Maternal gut microbiota reflecting poor diet quality is associated with spontaneous preterm birth in a prospective cohort study

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ABSTRACT

Background: A processed diet, high in fat and low in fiber, is associated with differences in the gut microbiota and adverse health outcomes in humans; however, little is known about the diet–microbiota relation and its impact on pregnancy. Spontaneous preterm birth (SPTB), a pregnancy outcome with serious short- and long-term consequences, occurs more frequently in black and in obese women in the United States.

Objectives: In a prospective, case-control sample matched for race and obesity (cases = 16, controls = 32), we compared the fecal gut microbiota, fecal and plasma metabolites, and diet in the late second trimester. We hypothesized that a Western diet would be associated with reduced microbiota richness and a metabolic signature predicting incidence of SPTB.

Methods: The fecal microbiota was characterized by 16S-tagged sequencing and untargeted metabolomics was used to analyze both plasma and fecal metabolites. Wilcoxon's rank-sum test was used for the comparison of microbiota genera, α-diversity, fecal and plasma metabolites, and dietary variables between term and SPTB. β-Diversity was analyzed using permutational multivariate ANOVA, and metabolite associations were assessed by module analysis.

Results: A decrease in α-diversity was strongly associated with the development of SPTB, especially in the taxonomic class of Betaproteobacteria. Of 824 fecal metabolites, 22 metabolites (mostly lipids) differed between cases and controls ($P < 0.01$), with greater DHA (22:6n–3) and EPA (20:5n–3) in cases [false discovery rate (FDR) < 0.2]. The most significant fecal metabolite module (FDR-adjusted $P = 0.008$) was dominated by DHA and EPA. Dietary saturated fat (primarily palmitate) intake was greater in cases (31.38 ± 7.37 compared with 26.08 ± 8.62 g, $P = 0.045$) and was positively correlated with fecal DHA and EPA ($P < 0.05$).

Conclusions: Reduced α-diversity of the gut microbiota and higher excretion of omega-3 (n–3) fatty acids in stool may provide a novel biomarker signature predicting SPTB in women with a low-fiber, high-fat diet. Further investigation of these markers in a larger sample is needed for validation. *Am J Clin Nutr* 2021;113:602–611.

Keywords: microbiome, metabolome, pregnancy, Western diet, precision nutrition

Introduction

Preterm birth (PTB) occurs before the 37th week of gestation and is one of the leading causes of neonatal morbidity and mortality. Evidence suggests that PTB is a multifactorial syndrome potentially mediated by infection, oxidative stress, inflammation, and maternal perinatal nutrition (1, 2); however, the relation is unclear and the biology of PTB remains poorly understood. Whereas the incidence of infants delivered before 37 weeks of gestation had been on the decline from 2004 to 2013,

Supported by the University of Pennsylvania March of Dimes Prematurity Research Center (to ME). The project was also supported in part by National Center for Research Resources and National Center for Advancing Translational Sciences, NIH grant UL1TR000003, the PennCHOP Microbiome Program, and Center for Molecular Studies in Digestive and Liver Disease grant NIH P30 DK050306. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. Neither funding source influenced the study design or findings.

Supplemental Figures 1–7 and Supplemental Tables 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: DHEA-S, dehydroepiandrosterone sulfate; DPA, docosapentaenoic acid; FDR, false discovery rate; HDoHE, hydroxydocosahexanoic acid; PERMANOVA, permutational multivariate analysis of variance; PTB, preterm birth; SPTB, spontaneous preterm birth; TOM, topological overlap measure; WGCNA, Weighted Gene Co-expression Network Analysis.

Received April 21, 2020. Accepted for publication November 11, 2020.

First published online January 29, 2021; doi: https://doi.org/10.1093/ajcn/nqaa361.

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the percentage of PTB deliveries has recently increased dramatically to 10% as of 2018, with especially high percentages among black women (14%) (3). Obese women are also at high risk of PTB (4). This is particularly concerning because PTB is responsible for nearly one-third of neonatal deaths in the United States (5), and infants born preterm may have serious long-term health conditions due to their immaturity (6), including respiratory distress syndrome, necrotizing enterocolitis, metabolic disease, and the potential for lifelong complications. Shockingly, rates of PTB in the United States are considerably greater than in other similarly developed societies, such as the Nordic countries which have a PTB rate of only 6% (7). Within this context, the identification of biomarkers that predict PTB would be of considerable utility, especially if they provide new insights into pathogenesis, which could lead to novel approaches to reduce risk.

There has been increasing interest in the gut microbiota and its association with health and disease in human populations. Studies in animal models suggest that there may be a cause-and-effect relation between the bacteria within the gastrointestinal tract and human health (8–11). Many studies demonstrate that an alteration in the composition of the human gut microbiota, its genome (referred to as the "metagenome"), and its metabolites (the "metabolome") are associated with disease states; however, there are fewer studies suggesting that these features can be used as a biomarker to predict the future development of disease. Analyses of samples matched to clinical outcomes have suggested that the gut microbiome might have predictive value in the treatment of inflammatory bowel disease (11) or in the prevention of cardiovascular disease (9), type 2 diabetes (8, 10), and metabolic syndrome (10). However, prospective studies are needed to validate the predictive value of these biomarkers. In metabolic syndrome, the most consistent feature associated with a disease state was decreased gut microbial richness; less microbial richness is associated with diet—specifically lower fiber and higher fat intake—and residence in an industrialized nation (10). There has been much interest in the impact of diet on the gut microbiota where effect sizes seem to be personalized and relatively modest (12, 13). Nevertheless, the high-dimensional nature of microbiota features may be helpful in the development of personalized dietary interventions to prevent and/or treat disease (14).

Using a case-control sample from a prospective cohort of pregnant women, we sought to determine whether, in the context of a high-fat, low-fiber diet, maternal fecal microbial diversity and metabolome during the second trimester contained features associated with future development of spontaneous PTB (SPTB). We hypothesized that a processed, high-fat, low-fiber diet would be associated with alterations in the gut microbiota, fecal metabolome, and plasma metabolome that could provide a metabolomic signature of eventual SPTB.

Methods

Sample

In accordance with an Institutional Review Board–approved protocol, informed consent was obtained from 301 primiparous women who were enrolled into the study at 20–26 weeks of gestation. The study participants received prenatal care from 1 of 2 hospital-affiliated obstetric practices, delivered at the same hospital, and remained enrolled in this observational study until after the delivery of their infant. The key exclusion criteria were women with multiple gestation, fetal chromosomal abnormality or major fetal anomaly, intrauterine fetal demise, maternal HIV, chronic kidney disease, transplantation, history of weight loss surgery, prior completed pregnancy, vegan diet, and chronic use of immunosuppressive medications or steroids.

The study design included a cross-sectional survey of diet, the fecal microbiome, and the fecal and plasma metabolome at 20–26 weeks of gestation, followed by prospective observation for the primary clinical outcome of spontaneous preterm compared with term delivery. The diagnosis of SPTB was obtained from medical record review, using American College of Obstetricians and Gynecologists definitions (15) and confirmed by a perinatologist. Gestational age at delivery was determined by best obstetric dating based on last menstrual period and confirmed by prenatal ultrasound.

An embedded case-control study (**Supplemental Figure 1**) was constructed by matching all women who underwent SPTB (cases, $n = 16$) to 2 women who delivered at term without complications such as gestational diabetes, gestational hypertension, or pre-eclampsia (controls, $n = 32$). Because of the recognized greater risk of PTB in black and obese women, cases were matched to controls by race (black/nonblack) and prepregnancy BMI group (obese/nonobese). Racial-ethnic identity was determined by self-report. Owing to the small number of women who identified as Asian or "other," they were included with Caucasians to form a nonblack category for comparison with women who self-identified as black. Prepregnancy BMI was calculated from height and measured weight at the initial prenatal visit (in kg/m^2). According to CDC guidelines (16), BMI $\geq$ 30 was used to categorize obesity. Gestational weight gain was calculated as the maternal weight at the last prenatal visit before delivery minus the weight at the first prenatal visit.

Three 24-h dietary recalls were obtained during weeks 20–26 of gestation via unannounced telephone calls within a 10-d interval before collection of the stool sample. A trained research nutritionist administered the recalls using the validated multipass method supported by Nutrition Data System for Research software (University of Minnesota Nutrition Coordinating Center) (17). Oral prenatal vitamin content was not included in the food recalls, but all patients reported taking these supplements. Food recall data were cleaned for implausible energy intake when energy intake was <600 or >3500 kcal/d (18). This excluded diet data in 1 case, where the energy intake was reported as 4801 kcal/d, 76 kcal · kg body weight^{-1} · d^{-1}.

After the third dietary recall was obtained, the study participants collected their next stool sample and shipped it to the laboratory. During the following obstetric clinic visit, a sample of $\leq$50 mL blood was drawn and processed to obtain plasma. Plasma and fecal samples were stored at −80°C until further analysis. The study protocol did not allow missing data. Study participants were then monitored prospectively for the timing of their delivery. Of the original 301 enrolled subjects, 4 delivered their infants in a different hospital that did not permit exact tracking of birth timing, and these subjects were excluded from the analyses.

The stool samples were assayed for gut microbial identity using 16S tagged gene sequencing processed by Quantitative

Insights Into Microbial Ecology (QIIME) 2, including demultiplexing, quality control, amplicon sequence variant identification, and taxonomic assignment. The maternal plasma and fecal water samples were assayed for untargeted metabolomics by Metabolon (Metabolon, Inc.).

Statistical and bioinformatics analysis

16S tagged sequencing data analysis.

The relative abundance per sample was summarized at the genus level. Richness, Shannon indexes, and Menhinick indexes were calculated to represent the microbiome α-diversity. The weighted (proportional abundance) and unweighted (presence-absence) Unifrac distances were calculated to represent the microbiome β-diversity. R package "vegan" was used for α- and β-diversity calculation. Two control samples had very low read counts and were removed in the analyses that were related to microbiome data. The mean number of reads per sample ($\pm$ SD) that could be assigned to the genus level was 30,409.71 $\pm$ 9733.2.

Differential abundance analysis.

Wilcoxon's rank-sum test was used for the comparison of microbiome genera, α-diversity, and stool and plasma metabolites between term and SPTB. Differences in dietary intake variables were compared between term and SPTB cases using a 2-tailed *t* test with unequal variance. Permutational multivariate analysis of variance (PERMANOVA) was applied for the comparison of β-diversity between term and SPTB. A false discovery rate (FDR) of 0.20 was used to select the bacterial genera or metabolites that were associated with SPTB. All analyses were performed using R version 3.5.1 (R Foundation). For our given sample size, we had a power of 80% to detect an effect size (as measured by Cohen's *d*) of 0.88 at an α level of 0.05 for the primary outcome of microbial diversity. For other secondary outcomes, owing to multiple comparison adjustments, only associations with a very large effect size could be detected.

Weighted Gene Co-expression Network Analysis metabolite module.

For the stool metabolites, Weighted Gene Co-expression Network Analysis (WGCNA) (19) was applied to *1*) identify functional modules of stool metabolites based on pairwise correlations, *2*) correlate the modules with SPTB based on principal component analysis, and *3*) find the most important metabolites within the module of interest. Such a network analysis accounts for high correlations among certain metabolites, provides effective dimension reduction, and leads to more biologically interpretable results. By using WGCNA, the correlations raised to a certain power (adjacencies) were calculated between all pairs of metabolites, so that the degree distribution would fit a scale-free model. The adjacencies were then transformed into topological overlap measures (TOMs) and TOM-based distance matrices. Finally, a dynamic branch-cutting method was applied to detect clusters (modules) of metabolites depending on the shape of the clustering tree. Pearson's correlation was calculated between each module (represented by the first principal component) and SPTB. The important metabolites in each module were defined as metabolites with high metabolite significance (represented by absolute Pearson's correlation with SPTB > 0.4) and high intramodular connectivity (represented by absolute Pearson's correlation with the first principal component of the module > 0.8). R package WGCNA was used for the analysis.

Results

Baseline characteristics

From a prospective cohort observation of 301 pregnant women, 16 women delivered spontaneously earlier than 37 weeks of gestation (Supplemental Figure 1). Four women were considered to have early SPTB (24–30 wk) and 12 were categorized as late SPTB (31–36 wk). All cases, including 8 black and 8 nonblack women (6 white and 2 Asian), were matched with 2 controls of the same self-reported race and obesity status (18 obese and 30 nonobese women). As expected, owing to the preterm delivery, gestational weight gain and gestational age at delivery were lower in cases, but gestational weight gain per week was similar for both groups (**Table 1**).

α-Diversity of fecal microbiota was reduced in women with SPTB

In order to determine the difference of diversity at the genus level in the fecal microbiota, 3 measures of α-diversity were evaluated: *1*) richness, the total number of different genera in a sample; *2*) Shannon diversity, which accounts for the proportion (evenness) of each genus in a sample; and *3*) Menhinick's index, the number of genera divided by the square root of the total number of individuals. The 3 α-diversity indexes were all significantly reduced in fecal microbiota collected between 20 and 26 weeks of gestation from women who ultimately had SPTB (**Figure 1**A, Shannon diversity $P = 0.043$; Figure 1B, richness $P = 0.009$; Figure 1C, Menhinick's index $P = 0.014$). To determine the taxonomic features responsible for this difference, Shannon diversity was calculated for each bacterial class (**Figure 2**A). The bacterial class of Betaproteobacteria was identified as showing a significant reduction in Shannon diversity in SPTB cases (Wilcoxon's rank-sum test, FDR < 0.2) (Figure 2B), where the AUC on logistic regression analysis was 0.71 (Figure 2C). No specific genera in Betaproteobacteria showed a statistically significant difference in relative abundance in cases compared with controls (Wilcoxon's rank-sum at FDR $= 0.2$) (**Supplemental Figure 2**).

Owing to the small sample size, to test for predictive performance using within-class diversity, we performed leave-one-out cross-validation analysis. The resulting AUC of Betaproteobacteria was 0.60 (**Supplemental Figure 3**).

β-Diversity of the fecal microbiome differed in mothers who had SPTB compared with full-term birth

Both weighted and unweighted Unifrac distance were significantly different between cases and controls (PERMANOVA, p=0.034 for weighted analysis, **Figure 3**A; p=0.013 for

TABLE 1 Baseline demographic and delivery characteristics of cases who developed spontaneous preterm birth and controls[1]

Variable	Cases ($n = 16$)	Controls ($n = 32$)	P value
Baseline demographic characteristics			
Age, y	28.88 ± 4.92	28.38 ± 5.77	0.756
Black	9 (56.25)	18 (56.25)	1.00
White	5 (31.25)	10 (31.25)	
Asian	2 (12.5)	4 (12.5)	
Private insurance	10 (62.5)	23 (71.9)	0.527
Medicaid	6 (37.5)	9 (28.1)	
First clinic BMI, kg/m^2	27.93 ± 6.32	27.82 ± 5.76	0.957
Normal or underweight	4 (25.00)	17 (53.13)	0.149
Overweight	6 (37.51)	3 (9.38)	
Obese	6 (37.51)	12 (37.50)	
Antibiotics within 8 wk before enrollment	2 (12.5)	3 (9.4)	0.738
Smoking within 8 wk before enrollment	1 (6.3)	0 (0)	0.153
Assisted reproduction therapy	1 (6.3)	3 (9.4)	0.712
First-trimester vaginal bleeding	2 (12.5)	4 (12.5)	1.00
Second-trimester vaginal bleeding	1 (6.3)	0 (0)	0.153
Delivery characteristics			
Gestational weight gain, kg	8.89 ± 5.44	13.29 ± 6.19	0.017
Gestational weight gain, kg/gestational age	0.265 ± 0.147	0.339 ± 0.157	0.122
Gestational age at delivery, wk	33.13 ± 3.77	39.44 ± 1.22	<0.0001

[1] Values are mean ± SD for continuous variables and the P value is based on the t test; values are n (%) for categorical variables and the P value is based on the chi-square test.

unweighted analysis, Figure 3B). We were unable to identify any statistically significant discriminatory taxa based on the relative abundances at the genus level (106 genera tested) at an FDR of 0.20.

SPTB was associated with an increase in 2 lipid metabolites in the fecal metabolome

To identify additional analytic features associated with SPTB, we performed untargeted metabolomics analysis on maternal fecal and plasma samples collected at the prenatal visit. Out of 824 fecal metabolites, 22 were different in abundance between cases and controls ($P < 0.01$, Wilcoxon's rank-sum test). The majority of fecal metabolite differences were found among fatty acids and cholesterol hormone metabolites (**Figure 4**A). After correction for multiple comparisons (FDR < 0.20), both DHA (22:6n–3) and EPA (20:5n–3) were increased in the fecal metabolites of women with SPTB (Figure 4A).

Similarly, out of 825 plasma metabolites, 4 [β-citrylglutamate, propionylglycine, caproic acid (6:0), and 14-hydroxydocosahexanoic acid (HDoHE)/17-HDoHE] were different in abundance ($P \leq 0.01$), although none were significant after correction for multiple comparisons (**Supplemental Table 1**).

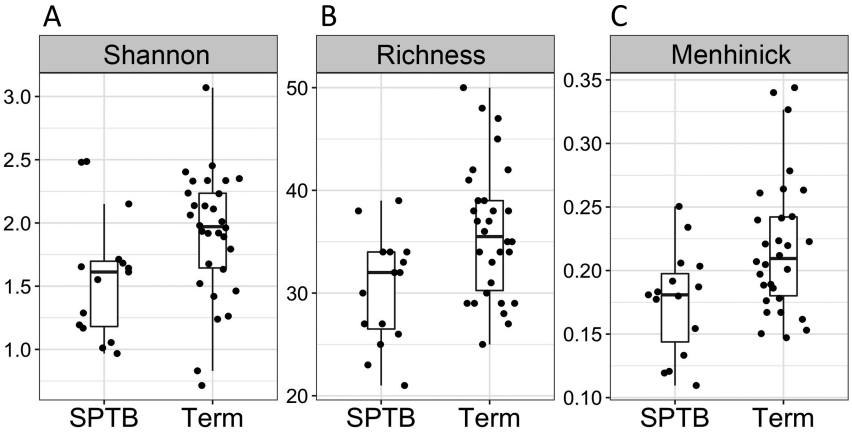

FIGURE 1 Shannon diversity and richness of the gut microbiota at 20–26 weeks of gestation in women who spontaneously delivered preterm ($n = 16$) relative to those with term deliveries ($n = 32$). (A) Shannon diversity (Wilcoxon's rank-sum test, $P = 0.043$). (B) Microbiota richness (Wilcoxon's rank-sum test, $P = 0.009$). (C) Menhinick's index (Wilcoxon's rank-sum test, $P = 0.014$). In each plot the bar is the median value, and the whiskers indicate the 25% and 75% distribution. SPTB, spontaneous preterm birth.

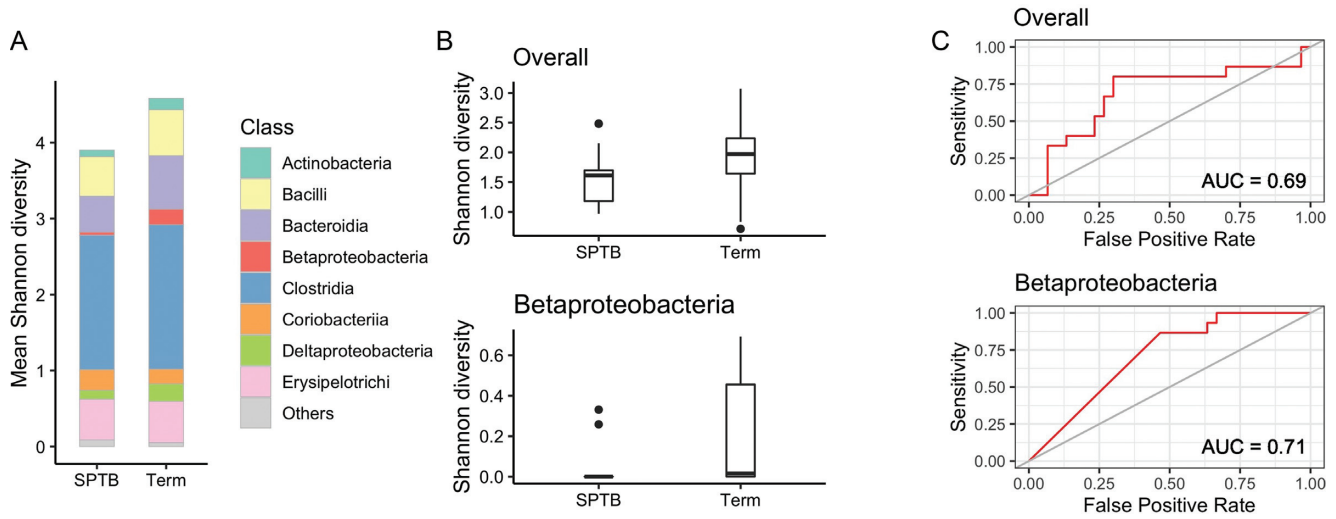

FIGURE 2 Shannon diversity of bacterial classes at 20–26 weeks of gestation in women who spontaneously delivered preterm ($n = 16$) relative to those with term deliveries ($n = 32$). (A) The composition of mean Shannon diversity by bacterial class. (B) Shannon diversity of Betaproteobacteria in SPTB compared with term (Wilcoxon's rank-sum test, false discovery rate < 0.2). In each plot the bar is the median value, and the whiskers indicate the 25% and 75% distribution. (C) AUC in a receiver operating characteristic analysis using a logistic regression model to predict SPTB with the contribution of Shannon diversity of Betaproteobacteria (AUC = 0.71) and of the overall microbiota (AUC = 0.69). SPTB, spontaneous preterm birth.

Cases with SPTB had greater intake of dietary fat—both saturated and polyunsaturated—than women who delivered at term

Given the previously described associations between dietary intake and the gut microbiota (12, 13, 20), we used the mean of three 24-h dietary recalls administered before collection of the stool sample to characterize the average diet of cases and controls (**Table 2**). Women with SPTB had greater intake of dietary fat (90.2 g compared with 75.2 g, $P = 0.028$) with a trend toward a higher percentage of calories from fat (37.4% compared with 34.6%, $P = 0.075$); however, both groups were close to the upper limit of the reference range for dietary fat intake based on the American Heart Association recommendations (20%–35% of calories from fat). The intake of saturated fat was higher in cases (31.4 g compared with 26.1 g, $P = 0.045$), predominantly palmitic acid (16:0) (16.4 g compared with 13.7 g, $P = 0.044$). There was no difference in intake of monounsaturated fat. PUFA intake was greater in women with SPTB (22.2 g compared with 16.5 g, $P = 0.018$; 9% compared with 8% kcal, $P = 0.047$). Both omega (ω)-3 fatty acids (2.7 g compared with 1.9 g, $P = 0.014$) and ω-6 fatty acids (21.7 g compared with 16.3 g, $P = 0.05$) were higher in mothers with SPTB. Both groups had a ratio of ω-6 to ω-3 higher than the recommended ratio of <4:1, which is consistent with the overall poor quality of diet in this cohort of women (8.3:1 for cases compared with 9.1:1 for controls, $P = 0.39$). All women reported taking prenatal vitamins that included 200 mg DHA. However, both groups had <0.2 g/d of the beneficial long-chain ω-3 fatty acids, DHA and EPA, from their diet. In contrast, both cases and controls met the minimum DRI (1.4 g/d during pregnancy) of the precursor ω-3 fatty

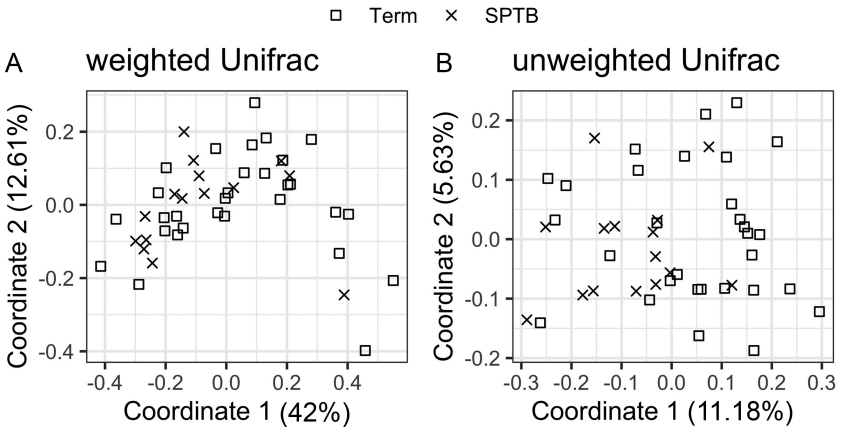

FIGURE 3 Principal components analysis plot of β-diversity between women who later experienced SPTB ($n = 16$) and term controls ($n = 32$). (A) The phylogenetic distance of proportional abundance (weighted Unifrac, $P = 0.067$) and (B) presence-absence (unweighted Unifrac, $P = 0.013$), analyzed by permutational multivariate ANOVA in SPTB and term. SPTB, spontaneous preterm birth.

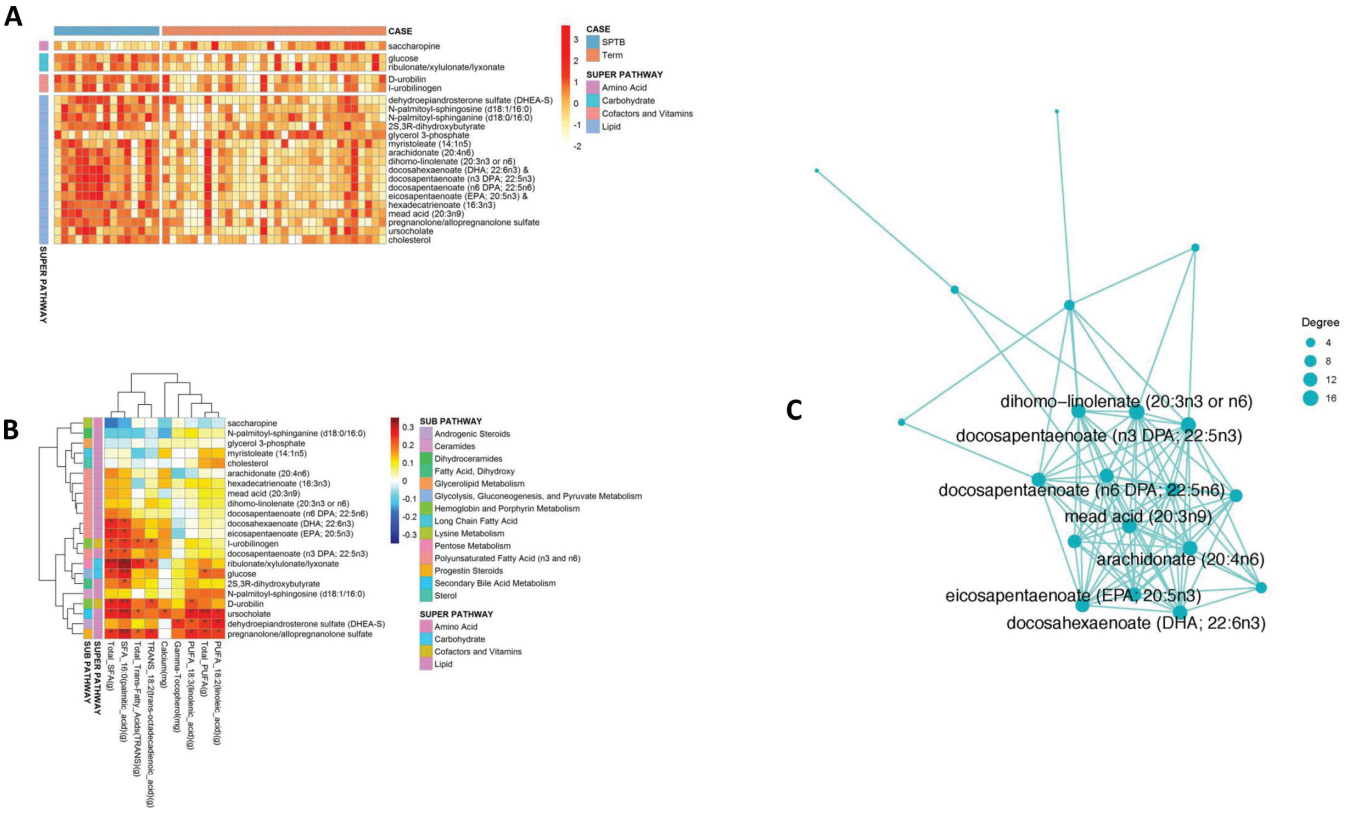

FIGURE 4 Untargeted fecal metabolites in cases and controls. (A) Of 824 fecal metabolites, 22 were differentially abundant in women with SPTB compared with term deliveries ($P < 0.01$ level and & = FDR < 0.2). (B) Heatmap comparing partial Kendall correlations between differentially abundant dietary micronutrients in columns and fecal metabolites in rows. *,**Significant correlation: $^{*}P < 0.05$; $^{**}P < 0.01$. (C) The most significantly associated module, built around ω-3 fatty acid metabolites, identified by Weighted Gene Co-expression Network Analysis of fecal metabolites (FDR = 0.008). Individual metabolites are represented by nodes, and are connected by edges whose topological overlap is above the threshold of 0.05. The centrality of a node (the number of adjacent edges) is represented by the node size. FDR, false discovery rate; SPTB, spontaneous preterm birth.

acid found in seed oils, α-linolenic acid (18:3n–3), with cases ingesting significantly more α-linolenic acid than controls (2.3 g compared with 1.7 g, $P = 0.009$). Intakes of ω-6 linoleic acid (18:2n–6) (19.1 g compared with 14.3 g, $P = 0.024$) and *trans*-octadecadienoic acid (18:2n–6) (0.45 g compared with 0.33 g, $P = 0.015$) were also higher in cases; these fatty acids are predominantly found in processed vegetable and seed oils. Consistent with higher intake of vegetable and seed oils, cases also had higher amounts of γ-tocopherol (17.0 mg compared with 12.0 mg, $P = 0.018$) and a trend toward higher amounts of Δ-tocopherol (4.2 mg compared with 2.9 mg, $P = 0.052$) (21).

We used partial Kendall correlation to examine the associations between the statistically significant dietary intake variables and fecal metabolites, which can be visualized in a heatmap (Figure 4B). Total saturated fat intake, the majority of which was palmitic acid, was positively associated with fecal metabolite concentrations of DHA, EPA, and docosapentaenoic acid (DPA; 22:5n–3) (each $P < 0.05$), as well as sugars (glucose, ribulonate/xylulonate/lyxonate), cholesterol breakdown (D-urobilin, ursocholate), and steroid hormone metabolites (pregnanolone/allopregnanolone sulfate) (each $P < 0.01$). Total PUFA intake, including both linolenic and linoleic acid, was positively associated with fecal concentrations of ursocholate, dehydroepiandrosterone sulfate (DHEA-S), and pregnanolone/allopregnanolone sulfate (all $P < 0.05$). These correlations provide objective analytic measures that parallel the dietary information collected by self-reported recall.

Four modules in the fecal metabolome were associated with SPTB

After individual tests on single metabolites, we used WGCNA to identify and evaluate whether specific metabolic modules or pathways were correlated with SPTB. A total of 20 modules were recovered from stool metabolomics data, and the module-level correlation analysis identified 4 modules highly correlated with SPTB (**Supplemental Figure 4**, Pearson correlation value > 0.3; P value < 0.05). The modules represented as connected graphs of metabolites are shown in Figure 4C and **Supplemental Figures 5–7**, where 2 metabolites are connected if the edge weight was >0.05. To summarize the metabolite measures within a module, the first principal component was calculated and was associated with SPTB. Members of the 4 most strongly associated modules included most of the differentially abundant metabolites identified from the individual variable tests (Wilcoxon's rank-sum, P value < 0.01). The 4 most associated modules included closely related metabolites that are similar in chemical structure and/or closely related in biological function. The module most strongly associated with SPTB included 26 metabolites and was enriched in ω-3 fats (Figure 4C, **Supplemental Table 2**).

TABLE 2 Mean daily dietary intake of selected nutrients in cases and controls[1]

Variable	Cases ($n = 15$)	Controls ($n = 32$)	P value
Energy, kcal	2163.02 ± 322.68	1943.84 ± 413.35	0.077
Energy, kcal/kg weight	30.16 ± 8.78	27.97 ± 9.54	0.456
Protein, g	87.52 ± 19.11	84.58 ± 26.16	0.699
Protein, % kcal	16.33 ± 3.56	17.41 ± 4.07	0.384
Protein, g/kg weight	1.19 ± 0.43	1.17 ± 0.41	0.849
Carbohydrate, g	258.07 ± 53.96	239.97 ± 58.46	0.317
Carbohydrate, % kcal	47.64 ± 5.83	49.65 ± 7.84	0.382
Total fiber, g	18.43 ± 6.27	18.64 ± 8.76	0.934
Soluble fiber, g	5.45 ± 1.58	5.33 ± 1.65	0.814
Insoluble fiber, g	12.89 ± 5.04	13.23 ± 7.47	0.875
Fat, g	90.17 ± 19.52	75.19 ± 21.68	0.028
Fat, % kcal	37.43 ± 5.32	34.55 ± 4.92	0.075
Saturated fat, g	31.38 ± 7.37	26.08 ± 8.62	0.045
Saturated fat, % kcal	13.01 ± 1.97	12.0 ± 2.82	0.218
Cholesterol, mg	339.26 ± 166.25	344.05 ± 162.99	0.926
Trans fat, g	2.57 ± 0.91	1.99 ± 1.05	0.071
Trans fat, % kcal	1.07 ± 0.34	0.893 ± 0.34	0.144
Monounsaturated fats, g	29.42 ± 7.17	26.26 ± 7.57	0.183
Monounsaturated fats, % kcal	12.24 ± 2.52	12.07 ± 1.94	0.800
Polyunsaturated fats, g	22.15 ± 9.17	16.52 ± 6.35	0.018
Polyunsaturated fats, % kcal	9.16 ± 3.38	7.56 ± 1.96	0.047
ω-3 Fatty acids, g	2.65 ± 1.05	1.89 ± 0.89	0.014
ω-6 Fatty acids, g	21.72 ± 9.16	16.25 ± 6.18	0.050
ω-6:ω-3	8.25 ± 2.06	9.14 ± 2.44	0.389
β-Carotene, retinol equivalents	3631.33 ± 3103.16	4058.27 ± 4551.32	0.744
Retinol, μg	509.76 ± 237.79	525.90 ± 324.09	0.864
Vitamin D, μg	7.46 ± 8.44	6.02 ± 4.68	0.457
β-Tocopherol, mg	0.48 ± 0.22	0.39 ± 0.16	0.116
γ-Tocopherol, mg	16.95 ± 7.87	11.97 ± 5.74	0.018
Δ-Tocopherol, mg	4.16 ± 2.45	2.88 ± 1.85	0.052
Vitamin K, μg	193.17 ± 145.54	143.52 ± 94.12	0.166
Solid fats, g	40.60 ± 11.69	33.37 ± 12.92	0.072
Sodium, mg	3320.89 ± 699.61	3047.50 ± 811.09	0.257
Calcium, mg	1197.62 ± 211.19	1001.51 ± 346.85	0.049
Magnesium, mg	314.36 ± 96.39	295.34 ± 95.12	0.528
Iron, mg	15.43 ± 3.27	16.44 ± 6.39	0.569
SFA 4:0 butyric acid, g	0.95 ± 0.44	0.74 ± 0.34	0.083
SFA 6:0 caproic acid, g	0.52 ± 0.27	0.42 ± 0.21	0.170
SFA 8:0 caprylic acid, g	0.42 ± 0.22	0.32 ± 0.29	0.062
SFA 10:0 capric acid, g	0.78 ± 0.34	0.65 ± 0.29	0.182
SFA 12:0 lauric acid, g	1.13 ± 0.59	1.05 ± 0.67	0.698
SFA 14:0 myristic acid, g	3.21 ± 1.19	2.58 ± 1.04	0.075
SFA 16:0 palmitic acid, g	16.38 ± 3.51	13.72 ± 4.32	0.044
SFA 17:0 margaric acid, g	0.16 ± 0.08	0.15 ± 0.13	0.781
SFA 18:0 stearic acid, g	6.87 ± 0.08	5.73 ± 1.99	0.157
SFA 20:0 arachidic acid, g	0.18 ± 0.07	0.14 ± 0.07	0.055
SFA 22:0 behenic acid, g	0.17 ± 0.12	0.12 ± 0.13	0.189
MUFA 14:1 myristoleic acid, g	0.11 ± 0.07	0.10 ± 0.09	0.627
MUFA 16:1 palmitoleic acid, g	1.32 ± 0.35	0.20 ± 0.48	0.411
MUFA 18:1 oleic acid, g	27.39 ± 6.83	24.26 ± 6.99	0.157
MUFA 20:1 gadoleic acid, g	0.36 ± 0.31	0.34 ± 0.34	0.805
MUFA 22:1 erucic acid, g	0.02 ± 0.03	0.01 ± 0.02	0.685
PUFA 18:2 linoleic acid, g	19.12 ± 8.42	14.30 ± 5.52	0.024
PUFA 18:3 linolenic acid, g	2.34 ± 0.87	1.73 ± 0.65	0.009
PUFA 18:4 parinaric acid, g	0.02 ± 0.04	0.01 ± 0.03	0.426
PUFA 20:4 arachidonic acid, g	0.25 ± 0.31	0.21 ± 0.23	0.634
PUFA 20:5 EPA, g	0.10 ± 0.17	0.04 ± 0.12	0.173
PUFA 22:5 docosapentaenoic acid, g	0.06 ± 0.10	0.04 ± 0.07	0.472
PUFA 22:6 DHA, g	0.18 ± 0.34	0.11 ± 0.25	0.370
Trans 18:1 *Trans* octadecenoic acid, g	2.01 ± 0.77	1.57 ± 0.91	0.113
Trans 18:2 *Trans* octadecadienoic acid, g	0.45 ± 0.16	0.33 ± 0.15	0.015
Trans 16:1 *Trans* hexadecenoic acid, g	0.06 ± 0.03	0.05 ± 0.03	0.277

[1] Values are mean ± SD or unpaired t test P values.

Three other modules with P value < 0.05 are in Supplemental Figures 5–7 and Supplemental Table 2. One module (Supplemental Figure 5, P value $= 0.01$, FDR-adjusted $P = 0.070$) contained a high representation of steroid hormones, including bile acids and DHEA-S for which elevated concentrations have recently been associated with preterm labor (22). Another module (Supplemental Figure 6, P value $= 0.03$, FDR-adjusted $P = 0.158$) was most strongly represented by ceramide and sphingosine signaling molecules that are recognized as proinflammatory. Further, sphingosine-1-phosphate has been identified for its role in myometrial contraction in response to LPS-induced infection (23), thus highlighting the potential role these modules may play in the etiology of SPTB. The module including sugars, fats, and alcohol was associated with SPTB overall (Supplemental Figure 7, P value $= 0.009$, FDR-adjusted $P = 0.07$), although no single member of the module was associated with SPTB with a Pearson's correlation value > 0.4.

Discussion

In this prospective, case-control study of pregnant women matched by nationally recognized variables associated with SPTB, we identified features of the fecal microbiota and metabolome that discriminate between women who eventually develop SPTB and those who carry their pregnancy to term. A decrease in α-diversity was strongly associated with the development of SPTB where a reduction in the taxonomic class of Betaproteobacteria performed moderately well in discriminating SPTB. Whereas there were no features in the maternal plasma metabolome associated with the development of SPTB after adjusting significance levels for FDR, the fecal metabolome had distinct increases in excretion of lipid and steroid hormone metabolites—specifically DHA, EPA, DHEA-S, and allopregnanolone—that were associated with greater dietary fat intake (especially SFAs, PUFAs, and *trans* fatty acids). These results not only suggest that the composition of the gut microbiota may ultimately have utility in predicting SPTB, but, together with the fecal metabolome, may also be congruent with previous epidemiologic studies showing that the consumption of a high-fat, low-fiber diet is associated with an increased risk of SPTB (5, 7, 24–26).

Few studies have examined the association between diet and the composition of the gut microbiota during pregnancy. In 2 studies of the same cohort of Norwegian pregnant women, higher dietary fiber and moderate fat intake were associated with increased microbial richness and fewer Bacteroidaceae (27), whereas higher intake of monounsaturated fat, cholesterol, and fat-soluble vitamins was associated with increased potentially proinflammatory bacteria of the genus *Proteobacteria* (28). Another study reported reduced Shannon diversity in postpartum fecal samples from women delivering preterm but did not report dietary intake or metabolomics data (29). Our study is the first that we know of to describe maternal gut microbiota and fecal metabolomics-based features that are associated with SPTB in a prospective cohort and to identify reduced microbial diversity in Betaproteobacteria. Although most Proteobacterial bacterial taxa associated with disease states belong to the class of Gammaproteobacteria, Betaproteobacteria taxa have also been associated with disease. For example, *Alcaligenes* spp. have been associated with inflammatory diseases in mice and humans (30) and both *Sutterella* and *Parasutterella* have been associated with both diet and disease (31, 32). We also noted weaker associations with plasma metabolites with potential relevance during pregnancy. 14-HDoHE is a lipoxygenase metabolite of DHA recognized for low activation of platelets (33). 17-HDoHE is a DHA-derived oxylipin signaling molecule that has been linked with reduced oxidative stress and oxidative damage in hepatocytes, and suggested as a possible novel biomarker of surgical systemic inflammatory stress (34). It has also been suggested to be a proresolving mediator and peroxisome proliferator–activated receptor-α/γ agonist in cell culture (35). In pregnancy, the concentration of plasma oxylipins and precursor fatty acids has been suggested as a potential predictor of SPTB (36). In the current study, the ratio of 14-HDoHE to 17-HDoHE was increased in women with SPTB; however, the meaning of this difference during pregnancy is not yet clear. Importantly, these analytic features were consistent with the subtle differences in diet between cases and controls based on dietary recall data.

Numerous researchers have shown that diet has significant influence on the diversity and richness of the gut microbiota (12, 20, 37–43). Greater intake of processed foods and proinflammatory fatty acids (reflected by higher SFAs, ω-6 PUFAs, and *trans* fatty acids) has been linked to obesity, diabetes, and cardiovascular disease, yet less is known about the impact of greater intake of processed and inflammatory fats during pregnancy. Large international cohort observations have linked a high-fat, low-fiber diet with increased risk of SPTB and a lower-fat, higher-fiber diet with reduced risk (7, 24–26). Similarly, highly processed, high-fat diets that are low in fiber are linked with decreased microbiota diversity, whereas those rich in fiber but low in fat are associated with increased diversity (12, 20). The diet in this study might be described as a comparison of a high-fat, low-fiber diet in controls with a yet worse quality diet in cases. Both were poor in quality but the diet in cases was worse and associated with SPTB in our cohort. Although fiber intake was low in both cases and controls, the predominant dietary difference was greater fat intake (especially SFAs, PUFAs, and *trans* fatty acids) in women with SPTB. The analytic data on the composition of the gut microbiota, together with 2 features in the fecal metabolome, were congruent with consumption of a poor-quality diet that includes higher amounts of proinflammatory dietary fat in mothers at risk of SPTB.

The dietary recall data in our study were suggestive of higher intake of plant and seed oils, including significantly greater intake of PUFAs—predominantly ω-6—as well as higher γ-tocopherol in processed foods. This signature was associated with higher maternal fecal concentrations of the steroid hormone metabolite DHEA-S. Both DHEA-S and allopregnanolone are fetal steroid molecules derived from breakdown of cholesterol that may signal intrauterine fetal stress. Intriguingly, maternal serum DHEA-S concentrations have been identified as one of the signals for cervical ripening (44) and indicators of successful induction of labor in term pregnancies (45). Similarly, the higher concentrations of fecal EPA and DHA paired with a decrease in α-diversity of the gut microbiota seen in women with SPTB were congruent with the dietary recall data showing that these women were consuming a higher-fat diet than controls. In addition, several bile acid metabolites (D-urobilin, L-urobilinogen, and ursocholate) cosegregated in the fecal metabolomics analysis, a finding that would be expected with greater dietary

fat intake and a subsequent increase in biliary secretions (Figure 4A, B).

Trans fatty acids are associated with cardiovascular disease and proinflammatory cytokine production by toll like receptor-4 activation, with the potential for deleterious effects on the fetus (46). *Trans* fatty acids have also been suggested as a risk factor for inadequate placental delivery of ω-3 DHA to the fetus. *Trans* fatty acids are inversely related to DHA and arachidonic acid (20:4n–6) concentrations in maternal plasma during pregnancy and at delivery and in fetal cord blood (47, 48), suggesting possible displacement by *trans* fatty acids of essential fatty acids in the case of placental transfer of this essential fatty acid for brain development to the fetus. In contrast to women in this cohort who delivered at term, *trans* fat intake in cases (2.7 g/d) exceeded the recommended intake amounts of <2 g/d and was also considerably greater than current Danish intake amounts (<1 g/d) where the rates of SPTB are lower than in the United States (48).

This study is limited by its small sample size, with only 16 cases of SPTB (5%). By contrast, national data include cases of medically indicated PTB due to pregnancy complications and women with a history of SPTB in prior pregnancies, both excluded from this study. However, this sample is an unusually rich representation of black women in a northeastern urban setting, a segment with limited previous description of diet and the gut microbiome during pregnancy. Even though black women have been recognized as at higher risk of SPTB (3), studies identifying novel predictive variables are limited, thus the current study is important. The limited generalizability of these findings to other samples with different racial and ethnic diversity or rural settings will require further study.

Nonetheless, the ability to validate self-reported dietary recall data by using independently determined quantifiable analytic features adds to the strength of our findings. It is also important to consider that both cases and controls had poor-quality, highly processed high-fat, low-fiber diets, so our ability to detect a dietary effect should have been modest. Yet, we observed differences in gut microbiota diversity and fecal metabolites. Future studies are needed comparing pregnant women eating a minimally processed healthy diet with those consuming a highly processed unhealthy diet to more adequately determine the potential impact of diet quality on the gut microbiota and birth timing during pregnancy.

In conclusion, data obtained several weeks before delivery suggest that reduced α-diversity of the fecal microbiota and increased fecal concentration of lipid (DHA, EPA, DPA) and steroid (DHEA-S and allopregnanolone) metabolites might provide a biomarker signature associated with future SPTB in women consuming a high-fat, low-fiber diet. Further investigation of these parameters in a larger sample is needed to evaluate the reproducibility of the findings. Translational studies in a mouse model of SPTB also might enlighten and enable full assessment of the absorption dynamics and any role that tissue concentrations of ω-3 fatty acids might play.

The authors' responsibilities were as follows—CWC, GDW, and ME: designed the research; CWC and ME: conducted the research; CWC, HL, YL, and GDW: analyzed the data or performed statistical analysis; CWC, GDW, YL, and VG: wrote the manuscript; CWC and GDW: had primary responsibility for the final content; and all authors: read and approved the final manuscript. The authors report no conflicts of interest.

Data Availability

Data described in the article, code book, and analytic code will be made publicly and freely available without restriction at https://github.com/yli0131/MGM.

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