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Authors

Cui, Jinrui

Ramesh, Gautam

Wu, Martin

et al.

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Butyrate-Producing Bacteria and Insulin Homeostasis: The Microbiome and Insulin Longitudinal Evaluation Study (MILES)

Jinrui Cui,[1] Gautam Ramesh,[2] Martin Wu,[3] Elizabeth T. Jensen,[4] Osa Crago,[4] Alain G. Bertoni,[4] Chunxu Gao,[5] Kristi L. Hoffman,[5] Patricia A. Sheridan,[6] Kari E. Wong,[6] Alexis C. Wood,[7] Yii-Der I. Chen,[8] Jerome I. Rotter,[8] Joseph F. Petrosino,[5] Stephen S. Rich,[9] and Mark O. Goodarzi[1]

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Gut microbiome studies have documented depletion of butyrate-producing taxa in type 2 diabetes. We analyzed associations between butyrate-producing taxa and detailed measures of insulin homeostasis, whose dysfunction underlies diabetes in 224 non-Hispanic Whites and 129 African Americans, all of whom completed an oral glucose tolerance test. Stool microbiome was assessed by whole-metagenome shotgun sequencing with taxonomic profiling. We examined associations among 36 butyrate-producing taxa (*n* = 7 genera and 29 species) and insulin sensitivity, insulin secretion, disposition index, insulin clearance, and prevalence of dysglycemia (prediabetes plus diabetes, 46% of cohort), adjusting for age, sex, BMI, and race. The genus *Coprococcus* was associated with higher insulin sensitivity (β = 0.14; *P* = 0.002) and disposition index (β = 0.12; *P* = 0.012) and a lower rate of dysglycemia (odds ratio [OR] 0.91; 95% CI 0.85–0.97; *P* = 0.0025). In contrast, *Flavonifractor* was associated with lower insulin sensitivity (β = −0.13; *P* = 0.004) and disposition index (β = −0.11; *P* = 0.04) and higher prevalence of dysglycemia (OR 1.22; 95% CI 1.08–1.38; *P* = 0.0013). Species-level analyses found 10 bacteria associated with beneficial directions of effects and two bacteria with adverse associations on insulin homeostasis and dysglycemia. Although most butyrate producers analyzed appear to be metabolically beneficial, this is not the case for all such bacteria, suggesting that microbiome-directed therapeutic measures to prevent or treat diabetes should be targeted to specific butyrate-producing taxa rather than all butyrate producers.

In recent decades, the prevalence of type 2 diabetes has increased dramatically (1). We refer to insulin sensitivity, insulin secretion, and insulin clearance as components of "insulin homeostasis," given that these traits encompass action, production, and removal of insulin. Dysfunction in insulin sensitivity and insulin secretion underlie the development of type 2 diabetes. The role of insulin clearance is less well established (2). Insulin clearance may contribute to diabetes pathogenesis in high-risk populations (3). A major goal of diabetes research is to determine mechanisms whereby insulin homeostasis deteriorates and predisposes to diabetes. Studies have implicated genetic factors, unhealthy diet, insufficient physical activity, suboptimal sleep, and obesogenic environmental factors. A recent addition is dysbiosis of the gut microbiome (4). The Microbiome and Insulin Longitudinal Evaluation Study

[1]Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, CA
[2]School of Medicine, University of California San Diego, La Jolla, CA
[3]Department of Biology, University of Virginia, Charlottesville, VA
[4]Department of Epidemiology and Prevention, Wake Forest School of Medicine, Winston-Salem, NC
[5]Alkek Center for Metagenomics and Microbiome Research, Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX
[6]Metabolon Inc., Morrisville, NC
[7]U.S. Department of Agriculture, Agricultural Research Service, Children's Nutrition Research Center, Baylor College of Medicine, Houston, TX
[8]Institute for Translational Genomics and Population Sciences, The Lundquist Institute for Biomedical Innovation and Department of Pediatrics, Harbor-UCLA Medical Center, Torrance, CA
[9]Center for Public Health Genomics, University of Virginia, Charlottesville, VA
Corresponding author: Mark O. Goodarzi, mark.goodarzi@cshs.org

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(MILES) seeks to define the effect of the gut microbiome on the three insulin homeostasis traits whose dysfunction leads to type 2 diabetes (5).

The gut microbiota consists of a diverse community of 10^{13} to 10^{14} bacteria, archaea, and eukaryotes (6). The number of genes represented by the gut microbiome (i.e., the combined genomes of the gut microbiota) is more than two orders of magnitude greater than the number encoded in the human genome (7). Although relatively stable in adults, the gut microbiome can be altered by diet and medications (8). High-throughput sequencing technology has facilitated large-scale microbiome profiling in individuals with prediabetes or diabetes versus controls, as recently reviewed (4,9). A recurrent finding in these studies was depletion of bacterial species that produce short-chain fatty acids (SFCAs), particularly butyrate, in individuals with type 2 diabetes.

Butyrate, propionate, and acetate are the most abundant SCFAs in the human gastrointestinal tract. Intestinal microbes generate SCFAs by fermenting dietary carbohydrates that humans cannot digest. SCFAs have been extensively reported to improve glucose homeostasis and metabolism in adipose, muscle, and liver tissues (10). Of the SCFAs, the greatest weight of evidence supports butyrate as a beneficial factor for metabolism. The relationships between butyrate-producing bacteria and distinct metrics of insulin homeostasis have not been thoroughly explored, and prior human studies (11,12) typically relied on surrogate measures based on fasting glucose and insulin, which do not clearly distinguish the aspects of insulin homeostasis (13). Thus, in the present study, we investigated the associations between butyrate-producing taxa (genus and species) and detailed indices of insulin sensitivity, secretion, and clearance measured using the oral glucose tolerance test (OGTT) in MILES. We hypothesized that butyrate-producing taxa would be associated with beneficial indices of insulin homeostasis and less frequent dysglycemia.

RESEARCH DESIGN AND METHODS

Study Participants

Ascertainment and recruitment of MILES participants were previously described (5). Three study visits are planned in MILES; the analyses reported here were performed using data collected at the first visit, which was completed by 353 individuals without known diabetes. Clinical and metabolic characteristics of these subjects have been previously described (2,5). Table 1 displays phenotypes used in the present report. None of the study participants had severe gastrointestinal illness or use of medications that could affect the microbiome (e.g., antibiotics, metformin, proton pump inhibitors [14,15]) or alter glucose homeostasis (e.g., glucocorticoids). Glucose tolerance (normal, prediabetes, diabetes) was defined using American Diabetes Association criteria (16). Prevalent diabetes at baseline (by history or point-of-care fasting glucose level $\geq$7.0 mmol/L) was an exclusion criterion; however, results of OGTT performed during the study showed that 25 individuals who initially self-identified as nondiabetic had 2-h glucose levels of 11.1 mmol/L or greater and an additional three individuals had fasting glucose levels slightly greater than 7.0 mmol/L. These 28 people were classified as having diabetes in this analysis. None of these individuals were taking antidiabetic medication, because they were not previously known to have diabetes. An additional 135 participants were classified as having prediabetes based on impaired fasting glucose (IFG; 5.6–6.9 mmol/L; n = 72 individuals) or impaired glucose tolerance (IGT; 2 h glucose level 7.8–11.0 mmol/L; n = 27 individuals) or both IFG and IGT (n = 36 individuals). To maximize power, those participants with diabetes and prediabetes were combined into a single dysglycemic group (n = 163 participants) and were compared with the 189 participants with normal glucose tolerance.

The study was approved by institutional review boards at participating centers. All participants gave written informed consent prior to participation.

Phenotyping Insulin Homeostasis

To achieve the best balance of quality phenotyping without undue burden to participants, the OGTT was used to obtain measures of insulin homeostasis. After an overnight fast, venous blood samples were obtained for the measurement of plasma glucose, insulin, and C-peptide levels before (fasting) and 30 and 120 min after the oral administration of a 75 g glucose load. Although several OGTT-derived indices of insulin sensitivity and resistance have been developed, we used the Matsuda insulin sensitivity index (ISI). Of the indices, the ISI is the most highly correlated with directly quantified (by euglycemic clamp) insulin sensitivity (r = 0.7–0.8) (17). Furthermore, the ISI can be calculated using fewer than five OGTT time points, without reduction in correlation with directly measured insulin sensitivity (18). Our measure of insulin secretion is the area under curve (AUC) for insulin from baseline to 30 min (AUC-Ins_{30}) divided by the corresponding AUC for glucose (AUC-Glu_{30}; i.e., AUC-Ins_{30}/AUC-Glu_{30}). This measure was found to be highly correlated with first-phase insulin secretion from the intravenous glucose tolerance test (r = 0.7) (17). In addition, this AUC-based insulin-secretion measure has been found to have a hyperbolic relationship with insulin sensitivity, consistent with the relationship found when insulin secretion and insulin sensitivity are measured with gold standard physiologic tests (19). This relationship allows calculation of the disposition index (DI_{30}), the product of insulin secretion and insulin sensitivity (herein, DI_{30} = ISI × AUC-Ins_{30}/AUC-Glu_{30}), which represents an index of insulin secretion that accounts for its degree of compensation for insulin resistance. Insulin clearance was measured as the AUC of C-peptide (AUC-Cpep) divided by the AUC of insulin (AUC-Ins; i.e., AUC-Cpep/AUC-Ins), a commonly used index

Table 1—Clinical and insulin homeostasis traits by glycemic category

	Normal glucose tolerance (n = 189)	Dysglycemia (n = 163)	P value*
Age (years)	58.0 (13.0)	62.0 (14.0)	0.0024
Female sex (%)	68.8	53.7	0.0035
African American race (%)	34.4	39.0	0.37
BMI (kg/m^2)	26.0 (7.2)	29.3 (7.5)	0.0002
Insulin sensitivity index	5.57 (4.59)	2.67 (2.23)	<0.0001
Insulin secretion (AUC-Ins$_{30}$/AUC-Glu$_{30}$)	0.35 (0.29)	0.36 (0.34)	0.44
Disposition index	1.93 (1.10)	1.02 (0.68)	<0.0001
Insulin clearance (AUC-Cpep/AUC-Ins)	0.11 (0.054)	0.095 (0.043)	<0.0001
Butyric acid (ng/mL)	28.61 (24.74)	28.96 (29.52)	0.82

Data are median (interquartile range) for quantitative traits percent. *P value for each row by t test or χ^2 test.

of hepatic insulin extraction, given that the liver clears insulin but not C-peptide (20).

Plasma Butyrate Measurement

Plasma butyrate was measured using liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Metabolon Method TAM148: "LC-MS/MS Method for the Quantitation of Short Chain Fatty Acid (C2 to C6) in Plasma and Serum"). Plasma samples were supplemented with stable, labeled internal standards and homogenized and subjected to protein precipitation with an organic solvent. After centrifugation, an aliquot of the supernatant was derivatized. The reaction mixture was injected onto an Agilent 1290/AB Sciex QTrap 5500 LC-MS/MS system equipped with a C18 reversed-phase ultra-high performance liquid-chromatography column. The mass spectrometer was operated in negative mode using electrospray ionization. The peak area of the individual analyte product ions was measured against the peak area of the product ions of the corresponding internal standards. Quantitation was performed using a weighted linear least squares regression analysis generated from fortified calibration standards prepared immediately prior to each run. LC-MS/MS raw data were collected using AB SCIEX software Analyst 1.6.3 and processed using SCIEX OS-MQ software, version 1.7.

Stool Collection and Microbiome Sequencing

Study participants collected a stool sample at home 1–2 days prior to the clinic visit. Collection was accomplished using a FecesCatcher (Tag Hemi), and stool was stored in the OMNIgene GUT collection kit to stabilize microbial DNA for gut microbiome profiling. Sampling with the OMNIgene GUT kit produces a microbial composition profile consistent with that of a direct stool sample (21). Stool samples were shipped from the clinical recruitment center at Wake Forest University to the Baylor College of Medicine Alkek Center for Metagenomics and Microbiome Research (CMMR).

Whole-metagenome shotgun sequencing was performed at CMMR at a depth to produce 10 gigabases/sample, providing species-level resolution of the microbial content of each sample. Briefly, libraries generated from total genomic DNA extracted from stool were sequenced on the HiSeqX (Illumina) platform using the 2 × 150-bp paired-end read protocol. Sequence data were processed using a series of publicly available tools, including Casava (Illumina) for the generation of fastq files, and the BBDuk (22) tool for quality trimming and filtering. After removal of low-quality reads and human reads, the mean number of final reads was 63,671,423 (range 36,818,000–115,800,576). Taxonomic profiling of the final read set was performed using MetaPhlAn3 (23). The default settings of MetaPhlAn3 were used.

Butyrate-Producing Taxa

Vital et al. (24) sought to comprehensively characterize all butyrate-producing bacteria in the human gut microbiota. This was accomplished in 2,387 metagenomic samples from 15 data sets. Butyrate producers were identified by the presence of genes coding for components of butyrate synthesis pathways, of which there are four main pathways. By requiring genes from entire pathways, this study more confidently identified butyrate producers versus assignment of butyrate-producing potential solely on the basis of terminal butyrate-producing enzymes. Although >1,600 butyrate-producing taxa were identified, 17 taxa, present in >70% of individuals, encompassed ∼85% of the butyrate-producing potential. Thus, we considered only these taxa in this study. The 17 taxa consisted of eight genera and nine species. In our data, we identified 27 species within the eight genera, such that a total of eight genera and 36 species (n = 44 taxa) were initially considered. One genus (*Butyricicoccus*) and seven species were present in <5% of the cohort and thus were eliminated from analysis, yielding seven genera and 29 species examined herein (n = 36 taxa).

Statistical Analysis

Phenotypes between the dysglycemic and normoglycemic groups were compared using Student's *t* tests (for quantitative traits) and χ^2 tests (for sex and race). For this and subsequent analyses, a rank-based inverse normal transformation was applied to normalize the distribution of ISI, AUC-Ins_{30}/AUC-Glu_{30}, DI_{30}, AUC-Cpep/AUC-Ins, and butyrate. However, median values (with interquartile range) are presented in Table 1 to facilitate interpretation.

The relative abundance of all genera and species generated from the whole-metagenome shotgun sequencing was subjected to the centered additive log-ratio (CLR) transformation. This transformation has the dual benefit of normalizing the distribution and removing the compositional aspect of the data. CLR-transformed abundance levels of the seven genera and 29 species were used for all statistical analyses.

Multiple linear regression was used to assess association of the 36 taxa with insulin sensitivity, insulin secretion, disposition index, and insulin clearance. These analyses were adjusted for the covariates age, sex, BMI, and race. Analyses were carried out in SAS, version 9.4 (SAS Institute, Cary, NC). Standardized regression coefficients (β coefficients) are reported from these analyses.

Association of the 36 taxa with the qualitative trait dysglycemia was assessed by analyzing each genus or species as an independent variable along with age, sex, BMI, and race in logistic regression analyses in which dysglycemia was the dependent variable. Exploratory race-stratified association analyses of the 36 taxa with insulin homeostasis traits and dysglycemia were carried out as outlined above, with the covariates being age, sex, and BMI.

Simple linear regression was used to characterize associations among abundance levels of 12 species associated with insulin homeostasis traits or dysglycemia. Association of taxa with blood butyrate level was assessed using multiple linear regression for 12 species manifesting association with insulin homeostasis traits or dysglycemia, with adjustment for age, sex, BMI, and race.

$P < 0.05$ were considered statistically significant. We did not correct for multiple testing, because this was a focused study exploring the hypothesis that butyrate-producing bacteria are associated with beneficial levels of insulin homeostasis and less frequent dysglycemia.

Data and Resource Availability

The data are not publicly available because participants did not give consent for the data to be publicly posted. Interested researchers should contact the corresponding author and submit their credentials to the Cedars-Sinai Institutional Review Board for determination of whether if they are eligible to have access to study data. Upon approval, a limited data set necessary for replication would be provided to the investigator.

RESULTS

Compared with those with normal glucose tolerance, the participants with dysglycemia were older, less frequently female, and had higher BMI (Table 1). The dysglycemic group also had lower insulin sensitivity, disposition index, and insulin clearance. Race, insulin secretion, and butyrate level were similar between the groups.

Association of Butyrate-Producing Taxa With Insulin Homeostasis Traits and Dysglycemia

Table 2 displays the association of seven genera with components of insulin homeostasis. *Coprococcus* was associated with increased insulin sensitivity and higher disposition index, and *Oscillibacter* was associated with increased insulin sensitivity. *Flavonifractor* was associated with reduced insulin sensitivity and decreased disposition index. *Coprococcus* was also associated with a decreased prevalence of dysglycemia, whereas *Flavonifractor* was associated with increased dysglycemia (Table 3). The genera *Odoribacter* and *Oscillibacter* were also associated with lower prevalence of dysglycemia. Table 2 and Table 3 present 12 species that were associated with at least one insulin homeostasis trait or prevalent dysglycemia. Supplementary Table 1 and Supplementary Table 2 list the associations for all species with insulin homeostasis traits and dysglycemia, respectively. In some cases, species association was more informative than genus association. For example, two *Anaerostipes* species manifested opposite associations with insulin sensitivity and disposition index that were not seen for the corresponding genus. The most frequently observed pattern of association of taxa was with increased insulin sensitivity, increased disposition index, and reduced rates of dysglycemia. However, two species, *Flavonifractor plautii* and *A. caccae,* associated with increased dysglycemia were associated with lower insulin sensitivity and lower disposition index, suggesting adverse metabolic effects. One species, *Odoribacter splanchnicus,* was associated with lower prevalent dysglycemia but did not manifest associations with insulin homeostasis traits. *Alistipes finegoldii, Anaerostipes hadrus, C. eutactus, Eubacterium hallii,* and *Oscillibacter* sp. PC13 were each associated with at least one insulin homeostasis trait but not with dysglycemia.

Correlation Among Species Associated With Insulin Homeostasis Traits or Dysglycemia

Given the varied association of butyrate-producing taxa with insulin homeostasis and dysglycemia, we hypothesized that this might reflect relationships among the species. To characterize the relationships among the 12 species associated with insulin homeostasis traits or dysglycemia, we assessed the correlation among abundance levels of these species (Fig. 1 and Supplementary Table 3). This revealed two inversely related abundance groups of butyrate producers. *F. plautii* and *A. caccae,* noted above to have adverse metabolic associations, were positively correlated

Table 2—Associations of genera and species with insulin homeostasis traits

	Insulin sensitivity		Insulin secretion		Disposition index		Insulin clearance	
	β	*P* value	β	*P* value	β	*P* value	β	*P* value
Genus								
Alistipes	0.028	0.52	−0.027	0.57	0.0096	0.85	0.0016	0.97
Anaerostipes	0.072	0.10	0.0001	0.99	0.093	0.058	0.013	0.78
Coprococcus	**0.14**	**0.0021**	−0.040	0.41	**0.12**	**0.012**	0.029	0.54
Flavonifractor	**−0.13**	**0.0039**	0.046	0.35	**−0.10**	**0.041**	0.024	0.61
Odoribacter	0.069	0.12	−0.030	0.53	0.054	0.28	0.033	0.48
Oscillibacter	**0.094**	**0.035**	−0.029	0.55	0.085	0.088	0.0086	0.85
Pseudoflavonifractor	0.028	0.52	−0.027	0.57	0.015	0.77	0.020	0.66
Species								
Alistipes finegoldii	−0.025	0.58	0.068	0.16	0.054	0.27	**−0.090**	**0.049**
A. onderdonkii	**−0.096**	**0.029**	0.043	0.39	−0.057	0.25	−0.073	0.11
Anaerostipes caccae	**−0.12**	**0.0076**	−0.0018	0.97	**−0.14**	**0.0036**	0.0052	0.91
A. hadrus	**0.089**	**0.043**	−0.021	0.66	0.088	0.073	0.022	0.64
C. comes	**0.11**	**0.015**	−0.011	0.82	**0.12**	**0.012**	0.012	0.80
C. eutactus	**0.14**	**0.0015**	−0.052	0.28	**0.11**	**0.032**	0.081	0.078
Eubacterium hallii	**0.094**	**0.033**	−0.050	0.30	0.058	0.24	−0.0003	0.99
Faecalibacterium prausnitzii	**0.088**	**0.045**	−0.049	0.315	0.054	0.28	−0.015	0.74
Flavonifractor plautii	**−0.15**	**0.0010**	0.051	0.30	**−0.12**	**0.019**	0.016	0.73
Odoribacter splanchnicus	0.067	0.13	−0.029	0.55	0.050	0.32	0.031	0.50
Oscillibacter sp. CAG 241	0.079	0.075	0.0010	0.98	**0.098**	**0.048**	−0.019	0.68
O. sp. PC13	**0.11**	**0.019**	**−0.11**	**0.023**	0.0072	0.89	**0.12**	**0.013**

Association was assessed using multiple linear regression, with CLR-transformed taxa abundance level and covariates (age, sex, BMI, and race) as independent variables and insulin homeostasis traits as dependent variables. Data in bold are statistically significant.

with each other ($r = 0.34$; $P < 0.0001$) and negatively correlated with many of the remaining 10 bacteria that were associated with beneficial effects on insulin homeostasis or dysglycemia. Numerous positive correlations were observed among the latter 10 species (Fig. 2). Among the abundance network of these 10 species, *C. comes* manifested the highest number of correlations with other members of the group ($n = 7$ correlations). Three other members of the network, *Oscillibacter* sp. CAG 241, *Faecalibacterium prausnitzii*, and *Alistipes finegoldii*, also exhibited high connectivity ($n = 6$ connections each).

Characterization of Key Taxa in Regard to Butyrate Production

Exploratory analyses were conducted to evaluate the potential role of butyrate production in the 12 species associated with insulin homeostasis traits or dysglycemia. We assessed the association of abundance of the 12 species with serum butyrate level (Supplementary Table 4). Three species associated with increased butyrate level were *Anaerostipes hadrus* (β = 0.11; $P = 0.027$), *E. hallii* (β = 0.11; $P = 0.034$), and *F. prausnitzii* (β = 0.13; $P = 0.011$), which were member species of the network associated with beneficial effects on insulin homeostasis. We also sought to determine whether different pathways of butyrate production (as previously described for each species [24]) were differentially present between the two groups of bacteria, but we found no clear pattern. All 13 bacteria contained genes for the acetyl CoA pathway. A few members of both groups harbored genes for multiple pathways; for example, both *Flavonifractor plautii* (adverse metabolism group) and *Odoribacter splanchnicus* (beneficial metabolism group) expressed genes for acetyl-CoA, lysine, and 4-aminobutyrate pathways.

Race-Stratified Analyses of Butyrate-Producing Taxa With Insulin Homeostasis Traits and Dysglycemia

In secondary analyses, we examined the association of butyrate-producing taxa with insulin homeostasis traits and dysglycemia separately in the two race groups. Supplementary Table 5 and Supplementary Table 6 list the associations of 36 taxa with insulin homeostasis traits in African Americans and non-Hispanic Whites, respectively. Comparison of the associations in each race with those of the combined cohort revealed the most consistent results for insulin sensitivity. The association of genus *Coprococcus* with insulin sensitivity was significant in the entire cohort as well as in both races. Additional signals significant in the entire cohort as well as in non-Hispanic Whites included associations with insulin sensitivity of the genus *Flavonifractor* and the species *A. caccae, A. hadrus*, *C. eutactus*, *E. hallii*, *F. plautii*, and *Oscillibacter* sp. PC13, with consistent directions of effect in African Americans, where the association was not significant. Associations with disposition index were less consistent, with no taxon significant in both races; however, the genus *Coprococcus* and the species *A. caccae*, *C. comes*, and *F. plautii* were associated

Table 3—Associations of genera and species with dysglycemia

	OR	95% CI	Adjusted *P* value
Genus			
Alistipes	0.94	0.88–1.00	0.067
Anaerostipes	0.97	0.89–1.07	0.54
Coprococcus	**0.91**	**0.85–0.97**	**0.0025**
Flavonifractor	**1.22**	**1.08–1.38**	**0.0013**
Odoribacter	**0.94**	**0.90–0.99**	**0.022**
Oscillibacter	**0.95**	**0.90–1.00**	**0.044**
Pseudoflavonifractor	0.99	0.91–1.08	0.86
Species			
Alistipes finegoldii	0.96	0.91–1.02	0.16
A. onderdonkii	1.07	0.99–1.15	0.074
Anaerostipes caccae	**1.26**	**1.07–1.53**	**0.010**
A. hadrus	0.98	0.90–1.06	0.57
Coprococcus comes	**0.90**	**0.85–0.96**	**0.0008**
C. eutactus	0.97	0.91–1.04	0.43
Eubacterium hallii	0.97	0.89–1.06	0.49
Faecalibacterium prausnitzii	0.96	0.88–1.06	0.46
Flavonifractor plautii	**1.18**	**1.07–1.32**	**0.0014**
Odoribacter splanchnicus	**0.95**	**0.90–1.00**	**0.040**
Oscillibacter sp. CAG 241	**0.92**	**0.86–0.97**	**0.0039**
O. sp. PC13	0.91	0.82–1.01	0.08

Association was assessed using multiple logistic regression, with CLR-transformed taxa abundance level and covariates (age, sex, BMI, and race) as independent variables and dysglycemia status as the dependent variable. Data in bold are statistically significant.

with disposition index in the entire cohort and in one of the two races, with consistent direction of effect in the other race.

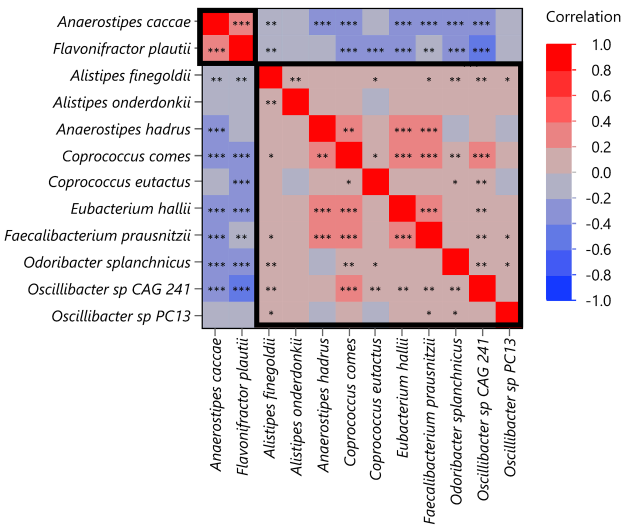

Figure 1—Correlations among abundance levels of 12 species associated with insulin homeostasis traits or dysglycemia. Correlation coefficients are displayed, with shades of red representing positive values and shades of blue representing negative values. The two clusters of species are outlined with squares. *$P < 0.05$ and >0.01; **$P < 0.01$ and ≥0.0001; ***$P < 0.0001$.

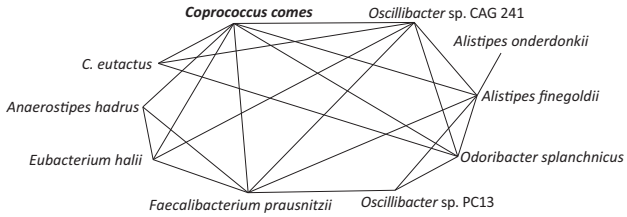

Figure 2—Network of species associated with beneficial effects on insulin homeostasis and dysglycemia. Each line connecting two species indicates a significant correlation in abundance ($P < 0.05$). *Coprococcus comes* (in bold) had the greatest number of connections to other species.

Supplementary Table 7 displays the association of 36 taxa with dysglycemia stratified by race. The associations of the genera *Coprococcus* and *Flavonifractor* and species *C. comes* and *Oscillibacter* sp. CAG 241 with dysglycemia were statistically significant in both African Americans and non-Hispanic Whites. Most of the other associations with reduced dysglycemia observed in the whole cohort (n = 4 of 5) were statistically significant in only one race, with the direction of effect being similar in the other race. Only the association of *Odoribacter splanchnicus* with reduced dysglycemia that was significant in the whole cohort was not significant in either cohort separately. Race-stratified analyses for dysglycemia revealed associations of genus *Alistipes* and species *Oscillibacter* sp. PC13 with reduced dysglycemia in non-Hispanic Whites that were not statistically significant (P = 0.067 and 0.080, respectively) in the whole cohort.

DISCUSSION

This study was a hypothesis-driven, focused analysis of only butyrate-producing taxa, assessing their association with insulin homeostasis traits and dysglycemia. We identified two groups of taxa, one associated with beneficial insulin homeostasis traits and the other with adverse insulin homeostasis associations. This may inform future microbiome-targeted modalities to prevent or treat diabetes.

The SCFA butyrate is one of the most widely studied gut microbial metabolites. A substantial body of literature has documented beneficial effects of SCFAs on glucose homeostasis and metabolism (10). By binding to G-protein–coupled receptors in the gut, SCFAs can stimulate secretion of glucagon-like peptide 1 and peptide YY, which promote satiety and improve insulin sensitivity, respectively (25,26). SCFAs also appear to stimulate leptin production in adipocytes (27). Among the SCFAs studied, the most consistent pattern of metabolic advantage has been seen for butyrate, whereas there is less consistent evidence of benefit for other SCFAs (namely, propionate and acetate) (28). Butyrate inhibits histone deacetylase, and this inhibition promotes β-cell development, proliferation, differentiation,

and function and inhibit apoptosis (29), In mice, addition of butyrate to a high-fat diet prevented the development of insulin resistance and obesity via increased energy expenditure and improved mitochondrial function (30). Therefore, we focused attention on butyrate-producing taxa in this study, specifically 44 taxa that express at least one of four pathways of butyrate production (24), of which 36 were evaluable in our data set.

Of the species analyzed, we identified two groups. The group with the greater number of species exhibited a pattern of association that suggests they exert metabolic protection against diabetes via improved insulin sensitivity and improved insulin-secretion response to insulin sensitivity (the latter represented by the disposition index). The members of this group were highly intercorrelated in abundance level, suggestive of a functional network. Three members of this group were associated with higher circulating butyrate levels, providing support for the hypothesis that this group of bacteria may improve metabolism by producing butyrate, which is then absorbed systemically where it can improve insulin homeostasis. Several members of this group, *Coprococcus comes*, *Oscillibacter* sp. CAG 241, *Alistipes finegoldii*, and *Faecalibacterium prausnitzii*, exhibited the highest numbers of connections with the other members. This raises the possibility that future diabetes prevention or treatment modalities (e.g., prebiotic or probiotic) could be most effective if they are targeted to promoting growth of these species as nodes in the network. We note that the relationships among species depicted in Fig. 2 are based on abundance. The functional basis of such relationships, and whether they are direct or indirect or symbiotic, will require further study.

We also found association of two butyrate-producing species (*Flavonifractor plautii* and *Anaerostipes caccae*) with adverse associations with insulin sensitivity or disposition index, results seemingly inconsistent with the concept of butyrate as metabolically beneficial. These species were correlated with each other and negatively correlated with the group of species associated with metabolic benefit. Similar findings have been observed in other studies in this field. In a recent gut microbiome study of nearly 1,500 individuals, Wu et al. (12) also observed two clusters of butyrate producers. Consistent with our results, Wu et al. found a higher number of butyrate-producing species were depleted in those with prediabetes or diabetes, whereas a smaller number of butyrate-producing species were enriched in these groups. They did not report association of *A. caccae* with dysglycemic states, whereas they found that abundance of *F. plautii* was lower in those with prediabetes (i.e., individuals with both IFG and IGT) but higher in those with diabetes, compared with those with normal glucose tolerance (12). Another recent gut microbiome study found a significant association between increased levels of *F. plautii* and type 2 diabetes (31). A possible explanation for enrichment of specific butyrate producers in individuals with dysglycemia is that these taxa may carry genes that code for other processes that counteract the beneficial effects of butyrate or otherwise adversely affect metabolism. Indeed, in the study by Wu et al. (12), the genomes of such butyrate-producing taxa were enriched for genes encoding virulence factors. Another possible explanation is that such butyrate producers cooccur with butyrate-consuming species or other taxa that produce harmful metabolites. Another possibility is that these taxa produce insufficient amounts of butyrate to exert beneficial effects on metabolism.

We found that the abundance of the two groups of butyrate producers were inversely associated, suggesting competition for the niche of butyrate production. This key observation implies that future therapies for diabetes should target specific networks of butyrate-producing taxa, rather than butyrate producers in general.

An advantage of our study is that our dysglycemic group consisted of individuals with prediabetes and newly recognized, and therefore untreated, diabetes. This avoids confounding effects of antidiabetic medication as well as potential secondary effects on the microbiome of severe hyperglycemia. Prior gut microbiome studies of more advanced diabetes found strong differences in the microbiome of people with and without diabetes (32,33) that, on subsequent analysis, were found to be mainly driven by metformin treatment (14). When individuals taking metformin were excluded, depletion of butyrate-producing taxa was observed in individuals with diabetes (14), findings later confirmed in a large study of treatment-naïve patients (12). This was the basis of our focus on butyrate-producing taxa. Although >30 studies have examined the gut microbiome in prediabetes or diabetes (recently reviewed by Gurung et al. [4] and by Zhu and Goodarzi [9]), relatively few (11,12,34,35) have focused on the defects in insulin homeostasis (i.e., insulin sensitivity, secretion, and clearance) that underlie the development of diabetes, and none has examined insulin clearance. Similar to prior findings, we found several associations with insulin sensitivity, suggesting a particular effect of butyrate producers on this trait. Consistent with this, researchers reported that humans with metabolic syndrome receiving small-intestine fecal transplants from lean donors had improved insulin sensitivity 6 weeks later that correlated with an increase in butyrate-producing bacteria (36). Unlike insulin sensitivity, we observed few associations with insulin secretion and insulin clearance.

A unique feature of MILES compared with most other microbiome studies is that it includes two races, providing an opportunity to explore disease-associated microbes that are relevant to both races as well as any that are specific to one race. The associations of butyrate-producing taxa with insulin sensitivity and dysglycemia appear to be generally similar in both races, given that several taxa were significantly associated with these traits in both groups. Several other taxa that were statistically significantly associated with these traits in the entire cohort

and in one race group manifested a consistent direction of effect in the other race group. For such taxa, it is possible that larger sample sizes would have yielded significance in both races. Another possibility, which we cannot resolve with data from the sample in the present study, is that those represent race-specific associations. We did not observe any strong associations significant in only one race but not in the entire cohort to suggest race-specific associations of the taxa examined herein.

This study shares some limitations with prior work in this field, specifically that these studies have been cross-sectional. Such studies can demonstrate association but do not answer the question of whether the observed gut microbiome differences are a cause or a consequence of diabetes. We presented herein results from the baseline visit of MILES. As a prospective study, MILES will ultimately be able to assess whether the baseline microbiome profile or changes in the microbiota over time influence changes in insulin homeostasis or incident diabetes, providing stronger evidence for potential causality of gut bacteria on dysglycemia. Another caveat of this study is the sample size. Although larger than most studies in this field, the sample size may still have limited power. We observed association of blood butyrate level with only three species. We believe that a larger sample size would result in the association of more members of the apparently beneficial group with butyrate level, as well as identify more members of both groups. It is also possible that butyrate does not reflect the mechanism whereby some of these taxa influence dysglycemia; in our data set, plasma butyrate was not associated with any of the insulin homeostasis traits or dysglycemia (data not shown), raising the possibility that intestinal butyrate or other metabolites need to be explored. Another caveat is that though the OGTT-based measures used herein correlate well (r = 0.7–0.8) with the components of insulin homeostasis (i.e., insulin sensitivity, insulin secretion, insulin clearance), they do not perfectly represent these traits as measured by direct procedures, such as clamp studies or the frequently sampled intravenous glucose tolerance test (17,18,37–39).

This study adds to the growing body of work implicating butyrate-producing bacteria in metabolic health. Importantly, although many such taxa are depleted in individuals with dysglycemia, this does not apply to all butyrate producers. Our observation of two inversely related groups of taxa warrants independent confirmation, given the potential implication that microbiome-directed therapeutic measures would need to promote proliferation of the beneficial network of bacteria rather than all butyrate producers. This would provide key information for the design of prebiotic (foods that promote proliferation of specific species), probiotic, or antibiotic trials to prevent and treat type 2 diabetes.

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