The American Journal of Clinical Nutrition

Diet, microbiota, and microbial metabolites in colon cancer risk in rural Africans and African Americans1–4


ABSTRACT

Background: Epidemiologic studies have suggested that most cases of sporadic colon cancer can be attributed to diet. The recognition that colonic microbiota have a major influence on colonic health suggests that they might mediate colonic carcinogenesis.

Objective: To examine the hypothesis that the influence of diet on colon cancer risk is mediated by the microbiota through their metabolites, we measured differences in microbial and metabolites in African Americans with a high risk and in rural native Africans with a low risk of colon cancer.

Design: Fresh fecal samples were collected from 12 healthy African Americans aged 50–65 y and from 12 age- and sex-matched native Africans. Microbiomes were analyzed with 16S ribosomal RNA gene pyrosequencing together with quantitative polymerase chain reaction of the major fermentative, butyrate-producing, and bile acid–deconjugating bacteria. Fecal short-chain fatty acids were measured by gas chromatography and bile acids by liquid chromatography–mass spectrometry.

Results: Microbial composition was fundamentally different, with a predominance of Prevotella in native Africans (enterotype 2) and of Bacteroides in African Americans (enterotype 1). Total bacteria and major butyrate-producing groups were significantly more abundant in fecal samples from native Africans. Microbial genes encoding for secondary bile acid production were more abundant in African Americans, whereas those encoding for methanogenesis and hydrogen sulfide production were higher in native Africans. Fecal secondary bile acid concentrations were higher in African Americans, whereas short-chain fatty acids were higher in native Africans.

Conclusion: Our results support the hypothesis that colon cancer risk is influenced by the balance between microbial production of health-promoting metabolites such as butyrate and potentially carcinogenic metabolites such as secondary bile acids.


INTRODUCTION

There are wide geographic variations in colorectal incidence around the world, and most of these differences have been attributed to diet (1). Within the continental United States, the African American population shoulders the major burden, with an incidence of ~65:100,000 and a death rate of 25:100,000 (2). In sharp contrast, rural Africans rarely get the disease (3). Studies of ours have ascribed this difference to higher meat and fat intakes in Americans and to higher resistant starch intakes in Africans (4).

Colonic microbiota are dependent on dietary residues that escape small intestinal digestion and absorption. Consumption of a normal balanced diet predominantly yields carbohydrate residues such as fiber, which stimulates saccharolytic fermentation and the production of the health-promoting short-chain fatty acids (SCFAs)5 acetate, propionate, and butyrate. Butyrate is the preferred energy source for the colonic mucosa, and all 3 SCFAs have anti-inflammatory and antiproliferative properties (5). Consumption of an unbalanced diet rich in meat and low in fiber increases the delivery of proteinaceous residues, which promote proteolytic fermentation with the production of ammoniac compounds and branched-chain fatty acids, which are inflammatory and may enhance colon cancer risk (5, 6).

The influence of dietary fat on cancer risk may also be determined by microbial metabolism, because it increases the hepatic synthesis of bile acids (BAs) and the quantity of BAs that escape the enterohepatic circulation and enter the colon. This provides substrate for microbes with 7a-dehydroxylating enzymes, which convert primary BA into secondary BAs, which are proinflammatory and have carcinogenic properties (7).

Digestion of food is fundamentally different in the small and large intestine. In the small intestine, the enzymic digestion rate is determined by substrate concentrations, according to the Michaelis-Menten equation. In the colon, the fermentation rate is

5 Abbreviations used: BA, bile acid; CA, cholic acid; LC, liquid chromatography; LCA, lithocholic acid; MS, mass spectrometry; qPCR, quantitative polymerase chain reaction; rRNA, ribosomal RNA; SCFA, short-chain fatty acid.
complex, termed autocatalytic, and is determined by both the substrate concentration and the microbe concentration. In autocatalytic reactions, the maximal rate of reaction occurs at an intermediate, rather than at the highest, reactant concentration (8). Thus, SCFA and secondary BA production is codetermined by the microbiota composition.

To test our hypothesis that the higher risk of colon cancer in African Americans than in native Africans is related to the influence of their diet on the microbiota composition and metabolic activity, we measured the differences in microbiota composition and specific bacteria known to influence SCFA and secondary BA production in fecal samples from these 2 populations.

SUBJECTS AND METHODS

Study design

The relative contents of SCFAs, BAs, and microbes of specific interest were measured in fresh fecal samples from 2 populations of varying colon cancer risk, namely high-risk African Americans (Americans) and low-risk rural Africans (Africans) (3). Middle-aged subjects were selected because colon cancer affects that group most. Microbial analysis was first untargeted, based on high-throughput 16S ribosomal RNA (rRNA) pyrosequencing, and secondly targeted based on quantitative polymerase chain reaction (qPCR) to measure numbers of microbes of specific interest, which included the major butyrate producers Faecalibacterium prausnitzii, Clostridium cluster IV, and XIVa (9); the major starch fermenters Succinivibrio spp. and Prevotella spp. (10); the Bacteroides fragilis group, lactic acid bacteria, and Lactobacillus spp. (11); and Bifidobacterium spp. (12). Finally, a functional gene analysis was performed to compare the potential for butyrogenesis, methanogenesis, hydrogen sulfide production, and secondary bile salt conversion.

Study populations

Normal healthy volunteers of either sex aged 50–65 y were selected on the basis of their medical history and results of a medical examination. African Americans rather than white Americans were chosen because their risk of colon cancer is higher and there is more genetic similarity. Subjects with a history of gastrointestinal disease or surgery were excluded, as were those with a history of antibiotic use within the past 2 wk. First-morning fecal samples were collected from 12 healthy Americans in the Pittsburgh area and from 12 age-, sex-, and BMI-matched Africans from a rural area outside the town of Empangeni in the KwaZulu-Natal Province of South Africa (Table 1). Our earlier study showed that the dietary intake patterns of these 2 populations were widely different, with Americans consuming twice as much protein and 3 times as much fat [mean (±SEM) protein: 94 ± 9 compared with 58 ± 4 g/d; mean (±SEM) fat: 114 ± 11 compared with 38 ± 3 g/d] (4). The protocol was reviewed and approved by the Institutional Review Boards of the University of Pittsburgh and the University of KwaZulu-Natal (Biomedical Research Ethics Committee).

Materials

All BA and SCFA analytic chemicals were purchased from Sigma-Aldrich. qPCR primers for different bacteria were synthesized by Sigma-Aldrich. qPCR master mix was purchased from Applied Biosystem. An Econo-Cap EC-1000 gas chromatographic capillary column was purchased from Grace Davison Discovery Science. Millex-GS 0.22-μm syringe filters were purchased, and microconcentrators were purchased from Millipore. The Luna C18 column (3 μm, 2.0-mm internal diameter × 150 mm) was purchased from Phenomenex.

Fecal collection, preservation, and transport

Freshly voided fecal samples were collected immediately into airtight plastic vials and were transported on ice to be stored frozen at −80°C within 2 h. The samples collected in South Africa were air-couriered frozen on dry ice to Pittsburgh for analysis.

SCFA assay

Fecal samples (0.1 g) were transferred into plastic tubes; 2,2-dimethylbutyric acid was added at 1 mmol/L as internal standard. After undergoing vortex mixing and centrifugation (1900 × g, 10 min), the supernatant fluid was filtered through a Millex-GS 0.22-μm syringe filter unit (Millipore). The solution was refiltered through a microconcentrator (Ultracel YM-10; Millipore) with a molecular mass cutoff of 10,000 Da, by centrifugation (7000 g for 1.5 h). The filtrate was then analyzed by using an Agilent Technologies 6890N Network GC System with a flame-ionization detector for SCFA based on the method described by Scheppach et al (23). Compounds were separated on a Grace EC-1000 (15 m in length, 1.20-μm film thickness, 0.53 mm internal diameter) capillary column (Grace Davison Discovery Science). The oven temperature of the gas chromatograph was programmed at 5 min from 80°C to 175°C, which was held for 10 min with a total running time of 25 min. The temperatures of both the detector and injector were 200°C. The inlet was operated in a splitless mode. High-purity helium was used as carrier gas.

A mixed-SCFA standard solution was prepared by using high purity (>99%) reagents (Sigma). SCFA concentrations were computed by using a peak area ratio of the sample profile relative to the internal standard. A good linear correlation was found between the peak area ratio and the corresponding standard SCFA ($r^2 > 0.99$ for all SCFAs). The interday and intraday CVs ranged from 2.4% to 3.9%. This method does not separate 2-methylbutyric and isovaleric acids.

BA assay

Fecal BA concentrations were measured by using the method described by Tagliacozzi et al (24), except that quantification was carried out by using liquid chromatography (LC)—mass spectrometry (MS) as opposed to LC-tandem MS. A 125-μL colonic evacuete was mixed with 400 μL acetonitrile, followed by 1 min of vortex mixing. After 15 min of centrifugation at 13,000 × g, 450 μL supernatant fluid was transferred to an autosampler vial and blown to dryness with nitrogen. The residue was dissolved with 125 μL methanol and water (1:1). Ten microliters of this solution was injected into a Shimadzu HPLC-MS (model 2010A) for quantification by using electrospray ionization in
negative ion mode by monitoring the (M-H)$^-$ ion. The analytic conditions for LC-MS were as follow: column, Luna 3u, C18, 100Å (2.0-mm internal diameter × 150 mm; Phenomenex); mobile phase A: 20% acetonitrile-water containing 10 mmol ammonium acetate/L; mobile phase B: 80% acetonitrile-water, gradient program; mobile phase B: 0–6 min 15%, 20 min 30%, 30 min 60%, 40 min 80%, 45 min 15%; flow rate: 0.2 mL/min; column temperature: 40°C; probe voltage: 4.5 kv; curved desolvation line temperature: 230°C. BA concentrations were calculated based on standard curves run with each sample set.

Microbial identification

DNA isolation

Fecal bacterial DNA was isolated and purified with the QIAamp DNA Stool Mini Kit (Qiagen) in combination with a bead-beating step (30 s at 30 Hz 3 times) by using the FastPrep-24 System (MP Biomedicals), as described by Zoetendal et al (25).

Microbiota composition

Samples for 454 FLX pyrosequencing were amplified with universal forward 519F (5′-Fusion A-Barcode -CAGCMGCC-GCGTGATATWC-3′) and reverse 926R (5′-Fusion B-Barcode-CGGTCAATTCMTTTRAGTT-3′) primer pairs (Roche). PCR reaction mixtures were set up with the TopTaq PCR kit (Qiagen) according to the manufacturer’s recommendations with 10 pmol/L, each of forward and reverse primers in 25 μL reaction. Amplification was carried out with 30 cycles of thermal program (denaturation, 95°C for 30 s; annealing, 55°C for 45 s; and extension, 72°C for 60 s). All amplicons were gel-excised, concentrated, and purified with the Gel Extraction kit (Qiagen), and

<table>
<thead>
<tr>
<th>Target group and primer</th>
<th>Sequence (5′-3′)</th>
<th>Annealing temperature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>All bacteria</td>
<td></td>
<td></td>
<td>60 (13)</td>
</tr>
<tr>
<td>Uni531F</td>
<td>TCCTACGGGAGGCAGCAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uni597R</td>
<td>GGACTACCAGGGTACTTCTTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyrate-production gene (BcoA)</td>
<td></td>
<td>60 (14)</td>
<td></td>
</tr>
<tr>
<td>BcoA-F</td>
<td>GCIGAICATTTTGCAGAGTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BcoA-R</td>
<td>CTTGCTGTTGACAAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium cluster IV$^1$</td>
<td></td>
<td>50 (15)</td>
<td></td>
</tr>
<tr>
<td>Sg-Clept-F</td>
<td>GCACAAGCAGTTGAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sg-Clept-R</td>
<td>CTTCCTCGTGTTCAGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium cluster XI$^1$</td>
<td></td>
<td>50 (15)</td>
<td></td>
</tr>
<tr>
<td>g-CocF</td>
<td>AAATGACGGTACCTGACTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g-Coc-R</td>
<td>CTTTGAGTTCATTCTTCCGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td></td>
<td></td>
<td>60 (16)</td>
</tr>
<tr>
<td>Bif164F</td>
<td>GGGTTGTAATGGCGGAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bif662R</td>
<td>CCACCGTTCACCGGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinivibrio</td>
<td></td>
<td></td>
<td>60 (17)</td>
</tr>
<tr>
<td>SuccDeX1F</td>
<td>CGTCAGCTCGTGCTTGAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SuccDeX1R</td>
<td>CCCGGTGCGCAGCAGCAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prevotella spp.</td>
<td></td>
<td></td>
<td>60 (17)</td>
</tr>
<tr>
<td>PreGen4F</td>
<td>GGTTCCTGAGGAAGGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PreGen4R</td>
<td>TCTTCACGACTACTTCTTCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroides fragilis group</td>
<td></td>
<td>60 (15)</td>
<td></td>
</tr>
<tr>
<td>g-Bfra-F</td>
<td>ATAGCCTCTTCGAAAGRAAGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g-Bfra-R</td>
<td>CCGATATCAACTGCAATTTTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td></td>
<td></td>
<td>60 (18)</td>
</tr>
<tr>
<td>LactoF</td>
<td>TGGAAAACAGRTGCATAATACCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LactoR</td>
<td>GTCCAATTGGAAAGATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faecalibacterium prauntrizii</td>
<td></td>
<td>60 (19)</td>
<td></td>
</tr>
<tr>
<td>FpF</td>
<td>CCCCCATCTGCGGCGACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FpR</td>
<td>GTCGCAGGATGTTCAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Functional gene for hydrogen sulfide</td>
<td></td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>DSR1F+</td>
<td>ASCACCTGGAAAGCACGCGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSR-R</td>
<td>GTGGMRCCGTCGAKRTHG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Functional gene for methanogenesis</td>
<td></td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>mcrA-F</td>
<td>TTTGCTTGGATCDARAGRC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mcrA-R</td>
<td>GBARTGCGWACCGTAGAATCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Functional gene for secondary bile acids</td>
<td></td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>BaiCD-F</td>
<td>CAGCCCCRCAGATGTTCCTTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BaiCD-R</td>
<td>GCATGGAATTCWACTGCTYCTC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$Number indicates phylogenetic cluster of Clostridium as defined by Collins et al (22).

$^2$J Ou, A Heather, J Riddlon, S Curry, and SJD O’Keefe, April 2012.
amplicon concentrations were measured with a Qubit fluorometer (Invitrogen). A 454 FLX Titanium was used for 454 pyrosequencing (454 Life Sciences; Roche Applied Science). The paired-end pyrosequencing services were provided by Roy J Carver Biotechnology Center, University of Illinois. A total of 248,594 16S rRNA sequences (also referred to as 16S pyrotags) were obtained from the 454 Titanium pyrosequencing run. The 16S pyrotags were sorted based on their respective barcodes and handled by using the QIIME pipeline (26). RDP Classifier was used for taxonomic assignments of the aligned 16S pyrotags at the 95% confidence level (27).

Real-time quantitative PCR
qPCR was performed with a 7900HT Fast Real-Time PCR System (Applied Biosystem). 16S rRNA gene-specific primers were used to target total and specific bacteria (F. prausnitzii, Clostridium cluster IV and XIVa, Lactobacillus spp., Succinivibrio spp., Prevotella spp., B. fragilis group, and Bifidobacterium spp.) (Table 1). Cloned 16S rRNA genes were used to construct standard curves: Clostridium leptum 29065 (representing Clostridium cluster IV), C. coccoides (representing Clostridium cluster XIVa), Clostridium s. sordidus, Biﬁdobacterium longum 15707, Lactobacillus delbrueckii 12315, Succinivibrio dextrinosolvens 19716, Prevotella ruminicola 19189, B. fragilis 25285, and F. prausnitzii 27766 (also a member of Clostridium cluster IV) (obtained from the American Type Culture Collection) were cultured on reinforced clostridial medium broth and incubated at 37°C in an anaerobic chamber. Genomic DNA was extracted from a 2-mL culture by using the QIAamp DNA stool mini kit (Qiagen), and bacterial 16S rRNA genes were amplified with their respective primers (Table 2). PCR products were purified by using the MinElute PCR purification Kit (Qiagen) and cloned into pCR 2.1 TOPO vector with a TOPOTA cloning kit (Invitrogen). Plasmid DNA was isolated with a QIAprep Spin Miniprep kit (Qiagen), and plasmid DNA concentrations were measured spectrophotometrically (NanoDrop 1000; Thermol Scientiﬁc). The number of target gene copies was calculated from the mass of DNA with consideration of the size of insert and plasmid. The plasmid standard for sulfate-reducing bacteria was obtained from a previous study (28).

Functional microbial genes
In the context of microbial metabolite production, analysis of functional genes rather than taxonomic groups based on the 16S rRNA gene allows better quantification (29). Thus, we also examined differences in the abundance of genes encoding the enzymes responsible for butyrate production, secondary BA synthesis, methanogenesis, and hydrogen sulfide production. The butyryl-coenzyme-A-CoA transferase (BcoA) gene was used for quantification of butyrate producers (14). For secondary BA conversion potential, we measured the baiCD gene, which encodes the enzyme that dehydroxylates the 7α-hydroxy group in primary BAs to form secondary BAs (30). To compare methanogenic potential, we measured the gene that encodes the enzyme methyl coenzyme-M reductase (mcrA), which catalyzes the crucial removal of hydrogen produced from fermentation into methane (31). Finally, we measured the gene encoding the enzyme dissimilatory (bi)sulfite reductase (dsrA) that catalyzes a step in the reduction of inorganic sulfate to hydrogen sulfide (32).

The primer sequences are listed in Table 1. All PCR experiments were done in triplicate with a reaction volume of 10 μL by using MicroAmp optical 384-well reaction plates sealed with MicroAmp optical adhesive film (Applied Biosystems). Each reaction contained 5 μL 2X Power SYBR Green PCR Master mix (Applied Biosystems), 1 μL bovine serum albumin (New England Biolabs) at 1 mg/mL (final concentration: 100 μg/mL), 0.5 μM of each primer, and 2 μL template DNA. The cycling conditions were as follows: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s, primer-specific annealing temperature (Table 1) for 20 s, and 72°C for 45 s. After amplification, a dissociation step was included to analyze the melting profile of the amplified products. Ten-fold dilution series of the plasmid standard for the respective bacterial group or species were run along with the samples. Sample DNA concentrations were calculated by using standard curves of the diluted standards containing the respective gene target for each set of primers. Data analysis was processed with SDS v2.3 software supplied by Applied Biosystems.

Statistical analysis
Statistical analysis was conducted by using SPSS 16.0 (SPSS Inc). The significance of group differences for normally distributed data was assessed with Student’s t test. The non-parametric data were analyzed with a Mann-Whitney U test. The significance of the association was evaluated with Spearman’s rank correlation test. A level of P ≤ 0.05 was accepted as statistically significant. Data are presented as means ± SEs. Pyrosequencing data were analyzed by several multivariate ordinations (principal component analyses, nonmetric multidimensional scaling), Kruskal-Wallis independent tests, and multivariate ANOVA with Bonferroni correction.

### RESULTS
Body weight and BMI were similar between the 2 groups (Table 2). Four of the 12 Americans were obese [ie, BMI (in kg/m²) >30], as were 5 of the 12 Africans. The composition of the fecal microbiota, as shown by pyrosequencing analysis, was fundamentally different between Africans and Americans, as summarized in Figure 1. Nonmetric multidimensional scaling showed an unequivocal distinction (multivariate ANOVA: P < 0.01) between the 2 groups based on their fecal microbiota (Figure 1). This difference appeared to override interindividual differences between the groups shown in Figure 2. The most distinct feature was a predominance of Prevotella species in most Africans and Bacteroides in most Americans (Figure 2). Thus, Africans correspond to enterotype 1 and Americans to enterotype 2. Africans also had higher proportions

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Demographic data of the research subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td>Native Africans</td>
<td>8</td>
</tr>
<tr>
<td>African Americans</td>
<td>9</td>
</tr>
</tbody>
</table>

1 Mean ± SEM (all such values).
of Succinivibrio and Oscillospira—microbes that might be involved, such as Prevotella—in starch, hemicellulose, and xylan degradation (Figure 3) (33, 34). The African gut microbiota were also characterized by a large number of sequences that could not be affiliated to reference taxa. Less than one-third of the taxa was detected in comparable abundance between the groups. The American gut microbiota were characterized by a greater abundance of potentially pathogenic proteobacteria (Escherichia, Acinetobacter). Interestingly, the American gut microbiota were more diverse (Simpson index: 0.7 compared with 0.8; \( P = 0.04 \)), which presumably reflects consumption of a more diversified diet.

The targeted qPCR analysis showed that total bacteria, Prevotella spp., Succinivibrio spp., F. prausnitzii, Clostridium cluster IV, and Clostridium cluster XIVa bacterial counts were all significantly higher in the fecal samples from Africans, but Lactobacillus spp. were more abundant in Americans (Table 3). With regard to functional gene analysis, the gene-encoding secondary BA production (baiCD) was detected in greater abundance in African Americans, whereas the functional genes targeting butyrate producers (BcoA), methane producers (mcrA), and hydrogen sulfide producers (dsrA) were more abundant in native Africans.

The chief SCFA products of saccharolytic fermentation, acetate, propionate, and butyrate were significantly higher in stool samples from Africans (\( P, 0.05 \); Figure 4), whereas the products of proteolytic fermentation, namely the branched SCFAs isobutyric and 2-methylbutyric/isovaleric acids, were...
significantly higher in Americans (isobutyrate: 1.73 ± 0.26 compared with 1.22 ± 0.24 μmol/g feces, \( P = 0.02 \); 2-methylbutyric/isovaleric acid: 1.49 ± 0.19 compared with 0.33 ± 0.19 μmol/g feces, \( P = 0.0002 \); Mann-Whitney \( U \) test).

To assess the relation between production of SCFAs and the abundance of specific microbial groups, correlation analyses of fecal components were performed. The results showed that the total bacterial abundance was significantly correlated (\( P < 0.05 \);

![Image](https://example.com/image.png)

**FIGURE 3.** Illustration of the similarities and differences in fecal microbial taxa between AAs and NAs. The solid circle encloses the taxa that were detected in NA and the dotted circle those identified in AA. The overlap between the 2 circles contains taxa common to both populations. Much of the shared taxa were significantly (independent Kruskal-Wallis tests) more enriched in one group than in the other, indicated by the boxed text in AAs on the left and underlined text in NAs on the right. AA, African American; NA, native African.

### TABLE 3

<table>
<thead>
<tr>
<th></th>
<th>Native African</th>
<th>African American</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>gene copies/g feces</td>
<td>gene copies/g feces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bacteria</td>
<td>( 7.1 \times 10^{10} \pm 4.5 \times 10^{10} )</td>
<td>( 1.8 \times 10^{10} \pm 6.3 \times 10^{10} )</td>
<td>0.046</td>
</tr>
<tr>
<td>Butyrate-production gene (BcoA)</td>
<td>( 5.4 \times 10^{10} \pm 1.1 \times 10^{10} )</td>
<td>( 1.4 \times 10^{10} \pm 2.7 \times 10^{9} )</td>
<td>0.022</td>
</tr>
<tr>
<td>Faecalibacterium prausnitzii</td>
<td>( 1.6 \times 10^{10} \pm 2.7 \times 10^{8} )</td>
<td>( 8.3 \times 10^{9} \pm 2.7 \times 10^{8} )</td>
<td>0.038</td>
</tr>
<tr>
<td>Clostridium cluster IV</td>
<td>( 5.1 \times 10^{7} \pm 8.7 \times 10^{6} )</td>
<td>( 2.9 \times 10^{8} \pm 7.3 \times 10^{6} )</td>
<td>0.052</td>
</tr>
<tr>
<td>Clostridium cluster XIVa</td>
<td>( 9.5 \times 10^{9} \pm 1.7 \times 10^{9} )</td>
<td>( 5.1 \times 10^{9} \pm 9.3 \times 10^{8} )</td>
<td>0.049</td>
</tr>
<tr>
<td>Succinivibrio spp.</td>
<td>( 1.1 \times 10^{7} \pm 6.4 \times 10^{5} )</td>
<td>( 4.5 \times 10^{6} \pm 1.3 \times 10^{6} )</td>
<td>0.021</td>
</tr>
<tr>
<td>Prevotella spp.</td>
<td>( 8.2 \times 10^{7} \pm 1.4 \times 10^{7} )</td>
<td>( 3.5 \times 10^{10} \pm 6.4 \times 10^{6} )</td>
<td>0.011</td>
</tr>
<tr>
<td>Bacteroides fragilis group</td>
<td>( 5.7 \times 10^{7} \pm 1.6 \times 10^{6} )</td>
<td>( 4.4 \times 10^{10} \pm 1.3 \times 10^{7} )</td>
<td>0.177</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>( 5.9 \times 10^{10} \pm 3.4 \times 10^{8} )</td>
<td>( 7.1 \times 10^{7} \pm 5.7 \times 10^{7} )</td>
<td>0.021</td>
</tr>
<tr>
<td>Butyrobacterium spp.</td>
<td>( 6.8 \times 10^{10} \pm 2.8 \times 10^{7} )</td>
<td>( 3.0 \times 10^{9} \pm 1.2 \times 10^{8} )</td>
<td>0.291</td>
</tr>
<tr>
<td>Hydrogen sulfide producers</td>
<td>( 1.1 \times 10^{10} \pm 1.5 \times 10^{8} )</td>
<td>( 4.1 \times 10^{6} \pm 1.4 \times 10^{6} )</td>
<td>0.048</td>
</tr>
<tr>
<td>Methane producers</td>
<td>( 3.1 \times 10^{7} \pm 2.1 \times 10^{6} )</td>
<td>UDL</td>
<td></td>
</tr>
<tr>
<td>Secondary bile acid producers</td>
<td>( 2.2 \times 10^{7} \pm 5.3 \times 10^{6} )</td>
<td>( 4.7 \times 10^{8} \pm 1.6 \times 10^{8} )</td>
<td>0.037</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SEMs. \(^*\) Mann-Whitney \( U \) test; the sample number was 12 for each group. 
\(^2\) UDL, under detection limit.
butyrate (Mann-Whitney concentrations were significantly greater in NAs (centrations of the major short-chain fatty acids in fecal samples. Con-
pionate (\(r^2 = 0.047\)). A significant correlation was also found between 7
Africans (Figure 6). The ratio of butyrate to LCA was signifi-
acid) were significantly higher (\(r^2 = 0.29, P = 0.047\)) and
lithocholic acid (LCA, formed from CA and chenodeoxycholic
primary BAs cholic acid (CA) and chenodeoxycholic acid and
the secondary BAs deoxycholic acid (formed from CA) and

Table 4) with concentrations of stool acetate (\(r^2 = 0.38\)), propionate (\(r^2 = 0.33\)), and butyrate (\(r^2 = 0.37\)) (Figure 5). Second,
stool butyrate concentrations were significantly correlated with
the abundance of the butyrate producers Clostridium cluster IV (\(r^2 = 0.29, P = 0.047\)) and Clostridium cluster XIVa (\(r^2 = 0.29, P = 0.047\)) (Table 4).

Amounts of the 4 major BAs in feces were significantly higher
\((P < 0.05)\) in Americans than in Africans (Figure 6). The fecal
primary BAs cholic acid (CA) and chenodeoxycholic acid and
the secondary BAs deoxycholic acid (formed from CA) and
lithocholic acid (LCA, formed from CA and chenodeoxycholic
acid) were significantly higher \((P < 0.05)\) in Americans than in
Africans (Figure 6). The ratio of butyrate to LCA was signifi-
cantly higher in Africans \((39.2 \pm 19.0\) compared with 6.0 ± 3.2;
\(P = 0.047\)). A significant correlation was also found between 7α-
dehydroxylating bacteria and secondary BA concentrations in
stool \((r^2 = 0.65, P = 0.01;\) Spearman’s test) as well (Figure 7),
which likely indicated a higher microbial 7α-dehydroxylating
capacity for colonic BAs in the Americans.

DISCUSSION

The findings of the current study support the hypothesis that
the balance between health-promoting and inflammatory mi-
rogen metabolites may determine colon cancer risk and that
their production is dependent on both the composition of food
and composition of the microbiota, as in an autocatalytic system. The
data provide evidence that saccharolytic fermentation was lower,
proteolytic fermentation was higher, and secondary BA pro-
duction was higher in African Americans than in Africans. From
our earlier studies in the same populations, it is reasonable to
postulate that these differences can be ascribed to differences in
diet, with African Americans eating more dietary meat and fat
and less complex carbohydrate and fiber (4). Fecal microbial composition was shown to be very different in
Africans and African Americans, with the pattern in the former
corresponding to enterotype 2 and the latter to enterotype 1.

Three enterotypes were recently described by a European con-
nsortium based on their pooled analysis of fecal samples obtained
from healthy adults from 4 European countries, the United States,
and Japan (10). From this, they proposed that all human pop-
ulations could be categorized into 1 of 3 enterotypes depending on
their common networks of co- and anticorrelating genera, which
are driven by the genus Bacteroides (type 1), Prevotella (type 2),
or Ruminococcus (type 3). Interestingly, the composition in our
African sample was remarkably similar to that shown in Central
African children from Burkina Faso by De Filippo et al (35),
dominated by Prevotella (type 2) and showing an overabundance of
microbes that are likely involved in starch and cellulose de-
gregation. They related these differences to differences in diet,
with African children consuming a diet rich in coarse grains and
vegetables that was somewhat similar to what Burkitt described as
the “traditional African diet” that was associated with a low risk
of colon cancer in the African child—such as in our study; the authors proposed that this might
help reduce the risk of enteric infections.

Interestingly in the current study, whereas the higher propor-
tions of Prevotella, Succinivibrio, and Oscillospira in the
African gut microbiome also represented higher numbers of these microbes in stool samples, as measured by qPCR,
the higher compositional representation of bacteroides in African
Americans did not appear to reflect higher numbers. It is pos-
sible that the total numbers of bacteroides were underestimated
because the qPCR assay used targeted the B. fragilis group and
not all human Bacteroides strains. On the other hand, the assay
does include the 2 major human gut species, namely B. fragilis
and Bacteroides thetaiotaomicron (15). Presumably, the greater
numbers of total bacteria in the Africans was a consequence of
the expansion of populations of microbes that degrade starches,
hemicelluloses, and xylans—notably Prevotella, Succinivibrio,
and Oscillospira—and those that ferment their products.

Our targeted microbe analysis showed that both the butyrate
production gene and the recognized major butyrate-producing
bacteria, including F. prausnitzii, and those contained within
Clostridium cluster IV and cluster XIVa were more abundant
in stool samples from native Africans. Evidence that these

| Sample number = 24. SCFA, short-chain fatty acid.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total SCFAs</td>
<td>Acetic acid</td>
<td>Propionic acid</td>
<td>Butyric acid</td>
</tr>
<tr>
<td>R</td>
<td>P</td>
<td>R</td>
<td>P</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>0.37</td>
<td>0.111</td>
<td>0.38</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>−0.23</td>
<td>0.112</td>
<td>−0.27</td>
</tr>
<tr>
<td>Faecalbacterium prausnitzii</td>
<td>−0.28</td>
<td>0.215</td>
<td>−0.17</td>
</tr>
<tr>
<td>Clostridium IV</td>
<td>0.26</td>
<td>0.074</td>
<td>0.25</td>
</tr>
<tr>
<td>Clostridium XIVa</td>
<td>0.26</td>
<td>0.074</td>
<td>0.27</td>
</tr>
</tbody>
</table>
differences were of functional significance was provided by our observation of positive correlations between the abundance of butyrate producers and butyrate concentrations in stool samples. Early cultural and molecular studies from Flint’s group (37, 38) in Aberdeen, Scotland, indicated that the most numerous butyrate-producing bacteria found in human feces were highly oxygen-sensitive anaerobes belonging to the Clostridium clusters IV and XIVa. Their studies also highlighted cross-feeding between bacteria, with most butyrate producers consuming acetate produced by other microbes. Higher rates of fermentation in Africans might also have been facilitated by the higher numbers of the hydrogen-utilizing microbes, ie, sulfate reducers and methanogens, measured in stool samples, because it has been shown that the removal of the end product hydrogen increases fermentation potential (39).

Butyrate, propionate, and acetate have all been shown in experimental models to have antineoplastic properties, but butyrate appears to be the most potent (5). Butyrate has a unique role in the maintenance of colonic mucosal health: first, because of its position as the preferred energy source, and second, because of its protean antineoplastic properties. A wealth of experimental evidence, recently reviewed by ourselves (40, 41), indicates the inhibitory effect of butyrate on tumorigenesis, possibly mediated by its antiinflammatory and immunomodulatory effects and downregulation of the key canonical Wnt-signaling pathway linked to colonic carcinogenesis (42). Butyrate may also play a role in primary prevention through the activation of different drug-metabolizing enzymes. This can reduce the burden of carcinogens, such as BAs, and therefore decrease the number of mutations, which reduces cancer risk. Experimental studies have shown that it regulates colonic epithelial growth and protects against carcinogenesis by inhibiting the proliferation and migration of neoplastic cells, restricting tumor angiogenesis, inducing apoptosis, and promoting differentiation of the neoplastic colonocytes (43–49).

FIGURE 5. The abundance of total bacteria correlated significantly (Spearman’s test) with the concentrations of acetate (□, dashed and dotted line; $r^2 = 0.38, P = 0.033$), propionate (▲, dashed line; $r^2 = 0.33, P = 0.039$), and butyrate (×, continuous line; $r^2 = 0.37, P = 0.042$) in fecal samples from African Americans and native Africans ($n = 24$).

FIGURE 6. Contents of major bile acids in feces were significantly lower in NAs ($n = 12$) than in AAs ($n = 12$): Mann-Whitney $U$ test for CA ($P = 0.027$), CDCA ($P = 0.016$), DCA ($P = 0.043$), and LCA ($P = 0.031$). *$P < 0.05$ compared with AA. AA, African American; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; NA, native African.

Food residues from an unbalanced diet deficient in fiber and high in meat promotes proteolytic rather than saccharolytic fermentation, with generation of the branched-chain SCFAs isobutyrate, isovaleric, and 2-methylbutyric acid (51, 52) and nitrogenous metabolites. Whereas little is known about the functional significance of branched SCFAs, proteolytic products such as hippurate, $p$-cresyl, ammonia, and phenols as well as sulfide metabolites have been shown to be inflammatory and carcinogenic in experimental models (53, 54). Thus, the lower risk of colon cancer in Africans could also be a consequence of lower proteolytic fermentation. We have also hypothesized that the high meat content of Westernized diets may increase colon cancer risk because of its stimulatory effect on sulfate-reducing
bacteria, which use the sulfur residues of meat to release hydrogen sulfide, which has been shown to be genotoxic in experimental models (54). Surprisingly, the opposite was observed, with higher numbers of sulfate-reducing bacteria in Africans, which suggests that increased saccharolytic fermentation with increased hydrogen production may be more stimulatory to these hydrogenotrophs.

Strong epidemiologic evidence links high fat consumption to increased colon cancer risk (55, 56). Whereas experimental evidence indicates that fat, particularly fat high in n-6 fatty acids, is proinflammatory and that a Westernized diet high in fat and deficient in vitamin D increases colonic tumors in normal mice (57), fat digestion and absorption are very efficient and little dietary fat enters the colon, which suggests that other mechanisms may be involved. Perhaps the strongest contender is BAs. Increased fat consumption stimulates the liver to synthesize more BAs. This in turn increases the quantity of BAs that escape enterohepatic recirculation and enter the colon. Once in the colon, primary BAs, notably CA and chenodeoxycholic acid, are converted to secondary BAs, namely deoxycholic acid and LCA, by specific bacteria that contain 7α-dehydroxylating enzymes. Our studies showed that not only were microbes containing this enzyme more common in high-risk African Americans, but so were fecal secondary BAs. Human studies have associated this enzyme more common in high-risk African Americans, but so were fecal secondary BAs. Human studies have associated higher numbers of sulfate-reducing bacteria in Africans, which suggests that increased saccharolytic fermentation with increased hydrogen production may be more stimulatory to these hydrogenotrophs.

In summary, our study supports the hypothesis that colon cancer risk is determined by the interaction between diet and gut microbiota, which are key factors in the pathogenesis of colon cancer. Further studies are needed to assess the functional significance of other differences noted in the pyrosequencing analysis (eg, Bacteroides) and the responsiveness of the identified microbial and metabolic differences to dietary change.

**REFERENCES**

21. Denman SE, Tomkins NW, McSweeney CS. Quantitation and diversity analysis of ruminal methanogenic populations in response to the...