Metformin Is Associated With Higher Relative Abundance of Mucin-Degrading *Akkermansia muciniphila* and Several Short-Chain Fatty Acid–Producing Microbiota in the Gut

Jacobo de la Cuesta-Zuluaga,[1] Noel T. Mueller,[2] Vanessa Corrales-Agudelo,[1] Eliana P. Velásquez-Mejía,[1] Jenny A. Carmona,[3] José M. Abad,[4] and Juan S. Escobar[1]

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OBJECTIVE

Recent studies suggest the beneficial effects of metformin on glucose metabolism may be microbially mediated. We examined the association of type 2 diabetes, metformin, and gut microbiota in community-dwelling Colombian adults. On the basis of previous research, we hypothesized that metformin is associated with higher levels of short-chain fatty acid (SCFA)–producing and mucin-degrading microbiota.

RESEARCH DESIGN AND METHODS

Participants were selected from a larger cohort of 459 participants. The present analyses focus on the 28 participants diagnosed with diabetes—14 taking metformin— and the 84 participants without diabetes who were matched (3-to-1) to participants with diabetes by sex, age, and BMI. We measured demographic information, anthropometry, and blood biochemical parameters and collected fecal samples from which we performed 16S rRNA gene sequencing to analyze the composition and structure of the gut microbiota.

RESULTS

We found an association between diabetes and gut microbiota that was modified by metformin use. Compared with participants without diabetes, participants with diabetes taking metformin had higher relative abundance of *Akkermansia muciniphila*, a microbiota known for mucin degradation, and several gut microbiota known for production of SCFAs, including *Butyrivibrio*, *Bifidobacterium bifidum*, *Megasphaera*, and an operational taxonomic unit of *Prevotella*. In contrast, compared with participants without diabetes, participants with diabetes not taking metformin had higher relative abundance of Clostridiaceae 02d06 and a distinct operational taxonomic unit of *Prevotella* and a lower abundance of *Enterococcus casseliflavus*.

CONCLUSIONS

Our results support the hypothesis that metformin shifts gut microbiota composition through the enrichment of mucin-degrading *A. muciniphila* as well as several SCFA-producing microbiota. Future studies are needed to determine if these shifts mediate metformin's glycemic and anti-inflammatory properties.

[1]*Vidarium—Nutrition, Health and Wellness Research Center, Grupo Empresarial Nutresa, Medellin, Colombia*
[2]*Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD*
[3]*Dinámica I.P.S.—Especialista en Ayudas Diagnósticas, Medellin, Colombia*
[4]*EPS y Medicina Prepagada Suramericana S.A., Medellin, Colombia*

Corresponding author: Juan S. Escobar, jsescobar@serviciosnutresa.com.

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Microbial communities (microbiota) and their associated genes (microbiome) constitute the interface of our environs and our cells, and their composition is believed to play a deterministic role in human health and disease. In particular, the development of type 2 diabetes, a disease rising in prevalence around the globe, has been linked in nonhuman (1) and human (2–5) studies to imbalances in microbiota of the intestinal tract (gut). However, the most recent human study on this topic found that the association was modulated in a potentially beneficial manner by metformin treatment (6).

Metformin (1,1-dimethylbiguanide hydrochloride) is the most frequent medication used to treat type 2 diabetes (7), and findings from recent studies suggest it may also prevent cancer (7) and cardiovascular events (8). Metformin has pleiotropic effects, yet the majority of mechanistic studies have focused on changes in liver function (7,9,10). Although metformin certainly alters hepatic glucose production via effects on AMPK, there is growing evidence that the genesis of its action is in the gut (11–15).

Metformin is ~50% bioavailable, allowing for near-equal intestinal and plasma exposure, but intestinal accumulation of metformin is 300 times that of the plasma (16), making the gut the primary reservoir for metformin in humans. Unlike oral administration, intravenous administration of metformin in humans does not improve glycemia (14). Moreover, in mice, oral administration of a broad-spectrum antibiotic cocktail with oral metformin abrogates metformin's glucose-lowering effect (12). Providing yet further evidence that the glucose-lowering effect of metformin may originate in the lower bowel, a delayed-release oral metformin, which targets the ileum, had a similar or greater glucose-lowering effect than immediate-release or extended-release metformin, despite the delayed-release metformin having lower systemic exposure than the other metformin formulations (15).

Recent studies in animals (11,12,13) and humans (6,17) provide evidence that metformin may partially restore gut dysbiosis associated with type 2 diabetes. In mice fed a high-fat diet, metformin treatment increased the relative abundance of *Akkermansia muciniphila* (11–13), a mucin-degrading bacteria that has been shown to reverse metabolic disorders (1,12). In humans, participants with diabetes taking metformin had similar abundance of *Subdoligranulum* and, to some extent, *Akkermansia* compared with control subjects without diabetes, suggesting that metformin may help ameliorate a type 2 diabetes–associated gut microbiome (6). It has also been shown that people with diabetes taking metformin had a higher relative abundance of *Adlercreutzia* (17), and metagenomic functional analyses demonstrated significantly enhanced butyrate and propionate production in people with diabetes using metformin (6). In contrast, people with diabetes who were not treated with metformin had a higher abundance of *Eubacterium* and Clostridiaceae SMB53 (17) and lower levels of short-chain fatty-acid (SCFA)–producers, such as *Roseburia*, *Subdoligranulum*, and a cluster of butyrate-producing Clostridiales (6). These findings provide evidence that gut microbes may contribute to the antidiabetes effects of metformin through pathways that include mucin degradation and SCFA production.

In this study we aimed to test the generalizability of previous observations concerning the influence of metformin on the association of type 2 diabetes and gut dysbiosis in a Colombian adult population. Given the considerable variation in the microbiota associated with type 2 diabetes and that the gut microbiota of Colombians is different to that of other populations (18), we hypothesized that the microbial taxa involved in the type 2 diabetes dysbiosis of Colombians are different to those observed in Chinese and European populations (2,3) but that the effect of metformin is similar, i.e., through enrichment of mucin-degrading and SCFA-producing microbiota.

RESEARCH DESIGN AND METHODS

Study Design

Between July and November 2014, we enrolled 459 men and women 18–62 years old, with BMI $\geq$18.5 kg/m^2, living in the Colombian cities of Medellin, Bogota, Barranquilla, Bucaramanga, and Cali. All participants enrolled in the study were insured by the health insurance provider EPS y Medicina Prepagada Suramericana S.A. (EPS SURA). We excluded pregnant women, individuals who consumed antibiotics or antiparasitics $<$3 months prior to enrollment, and individuals diagnosed with Alzheimer disease, Parkinson disease, or any other neurodegenerative diseases; current or recent cancer ($<$1 year); and gastrointestinal diseases (Crohn disease, ulcerative colitis, short bowel syndrome, diverticulosis, or celiac disease).

This study was conducted in accordance with the principles of the Declaration of Helsinki, as revised in 2008, and had minimal risk according to the Colombian Ministry of Health (Resolution 008430 of 1993). All of the participants were thoroughly informed about the study and procedures. Participants were assured of anonymity and confidentiality. Written informed consent was obtained from all the participants before beginning the study. The Bioethics Committee of Sede de Investigación Universitaria—University of Antioquia reviewed the protocol and the consent forms and approved the procedures described here (approbation act 14–24–588 dated 28 May 2014).

Anthropometric, Clinical, and Dietary Evaluations

We calculated BMI as weight (kg)/height squared (m^2) to classify participants as lean (18.5 $\leq$ BMI $<$ 25.0 kg/m^2), overweight (25.0 $\leq$ BMI $<$ 30.0 kg/m^2), or obese (BMI $\geq$30 kg/m^2). In addition, values of HDL, LDL, VLDL, total cholesterol, triglycerides, apolipoprotein B, fasting glucose, glycated hemoglobin (HbA_{1c}), fasting insulin, adiponectin, and hs-CRP were obtained (collection and measurement explained in Supplementary Data). Dietary intake was evaluated through 24-h dietary recalls (see Supplementary Data).

DNA Extraction and Sequence Analysis

Each participant collected their own fecal sample in a hermetically sealed, sterile receptacle provided by the research team. Samples were immediately refrigerated in household freezers and brought to an EPS SURA facility in each city within 12 h; receipt of samples occurred exclusively in the morning (6 A.M.–12 P.M.). As such, stools were collected between the evening of the day before and the morning of the day of sample receipt. Fecal samples were stored on dry ice and sent to a central laboratory via next-day delivery. Before DNA extraction, stool consistency was evaluated by trained laboratory technicians.

Total microbial DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, with a slight modification consisting in a bead-beating step with the lysis buffer (20 s at 15 Hz using a stainless steel bead with a 5-mm diameter). After extraction, we quantified DNA concentration using a NanoDrop spectrophotometer (Nyxor Biotech, Paris, France) and sent it to the Microbial Systems Laboratory, University of Michigan Medical School (Ann Arbor, MI). The V4 hypervariable region of the 16S rRNA gene was amplified using the F515 (5′-CACGGTCGKCGGCGCCATT-3′) and R806 (5′-GGACTACHVGGGTWTC TAAT-3′) primers and sequenced using the Illumina MiSeq sequencing platform with V2 chemistry and the dual-index sequencing strategy (19). In addition to DNA from fecal samples, we sequenced negative controls (ultrapure water and the QIAamp DNA Stool Mini Kit's EB elution buffer), a DNA extraction blank, and a mock community (HM-782D, BEI Resources, Manassas, VA) in each instrument's run. Sequences were curated following the MiSeq standard operating procedure implemented by Mothur v.1.36 (20) (see Supplementary Data). Raw sequences were deposited at the NCBI and can be accessed through the BioProject (accession number PRJNA325931).

Definition of Type 2 Diabetes and Selection of Control Subjects

We identified 28 participants in our study that had type 2 diabetes; 26 self-reported physician-diagnosed diabetes prior to the beginning of the study and 2 were diagnosed through laboratory testing (fasting blood glucose $\geq$126 mg/dL and $HbA_{1c} \geq 6.5\%$). Of the 28 participants with type 2 diabetes, 14 were under metformin treatment, 14 were not (1 was treated with insulin alone, 2 with glibenclamide, and 11 were under no pharmacological treatment for type 2 diabetes, including the 2 participants unaware of their diabetes status) (Supplementary Table 1).

We matched each participant with diabetes with three participants without diabetes in our study based on sex, age (to the closest possible age; maximum difference between case and control subjects 6 years; mean 1.5 years; median 1 year), and BMI category (lean, overweight, or obese). This left us with a total analytic sample of 112 study participants comprising 14 with type 2 diabetes using metformin (T2D-met$^+$), 14 with type 2 diabetes not using metformin (T2D-met$^-$), and 84 without diabetes (ND).

Statistical Analyses

Anthropometric and clinical variables were compared across study groups using ANOVA and *t* tests after checking for homoscedasticity and normal distribution of residuals (using Fligner-Killeen tests of homogeneity of variances and Shapiro-Wilk normality tests). When necessary, variables were appropriately transformed (natural log for unconstrained variables or arcsin square root for proportions). Sex ratio and stool consistency were compared using χ^2 tests. Statistical analyses were performed with R v.3.2.2 (21).

Curated DNA sequences ranged from 69 to 102,660 sequences per sample (median 28,699). To limit the effects of uneven sampling, we rarefied the data set to 4,091 sequences per sample, resulting in the exclusion of one T2D-met$^-$ participant with 69 reads. Although rarefaction may lead to missing low-abundance data, it is a powerful way to reduce the likelihood of detecting false positives, especially among those operational taxonomic units (OTUs) with very low abundance.

The gut microbiota structure and composition was assessed by quantifying and interpreting similarities based on intra- and intergroup diversity analyses (α and β diversity, respectively). For α diversity, we calculated Good's coverage and the number of OTUs of each sample using Mothur and constructed rarefaction curves. We compared these indices among groups of participants using analysis of similarity (ANOSIM) with 1,000 permutations using the Vegan package of R (22).

β Diversity was assessed using phylogeny-based generalized UniFrac distances (with the α parameter controlling weight on abundant lineages = 0.5) calculated with the GUniFrac package of R (23). For this, we first reduced the alignment and the OTU table to one representative sequence per OTU, then obtained a distance matrix from uncorrected pairwise distances between aligned sequences, and finally constructed a relaxed neighbor-joining phylogenetic tree using Mothur and Clearcut. Comparisons among groups of participants were performed using the adonis function (ANOVA using distance matrices) of the permutational multivariate ANOVA (PERMANOVA) implemented in the Vegan package of R (22).

We next used linear discriminant analysis (LDA) effect size (LEfSe) (24) to agnostically identify microbial biomarkers. LEfSe uses the nonparametric factorial Kruskal-Wallis sum rank test to detect individual OTUs with significant differential abundance among groups of participants, then performs a set of pairwise tests among groups of participants using the unpaired Wilcoxon rank sum test, and finally uses LDA to estimate the effect size of each differentially abundant OTU (24). The strength of LEfSe compared with standard statistical approaches is that, in addition to providing *P* values, it provides an estimation of the magnitude of the association between each OTU and the grouping categories (e.g., metformin, type 2 diabetes) through the LDA score. For stringency, microbial biomarkers in our study were retained if they had a $P < 0.05$ and a (log10) LDA score $\geq$3, i.e., one order of magnitude greater than LEfSe's default.

Finally, across groups, we tested for differences in relative abundance of the mucin-degrading *A. muciniphila* and major butyrate-producing microbial genera, including *Butyrivibrio*, *Roseburia*, *Subdoligranulum*, and *Faecalibacterium*. For this analysis, we pooled all OTUs classified in each of these phylotypes (4 for *A. muciniphila*, 11 for *Butyrivibrio*, 4 for *Roseburia*, 10 for *Subdoligranulum*, and 5 for *Faecalibacterium*) and tested for differences using ANOVA and *t* tests on arcsin square root transformed relative abundances. This pooling served to examine whether differences in relative abundance of these groups of bacteria occurred across all OTUs or only in specific OTUs.

Results from LEfSe and from the pooled analysis of phylotypes were corrected for multiple testing using the Bayesian approach implemented in the qvalue package of R (25). Tests were considered significant if they had a *P* value $\leq$ 0.05 and a q value $\leq$0.1.

RESULTS

In Table 1 we present the characteristics of T2D-met$^+$, T2D-met$^-$, and ND participants. There were no statistically significant differences (all *P* values $>$ 0.10) in demographic, anthropometric, or clinical parameters between T2D-met$^+$ and

Table 1—General characteristics of T2D-met$^+$, T2D-met$^-$, and ND participants among community-dwelling Colombian adults

	Group			*P**		
	T2D-met$^+$	T2D-met$^-$	ND	T2D-met$^+$ vs. T2D-met$^-$	T2D-met$^+$ vs. ND	T2D-met$^-$ vs. ND
n	14	14	84	—	—	—
Age (years)	50 ± 10	44 ± 9	47 ± 9	0.11	0.33	0.24
Sex (F/M)	0.36	0.50	0.43	0.70	0.83	0.84
Anthropometry						
BMI (kg/m^2)	31.88 ± 4.63	32.15 ± 6.36	31.11 ± 4.53	0.98	0.56	0.62
Body fat (%)	0.41 ± 0.05	0.41 ± 0.03	0.40 ± 0.04	0.96	0.68	0.58
Waist circumference (cm)	104.6 ± 9.8	102.8 ± 14.2	102.0 ± 11.3	0.70	0.39	0.85
Clinical parameters						
Total cholesterol (mg/dL)	178 ± 51	208 ± 44	187 ± 30	0.11	0.54	0.10
HDL (mg/dL)	40 ± 11	38 ± 6	44 ± 12	0.77	0.21	0.0136
LDL (mg/dL)	105 ± 35	128 ± 39	115 ± 28	0.11	0.31	0.26
VLDL (mg/dL)	38 ± 40	52 ± 52	31 ± 15	0.19	0.64	0.0471
Apolipoprotein B (mg/dL)	102 ± 30	108 ± 30	97 ± 26	0.56	0.55	0.18
Triglycerides (mg/dL)	176 ± 202	244 ± 253	154 ± 75	0.15	0.97	0.07
Fasting glucose (mg/dL)	127 ± 47	145 ± 76	90 ± 10	0.57	0.0047	0.0060
HbA_{1c} [% (mmol/mol)]	6.9 ± 1.4 (52.0 ± 15.3)	7.1 ± 1.7 (54.0 ± 18.6)	5.6 ± 0.3 (38.0 ± 3.3)	0.78	0.0026	0.0047
Fasting insulin (μU/mL)	22.24 ± 12.58	24.24 ± 12.86	15.20 ± 8.79	0.49	0.11	0.0029
Insulin resistance index	2.9 ± 1.5	3.7 ± 3.0	1.9 ± 1.1	0.39	0.0420	0.0018
Leptin (ng/mL)	7.39 ± 6.09	8.34 ± 5.40	7.89 ± 6.14	0.38	0.91	0.25
Adiponectin (μg/mL)	4.59 ± 1.97	4.96 ± 3.22	6.79 ± 3.90	0.91	0.0195	0.07
hs-CRP (mg/L)	2.70 ± 2.53	3.35 ± 2.76	4.04 ± 5.02	0.61	0.21	0.62
Dietary intake						
Energy intake (calories)	1,843 ± 268	1,865 ± 584	1,945 ± 572	0.75	0.61	0.57
Carbohydrate (g)	250 ± 37	260 ± 86	267 ± 88	0.92	0.52	0.68
Protein (g)	74.7 ± 9.1	69.7 ± 13.1	74.1 ± 13.8	0.26	0.85	0.26
Fat (g)	60.7 ± 13.0	60.2 ± 21.3	62.9 ± 17.1	0.64	0.76	0.51
Cholesterol (mg)	336 ± 31	330 ± 41	347 ± 38	0.67	0.28	0.18
Dietary fiber (g)	17.9 ± 4.8	18.6 ± 6.4	17.5 ± 4.8	0.88	0.73	0.64
Stool consistency [*n* (%)]				0.38	0.58	0.50
Hard	4 (28)	2 (14)	13 (15)	—	—	—
Normal	8 (57)	9 (64)	54 (64)	—	—	—
Mushy	2 (14)	1 (7)	13 (15)	—	—	—
Diarrheic	0 (0)	2 (14)	4 (5)	—	—	—

Values presented as mean ± SD. *All *P* values from *t* tests except in sex and stool consistency (χ^2 tests).

T2D-met$^-$ participants. Compared with ND participants, T2D-met$^+$ participants had higher fasting glucose, HbA_{1c}, and insulin resistance than ND participants and lower levels of the insulin-sensitizing hormone adiponectin ($P < 0.05$). No other demographic, anthropometric, or clinical parameters were statistically different.

16S rRNA Gene Sequencing

Gut microbiota communities were specific to each participant with marked intersubject differences (Fig. 1*A*) (overall interindividual generalized UniFrac distance = 0.720 ± 0.009). We found high coverage across all groups of participants (mean Good's coverage ± SD = 0.990 ± 0.001); 99% of OTUs were detected at least by two DNA reads, demonstrating thorough sampling of the gut microbiota. We next tested for differences in the number of observed OTUs across the groups of participants. We found no differences between participants with diabetes and ND participants (ANOSIM statistic R = 0.005, *P* value = 0.425) or between T2D-met$^+$ and T2D-met$^-$ participants (ANOSIM statistic R = −0.018, *P* = 0.557). The number of observed OTUs tended to be more similar between T2D-met$^+$ and ND than between T2D-met$^-$ and ND participants (Supplementary Fig. 1); however, these differences were not statistically significant (T2D-met$^+$ vs. ND: ANOSIM statistic R = 0.018, *P* = 0.348; T2D-met$^-$ vs. ND: ANOSIM statistic R = 0.009, *P* = 0.409).

We observed no significant differences in β diversity estimates among the three groups of participants (PERMANOVA: R^2 = 0.019, *P* = 0.335) (Fig. 1*A*) or between participants with diabetes and ND participants (R^2 = 0.009, *P* = 0.416) (Fig. 1*B*). However, the comparison between metformin and nonmetformin users reached significance (R^2 = 0.013, *P* = 0.036) (Fig. 1*C*), demonstrating differences in the bacterial community structure associated with metformin use. The difference was also significant when comparing T2D-met$^+$ and ND participants (R^2 = 0.015, *P* = 0.036) but not when comparing T2D-met$^-$ and ND participants (R^2 = 0.008, *P* = 0.943). These results suggested the microbial communities of T2D-met$^+$ versus T2D-met$^-$ were modestly phylogenetically dissimilar.

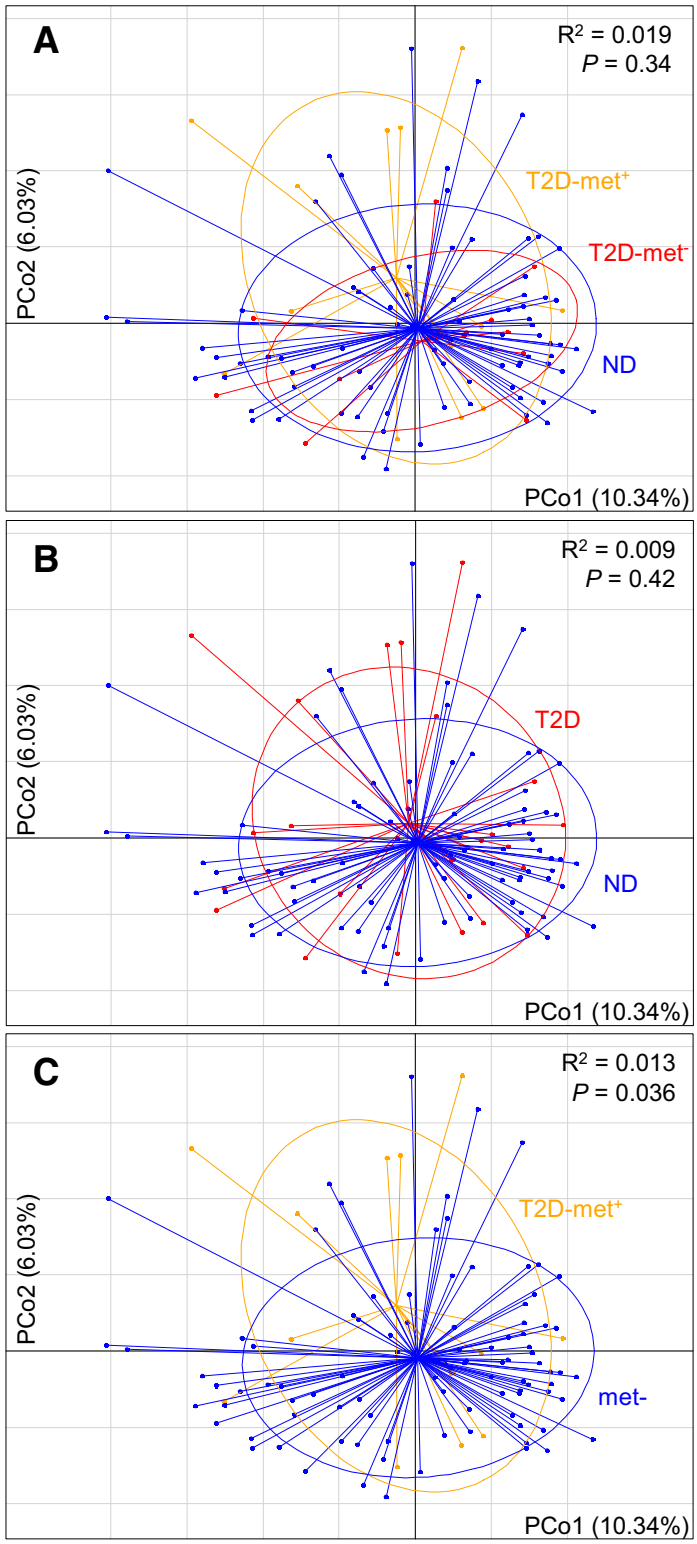

Figure 1—Principal coordinates analysis based on generalized UniFrac. *A*: Comparison among the three groups of participants. *B*: Comparison between participants with diabetes and ND participants. *C*: Comparison between T2D-met$^+$ and participants not taking metformin (T2D-met$^-$ and ND). Ellipses encompass 75% of data distribution in each group of participants. R^2 and *P* values from PERMANOVA. (A high-quality color representation of this figure is available in the online issue.)

We next used LEfSe to examine differences in the relative abundance of gut microbiota at the OTU level. Note that we were only interested in OTUs displaying strong associations in the LDA (represented by OTUs with [log10] LDA scores ≥3); such stringency resulted in fewer retained, but more likely biologically relevant, OTUs (19 displayed in Fig. 2 and

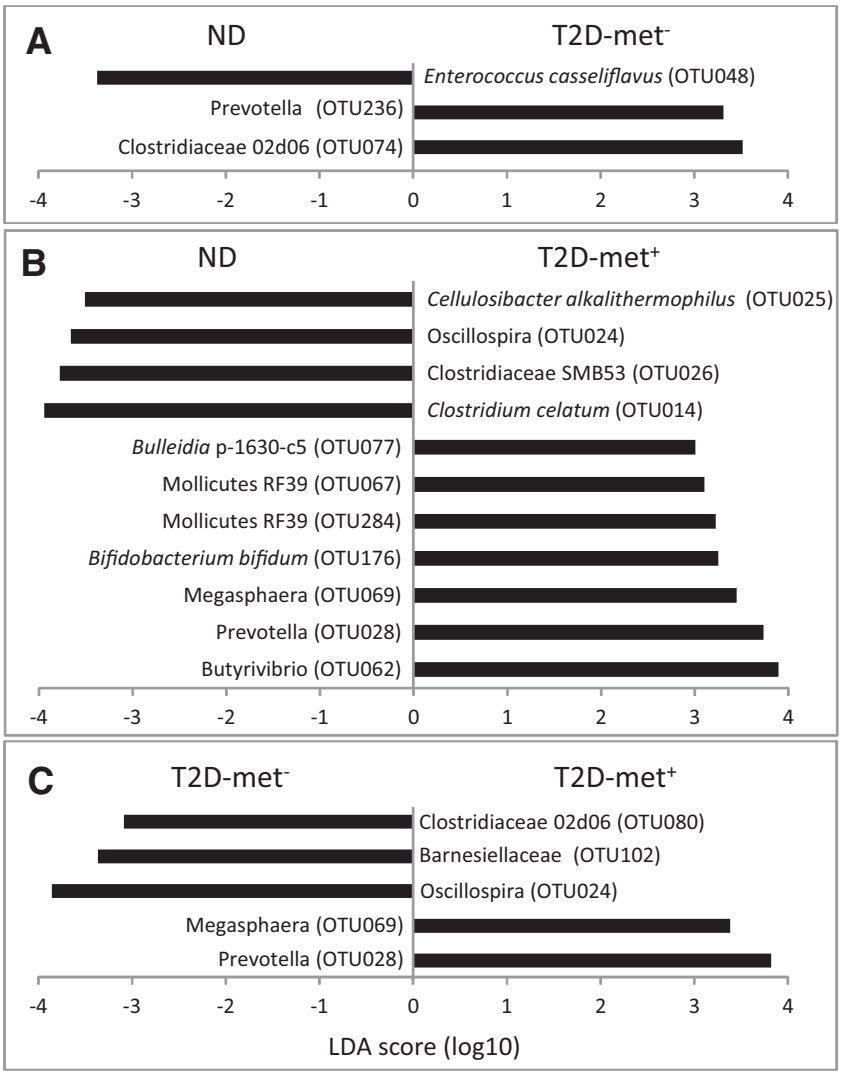

Figure 2—LDA scores (log10) of the OTUs displaying differences between pairs of groups of participants. ND vs. T2D-met$^-$ (*A*), ND vs. T2D-met$^+$ (*B*), T2D-met$^+$ vs. T2D-met$^-$ (*C*) participants.

Fig. 3 out of 273 statistically significant if not taking LDA scores into account). When comparing T2D-met$^-$ and ND participants, we found that OTUs belonging to Clostridiaceae 02d06 (Firmicutes|Clostridiaceae|OTU074) and *Prevotella* (Bacteroidetes|Prevotellaceae|OTU236) were overrepresented in T2D-met$^-$ participants, whereas *Enterococcus casseliflavus* (Firmicutes|Enterococcaceae|OTU048) was more abundant in ND participants (Figs. 2*A* and 3*A* and B). When comparing T2D-met$^+$ participants to ND participants, we found that OTUs of *Butyrivibrio* (Firmicutes|Lachnospiraceae|OTU062), a different OTU of *Prevotella* (Bacteroidetes|Prevotellaceae|OTU028), *Megasphaera* (Firmicutes|Veillonellaceae|OTU069), *Bifidobacterium bifidum* (Actinobacteria|Bifidobacteriaceae|OTU176), two OTUs of Mollicutes RF39 (Tenericutes|OTU284 and OTU067), and *Bulleidia* p-1630-c5 (Firmicutes|Erysipelotrichaceae|OTU077) were more abundant in T2D-met$^+$ than in ND participants. In contrast, four OTUs of Clostridiales including *Clostridium celatum* (Firmicutes|Clostridiaceae|OTU014), Clostridiaceae SMB53 (Firmicutes|Clostridiaceae|OTU026), *Oscillospira* (Firmicutes|Ruminococcaceae|OTU024), and *Cellulosibacter alkalithermophilus* (Firmicutes|Ruminococcaceae|OTU025) were more abundant in ND than in T2D-met$^+$ participants (Figs. 2*B* and 3*C* and *D*). OTUs from *Prevotella* (Bacteroidetes|Prevotellaceae|OTU028) and *Megasphaera* (Firmicutes|Veillonellaceae|OTU069) were enriched in T2D-met$^+$ compared with T2D-met$^-$ participants, whereas OTUs from *Oscillospira* (Firmicutes|Ruminococcaceae|OTU024), Barnesiellaceae (Bacteroidetes|OTU102), and a different OTU of Clostridiaceae 02d06 (Firmicutes|Clostridiaceae|OTU080) were enriched in T2D-met$^-$ compared with T2D-met$^+$ participants (Figs. 2*C* and 3*E* and *F*).

Finally, when we pooled mucin-degrading and butyrate-producing microbes, we found that *A. muciniphila* and *Butyrivibrio* were more abundant (3.4 and 4.4 times, respectively) in T2D-met$^+$ than in T2D-met$^-$ participants; differences were statistically significant for *A. muciniphila* ($F_{1,\ 109}$ = 9.46, P = 0.003, q value = 0.01) but not for *Butyrivibrio* ($F_{1,\ 109}$ = 3.03, P = 0.08, q value = 0.21) (Fig. 3*G* and *H*). There were no significant differences in the other groups of butyrate producers between metformin and nonmetformin users (*Roseburia*: $F_{1,\ 109}$ = 1.44, P = 0.23, q value = 0.39; *Subdoligranulum*: $F_{1,\ 109}$ = 0.001, P = 0.97, q value = 0.97; *Faecalibacterium*: $F_{1,\ 109}$ = 0.53, P = 0.47, q value = 0.59). There were no significant differences in any of these groups of bacteria between participants with diabetes and ND participants (all $P > 0.1$ and q values >0.2).

CONCLUSIONS

In our community-based sample of Colombian adults, we provide evidence consistent with previous literature (6,11–13,17) that the association between gut microbiota and type 2 diabetes is modified by metformin use. T2D-met$^+$ participants had higher relative abundance of purportedly beneficial mucin-degrading and SCFA-producing bacteria compared with T2D-met$^-$ and ND participants matched on age, sex, and BMI.

Several studies have demonstrated that type 2 diabetes is associated with gut microbiota composition, but findings on the taxa involved have been inconsistent (2–5). Some of the variance in previous study findings may be explained by confounding factors, including demographics, body weight, and treatment with drugs, such as metformin. After matching on age, sex, and BMI and stratifying comparisons on metformin, we found only modest associations between type 2 diabetes and gut microbiota composition. One OTU related to *Prevotella* was higher among participants with diabetes, whereas another OTU related to *E. casseliflavus* was lower among participants with diabetes.

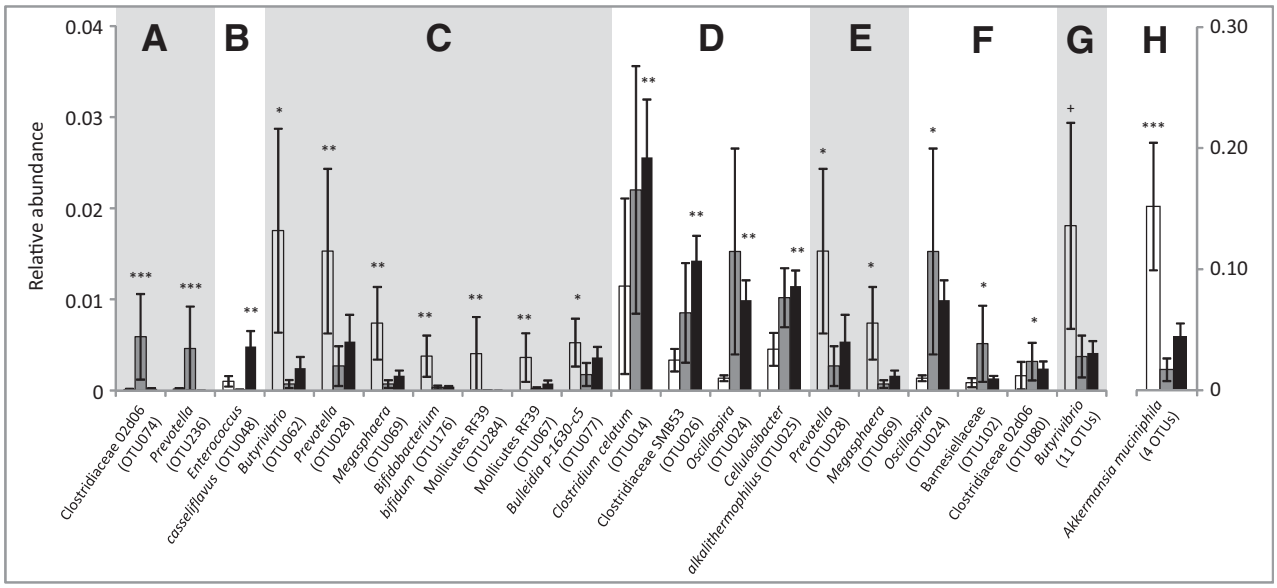

Figure 3—Relative abundance of the groups of bacteria displaying differences among participants (logLDA >3). Data presented as mean ± SE. Open bars = T2D-met$^+$; gray bars = T2D-met$^-$; black bars = ND. *A*: OTUs enriched in T2D-met$^-$ compared with ND. *B*: OTUs enriched in ND compared with T2D-met$^-$. *C*: OTUs enriched in T2D-met$^+$ compared with ND. *D*: OTUs enriched in ND compared with T2D-met$^+$. *E*: OTUs enriched in T2D-met$^+$ compared with T2D-met$^-$. *F*: OTUs enriched in T2D-met$^-$ compared with T2D-met$^+$. *G*: Eleven pooled OTUs classified as *Butyrivibrio* enriched in T2D-met$^+$ compared with T2D-met$^-$ and ND. *H*: Four pooled OTUs classified as *A. muciniphila* enriched in T2D-met$^+$ compared with T2D-met$^-$ and ND (note the change in scale). +$P < 0.1$; *$P < 0.05$; **$P < 0.05$ and q value <0.1; ***$P < 0.05$ and q value <0.05.

Prevotella has been associated with carbohydrate-based diets and degradation of complex polysaccharides (26), whereas *E. casseliflavus* is an opportunistic pathogen that may cause serious infections in immunosuppressed individuals (27).

We found associations between metformin use and gut microbiota composition that were largely consistent whether we compared T2D-met$^+$ participants to ND control subjects or to T2D-met$^-$ participants, suggesting that metformin may have direct microbial effects. Our findings are congruent with multiple lines of evidence indicating the gut-mediated glycemic effect of metformin stem from alterations in the gut microbiota composition. Cabreiro et al. (28) first demonstrated that metformin impacts the metabolism of the microbiota hosted by *Caenorhabditis elegans*. Three mouse studies have shown that metformin treatment in mice on high-fat diet shifts the microbiota composition toward that of mice fed normal chow by increasing abundance of *Akkermansia* spp. (11–13). Moreover, a follow-up experiment by Shin et al. (12) found that mice fed high-fat diets treated with either cultured *A. muciniphila* or metformin had similar improvements in mucin-producing goblet cells, proinflammatory interleukin-6, and glucose tolerance, suggesting that *A. muciniphila* alone may explain the beneficial effects of metformin.

In human studies, a small nonrandomized clinical trial of 12 patients with type 2 diabetes demonstrated that stopping metformin treatment for 7 days led to alterations in the gut microbiota and glucagon-like peptide 1 (17). A three-country cross-sectional metagenomic study found that metformin use was positively associated with SCFA-producing bacteria (6). Metformin has also been shown to enhance active and total glucagon-like peptide 1 (17,29,30), which is consistent with the hypothesis that it increases SCFA production through modification of the gut microbiota composition. There is also the possibility that metformin alters glucose metabolism through effects on bile acid secretion (31).

As we hypothesized, on the basis of the aforementioned nonhuman (11–13) and human (6,17) studies, metformin use in our study was associated with greater relative abundance of the mucin-degrading *A. muciniphila.* Through LEfSe biomarker discovery, we also found metformin was positively associated with the mucolytic bacterium *B. bifidum*. The higher abundance of the mucin-degrading bacteria *A. muciniphila* and *B. bifidum* in the gut microbiota of metformin users suggests that metformin's health benefits may derive from the strengthening of the intestinal mucosal barrier. *A. muciniphila* plays a crucial role in maintaining the integrity of the mucin layer, thereby reducing translocation of proinflammatory lipopolysaccharides and controlling fat storage, adipose tissue metabolism, and glucose homeostasis (1,12). Likewise, *B. bifidum* can grow on gastric mucin as a sole carbon source, and genome analysis revealed that this bacterium can use host mucins (32), potentially contributing to gastrointestinal health in the same way as *A. muciniphila*.

T2D-met$^+$ participants in our study also had higher relative abundance of some SCFA-producing bacteria, but not others (e.g., *Roseburia, Subdoligranulum, Faecalibacterium*). Those positively associated with metformin use included *B. bifidum*, *Prevotella* (an OTU distinct from the OTU enriched in T2D-met$^-$ participants), *Megasphaera* (a bacterium related to *Megamonas*), and *Butyrivibrio* (although this taxa was no longer significant after adjusting for multiple comparisons). These microbiota have been associated with production of SCFAs, including butyrate, propionate, and acetate (26,33–35). SCFAs may be beneficial for health. Butyrate, in particular, is one of the preferred energy sources of the colonic epithelium (36). Recent studies in

mice showed that an increase in the colonic production of SCFAs, especially butyrate and propionate, triggers intestinal gluconeogenesis, benefiting glucose and energy homeostasis and reducing hepatic glucose production, appetite, and body weight (37). Acetate produced by bifidobacteria improves the intestinal defense mediated by epithelial cells and protects the host against lethal infection (34). Also, SCFAs, particularly butyrate, stimulate epithelial metabolism and deplete intracellular O_2, resulting in stabilization of the transcription factor HIF-1 and increasing epithelial barrier function (38). In humans, low levels of butyrate-producing bacteria have been associated with colonic disease (e.g., inflammatory bowel disease), highlighting the role of SCFAs in disease resistance (39,40).

Our study is not without limitations. Our findings are based on observational data that cannot provide causal inference. We were able to reduce the potential for confounding by matching on age, sex, and BMI. Because our metformin–gut microbiota associations were largely consistent whether we used as a reference group the T2D-met$^-$ or ND participants, we believe our findings were not due to confounding by indication. However, we cannot rule out unmeasured or residual confounding. Another limitation to our study was the lack of information on dose and duration of metformin treatment. This limitation could have resulted in a weaker, more conservative, association (attenuation toward the null) between metformin and gut microbiota composition and structure. Future studies are warranted to determine the dose-response of the metformin-microbiota relationship. We also had a small sample size relative to a previous observational study on this topic (Forslund et al. [6] analyzed 784 gut metagenomes of Danish, Swedish, and Chinese participants, of which, 199 had type 2 diabetes) and thus may have been underpowered to detect statistical significance for measures of α diversity and associations of microbial composition that were smaller in magnitude. Nevertheless, our ability to largely confirm hypotheses generated from previous studies with our modest sample size and in a different population suggests that the effect of metformin on the gut microbiota is robust and replicable across diverse populations.

In conclusion, our study of Colombian adults provides evidence congruent with the hypothesis that metformin has direct effects on gut microbiota composition through augmentation of mucin-degrading *A. muciniphila* as well as several SCFA-producing bacteria. Randomized controlled trials are needed to determine whether the antidiabetes and anti-inflammatory effects of metformin are mediated by the changes to gut microbiota composition.

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Author Contributions. J.d.l.C.-Z. processed fecal samples and DNA sequences, performed analyses, and wrote the manuscript. N.T.M. conceived the study, put forward hypotheses to be tested, and wrote the manuscript. V.C.-A. designed the cohort study, recruited participants, coordinated field activities, collected fecal and blood samples, and measured anthropometric variables. E.P.V.-M. processed fecal samples and DNA sequences. J.A.C. coordinated field activities and transport and treatment of samples. J.M.A. coordinated participant recruitment and field activities. J.S.E. designed the cohort study, coordinated participant recruitment, supervised field activities and transport and treatment of samples, performed analyses, and wrote the manuscript. All authors read and approved the final version of the manuscript. J.S.E. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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References

1. Everard A, Belzer C, Geurts L, et al. Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci U S A 2013;110:9066–9071
2. Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 2012;490:55–60
3. Karlsson FH, Tremaroli V, Nookaew I, et al. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature 2013;498:99–103
4. Larsen N, Vogensen FK, van den Berg FWJ, et al. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PLoS One 2010;5:e9085
5. Zhang X, Shen D, Fang Z, et al. Human gut microbiota changes reveal the progression of glucose intolerance. PLoS One 2013;8:e71108
6. Forslund K, Hildebrand F, Nielsen T, et al; MetaHIT consortium. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature 2015;528:262–266
7. Pernicova I, Korbonits M. Metformin–mode of action and clinical implications for diabetes and cancer. Nat Rev Endocrinol 2014;10:143–156
8. Lamanna C, Monami M, Marchionni N, Mannucci E. Effect of metformin on cardiovascular events and mortality: a meta-analysis of randomized clinical trials. Diabetes Obes Metab 2011;13:221–228
9. Pryor R, Cabreiro F. Repurposing metformin: an old drug with new tricks in its binding pockets. Biochem J 2015;471:307–322
10. Rena G, Pearson ER, Sakamoto K. Molecular mechanism of action of metformin: old or new insights? Diabetologia 2013;56:1898–1906
11. Lee H, Ko G. Effect of metformin on metabolic improvement and gut microbiota. Appl Environ Microbiol 2014;80:5935–5943
12. Shin N-RR, Lee J-CC, Lee H-YY, et al. An increase in the *Akkermansia* spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut 2014;63:727–735
13. Zhang X, Zhao Y, Xu J, et al. Modulation of gut microbiota by berberine and metformin during the treatment of high-fat diet-induced obesity in rats. Sci Rep 2015;5:14405
14. Bonora E, Cigolini M, Bosello O, et al. Lack of effect of intravenous metformin on plasma concentrations of glucose, insulin, C-peptide, glucagon and growth hormone in non-diabetic subjects. Curr Med Res Opin 1984;9:47–51
15. Buse JB, DeFronzo RA, Rosenstock J, et al. The primary glucose-lowering effect of metformin resides in the gut, not the circulation: Results from short-term pharmacokinetic and 12-week dose-ranging studies. Diabetes Care 2016;39:198–205
16. Bailey CJ, Wilcock C, Scarpello JH. Metformin and the intestine. Diabetologia 2008;51:1552–1553
17. Napolitano A, Miller S, Nicholls AW, et al. Novel gut-based pharmacology of metformin in

patients with type 2 diabetes mellitus. PLoS One 2014;9:e100778
18. Escobar JS, Klotz B, Valdes BE, Agudelo GM. The gut microbiota of Colombians differs from that of Americans, Europeans and Asians. BMC Microbiol 2014;14:311
19. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 2013;79:5112–5120
20. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 2009;75:7537–7541
21. R Development Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria, R Foundation for Statistical Computing, 2012
22. Oksanen J, Blanchet FG, Kindt R, et al. *Vegan: Community Ecology Package. R package version 2.3-1*, R Foundation for Statistical Computing, 2015. p. 264.
23. Chen J, Bittinger K, Charlson ES, et al. Associating microbiome composition with environmental covariates using generalized UniFrac distances. Bioinformatics 2012;28:2106–2113
24. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. Genome Biol 2011;12:R60
25. Storey JD, Bass AJ, Dabney A, Robinson D. qvalue: Q-value estimation for false discovery rate control. R package version 2.2.2. Available from http://github.com/jdstorey/qvalue. Accessed 19 January 2016
26. Wu GD, Chen J, Hoffmann C, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science 2011;334:105–108
27. Murray BE. Vancomycin-resistant enterococcal infections. N Engl J Med 2000;342:710–721
28. Cabreiro F, Au C, Leung KY, et al. Metformin retards aging in *C. elegans* by altering microbial folate and methionine metabolism. Cell 2013;153:228–239
29. Maida A, Lamont BJ, Cao X, Drucker DJ. Metformin regulates the incretin receptor axis via a pathway dependent on peroxisome proliferator-activated receptor-α in mice. Diabetologia 2011;54:339–349
30. Mannucci E, Ognibene A, Cremasco F, et al. Effect of metformin on glucagon-like peptide 1 (GLP-1) and leptin levels in obese nondiabetic subjects. Diabetes Care 2001;24:489–494
31. Wahlström A, Sayin SI, Marschall H-U, Bäckhed F. Intestinal crosstalk between bile acids and microbiota and its impact on host metabolism. Cell Metab 2016;24:41–50
32. Turroni F, Bottacini F, Foroni E, et al. Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc Natl Acad Sci U S A 2010;107:19514–19519
33. Sakon H, Nagai F, Morotomi M, Tanaka R. *Sutterella parvirubra* sp. nov. and *Megamonas funiformis* sp. nov., isolated from human faeces. Int J Syst Evol Microbiol 2008;58:970–975
34. Fukuda S, Toh H, Hase K, et al. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 2011;469:543–547
35. Gargari G, Taverniti V, Balzaretti S, et al. Consumption of a *Bifidobacterium bifidum* strain for 4 weeks modulates dominant intestinal bacterial taxa and fecal butyrate in healthy adults. Appl Environ Microbiol 2016;82:5850–5859
36. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther 2008;27:104–119
37. De Vadder F, Kovatcheva-Datchary P, Goncalves D, et al. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Cell 2014;156:84–96
38. Kelly CJ, Zheng L, Campbell EL, et al. Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. Cell Host Microbe 2015;17:662–671
39. Machiels K, Joossens M, Sabino J, et al. A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. Gut 2014;63:1275–1283
40. Sokol H, Seksik P, Furet JP, et al. Low counts of *Faecalibacterium prausnitzii* in colitis microbiota. Inflamm Bowel Dis 2009;15:1183–1189