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# Significant differences in fecal microbiota are associated with various stages of glucose tolerance in African American male veterans

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## Abstract

The importance of gut microbiota in pathogenesis of diabetes remains unknown. This study investigated the relationship between microbiota and metabolic markers in African American men (AAM) with prediabetes and hypovitaminosis D. The study was ancillary to a randomized trial of vitamin D supplementation with weekly ergocalciferol (50,000 IU) conducted in AAM veterans over 12 months (D Intervention in Veterans Affairs). Glycemic groups (Gr) were characterized based on changes in oral glucose tolerance between baseline and exit. Subjects with stable normal glucose tolerance were assigned to Gr-1 and those with stable prediabetes (impaired glucose tolerance and impaired fasting glucose) to Gr-2. Microbiota composition was analyzed in stool collected at the exit (n = 115) and compared between Gr-1 and Gr-2, as well as between the lowest and highest quartiles of dietary intake of energy and fat, hemoglobin A1c, and serum 25-hydroxyvitamin D (25[OH]D) level. Differences between Gr-1 and Gr-2 included the Bacteroidetes/Firmicutes and Bacteroidales/Clostridia ratios and differences in genera such as Ruminococcus and Dialister. Changes in specific taxa associated with the lowest and highest quartiles of 25(OH) D (eg, *Ruminococcus*, *Roseburia*, *Blautia, Dorea*) were clearly distinct from those of dietary intake (eg, *Bacteroides*, *Bacteroides/Prevotella* ratio) or A1c (eg, *Faecalibacterium*, *Catenibacterium*, *Streptococcus*). These findings suggest a novel interaction between microbiota and vitamin D and a role for microbiota in early stages of diabetes development. Although results suggest that specific taxa are associated with glycemic stability over time, a causative relationship between microbiota makeup and dysglycemia is still to be demonstrated.

## BACKGROUND

The transition of normal glucose tolerance into type 2 diabetes mellitus (T2DM) includes contributions from genetic and environmental factors.[1] Intestinal microbiota existing in a

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state of symbiosis with the human host may be perturbed by environmental factors with unfavorable health consequences. Recently, the gut microbiota has become a focus of research as its manipulation could potentially help in managing various medical conditions including diabetes and obesity. Dysbiosis of the gut microbial community can trigger inflammation that plays substantial role in dysregulation of normal glucose tolerance into prediabetes and diabetes.[2-4] Prior studies have shown that in patients with T2DM, gut microbial composition has shown a number of significant alterations, including changes in the relative abundance of taxa within the dominant gut phyla Firmicutes and Bacteroidetes.[5] Prior studies also have demonstrated that the relative abundance of bacteria from the phylum Firmicutes and the class Clostridia has been significantly reduced in subjects with diabetes compared to those without diabetes.[5] Moreover, the ratio of Bacteroidetes to Firmicutes has been positively correlated with plasma glucose, whereas bacteria from the genera *Roseburia* and *Prevotella* were negatively and positively correlated with plasma glucose, respectively.[5] Bacteria from the genera *Roseburia*,[6,7] *Bifidobacterium*,[8] and *Akkermansia*[6,9,10] all have been implicated in glucose homeostasis and possibly in progression from normoglycemia to prediabetes and diabetes.[10] More broadly, obesity and diets high in calories and fat have been associated with significant differences in gut microbiota.[11-13] Obesogenic microbiota also seems to have increased energy harvesting capabilities.[14] In obese subjects, the ratio between phyla Firmicutes and Bacteroidetes has been inconsistently linked to body mass index (BMI) and dietary components.[13,15,16]

The wide range in relative abundance of these taxa in healthy humans,[17] the metabolic diversity contained by bacteria within each phylum, and functional redundancy present in microbial communities all increase the challenges in identifying diagnostic changes in the gut microbiome associated with T2DM. In addition, data from Han Chinese and European individuals have provided evidence for ethnicity and gender-related differences in the gut microbial makeup,[6,7] suggesting an uncertainty in generalizing findings, particularly to ethnic groups underrepresented in medical trials and research studies.[18,19]

Vitamin D deficiency may contribute to increased risk of diabetes and gut microbiota dysbiosis. In a prospective cohort study of 9,841 participants followed for up to 29 years, the risk of incident diabetes was 35% higher for the individuals from the lowest serum 25-hydroxyvitamin D (25[OH]D) level quartile relative to the highest quartile.[20] In mice, vitamin D deficiency at birth has resulted later in life in lower abundance of *Bacteroides* and *Prevotella*, increased expression of proinflammatory genes in the colonocytes, and higher serum lipopolysaccharide concentration.[21] Currently, there are no similar data on vitamin D and microbiota interaction in humans.

Here, we address T2DM and gut microbiota in an African American cohort. We examined the relationship of the gut microbiota with metabolic markers including dietary intake, glucose tolerance, and circulating vitamin D level to address the critical knowledge gap associated with gut microbial communities, vitamin D, and T2DM. The study focused on African American men (AAM), a major underserved population with poor dietary habits, relatively high burden of chronic disease and at risk for diabetes, and vitamin D deficiency.

## DESIGN AND METHODS

This study was ancillary to "D vitamin Intervention in Veteran Administration" trial described elsewhere.[22] Briefly, this double-blind placebo-controlled randomized clinical trial tested the effect of 12-month high-dose vitamin D supplementation on oral glucose insulin sensitivity in AAM with dysglycemia and hypovitaminosis D (clinicaltrials.gov registration NCT01375660). Participants had follow-up examinations every 3 months and the final assessment after 12 months of treatment. The main inclusion criteria were as follows: AAM veterans, age 35–85 years, BMI 28–39 kg/m$^2$, fasting glucose 95–125 mg/dL, and/or glycosylated hemoglobin (A1C) 5.7%–6.4% (38.8–46.5 mmol/mol), 25(OH)D 5.0–29 ng/mL. The criteria for the diagnoses of prediabetes and diabetes were based on the American Diabetes Association recommendations.[23] Participants who were diagnosed with diabetes during screening or intervention (A1C 6.5%–6.9% or 47.5–51.9 mmol/mol) were allowed in the study if they did not need to take antidiabetic medications and A1C remained <7% (<53 mmol/mol). All subjects received cholecalciferol (D3) 400 IU as multiple vitamin and in addition either weekly ergocalciferol (D2) 50,000 IU (Pliva Co) or placebo. Change in 25(OH)D level was calculated as delta equal exit minus baseline. A total of 2,067 subjects were prescreened, 205 randomized, and 173 (84%) came for the final visit. Of these 173 subjects, 115 (55 in placebo and 60 in vitamin D2 groups) had successful stool DNA extraction and PCR amplification for microbial community composition analysis. The research was carried out according to the principles of the Declaration of Helsinki. Participants provided informed consent, and the study was approved by Jesse Brown Veteran's Affairs Institutional Review Board.

### Glycemic control

Oral glucose tolerance test (OGTT) was performed at baseline and at 12 months. Glucose tolerance status was defined using the American Diabetes Association criteria for normal and impaired tolerance.[23] Using OGTT at the baseline and exit, n = 115 subjects were grouped depending on their glycemic control change during the study. Subjects who remained stable during the study were divided in 2 groups (Gr): (1) Gr-1 had normal glucose tolerance at the baseline and at the end of the study at 12 months; hence, they maintained normal glucose tolerance throughout the study (n = 35) and (2) Gr-2, prediabetes, had impaired fasting glucose, impaired glucose tolerance, or both (impaired fasting glucose/impaired glucose tolerance) at baseline and at the end of the study; hence, they maintained a prediabetes state throughout the study (n = 28).

### Dietary data collection and analysis

Dietary data were collected using two 24-hour dietary recall questionnaires administered during the study. A subgroup of 40 subjects provided 2–3 sets of dietary data to evaluate for dietary intake consistency. Their dietary intake was stable and therefore reported as average values Therefore, it was assumed that the study subjects maintained consistent dietary habits over 12 months, and their diet at exit was similar to the diet at any other point in the study. Data were analyzed using the Food Processor software version 10.12.0 (ESHA Research, Salem, Oregon) and presented as mean ± standard deviation and % Recommended Dietary Allowance (RDA) or % Adequate Intake. Body composition was determined from DXA

technology using a Lunar Prodigy (G. E. Medical Systems, Madison, Wisconsin). All the regional boundaries were automatically selected, and data were analyzed using the enCORE software version 11.0 (G. E. Medical Systems, Madison, Wisconsin). Percent fat for each region was calculated as fat % = (fat mass/[fat mass + lean soft mass + bone mineral content]) × 100.

### Biologic sample collection and analysis

A 3-hour OGTT and blood measurements were performed as previously described.[22] Stool samples were collected at home before the exit visit in sterile cups and stored at −80°C within 2 hours or after overnight refrigeration at home.

### Microbial community analysis

Genomic DNA was extracted from patient fecal samples using the Maxwell 16 Tissue DNA Purification kit (Promega, Madison, Wisconsin). Genomic DNA from the fecal samples was PCR amplified and prepared for high-throughput amplicon sequencing using a modified 2-step targeted amplicon sequencing approach, similar to that described previously.[24] PCR amplicons, generated with primers 515F and 806R, targeting the V4 variable region of microbial 16S ribosomal RNA (rRNA) genes[25] were prepared for sequencing using the Access Array Barcode Library for Ion Torrent Sequencers (Fluidigm, South San Francisco, California).

Pooled, diluted libraries were prepared for sequencing using automated emulsion PCR implemented with the Ion OneTouch 2 and OneTouch ES instruments, according to manufacturer's instructions. After emulsion cracking, spheres were loaded on an Ion Torrent Personal Genome Machine (PGM) 318 chip and run on a PGM according to the manufacturer's instructions. DNA extraction, library preparation, and sequencing were performed in the DNA Services facility at the University of Illinois at Chicago. Barcode sequences from Fluidigm were provided to the PGM server, and sequences were automatically binned according to 10-base multiplex identifier sequences. Raw reads were recovered from the PGM server as FASTQ files.

Raw sequence data were imported into the software package CLC genomics workbench version 7.0 (CLC Bio, Qiagen, Boston, MA). Sequences were quality trimmed (Q20), and reads shorter than 200 bases were removed. Subsequently, FASTA files were processed through the software package QIIME.[26] Briefly, sequences were screened for chimeras using the usearch61 algorithm,[27] and putative chimeric sequences were removed from the dataset. Subsequently, each sample sequence set was subsampled to the smallest sample size to avoid analytical issues associated with variable library size.[28] Subsampled data were pooled and renamed and clustered into operational taxonomic units (OTU) at 97% similarity. Representative sequences from each OTU were extracted, and these sequences were classified using the "assign_taxonomy" algorithm implementing the uclust consensus taxonomy assigner, using the Greengenes reference database. A biological observation matrix[29] was generated at taxonomic levels from phylum to genus using the "make_OTU_table" algorithm. The biological observation matrices were imported into the

software package Primer6 (Primer-E Ltd, Plymouth, United Kingdom) for calculation of alpha diversity indices and analysis of similarity calculations.[30]

### Statistical analysis

Statistical analysis of clinical characteristics was performed as described previously.[22] Before analysis, normal distribution and homogeneity of the variances were tested. Values were represented as means and standard deviations. The groups were specified a priori. For selected parameters, data were shown and analyzed as lowest and highest quartiles. Paired 2-tailed Student *t* test was used to compare baseline to exit characteristics, and significance level was set at $P < 0.05$. For each sample and taxon, relative abundance was calculated by dividing the absolute number of sequences by the rarefied number of sequences for each sample (6280 sequences). At the exit, differences in microbiota taxonomic abundance were tested using Kruskal–Wallis nonparametric test. False discovery rate–corrected *P* values were estimated as described by Benjamini and Hochberg[31] for all taxa comparisons. The significance was set at $P < 0.05$ for Kruskal–Wallis test. For false discovery rate, significance was determined at $P < 0.05$ (5% false positive allowed), and tendency for significance was at $P < 0.1$ (10% false positive allowed). The unpaired student's *t* test was used to compare Shannon's diversity index ($P < 0.05$). All statistical analyses were performed using SAS 9.2 statistical software.

### Data access

The amplicon sequence data from this study have been submitted to the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi) under the BioProject PRJNA274647.

## RESULTS

### Clinical characteristics

Subjects' characteristics at the end of the study were not significantly different from baseline, with the exception of serum 25(OH)D, which, as expected, was significantly increased from 14 ± 6 ng/mL to 36 ± 24 ng/mL ($P < 0.01$) in vitamin D–treated group. Subjects consumed daily an average of 2235 ± 978 kcals (77 ± 32% RDA), 95 ± 35 g (97 ± 59% RDA) total fat, 30 ± 19 g (96 ± 65% RDA) saturated fat, 377 ± 275 g (126 ± 92% RDA) cholesterol, 257 ± 157 g (61 ± 37% RDA) carbohydrate, 88 ± 34 g (115 ± 66% RDA) protein, and 18 ± 14 g (39 ± 30% RDA) fiber. Among micronutrients, subjects had low intake of vitamin D and exceeded the recommendations for sodium, selenium, and iron (data not shown; Table I).

### Fecal microbiota analysis in groups with different glycemic control

Comparison of microbial community composition, diversity, and abundance showed significant differences between Gr-1 and Gr-2. At the phylum level, the relative abundance of Bacteroidetes was higher, Firmicutes lower, and the ratio of Bacteroidetes to Firmicutes higher in Gr-1 vs Gr-2. The abundance of Proteobacteria was also higher in Gr-1 vs Gr-2. At the class level, the ratio between Bacteroidia and Clostridia was higher in Gr-1 vs Gr-2, as the relative abundance of Bacteroidia was higher in Gr-1 vs Gr-2 and Clostridia was lower in

Gr-1 vs Gr-2. Within the class Clostridia, Veillonellaceae and Ruminococcaceae were significantly higher in Gr-2 vs Gr-1. Further analysis to the next taxonomic level showed that bacteria from the genus *Ruminococcus*, from unclassified members of the family Ruminococcaceae, and from the genus *Dialister* were significantly more abundant in Gr-2 vs Gr-1. There was no significant difference in the ratio of *Bacteroides/Prevotella* between 2 groups (Tables II and III).

### Fecal microbiota analysis in quartiles of dietary energy and total fat intake, A1c, and 25(OH)D

A comparison of microbial community composition by dietary intake showed significant differences for energy and total fat (Tables IV and V) but not for other macronutrients (data not shown). At the taxonomic level of family, an increase in calorie (energy) intake was associated with reduced relative abundance of Bacteroidaceae but not Prevotellaceae. However, increase in fat intake was associated with significant decrease in Bacteroidaceae and increase in Prevotellaceae. These opposing trends led to a significantly reduced ratio of *Bacteroides/Prevotella* in subjects with increased caloric and fat intake. Additionally, Lachnospiraceae abundance was significantly lower with higher caloric but not fat intake. Among the Lachnospiraceae, genus *Ruminococcus* was significantly lower with increased caloric intake (Tables IV and V).

Comparison by A1c quartiles demonstrated significant differences in the relative abundance of several taxa at the family/genus level. Similarly to what was observed in glycemic groups, where Bacteroidaceae/*Bacteroides* abundance was lower in prediabetes vs normoglycemia cohorts, these taxa were also less abundant in high vs low A1c quartiles. Similarly, Ruminococcaceae/*Ruminococcus* abundance was higher in prediabtes vs normoglycemia, as well as in high vs low quartiles of A1c. Genera *Streptococcus*, *Catenibacterium*, and *Faecalibacterium* were all more abundant in high vs low A1c quartiles.

Comparison by serum 25(OH)D or delta 25(OH)D showed that the relative abundance of bacteria from the family Lachnospiraceae was significantly different between vitamin D–related quartiles (Tables IV and V). The relative abundance of sequences from several genera belonging to the Lachnospiraceae (eg, *Ruminococcus*, *Roseburia*, *Blautia*, and *Dorea*) was lower in high vs low vitamin D quartiles. In addition, the relative abundance of bacteria from the families Ruminococcaceae and Veillonellaceae was significantly different between groups of differing glycemic status (Tables II and III), whereas the relative abundance of bacteria of the family Lachnospiraceae was significantly different between quartiles of energy intake and vitamin D level (Tables IV and V). The ratio of the abundance of bacteria from the genera *Bacteroides* and *Prevotella* (*Bacteroides/Prevotella*) was found to be significantly different when comparing high and low quartiles of energy and fat intake but not with high and low quartiles of glycemic status, A1c, or vitamin D level.

## DISCUSSION

In this study, we observed significant associations between the physiological status of our subjects (ie, glycemic status, circulating vitamin D level) and their stool microbial composition, analyzed from the phylum to the genus level. In our experience, the taxonomic

resolution of the V4 variable region of the microbial 16S rRNA gene is not robust at the level of species. We further note that the observed differences in microbial community structure between the subjects with varied physiological status were found within the context of a high level of interindividual variation.[17]

This study suggests for the first time a microbiota—vitamin D—diabetes–diet interaction. Observational and randomized studies on vitamin D (blood levels or intake) and risk of T2DM have been contradictory. The observational studies suggest inverse association of plasma 25(OH)D and vitamin D intake with the risk of T2DM.[20] Recent randomized clinical trials have reported contradictory results on the effect of vitamin D supplementation on T2DM incidence and risk.[22,32,33] In a meta-analysis of 14 studies with a total of 72,204 participants and 4877 type 2 diabetes events, the odds ratio for T2DM was 1.50 (95% CI 1.33–1.70) for the bottom vs top quartiles of 25(OH)D.[20]

The study did not find association between microbiota composition and protein, fiber, or carbohydrates intake. This is in agreement with previous studies reporting increased metabolic endotoxemia only with high energy and fat intake but not with other macronutrients.[34,35] Fiber intake has been previously attributed beneficial health properties by the virtue of being a substrate for short-chain fatty acids (SCFA) production.[36,37] The lack of association between microbiota and fiber intake despite the 5-fold difference between the low and high fiber intake quartiles (6 ± 3 vs 34 ± 16 g/d) was likely the relatively modest fiber intake because 50 g/d fiber had been recommended for patients with diabetes.[38] In addition, soluble and fermented fiber more than nonfermented fiber was likely the source for SCFA, and a recent meta-analysis suggested that cereal but not fruit fiber as well as a lower BMI might be protective against diabetes.[39] However, the dietary fiber quality was not investigated in our dietary assessment.

There are no human data on microbiota—vitamin D—diabetes–diet interactions. There are preclinical studies showing vitamin D–related changes in microbiota and intestinal permeability as well as studies suggesting a pivotal role of microbiota-related inflammation in pathogenesis of diabetes.[2] Intestinal hyperpermeability and related inflammation may be a mutual point connecting microbiota, vitamin D, diabetes, and diet.

Pathophysiology of microbiota—vitamin D—diabetes–diet interactions remains to be elucidated but is likely multidirectional with dietary interactions playing particularly important role because diet substantially influences all segments of this axis, including vitamin D,[33,40] microbiota,[41,42] and diabetes.[2,43] Our research is an extension of the basic hypothesis that vitamin D deficiency is a significant contributor to risk of T2DM.[20,22] In particular, based on preclinical studies,[44-46] we hypothesized that vitamin D deficiency would further lead to an alteration in gut microbial community structure, and this would be observable in analyses of fecal samples.

## Gut microbiota associated with vitamin D status

This study demonstrates for the first time an association between serum vitamin D level and the composition of the gut microbial community structure in prediabetic individuals, whereas associations between microbiota and variety of other conditions including diabetes

have been previously suggested.[41,47,48] We report significant decrease in the relative abundance of *Roseburia*, *Blautia*, *Ruminococcus*, and *Dorea* (all belonging to the phylum Firmicutes, class Clostridia, family Lachnospiraceae) with increased vitamin D level. The significance of these findings is not clear.

Intestinal permeability regulation could be the pathogenic mechanism linking vitamin D status and microbiota composition.[41,49] Preclinical studies have confirmed the importance of vitamin D for local intestinal mucosal immunity, for maintaining tight junctions and function of colonic epithelium, whereas vitamin D deficiency has been associated with increased gut permeability, colon mucosa bacterial infiltration, and translocation of intestinal pathogens from the gut to the host interior resulting into subclinical inflammation (ie, metabolic endotoxemia).[41] We previously have reported that vitamin D supplementation raising 25(OH)D more than 50 ng/mL significantly decreased inflammation markers associated with gut microbiota–related endotoxemia (ie, zonulin and lipopolysaccharide binding protein; unpublished data).

An observational cohort study showed higher *Dorea* and *Blautia* abundance associated with increased intestinal permeability.[50] However, elsewhere, bacteria from the Lachnospiraceae, such as genera *Ruminococcus*, *Blautia*, and *Roseburia*, have been described as SCFA producers with beneficial effect on the intestinal barrier.[51]

VDR was described as localized to the surface epithelia of germ-free mice but to crypt epithelial cells in conventionalized mice. VDR expression, distribution, transcriptional activity, and target genes were regulated by commensal and pathogenic microbiota stimulation.[52] Furthermore, probiotic supplementation led to an increase in VDR expression in the colon and correlated with decreased richness and diversity of the mucosally adherent pathogenic microbiota and related inflammation.[45] Recently, *Clostridium*, a member of Clostridia class, was shown to be more abundant in VDR−/− mice compared with WT, suggesting an inhibitory effect of vitamin D on the abundance Clostridium.[53] This is consistent with our findings of lower abundance of members of Clostridia class in high quartile vs low quartile of serum vitamin D.

Increased abundance of members of Clostridia from the families Veillonellaceae and Lachnospiraceae (which include *Dorea*, *Blautia*, *Roseburia*, and some newly assigned species of *Ruminococcus*) has also been described to increase intestinal absorption of calcium and to have a beneficial role for bone health.[54] The decrease in the abundance of Lachnospiraceae in the high vitamin D quartile in our study may represent a decreased need in bacterial-driven calcium absorption as this process is mediated via the VDR. Additionally, Bora et al.[55] have reported that germ-free mice are less efficient in handling vitamin D absorption and metabolism compared with conventional mice suggesting that gut microbiota plays a role in regulating host vitamin D status beyond the VRD.

Together these findings suggest that vitamin D acts via VDR-dependent and VDR-independent (ie, microbiota modulation) mechanisms to maintain intestinal homeostasis and confer host protection from bacterial invasion and infection.

### Gut microbiota association with glycemic control

We observed that the relative abundance of bacteria from the phyla Bacteroidetes and Firmicutes was altered in that the relative abundance of Bacteroidetes was lower and Firmicutes higher in prediabetes vs normal glucose tolerance, and this is consistent with prior studies comparing diabetes and normal glucose control.[5,6,9,10] In addition, we observed a 2-fold higher Bacteroidia/Clostridia ratio in subjects who maintained normoglycemia, compared with those who remained prediabetic, suggesting a beneficial profile of a higher ratio. The magnitude of the Bacteroidetes/Firmicutes and Bacteroidia/Clostridia ratios was determined by a higher abundance of Firmicutes and Clostridia in hyperglycemia.

At the family level, the relative abundance of Ruminococcaceae and Veillonellaceae (both of class Clostridia) was higher in prediabetes compared with normoglycemia groups. Although bacteria belonging to the family Ruminococcaceae, known to harvest cellulose, glucose, and xylose and produce SCFA, have been previously associated with diabetes,[7,9,10] the observed association of Veillonellaceae with glycemia was novel. Bacteria from the genus *Veillonella* have been previously found in higher abundance in children with type 1 diabetes vs healthy controls[56] and in diabetic compared to nondiabetic subjects with periodontitis.[57] Members of the genus *Veillonella* reportedly play a role in gut microbiota development at an early age[58,59] and have been characterized as a lactate fermenter and shown to co-occur with genus *Streptococcus* at the same gastrointestinal levels. Genus *Streptococcus* has a role in the fermentation of sugars such as mannitol, yielding lactic acid as a predominant fermentation end product. In turn, *Veillonella* ferments this lactic acid to propionate and acetate.[60,61] This study showed that both Veillonellaceae (more abundant in prediabetes vs normoglycemia) and Streptococcaceae (more abundant in high vs low A1c quartiles) were concordantly more abundant with worsening glycemic control. This role may be partially explained by an increased production of propionate entering the portal circulation and further serving in hepatic gluconeogenesis.[62]

At the genus level, we observed significant correlations between glycemic control and relative abundance of the genera *Ruminococcus*, *Faecalibacterium*, and *Dialister*, where an increased abundance of all these SCFA-producing taxa[63,64] was found with worsened glycemia (ie, prediabetes, high A1c quartile). We speculate that this is possibly an adaptation of the gut microbiota necessary to maintain glycemia and prevent further worsening of the glycemic control. Reports on the association between increased abundance of *Dialister* and decreased proinflammatory IL-6 come to support this hypothesis.[63]

Comparison of microbiota by A1c quartiles showed results consistent with previous reports of less abundant Bacteroidetes and alteration in Bifidobacteriaceae, Ruminococcaceae, and Streptococcaceae with worsened glycemia.[7] Novel was the findings of more abundant Coprobacillaceae (genus *Catenibacterium*) in the highest A1c quartile. These taxa have been described to play a role in hepatic lipid metabolism in rodents and fatty liver development in humans and to be SCFA producers in the presence of high-fat and high-glucose diet, but limited data are available.[65-67]

We detected significant differences in the relative abundance of common gut microorganisms from the genera *Bacteroides* and *Prevotella* only between quartiles of

extreme energy and fat intake but not in relationship to dysglycemia or vitamin D levels. This could be related to the complex nature of human gut microbiota, particularly as it pertains to the relative abundance of *Bacteroides* and *Prevotella*[6,8,10] and possibly to the previously described bimodal distribution of *Prevotella* in the general population. The high variability of bacteria from these genera in the general population could mask differences associated with physiological conditions.[68]

We report for the first time that dysglycemia is associated with an increase in the abundance of Ruminococcaceae and Veillonellaceae, whereas high caloric intake and high vitamin D levels are associated with lower abundance of Lachnospiraceae. Again, the taxonomic resolution of the rRNA gene amplicons could limit our understanding of the role of taxa if multiple species within a genus serve different physiological roles.

In conclusion, this study is the first to demonstrate a relationship between gut microbiota and vitamin D deficiency in prediabetic individuals, within a controlled experimental setting. Importantly, this study also addresses a high-risk, but underserved community, AAM. Although this study has not evaluated functional changes or interactions of microbiota populations, it nonetheless has identified microbial taxa, which have been associated with diabetes in a number of previous studies across different ethnicities. We underscore that a cohort study can only indicate association, not causation. Furthermore, the single sex and ethnicity limit generalization, and a relatively small sample size decreases the ability to draw broad conclusions to other genders and ethnicities. Nonetheless, we highlight the observation indicating strong correlations between gut microbial community and dysglycemia at multiple taxonomic levels, along with significant correlations between vitamin D levels and specific genera. These findings suggest putative interactions of microbiota with glycemia, dietary intake, and vitamin D. Further controlled studies, in which finer taxonomic resolution of the gut microbiome is assessed, will be necessary to understand the complex interconnections among gut microbiome, the environment, and the development of prediabetes and T2DM.

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## Biography

**Irina Ciubotaru, MD, PhD,** is a Fellow in the Section of Endocrinology at the University of Illinois, Chicago. Her article is based on a poster presentation at the Combined Annual Meeting of the Central Society for Clinical Research and the Midwestern Section of the American Federation for Medical Research (CSCTR/MWAFMR), held in Chicago, Illinois in April 2014. Dr. Ciubotaru started her 3-year fellowship training in July 2013, and she is

expected to complete it in July 2016. The first two years of her fellowship training have been dedicated mainly to research training supported by a T32 grant.

## Abbreviations

| | |
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| **T2DM** | type 2 diabetes mellitus |
| **A1C** | glycosylated hemoglobin |
| **25(OH)D** | serum 25-hydroxyvitamin D |
| **AAM** | African American men |
| **NGT** | normal glucose tolerance |
| **IFG** | impaired fasting glucose |
| **IGT** | impaired glucose tolerance |
| **RDA** | Recommended Dietary Allowance |
| **AI** | Adequate Intake |
| **OGTT** | oral glucose tolerance test |
| **SCFA** | short-chain fatty acid |

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## AT A GLANCE COMMENTARY

Ciubotaru I, et al.

**Background**

Gut dysbiosis has been recognized as a potential player in diabetes pathogenesis. Although description of microbiota associated with diabetes has been reported, less is known about its makeup in prediabetes.

**Translational Significance**

We show that glycemic stability (stable normal glucose tolerance or stable prediabetes) is associated with specific microbiota makeup. There were significant correlations between glycemic control and the relative abundance of short-chain fatty acid producing genera *Ruminococcus*, *Faecalibacterium*, and *Dialister*. Higher abundance of these taxa was found in prediabetes and the highest glycosylated hemoglobin quartile, suggesting an adaptation for maintaining glycemia and preventing further worsening of the glycemic control.

**Table I**

Subject characteristics

| | Total (n = 115) | Quartile 1 | Quartile 4 |
|---|---|---|---|
| Characteristics | | | |
| Age (y) | 60 ± 7 | 51 ± 5 | 68 ± 5 |
| BMI (kg/m$^2$) | 32.1 ± 3.6 | 28.1 ± 1.1 | 37.2 ± 2.4 |
| HbA1c (%) | 6.1 ± 0.4 | 5.7 ± 0.1 | 6.6 ± 0.3 |
| FBG (mg/dL) | 101.9 ± 10.7 | 86.0 ± 3.9 | 120.4 ± 10.6 |
| 2 h-BG (mg/dL) | 133.6 ± 37.5 | 82.0 ± 11.0 | 199.0 ± 37.4 |
| OGIS (mL × min$^{-1}$ × m$^{-2}$) | 359.6 ± 60.0 | 259.8 ± 33.4 | 450.9 ± 60.2 |
| 25(OH)D (ng/mL) | 36 ± 24 * | 13 64 | 70 ± 20 |
| Delta 25OHD (ng/mL) | 21 ± 23 | −2 ± 5 | 56 ± 19 |
| Total body fat (%) † | 35 ± 5 | 28 ± 4 | 41 ± 3 |
| Android fat (%) † | 44 ± 8 | 34 ± 6 | 54 ± 3 |
| Diet ‡ | | | |
| Energy (kcals) | 2235 ± 978 | 1167 ± 270 | 3562 ± 748 |
| Total fat (g) | 95 ± 53 | 39 ± 12 | 168 ± 39 |
| Saturated fat (g) | 30 ± 19 | 12 ± 4 | 54 ± 19 |
| Carbohydrates (g) | 257 ± 157 | 111 ± 29 | 466 ± 155 |
| Protein (g) | 88 ± 34 | 48 ± 12 | 131 ± 20 |
| Fiber (g) | 18 ± 14 | 6 ± 3 | 34 ± 16 |

*Abbreviations:* 25(OH)D, serum 25-hydroxyvitamin D; BMI, body mass index; Delta 25(OH)D, exit minus baseline; HbA1c, hemoglobin A1c; OGIS oral glucose insulin sensitivity; SD, standard deviation.

Data are presented as mean ± SD. Quartiles 1 and 4 are the lowest and highest quartiles for each variable.

* $P < 0.01$.

† Body fat composition was measured by DXA and reported as % total fat and % android fat.

‡ Dietary data were collected using 24 h dietary recall questionnaires and reported as absolute daily intake. Significance between baseline and exit using paired *t* test.

**Table II**

Comparison of fecal microbiota between 2 groups of subjects who maintained their glycemic status over 12 months

| | **Gr-1 vs Gr-2; *P* value** |
|---|---|
| Phylum | |
| Bacterial composition [*] | 0.003 |
| Taxonomic diversity [†] | ns |
| Relative taxon abundance or ratio [‡] | |
| Bacteroidetes | 0.01 (0.01) [§] |
| Firmicutes | 0.00 (0.01) [//] |
| Proteobacteria | 0.04 (0.06) [§] |
| Bacteroidetes/Firmicutes ratio (1.9 vs 0.9) | 0.01 [§] |
| Family | |
| Bacterial composition [*] | ns |
| Taxonomic diversity [†] | 0.05 [//] |
| Relative taxon abundance or ratio [‡] | |
| Veillonellaceae | 0.03 (0.2) [//] |
| Ruminococcaceae | 0.01 (0.1) [//] |
| Class | |
| Relative taxon abundance or ratio [‡]: | |
| Bacteroidia/Clostridia ratio | 0.05 (2.0 vs 0.9) [§] |
| Genus | |
| Bacterial composition [*] | ns |
| Taxonomic diversity [†] | 0.02 [//] |
| Relative taxon abundance or ratio [‡] | |
| Ruminococcaceae; *Ruminococcus* | 0.03 (0.2) [//] |
| Ruminococcaceae; *g-* | 0.00 (0.03) [//] |
| Veillonellaceae; *Dialister* | 0.01 (0.1) [//] |
| *Bacteroides/Prevotella* ratio (2.7 vs 5.6) | ns |

*Abbreviations:* Gr, glycemic group; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; ns, not significant.

Data are presented as significant differences (*P* values)

Gr-1: n = 35 subjects who maintained NGT during the study; Gr-2: n = 27 subjects who remained IFG/IGT during the study.

[*] Bacterial composition was assessed by ANOSIM.

[†] Taxonomic diversity was assessed using Shannon index.

[‡] Relative taxonomic abundance between groups was compared using the Kruskal–Wallis test with significance set at $P < 0.05$. False discovery rate *P* values are shown in parenthesis.

§Taxonomic diversity or relative taxon abundance or ratio is higher in Gr-1 relative to Gr-2.

//Taxonomic diversity or relative taxon abundance or ratio is higher in Gr-2 relative to Gr-1.

## Table III

Comparison of relative abundance of taxa found to be significantly different at the genus level as shown in Table II. Relative abundance was calculated based on 6280 total sequences and reported as percentage (%)

| Genus | Gr-1 vs Gr-2 |
|---|---|
| Relative taxon abundance (%) | |
| Ruminococcaceae; *Ruminococcus* | 1.6, 3.0 |
| Ruminococcaceae; *g-* | 3.1, 6.8 |
| Veillonellaceae; *Dialister* | 0.7, 4.5 |

*Abbreviation:* Gr, glycemic group.

**Table IV**

Comparisonof fecal microbiota analyzedbyquartiles of energyandtotal fat intake, A1c,and25(OH)D

| | Energy intake | Fat intake | A1c | 25(OH)D | Delta 25(OH)D |
|---|---|---|---|---|---|
| Family | Q1 vs Q4 | Q1 vs Q4 | Q1 vs Q4 | Q1 vs Q4 | Q1 vs Q4 |
| Overall composition * | ns | 0.02 | ns | ns | 0.03 |
| Composition Q1 vs Q4 † | 0.02 | 0.003 | 0.04 | 0.05 | ns |
| Taxonomic diversity ‡ | ns | 0.02 ¶ | 0.04 ¶ | ns | ns |
| Relative taxon abundance § | | | | | |
| Bacteroidaceae | 0.02 (0.21) // | 0.00 (0.01) // | 0.05 (0.15) // | ns | ns |
| Prevotellaceae | ns | 0.03 (0.19) ¶ | ns | ns | ns |
| Lachnospiraceae | 0.03 (0.21) // | ns | ns | 0.00 (0.03) // | 0.00 (0.01) // |
| Ruminococcaceae | ns | ns | 0.02 (0.10) ¶ | ns | ns |
| Coprobacillaceae | ns | ns | 0.01 (0.10) ¶ | ns | ns |
| Streptococcaceae | ns | ns | 0.02 (0.10) ¶ | ns | ns |
| Bifidobacteriaceae | ns | ns | 0.05 (0.15) ¶ | ns | ns |
| Genus | | | | | |
| Overall composition * | ns | 0.01 | 0.05 | ns | 0.03 |
| Composition Q1 vs Q4 † | 0.02 | 0.002 | 0.03 | 0.03 | 0.02 |
| Taxonomic diversity ‡ | ns | ns | ns | ns | ns |
| Relative taxon abundance or ratio §: | | | | | |
| *Bacteroides* | 0.02 (0.21) // | 0.00 (0.01) // | 0.05 (0.23) // | ns | ns |
| *Prevotella* | ns | 0.02 (0.23) ¶ | ns | ns | ns |
| Lachno; *g-* | 0.03 (0.21) // | ns | ns | ns | 0.02 (0.09) // |
| Lachno; *Ruminococcus* | 0.03 (0.21) // | ns | ns | 0.01 (0.16) // | 0.00 (0.02) // |
| Lachno; *Blautia* | ns | ns | ns | 0.03 (0.22) // | 0.00 (0.02) // |
| Lachno; *Roseburia* | ns | ns | ns | 0.00 (0.03) // | 0.00 (0.01) // |
| Lachno; *Dorea* | ns | ns | ns | ns | 0.03 (0.11) // |
| Rumino; *Faecalibacterium* | ns | ns | 0.01 (0.10) ¶ | ns | ns |
| Copro; *Catenibacterium* | ns | ns | 0.01 (0.10) ¶ | ns | ns |
| *Streptococcus* | ns | ns | 0.02 (0.13) ¶ | ns | ns |
| *Bacteroides/Prevotella* ratio | 0.01 // (15.2 vs 2.7) | 0.01 // (12.4 vs 1.8) | ns (5.1 vs 2.4) | ns (5.9 vs 2.9) | ns (4.2 vs 2.2) |

*Abbreviations:* A1c, glycosylated hemoglobin; 25(OH)D, serum 25-hydroxyvitamin D; Delta 25(OH)D, exit minus baseline; ns, not significant.

Data are presented as significant differences (*P* values).

Bacterial taxa were analyzed at the taxonomic level of family and genus.

* Comparison of bacterial composition by ANOSIM among all quartiles (*P* value).

† Comparison of bacterial community composition between lowest (Q1) and highest (Q4) quartiles by ANOSIM (*P* value).

‡Taxonomic diversity was assessed by Shannon index and compared between Q1 and Q4 (*P* value).

§Comparisons of individual taxon relative abundance between Q1 and Q4 were performed using the Kruskal–Wallis test with significance set at $P < 0.05$. False discovery rate *P* values are shown in parenthesis. Ns, not significant.

//Taxonomic diversity or relative taxon abundance or ratio is higher in Q1 relative to Q4.

¶Taxonomic diversity or relative taxon abundance or ratio is higher in Q4 relative to Q1; energy intake (Kcals); total fat intake (g); A1c (%); 25(OH)D (ng/mL).

## Table V

Comparison of relative abundance of taxa found to be significantly different at the genus level as shown in Table IV. Relative abundance was calculated based on 6280 total sequences and reported as percentage (%)

| | Energy intake | Fat intake | A1c | 25(OH)D | Delta 25(OH)D |
|---|---|---|---|---|---|
| Genus | Q1 vs Q4 | Q1 vs Q4 | Q1 vs Q4 | Q1 vs Q4 | Q1 vs Q4 |
| Relative taxon abundance (%) | | | | | |
| *Bacteroides* | 45.4, 31.1 | 45.5, 24.4 | 44.6, 31.6 | ns | ns |
| *Prevotella* | ns | 3.7, 13.8 | ns | ns | ns |
| Lachno; *g-* | 3.8, 3.1 | ns | ns | ns | 4.1, 2.4 |
| Lachno; *Ruminococcus* | 1.6, 0.9 | ns | ns | 2.1, 1.0 | 2.2, 0.9 |
| Lachno; *Blautia* | ns | ns | ns | 2.7, 1.3 | 2.9, 1.2 |
| Lachno; *Roseburia* | ns | ns | ns | 3.2, 1.0 | 3.0, 0.8 |
| Lachno; *Dorea* | ns | ns | ns | ns | 2.2, 0.6 |
| Rumino; *Faecalibacterium* | ns | ns | 3.2, 15.9 | ns | ns |
| Copro; *Catenibacterium* | ns | ns | 0.6, 2.2 | ns | ns |
| *Streptococcus* | ns | ns | 0.7, 2.4 | ns | ns |

*Abbreviation:* ns, not significant.