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ABSTRACT

Background: Adverse childhood experiences (ACEs), such as abuse or chronic stress, program an exaggerated adult inflammatory response to stress. Emerging rodent research suggests that the gut microbiome may be a key mediator in the association between early life stress and dysregulated glucocorticoid-immune response. However, ACE impact on inflammatory response to stress, or on the gut microbiome, have not been studied in human pregnancy, when inflammation increases risk of poor outcomes. The aim of this study was to assess the relationships among ACE, the gut microbiome, and cytokine response to stress in pregnant women.

Methods: Physically and psychiatrically healthy adult pregnant women completed the Adverse Childhood Experiences Questionnaire (ACE-Q) and gave a single stool sample between 20 and 26 weeks gestation. Stool DNA was isolated and 16S sequencing was performed. Three 24-hour food recalls were administered to assess dietary nutrient intake. A subset of women completed the Trier Social Stress Test (TSST) at 22-34 weeks gestation; plasma interleukin-6 (IL-6), interleukin-1β (IL-1β), high sensitivity C-reactive protein (hsCRP), tumor necrosis factor α (TNF-α), and cortisol were measured at four timepoints pre and post stressor, and area under the curve (AUC) was calculated.

Results: Forty-eight women completed the ACE-Q and provided stool; 19 women completed the TSST. Women reporting 2 or more ACEs (high ACE) had greater differential abundance of gut *Prevotella* than low ACE participants (q=5.7x10^-13). Abundance of several gut taxa were significantly associated with cortisol, IL-6, TNF-α and CRP AUCs regardless of ACE status. IL-6 response to stress was buffered among high ACE women with high intake of docosahexaenoic acid (DHA) (p=0.03) and eicosapentaenoic acid (EPA) (p=0.05).

Discussion: Our findings suggest that multiple childhood adversities are associated with changes in gut microbiota composition during pregnancy, and such changes may contribute to altered inflammatory and glucocorticoid response to stress. While preliminary, this is the first study to demonstrate an association between gut microbiota and acute glucocorticoid-immune response to stress in a clinical sample. Finally, exploratory analyses suggested that high ACE women with high dietary intake of ω-3 polyunsaturated fatty acids (PUFAs) had a dampened inflammatory response to acute stress, suggesting potentially protective effects of ω-3s in this high-risk population. Given the adverse effects of inflammation on pregnancy and the developing fetus, mechanisms by which childhood adversity influence the gut-brain axis and potential protective factors such as diet should be further explored.

1. INTRODUCTION

History of adverse childhood experiences (ACEs), such as neglect, abuse, or chronic household dysfunction, are associated with impaired immune function (Carpenter et al., 2010; Danese et al., 2007; Fagundes et al., 2013; Gouin et al., 2012b; Kiecolt-Glaser et al., 2011) and dysregulated hypothalamic pituitary adrenal (HPA) axis function in adulthood (Ehrlich et al., 2016; Lovallo et al., 2012). While recent research from our laboratory indicates dysregulated HPA response among high ACE women postpartum (Morrison et al., 2017), ACE impact on inflammatory response to stress has not been studied during pregnancy. Maternal ACE history is associated with lower offspring gestational age and weight at delivery (Smith et al., 2016), and recent work suggests maternal cortisol (Gillespie et al., 2017) or elevated baseline interleukin (IL)-6 (Miller et al., 2017) may mediate this. Intriguingly, emerging rodent research indicates that early life stress also alters the gut microbiome (Jašarević et al., 2017, 2015), and the gut microbiome is known to influence inflammation and HPA axis function (Sudo, 2014). This raises the possibility that the relationship between ACE and dysregulated neuroendocrine-immune function may be mediated by the gut microbiota. However, ACE impact on gut microbiota has not been studied in humans, nor have associations between gut microbiota and glucocorticoid-immune function. The aim of this study was to examine the impact of ACE on proinflammatory cytokine and HPA response to stress during pregnancy, and to characterize potential mediators, namely, gut microbiota and diet.

1.1 Adverse Childhood Experiences and Inflammation

ACE history has been associated with elevated baseline C-reactive protein (CRP) (Danese et al., 2007; Taylor et al., 2006), IL-6 (Carpenter et al., 2010; Slopen et al., 2010), nuclear factor κB

(NF-κB) (Pace et al., 2012), and tumor necrosis factor (TNF)-α (Kiecolt-Glaser et al., 2011) in non-pregnant adults. Those who have experienced ACEs show an exaggerated inflammatory response to acute laboratory stress (Trier Social Stress Test; TSST) (Carpenter et al., 2010), larger IL-6 response to daily stressors (Gouin et al., 2012a), and larger ex vivo cytokine responses to microbial challenge or lipopolysaccharide (LPS) stimulation (Miller et al., 2011) compared with those without a history of childhood adversity. Individuals with exposure to childhood adversity also evidence dysregulated HPA axis function (Bunea et al., 2017), including blunted cortisol response to the TSST or Montreal Imaging Stress Task (MIST) (Carpenter et al., 2007; Lovallo, 2013; Suzuki et al., 2014; Voellmin et al., 2015), blunted cortisol awakening response (CAR) (Kumsta et al., 2017), greater heterogeneity in diurnal cortisol patterns (Gonzalez et al., 2009), low hair cortisol (Kalmakis et al., 2015), and blunted cortisol response to a separation stressor at six months postpartum (Morrison et al., 2017). During pregnancy specifically, high ACE women show lower baseline cortisol (Shea et al., 2007), greater hair cortisol levels (Schreier et al., 2015), increased cortisol response to daily stress (Bublitz and Stroud, 2012), elevated CAR in the context of poor current perceived family function (Bublitz et al., 2014), and greater hair cortisol associated with depressive or somatic symptoms (Bowers et al., 2018). Together, this suggests early life patterning of the central nervous system (CNS) and its bidirectional interactions with the neuroendocrine system and immune system. Poorly controlled inflammation during pregnancy may elevate risk for low birth weight (Atta et al., 2016; Marzi et al., 1996), low gestational age at delivery (Sorokin et al., 2010), or spontaneous preterm birth (Bastek et al., 2011; Gillespie et al., 2016; Pearce et al., 2010), making the impact of ACE on HPA and cytokine response to acute stress during pregnancy an important question.

1.2 The Gut Microbiome, Stress and Inflammation

The microbiota (bacteria, fungi, archaea, viruses) and their genetic material comprise the gut microbiome. Evidence suggests the gut microbiota influence inflammation and HPA axis function (Rea et al., 2016; Sudo, 2014). For instance, low diversity of gut microbial communities was associated with elevated inflammation in rodents (Bailey et al., 2011) and in humans (Röytiö et al., 2017). Relative abundance of specific gut bacteria including *Akkermansia*, *Flexibacter* and *Prevotella* have been associated with inflammation in rodents (Ganesh et al., 2013; Pusceddu et al., 2015), and *Megasphaera* and Proteobacteria were associated with elevated inflammatory markers in humans (Mukhopadhya et al., 2012; Schirmer et al., 2016). Conversely, *Dialister* and *Faecalibacterium* are associated with lower inflammation (Martínez et al., 2013; Sokol et al., 2008).

The gut microbiota are stress sensitive. Early life stress (ELS), the functional equivalent of ACE in rodent models, clearly alters the microbiome, persisting through adulthood (Jašarević et al., 2017, 2015). Male rats who were stressed neonatally had altered gut microbiota as adults, particularly elevated levels of *Enterobacteria* and *Bacteroides*, compared with pups who were not stressed (García-Ródenas et al., 2006). In addition to altered gut microbiota, neonatally stressed male rats exhibited elevated plasma corticosterone and elevated inflammatory response to LPS challenge as adults, compared with male rats who did not undergo the separation stressor (O'Mahony et al., 2009). Similarly in female rats, maternal separation stress at postnatal days two to twelve reduced the ratio of *Firmicutes* to *Bacteroidetes* in the adult gut, and increased taxa previously associated with inflammation, including *Akkermansia*, *Flexibacter* and

Prevotella (Pusceddu et al., 2015). Intriguingly, supplementation with the omega-3 (ω-3) PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in adulthood corrected the *Firmicutes:Bacteroidetes* ratio to that of non-ELS rats, increased levels of *Actinobacteria* and the butyrate-producing Butyrivbrio, and reduced abundance of *Proteobacteria* (Pusceddu et al., 2015). The authors proposed that EPA/DHA may thus exert its anti-inflammatory effects by modulating gut microbiota composition.

Stress also disrupts the gut microbiome in humans, although impact of ACE specifically is unknown (Kato-Kataoka et al., 2016; Knowles et al., 2008). In adults exposed to trauma, those who developed PTSD had lower total gut abundance of the phyla *Actinobacteria*, *Lentisphaerae*, and *Verrucomicrobia*, compared with those who did not develop PTSD (Hemmings et al., 2017). In this sample, a sub-analysis revealed that history of childhood trauma was associated with lower relative abundance of *Actinobacteria* and *Verrucomicrobia* (Hemmings et al., 2017). However, childhood trauma trended toward a significant association with PTSD diagnosis, making it difficult to determine the effect of childhood trauma independent of current psychiatric functioning. During pregnancy, maintenance of diversity in gut bacterial communities was associated with less systemic inflammation (Röytiö et al., 2017). Whether ACE is associated with altered gut communities during pregnancy is unknown, but a critical question, as maternal gut microbiota not only have a potential influence on inflammation (Schirmer et al., 2016), but produce metabolites necessary for the developing fetus (Gomez de Agüero et al., 2016), thus influencing fetal development in multiple ways.

1.3 Dietary Impact on Gut Microbiome and Inflammation

Diets high in saturated fats, trans fats, or omega-6 (ω-6) polyunsaturated fatty acids (PUFAs), and low in omega-3 (ω-3) PUFAs, are associated with elevated serum inflammatory markers such as IL-6, CRP, and TNF-α (Alfano et al., 2012; Kiecolt-Glaser et al., 2012, 2007; Lopez-Garcia et al., 2004). In addition to directly stimulating IL-6 and TNF-α production (Ajuwon and Spurlock, 2005; Suganami et al., 2005), dietary intake of saturated fat and ω-6 fatty acids impacts gut microbial community composition (Fava et al., 2013; Myles, 2014; Wu et al., 2011). Evidence suggests that altering the gut microbiome via diet may alter peripheral inflammatory markers (Macfarlane et al., 2013; Pusceddu et al., 2015), indicating that dietary intake of nutrients such as ω-3 PUFAs may modulate relationships between gut microbiota and inflammation.

1.4 Aims and Hypotheses

The aim of this study was to examine the impact of ACE on inflammation during pregnancy, including careful assessment of potential mediators of this relationship, namely, gut microbiota and diet. We hypothesized that 1) women with multiple ACE exposures (high ACE) would have an exaggerated plasma proinflammatory cytokine response and dampened plasma cortisol response to acute stress; 2) high ACE women would have different relative abundance of gut taxa compared with low ACE women; 3) abundance of particular gut taxa would correlate with proinflammatory cytokine response to acute stress; 4) high dietary intake of ω-3 PUFAs would dampen inflammatory cytokine response to acute stress.

2. METHODS

2.1 Study Design and Participants

Pregnant women ages 18-45 years old were recruited at 20 to 26 weeks gestation. Women were recruited from among participants in an ongoing cohort study of pregnant women (National Institute of Nursing Research RO1NR014784-01), a larger study examining pregnancy outcomes. Recruitment was targeted to balance high versus low ACE by administering the ACE scale at the screening visit, and the distribution was evaluated weekly. Participants were required to be fluent in written and spoken English and able to provide written informed consent. Exclusion criteria were current tobacco use, chronic steroid or immunosuppressive use, history of cancer or diabetes, chronic kidney disease, HIV, hypertension, preeclampsia, history of anemia, history of bariatric surgery, vegan diet, use of recreational drugs or psychiatric medications in the past 6 months, multiple gestation pregnancy or known fetal abnormalities. Participants provided written informed consent and underwent screening to ensure adherence to the inclusion and exclusion criteria.

2.2 Measures

2.2.1 Demographics and health. At the screening visit (20 to 26 weeks gestation), participants provided demographic and health data including age, education level, income, marital status, race, names and duration of current and past medications, vitamin and supplement use, significant illnesses/surgeries, age at menarche, menstrual cycle and obstetric history, caffeine and nicotine use. Weight and height were measured.

2.2.2 Childhood adversity. The Adverse Childhood Experiences Questionnaire (ACE-Q) was administered at screening to assess exposure to childhood adversity. The ACE-Q is a well validated scale that has been used in numerous studies to assess exposure to adversity before the age of 18 and associations with later health outcomes (Felitti et al., 1998). The 10-item questionnaire assesses history of emotional, physical or sexual abuse, childhood neglect, and household dysfunction. Scores range from 0-10 and reflect the occurrence(s) of event(s), not their frequency or severity. An ACE score >=2 is associated with increased risk of preterm birth (Christiaens et al., 2015) and altered HPA axis function during pregnancy (Bowers et al., 2018) and postpartum (Morrison et al., 2017). Thus, participants scoring 2 or greater were considered "high ACE" while subjects with ACE score of <2 were considered "low ACE."

2.2.3 Psychological assessments. At the screening visit, participants completed psychological assessments. A Structured Clinical Interview for DSM (SCID) (First et al., 2002) was administered to confirm healthy psychiatric status. Participants completed additional rating scales including Traumatic Life Events Questionnaire (TLEQ) (Kubany et al., 2000), Spielberger State Trait Anxiety Inventory (STAI; State -S, Trait -T) (Spielberger et al., 1983), Pittsburgh Sleep Quality Index (PSQI) (Buysse et al., 1989), Edinburgh Postnatal Depression Scale (EPDS) (Cox et al., 1987) and the Perceived Stress Scale (PSS) (Cohen et al., 1983).

2.3 Stool sampling and sequencing. Stool was collected at 20-26 weeks gestation. DNA was isolated from approximately 200 mg stool using the MoBio PowerSoil® DNA Isolation Kit (Catalog # 12888-50). 16S sequencing was performed; primers annealing to the V1-V2 region of the 16S bacterial gene were used for amplification as described previously (McKenna et al.,

2008). Purified products from the samples were pooled in equal amounts and sequenced using Illumina MiSeq; positive and negative controls were used (Kim et al., 2017). Sequence data was processed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.9 (Caporaso et al., 2010). Reads were joined to form a complete amplicon sequence for the V1-V2 region and sequences were filtered to remove low quality reads using default parameters in QIIME. Operational Taxonomic Units (OTUs) were generated at 97% sequence similarity using UCLUST v. 1.2.22 (Edgar, 2010). A phylogenetic tree was inferred from the OTU data using FastTree2 (Price et al., 2010). Similarity between samples was assessed by weighted and unweighted UniFrac distance (Lozupone and Knight, 2005). Global differences in bacterial community composition were visualized using principal coordinates analysis. Alpha diversity was measured in number of distinct OTUs at 10,000 read depth and Shannon index.

2.4 Food Recall. In the two weeks following the screening visit, approximately one week prior to stool sampling, participants received three unannounced phone calls from a research nutritionist at the university's Clinical Translational Research Center (CTRC). During each 15-minute phone call, participants completed a 24-hour food recall. The data from these recalls were analyzed using Nutrition Data System for Research software (Nutrition Coordinating Center, University of Minnesota), which provides quantitative information on 166 nutrients.

2.5 Laboratory stressor. At 22-34 weeks gestation, participants returned to the laboratory for the TSST (Kirschbaum et al., 1993). The TSST is a standardized laboratory stressor lasting 20 minutes that requires the participant to perform mental arithmetic and to give a speech in front of a panel of three panelists. Trained professional standardized patients from the Penn Medicine

Standardized Patient Program served as panelists. Participants were asked to refrain from eating, using caffeine and using nicotine for 90 minutes prior to initiation of the TSST. Timing was standardized so that participants initiated the TSST between 11:00 and 13:00. Prior to initiating the TSST, informed consent was confirmed and a catheter was placed (T-35) for serial blood draws. The participants rested for 30 minutes and completed questionnaires (EPDS, PSQI, PSS and STAI). Baseline blood was drawn 5 minutes prior to TSST; T-5); the TSST was initiated at T=0. The TSST lasted for 20 minutes, concluding at T+20. At T+20, participants completed the STAI-S. Blood was drawn 10, 45 and 120 minutes post TSST (T+30, T+65, T+140). At each blood collection timepoint, 3.5 mL blood was collected. Immune and HPA markers assessed were IL-6, IL-1β, CRP, TNF-α, and cortisol.

2.6 Cytokine and cortisol assays. Assessment of high sensitivity (hs) IL-1β, IL-6, and TNF-α in serum was performed using a solid phase protein immunoassay that uses spectrally encoded antibody-conjugated beads as the solid support (Luminex). Following manufacturer instructions, results were obtained by monitoring the spectral properties of the capture beads while simultaneously measuring the quantity of associated fluorophore. hsCRP and cortisol were assessed in serum samples using a solid phase sandwich enzyme linked-immuno-sorbent assay (ELISA). An antibody specific for each tested analyte was coated onto the wells of microtiter strips. Following manufacturer instructions, results were obtained by reading the absorbance of each well at a specified wavelength.

2.7 Statistical Methods

Data was examined descriptively and outcomes tested for normality using Kolmogorov-Smirnov test statistics and normal probability plots. Area under the curve (AUC) with respect to the ground was calculated as the total sum of the area from the first time point to the last time point, as a summary measure for cytokines and cortisol. Linear regression models were used to examine cortisol and cytokine AUC between ACE groups as well as correlations with dietary intake. Cortisol responder status was also calculated as a secondary measure of HPA axis response (Herbison et al., 2016; Maki et al., 2015; Schommer et al., 2003); cortisol responders were defined as those participants with a positive change from baseline to peak. Binomial generalized linear models were used to test associations with the binary cortisol responder outcome. Linear mixed effects models were also used to account for repeated measures of cytokines, cortisol, and STAI scores from pre to post stressor. Time was treated as a categorical variable to account for nonlinear trends. IL-6 AUC was transformed for normality and model fitting using the natural logarithm. Nutrition variables were log transformed when there was a benefit to model fit and normality. Participants with BMI greater than 40, classified as Class III or "extreme" obesity by the Centers for Disease Control (CDC), were discarded from the data in models with cytokine outcomes; obesity is associated with elevated proinflammatory cytokines and thus a BMI of 35-40 is often used as a cutoff in studies utilizing the TSST to induce a cytokine response to stress (Christian and Porter, 2014; Derry et al., 2013; McInnis et al., 2014). Models including nutrition variables were adjusted for total energy when appropriate. Total energy was scaled for convergence in mixed models with inflammatory dependent values and nutrition covariates. For microbiome analyses, community-level differences between sample groups was assessed with Permutation Multivariate Analysis of Variance (PERMANOVA) on the weighted and unweighted UniFrac distances; associations between microbiome composition,

ACE and proinflammatory cytokine response to TSST were assessed, controlling for gestational age at time of stool collection, BMI, and dietary fiber intake, as these are factors that have been shown in other research to impact the gut microbiome (Koren et al., 2012; Ottosson et al., 2018; Wu et al., 2011). Differential abundance was assessed in taxa with >1% mean abundance across samples using generalized linear models; multiple tests were corrected with the Benjamini-Hochberg method. Within the microbiota analyses, a false discovery rate (fdr, or q) < 0.05 were considered significant. In all other analyses, $p < 0.05$ was considered significant, and t-values are shown to indicate effect size. The study was powered such that a sample of n=48 would provide sufficient power to detect a moderate effect size (f^2 = 0.25, alpha = 0.05) of ACE on gut microbial communities in a regression model containing three predictors, and a sample of n=20 would provide sufficient power to detect a large effect size (f^2 = 0.45, alpha = 0.05) of ACE on inflammatory response to stress, based on effects observed in previous research (Carpenter et al., 2010). Analyses were performed with R Version 3.2.3 and 3.4.3.

3. RESULTS

3.1 Sample Characteristics

Forty-eight women completed the ACE-Q and other psychological measures and provided stool for sequencing; a subset of 19 women completed the TSST. Participant demographic features and baseline psychological measures are presented in Table 1. High (>=2) and low (<2) ACE participants were similar in demographics and psychiatric health (p's > 0.05), although high ACE participants were significantly younger (M = 25.98, SD = 4.99) than low ACE participants (M = 29.80, SD = 5.21) (t(45.92) = 2.60, p = 0.013) (Table 1). One woman who was taking

antibiotics in the week prior to stool collection was excluded from microbiome analysis. Two women with BMI > 40 were excluded from cytokine analyses.

3.2 Psychological Measures, ACE Exposure

The high ACE group had significantly higher total ACE score (*Median* = 3, *IQR* = 1.5) than the low ACE group (*Median* = 0, *IQR* = 01) (W = 0, *p* = 1.35e-9). Low and high ACE groups did not differ in scores on PSS, EPDS, STAI-S, STAI-T, or PSQI (*p*'s > 0.05) (Table 1). High ACE participants reported significantly more traumatic events on the TLEQ (*Median* = 7.0, IQR = 10.5) than low ACE participants (*Median* = 4.0, *IQR = 4.0*) (*W* = 165, *p* = 0.011) (Table 1).

3.3 Dietary Macronutrient Intake

There were no significant differences in dietary intake of daily total kilocalories, total fat, cholesterol, fiber, saturated fat, mono- or polyunsaturated fat, trans fats, total ω-3 PUFAs, nor specific ω-3 or ω-6 fatty acids (e.g. EPA, DHA) by ACE group (*p*'s > 0.05) (Table 2). Mean time from food recall to stool sampling was 0.65 weeks (*SD* = 0.67) which is < 5 days.

3.4 Subjective Response to Acute Stress

The TSST was subjectively stressful, increasing STAI-S score by 17 points on average (*t*(18.00) = 6.40, *p* = 5.01e-6). Pre to post change in STAI-S did not differ by ACE group (*t*(17.00) = 0.83, *p* = .420) (Table 3).

3.5 Cytokine, and Cortisol Response to Acute Stress

3.5.1 ACE Associations with Cytokine and Cortisol Response to Acute Stress

There were no significant differences between high and low ACE participants in terms of cytokine AUC response to stress (CRP: $t(13.48) = .156$, $p = 0.879$; IL-6: $t(10.27) = -.100$, $p = .922$; TNF-α: $t(13.46) = -.163$, $p = 0.873$). Similarly, there were no significant differences between high and low ACE women in cortisol AUC ($t(17) = -.848$, $p = .408$). However, high ACE women were less likely to be cortisol responders ($z(17) = -2.34$, $p=0.019$).

3.6 Gut Microbiome

3.6.1 ACE Associations with Gut Microbiota

Regarding ACE influence on the gut microbiome, there were no differences between ACE groups for richness ($p = 0.82$), Shannon index ($p= 0.58$), nor UniFrac distances (p's > 0.05). Controlling for gestational age at time of stool collection, BMI, and dietary fiber intake, high ACE participants had higher differential abundance of *Prevotella* (FDR-adjusted *p*-value, q=5.7x10^-13), and trend toward lower abundance of *Erysipelotrichaceae* (species previously in *Eubacterium* genus) (p=0.019, q=0.15) and *Phascolarctobacterium* (p=0.041, q=0.22) than low ACE participants (Figure 1).

3.6.2 Gut Microbiota Associations with Cytokine and Cortisol Response to Acute Stress

Controlling for gestational age at time of stool collection, BMI, and dietary fiber intake, IL-6 AUC was positively associated with abundance of *Bacteroides* (q<0.001), and negatively with *Clostridiales* (q=0.033), *Lachnospiraceae* (q=0.0033), *Dialister* (q=0.0038), and *Enterobacteriaceae* (q=0.0038) (Figure 2). Similarly, TNF-α AUC was positively associated with abundance of *Bacteroides* (q=0.0006), *Prevotella* (q=0.015), and *Megasphaera* (q=0.015) and negatively with *Ruminococcaceae* (q=0.0006) (Figure 2). CRP AUC was positively

associated with abundance of *Ruminococcaceae* (q=0.034) and *Megasphaera* (q=0.027) (Figure 2). Finally, cortisol response to stress was positively associated with abundance of *Rikenellaceae* (q=0.019) and *Dialister* (q<0.001), and negatively with *Bacteroides* (q=0.019) (Figure 2).

3.7 Dietary Fatty Acid Associations with Cytokine Response to Acute Stress

Among the proinflammatory cytokines measured, only TNF-α was significantly associated with dietary fatty acid intake. Dietary saturated fat (t(13) = -3.036, p = 0.009) was associated with smaller TNF-α AUC, while arachidonic acid (t(13) = 3.39, p = 0.004) and docosapentaenoic acid (DPA) (t(12) = 3.55, p = 0.003) were associated with greater TNF-α AUC. Exploratory analyses indicated that dietary intake of ω-3 PUFAs interacted with ACE to predict proinflammatory cytokine response to TSST. There was a significant ACE x DHA interaction for IL-6 AUC, in which high ACE women with high intake of the ω-3 PUFA DHA exhibited a lower IL-6 AUC (t(11) = -2.413, slope: low ACE =.106, high ACE = -.285, p = 0.03) (Figure 3). There was a trend-level interaction for ACE x EPA, so that in high ACE women, high intake of the ω-3 PUFA EPA was associated with lower IL-6 AUC (t(11) = -2.181, slope: low ACE = .093, high ACE = -.244, p = 0.05). Similarly, there was a trend-level stearidonic acid x ACE interaction for IL-6 AUC, in which high intake of the ω-3 PUFA stearidonic acid, a precursor of EPA, associated with lower IL-6 AUC among high ACE women (t(11) = -2.087, slope: low ACE = .027, high ACE = -.288, p = 0.06).

4. CONCLUSIONS

This study examined associations among ACE, gut microbiota, and inflammatory and cortisol response to stress during pregnancy. Our findings indicate that multiple childhood adversities are associated with dampened HPA response and altered gut microbiota composition during pregnancy. This is to our knowledge the first study to find associations between specific gut taxa and inflammatory and cortisol response to an acute laboratory stressor. While ω-3 PUFA intake did not dampen inflammatory cytokine response to acute stress in the overall sample, exploratory analyses indicated that high ACE women with high dietary intake of the ω-3 PUFA DHA, IL-6 response to stress was dampened, with a similar trend for EPA.

Our finding on ACE impact on HPA response to acute stress, that is, physically and psychiatrically healthy high ACE women at 22-34 weeks gestation were less likely to be cortisol responders than low ACE women, was consistent with our previous research in postpartum women (Morrison et al., 2017). Our previous translational study found that high ACE postpartum women exhibited a blunted cortisol response to acute stress, mirroring hyporesponsive HPA function in postpartum female mice exposed to ELS (Morrison et al., 2017). However, in the present sample there was no main effect of ACE on proinflammatory cytokine response to stress. This is discordant with past research suggesting that exposure to childhood adversity is associated with an exaggerated proinflammatory response to the TSST (Carpenter et al., 2010; Tell et al., 2018). These studies used different measures of childhood adversity (e.g. trauma exposure) (Carpenter et al., 2010) in different populations (e.g. breast cancer patients) (Tell et al., 2018), which may explain why our results differ. There is also wide variation in cutoff scores across studies when categorizing exposure to childhood adversity, although an ACE score of >=2 has been associated with altered HPA axis function during pregnancy (Bowers et al., 2018) and postpartum (Morrison et al., 2017), leading us to posit that this was an appropriate cutoff score. It

is also possible that, given the distribution of ACE scores within our sample (Table S2), we did not have sufficient participants with severe ACE exposure to have an effect. Most likely, pregnancy impacted cytokine response to stress; a similar study that utilized the TSST found that pregnant women had a dampened IL-6 response to the TSST compared with non-pregnant women (Christian et al., 2013). However, our result is similar to findings in which history of childhood abuse was not directly associated with baseline IL-6 during pregnancy, but mediated by another factor (in that case, BMI) (Mitchell et al., 2018). While not powered for a mediation analysis, exploratory analyses indicated that dietary intake of ω-3 PUFAs, particularly DHA, was associated with a dampened inflammatory response to acute stress among high ACE women. This is consistent with work in non-pregnant adults showing that dietary intake of ω-3 PUFAs (Alfano et al., 2012; Caughey et al., 1996; Rallidis et al., 2003), and similarly plasma and erythrocyte membrane ω-3 PUFA levels (Ferrucci et al., 2006; Kiecolt-Glaser et al., 2007; Maes et al., 2000) are negatively associated with a number of inflammatory markers. A recent study found that during pregnancy, a diet high in inflammation-promoting foods elevated levels of serum TNF-α, which was further exacerbated in women with high levels of stress (Lindsay et al., 2018). A low DHA:AA ratio in red blood cells during pregnancy was associated with greater inflammation and higher risk of preterm birth among African-American women (Christian et al., 2016), again underscoring potential risk of elevated peripheral inflammation to maternal-fetal health. During pregnancy, EPA-DHA supplementation reduced peripheral inflammation (Haghiac et al., 2015), placental inflammation (Lager et al., 2017), and salivary cortisol response to the TSST (Keenan et al., 2014) compared with placebo. Given these associations among inflammation, diet and stress, future studies should assess dietary ω-3 PUFA supplementation specifically in high ACE pregnant women.

Within the larger sample of forty-eight pregnant women, those who had experienced multiple ACEs had greater differential abundance of gut *Prevotella* than low ACE women, and trended toward lower *Erysipelotrichaceae* taxa and *Phascolarctobacterium* within the *Firmicutes* phylum, after controlling for gestational age at stool collection, BMI, and dietary fiber intake. Critically, these findings are consistent with rodent models indicating ELS produces a lasting impact on gut microbiota (García-Ródenas et al., 2006; O'Mahony et al., 2009; Pusceddu et al., 2015). Particularly, ELS increased *Prevotella* and reduced the *Firmicutes:Bacteroidetes* ratio in rats (Pusceddu et al., 2015), paralleling the elevated *Prevotella* and reduced abundances of *Firmicutes* taxa in our cohort. Intriguingly, among rodents exposed to ELS, EPA/DHA supplementation in adulthood corrected the *Firmicutes:Bacteroidetes* ratio to that of non-ELS rats, suggesting that EPA/DHA may exert anti-inflammatory effects by modulating gut microbiota composition (Pusceddu et al., 2015). Our findings align with rodent literature indicating that stress during early life provokes a stable and persisting influence on microbiota composition, and extends this to pregnancy. Whether modulation of inflammatory response by PUFAs in high ACE women is mediated by the gut microbiota remains a question for future research.

Relative abundance of several gut taxa were associated with cytokine and cortisol response to acute stress. *Dialister* was negatively associated, while *Bacteroides* and *Megasphaera* were positively associated, with proinflammatory cytokine response. *Ruminococcaceae* showed mixed relationships with proinflammatory cytokine response. Regarding cortisol response, *Dialister* was positively associated and conversely *Bacteroides* was negatively associated with cortisol.

While stress during pregnancy concurrently alters the gut microbiota and increases inflammation in mice (Gur et al., 2017), no prior human studies, in pregnant or non-pregnant samples, have reported on associations between gut microbiota and cytokine or cortisol response to acute stress. However, *Dialister* has been associated with lower baseline IL-6 in healthy adults (Martínez et al., 2013), and *Megasphaera* was associated with greater ex vivo stimulated IFN-γ production by peripheral blood mononuclear cells (PBMCs) (Schirmer et al., 2016). These results are consistent with findings in our sample, in which *Dialister* was negatively associated with proinflammatory cytokine response, and *Megasphaera* was positively associated with proinflammatory cytokine response. While discussion of potential mechanisms linking the gut-brain axis and inflammation is beyond the scope of this article, it is believed that gut microbiota metabolites such as neuropeptides and short chain fatty acids (SCFAs) interact with the CNS, activating microglia, which modulate HPA activity and in turn cytokine release (Rea et al., 2016). Although the present study provides information solely on associations among ACE, the gut microbiome, and stress response, this provides groundwork for future mechanistic or intervention studies.

4.1 Strengths and Limitations

A key strength of this study was careful screening to ensure a psychiatrically healthy sample. In previous studies, it has been difficult to parse the impact of ACE versus current psychiatric function, as individuals with significant ACE exposure are at greater risk for psychiatric diagnoses. However, this indicates a resilient sample of women, limiting the generalizability of these findings. There is an emerging literature on protective factors that may provide resilience to ACEs, including attachment style, positive childhood experiences, emotion regulation, and coping style (Beutel et al., 2017; Dagan et al., 2017; Gouin et al., 2017; Narayan et al., 2018;

Poole et al., 2017), which were not assessed in this study. In addition, there were relatively few women in the sample who had experienced severe adversity (i.e. ACE > 4), which may have impacted results. The study sample presented here was selected to have equal balance of low and high ACE women, and therefore does not reflect the distribution of ACE exposure in the general population. Therefore, estimated associations that do not adjust for ACE exposure represent associations in an enriched population and may not be generalizable. The sample included only pregnant women, making it difficult to generalize results to nonpregnant women or men. Another limitation was that blood levels of PUFAs were not measured directly. Regarding the subsample of women who completed the TSST, the mean time from stool sampling to TSST completion was six weeks. Ideally, stool sampling would have occurred in closer proximity to the TSST, to provide a more precise view of correlations between gut microbiota and inflammatory response to stress. However, we did control for gestational age at time of stool collection in microbiome analyses, to attempt to control for potential differences based on gestational timing. Further, a study that assessed microbiota weekly across pregnancy found no significant changes in alpha diversity, beta diversity or Shannon index from 10 to 40 weeks gestation (DiGiulio et al., 2015), suggesting that the microbiota we observed at stool sampling was likely similar to the community profile existing several weeks later at TSST administration. Finally, this study was not appropriately powered to test a mediation or moderation model of the relationships among ACE, the gut microbiome, and cytokine response to stress with potential modulation by dietary PUFA intake. This study examined associations among these factors respectively, but given the sample size, a full mediation or moderation model could not be tested. In particular, the sample size of the subset of participants who completed the TSST was small, and exploratory analyses of interactions between ACE and dietary PUFA were underpowered

and warrant additional investigation. Thus, replication is needed in a larger sample. A larger sample would allow mediation and moderation analyses to be performed, to examine the potential pathway from ACE to gut microbiome (with moderation by PUFA intake) to inflammation.

4.2 Conclusions

Our findings lend support to preclinical models suggesting that ACE may impact inflammation via the gut microbiome, modulated by PUFA intake. First, multiple adversities experienced during a woman's early life influenced gut microbiota composition, paralleling rodent experiments that demonstrate persistent effects of ELS on the gut microbiota (Pusceddu et al., 2015). Further, relative abundance of several gut taxa were associated with cytokine and cortisol response to TSST; to our knowledge, no human studies have reported associations between gut microbiota and acute glucocorticoid-immune response to stress, although rodent models indicate altered gut bacterial community composition is associated with peripheral inflammation during pregnancy (Gur et al., 2017; Jašarević et al., 2017). Finally, greater dietary intake of ω-3 PUFAs was associated with a diminished inflammatory response to acute stress among high ACE pregnant women, suggesting protective effects of ω-3s in this high risk population. Together, these findings support a model in which ACE impacts gut microbiota, and gut microbiota influences inflammation, with modulation by ω-3 PUFA intake. To advance this model, full mediation-moderation analyses should be performed in a larger sample to elucidate the relationships among ACE, the gut microbiome, diet and inflammation.

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TABLE AND FIGURE CAPTIONS

Table 1. Sample characteristics. Demographic and health information are presented for low and high ACE participants. Means and standard deviations are presented for normally distributed data. Medians and interquartile range (IQR) are reported for non-normally distributed data. P-values are presented for Chi-Square test, t-test or Wilcoxon-Mann-Whitney test comparing low and high ACE groups.

Table 2. Dietary macronutrient intake. Daily dietary intake is presented for low and high ACE participants. Daily dietary intake values were determined by taking the mean of three days of diet data obtained from 24-hour food recalls. Means and standard deviations are presented for normally distributed data. Medians and interquartile range (IQR) are reported for non-normally distributed data. P-values are presented for t-test or Wilcoxon-Mann-Whitney test comparing low and high ACE groups.

Table 3. Response to laboratory stressor. Subjective and physiological response to the Trier Social Stress Test (TSST) are presented for low and high ACE participants. Means and standard deviations are presented for normally distributed data. Medians and interquartile range (IQR) are reported for non-normally distributed data. P-values are presented for t-test or Wilcoxon-Mann-Whitney test comparing low and high ACE groups.

Figure 1. Relative abundance of gut taxa in low and high ACE participants. Controlling for gestational age at time of stool collection, BMI, and dietary fiber intake, high ACE participants

had higher differential abundance of *Prevotella* (FDR-adjusted *p*-value, *q*=5.7x10^-13), and trend toward lower abundance of *Eubacterium* (*p*=0.019, *q*=0.15) and *Phascolarctobacterium* (*p*=0.041, *q*=0.22) than low ACE participants.

Figure 2. Gut microbiota associations with cytokine and cortisol response to acute stress. Controlling for gestational age at time of stool collection, BMI, and dietary fiber intake, cortisol response to stress was positively associated with abundance of *Rikenellaceae* (*q*=0.019) and *Dialister* (*q*<0.001), and negatively with *Bacteroides* (*q*=0.019). IL-6 AUC was positively associated with abundance of *Bacteroides* (*q*<0.001), and negatively with *Clostridiales* (*q*=0.033), *Lachnospiraceae* (*q*=0.0033), *Dialister* (*q*=0.0038), and *Enterobacteriaceae* (*q*=0.0038). Similarly, TNF-α AUC was positively associated with abundance of *Bacteroides* (*q*=0.0006), *Prevotella* (*q*=0.015), and *Megasphaera* (*q*=0.015) and negatively with *Ruminococcaceae* (*q*=0.0006). Finally, CRP AUC was positively associated with abundance of *Ruminococcaceae* (*q*=0.034) and *Megasphaera* (*q*=0.027).

Figure 3. Dietary ω-3 PUFA associations with IL-6 response to stress in high and low ACE women. a) There was a significant ACE x DHA interaction for IL-6 AUC, in which high ACE women with high intake of the ω-3 PUFA DHA exhibited a lower IL-6 AUC ($t(11) = -2.413$, $p = 0.03$). b) There was a trend-level interaction for ACE x EPA, so that in high ACE women, high intake of the ω-3 PUFA EPA was associated with lower IL-6 AUC ($t(11) = -2.181$, $p = 0.05$). c) Similarly, there was a trend-level stearidonic acid x ACE interaction for IL-6 AUC, in which high intake of the ω-3 PUFA stearidonic acid, a precursor of EPA, associated with lower IL-6 AUC among high ACE women ($t(11) = -2.087$, $p = 0.06$).

FIGURES

Figure 1. Relative abundance of gut taxa in low and high ACE participants

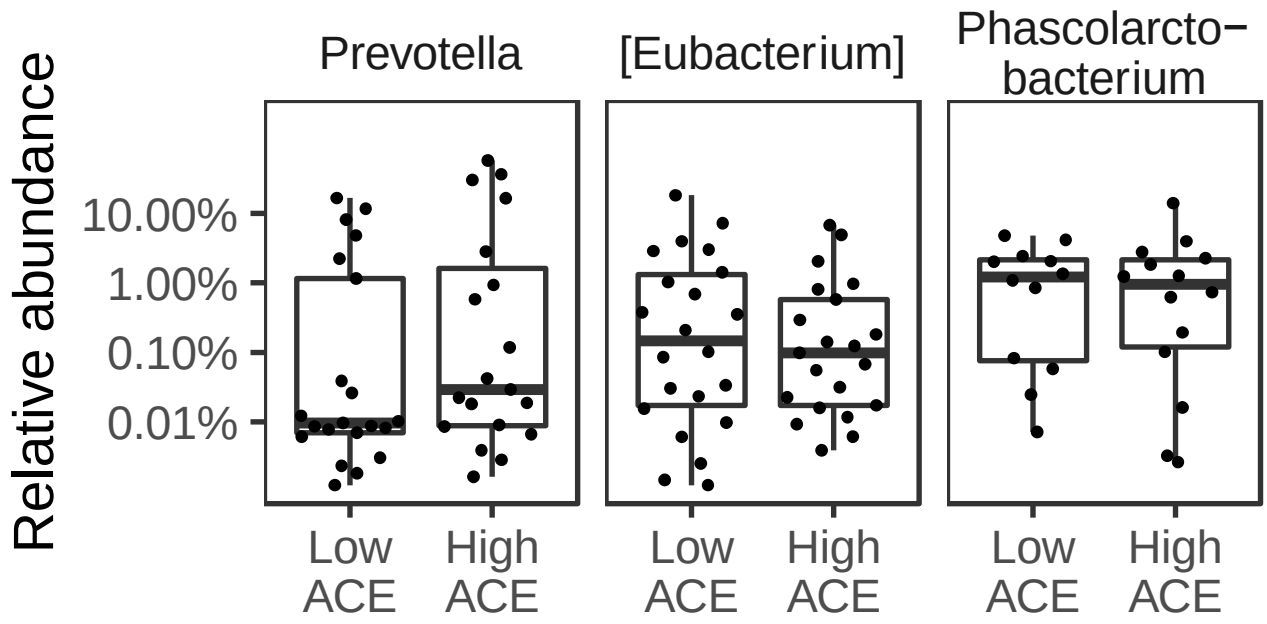


Figure 2. Gut microbiota associations with cytokine and cortisol response to acute stress

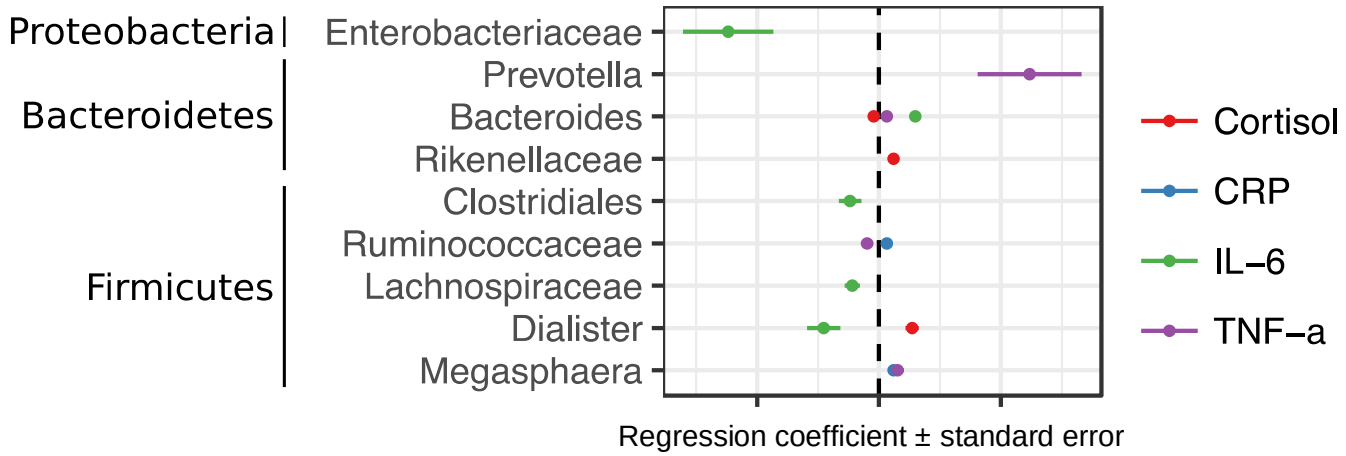
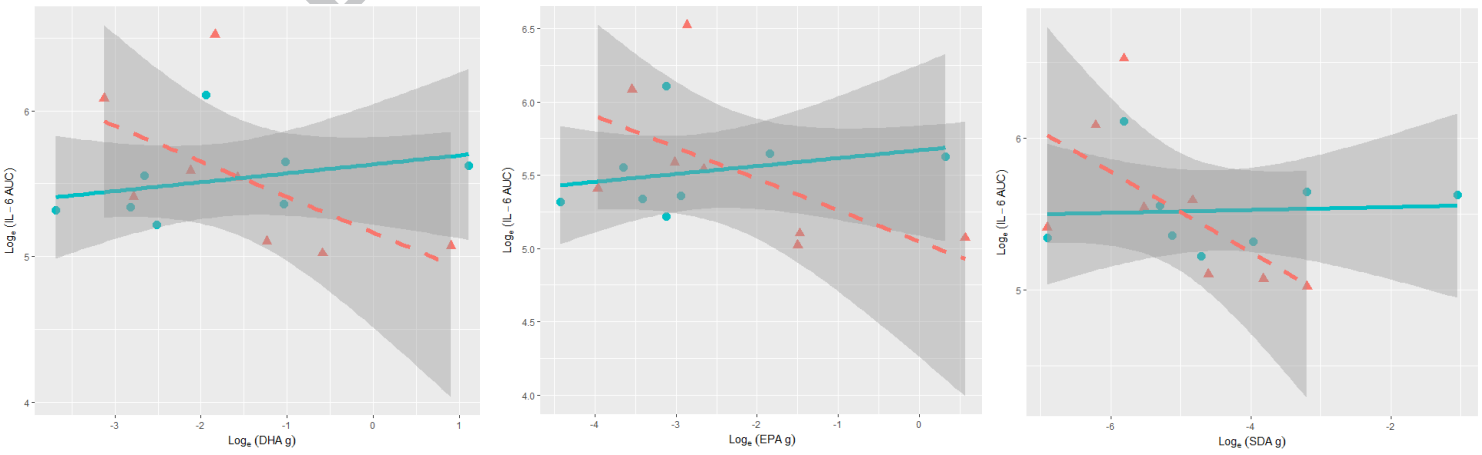


Figure 3. Dietary ω-3 PUFA associations with IL-6 response to stress in high and low ACE women

a. DHA

b. EPA

c. Stearidonic acid



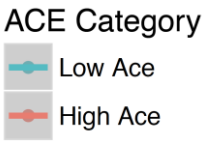


Table 1. Sample characteristics.

		Low ACE	High ACE	p-value
n		25	23	
Age, years (Mean (SD))		29.80 (5.21)	25.98 (4.99)	0.013
Weight, lbs (Mean (SD))		178.04 (44.81)	167.09 (27.16)	0.315
Prepregnancy BMI (Mean (SD))		27.98 (8.62)	25.53(3.72)	0.212
Gestational Age at Recruitment, weeks (Mean (SD))		27.31 (2.92)	27.20 (2.26)	0.885
Marital Status (n (%))	Single/Separated	13 (52.00)	16 (69.57)	0.25
	Married/Domestic Partnership	12 (48.00)	7 (30.43)	
Income (n (%))	Less than $50K	9 (36.00)	15 (65.22)	0.082
	Greater than $50k	16 (64.00)	8 (34.78)	
Employment (n (%))	Employed	20 (83.33)	15 (65.22)	0.193
	Unemployed	4 (16.67)	8 (34.78)	
Education (n (%))	Less than College Grad	10 (40.00)	15 (65.22)	0.094
	College Graduate or higher	15 (60.00)	8 (34.78)	
Race (n (%))	Caucasian	13 (52.00)	8 (34.78)	0.479
	African American	10 (40.00)	13 (56.52)	
	Other	2 (8.00)	2 (8.70)	
Ethnicity (n (%))	Non-Hispanic	24 (96.00)	19 (82.61)	0.18
	Hispanic	1 (4.00)	4 (17.39)	
ACE-Q (Median [IQR])		0 [0.0, 1.0]	3 [2.5, 4.0]	<.001
PSS (Median [IQR])		11 [7, 13]	10 [5.5, 12.5]	0.717

EPDS (Median [IQR])	5 [3, 6]	3 [1.5, 6.0]	0.258
STAI State (Median [IQR])	27 [22, 33]	23 [21, 27]	0.112
STAI Trait (Median [IQR])	30 [27.0, 36.0]	28 [23.5, 34.0]	0.225
TLEQ Events (Median [IQR])	4 [2.0, 6.0]	7[4.0, 14.50]	0.011
PSQI (Median [IQR])	5 [3.0, 6.0]	6 [4.0, 7.5]	0.429
Weight at Delivery, grams (Mean (SD))	3086.96 (708.57)	3188 (435.68)	0.566
Gestational Age at Delivery, weeks (Mean (SD))	39 (2.0)	39 (2.5)	0.641
Apgar Score, 1 minute (Median [IQR])	8 [8, 9]	8 [8, 9]	0.562
Apgar Score, 5 minutes (Median [IQR])	9 [9, 9]	9 [9 ,9]	0.969

Abbreviations: Adverse Childhood Experiences Questionnaire (ACE-Q), Body Mass Index (BMI), Edinburgh Postnatal Depression Scale (EPDS), interquartile range (IQR), Pittsburgh Sleep Quality Index (PSQI), Perceived Stress Scale (PSS), Standard Deviation (SD), State-Trait Anxiety Inventory (STAI), Traumatic Life Events Questionnaire (TLEQ)

Table 2. Daily dietary macronutrient intake.

	Low ACE	**High ACE**	**p-value**
n	25	23	
Energy (kilocalories)	1956.24 (452.77)	2001.29 (474.94)	0.738
Total fat (g)	78.17 [56.62, 99.36]	70.77 [63.22, 98.09]	0.942
Cholesterol (mg)	312.41 [231.03, 372.13]	305.03 [236.53, 397.12]	0.584
Dietary fiber (g)	16.23 [11.54, 24.88]	16.98 [11.85, 21.57]	0.992
Saturated fatty acids (g)	27.10 [17.99, 36.86]	26.70 [20.06, 30.99]	0.975
Trans fats (g)	2.00 [1.38, 2.63]	1.89 [1.31, 2.11]	0.57
Monounsaturated fats (g)	26.28 [20.79, 33.59]	24.09 [19.51, 30.62]	0.828
Polyunsaturated fatty acids (g)	16.99 [13.87, 21.61]	15.60 [13.43, 22.06]	0.893

Omega-3 fatty acids (g)	1.93 [1.54, 2.30]	2.11 [1.27, 2.91]	0.427
Linoleic acid 18:2 (g)	14.61 [11.61, 18.95]	12.86 [11.47, 18.60]	0.733
Linolenic acid 18:3 (g)	1.80 [1.20, 2.06]	1.64 [1.17, 2.57]	0.893
Arachidonic acid 20:4 (g)	0.16 [0.12, 0.19]	0.17 [0.14, 0.24]	0.216
Eicosapentaenoic acid (EPA) 20:5 (g)	0.01 [0.01, 0.02]	0.02 [0.01, 0.06]	0.063
Docosapentaenoic acid (DPA) 22:5 (g)	0.02 [0.01, 0.03]	0.03 [0.02, 0.04]	0.14
Docosahexaenoic acid (DHA) 22:6 (g)	0.04 [0.02, 0.10]	0.05 [0.03, 0.11]	0.337
Stearidonic acid 18:4 (g)	0.00 [0.00, 0.01]	0.00 [0.00, 0.02]	0.685

Abbreviations: grams (g), milligrams (mg)

Table 3. Response to laboratory stressor.

		Low ACE	**High ACE**	**p-value**
n		10	9	
Pulse Mid-stressor, bpm (mean (sd))		106.75 (13.96)	104.38 (26.23)	0.824
STAI State Pre-stressor (median [IQR])		24.00 [20.25, 31.25]	26.00 [21.00, 31.00]	0.537
STAI State Post-stressor (median [IQR])		44.50 [30.25, 48.50]	46.00 [41.00, 58.00]	0.307

Abbreviations: beats per minute (bpm), State-Trait Anxiety Inventory (STAI)

HIGHLIGHTS

- Adverse childhood experiences (ACEs) predicted higher gut *Prevotella* abundance.
- Cytokine response to acute stress was associated with abundance of specific gut taxa.
- Dietary ω-3 PUFA intake normalized cytokine response to stress in high ACE women.