Decreased dietary fiber intake and structural alteration of gut microbiota in patients with advanced colorectal adenoma[1–4]

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ABSTRACT

Background: Accumulating evidence indicates that diet is one of the most important environmental factors involved in the progression from advanced colorectal adenoma (A-CRA) to colorectal cancer.

Objective: We evaluated the possible effects of dietary fiber on the fecal microbiota of patients with A-CRA.

Design: Patients with a diagnosis of A-CRA by pathological examination were enrolled in the A-CRA group. Patients with no obvious abnormalities or histopathological changes were enrolled in the healthy control (HC) group. Dietary fiber intake was assessed in all patients. Short-chain fatty acids (SCFAs) in feces were detected by gas chromatography. The fecal microbiota community was analyzed by 454 pyrosequencing based on 16S ribosomal RNA.

Results: Lower dietary fiber patterns and consistently lower SCFA production were observed in the A-CRA group ($n = 344$). Principal component analysis showed distinct differences in the fecal microbiota communities of the 2 groups. *Clostridium, Roseburia*, and *Eubacterium* spp. were significantly less prevalent in the A-CRA group ($n = 47$) than in the HC group ($n = 47$), whereas *Enterococcus* and *Streptococcus* spp. were more prevalent in the A-CRA group ($n = 47$) (all $P < 0.05$). Butyrate and butyrate-producing bacteria were more prevalent in a subgroup of HC subjects with a high fiber intake than in those in both the low-fiber HC subgroup and the high-fiber A-CRA subgroup (all $P < 0.05$).

Conclusion: A high-fiber dietary pattern and subsequent consistent production of SCFAs and healthy gut microbiota are associated with a reduced risk of A-CRA. This trial was registered at www.chictr.org as ChiCTR-TRC-00000123. *Am J Clin Nutr* 2013;97:1044–52.

INTRODUCTION

Colorectal cancer (CRC)[5] is a leading cause of cancer-related mortality worldwide, and its incidence has increased rapidly in recent years in China (1, 2). Epidemiologic and experimental studies have indicated that dietary fiber is an important part of a healthy diet and may influence early events in the carcinogenic process that can result in advanced colorectal adenoma (A-CRA) or CRC. Dietary fiber offers potential benefits in preventing CRC by promoting the delivery of short-chain fatty acids (SCFAs), such as butyrate (fermented by the gut microbiota) to the distal colon (3–6).

Several studies of CRC and inflammatory bowel disease have reported that the presence of SCFAs in the colonic lumen is linked to a decreased incidence of CRC and (7–11). However, data supporting associations between A-CRA, diet, SCFAs, and gut microbiota are limited. Furthermore, the reliability of culture-independent methods, including clone libraries, quantitative real-time polymerase chain reaction (PCR), and denaturing gradient gel electrophoresis, traditionally used in these studies is limited by the fact that they exclude most microorganisms in the human intestine (12). The advent of 16S rRNA-based analyses

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[5] Abbrevations used: A-CRA, advanced colorectal adenoma; CRC, colorectal cancer; GC, gas chromatography; HC, healthy control; OTU, Operational Taxonomic Unit; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; SCFA, short-chain fatty acid.

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has allowed investigation of the human colonic microbiota at the level of phylotypes and bacterial species.

The current study was undertaken to evaluate possible differences and associations between dietary fiber intake, SCFAs, and gut microbiota in patients with A-CRA compared with those in healthy controls (HCs). We also attempted to identify microbial lineages and potential bacterial biomarkers of the development of A-CRA. It is hoped that these findings may provide a more comprehensive understanding of the role of diet and microbiota in preventing A-CRA and have potentially important implications in the design of fiber or probiotic-enriched diets.

SUBJECTS AND METHODS

Study population

Consecutive patients who had undergone colonoscopy in 5 medical centers in China (Renji Hospital, Tongji Hospital, Shanghai 10th Hospital, Ruijin Hospital, Shanghai East Hospital, and Xinhua Hospital) between 1 June 2010 and 30 September 2011 were eligible for the screening for the study.

Patients must have met the following inclusion criteria to enter the study: *1*) patients were ≥50 y of age, because current US and European guidelines as well as Asian guidelines' recommendation define the age threshold for endoscopy at 50 y; *2*) patients had a normal bowel frequency of a minimum of once every 2 d and a maximum of twice per day (13); *3*) patients had all received a relatively stable diet during the past 5 y; *4*) patients completed a questionnaire designed to provide information on health-related issues, including diet, lifestyle factors, and medical events; and *5*) patients underwent colonoscopies with adequate withdrawal time (14) by well-trained gastroenterologists using standard colonoscopy equipment. Patients with advanced adenoma (≥10 mm in diameter) with high-grade neoplasia, villous, or tubulovillous morphology (15) confirmed by pathological examination were included in the A-CRA group. Patients without obvious abnormalities were enrolled in the HC group.

None of the patients had a history of previous colorectal adenoma or carcinoma or irritable bowel syndrome. Patients with cirrhotic or portal hypertension gastropathy; severe cardiac, pulmonary, renal, liver, hematologic, or rheumatologic disorders; uncontrollable diabetes mellitus or hypertension; or CRC-related conditions, such as familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, ulcerative colitis, or Crohn disease, were excluded from the study. None of the patients had received systemic or oral topical corticosteroids, antibiotics, aspirin, other nonsteroidal antiinflammatory drugs (16), or health products that regulate intestinal microbiota within 6 mo before enrollment, and none were currently undergoing systemic cancer chemotherapy or receiving radiation.

All procedures were undertaken in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committees at each participating center. Informed consent was obtained from all of the subjects. An independent data and safety committee monitored the trial and reviewed the results.

Study design

The study adopted a cross-sectional design. Data on the demographics, colonoscopy results, lifestyle factors, and dietary fiber intake were collected on questionnaires by 2 qualified research nurses. Of the 5632 subjects enrolled this study, 688 eligible (344 in each of the HC and A-CRA groups) completed the food-frequency questionnaire and provided fecal samples. A score was assigned for smoking status, daily alcohol intake, frequency of weekly vigorous exercise, and intakes of fruit, vegetables, and grains (6, 17) (**Table 1**).

A semiquantitative, adjusted, and validated food-frequency questionnaire was used to estimate the average daily intake of dietary fiber during the previous 5 y (18–20). Participants were asked to identify the types and frequency of foods eaten. The foods were grouped into categories: fruit, vegetables, and grains (whole/refined) (21, 22). The intake of the individual dietary factors was computed from the reported consumption frequency of each specified unit of food by using data from the Chinese Society of Nutrition on the nutrient content of relevant fibers in the specified food types. The relevant fibers included nonstarch polysaccharides and resistant starch, such as whole-grain cereals, corn, seeds, nuts, wheat, barley, rice, and oats.

Patients and HCs (n = 344 per group) were asked to provide fresh stool samples, which were immediately stored at −80°C for further analysis. Fecal SCFAs in all study individuals were detected by gas chromatography (GC). Logistic regression was performed to explore the effect of multilifestyle factors and SCFAs on A-CRA. Furthermore, high-throughput 454 pyrosequencing of fecal samples was performed in 47 sex- and age-matched individuals in each of the 2 groups to characterize differences in microbial communities. The dietary fiber intake, SCFAs, and gut microbiota in patients with A-CRA were compared with those of HCs. We investigated the possible effects of dietary factors on the composition of the bacterial community in the host and the metabolic output of fibers (SCFAs) fermented by gut microbiota. A process flow chart for this study is shown in **Figure 1**.

GC analysis of fecal SCFA content

Duplicates of fecal samples (~1 g) were emulsified for 2 min and then centrifuged at 4800 × g for 15 min at 4°C. The supernatant fluid was decanted, filtered using a 0.2-μm filter, and frozen at −80°C for later analysis. Concentrations were measured by GC (Agilent Technologies 6890N Network GC System with a flame ionization detector fitted with an HP-wax; 30.0 m × 320 μm × 0.25 μm capillary column) by using a protocol adapted from a previously described method (23). A mixed-SCFA standard solution was generated by using reagents of the highest purity (99%) (Sigma-Aldrich Corporation). A 1-mmol/L concentration of 2,2-dimethyl butyric acid was used as an internal standard. SCFA values were computed by using the peak area ratio of the sample to the internal standard based on the standard curve.

Fecal sample preparation and DNA extraction

Frozen aliquots of each fecal sample (1 g) were suspended in 30 mL of 0.1 mol/L sodium phosphate buffer [pH 7.4; 57.7 mL 1 mol/L Na_2HPO_4 Tris and 42.3 mL 1 mol/L NaH_2PO_4] for 30 min. The suspension was centrifuged 3 times at 200 ×g (5 min) to remove coarse particles. The supernatant fluid was collected after centrifugation at 9000 × g for 3 min. The sediment was collected and washed 4 times with 30 mL sodium phosphate

TABLE 1
Dietary fiber and other lifestyle factor scores

Score	Vegetable	Grain	Fruit	Alcohol	Smoking	Weekly vigorous exercise
	g/d	*g/d*	*g/d*	*g/d*	*cigarettes/d*	
0	0–30	0–3	0–20	Current, >50	Current, >20	—[1]
1	>30 to <300	>3 to <30	>20 to <200	Current, ≤50	Current, ≤20	Low
2	300–500	30–50	200–400	Previously, >50	Previously, >20	Moderate
3	>500	>50	>400	Previously, ≤50	Previously, ≤20	High
4	—[1]	—[1]	—[1]	Never	Never	—[1]

[1] This variable was not considered in the determination of the score.

buffer, resuspended in 10 mL sodium phosphate buffer, and stored at −80°C for subsequent DNA extraction.

DNA was extracted from each fecal sample by using the bead-beating methods and phenol-chloroform purification as described previously (24). The DNA concentration was determined with the Gene Quant RNA/DNA Calculator (Amersham Pharmacia Biotech) and tested by 1% agarose gel electrophoresis. All DNA samples were stored at −20°C for subsequent PCR analysis. DNA purification was performed by using the AxyPrep DNA Gel Extraction Kit (catalog no. AP-GX-50; Axygen), and the TBS-380 Fluorometer was used for quantitative analysis.

Design of barcoded primers and PCR enrichment

The PCR enrichment of the 16S rRNA V1-V3 hypervariable region was performed with universal primers (27F 5′-AGAGTTTGATCCTGGCTCAG-3′, 533R 5′-TTACCGCGGCTGCTGGCAC-3′) incorporating the FLX Titanium adaptors and a sample barcode sequence (25) (synthesized by the Shanghai Sangon Biological Engineering Technology & Service Co). A 2-step PCR strategy was used (Takara Bio Inc). After the PCR reaction, electrophoresis was immediately performed to isolate the enriched V1-V3 region DNA fragments from the reaction mixture. All of the products were harvested by using a gel extraction kit (OMEGA Bio-tek) according to the manufacturer's instructions.

454 Pyrosequencing and analysis of the 16S rRNA sequences

The fecal samples (n = 47 per group) were pyrosequenced by using a Roche 454 GS FLX in accordance with the manufacturer's instructions. The gross sequencing data were arranged by the primer tags (26, 27). A total of 2,073,602 sequences were obtained and analyzed by using MOTHUR software (version 1.14). The sequences were binned into each sample according their barcodes and forward primers. Sequences that were <200 base pairs contained ambiguous bases or that contained >2 mismatches to the primers were removed. The aligned sequences were grouped into operational taxonomic units (OTUs) according to their pairwise distances by using the default cluster method in MOTHUR. Richness estimators (ACE and CHAO) and diversity estimators (Shannon and Simpson) were obtained. Similarities among the samples were calculated and shown as a dendrogram. Taxonomy information was obtained for each OTU sample by cross-referencing the SILVA database (28, 29). On the basis of this taxonomy information, differences among samples or between clinical groups were derived by Metastats and principal component analysis. Microsoft Excel 2007 was used to generate the tables and figures for presentation of the taxonomy results.

Real-time quantitative PCR assay

Fecal DNA from each sample was subjected to real-time quantitative PCR (qPCR) assays to determine the amounts of total bacteria, *Bacteroides* genus, and *Bifidobacteria* spp. through detection of 16S rRNA genes. The qPCR assay was performed with a SYBR Premix Ex Taq (Takara) on a DNA Engine Opticon 3 System (MJ Research). For each primer set (30–32), a constructed plasmid was chosen to create a 10-log-fold standard curve to directly quantify all samples. Each qPCR contained 10 μL SYBR Premix Ex Taq, 0.4 μL of a 10-μmol/L F/R primer mix, and 1 μL of the respective template DNA. Amplifications were performed under the following temperature profiles: one cycle at 95°C for 3 min, 40 cycles of denaturation at 95°C for 30 s, annealing for 40 s, and extension for 30 s. Fluorescence was measured after the extension phase of each cycle at an appropriate temperature for 10 s to avoid interference of primer dimers, secondary structure, or spurious priming. A final extension step was sustained for 5 min.

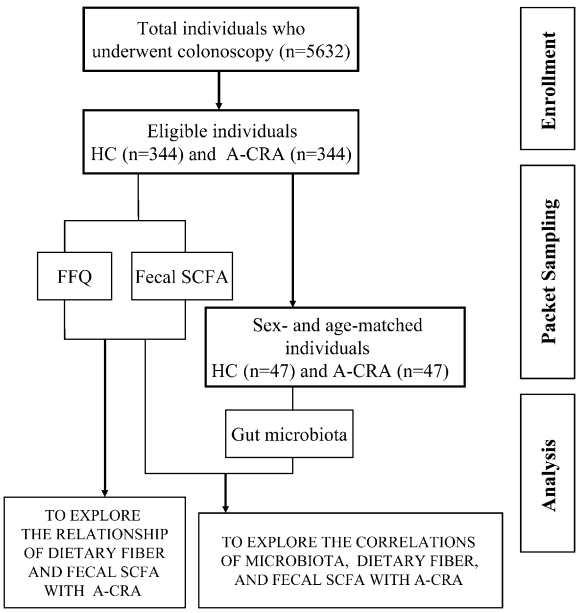

FIGURE 1. Flow diagram of the study. A-CRA, advanced colorectal adenoma; FFQ, food-frequency questionnaire; HC, healthy control; SCFA, short-chain fatty acid.

Statistics

Statistical analysis was performed by a biostatistician, who was blinded to sample identities, by using the SPSS version 17.0 software package (SPSS Inc). Data for continuous variables were reported as means ± SDs, means ± SEMs, or percentages. Student's *t* tests or Wilcoxon's rank-sum tests were used to analyze intergroup differences in continuous variables, and categorical variables were compared by using chi-square and Fisher's exact tests. Logistic regression models using 2 comparison groups were fitted to the data. The relative abundances of OTUs were calculated by using the Mann-Whitney *U* test. Two-sided *P* values <0.05 were considered statistically significant. The amounts of specific bacteria group measured by qPCR and pyrosequencing were compared by using Spearman's correlation (30).

TABLE 2
Multivariate comparison of environmental exposure data in 688 cases in the A-CRA (n = 344) and HC (n = 344) groups[1]

Factor	A-CRA (n = 344)	HC (n = 344)	*P*
Age [% (n)][2]			0.814
50–64 y	60.5 (208)	59.6 (205)	
≥65 y	39.5 (136)	40.4 (139)	
Sex [% (n)][2]			0.810
Male	50.6 (174)	50.0 (172)	
Female	49.4 (170)	50.0 (172)	
BMI (kg/m^2)	22.65 ± 3.45[3]	23.10 ± 2.97	0.068
Overweight [% (n)][2]	44.8 (154)	44.4 (153)	0.091
History of gastrointestinal cancer in directly-related family members [% (n)][2]	8.1 (28)	20.6 (71)	0.001
Smoking [% (n)][2]			0.155
0	9.0 (31)	6.1 (21)	
1	9.9 (34)	9.0 (31)	
2	1.2 (4)	0.6 (2)	
3	0.6 (2)	0.9 (3)	
4	79.3 (273)	83.4 (287)	
Alcohol [% (n)][2]			0.570
0	0.0 (0)	0.0 (0)	
1	5.2 (18)	3.8 (13)	
2	5.2 (18)	4.9 (17)	
3	1.8 (6)	2.0 (7)	
4	87.8 (302)	89.3 (307)	
Weekly vigorous exercise [% (n)][2]			0.520
1	44.5 (153)	45.1 (155)	
2	38.4 (132)	36.9 (127)	
3	17.1 (59)	18.0 (62)	
Vegetables [% (n)][2]			0.013
0	5.2 (18)	6.7 (23)	
1	52.3 (180)	44.2 (152)	
2	34.9 (120)	37.2 (128)	
3	7.5 (26)	11.9 (41)	
Grains [% (n)][2]			0.789
0	29.1 (100)	27.0 (93)	
1	40.7 (140)	36.9 (127)	
2	20.0 (69)	29.7 (102)	
3	10.2 (35)	6.4 (22)	
Fruit [% (n)][2]			0.333
0	13.1 (45)	13.1 (45)	
1	58.7 (202)	64.8 (223)	
2	21.2 (73)	18.0 (62)	
3	7.0 (24)	4.1 (14)	
Acetic acid[4]	0.84 ± 0.05	1.40 ± 0.11	0.001
Propionic acid[4]	0.50 ± 0.03	0.81 ± 0.06	0.001
Isobutyric acid[4]	0.08 ± 0.00	0.13 ± 0.02	0.001
Butyric acid[4]	0.45 ± 0.03	0.72 ± 0.07	0.001
Isovaleric acid[4]	0.13 ± 0.01	0.18 ± 0.02	0.001

[1] A-CRA, advanced colorectal adenoma; HC, healthy control.
[2] Categorical variables were compared by using a chi-square test or Fisher's exact test.
[3] Mean ± SD (all such values).
[4] Data were compared by using a 2-sample independent *t* test.

TABLE 3
Logistic regression in the A-CRA (n = 344) and HC (n = 344) groups[1]

	B	SE	Wald	df	P	Exponent (B)	95% CI for exponent (B) Lower	Upper
Acetic acid	−0.339	0.1	11.49	1	0.001	0.713	0.586	0.867
History of colorectal cancer in directly related family member	0.951	0.257	13.756	1	0.001	2.589	1.566	4.281
Vegetable	−0.518	0.172	9.034	1	0.003	1.679	1.197	2.353
Butyric acid	−0.172	0.075	5.253	1	0.022	0.842	0.726	0.975
Constant	0.025	0.246	0.01	1	0.919	1.025	—	—

[1] A-CRA, advanced colorectal adenoma; HC, healthy control.

RESULTS

Demographics, lifestyle factor analysis, and SCFAs

The results of a multivariate comparison of environmental exposure data in the A-CRA (n = 344) and HC (n = 344) groups are shown in **Table 2**. Multivariate logistic analysis identified 4 statistically significant factors associated with A-CRA. Intake of vegetables and high amounts of fecal acetic acid and butyric acid were protective factors, and a history of gastroenterological cancer in a directly-related family member was a risk factor for A-CRA (**Table 3**). The model indicated that when acetic acid concentrations were reduced by 1 μg/L, the probability of developing advanced adenoma increased by 71.3%; if the butyric acid content was reduced by 1 μg/L, the risk increased by 84.2%. An immediate family with a history of CRC increased the likelihood of developing advanced adenoma 2.59-fold.

There were 47 age- and sex-matched case-control subjects (24 men and 23 women) in each group. The mean ages were 57 ± 11 and 58 ± 11 y in the HC (n = 47) and A-CRA (n = 47) groups, respectively. BMIs (in kg/m^2) were 22.94 ± 2.17 and 22.85 ± 2.93, respectively, and there were 5 overweight individuals in the 2 groups [3 men in the HC group (n = 47) and 2 in the A-CRA group (n = 47)]. Dietary fiber intake in the A-CRA group (n = 47) differed from that in the HC group (n = 47), with ratios (scores from 0 to 3) of vegetables, grains, and fruit of 0:11:25:11 compared with 0:23:19:5 (P = 0.026), 4:22:18:3 compared with 9:19:9:10 (P = 0.030), and 2:23:12:10 compared with 2:27:13:5 (P = 0.560), respectively.

As shown in **Figure 2**, significantly lower yields of fecal SCFAs were found in the A-CRA group (n = 47) than in the HC group (n = 47). The major SCFA product was acetate, followed by butyrate and propionate.

Pyrosequencing data

A total of 2,073,602 pyrosequencing tags were obtained from stool samples from the case-control subjects (47:47) in each group. Secondary selection involving tag removal of short, long, or ambiguous bases or long homologous/repeat sequences resulted in 1,604,535 pyrosequencing candidates. The number of tag sequences per subject generated was 18,363 ± 4345 for the A-CRA group (n = 47) and 15,776 ± 3756 for the HC group (n = 47). The mean community richness, as estimated by the CHAO 1 index, was similar in the A-CRA (n = 47) and HC (n = 47) groups. Detailed statistical characteristics of the pyrosequencing data are summarized in the supplementary material (*see* Table S1 under "Supplemental data" in the online issue).

Rarefaction curve analysis was performed to determine whether all OTUs evaluated in the data set were recovered by pyrosequencing. There were no obvious differences between the rarefaction curve findings from samples from the A-CRA (n = 47) and HC (n = 47) groups. The analysis indicated the existence of unidentified OTUs (mean: 4.3% per sample). Whereas no rarefaction curves (*see* Figure S1 under "Supplemental data" in the online issue) plateaued with the current sequencing, the Shannon diversity estimates of all samples had already reached stable values at this sequencing depth, which suggests that, although identification of new phylotypes would be expected from additional sequencing, most of the diversity had already been captured (*see* Figure S2 under "Supplemental data" in the online issue).

Fecal microbiota

Principal component analysis showed altered fecal gut microbiota communities in patients with A-CRA (n = 47) compared with the HCs (separated from principal component 1 at 15.22% and principal component 2 at 10.34% of the explained variance, respectively). On the x axis PC1 direction, the intestinal microbiota of the HC group (n = 47) was relatively consistent and was distinct from that in the A-CRA group (n = 47). A similar

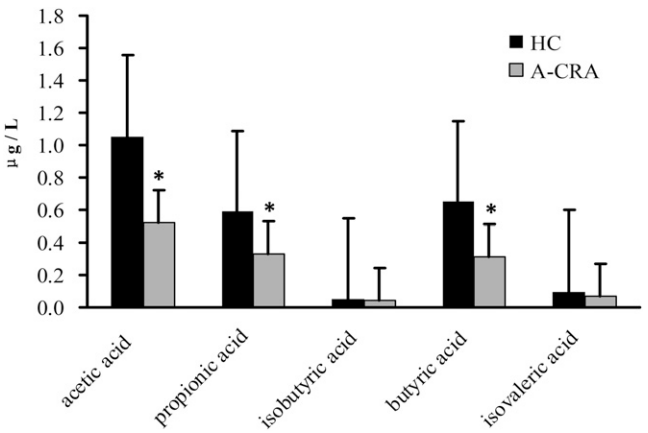

FIGURE 2. Short-chain fatty acids in the HC group (n = 47) and A-CRA group (n = 47). *Significantly different from the HC group, $P < 0.05$. A-CRA, advanced colorectal adenoma; HC, healthy control.

tendency was seen on the *y* axis adenoma–principal component 2 direction, with each sample in the A-CRA group (n = 47) showing bacterial communities similar to each other, together with the appearance of distinct bacterial communities in the A-CRA group (n = 47) compared with the HC group (n = 47) (**Figure 3**).

The sequences were principally distributed among 6 bacterial phyla, including *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria*, and *Verrucomicrobia* spp. Almost half of the microbial community [68.3% in the HC group (n = 47) and 67.9% in the A-CRA group (n = 47)] was composed of *Firmicutes* spp., followed by *Bacteroidetes*, which accounted for 24.8% in the HC group (n = 47) and 24.2% in the A-CRA group (n = 47). Furthermore, the proportions of *Proteobacteria* phylum was 2.98% in the HC group (n = 47) and 3.66% in the A-CRA group (n = 47). The proportions of each phylum for each individual in the HC group (n = 47) and in the A-CRA group (n = 47) are shown elsewhere (*see* Figures S3 and S4 under "Supplemental data" in the online issue). The 30 gut microbiota genera with the most significant individual differences in the A-CRA (n = 47) and HC (n = 47) groups are shown elsewhere (*see* Tables S2 and S4 under "Supplemental data" in the online issue, respectively). The 30 gut microbiota genera with the highest individual percentages in the A-CRA (n = 47) and HC (n = 47) groups are shown elsewhere (*see* Tables S3 and S5 under "Supplemental data" in the online issue, respectively).

Intestinal microbiota, including genera related to the fermentation of butyrate (*Clostridium, Roseburia*, and *Eubacterium* spp.), were significantly lower in the A-CRA group (n = 47) than in the HC group (n = 47), whereas the prevalence of *Enterococcus*, *Streptococcus*, and *Bacteroidetes* spp. was significantly higher in the A-CRA group (n = 47) than in the HC group (n = 47) ($P < 0.05$; **Table 4**).

Distinctive gut microbiota and butyrate in the fiber subgroups

To explore the correlations between the SCFAs, A-CRA, dietary fiber, and butyrate-producing bacteria, we further divided the HC (n = 47) and A-CRA (n = 47) groups into 2 subgroups according to vegetable and grain intakes because of their statistical differences of fiber intake score (<3 or ≥ 3) between the HC group (n = 47) and A-CRA group (n = 47) (chi-square test, P = 0.039). Subjects with dietary fiber intake scores <3 were assigned to the low-fiber subgroups, and those with scores ≥ 3 were assigned to the high-fiber subgroups.

Differences in butyrate and butyrate-producing bacteria in these 4 subgroups are shown in **Table 5**. In the HC group (n = 47), both butyrate and butyrate-producing bacteria (*Clostridium* and *Roseburia* spp.) were more abundant in subjects with a high fiber intake than in those with a low fiber intake. These differences were also apparent in the A-CRA subgroups. In the low-fiber subgroups, no differences in butyrate and the butyrate-producing bacteria (*Clostridium, Roseburia*, and *Eubacterium* spp.) were found between the HC and A-CRA groups; however, in the high-fiber subgroup, both butyrate and butyrate-producing bacteria were more enriched in the HC group than in the A-CRA group.

Real-time qPCR

Quantification of the *Bacteroides* genus by qPCR showed a high degree of consistency with pyrosequencing data (Spearman correlation r = 0.889, $P < 0.001$), which indicated that the differences at the genus level between the HC group (n = 47) and the A-CRA group (n = 47) found by pyrosequencing were reliable. Wilcoxon's signed-rank test suggested no significant difference between the 2 methods (P = 0.320), which further

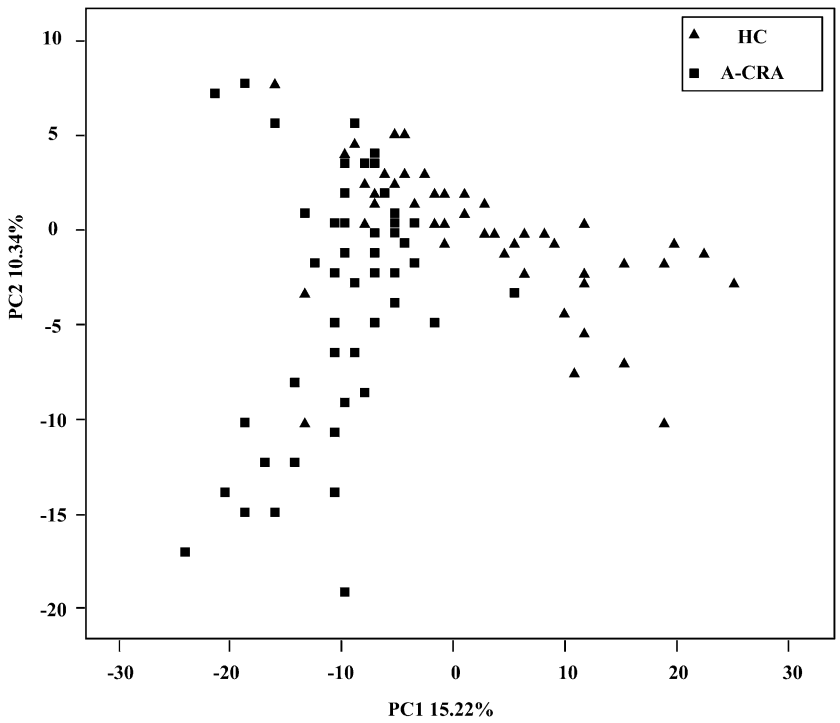

FIGURE 3. PC analysis plots based on the unweighted UniFrac. Separation between the microbiota in the HC (n = 47) and A-CRA (n = 47) groups was significant (P = 0.001, t test of permutation). A-CRA, advanced colorectal adenoma; HC, healthy control; PC, principal component.

TABLE 4
Significant differences in some genera identified between the HC (n = 47) and A-CRA (n = 47) groups[1]

Phylum	Genus	HC (n = 47)		A-CRA (n = 47)		P[4]	Tendency in 2 groups
		Relative contribution[2]	Mean ± SEM[3]	Relative contribution[2]	Mean ± SEM[3]		
		%	%	%	%		
Firmicutes	*Eubacterium*	0.08	0.07 ± 0.02	0.04	0.04 ± 0.02	0.020	HC > A-CRA
Firmicutes	*Roseburia*	2.12	1.99 ± 0.39	1.25	1.22 ± 0.27	0.035	HC > A-CRA
Firmicutes	*Clostridium*	2.17	2.07 ± 0.40	0.89	0.88 ± 0.20	0.001	HC > A-CRA
Firmicutes	*Enterococcus*	2.23	2.17 ± 0.44	3.24	3.75 ± 0.65	0.035	A-CRA > HC
Firmicutes	*Streptococcus*	16.04	14.04 ± 2.28	20.12	21.40 ± 2.93	0.049	A-CRA > HC
Bacteroidetes	*Bacteroides*	0.08	0.07 ± 0.02	0.04	0.04 ± 0.02	0.020	A-CRA > HC

[1] A-CRA, advanced colorectal adenoma; HC, healthy control.
[2] The relative contribution of a genus in the HC or A-CRA group was calculated as a percentage of the sequences of this genus to all sequences in this population.
[3] Values were calculated according to the percentage of the sequences of this genus to all sequences in each individual.
[4] The differences in relative abundances were calculated by using the Mann-Whitney U test. Two-sided P values <0.05 were considered statistically significant.

supported the reliability of pyrosequencing. However for *Bifidobacteria*, the results from pyrosequencing and qPCR displayed inconsistency. Spearman correlation analysis suggested low correlation between the 2 methods (r = 0.232, P = 0.188). Wilcoxon's signed-rank test showed a significant difference ($P < 0.001$) between the pyrosequencing range (quartile 25–quartile 75; 0.0–0.1%) and the qPCR range (quartile 25–quartile 75; 0.2–2.4%), which indicated that the quantification of this group by the pyrosequencing method was lower than that with qPCR.

DISCUSSION

Increasing evidence indicates that the intestinal microbial community plays an important role in the pathogenesis of the progression from A-CRA to CRC. The microbial community affects the fate of dietary fiber and its SCFA products. As far as we know, this is one of the first applications of 454 pyrosequencing of the V1-V3 region of the 16S rRNA gene for evaluation of the fecal microbiota in patients with A-CRA.

We clearly showed that yields of fecal SCFAs, including acetic acid, butyric acid, and propionic acid, were considerably lower in the A-CRA group (n = 344) than in the HC group (n = 344). SCFAs have been shown to prevent the development of colon cancer (4, 33). The higher yields of SCFAs in the HCs might therefore exert protective effects. Logistic regression also showed the protective effects of SCFAs. Furthermore, our results showed that individuals with a diet high in vegetables were less likely to develop A-CRA compared with individuals who ate fewer vegetables. Other studies have also proposed that a diet high in vegetable is associated with a modest reduction in the risk of colorectal adenoma (34).

We also showed that alterations in the structure of fecal microbe communities may contribute to the development of A-CRA. This leads to the hypothesis that the community changes observed between different fecal microbiota in the A-CRA group

TABLE 5
Butyrate and butyrate-producing bacteria in the HC (n = 47) and A-CRA (n = 47) subgroups[1]

Group	No. of subjects	*Clostridium*[2]	*Roseburia*[2]	*Eubacterium*[2]	Butyrate[3]
		%	%	%	%
HC low fiber	11	0.16 ± 0.03	0.79 ± 0.28	0.00 ± 0.00	0.49 ± 0.51
HC high fiber	36	2.65 ± 0.48	2.36 ± 0.48	0.10 ± 0.03	1.00 ± 0.83
P		0.001	0.001	0.001	0.031
A-CRA low fiber	19	0.59 ± 0.16	1.68 ± 0.60	0.05 ± 0.02	0.28 ± 0.15
A-CRA high fiber	28	1.08 ± 0.32	0.91 ± 0.19	0.03 ± 0.02	0.34 ± 0.30
P		0.001	0.026	0.024	0.553
HC low fiber	11	0.16 ± 0.03	0.79 ± 0.28	0.00 ± 0.00	0.49 ± 0.51
A-CRA low fiber	19	0.59 ± 0.16	1.68 ± 0.60	0.05 ± 0.02	0.28 ± 0.15
P		0.280	0.830	0.025	0.210
HC high fiber	36	2.65 ± 0.48	2.36 ± 0.48	0.10 ± 0.03	1.00 ± 0.83
A-CRA high fiber	28	1.08 ± 0.32	0.91 ± 0.19	0.03 ± 0.02	0.34 ± 0.30
P		0.001	0.001	0.001	0.002
P (group × fiber level)		0.045	0.025	0.033	0.048

[1] According to fiber intake, we divided the A-CRA and HC groups into 4 subgroups. In the HC group, the intake of fiber was low (fiber score <3) in 11 subjects and was high (fiber score ≥3) in 36 subjects. In the A-CRA group, 19 subjects had low fiber intake, and 28 subjects had high fiber intake (P = 0.039, χ^2 test). The P value for interaction of group × fiber level was generated by general linear model. $P < 0.05$ is statistically significant. A-CRA, advanced colorectal adenoma; HC, healthy control.
[2] Values are means ± SEMs.
[3] Values are means ± SDs.

($n = 47$) compared with the HC group ($n = 47$) are most likely to lead to A-CRA.

Bacterial metabolites, such as butyrate and other SCFAs, may reduce the risk of CRC (35). Production of butyrate in vivo is mediated largely by the butyrate-producing bacteria. These bacteria fall into 2 classes of the *Firmicute* phylum: clostridial cluster IV, represented by *Faecalibacterium prausnitzii* and *Clostridium* spp. and the Clostridial cluster XIVa, represented by *Eubacterium rectale/Roseburia* spp. (36–38). In our study, the populations of 3 genera of these butyrate-producing bacteria (*Clostridium, Roseburia*, and *Eubacterium*) were lower in the A-CRA group ($n = 47$) than in the HC group ($n = 47$). We therefore speculate that the smaller populations of these genera in A-CRA patients may be related to a reduction in SCFA fermentation in the human gut, which may in turn provide an explanation for the reduced SCFA concentrations in the A-CRA group ($n = 47$). This possibility is supported by 3 studies showing that the population densities of *Roseburia* spp. and *Eubacterium rectale* correlated strongly with fecal butyrate concentrations in response to an altered carbohydrate supply (39–41). Our findings suggest that the *Clostridium, Roseburia*, and *Eubacterium* genera play important roles in protecting hosts from A-CRA by modulating the fermentation of dietary fiber and the production of SCFAs, especially butyrate.

To explore the correlations between SCFAs, A-CRA, dietary fiber, and butyrate-producing bacteria, we also divided the HC ($n = 47$) and A-CRA ($n = 47$) groups into 2 subgroups according to dietary fiber intake. Our results suggest that a persistent deficiency in substrate dietary fiber results in a deficiency in butyrate-producing bacteria followed by fermentation of SCFAs, which in turn would contribute to the formation of A-CRA. When eliminating the effect of dietary fiber intake, the reductions in butyrate-producing bacteria and butyrate might suggest their important role in the occurrence and development of the intestinal advanced adenoma. It is possible, therefore, that changes in the intestinal microenvironment influence the constitution of the intestinal microbiota and SCFA production, which might further promote the development of A-CRA. However, the mechanisms involved and the causal relations require further investigations.

In our study, the structural imbalance in the intestinal microbiota communities in patients with A-CRA was also highlighted by the significant increase in a variety of opportunistic pathogens, represented by the genuses *Enterococcus* and *Streptococcus* spp. Collins et al (42) proposed a hypothesis for the mechanisms underlying the oncogenic potential of bacteria during the progression to CRC. These were suggested to include inflammation and the production of mutagenic toxins by opportunistic pathogens.

We measured both the quantity of dietary fiber and fecal SCFA concentrations to explore the association between dietary fiber and fecal fiber–fermented endproducts (ie, SCFAs). We used a pyrosequencing method to identify bacteria in an uncultured fecal sample to minimize bias that may be incurred by the use of alternative culture-dependent methods. We also attempted to identify possible target fecal microbiota from patients with A-CRA. The case-control subjects in our study had similar lifestyle characteristics and were matched for sex and age, which diminished confounding factors potentially affecting the composition of intestinal microbiota.

One limitation of this study was that the 454 pyrosequencing technique used only identifies bacteria at the genus level; further research is needed to develop a more efficient method for the identification of bacteria at the species level. It also appeared that the current primers were biased against high-GC *Actinobacteria* and *Bifidobacteria*. Further study is required to give a more accurate explanation. In addition, fecal SCFAs were detected in our study because it is clinically feasible and noninvasive. These data need to be interpreted with care because fecal SCFA concentrations, which correlate to not only the formation but also the uptake through the gut, do not necessarily reflect SCFA production.

In conclusion, by comparing the dietary fiber intake, fermentation production of SCFAs, and the intestinal microbiota composition between A-CRA patients and HCs, we have defined a difference in fecal SCFA concentrations and a structural imbalance in the gut microbiota, represented by the reduction of butyrate-produced bacteria and increased incidence of opportunistic pathogens in patients with A-CRA. Our data indicate that the reduced production of fecal SCFAs was the result of decreased dietary fiber intake and structural alteration of gut microbiota in patients with A-CRA. These findings provide evidence that a high-fiber dietary pattern, with subsequent and consistent production of SCFAs, with a healthy gut microbiota results in a reduced risk of A-CRA.

The authors' responsibilities were as follows—J-YF: designed the study; H-MC, Y-NY, J-LW, Y-WL, XK, and WZ: conducted the research; C-QY, LY, Z-JL,Y-ZY, and H-MC: collected the data; H-MC and Y-NY: analyzed the data and wrote the manuscript; and FL, J-XW, LZ, and D-CF: contributed to the data monitoring. All authors read and approved the final content. None of the authors declared a conflict of interest.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69–90.
2. Ji BT, Devesa SS, Chow WH, Jin F, Gao YT. Colorectal cancer incidence trends by subsite in urban Shanghai, 1972-1994. Cancer Epidemiol Biomarkers Prev 1998;7:661–6.
3. Duncan SH, Louis P, Flint HJ. Cultivable bacterial diversity from the human colon. Lett Appl Microbiol 2007;44:343–50.
4. D'Argenio G, Cosenza V, Delle CM, Iovino P, Delle VN, Lombardi G, Mazzacca G. Butyrate enemas in experimental colitis and protection against large bowel cancer in a rat model. Gastroenterology 1996;110:1727–34.
5. Bingham SA. Diet and colorectal cancer prevention. Biochem Soc Trans 2000;28:12–6.
6. Fuchs CS, Giovannucci EL, Colditz GA, Hunter DJ, Stampfer MJ, Rosner B, Speizer FE, Willett WC. Dietary fiber and the risk of colorectal cancer and adenoma in women. N Engl J Med 1999;340:169–76.
7. Macfarlane S, Macfarlane GT. Regulation of short-chain fatty acid production. Proc Nutr Soc 2003;62:67–72.
8. Wong JM, de Souza R, Kendall CW, Emam A, Jenkins DJ. Colonic health: fermentation and short chain fatty acids. J Clin Gastroenterol 2006;40:235–43.
9. Manning TS, Gibson GR. Microbial-gut interactions in health and disease. Prebiotics. Best Pract Res Clin Gastroenterol 2004;18:287–98.
10. Glade MJ. Food, nutrition, and the prevention of cancer: a global perspective. American Institute for Cancer Research/World Cancer Research Fund, American Institute for Cancer Research, 1997. Nutrition 1999;15:523–6.
11. Scheppach W, Weiler F. The butyrate story: old wine in new bottles? Curr Opin Clin Nutr Metab Care 2004;7:563–7.
12. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. Science 2005;308:1635–8.

13. Macfarlane GT, Macfarlane S. Human colonic microbiota: ecology, physiology and metabolic potential of intestinal bacteria. Scand J Gastroenterol Suppl 1997;222:3–9.
14. Winawer SJ, Zauber AG, Fletcher RH, Stillman JS, Obrien MJ, Levin B, Smith RA, Lieberman DA, Burt RW, Levin TR, et al. Guidelines for colonoscopy surveillance after polypectomy: a consensus update by the US Multi-Society Task Force on Colorectal Cancer and the American Cancer Society. CA Cancer J Clin 2006;56:143–59, quiz 184–5.
15. Kiedrowski M, Mroz A, Kaminski MF, Kraszewska E, Orlowska J, Olszewski W, Kupryjanczyk J, Requla J. Proximal shift of advanced adenomas in the large bowel—does it really exist? Pol Arch Med Wewn 2012;122:195–9.
16. Potter JD, Slattery ML, Bostick RM, Gapstur SM. Colon cancer: a review of the epidemiology. Epidemiol Rev 1993;15:499–545.
17. Beresford SA, Johnson KC, Ritenbaugh C, Lasser NL, Snetselaar LG, Black HR, Aderson GL, Assaf AR, Bassford T, Bowen D, et al. Low-fat dietary pattern and risk of colorectal cancer: the Women's Health Initiative Randomized Controlled Dietary Modification Trial. JAMA 2006;295:643–54.
18. Slattery ML, Potter J, Caan B, Edwards S, Coates A, Ma KN, Berry TD. Energy balance and colon cancer—beyond physical activity. Cancer Res 1997;57:75–80.
19. Tjonneland A, Haraldsdottir J, Overvad K, Stripp C, Ewertz M, Jensen OM. Influence of individually estimated portion size data on the validity of a semiquantitative food frequency questionnaire. Int J Epidemiol 1992;21:770–7.
20. Overvad K, Tjonneland A, Haraldsdottir J, Ewertz M, Jensen OM. Development of a semiquantitative food frequency questionnaire to assess food, energy and nutrient intake in Denmark. Int J Epidemiol 1991;20:900–5.
21. Lin J, Zhang SM, Cook NR, Rexrode KM, Liu S, Manson JE, Lee IM, Buring JE. Dietary intakes of fruit, vegetables, and fiber, and risk of colorectal cancer in a prospective cohort of women (United States). Cancer Causes Control 2005;16:225–33.
22. Bingham SA, Day NE, Luben R, Ferrari P, Slimani N, Norat T, Clavel-Chapelon F, Kesse E, Nieters A, Boeing H, et al. Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. Lancet 2003;361:1496–501.
23. Scheppach WM, Fabian CE, Kasper HW. Fecal short-chain fatty acid (SCFA) analysis by capillary gas-liquid chromatography. Am J Clin Nutr 1987;46:641–6.
24. Li M, Wang B, Zhang M, Rantalainen M, Wang S, Zhou H, Zhang Y, Shen J, Pang X, Zhang M, et al. Symbiotic gut microbes modulate human metabolic phenotypes. Proc Natl Acad Sci USA 2008;105:2117–22.
25. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. PLoS ONE 2012;7:e39743.
26. Meyer M, Stenzel U, Hofreiter M. Parallel tagged sequencing on the 454 platform. Nat Protoc 2008;3:267–78.
27. Meyer M, Stenzel U, Myles S, Prufer K, Hofreiter M. Targeted high-throughput sequencing of tagged nucleic acid samples. Nucleic Acids Res 2007;35:e97.
28. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glockner FO. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 2007;35:7188–96.
29. Schloss PD. A high-throughput DNA sequence aligner for microbial ecology studies. PLoS ONE 2009;4:e8230.
30. Wang T, Cai G, Qiu Y, Fei N, Zhang M, Pang X, Jia W, Cai S, Zhao L. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. ISME J 2012;6:320–9.
31. Chen Y, Yang F, Lu H, Wang B, Chen Y, Lei D, Wang Y, Zhu B, Li L. Characterization of fecal microbial communities in patients with liver cirrhosis. Hepatology 2011;54:562–72.
32. Ahmed S, Macfarlane GT, Fite A, McBain AJ, Gilbert P, Macfarlane S. Mucosa-associated bacterial diversity in relation to human terminal ileum and colonic biopsy samples. Appl Environ Microbiol 2007;73:7435–42.
33. Lu R, Wang X, Sun DF, Tian XQ, Zhao SL, Chen YX, Fang JY. Folic acid and sodium butyrate prevent tumorigenesis in a mouse model of colorectal cancer. Epigenetics 2008;3:330–5.
34. Millen AE, Subar AF, Graubard BI, Peters U, Hayes RB, Weissfeld JL, Yohochi LA, Zieqler RG, PLCO Cancer Screening Trial Project Team. Fruit and vegetable intake and prevalence of colorectal adenoma in a cancer screening trial. Am J Clin Nutr 2007;86:1754–64.
35. Scharlau D, Borowicki A, Habermann N, Hofmann T, Klenow S, Miene C, Munjal U, Stein K, Glei M. Mechanisms of primary cancer prevention by butyrate and other products formed during gut flora-mediated fermentation of dietary fibre. Mutat Res 2009;682:39–53.
36. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett 2009;294:1–8.
37. Louis P, Young P, Holtrop G, Flint HJ. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA: acetate CoA-transferase gene. Environ Microbiol 2010;12:304–14.
38. Louis P, Duncan SH, McCrae SI, Millar J, Jackson MS, Flint HJ. Restricted distribution of the butyrate kinase pathway among butyrate-producing bacteria from the human colon. J Bacteriol 2004;186:2099–106.
39. Devillard E, McIntosh FM, Duncan SH, Wallace RJ. Metabolism of linoleic acid by human gut bacteria: different routes for biosynthesis of conjugated linoleic acid. J Bacteriol 2007;189:2566–70.
40. Duncan SH, Belenguer A, Holtrop G, Johnstone AM, Flint HJ, Lobley GE. Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. Appl Environ Microbiol 2007;73:1073–8.
41. Ramirez-Farias C, Slezak K, Fuller Z, Duncan A, Holtrop G, Louis P. Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii. Br J Nutr 2009;101:541–50.
42. Collins D, Hogan AM, Winter DC. Microbial and viral pathogens in colorectal cancer. Lancet Oncol 2011;12:504–12.