SEROTONIN TRANSPORTER POLYMORPHISM AND STRESS EFFECTS ON GUT MICROBIOTA AT VARIOUS TIME POINTS IN PREGNANCY

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Serotonin Transporter Polymorphism and Stress Effects on Gut Microbiota at Various Time Points in Pregnancy

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ABSTRACT

Prenatal stress (stress experienced by the mother while pregnant) has been shown to greatly affect the health of a child. Still, not all offspring who experience prenatal stress will suffer poor outcomes. Maternal genetics and the timing of adverse events have been shown to interact to affect the likelihood that a child will be affected by prenatal stress. However, the mechanisms behind this interaction are not fully understood. One potential mechanism is the maternal gut microbiome (the community of bacteria residing in the mother's gut). The current study used a mouse model of genetic stress susceptibility, combined with a daily restraint stress during pregnancy to examine alterations in the maternal gut microbiome due to an interaction between genes and stress. While pregnancy was found to alter the microbiome differently at various time-points in pregnancy, there was no significant interaction between genes and stress. However, as the sample size was quite small and there were trending results, we believe there is good evidence to continue exploration of the effects of stress and genetics on the maternal microbiome.

Serotonin Transporter Polymorphism and Stress Effects on Gut Microbiota at Various Time Points in Pregnancy

During pregnancy, the experience of the mother and the health of the offspring are inextricably linked. Some easily quantifiable maternal stressors, such as nutrition or infection, have well understood direct effects on the fetus. However, it has also been shown that other, more subjective factors, such as the experience of maternal emotional stress, play a role in offspring development (Glover, 2011; Kinney, Munir, Crowley, & Miller, 2008; Lemaire, Koehl, Le Moal, & Abrous, 2000; Rice et al., 2010). However, while the correlation between maternal psychological stress and offspring outcomes is strong, it is important to determine the mechanism by which a subjective psychosocial stressor can influence fetal health.

One potential route of influence of stress on fetal health is the maternal microbiome. Recent research has discovered that the gut microbiome (the community of microorganisms that live in the gut) can impact many whole body systems and is related to several health outcomes such as obesity and anxiety (Foster & McVey Neufeld, 2013; Turnbaugh et al., 2009). While generally stable over time, microbial communities are greatly affected by host environmental stressors (Faith et al., 2013). Recently, evidence has shown that alterations of the maternal microbiome can affect offspring microbiome and development (Jašarević, Howerton, Howard, & Bale, 2015). As the gut microbiome influences and is influenced by full body systems and temporary states, such as stress and pregnancy, determining the changes in the maternal microbiome during pregnancy that could affect the development of offspring microbiome will shed light on the effects of stress and genetics on offspring health.

The current study examines how stress during pregnancy alters the maternal gut microbiota and whether or not there is a gene by environment interaction that drives these differences. Specifically, we use a mouse model of a human genetic polymorphism that affects the serotonergic system and evaluate the gut microbiota at three time-points in pregnancy. In this way, we hope to illuminate one potential route of influence for stress and genetics on fetal development.

Stress, Genetics, and a Critical Window

Human populations. Previous studies have shown that stress on the mother during pregnancy (prenatal stress) is associated with poorer outcomes for children. For instance, prenatal stress has been associated with an increase in ADHD diagnosis (Mulder et al., 2002; Rodriguez & Bohlin, 2005), decreased language and intellectual ability (Charil, Laplante, Vaillancourt, & King, 2010; Van Goozen, Fairchild, Snoek, & Harold, 2007), and decreased social skills (Van Goozen, Fairchild, Snoek, & Harold, 2007) in children. Children of mothers who experienced stress during pregnancy also had a greater risk for a diagnosis of Autism Spectrum Disorder (ASD) (D. Q. Beversdorf et al., 2005). However, not all studies have found an effect of maternal stress on offspring development (Li et al., 2009). Therefore, it is important, to take into account factors that may moderate the effects of prenatal stress. Previous research has shown strong evidence for two specific moderators: a genetic polymorphism on the serotonin transporter gene, and a critical time window in pregnancy in which prenatal stress is associated with poor outcomes.

There are several genetic mutations that are proposed to have influence on the effect of stress on individuals. The most frequently investigated is a deletion mutation found on the serotonin transporter gene (SERT, also known as the 5HTT gene). The

SERT gene is responsible for the tone of the serotonergic system (D. Bengel et al., 1998). The deletion on the SERT gene results in either a short (S) versus a long (L) allele with humans being either homogeneous for long or short alleles (LL or SS) or heterogeneous (LS). It was discovered that people carrying one or two copies of the short allele were more prone to depressive symptoms, depression diagnosis and suicidality following major life stressors than those homozygous for the long allele (Caspi et al., 2003). The short allele has also been implicated in lower cognitive function, mood and increased anxiety. Although there have been a few studies arguing that there is not a link between the 5HTT gene and depression/anxiety, a meta-analysis of 52 studies found that the literature overwhelmingly supports the relationship between the SERT variation and stress reactivity (Karg, Burmeister, Shedden, & Sen, 2011).

Along with carrying the short 5HTT allele, the timing of prenatal stress also plays a role in the health of an infant. Children of mothers who experienced stress during the fifth and sixth months of pregnancy were more likely to be born early compared to those who experienced stress at other times (Roesch, Schetter, Woo, & Hobel, 2004). An increase in recalled stressors during weeks 21-32 of pregnancy was also associated with an increase in ASD diagnosis (D. Q. Beversdorf et al., 2005). Using natural disaster data not vulnerable to biased recall, Kinney et al. found that experience of a hurricane in weeks 20-36 of pregnancy correlated with an increase in ASD diagnosis, and the more severe the hurricane, the more likely the diagnosis (Kinney, Miller, Crowley, Huang, & Gerber, 2008). Most impressively, a population study of over two million Swedish infants found that maternal stress in months five and six of pregnancy was tied to poorer outcomes for

children including poorer physical health, increased anxiety, and depression (Class, Lichtenstein, Långström, & D'onofrio, 2011).

There is also an important interaction between these genetic and environmental variables. In a multi-site study, children of mothers carrying the short allele and experiencing stress during weeks 21-32 of pregnancy had an increase in autism diagnosis compared to those carrying the long allele (D. Beversdorf, 2015).

In order to study this complex interaction, a simplified, controllable laboratory model is helpful. Researchers have created a mouse model of human 5HTT genetic polymorphism—a SERT knockout mouse—that is currently used to study depression, anxiety, and effects of stress susceptibility.

Animal model. As in humans, prenatal stress has been shown to affect mice offspring. Pups that experienced prenatal stress show greater signs of learned helplessness (Frye & Wawrzycki, 2003), increased anxiety (Estanislau & Morato, 2005), and poorer spatial learning and memory (Yang, Han, Cao, Li, & Xu, 2006). Prenatally stressed rat pups also had greatly reduced social interaction compared with rats born to mothers that were not stressed (Lee, Brady, Shapiro, Dorsa, & Koenig, 2007). As in humans, mice and rats also show the genetic influences that play into the offspring outcomes for pups exposed to prenatal stress.

Bengel et al. described the original genetic modification that produced SERT knockout mice with either one or both copies of the 5HTT allele missing (Dietmar Bengel et al., 1998). This modification decreased the serotonin uptake by 50% in heterozygous (+/-) knockout mice and eliminated the uptake in homozygous mice. The reduction in expression and function of SERT in heterozygous mice is similar to that of

humans homozygous for the short 5HTT allele, and heterozygous mice exhibited an abnormal behavioral phenotype characterized by increase anxiety, increased startle, and increased learned helplessness (Murphy et al., 2008).

Importantly, the alteration of the stress response system in mice interacts with the timing of prenatal stressors to influence pup outcomes. In one study, dams stressed in mid to late pregnancy, had pups that were more likely to be obese than when dams were stressed earlier in pregnancy (Mueller & Bale, 2007). Hecht et all (in press) found that pups born to dams of SERT knockout mice stressed at days 16-20 of pregnancy showed decreased development of GABAergic neurons compared to pups of wildtype unstressed dams.

With this controllable animal model, we can examine physical mechanisms behind effects of prenatal stress, taking into account genetics and timing. One proposed mechanism is the maternal gut microbiome.

Gut microbiome, stress, pregnancy

Microbiome The human (and animal) body plays host to a community of fungi, viruses and bacteria. One of the largest communities is found in the gut. In fact, bacterial cells in the gut are thought to far outnumber all human cells in the body, with some estimates as high as 10:1. Recent research has found links between gut bacterial colonies and many health outcomes including obesity (Bailey et al., 2014), inflammatory bowel disease (Marchesi et al., 2007), and blood pressure (Holmes et al., 2008). Surprisingly for many, the gut microbiome has also been shown to influence and be influenced by psychological processes through the “gut-brain axis”. For instance, manipulation of the gut microbiome is associated with reduction in depression and anxiety symptoms

(Benton, Williams, & Brown, 2006; Messaoudi et al., 2011; Rao et al., 2009). Inversely, stress has been shown to affect the gut microbiome. As an example, Bailey et al. (2009) found that a social stressor affects the gut microbiota of mice. Interestingly, while stress is shown to cause changes within the gut microbiome, the SERT knockout mouse (the model of the short 5HTT allele) that is associated with a greater susceptibility to stress has been found in a small pilot study to have some protection against stress-induced microbiome alterations (Kille et al., 2015). Pregnancy is also found to alter the microbiome. Multiple studies (DiGiulio et al., 2015; Koren et al., 2012; Walther-António et al., 2014) have found differences between pregnant and non-pregnant mothers, and temporal changes of the microbiota during the course of pregnancy.

Based on previous research, it is reasonable to hypothesize that microbiome changes associated with typical pregnancy would be altered based on an interaction between the 5HTT allele polymorphism and environmental stress. We therefore hypothesize that pregnancy will be associated with changes in the gut microbiome and that stress will exacerbate these changes. However, due to protection from the SERT knockout mutation, SERT knockout mice will not experience this exacerbation. We also predict that the greatest differences between groups will be seen at embryonic day 15.5 (E15.5) as this is the critical time period for prenatal stress effects on pups.

Methods

Mice

Ten male homozygous SERT knockout mice were bred to 20 wild type C57BL/6J (Jackson Laboratories) females. Resulting wild type female offspring were bred to heterozygous males to produce experimental females. Experimental wild type and

heterozygous (genotyped for Slc6a4 alleles with the EZ Fast Tissue/Tail PCR Genotyping Kit per manufacturer's instructions (EZ BioResearch)) females in proestrus and estrus were paired with wildtype males overnight—the morning a vaginal plug was identified was considered embryonic day 0.5 (E0.5).

First and second generation animals were initially housed in groups of 1-4 littermates separated by sex in 7 × 11.5 × 7.75 inch Plexiglas cages with aspen bedding and a cotton nestlet. They had free access to food and water. The room was held at 21°C on a 12:12 hour light:dark cycle with lights on at 7 a.m. Experimental dams were housed in pairs until E0.5 and then individually through pregnancy. (See Table1 for group sizes across conditions.)

Weight and food intake were evaluated at E0.5, E5.5, E10.5, E15.5, E18.5 and did not vary significantly between groups.

Procedures

Prenatal Stress. Beginning E12.5 and continuing through embryo collection, dams in the stress condition were subjected to restraint stress. Once per day, mice were transferred to the testing room, placed in a clear Plexiglass tube for two hours, then put back in the home cage and returned to the colony room. Dams in the control condition were only kept in the home cage.

Collection. At E13.5, E15.5 or E18.5, and day of delivery (PND0) dams were euthanized via decapitation, and fecal samples were transferred directly to aliquots and stored in -80°C until processing.

Microbiome DNA processing. Fecal microbial DNA was extracted according to a previously published protocol (Ericsson et al., 2015) Fecal samples were removed from

the -80°C freezer and thawed, homogenized for 3 minutes in a Qiagen Tissuelyser II in 800 µL of lysis buffer (500 mM NaCl, 50 mM tris-HCl, 50 mM EDTA, and 4% SDS), and incubated at 70°C for 20 minutes. Samples were spun at 5000 × g for 5 minutes. The supernatant was pipetted off and mixed with 200 µL of 10 mM ammonium acetate, and incubated on ice for 5 minutes, then centrifuged at 16,000 × g for 10 minutes at room temperature. 750 µL of supernatant was then mixed with an equal volume of chilled isopropanol and incubated for 30 minutes on ice. The tubes were centrifuged at 4°C for 15 minutes. The supernatant was pipetted off and the DNA pellet rinsed twice with 70% EtOH and re-suspended in 150 µL of tris-EDTA. 15 µL of proteinase-K and 200 µL of buffer AL (DNeasy kit, Qiagen) were added and tubes incubated at 70°C for 10 minutes. 200 µL of 100% EtOH was added and the entire contents of the tube were transferred to a Qiagen spin column before continuing with the manufacturer's instructions for DNA purification (DNeasy Kit, Qiagen). DNA was eluted in 125 µL of EB buffer (Qiagen). Yield of double-stranded DNA was determined via fluorometry (Qubit 2.0, Life Technologies, Carlsbad, CA) using Qubit® dsDNA BR assay kits (Life Technologies).

Sequencing of the V4 region of the 16S rRNA gene was performed on the Illumina MiSeq platform. Bacterial 16S ribosomal DNA amplicons were constructed by amplification of the V4 hypervariable region of the 16s rRNA gene with primers flanked by Illumina standard adapter sequences. Universal primers (U515F/806R) previously developed against the V4 region were used for generating amplicons. Oligonucleotide sequences were obtained at proBase. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system, quantified

with the Qubit flourometer using the quant-iT HS dsDNA reagent kit, and diluted according to the manufacturer's protocol for sequencing on the MiSeq.

Assembly, binning, and annotation of DNA sequences were performed at the MU Informatics Research Core Facility (IRCF). Contiguous sequences of DNA were assembled using FLASH software (Magoc and Salzberg, 2011) and contigs were culled if found to be short after trimming for a base quality less than 31. Qiime v1.7 (Kuczynski et al., 2011) software was used to perform chimera detection and removal, and remaining contigs were assigned to operational taxonomic units (OTUs) using a criterion of 97% nucleotide identity. Taxonomy was assigned to selected OTUs using BLAST (Altschul et al., 1997) against the Greengenes database (DeSantis et al., 2006) of 16S rRNA sequences and taxonomy.

Microbial community differences based on relative abundancies of OTUs were analyzed using principal component analysis (PCA) and permutation ANOVA (PERMANOVA) with Bonferroni correction in Paleontological Statistics (PAST) software (Hammer, Harper, & Ryan, 2001).

Results

A principal component analysis (Figure1) was conducted and showed no distinct separation regardless whether the samples were distinguished by genotype, stress condition, or pregnancy time point. Chao1 and Shannon indices were computed, and no significant difference was found in richness or diversity (also regardless of how samples were separated). Figures 2 and 3 illustrate Chao1 and Shannon indices for each time point with all treatment conditions combined. PERMANOVA analysis on OTU community revealed a significant effect of gestation period ($p < 0.01$), with post hoc analysis

showing a difference between pre-pregnancy and PND0. No other significant main effects or interactions were found. Individual relative OTU abundances found to vary between PP and PND0 time points were Bacteroidales S24-7 ($p < 0.01$), Lactobacillus ($p < 0.01$), and Streptococcus ($p < 0.01$), and are illustrated in Figure 4 with all treatment groups combined.

Discussion

While we did not find support for our general hypotheses, we believe this to be in large part due to a small sample size. It is also difficult to account for variance in microbiome changes caused by individual differences without a repeated measures design, which would have given us more power to identify changes due to genetics and stress. However, as the mice were sacrificed for other experimental procedures at collection, collecting multiple samples from each mouse was not an option.

Based on the finding of Kille et al. (2015) that the SERT knockout mutation decreases stress induced microbiome alterations, it is possible that the SERT knockout did dampen the microbiome changes caused by stress, and in doing so washed out any stress condition differences. In the future, we will be exploring this potentiality using larger sample sizes and Bayesian modeling, which will allow us to directly examine true null results.

Limitations aside, there are some positive results. The significant OTU changes observed in our sample are in line with previous research into microbiome alterations due to pregnancy. As is frequently the case in murine fecal samples, Bacteroidales S24-7 was found to be the most prevalent OTU in both PP and PND0 time points. However, the relative abundance of S24-7 decreased significantly at the postnatal time point, although

it was still the most prevalent OTU. Lactobacillus and Streptococcus also decreased from PP to PND0. In previous work (Jost, Lacroix, Braegger, & Chassard, 2014; Puapermpoonsiri et al., 1996; Verstraelen et al., 2009), these OTUs have been found to not only change throughout pregnancy, but have also been associated with fetal health outcomes.

With both the limitations of the study and the evidence we did find in mind, it is important to not overstate the implications of the lack of positive results. Given a larger sample size and Bayesian statistical methods, we believe this model will be able to show support for the gene x environment interaction on the pregnant gut microbiome.

Future Directions Future work should expand on this study in three specific ways. First, as has been shown by Hecht (2014), the current gene x prenatal stress model does effectively alter offspring development. It is plausible that the paradigm also affects dams, and we therefore suggest not only using a sample size that will provide sufficient power to observe the gene x stress interaction in the maternal microbiome, but also employing a repeated measures design to account for individual differences. This will provide a more in depth look at the changes that happen at specific time points in pregnancy, especially the critical window of embryonic days 16-20. Second, recent studies have discovered that the infant virome shows much earlier and more drastic changes than the bacterial microbiome (Lim et al., 2015) and could be linked to many health outcomes including the healthy maturation of the bacterial microbiome. It is reasonable that maternal stress could affect this virome during fetal development through either direct infection or another mechanism, such as inflammation. The changes observed in the virome will be important to map, both in clinical and typical populations.

Third, the placental microbiome could also be affected by maternal stress. Until fairly recently, the placenta was thought to be a sterile environment. However, researchers have found this not to be the case, and that the placental microbiome may be implicated in some pre-term births (Prince, Antony, Chu, & Aagaard, 2014). These three future avenues will help to elucidate the many ways the stress, genetics and the microbiome could be implicated in fetal health and development.

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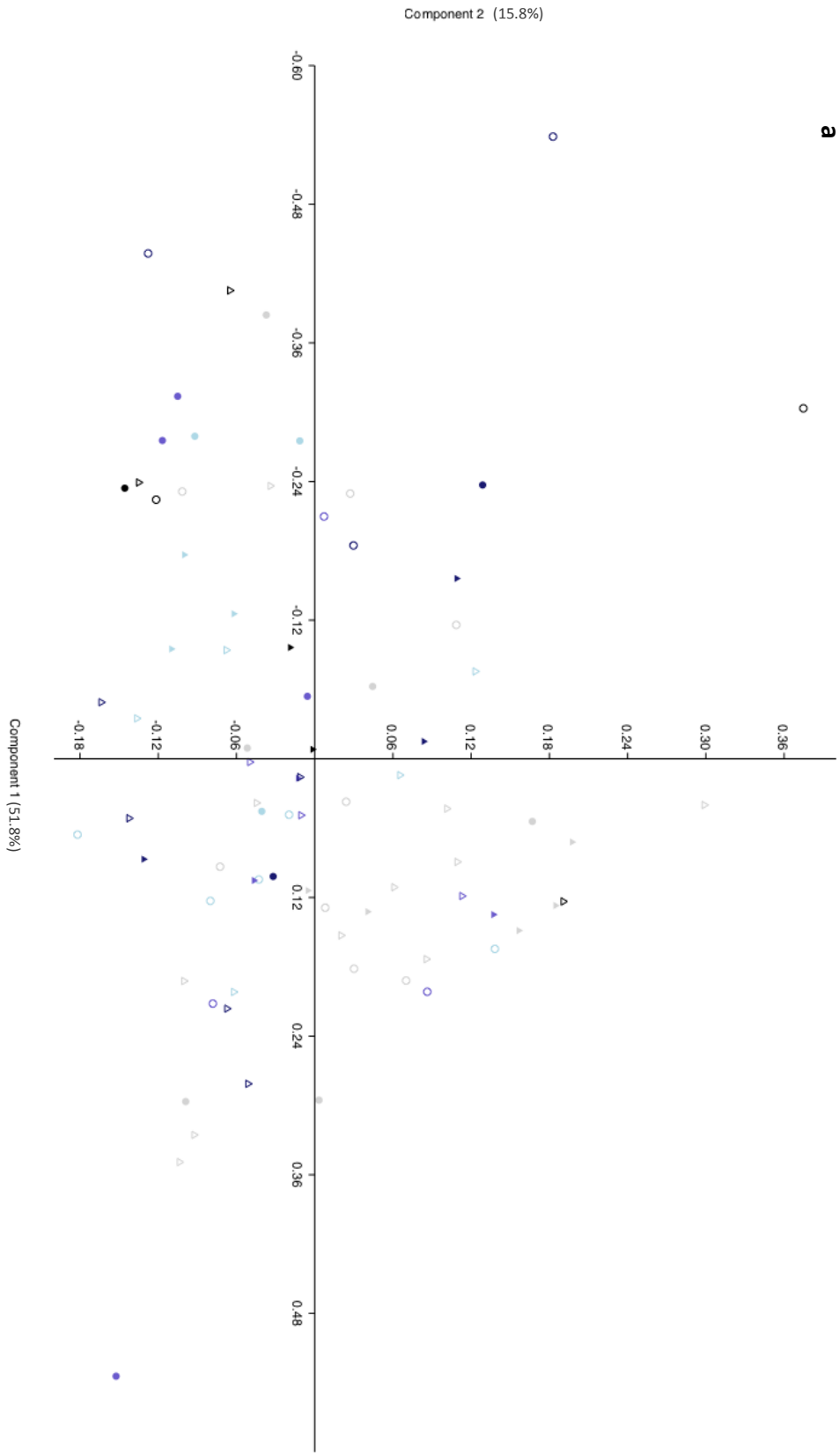
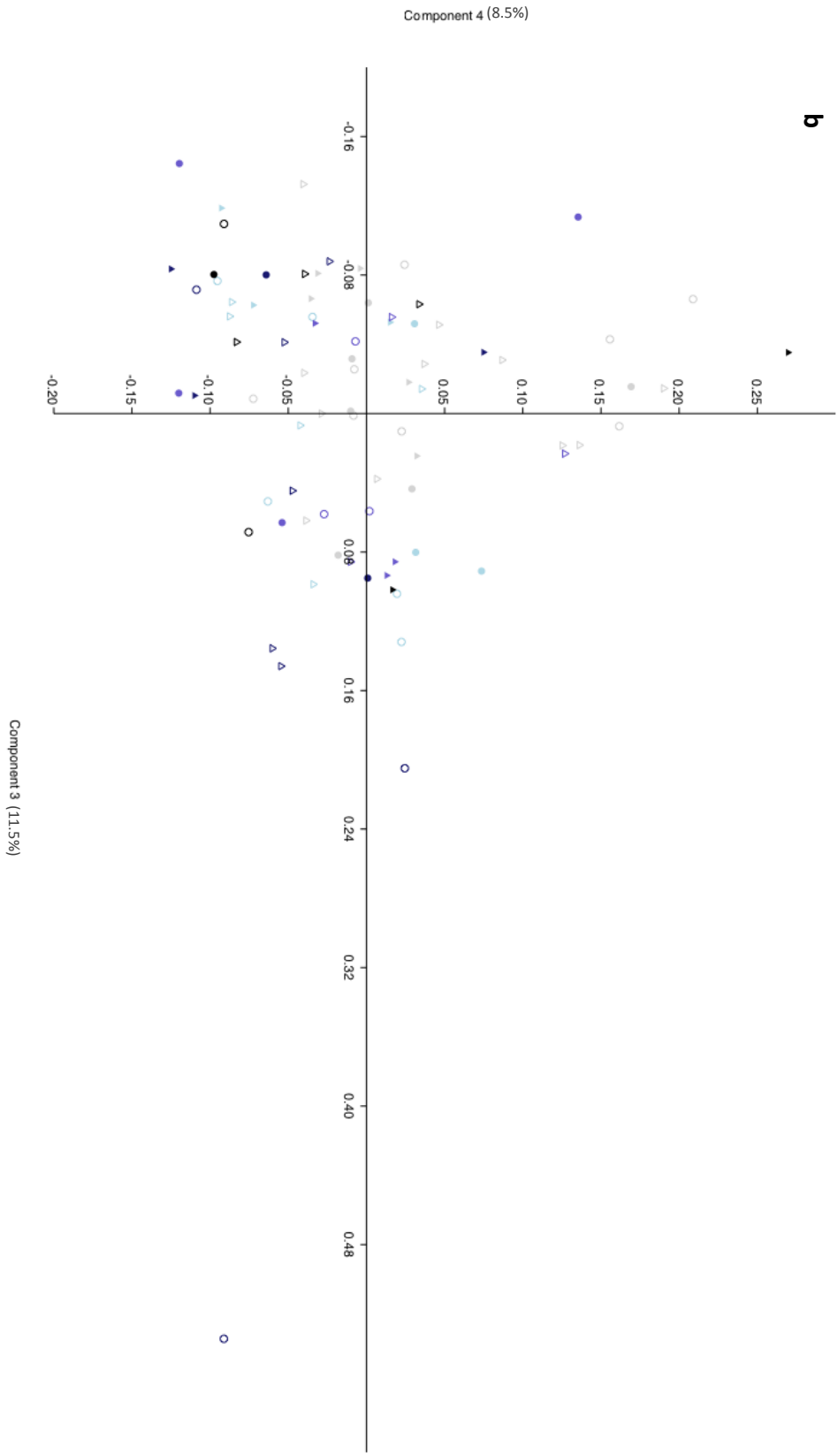
	Wild Type		SERT Knockout	
	Stress	No Stress	Stress	No Stress
Pre Pregnancy	11	5	8	6
E13.5	6	2	5	3
E15.5	3	3	3	4
E18.5	5	3	4	2
Postnatal Day 0	3	2	2	1

Table 1. n per sample group.


a
Component 2 (15.8%)
Component 1 (51.8%)
-0.60
-0.48
-0.36
-0.24
-0.12
0.12
0.24
0.36
0.48
-0.18
-0.12
-0.06
0.06
0.12
0.18
0.24
0.30
0.36


b
Component 4 (8.5%)
Component 3 (11.5%)
-0.16
-0.08
0.00
0.08
0.16
0.24
0.32
0.40
0.48
-0.20
-0.15
-0.10
-0.05
0.05
0.10
0.15
0.20
0.25

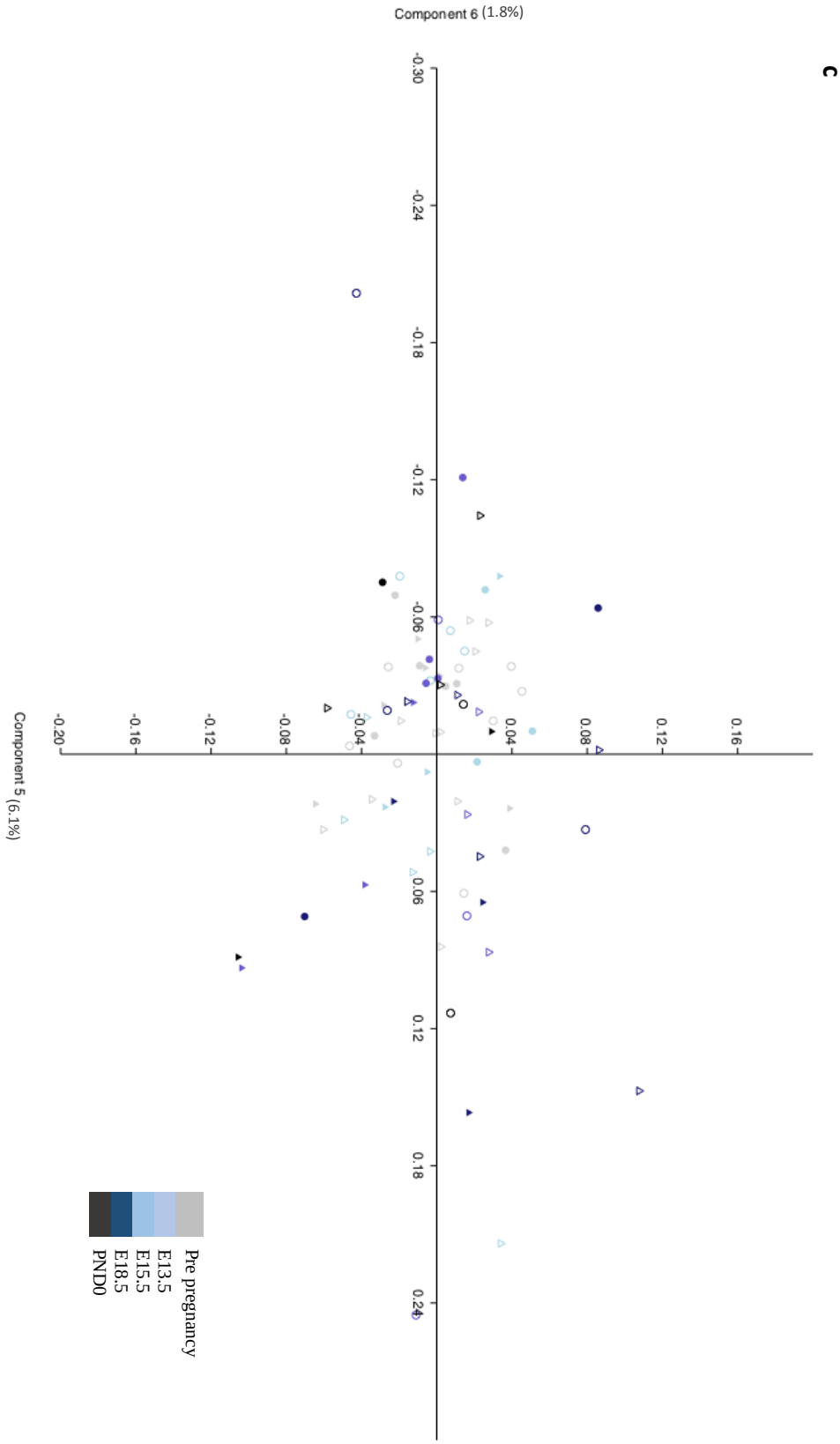


Figure 1a-c. PCAs accounting for ~96% of variance. Filled triangle = wild type x no stress. Empty triangle = wild type x stress. Filled circle = SERT knockout x no stress. Empty circle = SERT knockout x stress.

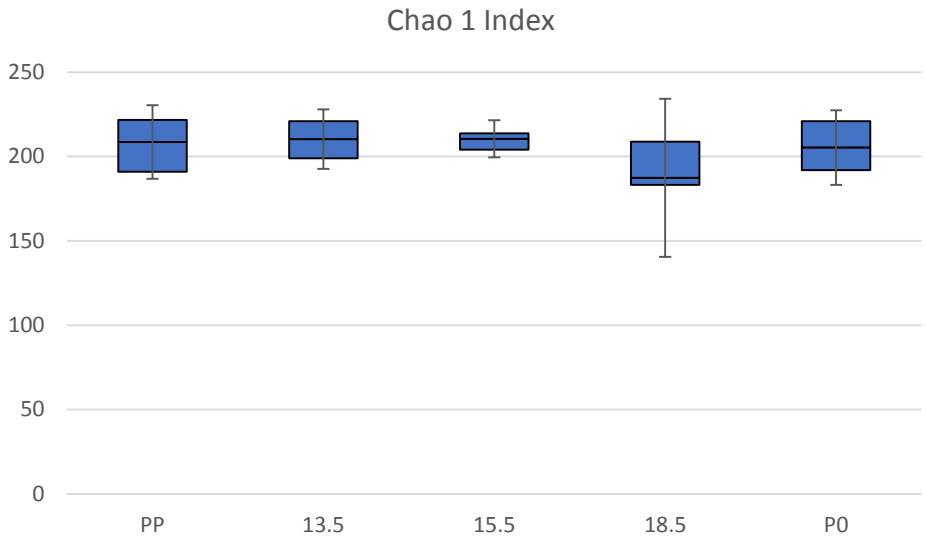


Figure 2. Chao1 Index of richness at each sampled time-point. All treatment groups combined.

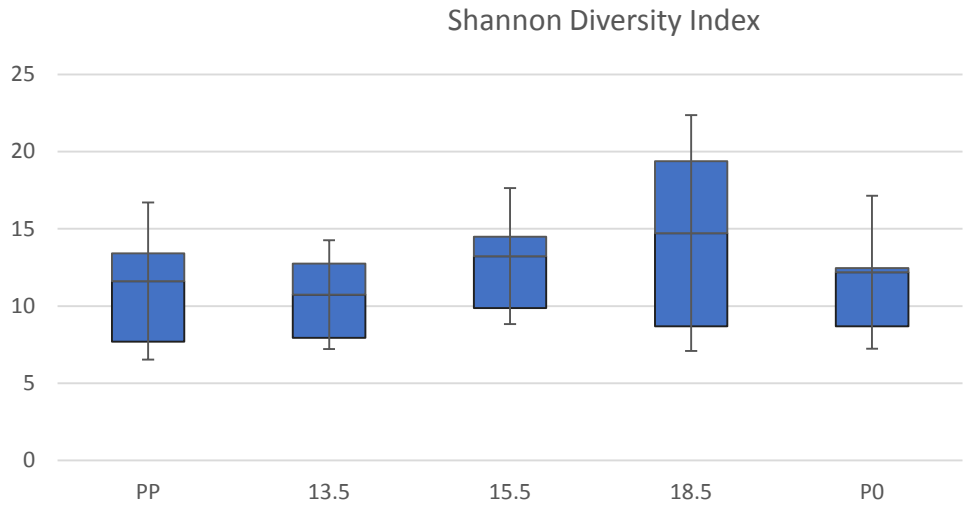


Figure3. Shannon Diversity Index at each sampled time-point. All treatment groups combined.

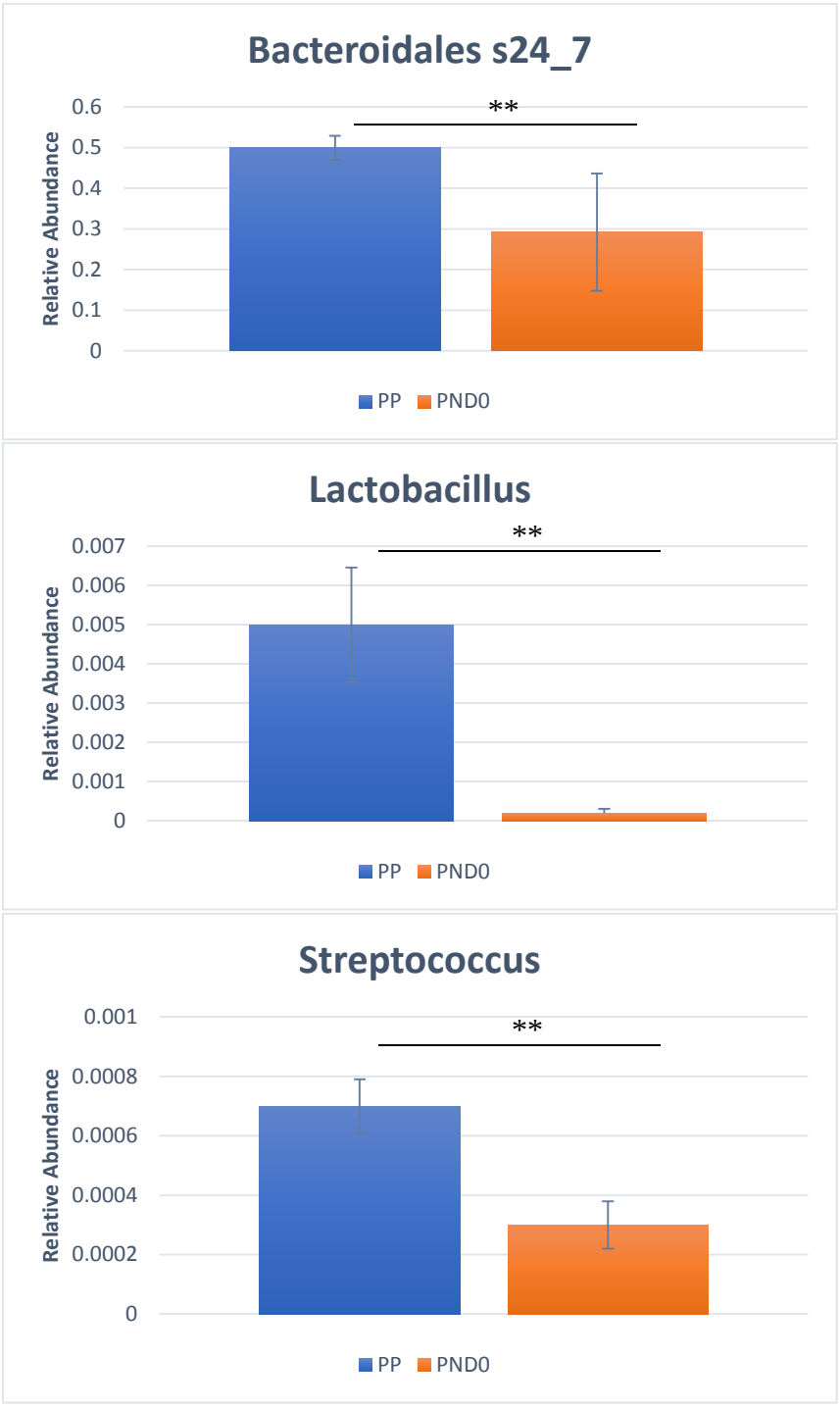


Figure 4. Relative abundance for individual OTUs found to significantly differ between pre-pregnancy and day of delivery time-points. (**p < 0.01) All treatment groups combined.