INTRODUCTION

Pregnancy is very often stressful, and some women experience stress and symptoms of depression and anxiety, including pregnancy-related anxiety (Glover, 2015). It has been reported that 15%–28% of women show signs of anxiety or depression during pregnancy (Gaynes et al., 2005; Melville, Gavin, Guo, Fan, & Katon, 2010; Verreault et al., 2014). Furthermore, considerable evidence shows that if the mother is stressed, anxious, or depressed during pregnancy, her child is more likely to have a suboptimal developmental trajectory with a range of physical and mental health problems (Entringer, Buss, & Wadhwa, 2011; Field, 2011; van den Bergh, Mulder, Mennes, & Glover, 2005). However, the underlying mechanisms that explain the link are still unclear. A review of recent literature shows that maternal psychosocial stress during pregnancy is a complex phenomenon that affects mothers’ emotions, behaviors, and physiology in many different ways that may influence the neurodevelopment of the fetus through a network of pathways (Glover, 2015).
2 | METHODS

2.1 | Subjects and samples

The study was approved by the Institutional Review Boards at the Icahn School of Medicine at Mount Sinai, New York Presbyterian Hospital, and Queens College, CUNY. The sample set came from 75 newborns whose mothers participated in a longitudinal birth cohort recruited at the prenatal obstetrics and gynecology clinics at Mount Sinai Hospital and New York Presbyterian/Queens Hospital. A detailed description of the population is published in Finik and Nomura (2017). Both sites serve predominantly low-income ethnic minority populations residing in New York City. Exclusion criteria for participation included HIV infection, maternal psychosis, maternal age < 15 years, life-threatening medical complications of the mother, and congenital or chromosomal abnormalities of the fetus. All subjects were consented per protocol approved by the local IRBs. Participating mothers were followed from their second trimester to delivery. The first neonate meconium passed within 48 hr after birth and was transferred by the research staff to sterile Falcon tubes using a sterile tongue depressor and stored at −80°C until processing. A detailed description can be found elsewhere (Hu et al., 2013).

2.2 | Demographic and clinical data collection

2.2.1 | Demographics

Participating mothers reported age, education, parity, and marital status at the time of enrollment to the study.

2.2.2 | Obstetric history and pregnancy outcomes

Gestational age, birth weight, ponderal index (PI) calculated as a relationship between weight and height, and past and current birth complications including obstetric (e.g., forceps delivery or premature rupture of membrane) and/or neonatal problems (e.g., jaundice, admission to the neonatal intensive care unit, and shoulder dystocia) were obtained from the mother’s electronic medical records. The cumulative sum of obstetric and neonatal problems was used as the birth complication index.
2.2.3 | Maternal stress during the second trimester

Using a battery of well-validated questionnaires, the mother’s level of stress was measured by (a) symptoms of depression, (b) symptoms of general anxiety, (c) pregnancy-related anxiety, (d) perceived pre-natal stress, and (e) self-reported stressful life events experienced during the second trimester. A dimensional measure for each scale for stress was used to evaluate the relative abundance and a dichotomous (median split) measure was used to evaluate the overall microbiota dissimilarities (beta diversity) analysis.

Symptoms of depression

Maternal depression symptomatology was measured during the second trimester by the Edinburgh postnatal depression scale (EPDS) (Murray & Carothers, 1990), a well-utilized self-report inventory that measures depression symptomatology. The participants were asked to report how they had felt in the past 7 days on a 4-point Likert scale. Response options include "yes, all the time," "yes most of the time," "no not very often," and "no, not at all." Some questions were reverse coded, and the sum score constituted the "maternal depression" scale. The sum was split at the median score of 7, which constituted the dichotomous low and high depression score. The inventory is well validated in different languages and has acceptable reliability ranging from 0.79 to 0.86 (Kheirabadi, Maracy, Akbaripour, & Masaeli, 2012; Mazarhi & Nakhaee, 2007; Montazeri, Torkan, & Omidvari, 2007; Small, Lumley, & Toomey, 2006), and satisfactory sensitivity (79%) and specificity (85%) (Kheirabadi et al., 2012).

Symptoms of general anxiety

The state-trait anxiety inventory (STAI) (Spielberger, 1989) measures the mother’s temporary condition of “state anxiety” and long-standing condition of “trait anxiety.” Each type (trait and state) is assessed by 20 statements that may or may not describe the participant, who responded to the statements on a 4-point Likert scale ranging from 1 “not at all” to 4 “very much so.” The sum was split at the median score of 35 for state and 37 for trait anxiety, which constituted the dichotomous low and high state and trait anxiety scores. A meta-analysis of 45 articles reporting Cronbach’s alpha for internal consistency reliability for this inventory determined the mean to be 0.92 (Barnes, Harp, & Jung, 2002).

Pregnancy-related anxiety

The pregnancy-related anxiety questionnaire-revised (PRAQ-R) (Huijzing, Mulder, Robles de Medina, Visser, & Buitelaar, 2004) measures specific fears and worries related to pregnancy. It comprised of three subscales of perinatal anxiety related specifically to pregnancy, including fear of giving birth, fear of bearing a mentally or physically handicapped child, and worries about changes in appearance due to pregnancy. The participants were asked to describe their feelings and thoughts using a 5-point Likert scale (1 = absolutely not relevant; 2 = hardly ever relevant; 3 = sometimes relevant; 4 = reasonably relevant; and 5 = very relevant). The mean item scores for each of the three dimensions were first computed, with a minimum of 1 and maximum of 5. The sum of the three dimensional scores comprised of Pregnancy-Related Anxiety Questionnaire-total (PRAQ-total) score. PRAQ-total, with a theoretical range of 15, was split at the median score of 6, which constituted the dichotomous low and high pregnancy-related anxiety scores. A score of 6 or greater was defined as high pregnancy-related anxiety and a score of <6 was defined as low. PRAQ-total is generally independent of other general anxiety measures such as STAI (Huizink, Robles de Medina, Mulder, Visser, & Buitelaar, 2003).

Perceived prenatal stress

Perceived stress scale (PSS-14) (Cohen, Kamarck, & Mermelstein, 1983) was used to ask about the mother’s feelings and thoughts within the last months as an indicator of perceived stress during pregnancy. The sum was split at the median score of 37, which constituted the dichotomous low and high perceived stress scores.

Stressful life events

The psychiatric epidemiology research interview life events scale (PERI) (Dohrenwend, Yager, Egri, & Mendelsohn, 1978) assesses the occurrence of stressful events in five major areas of life: relationships, health, legal matters, work and financials, and friendships. Mothers reported their experiences during the second trimester of specific stressful life events in those five areas of life, and reported the valence associated with each. This measure is widely used, has been shown to have good validity with narrative reports of life events, and has low intracategory variability (see review Dohrenwend, 2006). We used the total number of negative life events reported by the mothers as our measure of stressful life events. The score was also split at the median score of 3, which constituted the dichotomous low and high stressful life events scores.

2.3 | Sample processing

On average, meconium samples were collected after 50 min (ranging from 0 to 5.5 hr, SD = 11) of passing. The meconium was transferred from the diaper to sterile 15-ml Falcon tubes using a sterile tongue depressor by the research staff and stored at ~80°C until processing. During the sample collection and processing, extreme precaution was taken to avoid possible environmental contaminations. Bacterial DNA was extracted using Qiagen DNA stool mini kit (Qiagen, CA). Total DNA concentration was determined with Qubit 2.0 Fluorometer (Life Technologies, Norwalk, CT). The phylogenetically informative V3-V4 region of 16S ribosomal RNA (rRNA) gene was amplified using the universal primer set 347F/803R. The primers were synthesized by IDT (Integrated DNA Technology, Coralville, IA). A pair of 8-mer error-correcting Golay barcodes was added to both the reverse and forward PCR primers.

2.4 | 16S ribosomal RNA (rRNA) sequencing and quality control

We used dual barcoding to label the 16S rRNA amplicons from each sample as described previously (Hu et al., 2013). The bacterial 16S
rRNA gene in the sequence analysis is regarded as a preferred culture-independent approach to identify bacterial taxa. The 16S rRNA amplicons were further pooled with equal molarity and submitted for MiSeq 2 x 300 pair-end sequencing at high depth. The paired sequence reads were merged and filtered by size (>400 bp) and quality score (>Q30) using PANDAseq. The processed reads were further split by dual barcodes for each sample and assigned taxonomic classification using QIIME pipeline 1.9.0 (Caporaso et al., 2010). Three duplicate samples were included to assess sequencing reproducibility. Finally, a total of 4,105,299 (mean 54,737; SD 38,942; min 9,134; and max 197,210) sequences remained and each sample was normalized to 3,000 sequences for further analysis. After processing, QIIME provided detailed operational taxa unit (OTU) tables containing the microbiota composition and relative abundance for each individual sample.

2.5 | Data analysis

Firstly, using QIIME pipeline, we summarized the microbiota composition and measured the average relative abundances and the standard deviations for each bacteria taxon from phylum to genus level across all meconium samples. Secondly, we accessed the diversity of the overall microbiota communities within or across each sample. The alpha-diversity, or bacterial richness within each sample, was measured using the Shannon index. The beta-diversity, representing taxonomic relative abundance across samples, was measured using Bray–Curtis distance matrices computed from genus level OTU tables (Werner et al., 2012). The univariate and multivariate PerMANOVA tests with permutation 999 were performed to analyze the variance using the Bray–Curtis distance as the dependent variable and the clinical/psychological categorical or continuous variables as independent variables. A Spearman correlation between bacterial taxa and the maternal stress score was measured at phylum, family, and genus level. Strongly correlated taxa were further examined while adjusting for potential confounders in a regression model, which included maternal ethnicity/race, age, education, marital status, time of sampling (time between birth and sample collection), the mode of delivery (Cesarean, or C-section vs. vaginal), and antibiotic use during pre- and perinatal periods.

3 | RESULTS

3.1 | Demographic characteristics

Descriptive statistics for the demographic characteristics are presented in Table 1. The mean (±SD) age of the mothers was 28 (±6) years. Approximately 52% of the mothers were Hispanic/Latino, 27% Black, 5% White, 4% Asian, and 12% identified their race/ethnicity as other. A little over 29% had not completed high school. The majority (64%) reported being single. There was a general balance of the gender of their offspring with 53% male and 47% female. The mean (±SD) gestational age at birth and birthweight were 38.8 (±3.2)
weeks and 3.1 (±0.6) kg, respectively. Approximately a quarter of the participants delivered via C-section and one-eighth of the babies were admitted to neonatal intensive care unit. Note that the C-section mode of delivery was 98.6% (p = 7.4e-5) overlapped with antibiotic usage prior to birth in our studied population.

3.2 | Survey of meconium microbiota

From pooled barcoded PCR amplicons from 75 meconium samples, we obtained 4.5 million high-quality reads. After splitting by barcodes, we obtained an average of 60,362 reads (min = 10,499, max = 208,918) per sample.

Figure 1 shows the relative abundance of bacterial taxa at the phylum, family, and genus levels in the meconium. We found that the dominant phylum included Actinobacteria (mean = 17%), Bacteroidetes (mean = 9.3%), Firmicutes (mean = 20%), and Proteobacteria (mean = 71%). At the family and genus level, we found that most of the assigned taxa were from Firmicutes and Proteobacteria. The most abundant taxa were Enterobacteriaceae family with a nonidentified genus in Enterobacteriaceae family (mean = 46%). After further blasting the 16S reads of this Enterobacteriaceae genus, we found that its nearest neighbors included three strains from Escherichia fergusonii and one strain from each Shigella sonnei, Brenneria alni, Escherichia coli, Shigella flexneri, or Escherichia vulneris.

3.3 | The meconium microbiome composition by maternal pregnancy-related anxiety (praq-total)

We observed significant differences in the meconium microbiota beta-diversity by high versus low PRAQ-total scores (Figure 2a). The univariate PerMANOVA test using the beta-diversity distance matrix showed that the overall meconium microbiota at genus level were significantly associated with maternal PRAQ-total as a continuous variable (p = 0.001), delivery mode (p = 0.033), and two subscales of maternal PRAQ (i.e., fear of appearance changes and fear of childbirth; p = 0.003 and p = 0.005, respectively; Table 2). However, when adjusted in multivariate analysis, only PRAQ-total and the delivery mode remained significant. In addition, a trend for association between the increased microbial alpha-diversity and the increased PRAQ-total was detected (p = 0.07; Figure S1).
3.4 | Association between praq-total and the relative abundance of particular taxa in the meconium

We further examined the association between PRAQ-total and the individual taxa from phylum to genus level (Figure 2b, Table 3). We found that the Proteobacteria phyla, its Enterobacteriaceae family, and an unidentified genus from Enterobacteriacea family showed inverse correlations with PRAQ-total after adjustment for potential confounders (Spearman's $r = -0.40, -0.43, \text{ and } -0.54; p = 0.002, 2e-4, \text{ and } 1.5e-4, \text{ respectively})$, suggesting that PRAQ-total was associated with lower relative abundance in those taxa from phylum to genus level.

4 | DISCUSSION

In this study, we demonstrated that the low biomass first neonatal stool discharge, meconium, possessed diversified microbiota and that higher pregnancy-related anxiety was associated with the higher microbiota diversity in the offspring. Moreover, certain taxa from the Enterococcaceae family were inversely correlated with PRAQ-total. These findings contribute to the ongoing discussion on the potential role of maternal exposures during pregnancy in the initial bacterial colonization in the gut. Early-life microbiome has been shown to significantly impact the priming of the immune system and determine immediate and long-lasting health outcomes (Romano-Keeler & Welkamp, 2015) with altered early-life microbiota reportedly linked to the risk of developing asthma, eczema (Hong, Lee, & Aw, 2010), allergy (Johansson, Sjögren & Sverremark-Ekstrom, 2011), autism (Wang, Christophersen, Sorich, Gerber, Angley, & Conton, 2011), and other immune-mediated diseases (Sjögren, Tomicic, Lundberg, Bottcher, Bjoksten, Svermark-Skstorm, & Jenmalm, 2009).

We confirm prior findings that the meconium is already colonized with Enterococcaceae, Bacteroidetes, Firmicutes, and Proteobacteria. The relative abundances of these dominant phyla resembled that reported in our earlier study (Hu et al., 2013) and showed a significantly higher relative abundance of Proteobacteria and lower relative abundance of Bacteroidetes and Firmicutes compared to adult stool. While some studies have shown the presence of bacteria in the newborn's microbiome, suggesting a possible transfer of microbiota between mothers and children (Chu et al., 2017; Hu et al., 2013; Valles, Gosalbes, de Vries, Abelián, & Francino, 2012), to date, the possibility that colonization of microbiome happens in utero remains controversial (Perez-Muñoz et al., 2017). A few recent studies that support the "sterile womb" hypothesis (Lauder et al., 2016; Lim, Rodrigue, & Holtz, 2018) argue that the positive bacterial sequencing results in pregnancy-related samples, including the meconium, may be partially or fully due to environmental contamination, pointing to the establishment of germ-free animals as a proof of the sterility of the fetal environment in mammals (Perez-Muñoz et al., 2017). On the other hand, accumulating evidence has shown the presence of viable bacterial cells in the fetal environment in the amniotic fluid (Bearfield, Davenport, Sivapathasundaram, & Allaker, 2002; Jiménez et al., 2008; Rautava et al., 2012), placental tissues (Aagaard et al., 2014), umbilical cord blood (Jiménez et al., 2005), and fetal membranes (Rautava et al., 2012; Steel et al., 2005), without any indication of infection and inflammation. Even though the exact timing of establishment and the initial source of the microbes in the infant microbiome remain unknown, our findings support the notion that the initial colonization of the gut may start in utero (Rodríguez et al., 2015).

Our study also revealed that pregnancy-related anxiety, but not a general state and trait anxiety, was the significant predictor of the overall meconium microbiota composition, suggesting that the specific types of stress that mothers experience concerning their pregnancy may have an impact on the initial bacterial colonization in the offspring. At taxa level, the higher maternal PRAQ-total was associated with a lower relative abundance of Proteobacteria phylum in the meconium. Several studies have demonstrated an increased relative
abundance of bacterial members belonging to *Proteobacteria* phylum in diseases sustained by various degree of inflammation, including metabolic disorders and inflammatory bowel disease (reviewed in Rizzatti, Lopetuso, Gibiino, Binda, & Gasbarrini, 2017). Interestingly, prior research (Zijlmans et al., 2015) indicated that the cumulative stress mothers experienced during pregnancy was positively associated with the level of *Proteobacteria* groups in infants' stool samples during the first 4 months of life. However, the significant correlation between a lower relative abundance of pro-inflammatory *Proteobacteria* in the meconium and higher pregnancy-related anxiety found in our study contradicts that of Zijlmans et al. (2015). The discrepancy in the direction of the association may be due to sampling differences (baby stools vs. meconium), microbiome surveying technique (microarray vs. 16S rRNA sequencing), or different measures of maternal stress during pregnancy. It may also be due to the fact that the total bacterial loading in the meconium is very low and the relative abundance of the *Proteobacteria* is high, with unknown microbiota viability. Future studies using culturing techniques are warranted to profile the live versus dead bacteria.

Other studies have also provided evidence that pregnancy-related anxiety, but not general anxiety, is associated with altered molecular programming in newborn relevant to long-term health as suggested by methylation of the promoter region of the stress-related glucocorticoid receptor gene (*NR3C1*) in cord blood (Hompes, et al., 2013) and shorter telomere length (Entringer, de Punder, Buss, & Wadhwa, 2017; Shalev, et al., 2013). Our findings add to this emerging evidence that maternal pregnancy-related anxiety may influence the health of the offspring through the effects on the initial colonization of gut microbiome.

Our data also showed the beta diversity of meconium microbiome is significantly associated with delivery mode. It is contradictory to the previous reports (Dong, et al., 2018; Hu, et al., 2013) that the mode of delivery is unlikely a major contributor to shape this initial bacterial community as the meconium microbiota is mostly derived from the in utero environment. One possible reason for our result is the almost 100% overlapping between the delivery mode via C-section and antibiotic usage, in which the latter may affect the meconium microbiome composition significantly.

The strengths of this study include the longitudinal design that made it possible to collect information about the stress mothers experienced during pregnancy prospectively before the baby was born. Also, we profiled the bacterial 16S rRNA gene in the meconium, the first newborn fecal discharge, which is believed to be formed in utero. However, the study also has limitations. First, although maternal ethnicity/race, age, education, marital status, time of sampling, and mode of delivery were included as potential confounders in the model, our sample size restricted the ability to consider a broader range of covariates. Second, this study lacks information on dietary intakes, while recently several studies show the important role of diet in shaping gut microbiome (Gohir, et al., 2015; Lundgren, et al., 2018; Makki, Deehan, Walter, & Bäckhed, 2018). Third, this study lacks biological markers of HPA-axis functioning such as cortisol levels, which are known to correlate with stressful life events and psychopathology (Rubinow, Post, Savard, & Gold, 1984). Having a biological measure of maternal stress during pregnancy might help to better understand the underlying biological mechanisms of the observed associations. Fourth, evidence that tracks the newborn meconium microbiome to the maternal source (gut, oral, placenta, or vaginal) is lacking as we

<table>
<thead>
<tr>
<th>Variables</th>
<th>PerMANOVA test*</th>
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<tbody>
<tr>
<td></td>
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<td>Maternal variables</td>
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<td>Trait anxiety</td>
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<td>Fear of having handicapped child</td>
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<td>Fear of appearance change</td>
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<td>Fear of birth/delivery</td>
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*PerMANOVA test was performed at genus level. Bold values represent p-values.
do not have access to the matched maternal microbiome samples, preventing us from determining the maternal origin of the initial microbiome, if any. Future studies comparing the bacterial composition of maternal and neonatal microbiome are warranted to determine the major source of the newborn microbiota. Although the 16S sequencing-based taxonomy analysis is cost efficient and allows us to compare the overall microbiome composition, it only profiles bacterial taxa to the genus level so that no conclusion can be made as to which particular strains are correlated with pregnancy-related anxiety. Readers should also be reminded that the microbiome analysis using 16S sequencing data in this study should be interpreted with caution as it only assessed the relative abundance of each taxa, not the absolute bacterial counts, which require additional qualification by real-time PCR (Nagpal et al., 2016) or flow cytometry (Vandeputte et al., 2017). Moreover, the time of passing the meconium ranged from 0 to 5.5 hr post-delivery, raising the possibility of some bacteria being introduced from environmental influences, such as delivery mode, breast milk or formula feeding, etc. However, we adjusted for the time until meconium passing in regression models and found that it was not statistically significantly related to microbiome diversity and relative abundance.

In summary, while we acknowledge the limitations above, this study adds to the growing body of literature supporting a link between maternal pregnancy-related stress and the microbiome in the offspring. Understanding the dynamics of the gut-brain axis during early life may help develop novel targets to promote a healthy microbiome and optimal neurobehavioral development.

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**REFERENCES**


**TABLE 3**  Bacteria associated with the pregnancy-related anxiety accessed by PRAQ-total score

<table>
<thead>
<tr>
<th>Taxa⁵</th>
<th>Correlations⁶</th>
<th>p-value 1c</th>
<th>p-value 2d</th>
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<td>Family level</td>
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<td>Hemophilus</td>
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⁵Taxa with less than 5% relative abundance was removed from the analysis. ⁶Spearman's correlation rho by R command cor(). ⁷p-value 1 was obtained from R command cor.test() to test for association between paired samples, using Spearman’s rho. ⁸p-value 2 was obtained from linear regression model using relative abundance of selected taxa as dependent variable and PRAQ-total score and mode of delivery as independent variables. Bold values represent p-values.


SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.