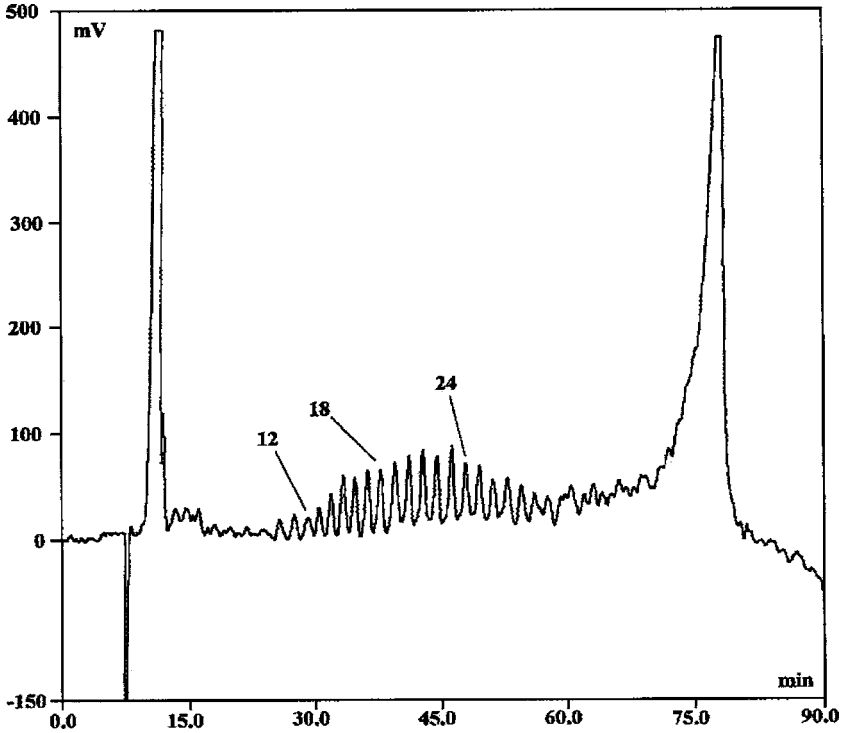

Figure 3. HPAEC chromatogram of RS from modified potato starch (RSB) after its enzymatic debranching using pullulanase. Numbers above the peaks mark glucose units of the maltooligo- and polysaccharides.

polymers of degraded amylopectin. The chain lengths of the glucans corresponded to 10−35 glucose units. In the high molecular weight fraction, the degree of polymerization (DP) was between 35 and 100. RSB contained only high molecular weight polymers of degraded amylose and amylopectin with a DP of 35−100. The HPAEC chromatogram (**Figure 2**) indicates that only a small part of these glucans could be debranched by pullulanase (**Figure 3**).

Food Intake and Body Weight Gain. All rats were in good health. No significant differences were found in food intake between the animal groups. Rats of the control group consumed 18.2 ± 1.3 g, and those of the RS3 groups consumed 18.7 ± 1.08 (RSA) and 18.2 ± 1.06 g (RSB) per day. The daily RS3 intake per rat was 0 (control), 1.06 ± 0.06 (RSA), and 1.02 ± 0.06 g (RSB). The RS3 supply did not influence the growth of the rats. The daily increase in body weight per rat was 2.37 ± 0.32 (control), 2.42 ± 0.35 (RSA), and 2.43 ± 0.28 g (RSB).

Nitrogen and Energy Turnover. The mean daily nitrogen intake per rat was 353 ± 40 (control), 359 ± 46 (RSA), and 326 ± 23 mg (RSB); the energy intake corresponded to 257 ± 29 (control), 264 ± 32 (RSA), and 245 ± 17 kJ (RSB). The influence of RSA and RSB on energy and nitrogen digestibility is summarized in **Table 2**. As compared with the control groups, fecal nitrogen excretion increased significantly in animals fed both RSA and RSB. Nitrogen output in urine was lower in animals fed RS3 as compared with the control group, although this was not statistically significant. The overall nitrogen output in urine and feces was numerically higher in animals fed RS3. These values resulted in a significant reduction in nitrogen digestibility for RSA and RSB groups as compared with the control group. The lowest nitrogen digestibility was observed for animals fed RSA, which was significantly lower than both control and RSB-fed animals. The intake of RSA resulted in a significant increase in energy loss in feces, and as a result, a decreased energy digestibility was noted for this group as compared with the other groups.

Table 2. Effects of RSs on Energy and Nitrogen Digestibility[a]

	control group	RSA group	RSB group
nitrogen in urine (mg/day/rat)	231.0 ± 31.6	213.0 ± 49.2	217.0 ± 20.4
nitrogen in feces (mg/day/rat)	26.4 ± 2.6	56.6 ± 11.2 b	32.8 ± 4.8 a,**
nitrogen in feces + urine (mg/day/rat)	257.0 ± 49.2	268.0 ± 55.0	250.0 ± 25.0
energy in feces (kJ/day/rat)	19.1 ± 1.5	25.4 ± 3.8 b	19.2 ± 2.0
nitrogen digestibility (%)	92.5 ± 0.23	85.3 ± 0.21 b	89.9 ± 0.99 b,**
energy digestibility (%)	92.5 ± 0.5	90.4 ± 0.9 b	92.2 ± 0.5 **

[a] Values are means ± SD; $n = 6$. Mean values were significantly different from control group: [a]$P < 0.05$ and [b]$P < 0.01$. Mean values were significantly different from group RSA: *$P < 0.05$ and **$P < 0.01$.

Fecal Output, Digesta and Tissue Weights, Digesta Starch Content, and pH. In **Figure 4**is shown the diet-mediated influence on feces output. The intake of RSA enhanced the feces excretion significantly as compared with the RSB and control groups ($P < 0.001$). The wet weights of the whole ileum, cecum, and colon and the weights of tissue and contents at the end of the experimental period are shown in **Figure 5**. Although RSA and RSB both caused an increase in the weight of colon contents, only RSA elevated both wet cecum tissue and cecal contents weight significantly ($P < 0.001$). This effect was associated with opposite changes in the dry matter contents, with the following proportion of dry matter in cecal contents: control group = 23.5 ± 1.18%, RSA group = 20.6 ± 0.79%, and RSB group = 21.1 ± 0.90%. Similarly, the proportion of dry matter was lower in colonic contents of both groups fed RS: control group = 40.1 ± 2.73%, group RSA = 27.1 ± 1.38% ($P < 0.001$), and RSB = 37.2 ± 1.38%.

In contrast, the total content of ileal dry matter was significantly higher in rats fed RS3 as compared with the control

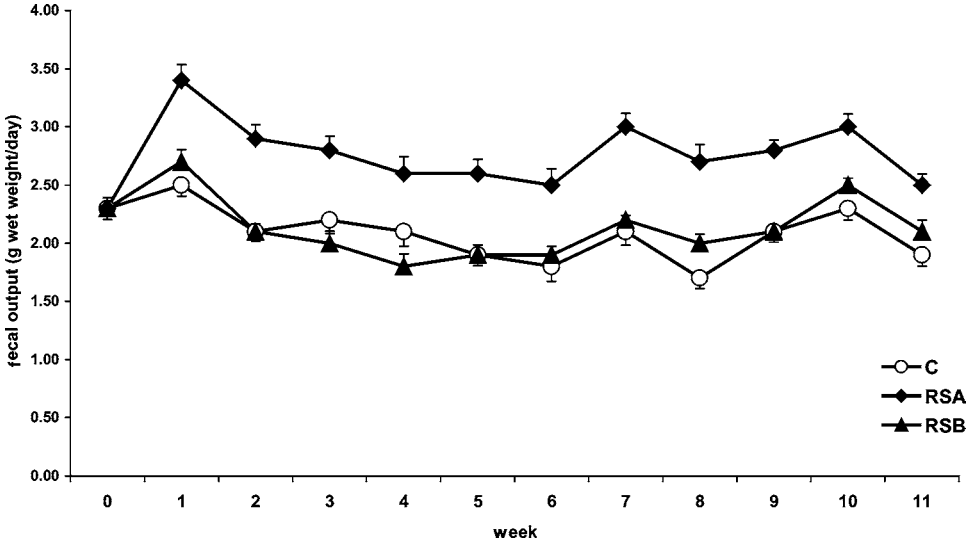

Figure 4. Feces output of rats fed the control diet C or the diets supplemented with the RS preparations RSA and RSB during the feeding period. Values are means ± SEM; $n = 10$.

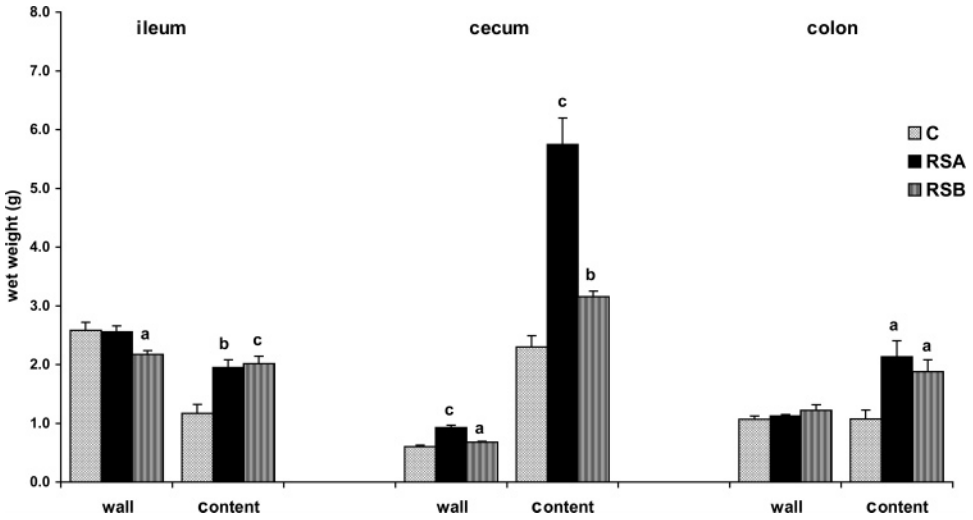

Figure 5. Wet weight of ileum, cecum, and colon walls as well as of ileal, cecal, and colonic contents of rats fed the control diet C or the diets supplemented with the RS preparations RSA and RSB at day 70. Values are means ± SEM; $n = 9$–10. Mean values were significantly different from the control group: $^{a}P < 0.05$, $^{b}P < 0.005$, and $^{c}P < 0.001$.

group: control group = 0.23 ± 0.031 g, RSA group = 0.45 ± 0.048 g ($P < 0.005$ as compared with control group), and RSB group = 0.34 ± 0.029 g ($P < 0.05$ as compared with control group). Likewise, for whole ceca, the total dry matter content was significantly higher in RS-fed rats: control group = 0.53 ± 0.036 g, RSA group = 1.20 ± 0.117 g ($P < 0.001$ as compared with control group), and RSB group = 0.67 ± 0.040 g ($P < 0.05$ as compared with control group). The differences in total dry matter were smaller in the colon, but the same trends were observed as follows: control group = 0.50 ± 0.058 g, RSA group = 0.58 ± 0.068 g, and RSB group = 0.73 ± 0.106 g. Starch concentrations, measured in ileum, cecum, and colon contents, are shown in **Figure 6**. The starch concentration was highest in the ileum, where it was more than three times higher in rats fed RSA than RSB ($P < 0.001$). The lowest starch concentrations were found in ileum of rats given the control diet. The starch concentration dropped substantially in the large bowel contents of both RS3 groups. **Figure 7** shows the pH of cecal and colonic contents at the end of the experimental period. Whereas the pH did not differ in cecum and colon of the control group (pH 7.50), it was significantly reduced in cecal contents and even more so in colonic contents of both RS3 groups. **Figure 8** shows the pH changes in feces throughout the experimental period. The fecal pH of the control group was not different during the experiment (7.00 ± 0.03). RS3 decreased the fecal pH significantly as compared with the control group ($P < 0.001$ between days 3 and 70), and this effect was more strongly expressed in the RSA than in the RSB group ($P < 0.001$ for differences between the two RS groups).

SCFAs. Consumption of RS3 significantly enhanced total SCFA levels in cecum and colon ($P < 0.001$; **Figure 9**). Intake of RSA caused the highest increase of SCFA concentrations. The cecal total SCFA concentration was 135.2 ± 9.0 μmol/g dry matter in control rats and was nearly doubled after intake of RSA. Consumption of RSB resulted in a SCFA concentration of 207.4 ± 10.9 μmol/g dry matter, which was significantly lower than in the RSA group ($P < 0.005$).

Likewise, the total SCFA concentration was greater in colonic contents of the RSA group as compared with RSB ($P < 0.05$). The molar ratio of acetate:propionate:butyrate was 67:25:8 (control), 70:15:15 (RSA), and 66:22:12 (RSB) in cecal contents and 72:20:8 (control), 74:13:13 (RSA), and 68:22:12 (RSB) in colonic contents.

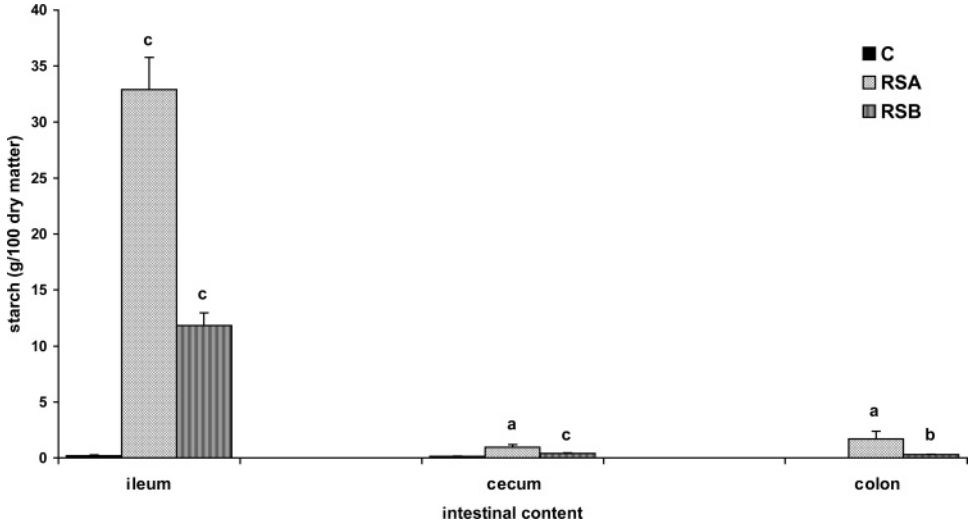

Figure 6. Concentration of starch in ileal, cecal, and colonic contents of rats fed the control diet C or the diets supplemented with the RS preparations RSA and RSB at day 70. Values are means ± SEM; $n = 9–10$. Mean values were significantly different from control group: $^{a}P < 0.05$, $^{b}P < 0.005$, and $^{c}P < 0.001$.

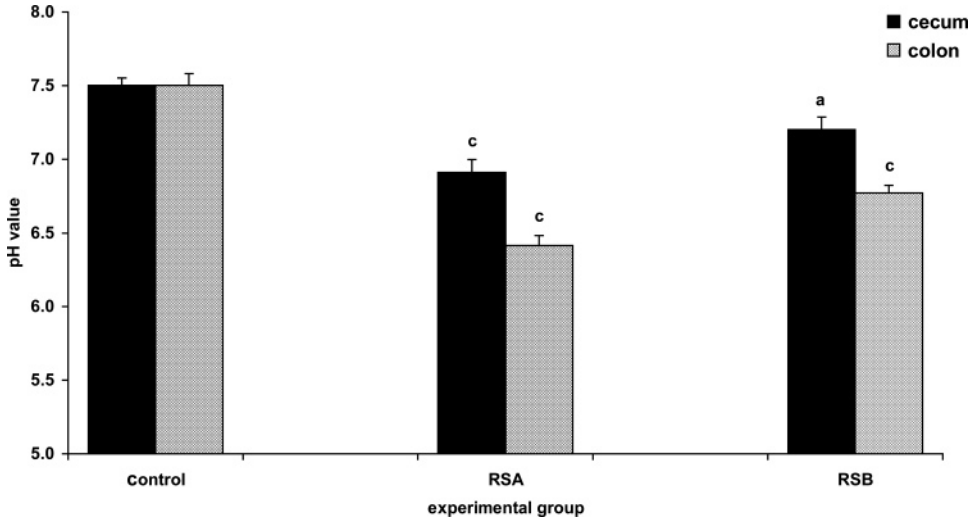

Figure 7. pH values in cecal and colonic contents of rats fed the control diet or the diets supplemented with the RS preparations RSA and RSB at day 70. Values are means ± SEM; $n = 9–10$. Mean values were significantly different from control group: $^{a}P < 0.05$ and $^{c}P < 0.001$.

The butyrate concentration of cecal contents was between 2- and 4-fold higher for RSA or RSB as compared with the control group ($P < 0.001$) and was significantly higher for RSA as compared with RSB ($P < 0.005$). Likewise, more butyrate was found in colonic contents of rats fed RS-containing diets (μmol/g dry matter), control group = 4.6, RSA = 25.0, and RSB = 14.3 ($P < 0.001$), and the value for RSA was significantly higher than that for RSB ($P < 0.05$).

The fecal concentrations of total SCFA and butyrate of rats fed RSA were higher throughout the experiment as compared with control with the highest concentrations measured between day 3 and day 18 ($P < 0.001$ as compared with control group) (**Figure 10**). The molar ratio of acetate:propionate:butyrate was 85:5:9 (control), 73:13:14 (RSA), and 84:8:8 (RSB) at the end of the experiment in fecal contents. Throughout the experiment, more total SCFA were excreted in rats fed RSA as compared with RSB ($P < 0.001$).

Effects of RS3 on Concentrations and Composition of Bile Acids in the Large Bowel and in Feces. An exchange of 10% digestible starch by RS3 in the diet was sufficient to provide enough substrate for bacterial fermentation in the large bowel if the structures of the used RS3 samples are resistant to degradation in the small intestine. Both RS3 preparations applied in this study differed in the stability of their resistant structures, and a higher proportion of RSA than of RSB entered the large bowel. Assuming that RS3 can bind bile acids (*12*), higher bile acid concentrations would have been expected in the cecum during intake of RSA. The cecal bile acid concentrations, however, calculated on a dry matter basis, were 4 and 11% lower in the RSB and RSA groups, respectively. Because cecum is the main site of bacterial fermentation in rats, RS3 caused an increase in wet weight of the cecal content. This resulted in a decrease of the bile acid concentration in the cecum calculated on fresh weight. The bile acid level was diminished by 11% in rats fed RSB but by 19% in animals given RSA, although much more RS3-bound bile acids arrived in the large bowel.

The higher bile acid concentrations observed in the colonic contents were as a result of the influx of bile acids and the water reabsorption. The intake of RS3 induced a small increase in bile acids in the colonic contents. Calculated on dry matter basis, this effect was more pronounced and was related to the starch concentration in the colon. Surprisingly, bile acid concentrations were highest, calculated on fresh weight, in the RSB-fed rats. This may be due to the very low starch content, which may

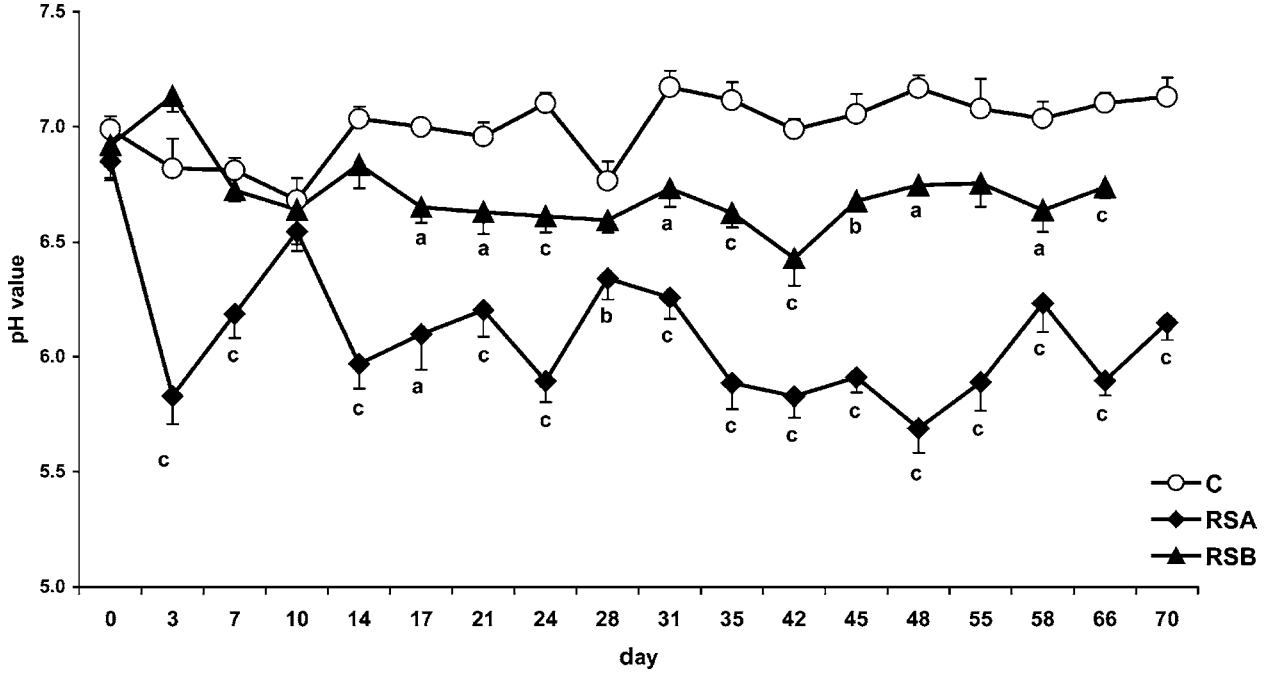

Figure 8. Behavior of the pH values in feces during the experimental period of rats fed the control diet C or the diets supplemented with the RS preparations RSA and RSB. Values are means ± SEM; $n = 4$–10. Mean values were significantly different from control group: $^{a}P < 0.05$, $^{b}P < 0.005$, and $^{c}P < 0.001$.

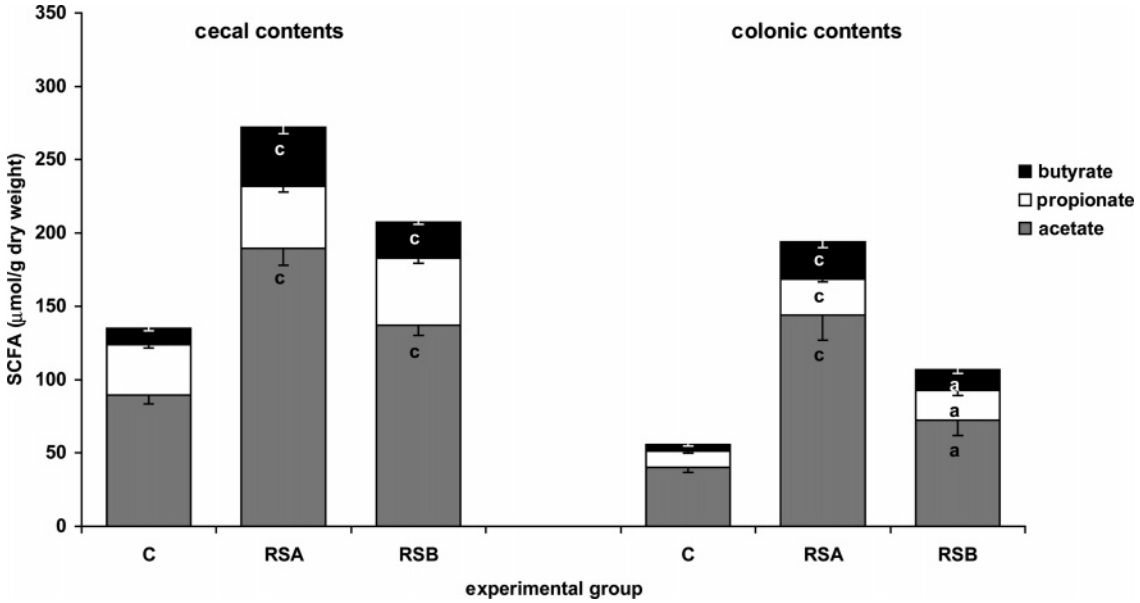

Figure 9. Concentration of individual SCFAs in cecal and colonic contents of rats fed the control diet C or the diets supplemented with the RS preparations RSA (**A**) and RSB (**B**). Values are means ± SEM; $n = 8$–10. Mean values were significantly different from control group: $^{a}P < 0.05$ and $^{c}P < 0.001$.

have limited fermentation and induced only a small increase in wet volume of the colonic content. In total, 4.55 μmol bile acids/g fresh weight were found in the control group, 5.32 in the RSB group, and 4.88 in the RSA group.

Similar results were found in the fecal excretion of bile acids. The lowest excretion was found in the control rats (0.543 μmol bile acids/g fresh weight) followed by the RSB and RSA group with 22 and 16% higher concentrations. However, because fecal output was greatest for rats fed RSA, the total daily, bile acid excretion was highest in this group. The effect of RS3 on fecal bile acid concentrations changed during the experimental period. The fecal bile acid concentrations were much higher in the first week than at the end of the study. As the experimental period progressed, the daily output of feces increased. Simultaneously, the fecal total bile acid level per gram fresh weight diminished to about 60% of the starting values by day 38 and was similar to control rats at this point (**Table 3**).

A spectrum of individual bile acids was evident in intestinal contents and feces. Thus, in cecal contents, a small amount of tauro-conjugated bile acids was present (0.35% in control group; 0.29 and 0.21% in groups RSA and RSB, respectively). In feces, changes in concentration of the individual bile acids through the course of experiment were observed. Whereas the concentration of total bile acids did not differ in feces if expressed in μmol/g dry matter, more bile acid was found in groups fed the RS-containing diets.

The effect of consumption of RS3 on the concentration of the muricholic acids (MCA) and CA is of special interest,

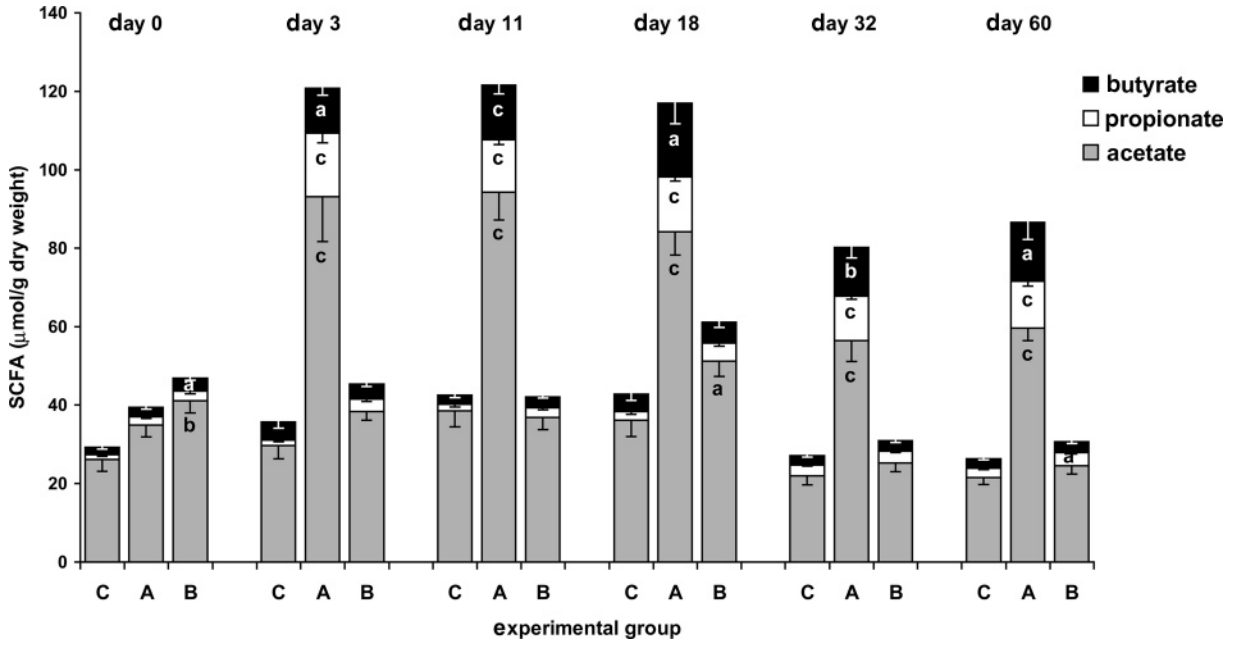

Figure 10. Concentration of SCFAs in feces during the experimental period of rats fed the control diet C or the diets supplemented with the RS preparations RSA (**A**) and RSB (**B**). Values are means ± SEM; $n = 9–10$. Mean values were significantly different from control group: $^{a}P < 0.05$, $^{b}P < 0.005$, and $^{c}P < 0.001$.

Table 3. Influence of RS Preparations RSA and RSB and of Experimental Day on the Concentration of Total Bile Acids in Feces[a]

experimental	mmol/g wet weight		
day	control group	RSA group	RSB group
0	0.933 ± 0.148	0.933 ± 0.148	0.933 ± 0.148
7	0.583 ± 0.029	1.102 ± 0.133 b	0.600 ± 0.026 **
14	0.675 ± 0.034	0.766 ± 0.038 a	0.516 ± 0.035 a,***
38	0.543 ± 0.029	0.633 ± 0.039	0.663 ± 0.036 a

[a] Values are means ± SEM; $n = 9–10$; day 0, $n = 39$. Mean values were significantly different from control group: $^{a}P < 0.05$ and $^{b}P < 0.005$. Mean values were significantly different from group RSA: $^{**}P < 0.005$ and $^{***}P < 0.001$.

because these bile acids are substrates for the formation of secondary bile acids. The concentration of these bile acids was increased slightly following RS3 consumption, and the response was most evident in rats fed RSA (**Table 4**).

Consumption of both RS3 sources resulted in a significant decrease in the concentrations of secondary bile acids in the cecum as compared with the control group ($P < 0.001$), and this effect was greatest for RSA. This result is due to the decrease of cecal pH caused by the enhanced formation of SCFA (**Figure 9**). As compared with the control group, the concentrations of DCA and HDCA were approximately 40% lower and for LCA approximately 70% lower in the cecum of rats fed RSA. These results were strongly correlated with pH, which were 7.5 for the control group and 7.2 and 6.9 for the RSB and RSA groups, respectively. Because the bacterial 7-dehydroxylase is sensitive to an increase in proton concentration, the formation of secondary bile acids was reduced in line with the decrease in pH. In comparison with the cecum, the concentration of secondary bile acids was increased in the colon more than 3-fold in the control group and 5-fold in the RS3-fed rats (**Table 5**). The increase was greatest for the HDCA followed by DCA and LCA. Additionally, KLCA was found in colon, whereas this secondary bile acid was not formed in the cecum. Intake of RS3 resulted in an increase in the concentration of HDCA and DCA, but this effect was lower in the RSA than in the RSB group. Furthermore, RSA decreased the formation of KLCA and LCA in comparison with the control. The lowest concentration of secondary bile acids resulted in the cecum from a supply of RSA. This affected the concentration of all secondary bile acids. The numerical lower secondary bile acid concentrations in RSA-fed rats are not significant. DCA and LCA were most diminished. It can be concluded that dietary RSA administration lowers the formation of secondary bile acids indirectly by both a decrease of the colonic pH and by an increase of the concentrations of butyrate.

From the time−course of the fecal secondary bile acids excretion, it can be proposed that at least 4 weeks are necessary to adapt the intestinal microflora to RS3 supply, because the benefit is dependent from high rates of fermentation resulting in low pH values and a high output of butyrate. Up to this time, fecal secondary bile acid concentrations decrease continuously. The results of this animal study provide evidence that the intake of a RS3 preparation stable to digestion but well fermentable is to be recommended because the tumor-promoting effects of secondary bile acids can be partially inhibited despite an enhanced turnover of bile acids in the large bowel.

DISCUSSION

Carbohydrates entering the large bowel can alter the colonic physiology by fermentation-coupled mechanisms and by the osmotic effects of indigested oligosaccharides. SCFAs produced by bacterial fermentation are the major anions in the cecal and colonic lumen. SCFAs, particularly butyrate, have been shown to regulate colonic fluid and electrolyte absorption. Butyrate is important in maintaining homeostasis of the colonic mucosa by regulation of its proliferation and differentiation as well as by promotion of growth resulting in a larger membrane mucosal surface of the large bowel (*3*). SCFAs stimulate colonic transit time via intraluminal 5-hydroxytryptamine, which is transported to the vagal efferent system, where it stimulates the release of acetylcholine from the colonic myenteric plexus. This results in muscle contraction at the proximal colon, which migrates to the mid- and distal colon (*25*). A further advantage of RSs such as RS3, which enter the large intestine, is related to the lower

Table 4. Influence of the RS Preparations RSA and RSB on the Concentrations of MCA Isomers and CA in Cecal and Colonic Contents and Feces at Day 71 (in μmol/g Wet Weight)[a]

	sum of α-, β- and ω-MCAs			CA		
	control group	RSA group	RSB group	control group	RSA group	RSB group
cecal content	1.414 ± 0.039	1.256 ± 0.057 a	1.334 ± 0.035	0.468 ± 0.012	0.424 ± 0.020 c	0.433 ± 0.014 c,*
colonic content	1.526 ± 0.090	1.641 ± 0.073	1.595 ± 0.054	0.291 ± 0.020	0.496 ± 0.023	0.437 ± 0.011
feces	0.176 ± 0.011	0.220 ± 0.015 a	0.206 ± 0.012	0.035 ± 0.002	0.060 ± 0.003 c	0.054 ± 0.003 c

[a] Values are means ± SEM; n = 9–10. Mean values were significantly different from control group: [a]$P < 0.05$ and [c]$P < 0.001$. Mean values were significantly different from group RSA: *$P < 0.05$.

Table 5. Concentration of Secondary Bile Acids in Cecal and Colonic Contents and Feces in Control Group C and in Groups Fed the RS Preparations RSA and RSA[a]

		mmol/g wet weight				
content	group	DCA	KLCA	LCA	HDCA	total secondary BA
cecum	C	0.362 ± 0.014	0	0.014 ± 0.002	0.382 ± 0.016	0.757 ± 0.027
	RSA	0.217 ± 0.011 c,**	0	0.004 ± 0.001 c	0.237 ± 0.011 c	0.459 ± 0. 21 c,***
	RSB	0.271 ± 0.009 c	0	0.008 ± 0.001 a	0.301 ± 0.014 b,**	0.581 ± 0.022 b
colon	C	0.797 ± 0.047	0.338 ± 0.019	0.464 ± 0.028	0.920 ± 0.060	2.519 ± 0.147
	RSA	0.750 ± 0.033	0.314 ± 0.018	0.403 ± 0.021	0.915 ± 0.040 *	2.382 ± 0.109
	RSB	0.271 ± 0.027 a,***	0.438 ± 0.015 b,***	0.534 ± 0.017 c	1.070 ± 0.034	2.981 ± 0.109 a,***
feces	C	0.099 ± 0.006	0.040 ± 0.002	0.061 ± 0.003	0.113 ± 0.006	0.312 ± 0.016
	RSA	0.097 ± 0.006	0.039 ± 0.002	0.059 ± 0.003	0.117 ± 0.007	0.312 ± 0.018
	RSB	0.114 ± 0.006	0.053 ± 0.003 b,**	0.073 ± 0.004	0.134 ± 0.007 c	0.374 ± 0.022 a,*

[a] Values are means ± SEM; n = 9–10. Mean values were significantly different from control group: [a]$P < 0.05$, [b]$P < 0.005$, and [c]$P < 0.001$. Mean values were significantly different from group RSA: *$P < 0.05$, **$P < 0.005$, and ***$P < 0.001$.

energy load of these products because SCFAs generate less than 10% of body energy serves (*26*). Consumption of RS3 resulted in a shift in nitrogen excretion from urine to feces. The increase in fecal nitrogen output is due to stimulation of bacterial growth in the large bowel by RS3 supply. Butyrate is well-known to be the preferred source of energy for the colonic epithel cells (*27*). However, the mechanisms of butyrate absorption and regulation are still not fully understood. Two mechanisms are proposed as to how SCFA crosses the intestinal mucosa: anionic diffusion of protonated SCFA and/or a carrier-mediated anion exchange. The assumption of a passive diffusion as the predominant mechanism of absorption is difficult to conceive, because SCFAs are ionized (pK_a approximately 4.8) at the normal colonic luminal pH. Therefore, only minimal diffusion is possible. A fermentation-dependent decrease in pH such as occurs following consumption of RS3 does not change this situation significantly. The use of a bicarbonate-dependent, carrier-mediated anion exchange mechanism is more probably responsible for the SCFA uptake in the apical membranes of the large bowel. Distinct electroneutral Cl^-/HCO_3^- (*28*) and SCFA/HCO_3^- exchangers (*29*) have been identified in the proximal colonic apical membrane vesicles. Results, obtained by utilizing a rapid filtration *n*-(^{14}C) butyrate uptake technique, revealed also the existence of a HCO_3^-/SCFA exchanger in colonic basolateral membrane, which regulates the uptake of SCFA in the blood stream via portal vein (*30*). A single order of magnitude of higher K_m for the butyrate uptake in the basolateral membrane than in the luminal apical membrane permits colonocytes to metabolize most of the absorbed butyrate.

The name RS characterizes starches and starch degradation products, which resist amylolysis in the small intestine and enter the large bowel (*21*). RS3 is an example of a RS and comprises retrograded starches (*31*). To utilize RS3 as a substrate for the microflora in functional foods requires an exact knowledge of parameters, which determine the stability and microbial degradation of RS. The results of this study suggest that both parameters depend on the RS3 structure. The applied RS3 samples differed significantly in their composition of polymers; RSB consisted of a mixture of linear and branched polysaccharides with a higher DP (DP 35−100) as compared with RSA, which was composed of a linear (DP 10−35) fraction together with a fraction with a DP > 35. The intention was to use debranched maltodextrins in order to provide a substrate with linear, small recrystallizable chains of α-1,4-D-glucans. RS3 stability is related in part to the crystallinity of the molecule, which increases with a higher proportion of chain lengths with 10−35 glucose units in the gel (*18*, *32*). Such a mixture of small glucan polymers will lead to a pronounced acceleration of the formation of resistant structures with RS contents of up to 90%. The prebiotic properties of RS3 rise with the content of B type microcrystalline filaments, which are also determined by this optimal composition of the glucan chain length. Therefore, from these criteria, only RSA fulfills the requirement of an effective RS3 product with health-promoting properties.

An increase in the rate of intestinal fermentation was linked with a higher butyrate output and was accompanied by an elevation in the wet weight of cecal and colonic contents, an increase in tissue weight, and a lowering of luminal pH values. RSA consumption resulted in an increase in both fecal volume and bile acid excretion. Whereas bile acid excretion did not change in the control group during the experimental period, as expected, RS3 consumption increased this process by approximately 15 and 25% in rats fed RSB or RSA, respectively. Parallel with the increase of fecal volume (maximal 28%), both total and secondary bile acid concentrations calculated on fresh weight basis decreased continuously throughout the experiment. At the end of the experimental period, their concentrations were 40% lower than those after 1 week of RSA intake, and concentration of secondary bile acids did not differ from the control; only the total bile acid concentrations exceeded those of control rats by approximately 16%. The decrease was most strongly expressed for LCA, HDCA, DCA, KLCA, and ωMCA. In general, RSs may cause a cholesterol-lowering effect because bile acid biosynthesis is feedback-inhibited at the transcriptional

level of hydrophobic bile acids (*33*). They are ligands for the farnesoid X receptor activation, which is the rate-limiting step catalyzed by cholesterol 7α-hydroxylase, and are repressed in hepatocytes. Lower fecal bile acids concentrations have also been described in rats fed diets containing 46% of starch or raw high amylose corn starch or Hylon VII (*34*, *35*) and by acarbose-mediated malabsorption of starch (*36*). Inhibition of α-glucosidase by acarbose leads to an extremely high excretion of bile acids particularly of CA; the removal of neutral sterols and the secondary bile acids DCA and LCA was not influenced so much. Higher bile acid excretion with consequences on the cholesterol and the bile acid metabolism have also been described as results of diet supplementation with raw high amylose corn starch, freeze-dried raw potatoes (granular starch; RS2), freeze-dried, cooked, retrograded potato starch (RS3), and cellulose (*34*, *35*, *37*, *38*). These studies showed a higher bile acid excretion (in mg/g dry matter) in rats fed RS2, especially a higher loss of the secondary bile acids LCA and HDCA, but the daily total bile acid excretion was similar in the RS2 and cellulose group. Other studies have also confirmed that an intake of RS2 does not change significantly bile acid excretion or the ratio of primary to secondary bile acids in the large bowel (*35*, *39*). These findings agree with the observation that RS2 is only poorly fermented. RS3 is a much better source for bacterial fermentation than RS2. Cecal and colonic SCFA concentrations fall in starved rats especially in the colon but may be restored and elevated by a supply of high quality RS3 (*39*). As shown in this paper, the quality of RS3 preparations may differ strongly in the reassociated linear regions of the polymers, which form the insoluble crystallites, which are responsible for the resistance against α-amylases. RS3 with exclusively B type crystallites shows the best fermentation properties; with a higher proportion of A type crystallites, bacteria degrade RS3 more slowly and lower quantities of SCFA and butyrate are produced. It can be assumed from the composition analysis that both the portion of crystallinity and that of B type crystallites is much higher in RSA than in RSB. Therefore, the resistant structures of RSA are more stable and favor the formation of both SCFA and butyrate.

Feeding the RSA-containing diet induced a negative correlation between butyrate and secondary bile acids in cecum (correlation coefficient $r = -0.040$). In contrast, a positive correlation seems to exist between bile acids and propionate. These data are in accordance with an inhibition of secondary bile acid formation at low pH and high butyrate levels (*40*). The relationship between bile acids, large bowel SCFA, butyrate, and cancer risk may be important because secondary bile acids are thought to promote colon carcinogenesis in setting apc mutations (*24*). High butyrate and SCFA production may lower the cytotoxic potential of bile acids. The distal colon and rectum are the regions of the large bowel with the most limited sources for SCFA formation and consequently are the sites of widespread diseases such as ulcerative colitis and cancer. In this connection, it should be emphasized that butyrate formed in vivo by prebiotic degradation of RS3 exerts a specific health-promoting effect on colon mucosa; colon cancer and inflammatory diseases can be effectively suppressed (*10*, *11*), whereas in animals treated with the genotoxic carcinogen 1,2-dimethylhydrazine, RS type 2 did not significantly prevent tumorigenesis (*41*). Therefore, only an intake of butyrogenic RS3 preparations that are well-fermented in the colon can be recommended.

ABBREVIATIONS USED

BMC, 4-bromomethyl-7-methoxycoumarin; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; DP, degree of polymerization; HDCA, hyodeoxycholic acid; HPAEC, high-performance anion exchange chromatography; KLCA, 12-ketolithocholic acid; LCA, lithocholic acid; MCA, muricholic acid; RS, resistant starch; RSA, RSB, used resistant starch preparations; RS3, resistant starch type 3; SCFA, short chain fatty acid.

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