

Diet, microbiota, and microbial metabolites in colon cancer risk in rural Africans and African Americans^{1–4}

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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 colonic microbes and their 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. *Am J Clin Nutr* 2013;98:111–20.

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 antiinflammatory 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 7 α -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

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³ Supported by Public Health Service grant CA-135379 from the NIH.

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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.

Received December 19, 2012. Accepted for publication April 5, 2013.

First published online May 29, 2013; doi: 10.3945/ajcn.112.056689.

complex, termed autocatalytic, and is determined by using 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 6 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 (\pm SEM) protein: 94 ± 9 compared with 58 ± 4 g/d; mean (\pm 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- μ m syringe filters were purchased, and microconcentrators were purchased from Millipore. The Luna C₁₈ column (3 μ m, 2.0-mm internal diameter \times 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 ($1900 \times g$, 10 min), the supernatant fluid was filtered through a Millex-GS 0.22- μ m 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 ($7000 \times g$ at 4°C 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- μ m film thicknesses, 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- μ L colonic evacuate was mixed with 400 μ L acetonitrile, followed by 1 min of vortex mixing. After 15 min of centrifugation at $13,000 \times g$, 450 μ L supernatant fluid was transferred to an autosampler vial and blown to dryness with nitrogen. The residue was dissolved with 125 μ L 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

TABLE 1

Real-time quantitative polymerase chain reaction primers used to determine the density of specific microbes and functional microbial genes

Target group and primer	Sequence (5'-3')	Annealing temperature	Reference
All bacteria		°C	
Uni331F	TCCTACGGGAGGCAGCAGT	60	(13)
Uin797R	GGACTACCAGGGTATCTATCCTGTT		
Butyrate-production gene (<i>Bcoa</i>)		60	(14)
Bcoa-F	GCIGAICATTTCACITGGAAAYWSITGGCAYATG		
Bcoa-R	CCTGCCTTTGCAATRTCIACRAANGC		
<i>Clostridium</i> cluster IV ¹		50	(15)
Sg-Clept-F	GCACAAGCAGTGGAGT		
Sg-Clept-R3	CTTCTCCGTTTTGTCAA		
<i>Costridium</i> cluster XIVa ¹		50	(15)
g-Ccoc-F	AAATGACGGTACCTGACTAA		
g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA		
<i>Bifidobacterium</i> spp.		60	(16)
Bif164F	GGGTGGTAATGCCGGATG		
Bif662R	CCACCGTTACACCGGGAA		
<i>Succinivibrio</i>		60	(17)
SucDex1F	CGTCAGCTCGTGCTGAGAGA		
SucDex1R	CCCGCTGGCAACAAAGG		
<i>Prevotella</i> spp.		60	(17)
PreGen4F	GGTTCTGAGAGGAAGGTCCCC		
PreGen4R	TCCTGCACGCTACTTGGCTG		
<i>Bacteroides fragilis</i> group		60	(15)
g-Bfra-F	ATAGCCTTTTCGAAAAGRAAGAT		
g-Bfra-R	CCAGTATCAACTGCAATTTTA		
<i>Lactobacillus</i> spp.		60	(18)
LactoF	TGGAAACAGRTGCTAATACCG		
LactoR	GTCCATTGTGGAAGATTCCC		
<i>Faecalibacterium prausnitzii</i>		60	(19)
FpF	CCCTTCAGTGCCGCAGT		
FpR	GTCGCAGGATGTCAAGAC		
Functional gene for hydrogen sulfide		60	
DSR1F+	ACSCACTGGAAGCACGGCGG		(20)
DSR-R	GTGGMRCCTGCAKRTTHG		(20)
Functional gene for methanogenesis		60	
mcrA-F	TTCGGTGGATCDCARAGRGC		(21)
mcrA-R	GBARGTCGWAWCCGTAGAATCC		(21)
Functional gene for secondary bile acids		60	
BaiCD-F	CAGCCRCAGATGTTCTTTG		(unpublished observation) ²
BaiCD-R	GCATGGAATTCWACTGTCYTC		

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.

negative ion mode by monitoring the (M-H)⁻ ion. The analytic conditions for LC-MS were as follow: column, Luna 3u, C₁₈, 100A (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-GCGTAATWC-3') and reverse 926R (5'-Fusion B-Barcode-CCGTCAATTCMTTTRAGTT-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

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), *Clostridium coccoides* (representing *Clostridium* cluster XIVa), *Clostridium sindense*, *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 TOPO-TA 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 7 α -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 μ L by using MicroAmp optical 384-well reaction plates sealed with MicroAmp optical adhesive film (Applied Biosystems). Each reaction contained 5 μ L 2X Power SYBR Green PCR Master mix (Applied Biosystems), 1 μ L bovine serum albumin (New England Biolabs) at 1 mg/mL (final concentration: 100 μ g/mL), 0.5 μ M of each primer, and 2 μ L 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 $P \leq 0.05$ was accepted as statistically significant. Data are presented as means \pm 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 Africans 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 2
Demographic data of the research subjects

	Female	Male	Age	Weight	BMI
			y	kg	kg/m ²
Native Africans	8	4	57 \pm 1.9 [†]	80 \pm 4.7	32 \pm 2.4
African Americans	9	3	58 \pm 2.5	74 \pm 4.0	28 \pm 1.8

[†] Mean \pm SEM (all such values).

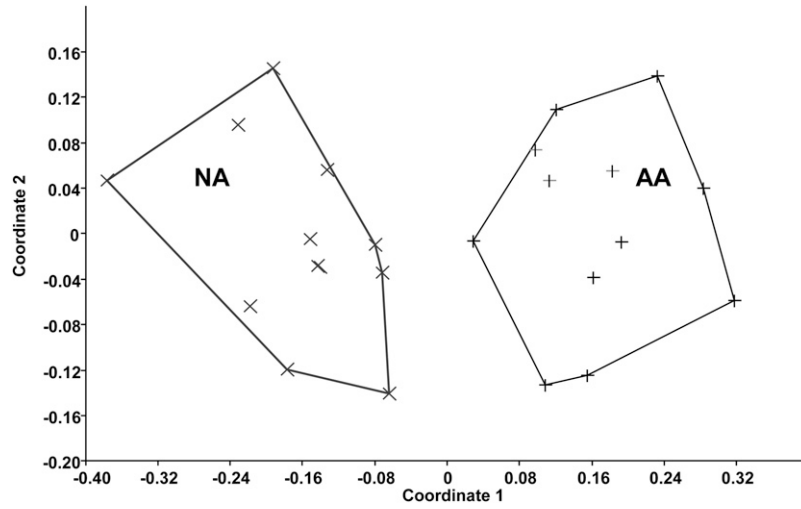


FIGURE 1. Illustration of the marked phylogenetic differences in microbiota composition between AAs ($n = 12$) and NAs ($n = 12$) detected by 16S-rRNA-based taxonomic pyrosequencing. Nonmetric multidimensional scaling shows strong clustering (multivariate ANOVA: $P < 0.01$) according to ethnic group. AA, African American; NA, native African; rRNA, ribosomal RNA.

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

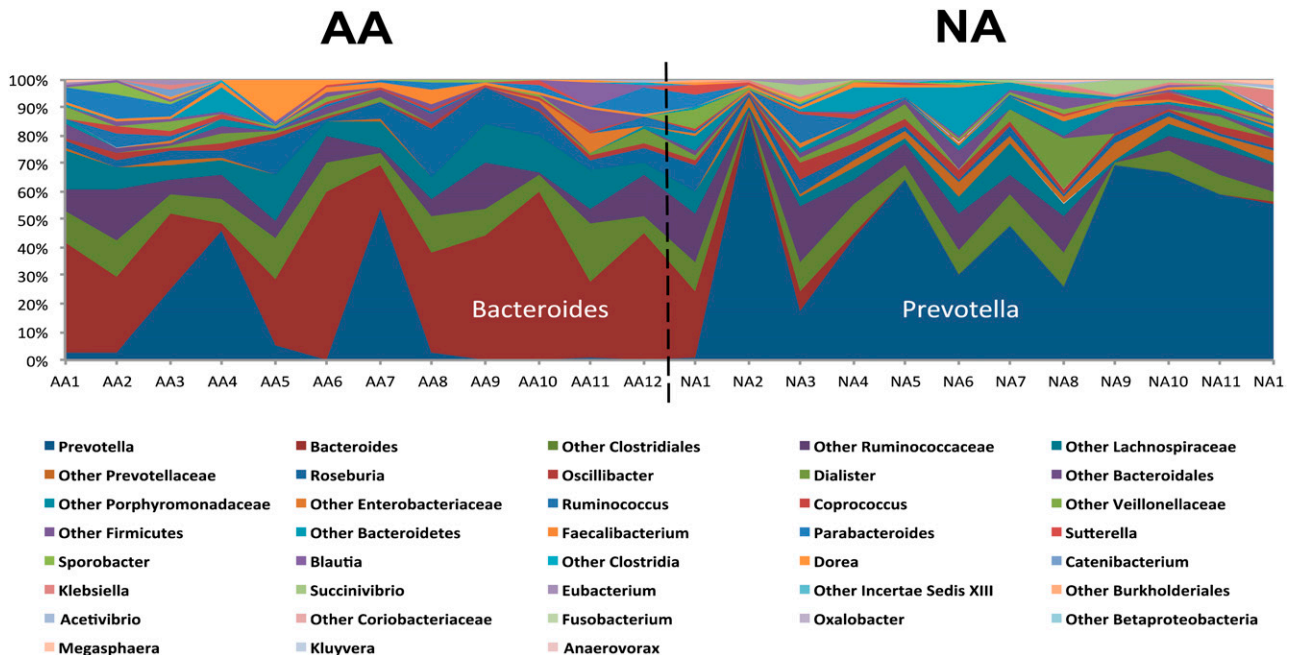


FIGURE 2. Composition was dominated by *Bacteroides* in the 12 AAs, which indicated that they belonged to enterotype 1, and was dominated by *Prevotella* in the 12 NAs, which categorized them as enterotype 2 (10). AA, African American; NA, native African.

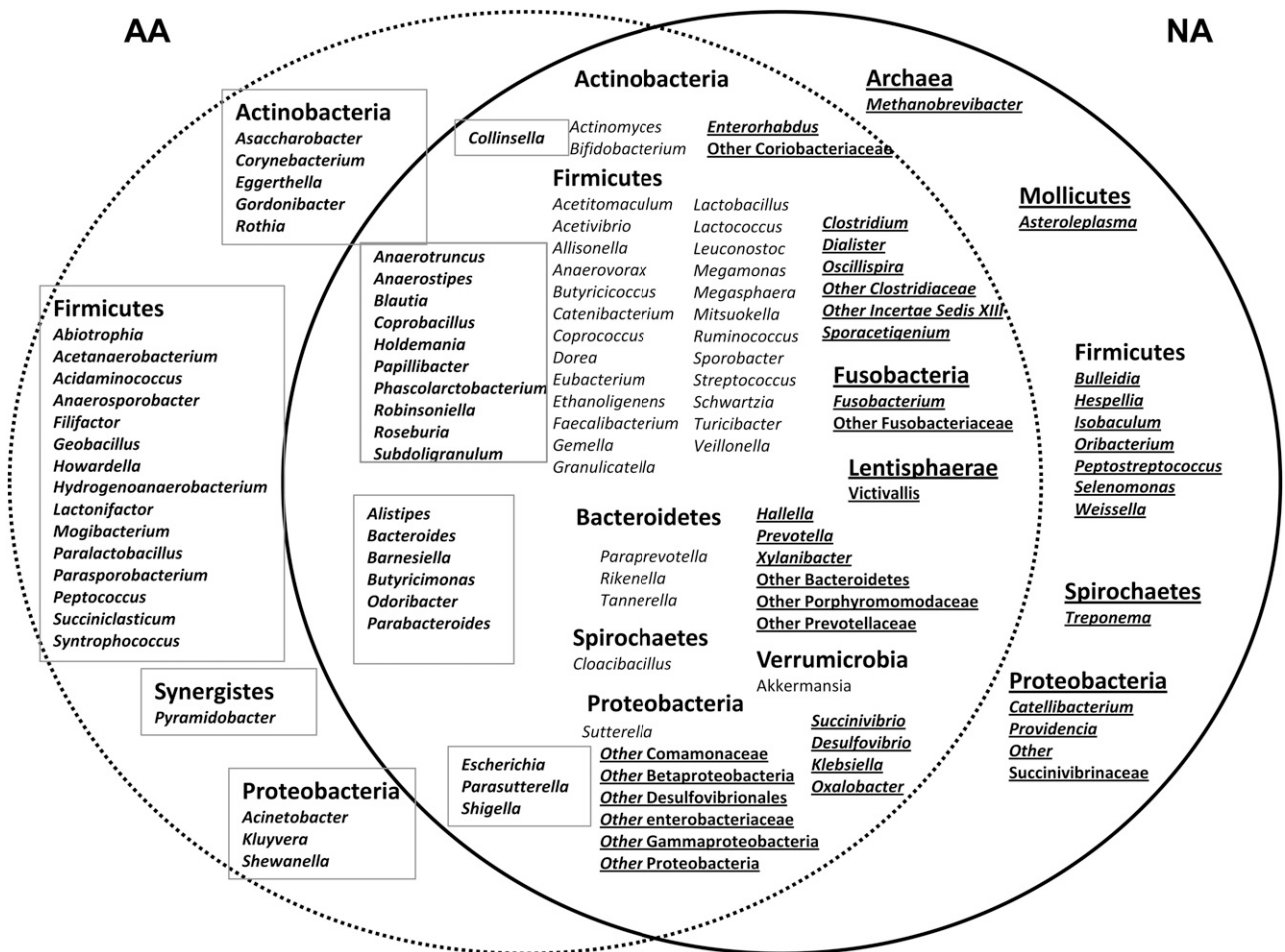


FIGURE 3. Illustration of the similarities and differences in fecal microbial taxa between AAs and NAs. The solid circle encloses the taxa that were detected in NA and the dotted circle those identified in AA. The overlap between the 2 circles contains taxa common to both populations. Much of the shared 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.

significantly higher in Americans (isobutyrate: 1.73 ± 0.26 compared with 1.22 ± 0.24 $\mu\text{mol/g}$ feces, $P = 0.02$; 2-methylbutyric/isovaleric acid: 1.49 ± 0.19 compared with 0.33 ± 0.19 $\mu\text{mol/g}$ 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 ($P < 0.05$;

TABLE 3
 Bacterial abundance in fecal samples from native Africans and African Americans¹

	Native African	African American	P value
	<i>gene copies/g feces</i>	<i>gene copies/g feces</i>	
Total bacteria	$7.1 \times 10^{11} \pm 4.5 \times 10^{10}*$	$1.8 \times 10^{11} \pm 6.3 \times 10^{10}$	0.046
Butyrate-production gene (<i>Bcoa</i>)	$5.4 \times 10^{10} \pm 1.1 \times 10^{10}*$	$1.4 \times 10^{10} \pm 2.7 \times 10^9$	0.022
<i>Faecalibacterium prausnitzii</i>	$1.6 \times 10^9 \pm 2.7 \times 10^8*$	$8.3 \times 10^8 \pm 2.7 \times 10^8$	0.038
<i>Clostridium</i> cluster IV	$5.1 \times 10^9 \pm 8.7 \times 10^8*$	$2.9 \times 10^9 \pm 7.3 \times 10^8$	0.032
<i>Clostridium</i> cluster XIVa	$9.5 \times 10^9 \pm 1.7 \times 10^9*$	$5.1 \times 10^9 \pm 9.3 \times 10^8$	0.049
<i>Succinivibrio</i> spp.	$1.1 \times 10^8 \pm 6.4 \times 10^7*$	$4.5 \times 10^6 \pm 1.3 \times 10^6$	0.021
<i>Prevotella</i> spp.	$8.2 \times 10^{10} \pm 1.4 \times 10^{10}*$	$3.5 \times 10^{10} \pm 6.4 \times 10^9$	0.011
<i>Bacteroides fragilis</i> group	$5.7 \times 10^9 \pm 1.6 \times 10^6$	$4.4 \times 10^{10} \pm 1.3 \times 10^7$	0.177
<i>Lactobacillus</i> spp.	$5.9 \times 10^6 \pm 3.4 \times 10^6*$	$7.1 \times 10^7 \pm 5.7 \times 10^7$	0.021
<i>Bifidobacterium</i> spp.	$6.8 \times 10^7 \pm 2.8 \times 10^7$	$3.0 \times 10^8 \pm 1.2 \times 10^8$	0.291
Hydrogen sulfide producers	$1.1 \times 10^7 \pm 1.5 \times 10^7*$	$4.1 \times 10^6 \pm 1.4 \times 10^6$	0.048
Methane producers	$3.1 \times 10^6 \pm 2.1 \times 10^6$	UDL ²	
Secondary bile acid producers	$2.2 \times 10^7 \pm 5.3 \times 10^6*$	$4.7 \times 10^8 \pm 1.6 \times 10^8$	0.037

¹ Values are means \pm SEMs. *Mann-Whitney U test; the sample number was 12 for each group.

² UDL, under detection limit.

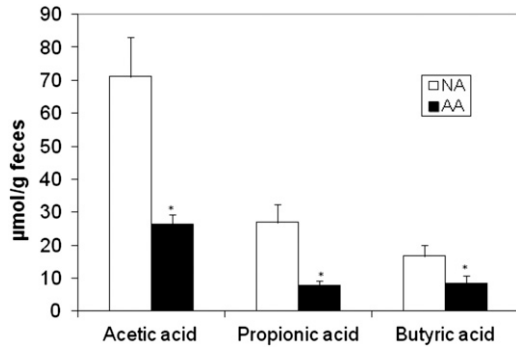


FIGURE 4. Summary of the differences in mean (\pm SE) group concentrations of the major short-chain fatty acids in fecal samples. Concentrations were significantly greater in NAs ($n = 12$) than in AAs ($n = 12$): Mann-Whitney U test for acetate ($P = 0.001$), propionate ($P = 0.003$), and butyrate ($P = 0.049$). AA, African American; NA, native African.

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 significantly higher in Africans (39.2 ± 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 microbial 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 production 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 consortium 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 populations 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 degradation. 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 colonic chronic diseases and colon cancer (36). Importantly, butyrate and the other SCFAs were also higher in the African children—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 proportions 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 possible 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 thetaiotamicron* (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

TABLE 4 Nonparametric Spearman correlations (R) between fecal short-chain fatty acid concentrations and copy numbers of bacteria¹

	Total SCFAs		Acetic acid		Propionic acid		Butyric acid	
	R	P	R	P	R	P	R	P
Total bacteria	0.37	0.011	0.38	0.010	0.33	0.026	0.37	0.042
<i>Bifidobacterium</i> spp.	-0.23	0.112	-0.27	0.066	-0.17	0.234	-0.19	0.197
<i>Faecalibacterium prausnitzii</i>	-0.28	0.215	-0.17	0.105	-0.27	0.265	-0.20	0.246
<i>Clostridium</i> IV	0.26	0.074	0.25	0.083	0.17	0.234	0.29	0.047
<i>Clostridium</i> XIVa	0.26	0.074	0.27	0.066	0.19	0.197	0.29	0.047

¹ Sample number = 24. SCFA, short-chain fatty acid.

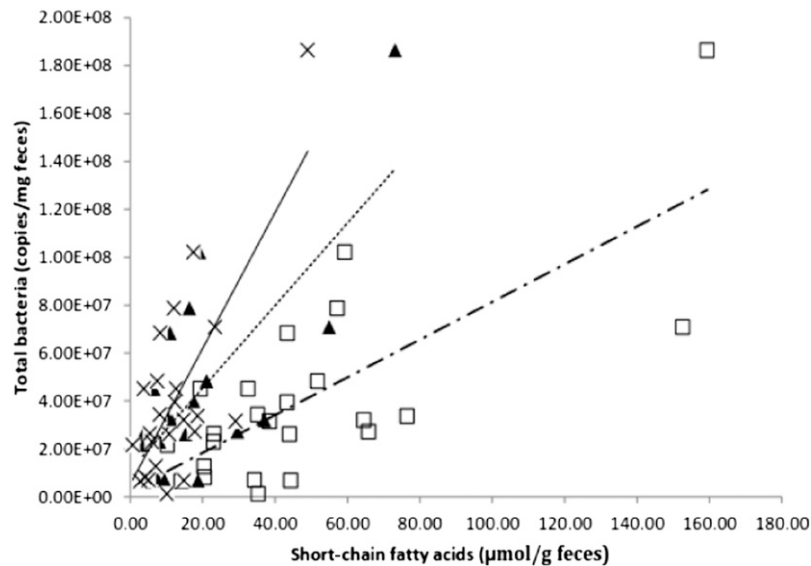


FIGURE 5. The abundance of total bacteria correlated significantly (Spearman's test) with the concentrations of acetate (\square , dashed and dotted line; $r^2 = 0.38$, $P = 0.033$), propionate (\blacktriangle , dashed line; $r^2 = 0.33$, $P = 0.039$), and butyrate (\times , continuous line; $r^2 = 0.37$,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

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).

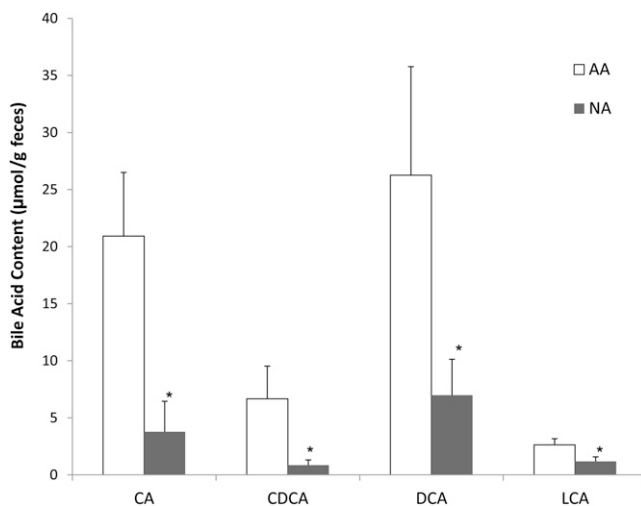


FIGURE 6. Contents of major bile acids in feces were significantly lower in NAs ($n = 12$) than in AAs ($n = 12$): Mann-Whitney U test for CA ($P = 0.027$), CDCA ($P = 0.016$), DCA ($P = 0.043$), and LCA ($P = 0.031$). * $P < 0.05$ compared with AA. AA, African American; CA, cholic acid; CDCA, chenodeoxycholic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

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). *F. prausnitzii*, a member of *Clostridium* cluster IV, is one of the most dominant butyrate producers, but also may have independent antiinflammatory properties related to secreted metabolites, which have been shown to block nuclear factor κ B activation and IL-8 production (50).

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

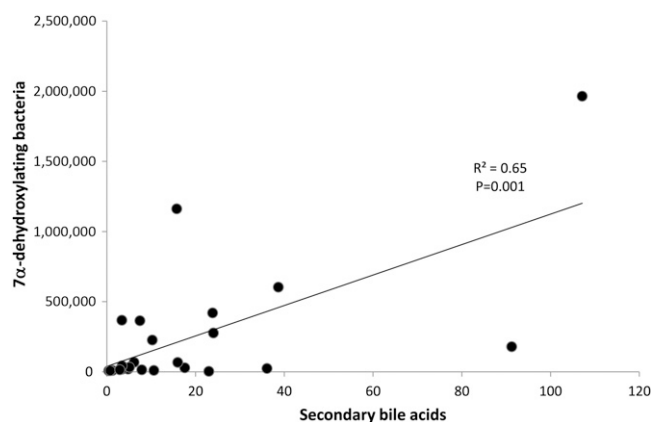


FIGURE 7. The abundance of 7 α -dehydroxylating bacteria, estimated from detection of their functional genes by polymerase chain reaction, correlated significantly with the concentration of secondary bile acids in fecal samples ($n = 24$). $r^2 = 0.65$, $P = 0.001$ (Spearman's test).

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 7 α -dehydroxylating enzymes. Our studies showed that not only were microbes containing this enzyme more common in high-risk African Americans, but so also were fecal secondary BAs. Human studies have associated increased fecal secondary BAs with colon polyps (58) and colon cancer (59), and there is substantial experimental evidence that they have carcinogenic properties (7). A further potential mechanism recently reported in a mouse model was the ability of a high-fat diet to stimulate the delivery of sulfur-rich taurine conjugates of BAs to the colon, where they produced a blossom of *Biophila wadsworthia*, which released hydrogen sulfide from taurine that lead to acute inflammation and colitis (60).

In summary, our study supports the hypothesis that colon cancer risk is determined by the interaction between diet and gut microbiota (61, 62) and that the higher risk in African Americans could be attributed to their chronically lower consumption of fiber and resistant starch and their higher consumption of dietary fat. 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 metabolomic differences to dietary change.

The authors' responsibilities were as follows—JO, SJDO, KN, and HRG: participated in the design and conduct of the study; JPD: conducted the BA and SCFA analyses; FC, EGZ, and MW: assisted with the microbiota analysis; and JO, FC, and SJDO: performed the data analysis. All authors were involved in finalizing the manuscript. None of the authors had any conflicts of interest to declare.

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