Limited effect of consumption of uncooked (RS2) or retrograded (RS3) resistant starch on putative risk factors for colon cancer in healthy men1–3

Marie-Louise A Heijnen, Johan MM van Amelsvoort, Paul Deurenberg, and Anton C Beynen

ABSTRACT To investigate whether resistant starch (RS) affects putative risk factors for colon cancer, 24 healthy men consumed a daily RS supplement for 4 wk in addition to their habitual diet in a single-blind, randomized, balanced multiple crossover trial. During the first week, all subjects consumed the control supplement containing glucose. Subsequently, each subject consumed, in random order, a supplement with RS2 (uncooked high-amylose cornstarch), RS3 (extruded and retrograded high-amylose cornstarch), and glucose, each for 1 wk. The RS2 and RS3 supplements provided 32 g RS/d. Lithium was added to the supplements to measure compliance. Feces, 24-h urine, and breath samples, as well as a 24-h food-consumption recall were obtained weekly from each subject. Compliance as measured by urinary lithium recovery was satisfactory. The mean composition of the background diet did not differ between the various supplementation periods. Breath-hydrogen excretion, stool weight, and fecal starch excretion were significantly higher during RS than during glucose supplementation, but did not differ during RS2 and RS3 supplementation. There were no significant differences in fecal dry weight, pH, or short-chain fatty acid concentrations, nor in the pH, bile acid concentrations, cytotoxicity, or osmolality of fecal water. It is concluded that in healthy men, supplementing the habitual diet for 1 wk with 32 g RS2 or RS3/d compared with glucose had no effect on putative risk factors for colon cancer, except for increasing stool weight and colonic fermentative activity. There were no significant differences between the effects of RS2 and RS3 on the indexes studied. Am J Clin Nutr 1998;67:322–31.

KEY WORDS Resistant starch, uncooked starch, retrograded starch, men, breath hydrogen, fecal bile acids, fecal pH, short-chain fatty acids, cytotoxicity, colon cancer risk

INTRODUCTION Because resistant starch (RS) is not absorbed in the small intestine of healthy individuals (1), but is partly fermented in the colon, it may have positive effects on putative risk factors for colon cancer by analogy with dietary fiber. Colonic fermentation of RS may lead to the production of short-chain fatty acids (SCFAs) (2–4). Some studies indicate that fermentation of RS leads specifically to an increase in butyrate (5–7). Butyrate putatively has a protective effect against colon cancer (8–11). SCFAs, which are physiologically active in the large intestine, induce a decrease in colonic pH resulting in reduced solubility of bile acids (12). Furthermore, the initial, irreversible step in bacterial conversion of primary into secondary bile acids (7α-dehydroxylation) is inhibited at a pH < 6.5 (13, 14). The amount of soluble long-chain fatty acids and soluble bile acids (15), particularly secondary bile acids (16), may affect the cytotoxicity, ie, the cell-damaging properties, of fecal water. Fecal water is the fraction of feces containing the water-soluble, unbound components of the feces (17) that are in contact with the colonic mucosal cells (12, 18, 19). A diet-induced increase of the bile acid concentration in fecal water resulted in a higher cytotoxicity of fecal water in humans (20) and rats (21) and was associated with higher colonic cell proliferation in rats (22). In healthy volunteers, proliferation of mucosal cells from a rectal biopsy was increased and associated with an increase in the concentrations of total and secondary bile acid in fecal water after consumption of fat as a bolus (23). However, there is no conclusive evidence for a causal relation between cytotoxicity of fecal water and mucosal proliferation. Hyperproliferation of colonic epithelial cells is suggested to be an important biomarker of increased susceptibility to colon cancer (24). However, colonic epithelial proliferation was not found to be a reliable predictor of tumor formation in rats (25). In addition, studies in rats indicate that the secondary bile acids deoxycholic and lithocholic acids may be colon tumor promoters (26–28).

The hypothesis that RS consumption may protect against colon cancer is supported by some epidemiologic and experimental studies. In an ecologic study, strong inverse associations were found...
RESISTANT STARCH AND COLON CANCER

between large bowel cancer incidence and consumption of starch or nonstarch polysaccharides in combination with RS, which was estimated to be 5% of total starch intake (29). Supplementation with 28 g RS/d for 2 wk resulted in an increase in breath-hydrogen excretion (a semiquantitative measure of colonic fermentation) (30) and fecal SCFA excretion, a decrease in secondary bile acid concentration and cytotoxicity of fecal water, and a decrease in colonic mucosal proliferation in rectal biopsies (6). However, this study lacked a control group and only RS derived from uncooked granular starch (RS₃) (31) was studied. Cummings et al (32) found that RS₂ gave greater proportions of acetate in feces, but RS₃ (retrograded resistant starch) (31) gave more propionate. However, the amounts of RS consumed in their experiment differed depending on the type of RS studied, and fecal bile acids, cytotoxicity of fecal water, and mucosal proliferation were not measured. These indexes were also not measured in a study with a diet containing 39 g/d of a mixture of RS₁ (ie, starch physically inaccessible to α-amylase) (31), RS₂, and RS₃ (7). This high-RS diet induced a lower fecal pH and increased fecal concentration and excretion of butyrate and acetate compared with a diet containing only 5 g RS/d. Recently, three studies were published that investigated the effect of dietary RS on chemically induced colon cancer in rats (25, 33, 34). However, in these studies the amount and the type of RS used is unclear.

Because the studies mentioned above are not conclusive, we investigated whether supplementing the habitual diet with 32 g/d of either RS₂ or RS₃ compared with an equivalent amount of glucose would affect putative risk factors for colon cancer in 24 healthy men in a randomized, balanced multiple-crossover trial. Characteristics of the subjects (x ± SD) were as follows: age > 18 y; weight fluctuation during the previous 3 mo of ≤ 2.5 kg; no diseases of the kidneys or the gastrointestinal tract; no diabetes mellitus; no history of stomach or bowel surgery other than removal of the appendix; no complaints of diarrhea, obstipation, or abdominal pain; no use of antibiotics or laxatives during the previous 3 mo; and preferably a good appetite and daily stool passage. One subject took antibiotics during week 2: his data were excluded from statistical analysis.

SUBJECTS AND METHODS

Subjects

Twenty-four apparently healthy men were recruited by advertisements in local newspapers and posters mounted in public buildings in Wageningen. The inclusion criteria were as follows: age > 18 y; weight fluctuation during the previous 3 mo of ≤ 2.5 kg; no diseases of the kidneys or the gastrointestinal tract; no diabetes mellitus; no history of stomach or bowel surgery other than removal of the appendix; no complaints of diarrhea, obstipation, or abdominal pain; no use of antibiotics or laxatives during the previous 3 mo; and preferably a good appetite and daily stool passage. One subject took antibiotics during week 2: his data were excluded from statistical analysis.

Characteristics of the subjects (x ± SD) were as follows: age, 23 ± 2 y; height, 1.84 ± 0.07 m; body weight, 76.9 ± 7.5 kg; and body mass index (in kg/m²), 22.7 ± 1.8. The experimental design of the study and possible discomforts resulting from the consumption of the supplements were explained to the subjects before they gave their written, informed consent. The study protocol was approved by the Medical-Ethical Committee of the Department of Human Nutrition of the Wageningen Agricultural University. Subjects were paid for their participation after they had completed the experiment.

Study design

The 24 subjects were randomly divided into six groups before the experiment started. Each group consumed the supplements in one of the six possible sequences to eliminate variation due to residual effects of the previous supplement or to drift of variables over time (36). The groups were not different with respect to age, height, body weight, or body mass index (data not shown). The subjects consumed a daily supplement for 4 wk in three portions per day in addition to their habitual diet in a single-blind, randomized, balanced, multiple crossover trial using an orthogonal Latin-square design for three treatments. During the first week (run-in period), each subject consumed the control supplement containing glucose. Subsequently, each subject consumed a supplement containing RS₂, RS₃, or glucose; each type of supplement was consumed for 1 wk.

During days 5–7 of weeks 2, 3, and 4, the subjects defecated twice at the Department of Human Nutrition. The fecal samples were weighed and frozen immediately at −20 °C. With each supplement portion, the subjects swallowed 10 radioopaque barium sulfate-impregnated polyethylene rings (TD Medical BV, Eindhoven, Netherlands) (30 rings/d throughout the study) to serve as a marker for feces collection. All stools were X-rayed before sampling to count the polyethylene rings in each frozen stool. On days 6 and 7 of weeks 2, 3, and 4, the subjects collected 24-h urine samples, used for the determination of lithium. Weekly, a 24-h food-consumption recall was obtained from each subject to check whether the amount and composition of the habitual diet had remained constant. Subjects were weighed twice a week while wearing light indoor clothing with empty pockets and no shoes.

Dietary supplements

The daily supplements consisted of a mixture of 144 g skim yogurt, 216 g skim milk, 123–183 g mashed canned fruit (the amount depending on the type of fruit used), and either one of the following carbohydrate preparations (obtained from Cerestar, Vilvoorde, Belgium): 110.4 g glucose (glucose monohydrate, dry wt 91.6%) for the glucose supplement, 58.5 g glucose plus 47.4 g uncooked high-amylase cornstarch [Hylon VII; Cerestar, dry wt 90.3%, 63.3% RS by wt as measured in vitro (31)] for the RS₁ supplement, and 100.2 g retrograded high-amylase cornstarch [extruded and retrograded Hylon VII, dry wt 90.8%, 29.9% RS by wt as measured in vitro (31)] for the RS₃ supplement. These preparations were calculated to provide 30 g RS in the RS₁ and the RS₃ supplements and equal amounts of glucose units in the three supplements. Corrections were made for the different water contents of the carbohydrate preparations and for the water lost during formation of glycosidic bonds. The supplements had identical nutrient compositions except for the type of carbohydrate (Table 1). In vitro analysis (31) confirmed that the control supplement contained mostly digestible carbohydrate and only 4 g RS, whereas the RS₁ and the RS₃ supplements contained 32 g RS (Table 1). Eighty micromoles lithium chloride was added to each supplement portion, and lithium recovery in 24-h urine samples was measured by atomic-absorption spectrophotometry (38) to measure compliance. We did not try to equalize the gross energy content of the supplements because there is no accurate estimate of the amount of energy that RS supplies. At most, the glucose supplement contained 500 KJ (=4% of total energy intake in this group of subjects) more than the RS supplements, assuming that RS supplies no energy at all.
TABLE 1
Composition of the three dietary supplements

<table>
<thead>
<tr>
<th>Dietary supplement</th>
<th>Glucose</th>
<th>RS_1</th>
<th>RS_3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS (g/d)(^2)</td>
<td>4 ± 4.5</td>
<td>32 ± 2.9</td>
<td>32 ± 2.5</td>
</tr>
<tr>
<td>Carbohydrate (g/d)</td>
<td>138</td>
<td>136</td>
<td>136</td>
</tr>
<tr>
<td>Mono- and disaccharides (g/d)</td>
<td>132</td>
<td>90</td>
<td>42</td>
</tr>
<tr>
<td>Digestible starch (g/d)</td>
<td>0</td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>Dietary fiber (g/d)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Fat (g/d)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Energy (MJ/d)(^3)</td>
<td>2.46</td>
<td>1.98</td>
<td>1.95</td>
</tr>
<tr>
<td>Lithium chloride (µmol/d)</td>
<td>240</td>
<td>240</td>
<td>240</td>
</tr>
</tbody>
</table>

\(^1\) Calculated by using a computerized food-composition table (37). RS, resistant starch; RS_1, RS from uncooked high-amylose cornstarch; RS_3, RS from retrograded high-amylose cornstarch.

\(^2\) x ± SD. Measured in vitro according to the procedure of Englyst et al (31). For each type of supplement (two fresh samples and two samples after storage at 4 °C for 3 d).

\(^3\) Does not include the energy that RS provides when it is fermented in the colon.

The supplements were prepared in the kitchen of the Department of Human Nutrition three times a week, and the subjects took them home for consumption. Supplements were stored at 4 °C until consumed. The supplements were consumed in three equal portions of ∼200 g with breakfast, lunch, and dinner, totaling ∼600 g/d.

Food consumption

The subjects were free to eat and drink what they wanted in addition to the supplements, but they were encouraged to maintain their habitual diet as much as possible. Subjects were instructed to minimize consumption of foods presumed to contribute significantly to their RS intake, such as pulses and unripe bananas. A 24-h food-consumption recall was obtained weekly from each subject by one of the three dietitians involved. Each subject was interviewed by the same dietitian throughout the study, and each interview was conducted on a different day of the week. The interviews were spread equally over the days of the week, but the dietary intake on Saturday was never recalled. The methods of interviewing the subjects and coding the foods were standardized. Energy and nutrient intakes were calculated by using a computerized food-consumption table (37).

Diary

The subjects noted the times of consumption of the supplement portions and consumption of foods relatively rich in RS daily in a diary. Furthermore, they were asked to record deviations from their habitual diet or activity pattern, illness, medicine use, and time of defecation each day. They also rated the consistency of their feces from “watery” (rank of 1) to “like pellets” (rank of 8). A mean consistency score was calculated per subject for each supplement period. In addition, the subjects were asked to report the occurrence of flatulence, bloated feelings, or other gastrointestinal discomforts such as belching, nausea, diarrhea, constipation, vomiting, or bellyache. The number of days in a supplementation period that a subject reported flatulence, bloated feelings, or other gastrointestinal discomforts was counted.

Fecal analysis

Feces samples were thawed overnight at 4 °C, pooled per subject per week, and mixed, and pH was determined. To measure SCFAs, a 10% (wt:vol) homogenate of 0.5 g thawed mixed feces in 9 volumes of doubly distilled water was prepared by sonification. The homogenate was mixed with 2.5 µmol of the internal standard 2-ethylbutyric acid and the mixture was centrifuged at 2500 × g for 15 min at 4 °C. Subsequently, the supernate was mixed with 5 volumes of ethanol at 0 °C to precipitate impurities. After centrifugation for 30 s at 8000 × g, 1 mL supernate was mixed with 30 µL of 0.75 mol NaOH/L to convert the SCFAs into sodium salts. After concentration by centrifugation, the sodium salts in the pellet were converted into fatty acids again by the addition of 50 µL of 1 mol phosphoric acid/L and were analyzed by gas chromatography.

A portion of the mixed feces was freeze-dried and ground. Dry matter content was determined by drying to constant weight. Starch and its degradation products (corrected for free glucose) were measured in samples of freeze-dried feces by the procedure of Björck et al (39) with slight modifications.

To obtain fecal water, another portion of the mixed feces was centrifuged at 26000 × g for 90 min at room temperature. The supernate was carefully removed and stored at −20 °C until pH, cytotoxicity, and osmolality were determined, and bile acids were measured. Cytotoxicity was measured as the release of potassium from erythrocytes due to lysis as described by Govers et al (40). The cytotoxicity of fecal water measured with use of this method was significantly correlated with colonic cell proliferation in rats (22). Furthermore, the lytic effects of bile acids, fatty acids, and mixtures of bile acids and fatty acids on erythrocytes and Caco-2 cells agreed well (41). Osmolality was determined by measuring freezing point depression with saline as a standard (Osmomat 030; Gonotec, Berlin). Bile acids were determined as described elsewhere with minor modifications (42, 43). The bile acid derivatives were analyzed on a capillary fused-silica column (length 25 m, internal diameter 0.20 mm) coated with 0.11 µm HP Ultra-1 phase (Hewlett-Packard Co, Palo Alto, CA) by using a Hewlett-Packard gas chromatograph (model 5890 series II) equipped with a liquid sampler (model HP-7673) and a mass selective detector (model HP-5971) operating in electron impact (70 eV) and selected ion mode. One microliter was injected in split mode (split ratio 1:100). Helium gas was applied as the carrier gas with a constant flow rate of 0.7 mL/min. The oven temperature, which was initially 150 °C, was gradually increased to 275 °C and maintained at this temperature for 30 min. The temperature of the injection port was 325 °C and that of the direct mass spectrometer interface was 275 °C. Instrument control and data acquisition were performed with Hewlett-Packard MS-ChemStation version C.03.00 software on a Hewlett-Packard Vectra 486/25T computer. The amount of each bile acid was calculated from area response by using the internal standard method with a five-point multilevel braqueting calibration with pure standards.

Breath hydrogen

Assuming that the amount of hydrogen in breath is directly related to the extent of colonic fermentation in vivo (30), end-expiratory breath samples were obtained to test the hypothesis that RS_3 is more fermentable than RS_1. Unfortunately, because of unforeseen technical problems a considerable number of the breath samples could not be analyzed. Therefore, another experiment was carried out with 15 apparently healthy nonsmoking men with the same characteristics as those in the initial study.
using exactly the same supplements, study design, and inclusion and exclusion criteria as in the first study, but omitting the run-in period. To prevent excess hydrogen from being channeled into methane production, only subjects that were non-methane excreters were enrolled. A subject was classified as a non-methane excreter if on 3 different days before the study started the methane concentration in his breath was < 3 ppm above the concentration in ambient air (30, 44). All subjects completed the study successfully. They reported in their diaries that 98% of the supplements were consumed. The frequency of defecation, the rated consistency of the feces, and the number and severity of gastrointestinal discomforts were very similar to those in the main experiment (data not shown). The body weight of the subjects remained constant throughout the study.

To investigate whether an adaptation to RS as substrate for colonic fermentation took place, end-expiratory breath samples were collected from subjects both on days 2 and 7 of each supplementation period. On each sampling day, one sample was collected between 0730 and 0830, one between 1200 and 1300, one between 1630 and 1730, one between 2000 and 2100, and one the next morning between 0730 and 0830. Breath samples were taken and stored in 60-mL plastic syringes equipped with a cap (Plastipak; Becton Dickinson, Dublin). Immediately after all samples at one time point had been collected, the hydrogen content of all the samples was measured with an electrochemical measurement cell (Exhaled Hydrogen Monitor; Gas Measurements Ltd, Renfrew, Scotland). The measurement cell was calibrated before each run with ambient air and 100 ppm hydrogen-in-air gas (Intermar BV, Breda, Netherlands). The 24-h integrated breath-hydrogen excretion was estimated by calculating geometrically the area under the curve of breath hydrogen content versus time (45).

### Statistical analysis

Differences between group means for each variable were evaluated by repeated-measures analysis of variance (ANOVA) with dietary supplement as the trial factor with the general linear models procedure of SAS (release 6.09; Statistical Analysis Systems Institute Inc, Cary, NC). When the ANOVA indicated a significant effect of a supplement (P < 0.05), Tukey’s Studentized range test was used for pairwise comparison of the group means for each variable as induced by the three supplements. This method encompasses a downward adjustment of the significance limit for multiple testing. The 24-h integrated breath-hydrogen excretion on day 2 of a supplementation period was compared with that on day 7 by using a paired Student’s t test. Differences between means of the groups that can be detected by ANOVA when testing at \( P = 0.05 \) with powers of 80% and 90% are shown in Table 2. The calculations were performed a priori for the variables for which data on variability were available in the literature, and a posteriori based on the variability as found in the present study.

### RESULTS

#### Compliance

According to the diaries, subjects did not consume 0.4% of the glucose supplements, 1.4% of the RS2 supplements, and 1.9% of the RS3 supplements. Mean (± SEM) urinary lithium recovery was 96 ± 3% during glucose supplementation, 100 ± 3% during RS2 supplementation, and 92 ± 2% during RS3 supplementation.

#### Food consumption and body weight

No significant differences were found in reported energy and nutrient intakes among the three various supplements (Table 3). Body weight remained constant throughout the study (\( P = 0.06, \) ANOVA).

#### Breath hydrogen

Supplementation with RS2 and RS3 led to significantly more hydrogen excretion in breath than did supplementation with glucose both on day 2 (\( P < 0.01 \)) and day 7 (\( P < 0.05 \); Table 4). Breath-hydrogen excretion during RS2 and RS3 supplementation was similar. For each supplementation, hydrogen excretion was similar on days 2 and 7.

#### Frequency and consistency of feces

More defections (\( \bar{x} \) ± SEM: 10.2 ± 0.7 stools/wk) were reported during supplementation with RS3 than during supplementation with glucose (8.9 ± 0.5 stools/wk; \( P < 0.05 \)). The number of stools during RS2 supplementation (9.6 ± 0.5 stools/wk) did not differ significantly from the number during either glucose or RS3 supplementation. The mean rated consistency of the feces (scored on a scale from 1 to 8) did not differ during glucose (5.6 ± 0.2), RS2 (5.4 ± 0.2), and RS3 (5.5 ± 0.2) supplementation. This finding is consistent with the lack of differences in percentage dry weight of the feces (see below).

#### Fecal output

Stool weight was higher during RS supplementation than during glucose supplementation (Table 5). If the data of two subjects with high stool weights are omitted, mean (± SEM) stool weight during RS3 supplementation changes from 301 ± 29 to 267 ± 15 g/d, which is similar to the stool weight after RS2 supplementation (277 ± 20 g/d). Fecal dry weight and pH did not differ significantly between the three supplementation periods (Table 5). After RS supplementation, more stoch was found in feces than after glucose supplementation, the highest amount being 18% of the amount supplemented (RS3 in this case). The pH, cytotoxicity, and osmolality of fecal water were not different during the three supplementation periods (Table 5).

#### SCFAs in feces

Total SCFA concentration in feces did not differ significantly during the three supplementation periods (Table 6). The molar ratio of acetate:propionate:butyrate (the main SCFAs) was 4:1:1 during the three supplementation periods. The sum of isobutyric, valeric, isovaleric, and caproic acids was only 7.9 ± 0.6% (\( \bar{x} \) ± SEM) of the total amount of fecal SCFAs during glucose supplementation and 7.5 ± 0.6% during RS2 and RS3 supplementation (NS). Isovalerate comprised a significantly lower (\( P < 0.05 \)) percentage of total SCFAs during RS3 supplementation than during glucose supplementation and caproate comprised a significantly higher (\( P < 0.05 \)) percentage. Daily total SCFA excretion in feces did not differ significantly during the three supplementation periods.

#### Bile acids in fecal water

The concentration of total, primary (sum of cholic and chenodeoxycholic acids), secondary (sum of deoxycholic, isodeoxy-
TABLE 2
Differences between group means that can be detected by ANOVA (Tukey’s Studentized range test) when testing at \( P = 0.05 \) with powers of 80% and 90% for variables for which data on variability were available in the literature

<table>
<thead>
<tr>
<th>Variable</th>
<th>SD(^1)</th>
<th>Detectable differences between means</th>
<th>Power 80%</th>
<th>Power 90%</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A priori ((n=24))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short-chain fatty acids in feces ((\text{mmol/d}))</td>
<td>5.2</td>
<td>3.6</td>
<td>4.1</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>(\text{(\mu\text{mol/g})})</td>
<td>30</td>
<td>21</td>
<td>23</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>pH of fecal water</td>
<td>0.36</td>
<td>0.25</td>
<td>0.28</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Bile acids in fecal water ((\text{(\mu\text{mol/L})})</td>
<td>132</td>
<td>91</td>
<td>103</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Cytotoxicity of fecal water (%)(^1)</td>
<td>32</td>
<td>22</td>
<td>25</td>
<td>6, 17</td>
<td></td>
</tr>
<tr>
<td>A posteriori ((n=23))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short-chain fatty acids in feces ((\text{mmol/d}))</td>
<td>19.9</td>
<td>13.9</td>
<td>15.9</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>(\text{(\mu\text{mol/g})})</td>
<td>30</td>
<td>21</td>
<td>24</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>pH of fecal water</td>
<td>0.48</td>
<td>0.34</td>
<td>0.38</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Bile acids in fecal water ((\text{(\mu\text{mol/L})})</td>
<td>618</td>
<td>433</td>
<td>492</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Cytotoxicity of fecal water (%)(^1)</td>
<td>40</td>
<td>28</td>
<td>32</td>
<td>6, 17</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) For the a priori power calculations, an estimate for SD was derived from the SEM of the difference for the variables short-chain fatty acids, pH, and bile acids, and from the between subject SEM for the variable ‘cytotoxicity’ as reported in the literature. For the a posteriori power calculations, an estimate for SD was derived from the square root of the error mean square from the repeated-measures ANOVA of the present study, times \(v^2\).

\(^2\) SM de Boer and MCJF Jansen, unpublished observations, 1992.

\(^3\) Measured as release of potassium from erythrocytes as a result of lysis (40).

TABLE 3
Energy and nutrient intakes during supplementation of the habitual diet with 32 g/d of glucose, RS\(_2\), or RS\(_3\), for 1 wk.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Glucose(^1)</th>
<th>RS(_2)</th>
<th>RS(_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (MJ/d)</td>
<td>13.0 ± 1.0</td>
<td>13.6 ± 1.0</td>
<td>13.3 ± 0.6</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>97 ± 7</td>
<td>105 ± 9</td>
<td>106 ± 5</td>
</tr>
<tr>
<td>Fat (g/d)</td>
<td>126 ± 12</td>
<td>123 ± 11</td>
<td>132 ± 8</td>
</tr>
<tr>
<td>Carbohydrate (g/d)</td>
<td>355 ± 26</td>
<td>366 ± 28</td>
<td>357 ± 18</td>
</tr>
<tr>
<td>Alcohol (g/d)</td>
<td>22 ± 7</td>
<td>36 ± 11</td>
<td>18 ± 7</td>
</tr>
<tr>
<td>Dietary fiber (g/d)</td>
<td>39 ± 3</td>
<td>34 ± 3</td>
<td>34 ± 2</td>
</tr>
</tbody>
</table>

\(^1\) \(\overline{X} \pm \text{SEM}; n = 23\). Supplements were not included in the calculations. Calculated by using a computerized food-composition table (37). There were no significant differences by ANOVA. Amounts of resistant starch were measured in vitro (31). RS\(_2\), RS from uncooked high-amylose cornstarch; RS\(_3\), RS from retrograded high-amylose cornstarch.

\(^2\) One subject reported having consumed two supplement portions only on the day of the 24-h food-consumption recall; his data were excluded from the analysis.

Gastrointestinal discomfort

Ninety-one percent of the subjects during RS\(_3\) supplementation and 82% of the subjects during RS\(_2\) supplementation reported flatulence, compared with 55% of the subjects during glucose supplementation. Of those mentioning flatulence, half reported flatulence on ≥4 d/wk during RS\(_2\) and RS\(_3\) supplementation, and only 18% did so during glucose supplementation. Bloating feelings were reported by 41% of the subjects during RS\(_3\) supplementation, by 28% of the subjects during RS\(_2\) supplementation, and by 9% of the subjects during glucose supplementation. Subjects reported bloated feelings for only 1 d/wk during glucose and RS\(_2\) supplementation, whereas 33% of the subjects who suffered from bloated feelings reported them ≥4 d/wk during RS\(_3\) supplementation. Fewer gastrointestinal discomforts were reported: on only 1 d/wk by 5% of the subjects during glucose supplementation, by 18% of the subjects during RS\(_2\) supplementation, and by 14% of the subjects during RS\(_3\) supplementation. Severe side effects were not reported.

Awareness of the nature of the supplements

The subjects were not informed about the sequence in which they would receive their supplements until they completed the experiment. At the end of the study the participants were asked to indicate the supplement sequence as perceived. The sequence was correctly perceived by 2 of the subjects (8%); 10 subjects (42%) were able to discern the glucose from the RS supplements but could not discern between the RS\(_2\) and RS\(_3\) supplements. The other 12 subjects (50%) incorrectly perceived the sequence of their supplements.

DISCUSSION

This study showed that in healthy men, 1 wk of supplementation with 32 g/d RS\(_2\) or RS\(_3\) compared with glucose increased hydrogen excretion in breath, fecal starch excretion, and stool...
mass. Supplementation had no effect on fecal pH and SCFA concentrations, nor on bile acid concentrations and cytotoxicity of fecal water. No differences in results were found between RS 2 and RS 3. Other studies have shown that a dietary change for 1 wk is sufficiently long to detect changes in fecal pH (40), fecal bile acids (20, 23, 40), cytotoxicity of fecal water (20, 40), and mucosal proliferation (23). In our study, both reported compliance and compliance as assessed by urinary lithium recovery were > 90%. Lithium recovery during RS3 supplementation was slightly lower than during glucose and RS 2 supplementation, possibly because most subjects considered the RS 3 supplement to be the least palatable (47). Despite differences in palatability, only half of the subjects were able to discern the glucose from the RS supplements. It is unlikely that awareness of the nature of the supplements or the small difference in lithium recovery could have affected the outcome of the study with regard to fecal composition and breath-hydrogen excretion. As in other studies with comparable supplements (6, 37, 48), no changes were observed in the composition of the background diet during the study. Therefore, it may be assumed that differences in the outcome variables are caused by consumption of the supplements. The RS content of the supplements was analyzed according to the procedure of Englyst et al (31). Because there was no source of added starch in the glucose supplements, the 4 g RS/d measured in these supplements must have been derived from other ingredients in the supplements. The supplementation dose of 32 g RS/d is estimated to be about six times the current average intake of RS in the Netherlands (49). This dose was tolerated well because reports of flatulence and bloated feelings were few. The mean dietary fiber intake of this group of volunteers was relatively high (\(<35\) g/d), and consequently, the mean fecal output was relatively high (232 g/d during glucose supplementation). Subjects unaccustomed to such a high amount of fermentable material in their colon may react differently to a dose of 32 g RS/d. Therefore, the conclusions of this study are limited to people with a high dietary fiber intake.

**TABLE 4**

Area under the 24-h curve of hydrogen concentration in end-expiratory breath versus time during supplementation of the habitual diet with 32 g/d of glucose, RS 2, or RS 3 for 1 wk

<table>
<thead>
<tr>
<th>Dietary supplement</th>
<th>Area on day 2 (ppm-h)</th>
<th>Area on day 7 (ppm-h)</th>
<th>Area, mean of day 2 and 7 (ppm-h)</th>
</tr>
</thead>
</table>
| Glucose  
2 | 420 ± 39 | 454 ± 58 | 432 ± 43 |
| RS 2 | 683 ± 73  
3 | 634 ± 64  
3 | 658 ± 61  
3 | 660 ± 71  
3 | 656 ± 67  
3 | 662 ± 64  
3 |

\footnote{\(\bar{x} \pm \text{SEM}; n = 15\). This was a separate study as described in Methods. Amounts of resistant starch (RS) were measured in vitro (31). RS 2, RS from uncooked high-amylose cornstarch; RS 3, RS from retrograded high-amylose cornstarch.}

\footnote{\(n = 14\) because of missing values.}

\footnote{Significantly different from glucose, \(P < 0.05\).}

**TABLE 5**

Fecal indexes after supplementation of the habitual diet with 32 g/d of glucose, RS 2, or RS 3 for 1 wk

| Dietary supplement | Glucose  
2 | RS 2  
3 | RS 3  
4 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed wet feces</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Wet weight (g/d)   | 232 ± 19  
3 | 277 ± 20 | 301 ± 29  
4 |
| Dry weight (g/d)   | 55 ± 3     | 66 ± 5     | 66 ± 4     |
| (% )               | 24.9 ± 0.9  | 24.3 ± 0.7  | 23.2 ± 0.9  |
| Starch (g/d)       | 1.1 ± 0.4  
5 | 4.5 ± 1.7  | 5.6 ± 2.0  
5 |
| pH                 | 6.8 ± 0.1  | 6.7 ± 0.1  | 6.6 ± 0.1  |
| Fecal water pH     | 6.7 ± 0.1  | 6.6 ± 0.1  | 6.5 ± 0.2  |
| Cytotoxicity (\%)  | 53 ± 8     | 59 ± 9     | 49 ± 9     |
| Osmolality (mosmol/kg) | 553 ± 24 | 528 ± 17 | 528 ± 19 |

\footnote{\(\bar{x} \pm \text{SEM}; n = 23\). Amounts of resistant starch (RS) were measured in vitro (31). RS 2, RS from uncooked high-amylose cornstarch; RS 3, RS from retrograded high-amylose cornstarch.}

\footnote{\(n = 22\) because of missing values.}

\footnote{Significantly different from RS 3, \(P < 0.05\).}

\footnote{Significantly different from glucose, \(P < 0.05\).}

\footnote{Measured as starch and its degradation products (corrected for free glucose) according to the procedure of Björck et al (39).}

\footnote{Measured as release of potassium from erythrocytes as a result of lysis (40).}
As found by others (2, 6, 48), breath-hydrogen excretion increased during consumption of RS. On the basis of this semiquantitative measure (30), there did not seem to be a difference in fermentability between RS2 and RS3. However, the existence of a difference between RS2 and RS3 in the rate of fermentation or location of fermentation in the colon cannot be excluded on the basis of the results of this study (50). The latter may have important consequences for colon cancer risk (11). We may not have been able to measure the optimal fermentability of RS if hydrogen excretion plateaued, for example, because the amount of bacterial enzymes was limiting. Our results seem to disagree with in vitro data (3), a study in rats (35), and two studies in humans (51; M Champ unpublished observations, 1994) that suggest that RS2 is more easily or more quickly fermentable than RS3, or both. However, the in vitro and human studies are difficult to interpret because not only the type of RS but also the amount of RS differed. Furthermore, in vitro studies may show inconsistent results depending on the inocula used: some subjects fermented one kind of RS well and another kind poorly, implying that different intestinal flora ferment various RS sources differently (32). In the study in rats (35), the RS dose provided per kg metabolic body weight was about five times larger than the maximum dose that is tolerated well in humans. Thus, the specific type and amount of RS and factors relating to the subject must be determined whether or not and to what extent the amount of hydrogen in breath increases after consumption of RS.

During consumption of the glucose supplements, there was also a significant hydrogen excretion in breath, probably because the background diet provided a considerable amount of dietary fiber. Compared with glucose, RS supplementation increased stool weight by 1.4 g/g RS2 and by 2.2 g/g RS3. Because others reported similar results (6, 7, 32), it can be concluded that RS2 and RS3 have a mild laxative effect. This may be positive for human health because an inverse relation has been reported between stool weight and colon cancer incidence (52). Burkitt (53) proposed a protective mechanism by dilution of the intestinal contents and reduction of intestinal transit time, thus reducing the contact of carcinogens with the colonic mucosa. Only 7% of the increase in stool mass cannot be explained by the increase of RS in the feces. The increase in fecal mass cannot be explained by an increase in water content because, in agreement with others (7, 32, 54), no differences in percentage dry matter of the feces were found. Therefore, stool weight was most likely increased mainly by an increase in bacterial mass, which is supported by the reported increase in fecal nitrogen excretion after RS consumption (32, 55). Further support is provided by a study...

### Table 6

<table>
<thead>
<tr>
<th>Dietary supplement</th>
<th>Glucose (mmol/d)</th>
<th>RS2 (mmol/d)</th>
<th>RS3 (mmol/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>25.4 ± 2.8</td>
<td>33.0 ± 2.9</td>
<td>34.5 ± 4.3</td>
</tr>
<tr>
<td>Acetate (%) of total</td>
<td>59.4 ± 0.9</td>
<td>59.8 ± 1.4</td>
<td>59.5 ± 1.7</td>
</tr>
<tr>
<td>Propionate (%) of total</td>
<td>16.2 ± 0.7</td>
<td>15.1 ± 0.6</td>
<td>15.4 ± 1.3</td>
</tr>
<tr>
<td>Buturate (%) of total</td>
<td>16.5 ± 0.5</td>
<td>17.7 ± 1.2</td>
<td>17.6 ± 0.8</td>
</tr>
<tr>
<td>Valurate (%) of total</td>
<td>1.7 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Isobutyrate (%) of total</td>
<td>2.4 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Butyrate (%) of total</td>
<td>2.6 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>Caproate (%) of total</td>
<td>2.3 ± 0.4</td>
<td>2.3 ± 0.2</td>
<td>2.1 ± 0.3</td>
</tr>
</tbody>
</table>

1. x ± SEM; n = 23. Amounts of resistant starch (RS) were measured in vitro (31). RS2, RS from uncooked high-amylose cornstarch; RS3, RS from retrograded high-amylose cornstarch.
2. n = 22 because of missing values.
3. n = 21 because of missing values.
4. Significantly different from glucose, P < 0.05.
5. Significantly different from RS3, P < 0.05.

### Table 7

<table>
<thead>
<tr>
<th>Dietary supplement</th>
<th>Glucose (mmol/L)</th>
<th>RS2 (mmol/L)</th>
<th>RS3 (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bile acids</td>
<td>384 ± 61</td>
<td>485 ± 131</td>
<td>480 ± 189</td>
</tr>
<tr>
<td>Total primary bile acids</td>
<td>34 ± 14</td>
<td>47 ± 8</td>
<td>40 ± 12</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>24 ± 11</td>
<td>31 ± 6</td>
<td>28 ± 9</td>
</tr>
<tr>
<td>Cheno deoxycholic acid</td>
<td>11 ± 3</td>
<td>16 ± 2</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>Total secondary bile acids</td>
<td>350 ± 62</td>
<td>439 ± 131</td>
<td>441 ± 181</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>196 ± 34</td>
<td>226 ± 60</td>
<td>224 ± 93</td>
</tr>
<tr>
<td>Isodeoxycholic acid</td>
<td>65 ± 12</td>
<td>54 ± 12</td>
<td>60 ± 17</td>
</tr>
<tr>
<td>Lithocholic acid</td>
<td>47 ± 13</td>
<td>101 ± 38</td>
<td>92 ± 41</td>
</tr>
<tr>
<td>Isolithocholic acid</td>
<td>19 ± 8</td>
<td>37 ± 19</td>
<td>42 ± 27</td>
</tr>
<tr>
<td>12-ketolithocholic acid</td>
<td>15 ± 3</td>
<td>11 ± 3</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>12-keto-isolithocholic acid</td>
<td>5 ± 2</td>
<td>6 ± 2</td>
<td>6 ± 4</td>
</tr>
<tr>
<td>Ursodeoxycholic acid</td>
<td>2 ± 0.5</td>
<td>3 ± 0.6</td>
<td>3 ± 0.8</td>
</tr>
<tr>
<td>7-ketodeoxycholic acid</td>
<td>1 ± 0.3</td>
<td>2 ± 0.3</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>Secondary bile acids (% of total)</td>
<td>89 ± 3</td>
<td>84 ± 3</td>
<td>87 ± 3</td>
</tr>
</tbody>
</table>

1. x ± SEM; n = 23. Amounts of resistant starch (RS) were measured in vitro (31). RS2, RS from uncooked high-amylose cornstarch; RS3, RS from retrograded high-amylose cornstarch.
2. n = 22 because of missing values.
3. n = 21 because of missing values.
in which starch malabsorption was induced by the α-amylase inhibitor acarbose, which caused an increase in fecal bacterial mass and nitrogen excretion as well as in bacterial nitrogen and diaminopimelic acid in feces (54).

Bacterial mass and colonic fermentation may increase as a result of the availability of more substrate (RS) in the colon. This was confirmed by the increase in breath-hydrogen excretion and the amount of starch in the feces, representing only 15–18% of the RS supplemented. Others reported an overall RS digestibility of 80–90% (7, 32), and one study found almost 100% digestibility (6). No significant differences between RS2 and RS3 were found with respect to fecal starch excretion, a finding that largely agrees with the findings of Cummings et al (32). The likely increase in colonic fermentation was not reflected by a drop in fecal pH, which agrees with some studies (6) but not with others (7, 56). The pH of the colonic contents has been shown to rise gradually during the passage from the cecum to the sigmoid colon (50, 57, 58), which is probably due to the rapid absorption of SCFAs by the colonic epithelium (50, 57, 59). Thus, fecal pH is not necessarily a good indicator of fermentation and acidity in the proximal colon, which may explain the absence of a change in fecal pH.

This rapid absorption of SCFAs from the colon may also be the explanation for the lack of differences in fecal SCFA concentrations among the supplementation periods in the present study, and others (6, 7, 32). In contrast, starch malabsorption induced by acarbose resulted in increased fecal SCFA excretion (5). The total amount of SCFA excreted tended to increase after RS supplementation both in this study and the one by Cummings et al (32) and was significantly increased after RS consumption in two other studies (6, 7). This was probably related to the higher stool mass after RS consumption. Of the total amount of SCFAs, 0.60 was acetate, 0.15 was butyrate, and 0.17 was propionate. These proportions are in the same range as reported before (59) and did not differ among the three supplementation periods. The present study did not show a clear increase in fecal butyrate excretion after RS consumption, whereas some others did (5–7, 56). Cummings et al (32) found a significant higher molar ratio of butyrate to acetate and propionate, but only after consumption of RS3 from potatoes. Failure to measure an increase in fecal butyrate, however, does not exclude the possibility that in vivo fermentation of RS specifically increases butyrate production. Obviously, it is very difficult to measure SCFA production in the human colonic contents in vivo.

In the present study, bile acid concentrations in fecal water did not differ between RS and glucose supplementation, nor between RS2 and RS3. This agrees with the lack of a difference in cytotoxicity of fecal water. However, the fecal sampling method used may not have been optimal (60) and the within-subject variability was large (Table 2), which may explain why the tendency of RS2 and RS3 to increase the concentration of total and secondary bile acids in fecal water was not significant. In contrast, van Munster et al (6) reported a decrease in total bile acid concentration in fecal water, mainly due to a decrease in deoxycholic acid concentration after consumption of 28 g/d RS3 for 2 wk. In that study, the primary bile acid concentration in freeze-dried feces rose significantly, whereas the secondary bile acid concentration tended to decrease. Concomitantly, cytotoxicity of fecal water and mucosal proliferation in rectal biopsies decreased significantly. Unfortunately, however, there was no control group in that study. Consumption of 17–25 g RS/d for 4 wk tended to lower the concentration of deoxycholic acid in fecal water in hypertriglyceridemic subjects (56). Starch malabsorption as induced by acarbose significantly decreased the secondary bile acid concentration and excretion in feces, and significantly increased the primary bile acid concentration and excretion in feces (61).

In conclusion, the present findings do not support the initial hypothesis that 1 wk supplementation of a habitual high-fiber diet with 32 g/d of either RS2 or RS3 from high-amylose corn-starch compared with an equivalent amount of glucose positively affects putative risk factors for colon cancer in healthy men. No differences were found between RS2 and RS3. Neither does this study support a difference in fermentability between RS2 and RS3 as evaluated by breath-hydrogen excretion. No information could be obtained about the colonic location of fermentation of RS2 and RS3 nor about the magnitude of butyrate production in situ. Both factors may be important regarding colon cancer risk. In addition, because consumption of RS2 and RS3 increased stool mass, a protective but perhaps limited effect of long-term RS consumption toward colon cancer is still feasible.

We are indebted to the volunteers for their cooperation. We thank Edith Hobbel, Nicole de Roos, Gera Woestenek, Emmy Wouters, and Liesbeth Zandstra for their help in conducting the experiment; Meijke Booj and Jan Harryvan (Department of Human Nutrition, Wageningen Agricultural University), Yvonne Gielen (Unilever Research Laboratory, Vlaardingen), Denise Tertmond (Netherlands Institute for Dairy Research, Ede), and Inez Lemmens (Department of Laboratory Animal Science, Utrecht University) for laboratory analyses; Saskia Meyboom (Department of Human Nutrition) for dietary advice and interviewing; and Jan Burema (Department of Human Nutrition) for statistical advice.

REFERENCES