Archival Report

Biological Psychiatry: GOS

Investigating the Microbiome in Relation to Mental Distress Across Two Points During Pregnancy: Data From U.S. and Swedish Cohorts

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ABSTRACT

BACKGROUND: In this study, we aimed to characterize the gut microbiome and its potential functioning in 2 populations at 2 time points during pregnancy in relation to mental distress.

METHODS: During the second and third trimester, individuals from the United States and Sweden completed the Edinburgh Postnatal Depression Scale and provided fecal samples for whole-genome metagenomics. A total of 832 and 161 samples were sequenced and analyzed from the Swedish cohort and the U.S. cohort, respectively. Multiple characterizations of the microbial community were analyzed in relation to distress measured using the Edinburgh Postnatal Depression Scale. Principal coordinate analysis and distance-based redundancy analysis assessed variation in functional gut-brain modules. For the U.S. cohort, the Trier Social Stress Test was administered 8 weeks postpartum while collecting salivary cortisol.

RESULTS: Principal coordinate analysis identified 4 sample clusters based on the gut-brain modules distinguished by functions such as short-chain fatty acid synthesis and cortisol degradation. While with distance-based redundancy analysis, mental distress subtypes did not significantly contribute to variation in gut-brain modules (p = .085 for Sweden, p = .23 for the U.S.), a U.S. sample cluster distinguished by lower cortisol degradation from another cluster with higher gut microbial cortisol degradation abundance had significantly higher odds of being associated with depression (p = .024). The U.S. sample cluster with lower gut microbial cortisol degradation abundance also had significantly higher cortisol levels after a postpartum social stressor.

CONCLUSIONS: Further studies are warranted to investigate the potential for the gut microbiome to serve as biomarkers of gut-brain axis health during pregnancy across disparate populations.

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Depression is thought to affect 10% to 20% of individuals during pregnancy and the postpartum period; however, estimates have been as high as 25% of pregnant individuals experiencing elevated depressive symptoms during the third trimester (1,2). Anxiety is highly comorbid with perinatal depression (3), making it important to consider anxiety symptoms along with depression symptoms. Pregnancy is a critical time for the future mental health of both parent and child; elevated mental distress, including depression and anxiety, during pregnancy are associated with worse outcomes, including higher rates of suicide, for parent and child during the postpartum period and later in life (4–10). The etiologies of perinatal depression and anxiety are multifactorial and individualized based on the individual's environment and biological makeup, making diagnosis and personalized treatment difficult (11).

An evolving area of study that has the potential to reflect the environment and the individual's biologic makeup is the microbiome, which refers to the microbes including bacteria, viruses, and fungi that live within us (12). The hypothalamic-pituitary-adrenal axis and immune systems are implicated in depression and anxiety and are thought to regulate the microbiota, while the microbiota also trains the immune and hypothalamic-pituitary-adrenal systems (13–15). Greater diversity of bacteria in the adult gut is thought to result in an increase in beneficial metabolites, and diversity provides functional redundancy and resilience (16–18). Aspects of the gut microbiome have been associated with major depression and anxiety disorders (19–21), supported by evidence that the microbiome and metabolites from microbial pathways interact with the peripheral and central nervous systems (22,23).

There are several ways to characterize the microbiome including how many different types of bacteria make up the community and how evenly distributed the different types are (the within-person diversity known as alpha diversity) and how much the microbial communities as a whole are distinct from other microbial communities (beta diversity). Originally, the microbiome was characterized by the types of bacteria that

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make it up, identified from sequencing of microbial genes and then matching to types of bacteria. There have been conflicting results in the literature about whether alpha diversity of the gut microbiota, on average, decreases, remains the same, or even increases from earlier to later in pregnancy (12,24,25). Research on the microbiome and gestational diabetes suggests that the potential functioning of the microbiome may be an important new direction for assessing the microbiome in relation to pregnancy health (26). Potential functioning can be identified based on what is known about the microbial genes identified in the entire community without needing to assign bacterial types.

There has been limited study of the gut microbiome across pregnancy in relation to mood and anxiety. In a small study of 28 individuals with postpartum depression and 16 control participants, there were no significant differences in alpha diversity, but the microbial communities of individuals with postpartum depression were significantly different from the microbial communities of individuals without postpartum depression (27). Alpha diversity as assessed by Shannon diversity earlier in pregnancy was positively correlated with coping better with stress postpartum (28). Bernabe *et al.* did not find differences in alpha diversity or beta diversity based on perceived stress, but they did find that a relative abundance of specific bacteria distinguished individuals with higher levels of distress from individuals with self-efficacy (29). In a study of 140 pregnant individuals, higher mental distress scores were associated with lower species richness of the gut microbiota (30). In a small study of pregnant individuals, metabolites created by the microbiome and in the blood of the individual changed from the second trimester to the third trimester, which was associated with less bacterial diversity, and were significantly correlated with a bacteria associated with producing short-chain fatty acids (SCFAs) (31). Further studies are warranted.

One of the most commonly used tools for assessing mental distress during pregnancy and the postpartum period is the Edinburgh Postnatal Depression Scale (EPDS) (32). The EPDS attempts to assess not only depression but also anxiety, making cutoff scores a reflection of a multifaceted mental distress (33,34). Factor analysis has shown that the EPDS can be divided into 3 dimensions of anhedonia, depression, and anxiety (35).

Our aim in this study was to assess the maternal gut microbiome in terms of species composition and its associated predicted functioning based on the microbial genetics at 2 time points during pregnancy and in relation to self-reported symptoms of mental distress as assessed by the EPDS, including the subscales reflecting anhedonia, depression, and anxiety, in both a Swedish cohort and a cohort from the United States.

METHODS AND MATERIALS

Data

BASIC Cohort, Uppsala, Sweden. BASIC (Biology, Affect, Stress, Imaging and Cognition) is a population-based, prospective cohort study that recruited participants at the Department of Obstetrics and Gynecology at Uppsala University Hospital from 2009 to 2018 (36). Women listed for routine ultrasound examination around pregnancy week 18 were invited to participate. Exclusion criteria were age below 18 years, insufficient ability to read and understand Swedish, protected identity, known blood-borne infections, and/or nonviable pregnancy as diagnosed by routine ultrasound. Invited women received written information about the BASIC study by mail, together with the telephone number and e-mail address of the study personnel. Women who agreed to participate returned their written informed consent by mail separately for every modality that they wished to participate in (e.g., questionnaires, blood samples, and fecal samples). The participants were asked to complete online surveys at gestational weeks 17 and 32 and at 6 weeks postpartum. BASIC gathered demographic and medical information from surveys and was able to gather additional information through linkage to medical records (36). Starting in 2016, participants were invited to provide fecal samples for analyses of the gut microbiota twice during pregnancy, which were sent to participants after they had filled out the surveys.

Mental distress symptoms were assessed with the Swedish version of the EPDS (32,37) at gestational weeks 17 and 32. At gestational week 17, participants reported their age, education, employment, country of birth, and height and weight before pregnancy for the calculation of body mass index. Questions about use of selective serotonin reuptake inhibitors and other medications were answered at both week 17 and week 32.

Participants collected fecal samples at home and were instructed to sample from toilet paper with a spoon attached to the lid of the tube and put in the DNA/RNA shield (Zymo Research Corporation). Samples were mailed to the hospital and stored at -80° C until DNA extraction.

University of North Carolina at Chapel Hill. The U.S. cohort was prospectively recruited to study the microbiota-gut-brain axis and perinatal mood and anxiety disorders. Individuals who were at least 18 years old and at <28 gestational weeks of a singleton pregnancy were recruited through outreach to the University of North Carolina (UNC) Department of Obstetrics & Gynecology and the surrounding community. Data collection occurred from April 2017 to November 2019. Exclusion criteria included being a non-English speaker; having a history of bipolar disorder or psychosis confirmed by the Structured Clinical Interview for DSM-5 (38); alcohol or drug abuse during the past 90 days, which was also confirmed by the Structured Clinical Interview for DSM-5; inflammatory bowel disease (i.e., ulcerative colitis or Crohn's disease); celiac disease; and major gastrointestinal surgery (i.e., other than appendectomy or cholecystectomy), the latter per self-report. Participants in the U.S. cohort provided demographic data through a secure Qualtrics web survey with additional information asked at the research visit. Weight and height were recorded at the research visit. Information on medication use was obtained during each study visit. The EPDS was collected at the initial study visit (before week 28) and then again at the third trimester study visit (range week 30–38, mean week 34).

The U.S. participants were given a hat (Fisher brand Commode Specimen Collector) to collect fecal matter at their homes and a spatula to put the sample into a tube with a DNA/RNA shield. These were put in a cooler with a frozen ice pack

and transported in the cooler within 24 hours for processing by the study team. Samples were homogenized and stored at −80° C. Samples were shipped to Sweden with dry ice to be analyzed alongside the Swedish samples.

The mothers' salivary cortisol levels were measured during 1) baseline, defined as 10 minutes before beginning a stressor; 2) a stressor interval using the Trier Social Stress Test (TSST); and 3) a recovery period of 40 minutes of quiet alone time. The TSST involves participants experiencing 2 social stressors, giving a speech and serial subtraction out loud, in front of a committee of laboratory personnel instructed to correct the individual and maintain a strict countenance (39). Saliva was then assayed by enzyme-linked immunosorbent assay to obtain cortisol levels.

The U.S. participants were asked to complete a 3-day diet journal before each visit. Dietary components including total dietary fiber, soluble dietary fiber, insoluble dietary fiber, and pectins were analyzed from the journals using NDSR Software available through the Nutrition Coordinating Center.

We did not exclude individuals in either cohort based on medication usage, including antibiotic, selective serotonin reuptake inhibitor, and gastrointestinal medication usage, but we did gather data on medication usage.

Ethics

The Swedish part of the project followed the ethical guidelines set out by the Swedish Ethical Review Authority and General Data Protection Regulation requirements. Ethical permits were obtained for the BASIC study (EPN Uppsala 2009/171 with amendment 2009/171/4 from 2016). The U.S. part of the project was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (#16–0959; #16–2783). All participants provided written informed consent before joining the study.

EPDS Groups and Symptom Clusters

Individuals were dichotomized into higher or lower distress based on having an EPDS score >11 or having an EPDS score ≤ 11, respectively. This cutoff was chosen because it was found in a meta-analysis to optimize sensitivity and specificity (40). Principal component analysis and K-means were used to assess which EPDS questions clustered together. This was done in the BASIC and in the UNC cohort, each separately and at both time points during pregnancy.

Group Definitions and Data Processing

Details on DNA extraction, sequencing, and annotation are provided in the Supplement. All statistical analyses were performed in R version 4.2.2, while graphical representations were created using ggplot2 (41). For continuous, normally distributed demographic and clinical variables of the individuals with higher distress and lower distress, the Student's t test was used; for variables not normally distributed, the Mann–Whitney U test was used. Both are shown as mean ± SD. For categorical variables, the χ^2 test of independence was used. For all analyses, $p < .05$ was considered statistically significant.

RESULTS

Demographic and Other Relevant Characteristics of Study Participants

There were 403 participants from the Swedish cohort and 83 participants from the U.S. cohort with sequenced samples taken during the second trimester and 429 and 78 participants from the Swedish cohort and the U.S. cohort, respectively, during the third trimester. Table 1 shows the demographic variables by cohort and time point. p Values were included to indicate whether there were significant differences in demographic variables between the 2 groups of mental distress created based on an EPDS score >11 or an EPDS score ≤ 11.

Mental Distress in the Two Cohorts

Table 2 shows the percentages of individuals in each group based on EPDS scores. The mean EPDS score was lower for U.S. participants than for Swedish participants during the second trimester (mean [SD] = 4.1 [3.6] vs. 5.8 [5.0]), but the mean scores were more similar during the third trimester (5.5 [4.6] vs. 5.6 [4.6]). In the U.S. cohort, only 2 participants had an EPDS score >11 during the second trimester.

Microbial Diversity

As is shown in more detail in the Supplement, both cohorts demonstrated decreased alpha diversity during late pregnancy, although a significant reduction in both richness and diversity was observed exclusively in the less distressed Swedish mothers. After adjusting for time point, the difference in microbial composition between more distressed and less distressed mothers was identified only in the Swedish cohort. Nevertheless, microbial composition was significantly associated with the EPDS total score and the EPDS depression score solely in the U.S. cohort with time point adjusted.

Microbial Functioning (Gut-Brain Modules)

As described in Supplemental Methods, 4 clusters for each cohort (see Figure 1A for Sweden and Figure 2A for the U.S.) were generated based on the clusters found when samples from different time points were split (see Figure 1B for Sweden and Figure 2B for the U.S.). By summing the squared loading of the gut-brain modules (GBMs) on the first 2 axes in distance-based redundancy analysis, cortisol degradation and acetate synthesis contributed to the variance in microbial communities in both cohorts. Additionally, in the Swedish cohort (Figure 3), cortisol degradation distinctly separated cluster A and cluster D from cluster B and cluster C by pointing toward clusters A and D, indicating that a higher activity of cortisol degradation is shared by gut microbiota of samples in clusters A and D compared with clusters B and C. In contrast, in the U.S. cohort (Figure 4), ClusterFour stood apart from the other clusters because the direction of the cortisol degradation vector deviated from that of ClusterFour, suggesting that gut microbiota of the samples in ClusterFour were less active in cortisol degradation. Menaquinone synthesis I also contributed to variance in the Swedish cohort, and inositol synthesis contributed to variance in the U.S. cohort. Similar patterns can also be seen in Figures S10A–D, which indicate the presence/

Table 1. Self-Reported Demographic and Other Relevant Characteristics of the Two Cohorts Included in Analyses for the Second and Third Trimesters

	Sweden			United States		
	Second Trimester, *n* = 403	Third Trimester, *n* = 429	EPDS ≤11 vs. >11, *p* Value	Second Trimester, *n* = 83	Third Trimester, *n* = 78	EPDS ≤11 vs. >11, *p* Value
Age, Years						
Mean (SD)	32 (4.3)	32 (4.3)	.98	32 (3.8)	32 (3.8)	.059
Missing	3 (0.7%)	1 (0.2%)	–	3 (3.6%)	3 (3.8%)	–
Birthplace						
Other	37 (9%)	36 (8%)	.83	NA	NA	NA
Scandinavia	339 (84%)	365 (85%)		NA	NA	–
Missing	27 (6.7%)	28 (6.5%)		–	–	–
Ethnicity						
Hispanic	NA	NA	NA	7 (8%)	7 (9%)	.64
Non-Hispanic	NA	NA	–	74 (89%)	70 (90%)	
Missing	–	–	–	2 (2.4%)	1 (1.3%)	
Race						
Not White	NA	NA	NA	16 (19%)	13 (17%)	.25
White	NA	NA	–	65 (78%)	64 (82%)	
Missing	–	–	–	2 (2.4%)	1 (1.3%)	
Antibiotics Second Trimester						
No	369 (92%)	331 (77%)	.10	79 (95%)	74 (95%)	1.0
Yes	13 (3%)	11 (3%)		4 (5%)	4 (5%)	
Missing	21 (5.2%)	87 (20.3%)		0 (0%)	0 (0%)	
Antibiotics Third Trimester						
No	305 (76%)	396 (92%)	.18	76 (92%)	72 (92%)	.65
Yes	13 (3%)	14 (3%)		7 (8%)	6 (8%)	
Missing	85 (21.1%)	19 (4.4%)		0 (0%)	0 (0%)	
Body Mass Index Reported Prepregnancy						
Mean (SD)	24 (4.1)	24 (4.0)	.84	NA	NA	NA
Missing	28 (6.9%)	30 (7.0%)	–	NA	NA	–
Body Mass Index at Second Trimester Visit						
Mean (SD)	NA	NA	NA	27 (5.8)	27 (6.5)	.43
Missing	–	–	–	5 (6.0%)	8 (10.3%)	–
Body Mass Index at Third Trimester Visit						
Mean (SD)	NA	NA	NA	30 (5.8)	30 (6.1)	.055
Missing	–	–	–	9 (10.8%)	9 (11.5%)	–
Parity						
Multiparous	129 (32%)	133 (31%)	.923	45 (54%)	46 (59%)	.40
Nulliparous	182 (45%)	187 (44%)		37 (45%)	32 (41%)	
Missing	92 (22.8%)	109 (25.4%)		1 (1.2%)	0 (0%)	
SSRI Use Second Trimester						
No	288 (71%)	293 (68%)	.51	76 (92%)	71 (91%)	<.001
Yes	19 (5%)	24 (6%)		7 (8%)	7 (9%)	
Missing	96 (23.8%)	112 (26.1%)		0 (0%)	0 (0%)	
SSRI Use Third Trimester						
No	235 (58%)	247 (58%)	.02	75 (90%)	70 (90%)	<.001
Yes	21 (5%)	25 (6%)		8 (10%)	8 (10%)	
Missing	147 (36.5%)	157 (36.6%)		0 (0%)	0 (0%)	
Gestational Days at Second Trimester						
Mean (SD)	150 (34)	150 (37)	.093	150 (33)	150 (33)	.054
Missing	116 (28.8%)	191 (44.5%)	–	1 (1.2%)	2 (2.6%)	–
Gestational Days at Third Trimester						
Mean (SD)	240 (9.9)	240 (11)	.838	240 (12)	240 (11)	.28
Missing	171 (42.4%)	142 (33.1%)	–	2 (2.4%)	3 (3.8%)	–

Table 1. Continued

	Sweden			United States		
	Second Trimester, n = 403	Third Trimester, n = 429	EPDS ≤11 vs. >11, p Value	Second Trimester, n = 83	Third Trimester, n = 78	EPDS ≤11 vs. >11, p Value
Bowel Medications						
No	366 (91%)	390 (91%)	.64	67 (81%)	64 (82%)	1.0
Yes	37 (9%)	39 (9%)		16 (19%)	14 (18%)	

The third columns for Sweden and the United States show the p values obtained when comparing individuals with elevated EPDS scores and individuals with lower EPDS scores based on the characteristic.

EPDS, Edinburgh Postnatal Depression Scale; NA, not available; SSRI, selective serotonin reuptake inhibitor.

absence of different functions from the GBM and in relation to higher and lower levels of distress.

Variance in GBM composition explained by the 4 EPDS subgroups in both cohorts was 0.7% and 2.9% for the Swedish and U.S. cohorts, respectively, according to the analysis of variance test on the distance-based redundancy analysis. In both cohorts, EPDS subtypes are not likely to account for the variance in GBM composition (Sweden, p = .085; U.S., p = .232). Exploratory analyses including all MetaCyc pathways (42) identified in the samples were also conducted, but no significant correlation was found between the total genetic makeup of the fecal samples and the EPDS total score or EPDS subscores.

Sample Clusters Found From GBM Data

Logistic regression models were built to examine the association between the dichotomous EPDS score and GBMs. In the U.S. cohort, ClusterFour was found to have a significantly higher likelihood of elevated EPDS scores compared with ClusterThree (p = .024, standard error = 0.78), with a log odds ratio of 1.76 (see Table S4 for details).

Knowing that samples in ClusterFour had a significantly higher chance of being linked to depression than samples in ClusterThree, we then explored which GBMs/species were significantly different between the 2 clusters. To explore this, analysis of compositions of microbiomes with bias correction (ANCOMBC) was applied to both species and GBM abundance matrices, using ClusterThree (reference) and ClusterFour as groups and with age and body mass index as confounding factors. For GBM, cortisol degradation ($p = 2.0 \times 10^{-75}$, log2 fold change [log2FC] = −8.6), isovaleric acid synthesis I (KADH pathway) ($p = 1.6 \times 10^{-5}$, log2FC = −3.6), GABA (gamma-aminobutyric acid) synthesis II ($p = 3.1 \times 10^{-8}$, log2FC = −1.9), GHB (gamma-hydroxybutyric acid) degradation ($p = 2.3 \times 10^{-2}$, log2FC = −1.5), acetylcholine synthesis ($p = 3.3 \times 10^{-4}$, log2FC = −1.3), and propionate degradation I ($p = 2.3 \times 10^{-3}$, log2FC = −0.81) were all found to be significantly more abundant in ClusterThree (Figure 5). No species remained significant after multiple-testing correction in the comparison. Additionally, samples in ClusterFour had a significantly lower species richness than samples in ClusterThree (Figure 6).

Given the significant differences in microbial function and alpha diversity between samples from ClusterThree and ClusterFour in the U.S. cohort, saliva cortisol levels were measured from the individuals in the U.S. cohort in these 2 clusters through a TSST at approximately 5 weeks postpartum to detect whether individuals from the 2 clusters reacted differently to stress. Saliva cortisol levels of participants from ClusterFour were found to be significantly higher than those of participants from ClusterThree at all 6 sampling points (see Figure 7).

Table 2. EPDS Scores in the Two Cohorts Separately and in the Total Sample by Trimester Analysis

	Sweden		United States		Overall	
	Second Trimester, n = 403	Third Trimester, n = 429	Second Trimester, n = 83	Third Trimester, n = 78	Second Trimester, n = 486	Third Trimester, n = 507
Second Trimester EPDS						
Mean (SD)	5.8 (5.0)	NA	4.1 (3.6)	NA	5.5 (4.8)	NA
Missing	27 (6.7%)	NA	4 (4.8%)	NA	31 (6.4%)	NA
Second Trimester EPDS Group						
0–11	325 (81%)	352 (82%)	77 (93%)	70 (90%)	402 (83%)	422 (83%)
12–30	51 (13%)	48 (11%)	2 (2%)	5 (6%)	53 (11%)	53 (10%)
Missing	27 (6.7%)	29 (6.8%)	4 (4.8%)	3 (3.8%)	31 (6.4%)	32 (6.3%)
Third Trimester EPDS						
Mean (SD)	NA	5.6 (4.6)	NA	5.5 (4.6)	NA	5.6 (4.6)
Missing	NA	27 (6.3%)	NA	3 (3.8%)	NA	30 (5.9%)
Third Trimester EPDS Group						
0–11	332 (82%)	361 (84%)	71 (86%)	64 (82%)	403 (83%)	425 (84%)
12–30	41 (10%)	41 (10%)	9 (11%)	11 (14%)	50 (10%)	52 (10%)
Missing	30 (7.4%)	27 (6.3%)	3 (3.6%)	3 (3.8%)	33 (6.8%)	30 (5.9%)

EPDS, Edinburgh Postnatal Depression Scale; NA, not available.

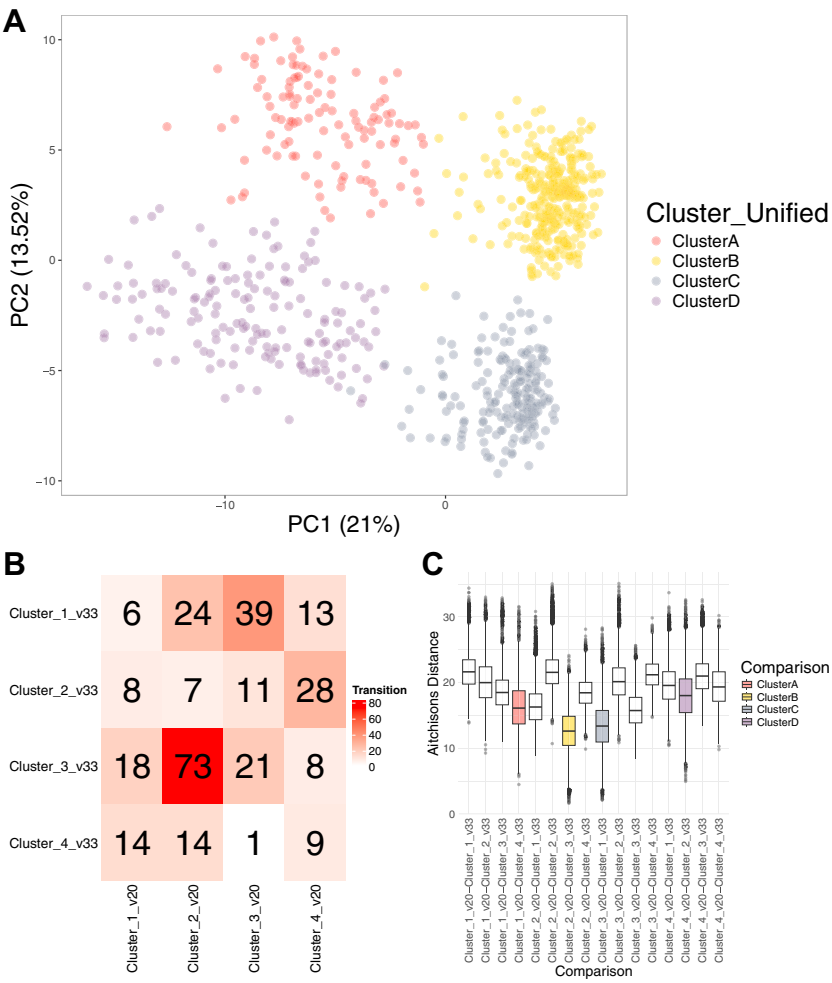

Figure 1. Four distinguishable clusters were found in the Swedish cohort using gut-brain module abundances. **(A)** Principal coordinate analysis plot of Swedish samples at 2 time points, performed using Aitchison distance. Samples are color-coded according to the 4 cluster pairs identified in the Aitchison distance boxplot shown in panel **(C)**. **(B)** Heatmap illustrating the number of shared individuals between clusters at week 20 and week 33. **(C)** Boxplots depicting the Aitchison distances between samples from each cluster at week 20 and week 33. The y-axis represents distance values, while the x-axis denotes the cluster comparisons (e.g., cluster_1_v20-Cluster_1_v33 represents the comparison between cluster 1 at week 20 and cluster 1 at week 33). The most similar cluster pairs are highlighted in color, while other clusters are not. Samples from cluster 1 at week 20 and cluster 4 at week 33 were combined and designated as cluster A, samples from cluster 2 at week 20 and cluster 3 at week 33 were designated as cluster B, samples from cluster 3 at week 20 and cluster 1 at week 33 were designated as cluster C, and samples from cluster 4 at week 20 and cluster 2 at week 33 were designated as cluster D. PC, principal component.

Specifically, it was at the end of the stress test when the difference in cortisol levels between individuals from ClusterThree and ClusterFour were the most significant (adjusted p value = .0098). There were no significant differences between ClusterThree and ClusterFour on any of the 4 measures of dietary fiber.

DISCUSSION

This study utilized multiple methods of characterizing the gut microbiome: alpha diversity, beta diversity, differential abundance of bacterial species between individuals with higher and lower levels of distress, and assessing the variation in potential functioning as predicted by the genetic makeup of the individual's microbial community in relation to the potential functioning of another's community. The results indicate that changes in alpha diversity across pregnancy may differ based on the amount of mental distress experienced by the mother, but the results also indicate the difficulty of using alpha diversity measures to characterize individual microbial communities as a biomarker of mental distress during pregnancy. Individuals with lower distress levels at both time points seemed to have a decrease in alpha diversity from the second to the third trimester in both cohorts, although there was a lot of variation within the group of individuals with lower distress levels. There was less variation in the group with higher distress levels, but this was also a much smaller group. Similarly, the results for beta diversity are included in the Supplement and may indicate differences based on higher or lower levels of distress, but these findings were not strong.

This work suggests that it is important to consider characterization of the microbiome beyond composition and analyze the functioning of the microbial community during pregnancy. The 4 distinct clusters of gut-brain axis clustered microbial pathways were differentially associated with specific functions, with SCFA acetate synthesis and cortisol degradation contributing to most of the variation. Crucially, these functional clusters were identified in both cohorts separately, suggesting that it may be a more robust finding than species-level differences. An unsupervised clustering method revealed that individuals in ClusterThree of the U.S. cohort had a significantly lower likelihood of mental distress as measured by the EPDS than individuals in ClusterFour. This difference may be attributed to the higher alpha diversity and greater abundance of beneficial gut microbial functions observed in ClusterThree.

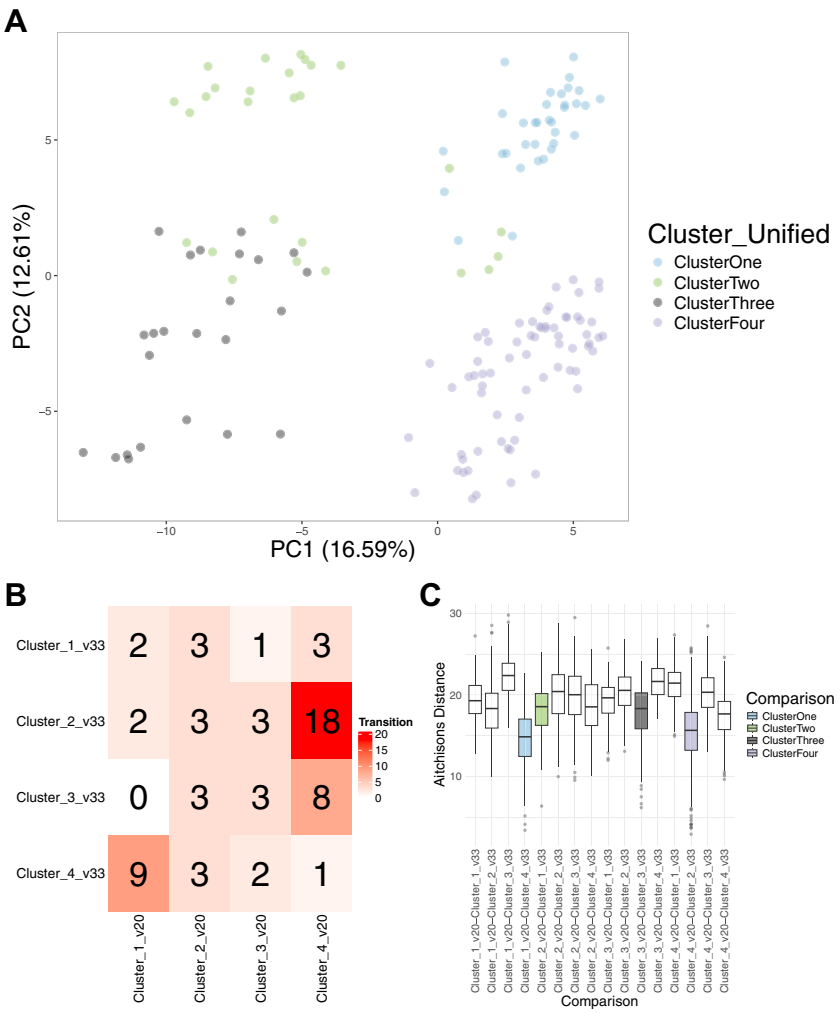

Figure 2. Four distinguishable clusters were found in the U.S. cohort using gut-brain module abundances. **(A)** Principal coordinates analysis plot of U.S. samples at 2 time points, performed using Aitchison distance. Samples are color-coded according to the 4 cluster pairs identified in the Aitchison distance boxplot shown in panel **(C)**. **(B)** Heatmap illustrating the number of shared individuals between clusters at week 20 and week 33. **(C)** Boxplots depicting the Aitchison distances between samples from each cluster at week 20 and week 33. The y-axis represents distance values, while the x-axis denotes the cluster comparisons (e.g., Cluster_1_v20-Cluster_4_v33 represents the comparison between cluster 1 at week 20 and cluster 4 at week 33). The most similar cluster pairs are highlighted in color, while other clusters are not. Samples from cluster 1 at week 20 and cluster 4 at week 33 were combined and designated as ClusterOne, samples from cluster 2 at week 20 and cluster 1 at week 33 were combined and designated as ClusterTwo, samples from cluster 3 at week 20 and cluster 3 at week 33 were combined and designated as ClusterThree, and samples from cluster 4 at week 20 and cluster 2 at week 33 were combined and designated as ClusterFour. PC, principal component.

Furthermore, individuals in ClusterThree demonstrated increased resilience to a stressor compared with individuals in ClusterFour as indicated by lower cortisol levels, particularly during recovery from the stressor. Together, these results highlight potential links between microbial diversity, gut microbial function, and mental health and that distinguishing individuals by the predicted functions of their microbial community genetics may be a new way to understand which gut-brain processes may be important to different individuals navigating pregnancy.

Cortisol management is critical to the developing fetus. Although studies of cortisol in relation to depression during pregnancy have yielded mixed results (42–44), further investigation of microbially driven cortisol degradation may be a missing component in understanding variation in cortisol metabolism in pregnancy (45). Higher cortisol levels and less recovery during the TSST have been associated with major depressive disorder (46). The social stressor was only administered during the postpartum period in the current study, but this may suggest that cortisol degradation of the microbiome during pregnancy sets the stage for postpartum depression in a subset of individuals.

Our findings regarding SCFA acetate are supported by significant interest in the literature. Altered levels of SCFAs have been implicated in relation to mental health and pregnancy complications (47–51). In animal models, supplementation with SCFA, including acetate, decreased anxiety-like and depression-like behavior, particularly in relation to social defeat stress, but not for animals stressed during early life (22,51,52). While analysis of the U.S. cohort did not show differences in dietary fiber intake between ClusterThree and ClusterFour, which is important in microbial SCFA production, the study was not powered to assess individual components of diet. We lack dietary data from the Swedish cohort to compare U.S. and Swedish dietary patterns and study dietary factors between the clusters based on microbial community functioning in the larger cohort. These preliminary findings may indicate that future research should include characterizations of whole diet for individuals such as calories received from processed sugars versus calories from nonprocessed foods and how that may change over time during pregnancy. Factors such as exposure to early-life stress should be assessed in future studies of clusters distinguished by microbial acetate synthesis (22,53–56).

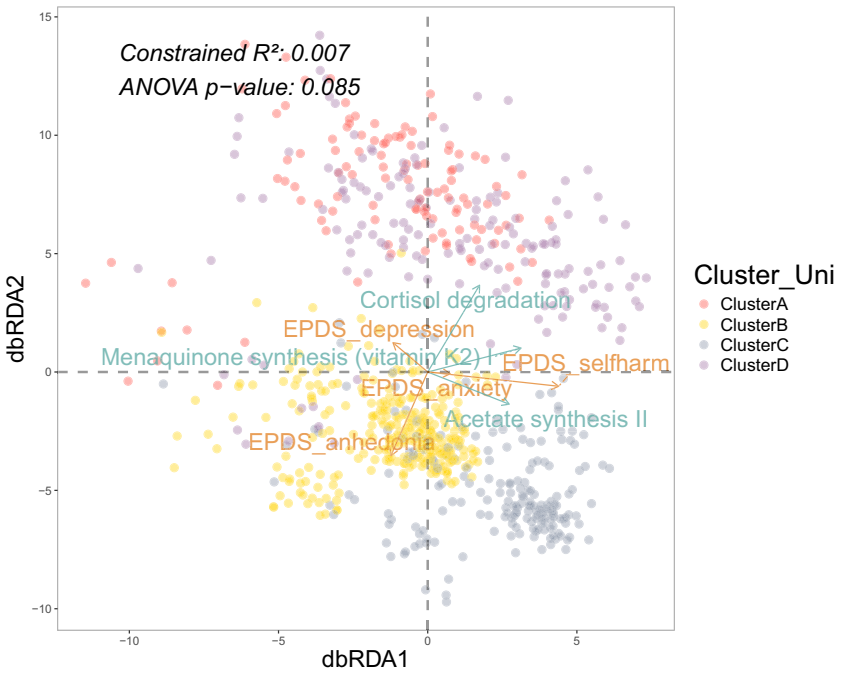

Figure 3. Edinburgh Postnatal Depression Scale (EPDS) components are not significantly correlated with the gut-brain module (GBM) composition of the Swedish cohort. Distance-based redundancy analysis (dbRDA) triplots showing the beta diversity at 2 time points in the Swedish cohort. Dots are colored according to the 4 unified clusters in Figure 5A. The top 3 GBMs that contributed the most to dbRDA1 and dbRDA2 (cortisol degradation, menaquinone synthesis I, and acetate synthesis II) were plotted with light blue vectors as described in Supplemental Methods. The 4 EPDS subgroups were plotted with orange vectors according to their loadings. Four EPDS subgroups explained 0.7% of the total variance in GBM composition among all samples, with a nonsignificant *p* value (.085) from a post hoc analysis of variance (ANOVA) test. Both the EPDS self-harm and EPDS anhedonia subscales were found to be more likely to drive the variation in GBM composition than the EPDS anxiety or EPDS depression subscales from the graph. EPDS anhedonia and EPDS anxiety demonstrate a negative correlation. Cluster A and cluster D are more abundant in cortisol degradation, while cluster B and cluster C are more abundant in acetate synthesis II and menaquinone synthesis I.

Strengths and Limitations

Strengths of this study are that it compared and contrasted 2 cohorts from distinct settings—one from the U.S. and one from Sweden, that these cohorts followed the same individuals at 2 time points during pregnancy, and that the microbiome data from both cohorts were analyzed together. There are slight differences in collection to note that might have led to differences in the microbiome findings, but these are difficult to take into account. For example, collection in a hat versus with toilet paper may lead to more skin flora being included in the toilet

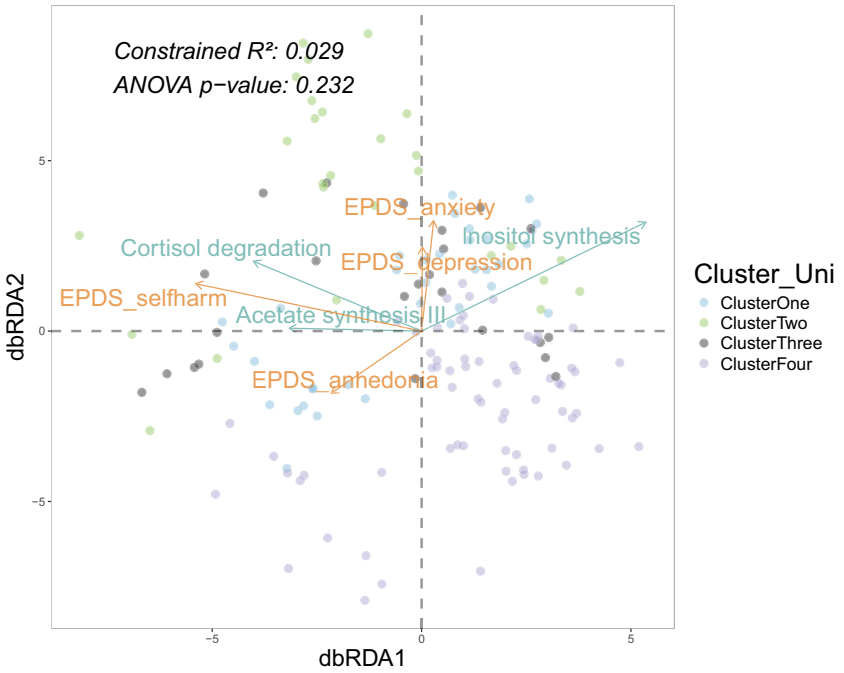

Figure 4. Edinburgh Postnatal Depression Scale (EPDS) components are not significantly correlated with the gut-brain module (GBM) composition of the U.S. cohort. Distance-based redundancy analysis (dbRDA) triplots showing the beta diversity at 2 time points in the U.S. cohort. Dots are colored according to the 4 unified clusters in Figure 6A. The top 3 GBMs that contributed the most to dbRDA1 and dbRDA2 (cortisol degradation, inositol synthesis, and acetate synthesis III) were plotted with light blue vectors as described in Methods and Materials. The 4 EPDS subgroups were plotted with orange vectors according to their loadings. Four EPDS subgroups explained 2.9% of the total variance in GBM composition among all samples, with a nonsignificant *p* value (.232) from a post hoc analysis of variance (ANOVA) test. EPDS self-harm was more likely to drive the variation in GBM composition than the other 3 subgroups. EPDS anhedonia and EPDS anxiety also demonstrate a negative correlation in the U.S. cohort. ClusterTwo and ClusterThree are more abundant in the context of cortisol degradation as well as acetate synthesis III, while ClusterOne is more abundant in inositol synthesis

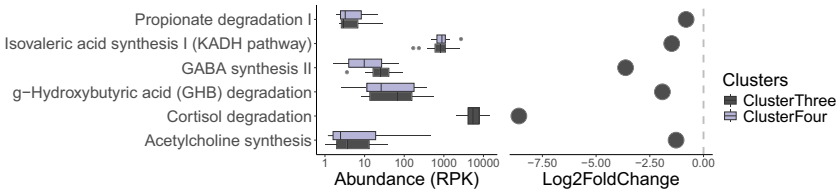

Figure 5. Significantly abundant gut-brain modules (GBMs) between ClusterThree and ClusterFour in the U.S. cohort. Plots displaying GBMs with significant differential abundance (q value $<$ .05) identified by ANCOMBC2 between ClusterThree and ClusterFour, adjusted for age and body mass index. The left panel shows boxplots of the relative abundance of the significantly abundant GBMs in the 2 clusters, with the x-axis representing estimated abundance and the y-axis displaying the GBM name. Box colors indicate the corresponding clusters. The right panel presents a dot plot of the $\log_2$ fold change (effect size) for the GBM by comparing ClusterFour with ClusterThree (reference group), with dot color representing clusters and a dashed line indicating the borderline for the direction of enrichment. ANCOMB, analysis of compositions of microbiomes with bias correction; GABA, gamma-aminobutyric acid; RPK, reads per kilobase.

paper sample. It is also a limitation that only participants in one cohort, the smaller cohort, were asked about dietary patterns and stool consistency at the time of collection. Another limitation is that the first samples were collected during the second trimester instead of the first trimester. The U.S. cohort offered entrance into the study during the first trimester, but we found that most individuals did not enroll until the second trimester; it may be that patients did not want to engage in research until they were sure that the pregnancy would not result in miscarriage. However, we hope that this is the foundation for microbiome research that is integrated into clinical care that starts during the first trimester, making earlier collection of samples more possible. There were limitations to combining the data from the 2 cohorts due to the differences in sample size. For example, our findings that the grouping of subtypes of symptoms from the EPDS in the U.S. cohort differed during the third trimester from the Swedish subtypes at both points and the U.S. subtypes during the second trimester may be a result of the smaller U.S. sample size. However, other studies also suggest that tools for assessing mental distress may be interpreted differently depending on the population in which the tool is being used, and this must be considered in studies with 2 populations (29). Another limitation is that both cohorts predominantly included individuals with higher education levels because participants were recruited from university-centric suburban cities, making generalizability to populations with less access to education and settings such as densely populated urban areas or sparsely populated rural areas difficult. The Swedish and U.S. cohorts are 2 of the largest cohorts that have been used to study mental health in pregnancy and the microbiome. However, our findings indicate that because of significant variation in trajectories of symptoms, even larger cohorts will be needed to group individuals by symptom type and trajectories of mental distress. Furthermore, larger sample sizes, aided by additional time points per individual and with computational modeling, will enable consideration of more factors that may be contributory and how they change over time. For example, Long *et. al.* (28) found during the third trimester that higher diversity was associated with fewer bowel symptoms. Another limitation is that the EPDS was developed as a screening tool and cannot substitute a clinical diagnostic interview for a major depression or anxiety disorder diagnosis, although there is some evidence that women with a score of 12 or above are more often found to have a psychiatric diagnosis of depression when followed up with a clinical interview (32,57). We did preliminary analyses of different ways to characterize psychiatric history, but methods of obtaining history differed in the 2

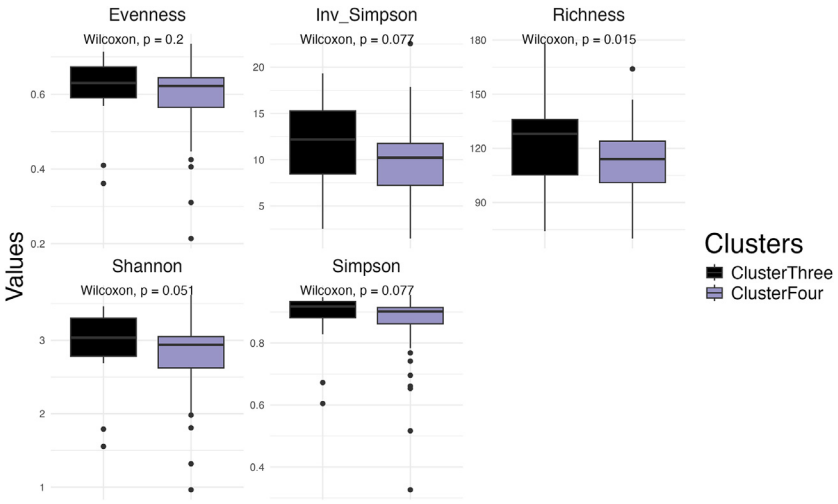

Figure 6. Samples in ClusterFour have significantly lower species richness than samples in ClusterThree. Boxplots displaying the alpha indices of samples in ClusterThree and ClusterFour. Five alpha indices (evenness, inverse Simpson, richness, Shannon, and Simpson) of samples in ClusterThree and ClusterFour were compared using the Wilcoxon test. Boxes on each panel were colored according to clusters. Species richness was found to be significantly higher in ClusterThree than in ClusterFour (p = .015).

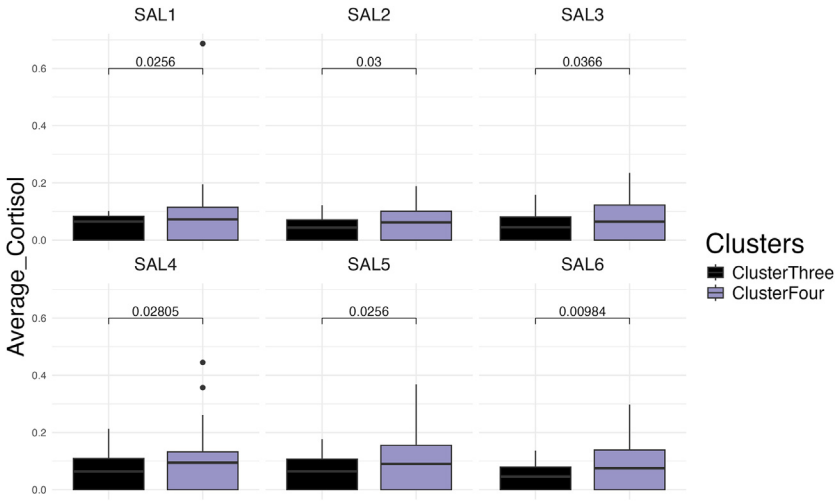

Figure 7. Higher saliva cortisol level was noted in individuals from ClusterFour than in individuals from ClusterThree. Boxplots displaying the saliva cortisol level from ClusterThree and ClusterFour saliva samples of individuals who took part in the Trier Social Stress Test, collected at 6 different sampling points. Cortisol levels of individuals from ClusterThree and ClusterFour were compared at each of the 6 sampling points using a *t* test. The Benjamini-Hochberg procedure was conducted for post hoc *p* adjustment. Boxes on each panel were colored according to clusters. Cortisol levels at all 6 sampling points were found to be significantly higher in ClusterFour than in ClusterThree after Benjamini-Hochberg adjustment.

cohorts. This study utilized whole-genome sequencing that allowed for deeper resolution in identifying taxa and greater ability to assess functional potential.

Practical Implications and Conclusions

A biomarker has been defined as "an indicator of normal biological processes, pathogenic processes or responses to an exposure or interventions" (58). While many of the characterizations of the microbiome suggest some differences between individuals with higher levels of mental distress and individuals with lower levels of mental distress during pregnancy, the heterogeneity seen in the low distress group may be a limitation to their use as biomarkers. This work indicates that microbial functioning from whole-genome sequencing has the potential to cluster individuals into groups and identify functions that contribute to each group; these functions may have the potential to serve as biomarkers for how different individuals navigate pregnancy, and the role components of the gut-brain axis may play for different groups. For example, this work indicates functions of the gut-brain axis related to the hypothalamic-pituitary-adrenal axis may be important in distinguishing a group of individuals with perinatal depression. It may be possible to cluster pregnant individuals by potential functioning of the microbial community predicted by microbial genetics, and these functions may be targets for more precise interventions to improve outcomes for both parents and children.

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