Dietary Guar Gum Alters Colonic Microbial Fermentation in Azoxymethane-Treated Rats[1,2,3]

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ABSTRACT To assess the effects of guar gum on colonic microbial fermentation and cancer development, azoxymethane-treated rats were fed a partially hydrolyzed guar or control diet. Anaerobic fecal incubations were conducted at 8-wk intervals, either without added substrate or with cornstarch or hydrolyzed guar as substrates. Short-chain fatty acids in colonic contents and colonic carcinoma areas were measured at 27 wk. Fecal in vitro fermentation rates were higher for guar-fed rats than for control rats [three-way ANOVA (diet, time, in vitro substrates), $P = 0.002$]. Fecal in vitro butyrate production was greater for guar-fed rats than for control rats after 3–11 weeks of diet treatment (three-way ANOVA, $P = 0.027$). Butyrate concentrations of colonic contents at 27 wk were higher in guar-fed than in control rats and higher in the cecum than in the post-cecal colon (two-way ANOVA, $P = 0.0001$). A regression equation predicting colonic carcinoma area ($r^2 = 0.279$) using propionate and butyrate concentrations of the contents of the post-cecal colon showed propionate as a positive predictor ($P < 0.001$) and butyrate as a negative predictor ($P = 0.033$). Our results show that patterns of short-chain fatty acid production may affect the results of fiber-carcinogenesis experiments. Dietary addition of hydrolyzed guar is associated with fecal fermentation low in propionate and high in butyrate; short-chain fatty acid concentrations are greater proximally than distally. These results suggest that butyrate protects against colonic neoplasia, whereas propionate enhances it, and demonstrate that colonic microbiota adapt to produce more butyrate if given time and the proper substrate. J. Nutr. 126: 1979–1991, 1996.

INDEXING KEY WORDS:

• *rats* • *guar gum* • *colonic neoplasms*
• *butyric acid* • *fermentation*

Many rodent-carcinogen experiments have sought preventive effects of dietary fibers and other agents. Until recently (Bianchini et al. 1992, Caderni et al. 1994, McIntyre et al. 1993, Sakamoto et al. 1996), these studies did not consider the effect of the agent being studied on the products of colonic microbial fermentation, or its effects on the microbial community and the fermentation process. The principal products of anaerobic fermentation are the short-chain fatty acids (SCFA),[5] acetic, propionic and butyric acids, and the gases hydrogen and carbon dioxide. The proportions of the SCFA produced from in vitro fermentation vary with the type of carbohydrate substrate present; for example, more butyrate is produced from cornstarch (CS) than from cabbage cellulose (Weaver et al. 1992).

Dietary carbohydrate has been shown to change the concentrations or proportions of SCFA in the colon in animal studies. The ratio of acetate to propionate decreased in the large intestine of pigs fed a high starch diet (Argenzio and Southworth 1974, Imoto and Namioka 1978). Goodlad and Mathers (1990) showed a progressive increase in the percentage of cecal butyric acid in rats fed increasing proportions of raw peas. A wheat-

[1] Presented at the Scientific Session of the American Gastroenterological Association meeting, May 16, 1994, New Orleans, LA [Weaver, G., Tangel, C., Krause, J., Alpern, H., Jenkins, J., Stragand, J., & Parfitt, M. (1994) Colonic fermentation products in azoxymethane-treated rats fed a fiber-free diet or a 5% partially hydrolyzed guar gum diet. Gastroenterology 106: A459 (abs.)].

[2] Supported by Sandoz Nutrition Corporation, Minneapolis, MN 55440.

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[5] Abbreviations used: AOM, azoxymethane; CS, cornstarch; FF, fiber-free; PHGG, partially hydrolyzed guar gum; SCFA, short-chain fatty acids (acetic, propionic and butyric).

0022-3166/96 $3.00
Manuscript received 29 February 1996. Initial review completed 1 April 1996. Revision accepted 30 April 1996.

bran diet has been shown to increase butyrate in distal-colonic contents in rats in comparison with other fiber diets (McIntyre et al. 1991). In another study, rats fed a starch diet had lower ratios of acetate:butyrate in cecal contents and feces than did rats fed a sucrose diet (Bianchini et al. 1992). Because changes in SCFA production occur with introduction of different carbohydrates into a fermentation system, it is reasonable to consider whether SCFA could be the primary effectors of dietary fiber on colon cancer development.

Of the principal products of fermentation, butyrate has received particular attention. Butyrate is partly responsible for maturation or differentiation of the rumen mucosa (Sakata and Tamate 1978, Sander et al. 1959, Van Soest 1982) and is a preferred colonocyte energy source (Roediger 1980). Colon cancer cell culture studies have shown that butyrate has a differentiating effect on malignant cells (Kruh 1982). These effects include the induction of proteins and peptide hormones, reversal of the morphology of transformed cells, formation of a normal cytoskeleton, and arrest of cell growth in G1 of the cell cycle (Kruh 1982). Comparison of SCFA in colonic contents of subjects with normal colons and those with polyps or colon cancer showed that the percentage of butyric acid was significantly higher in those with normal colons (Kashtan et al. 1992, Weaver et al. 1988). Fecal fermentation studies also support increased butyrate production in normal subjects compared with those with polyps (Clausen et al. 1991).

Preliminary studies in our laboratory suggested that partially hydrolyzed guar gum (PHGG) fed to rats short term had a propensity to increase butyrate production in colonic contents. Because butyrate has the potential to suppress neoplasia, this study was conducted with the following aims: *1*) to determine whether PHGG could decrease the occurrence of colon cancers in carcinogen-treated rats; *2*) to determine whether butyric acid concentrations correlate negatively with colonic neoplasia area; *3*) to determine in vitro fermentation differences between rats consuming a PHGG-supplemented diet and rats consuming a fiber-free (FF) control diet.

METHODS

Animals. The study was approved by the Institutional Animal Care and Use Committee of the Mary Imogene Bassett Hospital June 9, 1992. This committee followed the guidelines of the *Guide for the Care and Use of Laboratory Animals* (NRC 1985).

Sixty female Fisher F344 rats were entered into the azoxymethane (AOM) protocol outlined below. Four-week-old rats were obtained from Harlan Sprague Dawley, Indianapolis, IN, in biweekly shipments of 12 with half of each shipment placed on each diet.

TABLE 1

***Diet composition*[1]**

Ingredient	FF diet	PHGG diet
	g/kg diet	
Casein	200	200
DL-Methionine	3	3
AIN vitamin mix 76A	10	10
Choline bitartrate	2	2
Cornstarch	220	220
AIN mineral mix 76	35	35
Dextrose monohydrate	240	215
Sucrose	240	215
PHGG	—	50
Corn oil	50	50

[1] Abbreviations used: FF, fiber-free; PHGG, partially hydrolyzed guar gum.

Rats were given unique study numbers identified by ear-notch markings and housed in pairs in a Thoren Maxi-Miser Caging System (Thoren Caging Systems, Hazleton, PA). Wire-mesh floor inserts were placed in the cages and bedding removed the day before fecal collections. Rats were weighed initially, at the time of AOM injections and then every 8 wk. Thirty rats were given free access to the pelleted 5 g/100 g PHGG diet and 30 rats were given free access to the FF diet. Except for fecal collection periods, the rats were in contact with wood chip bedding so that it is likely that they consumed some wood fiber. Sugar maple (*Acer saccharum*) wood chips, Sani-Chips®, (P.G. Murphy Forest Products, Montville, NJ) were used for bedding.

Diets. The FF and 5 g/100 g PHGG pelleted diets were made by Bioserve, Frenchtown, NJ. The composition of the diets is shown in **Table 1.** PHGG (Benefiber®) was supplied by Sandoz Nutrition, Minneapolis, MN. PHGG may be prepared by treating guar gum with galactomannase of *Aspergillus niger* origin (Ide et al. 1991).

Carcinogen protocol. The protocol for azoxymethane administration was based on the methods of Watanabe et al. (1979). The 4-wk-old rats were fed their respective diets for 3 wk before azoxymethane was given at a dose of 8 mg/kg in 9 g/L saline subcutaneously each week for 10 wk. The rats were killed by asphyxiation in a carbon-dioxide chamber 24 wk after the first does of azoxymethane.

Fecal collection and pooling. Feces from three cages (6 rats) were pooled for SCFA determinations at 8-wk intervals. The fecal pools were always from the same animals. If animal deaths occurred, some fecal pools contained feces from fewer than six rats. The feces collected at each fermentation period for each fermentation group were added to 45 mL of anaerobic dilution solution (Bryant and Burkey 1953). Aliquots of these suspensions were used for the in vitro fermentations and dry matter determinations. Separate suspensions of cecal contents and post-cecal colon (all of the colon

excluding the cecum) contents were made from each rat at the completion of the study for individual SCFA and dry matter determinations. Aliquots for dry weight determination were dried to a constant weight (Weaver et al. 1986).

Fermentations. Fermentations of rat feces were conducted at the initiation of AOM injections (3-wk fermentation period) and then at 8-wk intervals. Fermentations included: *1*) fermentation without added substrate (endogenous) for 0, 6, and 24 h, *2*) fermentation with PHGG (100 mg) for 6 and 24 h, *3*) fermentation with CS (100 mg) for 6 and 24 h. Fermentations were conducted in sealed 20-mL serum bottles as previously described (Weaver et al. 1992). In brief, PHGG or CS was added as dry powder to substrate fermentation bottles followed by 5 mL of fecal suspension and 4.8 mL of phosphate and bicarbonate-carbon dioxide–buffered basal medium (pH 7.0) under a stream of 80% N_2: 20% CO_2. The bottles were stoppered anaerobically and 0.2 mL of cysteine · HCl + $Na_2S \cdot 9H_2O$ solution was added (Weaver et al. 1989). The bottles were incubated at 37°C for 6 or 24 h on a shaker. Reactions were terminated by boiling. Boiled samples without incubation were used to determine base-line SCFA concentrations of fermentations.

Analyses. Gas liquid chromatography (GLC) was used to determine SCFA (principal SCFA: acetic, propionic, butyric; minor SCFA: isobutyric, isovaleric, valeric; total SCFA refers to principal plus minor fatty acids) amounts on sulfuric acid–acidified supernatants after centrifugation by a modification of our previously described method (Weaver et al. 1989). The GLC method used a Nukol fused-silica capillary column (15 m × 0.53 mm i.d. with a 0.5-μm film) (Supleco, Supelco Park, Bellefonte, PA). Injector and detector temperatures were 145 and 175°C, respectively. The initial oven temperature was 100°C and was increased by 10°C/min and then held at 130°C for 1 min. The carrier gas was helium with a flow of 8.7 mL/min. Sample injection volume was 1 μL. Chromatograms were integrated with a Perkin Elmer/Omega system (Norwalk, CT).

Headspace gas volume calculations were based on headspace pressure, fixed headspace volume, temperature and atmospheric pressure. Hydrogen was determined as previously described (Weaver et al. 1986) using a Gow-Mac Series 55OP thermal conductivity gas chromatograph (Bound Brook, NJ) with a 1.8 m × 1 cm stainless steel column packed with 60/80 mesh silica gel (Alltech Associates, Deerfield, IL). The carrier gas was argon at a flow rate of 20 mL/min. Injector and column temperatures were 80°C, detector temperature was 30°C and bridge current was 90 mA. A 2-mL sample was used. Methane was not present in the headspace gas.

Fermentation product and rate units. The data for 24-h fermentations with 100 mg of added substrate are expressed as micromoles of product produced in 24 h. The 24-h fermentations are sufficiently long so that most added substrate is utilized. Consequently, moderate differences in amounts of dry matter between fermentations have little effect on product amounts or proportions. Product amounts from 24-h fermentations without added substrate can be affected by differences in amounts of dry matter but have been reported as micromoles of product produced in 24 h for ease of comparison with 24-h fermentations with added substrate. Parallel fermentations with and without added substrate for a specific fermentation group contained equal wet and dry weight amounts of feces.

The products from 6-h cumulative-rate fermentations have been expressed on a μmol/g dry matter basis because substrate excess is present at 6 h, so that differences in dry matter in the fermentations lead to differences in rate; therefore the values are normalized by dry weight.

Cancer assessment. Rats were killed in a carbon-dioxide chamber and then inspected for external cancers. The abdomen and thorax were opened and inspected. The colon was removed, opened longitudinally, emptied of fecal material and washed with tap water. The colon was oriented on a styrofoam wafer, photographed and copied on a photocopy machine with enlargement. Gross tumors were noted on the photocopy. The colon was then suspended in 10% neutral-buffered formalin for 2 h, removed, divided longitudinally, cut into short segments, placed into embedding cassettes and then returned to the formalin. Each colon section was indexed on the colon photocopy. Sections (3–4 μm) were cut from paraffin-embedded tissues, transferred to microslides, dried, and deparaffinized in a series of xylene and alcohol graded washes. Sections from each block known to contain tumor were serially cut and stained with eosin/hematoxylin stain until each tumor was histologically proven.

Rat colon tumors were classified by the system utilized by Watanabe et al. (1979) and Tatsuta et al. (1992) as follows:

1) Adenoma: benign neoplastic epithelial tumors with mild or moderate epithelial atypia if indicated.
2) Carcinoma in situ: tumors with severe epithelial atypia confined to the mucosa without invasion through the muscularis mucosa.
3) Adenocarcinoma: frankly malignant tumors showing definite invasion through muscularis mucosa.

Tumor areas were determined both by digitizing tumor areas on photographs of the colons and by digitizing the same areas on photocopies (Sigma Scan digitizing system, Jandel Scientific Software, San Rafael, CA). The two areas were averaged to determine tumor size. Only tumors determined histologically to be neoplastic were scored for area. Only adenocarcinomas and areas of carcinoma in situ were used in analyses comparing the two diet groups and for regression analysis. Cross-sectional tumor areas on histologic slides were also

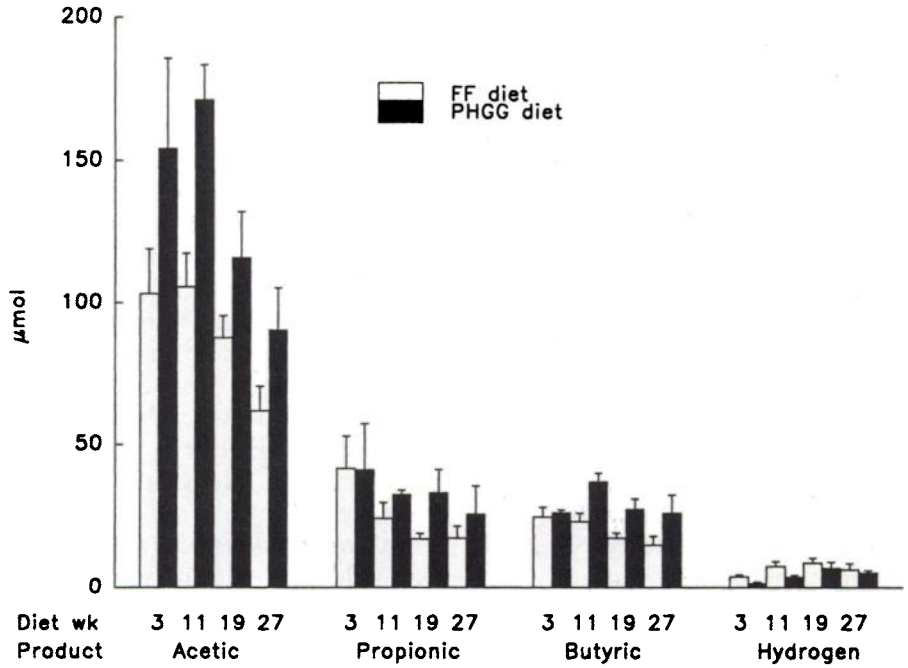

FIGURE 1 Short-chain fatty acids and hydrogen from in vitro fermentation of rat feces for 24 h without additional substrate. Values are means ± SEM, $n = 5$. Paired bars [fiber-free (FF) diet and partially hydrolyzed guar-gum (PHGG) diet] show products for each of the four fermentation periods 3, 11, 19, and 27 wk after starting FF or PHGG diet. Two-way ANOVA results for each product (with fermentation periods 3–27 wk as repeated measures and diet groups FF and PHGG as nonrepeated measures) are shown in Table 2.

digitized and the areas measured for comparison with photocopy and photographic areas using the Sigma Scan digitizing system.

Statistical methods and data management. To estimate the number of rats needed, a cancer incidence of 1.57 in control rats and 0.55 in PHGG rats with a common variance of 2 was used. These estimates were based on a review of studies of carcinogen-induced colon cancer in rats that were primarily dietary studies. Based on these estimates, a one-tailed independent t test with 25 rats in each group would have a power of 0.82 of detecting a 65% reduction in cancer occurrence.

SAS (Statistical Analysis System, Version 6.08, SAS Institute, Cary, NC) procedures (General Linear Models Procedure, GLM) were used for ANOVA to examine SCFA differences between diet groups. Mixed models of ANOVA with repeated measures applied to repeated measures of fermentation substrate and fermentation period or sample site within each diet group were used. The main effect terms for two-way ANOVA used for fermentations without added substrate were for diet and fermentation period. The main effect terms for three-way ANOVA for in vitro fermentations with added substrate were for diet, fermentation period, and fermentation substrate. The main effect terms for two-way ANOVA for SCFA of colonic contents and feces at completion of the study were for diet and sample source (feces, cecum or post-cecal colon).

The cancer areas of the PHGG-diet group and fiber-free control group were compared by a t test for independent samples. The ordinary least-squares method in SAS was used for regression analysis to predict a relationship between cancer size across both groups of rats and the principal SCFA.

RESULTS

Fermentation products at 24 h. Fermentation products (μmol) from endogenous substrate, CS (100 mg), and PHGG (100 mg) after 24 h of fermentation are shown in **Figures 1, 2,** and **3,** respectively. **Table 2** shows the two-way ANOVA results for data in Figure 1. When the data in Figure 1 are expressed on a μmol/g dry matter basis, the same trends are present but values between the two diet groups are not significantly different. Fecal dry matter values were slightly higher in fermentations from rats fed the PHGG. ANOVA results in Table 2 based on the micromole amounts shown in Figure 1 are consistent with more acetic and butyric acid production from endogenous substrate by samples from PHGG-fed rats compared with samples from rats fed the FF diet. It is likely that these differences are due to slightly greater dry-weight amounts in the sample fermentations from PHGG-fed rats compared with the sample fermentations of rats fed the FF diet. Propionate values for sample fermentations from PHGG-fed rats shown in Figures 2 and 3

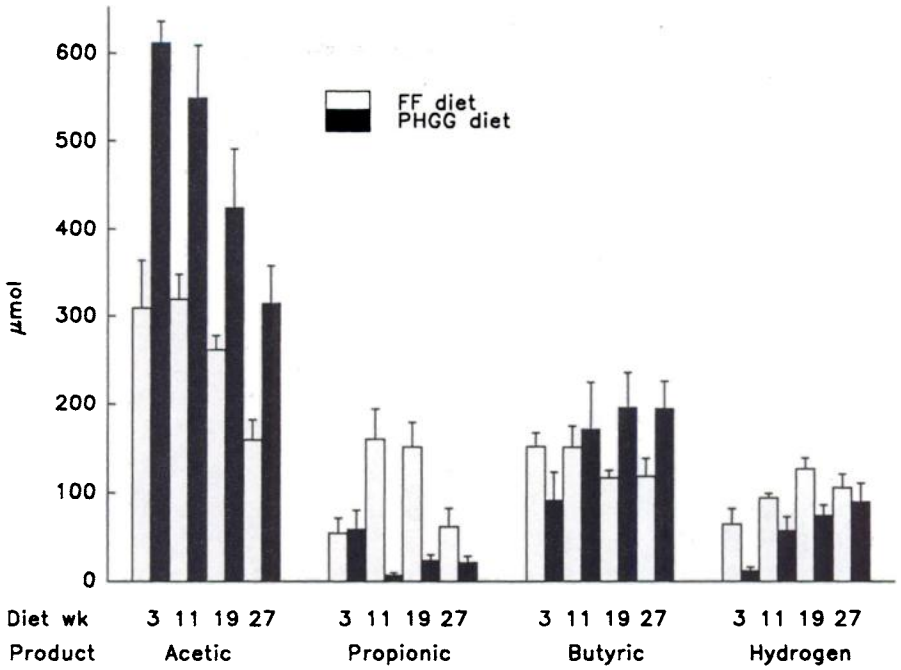

FIGURE 2 Short-chain fatty acids and hydrogen from in vitro fermentation of rat feces for 24 h with 100 mg of cornstarch (CS). Values are means ± SEM, $n = 5$. Paired bars [fiber-free (FF) diet and partially hydrolyzed guar-gum (PHGG) diet] show products for each of the four fermentation periods 3, 11, 19, and 27 wk after starting FF or PHGG diet. Three way ANOVA results for each product (with fermentation substrates, CS, this figure, and PHGG, Figure 3, as repeated measures, fermentation periods 3–27 wk as repeated measures, and diet groups FF and PHGG as nonrepeated measures) are shown in Table 3.

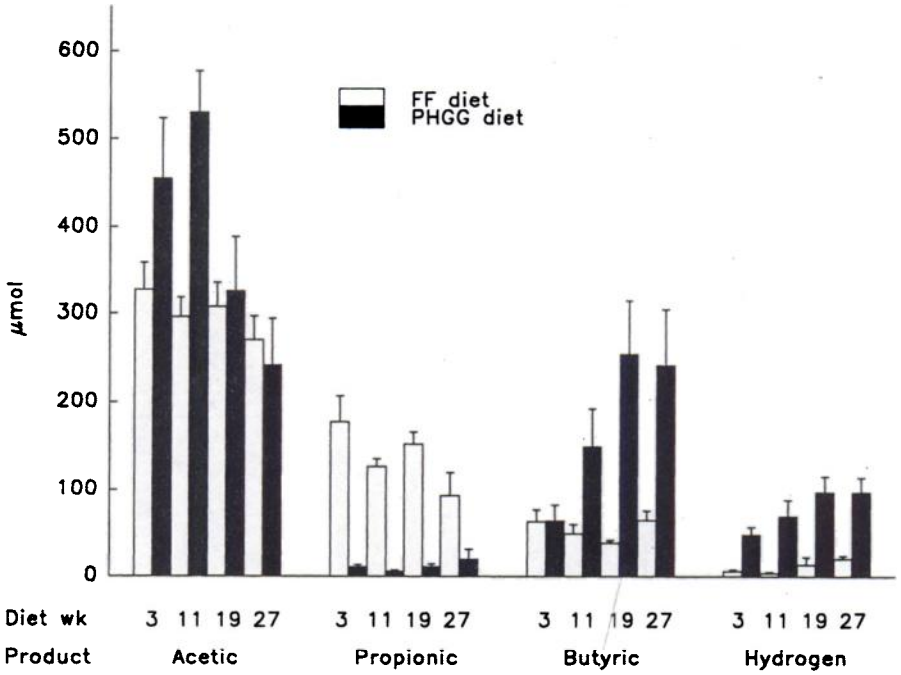

FIGURE 3 Short-chain fatty acids and hydrogen from in vitro fermentation of rat feces for 24 h with 100 mg of partially hydrolyzed guar gum (PHGG). Values are means ± SEM, $n = 5$. Paired bars [fiber-free (FF) diet and PHGG diet] show products for each of the four fermentation periods 3, 11, 19, and 27 wk after starting FF or PHGG diet. Three-way ANOVA results for each product (with fermentation substrates, PHGG, this figure, and cornstarch, Figure 2, as repeated measures, fermentation periods 3–27 wk as repeated measures, and diet groups FF and PHGG as nonrepeated measures) are shown in Table 3.

TABLE 2

ANOVA values based on micromole amounts for acetic, propionic, and butyric acids and hydrogen at 24 h from endogenous substrate (Fig. 1)[1,2]

	Acetic		Propionic		Butyric		Hydrogen	
Variables	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Diet	7.66	0.024	1.51	0.255	9.51	0.015	2.29	0.169
Period	7.13	0.001	2.17	0.118	2.85	0.059	6.6	0.002
Diet × Period	0.72	0.55	0.33	0.806	1.42	0.262	0.45	0.721

[1] Values for each acid and hydrogen are from separate two-way ANOVA (periods, 3–27 wk, as repeated measures).

[2] Abbreviations used: Diet, fiber-free diet or partially hydrolyzed guar-gum diet; Period, fermentation period (3–27 wk); Diet × Period, Diet and fermentation period interaction.

are generally lower than sample fermentations from PHGG-fed rats in Figure 1.

Table 3 shows the three-way ANOVA results for the within and between comparisons for Figures 2 and 3. Butyrate production from PHGG fermentation by samples from the PHGG-fed rats did not increase until after the diet was consumed for 3 wk and not until after 11 wk for CS fermentation compared with the FF-fed rats. Butyrate production from CS fermentation was initially greater for the FF-fed rats than the PHGG-fed rats. Butyrate ANOVA values for diet, period, diet and period interaction, and diet and substrate interaction are consistent with the observations above. Even though butyrate production was not greater initially, other fermentation changes were underway, with greater acetate production by samples from PHGG-fed rats compared with those of FF-fed rats. ANOVA values are consistent with acetate production differences between the two diet groups with significant interactions between diet and fermentation period and between diet and substrate. Propionate production for sample fermentations from PHGG-fed rats was less than that for samples from FF-fed rats.

Rate of fermentation. Products after 6 h, expressed as μmol/g dry matter for the endogenous, CS, and PHGG fermentations, are shown in **Figures 4, 5, and 6,** respectively. There were no significant differences between the two diet groups for products from endogenous fermentation in 6 h (Fig. 4). **Table 4** shows the three-way ANOVA results for the comparisons within and between Fig. 5 and 6. Fermentation rates based on total SCFA (data for minor SCFA are not shown) production were significantly greater for the samples from PHGG-fed rats for both the PHGG and CS fermentations (ANOVA for comparison of PHGG and FF diets $P = 0.0002$ and for diet × substrate interaction $P = 0.002$). On an individual SCFA basis (Fig. 5, 6), more rapid production of acetate, butyrate, and excess hydrogen was present for samples from PHGG-fed rats compared with those of FF-fed rats.

Concentrations of short-chain fatty acids in fecal, post-cecal-colonic, and cecal contents at 27 weeks.

TABLE 3

ANOVA values based on micromole amounts for acetic, proprionic, and butyric acids and hydrogen at 24 h from cornstarch and partially hydrolyzed guar-gum fermentations (Fig. 2, 3)[1,2]

	Acetic		Propionic		Butyric		Hydrogen	
Variables	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Diet	23.73	0.001	69.71	0.0001	7.34	0.027	3.62	0.094
Period	15.94	0.0001	3.37	0.025	4.16	0.01	12.15	0.0001
Diet × Period	3.93	0.013	5.39	0.003	8.14	0.0001	1.43	0.243
Substrate	1.35	0.251	0.69	0.409	5.47	0.023	29.14	0.0001
Diet × Substrate	9.26	0.004	8.02	0.006	11.37	0.001	72.10	0.0001
Period × Substrate	0.77	0.517	2.11	0.11	1.16	0.334	1.39	0.255
Diet × Period × Substrate	1.16	0.334	7.02	0.004	0.33	0.802	0.67	0.575

[1] Values for each acid and hydrogen are from separate three-way ANOVA (periods, 3–27 wk, and substrates CS and PHGG as repeated measures).

[2] Abbreviations used: Diet, fiber-free diet or partially hydrolyzed guar-gum diet; Period, fermentation period (3–27 wk); Diet × Period, diet and fermentation period interaction; substrate, corn starch or partially hydrolyzed guar gum; Diet × Substrate, diet and substrate interaction; Period × Substrate, fermentation period and substrate interaction; Diet × Period × Substrate, diet, fermentation period, and fermentation substrate interaction.

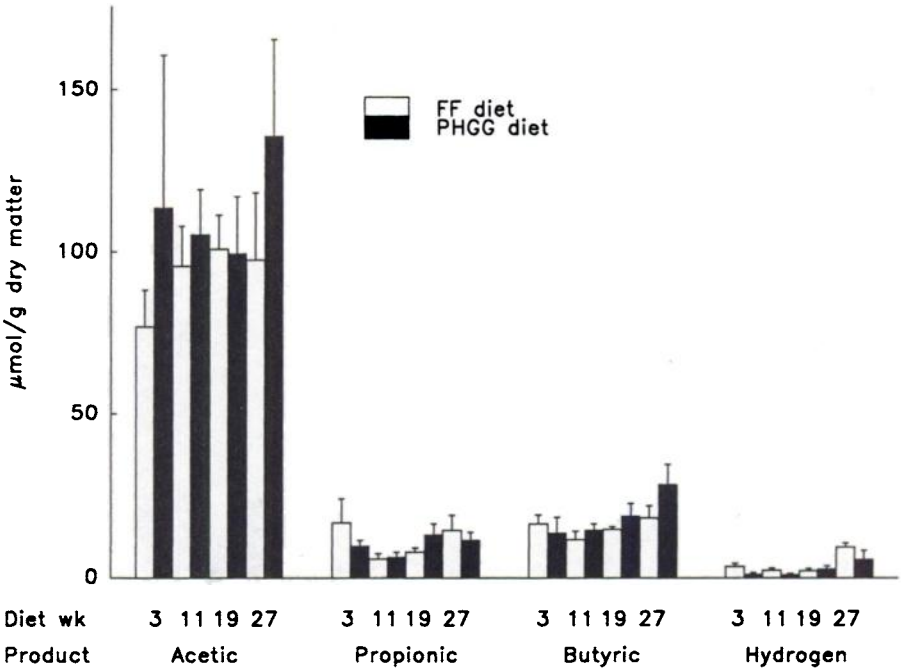

FIGURE 4 Short-chain fatty acids and hydrogen from in vitro fermentation of rat feces for 6 h without additional substrate. Values are means ± SEM, $n = 5$. Paired bars [fiber-free (FF) diet and partially hydrolyzed guar-gum (PHGG) diet] show products for each of the four fermentation periods 3, 11, 19, and 27 wk after starting FF or PHGG diet. Two-way ANOVA (with fermentation periods 3–27 wk as repeated measures and diet groups FF and PHGG as nonrepeated measures) showed no significant differences between the two diet groups.

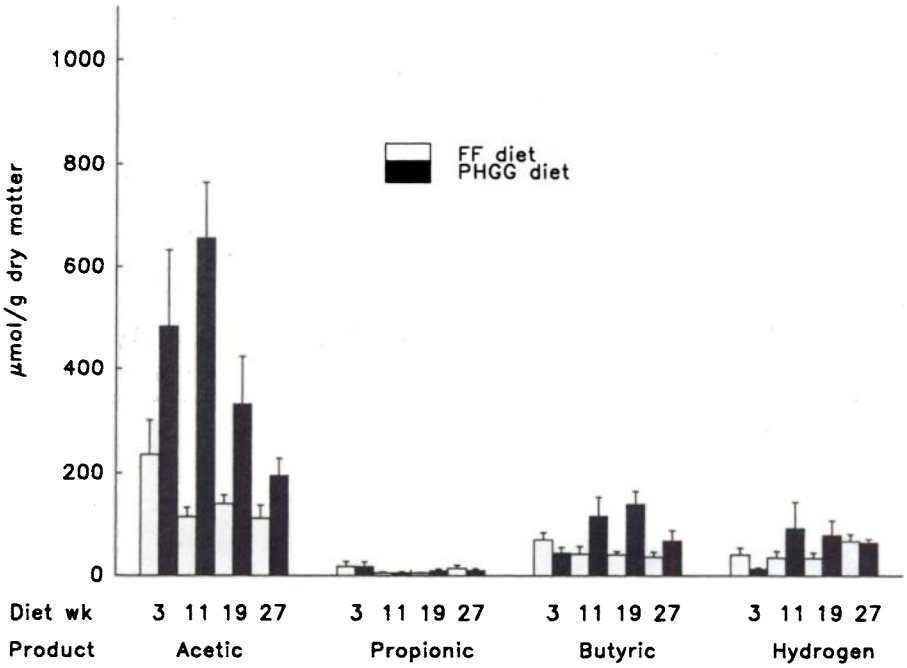

FIGURE 5 Short-chain fatty acids and hydrogen from in vitro fermentation of rat feces for 6 h with 100 mg of cornstarch (CS). Values are means ± SEM, $n = 5$. Paired bars [fiber-free (FF) diet and partially hydrolyzed guar-gum (PHGG) diet] show products for each of the four fermentation periods 3, 11, 19, and 27 wk after starting FF or PHGG diet. Three-way ANOVA results for each product (with fermentation substrates, CS, this figure, and PHGG, Figure 6, as repeated measures, fermentation periods 3–27 wk as repeated measures, and diet groups FF and PHGG as nonrepeated measures) are shown in Table 4.

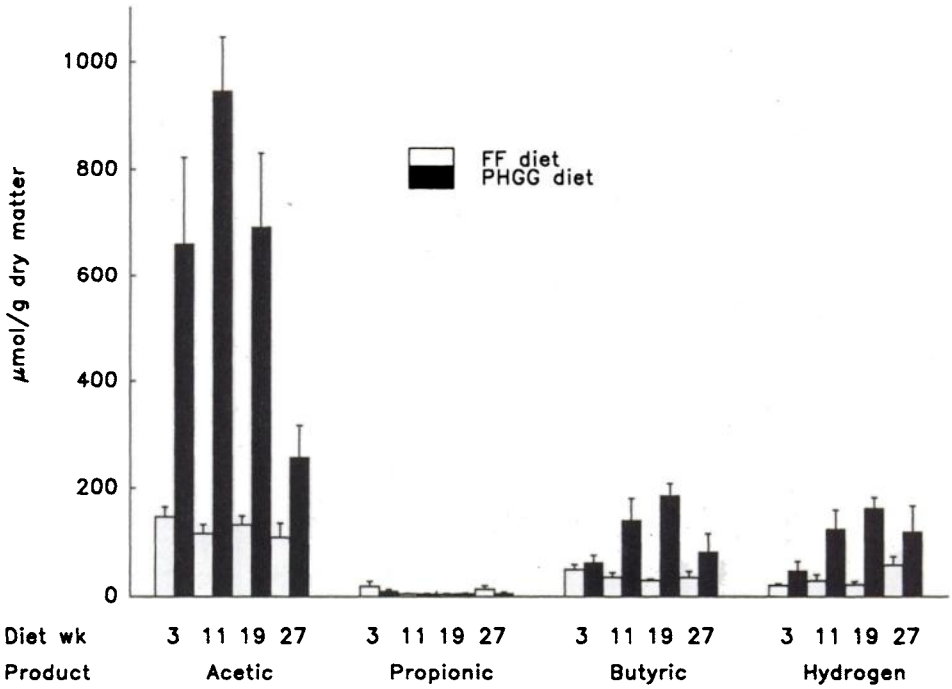

FIGURE 6 Short-chain fatty acids and hydrogen from in vitro fermentation of rat feces for 6 h with 100 mg of partially hydrolyzed guar gum (PHGG). Values are means ± SEM, $n = 5$. Paired bars [fiber-free (FF) diet and PHGG diet] show products for each of the four fermentation periods 3, 11, 19, and 27 wk after starting FF or PHGG diet. Three-way ANOVA results for each product (with fermentation substrates, PHGG, this figure, and cornstarch, Figure 5, as repeated measures, fermentation periods 3–27 wk as repeated measures, and diet groups FF and PHGG as nonrepeated measures) are shown in Table 4.

SCFA concentrations of post-cecal colonic and cecal contents are shown in **Figure 7**, as well as fecal SCFA concentrations of the last fecal samples. Two-way ANOVA results for Fig. 7 are shown in **Table 5.** Individual values for cecal and post-cecal colonic contents of each group of six rats were used to create an average for each group for comparison with the biological fecal pools used for serial fermentations for the same group of six rats for ANOVA comparison across cecal contents, post-cecal-colonic contents, and fecal samples. Little difference in SCFA concentrations was seen between diet groups for fecal samples whereas differences for acetate and butyrate were present for cecal and post-cecal-colonic contents. There was a gradient from high SCFA concentration in the cecal contents to lower SCFA concentrations in the post-cecal-colonic con-

TABLE 4

ANOVA values based on μmol/g dry matter concentrations for acetic, propionic, and butyric acids and hydrogen at 6 h from cornstarch and partially hydrolyzed guar-gum fermentations (Fig. 5, 6)[1,2]

	Acetic		Propionic		Butyric		Total SCFA		Hydrogen	
Variables	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Diet	36.43	0.0003	0.24	0.638	20.87	0.002	42.44	0.0002	16.03	0.004
Period	11.04	0.0001	4.79	0.005	4.65	0.006	8.93	0.0001	3.91	0.013
Diet × Period	9.96	0.0001	0.71	0.552	8.96	0.0001	9.2	0.0001	3.77	0.016
Substrate	7.13	0.01	0.63	0.431	0.59	0.447	5.8	0.019	2.8	0.1
Diet × Substrate	11.2	0.001	0.86	0.358	3.11	0.083	10.3	0.002	7.51	0.008
Period × Substrate	0.97	0.412	0.04	0.987	0.16	0.922	0.87	0.463	0.29	0.836
Diet × Period × Substrate	0.76	0.521	0.13	0.94	0.21	0.89	0.72	0.546	0.29	0.832

[1] Values for each acid, total acids, and hydrogen are from separate three-way ANOVA (periods, 3–27 wk, and substrates cornstarch and partially hydrolyzed guar gum as repeated measures).

[2] Abbreviations used: Total SCFA, sum of acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids (isobutyric, valeric, and isovaleric acids are not shown in the figures); Diet, fiber-free diet or partially hydrolyzed guar-gum diet; Period, fermentation period (3–27 wk); Diet × Period, diet and fermentation period interaction; Substrate, cornstarch or partially hydrolyzed guar gum; Diet × Substrate, diet and substrate interaction; Period × Substrate, fermentation period and substrate interaction; Diet × Period × Substrate, diet, fermentation period, and fermentation substrate interaction.

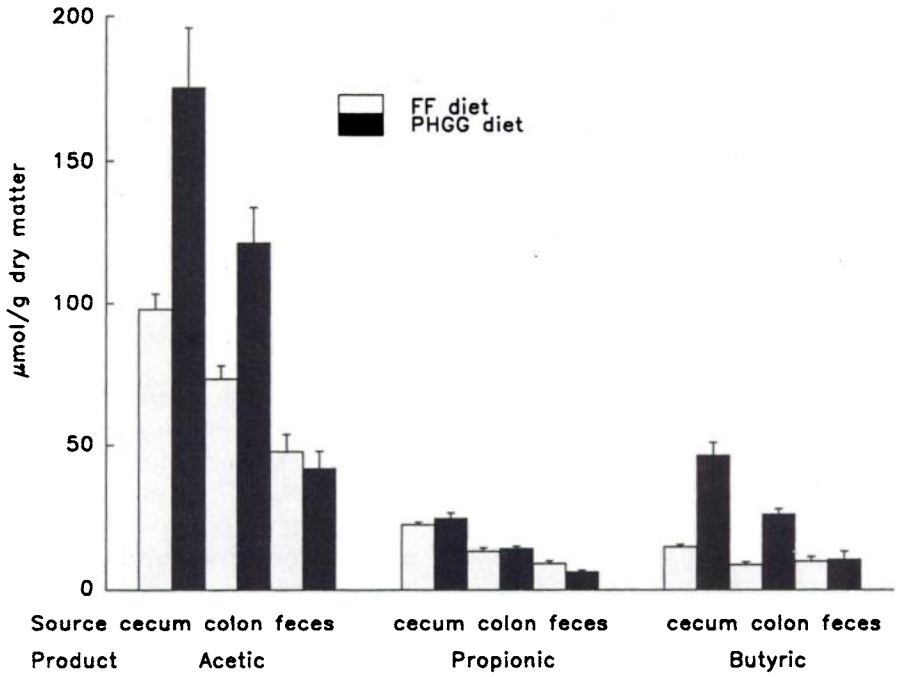

FIGURE 7 Short-chain fatty acids cecal and post-cecal-colonic contents at the end of the study and feces from the 27-wk fermentations. Values are means ± SEM, $n = 5$. Paired bars [fiber-free (FF) diet and partially hydrolyzed guar-gum (PHGG) diet] show products for each sample source (cecum = cecal contents, colon = post-cecal colon contents, and feces) for the three products (acetic, propionic, and butyric acids). Two-way ANOVA [with sample source as a repeated measure and diet group (FF or PHGG) as a nonrepeated measure] results are shown in Table 5. Individual cecal and post-cecal colon values of each group of six rats were used to create an average for each group for comparison by ANOVA with the biological fecal pools used for serial fermentations for the same groups of six rats (5 groups for each diet).

tents and feces. ANOVA results support these observations and indicate that butyrate is significantly greater in the cecal contents than in the post-cecal-colonic contents and less in the feces than in the post-cecal-colonic contents, and that butyrate is greater in rats fed the PHGG diet than in those fed the FF diet.

Cancer incidence. The mean weights of the rats at 31 wk of age for the FF and PHGG diets were 210.0 (SEM 4.6) and 212.3 (SEM 2.6) g, respectively. The colon weights (g) were 2.32 (SEM 0.29) and 2.29 (SEM 0.07) for the FF and PHGG rats, respectively. One adenoma was identified in the PHGG-fed group and three adenomas in the FF-fed group. **Table 6** compares cancer areas and incidence for the two diet groups. Mean cancer areas were larger in the FF group but the difference between the two diet groups was not significant. In the FF group, two of seven rats that died before study completion had invasive colon cancers. These rats are not included in Table 6. None of the five rats fed the PHGG that died before the end of the study had colon cancer. Inclusion of the two rats with cancer that died before completion of the study gives a mean cancer area of 7.46

TABLE 5

ANOVA values based on μmol/g dry matter concentrations for cecal, post-cecal-colonic, and fecal short-chain fatty acids at 27-wk fermentation period (Fig. 7)[1,2]

	Acetic		Propionic		Butyric	
Variables	*F*	*P*	*F*	*P*	*F*	*P*
Diet	10.83	0.011	0.04	0.845	42.58	0.0002
Source	67.97	0.0001	167.02	0.0001	56.97	0.0001
Diet × Source	14.25	0.003	4.49	0.028	32.56	0.0001

[1] Values for each acid are from separate two-way ANOVA (sources cecum, post-cecal colon, and feces as repeated measures).

[2] Abbreviations used: Diet, fiber-free diet or partially hydrolyzed guar-gum diet; Source, samples from the cecum and post-cecal colon at the end of the study and fecal values from the 27-wk fermentations. Fecal sample values were from the fecal pools of six rats (10 groups of 6 rats) used for the serial fermentations. Individual post-cecal colon and cecal values at the end of the study were used to find the average for each group of six rats for comparison with fecal pool values for each group of six rats from the 27-wk fermentations; Diet × Source, Diet and source interaction.

TABLE 6

Colon cancer incidence and area (mm²) in the two diet groups 27 wk after azoxymethane initiation[1,2,3]

	Number of tumors	Number of rats with tumors	Total tumor area by photo	Total tumor area by histologic cross section
FF diet (n = 23)	13	9	13.15 ± 6.2	3.39 ± 1.67
PHGG diet (n = 25)	12	10	7.74 ± 2.82	1.9 ± 0.61

[1] Carcinoma in situ and adenocarcinoma.
[2] Abbreviations used: FF, fiber-free; PHGG, partially hydrolyzed guar-gum.
[3] Values are means ± SEM. There were no significant differences in tumor incidence or size between the two diet groups.

mm^2 (SEM 4.14) by histologic analysis for the FF group. Inclusion of these two rats fed the FF diet in the statistical analysis does not cause the mean cancer area of the FF-diet group determined by histology to become significantly different from the histologic mean cancer are of the PHGG-diet group (P = 0.19). **Figure 8** shows a scatter plot comparing the two methods of cancer area estimation. The two methods of determining cancer area were significantly correlated (r = 0.706, P = 0.0001), but two points appear to be outliers. The outliers are one cancer that had marked submucosal infiltration not apparent on the gross photo/photocopy and one cancer which was large and flat so that photo/photocopy area was large relative to the histologic cross-sectional area. Histologic cross-sectional areas are smaller proportionally than the photo/photocopy areas because of shrinkage with histologic processing and the tendency for the tumors to be more flat than spheroid.

To seek a relationship between cancer size and SCFA, a regression equation was developed using SCFA concentrations of post-cecal-colonic contents to predict cancer area (photo/photocopy); cancer area in mm^2 = −19.7 + 2.9 (μmol propionic acid/g dry matter) −0.55 (μmol butyric acid/g dry matter). Only butyrate and propionate were significant predictors. Butyrate (P = 0.0335) was a negative predictor and propionate (P = 0.0002) was a positive predictor of cancer area; r^2 was 0.279, suggesting that the equation explains only about 28% of cancer variance.

DISCUSSION

Differences in fermentation and short-chain fatty acids between rats fed partially hydrolyzed guar-gum and fiber-free diets. We conducted in vitro studies to attempt to document fecal fermentation differences that might develop between rats fed the FF diet and

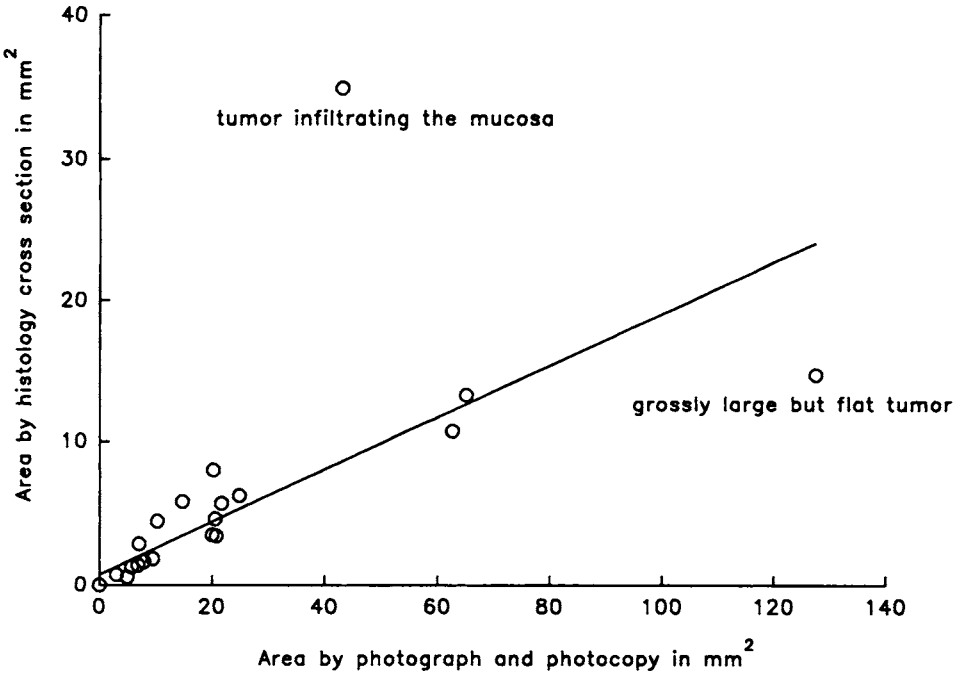

FIGURE 8 Comparison of two methods for measurement of tumor areas, measurement by photograph and photocopy areas and measurement by cross section of histologic slides. The two methods were correlated r = 0.706, P = 0.0001. A least-squares regression line and two probable outliers are noted on the figure.

those fed the PHGG diet. Except for fecal collection periods, the rats were in contact with wood chip bedding so that it is likely that they consumed some wood fiber. The highly lignified cellulose in wood is resistant to fermentation; coupled with the limited ability of the rat and its microbiota to ferment cellulose (Van Soest 1988), this suggests that the ingestion of wood chips would add little to the measured fermentation products. It was anticipated that greater butyric acid production would result from fermentations of samples from rats fed the PHGG diet, with the hypothesis that prolonged feeding of a fiber that has potential for butyric acid production would select for butyrate-producing bacteria. This occurred in the 24-h fermentations of PHGG shown in Figure 3, but unexpectedly the increase in butyric acid production from in vitro PHGG fermentation did not occur until after the 3-wk fermentation experiment, that is, between 3 and 11 wk of the diet treatment. However, other fermentation differences between the two groups of rats were already emerging. For the PHGG fermentation, acetate production appears greater for samples from rats fed the PHGG diet and propionate greater for samples from rats fed the FF diet. Fermentations of CS for samples from rats fed the FF diet at 3 wk had greater production of butyric acid than did fermentation samples from rats fed the PHGG-diet. Considering both the CS and PHGG fermentations, there was little difference in butyrate production between the two groups of rats until after 11 wk of consuming the diet (8 wk after initiation of AOM). For example, expressing the most contrasting early butyrate amounts in Figures 2 and 3 as percentages of the principal SCFA, the butyrate percentages for CS fermentation by samples from rats fed the FF diet at 3 and 11 wk were 31.4 and 24.7% compared with 13.5% and 21.7% for PHGG fermentations by samples from PHGG-fed rats. The 19- and 27-wk fermentations are consistent with significantly more butyrate production from fermentations of samples from PHGG-fed rats. Our intent was for the two groups of rats to have dramatically different levels of butyrate production by the initiation of AOM treatment. It is unlikely that this occurred until late in the experiment.

Evolution of fermentation changes with changing proportions of SCFA may have occurred in other dietary fiber-feeding experiments. Bianchini et al. (1992) found lower acetate:butyrate ratios in rat cecal contents after 105 d on a CS diet compared with acetate:butyrate ratios at 30 d, suggesting an evolving SCFA pattern. Lack of a constant proportion of SCFA during a feeding experiment could confound the isolated effect of one fatty acid or the effect of constant proportions of SCFA.

Six-hour fermentations were done to assess changes in rates of fermentation that may reflect changes in concentrations of bacteria capable of fermenting PHGG due to colonic microbial exposure to PHGG. The six-hour fermentations shown in Figures 5 and 6 show significantly greater total fatty acid (principally acetate and butyrate) production by fermentations from the PHGG-fed rats than do fermentations from the FF-fed rats. This is consistent with a greater viable fecal PHGG-fermenting population in the PHGG-fed rats than in the FF-fed rats. Greater SCFA production from CS by fermentations of samples from PHGG-fed rats than of samples from FF-fed rats suggests versatility in the population of bacteria induced by PHGG feeding. The higher acid production by fermentations from PHGG-fed rats compared with fermentations from FF-fed rats is due initially to acetate production with increased butyrate production also contributing after the 3-wk fermentation period. This is similar to the evolution of the SCFA pattern seen with the 24-h fermentations.

Higher SCFA production by in vitro fermentations of samples from PHGG-fed rats than by those from FF-fed rats predicts that greater amounts of SCFA are produced in vivo in PHGG-fed rats than in FF-fed rats. This prediction is supported by the higher SCFA concentrations of cecal and post-cecal-colonic contents of PHGG-fed rats at the end of the study compared with the FF-fed rats (Fig. 7). The decreasing SCFA concentrations in colonic contents from the cecum to the post-cecal colon to the feces are consistent with more substrate and fermentation in the proximal colon compared with the distal colon, as well as with the progressive absorption of SCFA leading to little difference in fecal-SCFA concentrations. Even though base-line fecal-SCFA concentrations are similar between the two diet groups (Fig. 7, feces), in vitro fermentations of fecal samples without added substrate (Fig. 1) suggest differences in SCFA production. Taken together, the in vitro fermentation results and the SCFA values for cecal and post-cecal-colonic contents suggest that the fecal sample without further in vitro fermentation is the least likely sample or method to show SCFA differences between diet groups or perhaps between diagnostic groups.

Short-chain fatty acids and tumor size. These observations showed a trend towards smaller cancer areas in the PHGG-fed rats but did not define a significant cancer-suppressive effect of the PHGG diet. Accepting the absence of a statistically significant PHGG antitumor effect should be done in the context of the experimental model. The experimental model may have confounded the possibility of demonstrating a significant tumor-inhibiting effect for PHGG. The in vitro fermentation results for PHGG fermentations suggest that increased butyrate production from PHGG did not occur until after completion of AOM treatment, so that colonic microbial butyrate production was probably similar in the two diet groups during tumor induction. In retrospect, a better experimental design would have compared the PHGG diet with a diet containing another fiber with a different fermentation SCFA profile such as pectin. Additionally, a longer diet adaptation

period before carcinogen exposure would be more likely to test for effects of differences in profiles of steady-state SCFA fermentation. Contact of the rats with their wood-fiber bedding, allowing them a potential source of nonfermentable fiber, may also have served to reduce carcinoma development in both diet groups. A significant negative effect of colonic-contents butyrate on cancer size in combination with a positive effect of propionate was demonstrated. These effects are similar to those shown by McIntyre et al. (1993) and have to be interpreted in the context of this specific rat model. As we have discussed above, it is likely that relatively high concentrations of butyrate did not develop until after tumor induction. SCFA amounts after endogenous fermentation at 24 h were slightly greater for samples from PHGG-fed rats than from the FF-fed rats when expressed in micromoles. If expressed as micromoles per gram of dry matter, the SCFA amounts were not significantly greater for the samples from PHGG-fed rats (data not shown) than samples from the FF-fed rats. Thus it is unlikely that significant amounts of PHGG were present in the post-cecal colon, that is, PHGG was probably used principally in the cecum and proximal colon.

Concentrations in Figure 7 are consistent with a SCFA gradient from the cecum to the feces. This would suggest that this experiment tested the effect of butyrate principally on the proximal colon after initial tumor induction but did not test the effect of butyrate production during tumor induction or throughout the post-cecal colon. The positive prediction of cancer size by propionate in combination with negative prediction by butyrate offers a potential explanation for a lack of cancer protection by some soluble fibers such as pectin (Bingham 1990, Jacobs 1986), because fermentation of some carbohydrates may enhance propionate production. Two studies have been cited as showing no protective effect for butyrate (Deschner et al. 1990, Freeman 1986). However it is possible that the forms of butyrate given in these studies made it unavailable for colonic absorption.

Several other studies that have considered SCFA in the protective effects of fiber have shown inconclusive results (Caderni et al. 1994, Sakamoto et al. 1996). In a study (Caderni et al. 1994) comparing dietary sucrose, CS, and high amylose-CS in azoxymethane-treated rats, tumor size was smaller in rats consuming the CS diet than in those consuming the sucrose diet. The concentrations of the colonic contents of SCFA were not given, but nondigested CS would typically tend to increase butyrate production by colonic microbiota. A study comparing resistant-starch diets with cellulose diets in dimethylhydrazine-treated rats (Sakamoto et al. 1996) showed that a 10% cellulose-diet offered some protection compared with a basal diet, whereas the group consuming the 10% resistant-starch diet had a smaller mean tumor volume than the basal-diet group; the difference was not significant. Because amylase-resistant starch is also resistant to fermentation by the colonic microbial community (Granfeldt et al. 1993), a lesser benefit of a resistant-starch diet could be rationalized by the delayed production of butyrate as the microbial community evolved to metabolize resistant starch. Fiber fermentability during dimethylhydrazine use could affect results as well. For example, a nonfermentable fiber might tend to enhance carcinogen excretion, while a fermentable fiber might enhance or inhibit bacterial metabolism of dimethylhydrazine, leading to differing carcinogenesis potentials because dimethylhydrazine is less carcinogenic in germ-free rats (Reddy et al. 1975).

Our study demonstrates fermentation changes consistent with permutations in the concentrations of bacteria in the colonic microbial community that evolve with continued use of two different diets. These permutations are of interest in the interpretation of this and other dietary fiber studies. The slow evolution of SCFA changes with diet in this experiment may partially explain the lack of a significant anti-cancer effect of the PHGG diet in this protocol. This study also points out the lack of fecal SCFA changes compared with changes that occur with in vitro fermentations and with direct measurement of colonic contents. Finally, this study adds support for a protective effect of butyrate in an experimental model of colon cancer in rats.

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