Exploring the role of the gut-brain axis in infant brain and behavioral development

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Table of Contents

Dedication

To all of the babies, may your lives be filled with happiness and simple pleasures.

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Abstract

Background: Research is beginning to accumulate suggesting that the gut microbiome may play an important role in early postnatal brain and behavioral development. Incorporating information regarding the gut microbiome into psychobiological research thus promises to shed new light on how individual differences in brain and cognitive development emerge. Here, we examined the link between gut microbiome diversity, functional brain network connectivity, and behavioral temperament in newborn infants.

Method: Newborn infants were recruited from a local hospital as part of a larger longitudinal study. Stool samples were collected and sequenced using shotgun metagenomic sequencing. Using a resting-state functional near-infrared spectroscopy (rs-fNIRS) procedure, we measured brain connectivity in three functional brain networks (fronto-parietal network, default mode network, homologous-interhemispheric network) and one (non-functional) control network. Infant temperament was assessed using parental report.

Results: Our results show that functional connectivity networks are linked to behavioral temperament traits already within the first weeks of life. Moreover, we test and provide evidence for a potential mechanism by which the gut microbiome composition is linked to functional connectivity networks in the brain that in turn influences infant behavioral temperament.

Conclusions: The current results suggest that already in newborns a positive association between gut-microbiome diversity and functional brain connectivity patterns exists, highlighting the potential importance of the microbiome in human brain function. This study should thus be considered a first proof-of-principle study with newborns, which may serve as the foundation for systematic longitudinal work, deciphering the role of the gut microbiome in early human development and infant mental health.

Project Narrative

Within the human body, bacteria outnumber our own human body cells. These microscopic gut bacteria are not only crucial to everyday physiological functioning but also affect brain function and mental health through the gut-brain axis. From birth to age three, the gut microbiome changes from a relatively sterile environment to a diverse eco-system, yet little is known about how the microbiome affects developing brain function and psychological health during this sensitive period of development. This thesis aims to fill this gap by investigating whether and how the gut microbiome influences developing brain function and behavior.

Journal Publication Venues

Manuscript 1: A primer on investigating the role of the microbiome in brain and cognitive development
Published in *Developmental Psychobiology* in 2019
Link to manuscript: https://onlinelibrary.wiley.com/doi/abs/10.1002/dev.21778

Manuscript 2: A call for mapping the development of the microbiota-gut-brain axis during human infancy
Commentary published in *Brain and Behavioral Sciences* in 2019
Link to manuscript: https://search.proquest.com/docview/2257569672?pq-origsite=gscholar

Manuscript 3: Resting-state functional brain connectivity is associated with differences in newborn behavioral temperament
Currently Under Review at the *Journal of Child Psychology and Psychiatry*

Manuscript 4: Gut microbiota composition is linked to newborn functional brain connectivity and behavioral temperament
Currently in preparation

A primer on investigating the role of the microbiome in brain and cognitive development

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Abstract

Incorporating information regarding the gut microbiota into psychobiological research promises to shed new light on how individual differences in brain and cognitive development emerge. However, the investigation of the gut-brain axis in development is still in its infancy and poses several challenges, including data analysis. Considering that the gut microbiome is an eco-system containing millions of bacteria, one needs to utilize a breadth of methodologies and data analytic techniques. The present review serves two purposes. First, this review will inform developmental psychobiology researchers about the emerging study of the gut-brain axis in development and second, this review will propose methodologies and data analytic strategies for integrating microbiome data in developmental research.

Keywords: Brain development, Cognitive development, Gut-brain axis, Microbiome

A primer on investigating the role of the microbiome in brain and cognitive development

Within the human body, microorganisms, collectively called the microbiome, outnumber our own human body cells (Greenhalgh, Meyer, Aagaard, & Wilmes, 2016; Walker, 2013). In the gut microbiome alone, there are more than 1000 species that encode 200 times as many genes as the entire human genome (D'Argenio & Salvatore, 2015). This new knowledge about the human microbiome challenges existing views in multiple disciplines beyond biology, including concepts about the individual nature of the self (Rees, Bosch, & Douglas, 2018). Moreover, incorporating questions about the influence of the microbiome into research programs has the potential to significantly change the scientific landscape. The gut microbiome is thought to play a crucial role in everyday physiological functioning; and yet, relatively little is known about its specific contributions to health and disease (D'Argenio & Salvatore, 2015). With the emergence and improvement of next-generation genetic sequencing technology in recent years, the study of the microbiome has become feasible and more affordable. This increased access has kick-started large-scale scientific efforts to map the human microbiome, such as the *Human Microbiome Project,* funded by the National Institutes of Health (https://commonfund.nih.gov/hmp). These and future research efforts will help to uncover the role of the microbiome in human health and disease.

Given these advances in mapping the human microbiome, there is also growing interest in investigating how the microbiome affects developmental processes, specifically, brain and cognitive development (Borre et al., 2014; Carlson et al., 2018). Adding approaches that enable the study of gut microbiota to the developmental scientist's toolkit promises to shed new light on how individual differences psychological processes emerge. However, the investigation of how

the gut-brain axis influences development is still in its infancy and poses several challenges, including data analysis strategies. Considering that the gut microbiome is an eco-system containing millions of bacteria, applying traditional data analysis strategies is thus of limited use. The goals of the current review are: 1) to review the existing research investigating the role of the gut microbiome in brain and cognitive development, and 2) to provide an overview of research methodology and data analytic approaches utilized in the study of how the microbiome influences brain and cognitive development.

Investigating the gut-brain axis using animal models

A host of experimental evidence for the influence of the gut microbiome on brain development comes from animal studies comparing germ-free mice to conventional mice reared in a pathogenic-free environment (Heijtz et al., 2011). Germ-free mice do not have a microbiome, meaning that they are bacteria-, fungi-, and virus- free. These mice are created and maintained through specialized husbandry procedures, including birth through cesarean section and housing in sterile environments (Faith et al., 2010; Stilling, Dinan, & Cryan, 2014). Pathogen-free mice are reared through the most common husbandry practices, which includes screening to ensure that they are free from a specific list of disease-causing agents that would interfere with mouse health. In contrast to germ-free mice, pathogen-free mice are housed in bacteria-rich environments and maintain a diverse microbiome.

Germ-free mice exhibit marked physiological and behavioral differences from their conventional, pathogenic-free counterparts. More specifically, germ-free mice show a number of brain differences when compared to conventional mice, such as a significantly increased brain volume, decreased integrity of the blood brain barrier, increased serotonin synthesis, and increased myelination (Heijtz et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011).

Furthermore, a series of studies have shown that, compared to conventional mice, germ-free mice display significantly reduced levels of anxiety-like behaviors resulting in increased risk-taking behaviors, such as increased open field exploration (De Palma et al., 2015; Hsiao et al., 2013).

The use of the germ-free animal model has illustrated the importance of the microbiome in psychobiological development. Specifically, the microbiome has been implicated in behavioral responses to early life stress. For example, germ-free mice but not pathogen-free control mice exhibit reduced species-typical anxiety-like behaviors in response to maternal separation (De Palma et al., 2015). This lack of anxiety seen in germ-free mice is thought to represent an aberrant response to the real-life threat of maternal separation. Moreover, the germ-free animal model has been used to study psychobiological effects of particular bacterial genera through administration during key phases during development. For example, Sudo and colleagues (2004) found that germ-free mice had increased release of corticosterone in response to an acute restraint stressor when compared to specific pathogen-free control mice. This study further showed that, in the germ-free mice, the HPA-axis response returned to normal levels after administration of *Bifidobacterium infantis*, whereas administration with *Escherichia coli* was associated with hyperactivity of the HPA axis. Critically, Sudo and colleagues also found that when during postnatal development administration occurred played an important role. In particular, this study showed that the administration of *Bifidobacterium infantis* was only able to return HPA axis activity to normal levels if administered to the mouse pups by six weeks (prior to sexual maturity) but not later during development (at eight weeks of age; the onset of sexual maturity). This research thus points to the possibility that there might be a sensitive period, prior to adolescence, when the microbiome may have the greatest impact on the development of the

stress system. Considering this kind of evidence it appears important to systematically investigate the role of the gut-brain axis in early psychobiological development.

Evidence for gut-brain axis in human models

Preliminary evidence for the existence of the gut-brain axis in humans comes from correlational studies, showing that delivery method and breastfeeding influences the colonization of the gut with bacteria. These differential patterns in colonization may in turn, have downstream effects on psychological development. For example, vaginal birth has been shown to expose infants to a larger diversity of bacteria in the birth canal than seen in infants delivered by caesarean section, who predominately receive bacteria from their mothers' skin (Dominguez-Bello et al., 2010). Feeding method also appears to be a contributor to the type and diversity of bacteria that inhabit the infant gut. For instance, breastfeeding provides infants with both bacteria and prebiotics, or nutrients that support bacterial growth, leading to a larger number of keystone (health-promoting) bacteria in breastfed when compared to formula-fed infants (Heikkilä & Saris, 2003).

It is important to emphasize that direct causal links between gut bacteria, brain, and cognitive development have not yet been established in humans. Nonetheless, there is correlational evidence from epidemiological studies suggesting that delivery method and breastfeeding, which as outlined above affect changes in the microbiome, also impact brain and cognitive development in infants. A recent meta-analysis found that infants delivered by cesarean section, when compared to those delivered vaginally, show a modest increase in the risk of developing Autism Spectrum Disorder (ASD) and Attention Deficit/Hyperactivity Disorder (ADHD) (Curran et al., 2015). Similarly, in animal models, mice that are born through cesarean section when compared to mice delivered vaginally, display increased repetitive behaviors and

atypical social behaviors, characteristic of these neurodevelopmental disorders (Borre et al., 2014). Moreover, this is in line with the human studies showing differences in bacteria composition between children with ASD when compared to neurotypical children. Specifically, children with ASD show distinct patterns of broad classes of bacteria composition with an increase in some toxin-producing bacteria, such as *Clostridia* (Finegold et al., 2002; Parracho, Bingham, Gibson, & McCartney, 2005). However, research concerning ASD is inconsistent because other bacteria genera, such as *Bacteriodetes,* are reported as increased prevalence in ASD children one study and not increased in another study (Son et al., 2015; Tomova et al., 2015).

Breastfeeding, in addition to affecting the colonization of the infant gut with microbes, has also been shown to impact brain and cognitive development in infants (see Krol & Grossmann, in press, for a review). Specifically, there is evidence suggesting that the absence or short duration of exclusive breastfeeding might be associated with the development of ASD. For example, a recent meta-analysis reports that those children diagnosed with ASD were significantly less likely to have been breastfed when compared to typically developing children (Tseng et al., 2017).

These associations seen between delivery and feeding experiences among infants and developmental outcomes obviously do not provide direct evidence that the microbiome is influencing brain and cognitive development. Moreover, there could be a host of alternative explanations for these results, one being that both breastfeeding and delivery method may impact the development of the oxytocin system; and consequently, the neurohormone oxytocin has been linked to various outcomes in social behavior (Carter, 2014). Nonetheless, given that changes in the microbiome are associated with breastfeeding and vaginal birth, it is likely that the associated

microbiome changes are relevant to psychological development in infancy and beyond. Developmental work, which directly assesses the role of the microbiome in early brain and cognitive development, is needed to arrive at a more mechanistic understanding of how the gut-brain axis functions in early development.

Direct assessment of how the human gut microbiome impacts cognitive development in infancy

To date, there is very little work in humans that has directly assessed the relation between the gut microbiome and early brain and cognitive development. Only very recently, Carlson and colleagues (2018) took a first step by assessing gut microbiome composition at 1 year of age, and testing the association with cognitive and motor development (measured by the Mullen Scales of Early Learning), and with brain volume (measured using structural Magnetic Resonance Imaging [MRI]), at both 1 and 2 years of age. This study characterized the gut microbiome composition in two ways: 1) using the mean bacteria species diversity per individual (alpha diversity) and 2) using cluster analysis, which identified three major groupings across infant microbial composition based on differences in the abundance of three key bacteria genera *Faecalibacterium, Bacterioides,* and *Ruminococcacea* (grouped with unclassified genera). Carlson et al.'s (2018) study shows that infants' overall score on cognitive and motor development tasks, the Early Learning Composite Score, differed significantly between the three groups. Specifically, infants with a relatively high abundance of *Bacterioides* achieved the highest score, whereas infants with a relatively high abundance of *Faecalibacterium* showed the lowest score with respect to their cognitive and motor development. Moreover, findings revealed that when the analysis was focused on specific subscales of the Mullen Scales of Early Learning, the difference across groups was most prominent with respect to their receptive language scores.

In addition, Carlson et al. (2018) report structural brain differences, whereby infants in the group with a relatively high abundance of *Bacterioides* showed a larger right superior occipital gyrus at age one but smaller caudate nucleus when compared to infants in the other two groups. On the one hand this study suggests that there are some specific structural brain differences; however, it should be noted that the majority of structural brain measures such as intracranial volume, total white or gray matter, total cerebrospinal fluid, or lateral ventricle volume did not reveal any differences between infants in the different bacterial composition groups. Moreover, from these data it is unclear how these differences in brain structure are linked to brain and cognitive function.

Carlson et al.'s (2018) study also showed that gut microbial diversity at the age of one year predicted cognitive development at the age of two years. The longitudinal association found in this study was such that increased microbial diversity was associated with lower cognitive performance as measured in the Early Learning Composite Score and lower scores on the specific subscales of visual reception and expressive language. This finding is surprising considering that higher microbial diversity in adults has typically be shown to be predictive of positive health outcomes (Abrahamsson et al., 2014; Kostic et al., 2015). Carlson and colleagues (2018) suggest that microbial diversity may affect cognitive functions differently in infancy than later in development. This points to the importance of developmental research which maps associations between microbial characteristics and brain and cognitive development across the human lifespan. In the following, we would like to briefly outline how researchers may use new methodological and statistical approaches to explore the influence of the microbiome on brain and cognitive development.

Generating microbiome data

After reviewing existing research on the role that the microbiome may play in brain and cognitive development, this section of the review will discuss sampling, data collection, and genomic sequencing of microbiome data for use in psychobiological research. Here, we outline how the microbiome is collected, sequenced, and processed in a data stream to address questions about composition and function of microbes.

Collecting microbiome data

There are several methods for collecting microbiome samples from study participants. The two major collection methods for assessing the microbiome of the distal gastrointestinal tract, a proxy for understanding the community structure of the gut, are rectal swabbing and collection of a stool/fecal samples. A rectal sample includes the participant utilizing a small q-tip swab after a recent bowel movement to collect the microbiota that are more focused at the rectum. Participants should insert the swab approximately 1-2 centimeters beyond the rectum for optimum collection (Bassis et al, 2017). For fecal samples, sterilized containers with small scoops should be used for cleanliness. Tools, such as toilet inserts to catch samples, can help participants to feel comfortable with collection. Moreover, infant researchers may ask parents to bring in a diaper (note, researchers may choose to provide a sterile plastic insert to parents to put into the diapers to optimize collection) and transfer the sample from the diaper to a storage container in the lab. Both fecal samples and swabs should be labeled appropriately with date and time of collection and study identification number. In the interest of gathering more robust data for fecal samples, charts such as the Bristol Stool chart (BSC) can be used to allow participants to classify their sample into 7 distinct types illustrated by representative pictures of various textures and consistencies. Classification of the types using the BSC, including amount and consistency, is important because early research has identified stool consistency being associated

with gut microbiota richness and composition (Vandeputte et al, 2016). Complete sampling kits, with all the necessary materials, are available for purchase from a wide range of medical and research distributors. Both methods, including swabs or fecal samples, should be considered for research and the specific method should be selected on the basis of participant population (e.g, for infants, fecal samples may be easier to collect and may have higher compliance from families as compared to swabs), resources available to data collection team, and consultation with the data sequencers.

After collection, storage options can vary depending on study question and availability. Options include liquid buffers to help stabilize samples and long-term freezing (typically in temperatures ranging from -80 to -4 degrees celsius). Most importantly, consideration should be used when freezing and thawing samples as this could have an effect on bacterial growth and/or DNA damage (Hugerth & Andersson, 2017). With the expansion of large-scale cohort studies and biospecimen banking, long-term freezing is common to ensure sample availability for future research. A recent comparison study found that these storage methods, including freezing temperature and stabilizing agents, can be used interchangeably with similar diversity metrics (Bassis et al, 2017). However, for consistency, studies should utilize the same technique for all samples.

Sequencing microbiome and processing samples

A gut microbiome sample, either a stool sample or a rectal swab, can be sequenced in two major protocols that result in different types of output data. The first and most common method to studying taxonomy and phylogeny of the microbiome, due to cost and efficiency, is 16s RNA sequencing (Janda & Abbott, 2007). In this method, short strands of DNA called primers, which are designed to target specific variable regions of the 16s ribosomal RNA gene, are used to

classify taxonomic units of microbiota within a sample (Illumina, 2018). The 16s RNA gene is highly conserved, or passed through generations, and acts as a microbe-specific genetic signature. The protocol begins with purified DNA from the fecal samples (Illumina, 2018). Primers are tagged with indexing barcodes and samples are pooled into a single library, or a collection of the primer nucleic acid targets, for sequencing (Illumina, 2018). Taxonomic profiling on the Illumina MiSeq system, a type of popular sequencing equipment and the industry standard platform, is typically cycled to generate paired 250-base pair reads in each protocol (Illumina, 2018). Other platforms include the Roche 454 GS FLX and the Ion Torrent PGM which both include different library preparations, procedures for barcodes and adapters as well as amplification (Allali et al, 2017). A recent study found that microbiome community profiles were comparable across platforms but that the relative abundance of specific microbiota varied depending on the sequencing platform, library preparation procedures, and analytic approach (Allali et al, 2017). However, the longer read lengths provided by the Illumina platform offer a high-quality analysis of the rRNA gene to ensure the most accurate classification available. Additionally, because chimeric sequences, sequences originating from two transcripts, and mismatched primers are considered to be contaminant within the analysis, they are filtered out using the standard Human Microbiome Project search and clustering program, USEARCH (Shaw et al, 2017). Raw sequence data in the form of fastq files are the output product of this processing pipeline, which can then be entered for further analysis.

Once the sequencing is completed, 16S rRNA gene sequence data in the form of fastq will be input into the Quantitative Insights Into Microbial Ecology (QIIME) 1.8.0 software package (Caporaso et al., 2010). QIIME is a big data, open-source software built for microbiome analysis from raw fasq sequencing data on Illumina platforms. QIIME groups the genomic

sequence into operational taxonomic units (OTUs). OTUs are groups of similar 16s sequences that become proxies for a species of a microbe (Caporaso et al., 2010). OTUs group the genomic sequences to identify which taxonomic group it belongs to. Genome reference databases such as Green Genes should be used to provide standardization of OTU assignment with publically available and published taxonomies (DeSantis et al., 2006). In addition to QIIME, several other bioinformatics packages are available including mothur and MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST). Both MG-RAST and mothur offer a comparable data processing pipeline for 16s microbial comparisons. Another recent bioinformatic comparison study found that researchers arrived at largely comparable results regardless of which of the three existing pipelines were used (Plummer et al, 2015). It is worth noting that the main difference revealed by the pipeline comparison carried out in this study was the significantly increased computational speed for QIIME compared to mothur and MG-RAST, taking approximately 1 hour, 10 hours, and 2 days respectively for processing a sample of 35 specimens (Plummer et al, 2015). Similar to the sequencing methods, for the purposes of this primer, the focus will be on processing with QIIME due to its widespread use.

From QIIME, data can be read into R to be manipulated using a package called ‘phyleoseq’. A full microbial analysis workflow is provided open access through Bioconductor (Callahan et al, 2016). Bioconductor in R is the most common package for bioinformatic analysis with inclusion of packages such as ‘dada2’, ‘phyloseq’, ‘DESeq2’, ‘ggplot2’ and ‘vegan’ to normalize, visualize, test, and compare microbial data samples. At this point, questions about community analysis, including which microbes are present and how do they compare in abundance to others, can be elucidated.

SixteenS rRNA sequencing is not the only method for extracting valuable insight from microbiome data. Metagenomic sequencing, also known as shotgun metagenomics, uses next-generation sequencing technology to understand functional gene composition rather than just viewing the 16s RNA conserved gene (Thomas et al., 2012). The sequence pathway begins with extracting DNA from the fecal samples similar to 16s. By sequencing the community DNA and comparing it to reference gene catalogs, metagenomics offers improved precision and allows for genetic observation of variants such as single nucleotide polymorphisms (Morgan & Huttenhower, 2012). Function can then be assessed and assigned using other publicly-available databases like the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Kanehisa et al, 2008). The ability to sequence the entire genome comes at a significant expense, which may double the costs of 16S sequencing. With the additional cost and expertise comes the ability to generate more data to elucidate information on not only community structure and prevalence of microbiota but also on the function of the microbes present (Sharpton, 2014). Metagenomic profiling can offer answers to questions of not only 'what microbiota are present?' but also 'what can the community do?'. For the purposes of this primer, we have outlined methodologies to approach microbiome research through the cost-effective and widespread use of 16S sequencing.

Outlining the process of microbiome collection, sequencing, and the bioinformatics pathway for the raw data analysis as done here in brief is important in preparing microbiome data to be used in a research study. Once the taxonomic data, including which species of bacteria are present within the samples, has been identified, further data analysis can be pursued to answer questions of developmental and clinical relevance.

Analysis using data science methods to analyze the human microbiome

In the following section, we will discuss suitable research data science methods including machine learning, data mining, and deep learning that can be applied to explore heterogeneous microbiome data sets. The introduction of such analysis techniques to study the role of the microbiome in human development has the potential to capture the complexity, allow for relatively unbiased statistical inferences, generate testable predictions, and ultimately, may result in clinical applications.

Traditional approaches and correlational statistics

The primary output from microbiome sequencing processing is counts of genomic reads that are taxonomically assigned to specific microbiota through reference genome sets. This count data is often normalized or processed additionally to remove systematic variance in the data. The count data is then considered for each microbiota in terms of its abundance within a certain feature, in the case of 16S sequencing it is in terms of the taxonomic classification. The counts for each classification are typically reported as proportions, which reflect fractions of specific species rather than absolute abundances (Lovell et al, 2015). Unfortunately, these proportions are difficult to predict and interpret in relation to the absolute abundance and confounding factors within an environment (Gloor et al, 2017; Friedman et al, 2012). This is a particularly challenging problem for the investigation of maternal microbiome samples because of the known community and diversity changes in pregnancy related to hormonal fluctuations, which occur independently from microbial dysbiosis or pathology.

More than just understanding the role of read counts as data output and microbiota proportions, there are several other traditional methods that should be reconsidered as the science moves toward understanding more than just community composition. One particular issue is the reliance on proportions as the major data analytic method when analyzing microbiome data --

this results in the assumption that abundance, or relative low abundance, is the key driver for functional differences. In addition, many standard statistical approaches assume an independence between microbiota, which does not exist (see Xia & Sun, 2017).

Data mining

As an overarching method comprised of several approaches in data science, data mining has become a popular computing and statistical process to discover patterns in large, mixed source datasets (Hendler, 2014). Data mining helps to explore information in large datasets where patterns emerge, which cannot be adequately captured by traditional linear regression models because of the highly non-linear complexity present within the data (Zhang & Zaki, 2006). We will now briefly describe some of the existing data mining techniques and in turn discuss the use of simulated data, time analysis, and clustering.

Simulated data. Simulated data is a particularly powerful data mining technique when applied prior to large-scale, cost-intensive experimental studies as it can help formalize conceptual models that can then be tested empirically. Step-by-step workflows are available to assist researchers in creating simulated data for specific purposes (Hallgren, 2013). Available coding software, such as R and python, can be used to simulate data according to a formalized model before investing in large-scale experimental studies. Implementation of simulated data in the context of a relevant research questions can help with answering specific questions in model building, estimation of beta coefficients, and better tuning of parameters of machine learning algorithms such as gamma values or learning rates (Schloss, 2008; Chen, 2012). For example, simulations of what parameters of the microbiome in an animal model (mouse) impact a given outcome such as social behaviors seen in ASD, could be used to help design experimental studies with humans. The largest limitation of this method is that the use of simulated data critically

relies on prior information, which is needed to build a simulation model. Considering that, apart from animal models, very little prior information is available in human translational research limiting the utility of this technique until more literature is published in this area.

Time Analysis. Previous research in humans and model organisms have predominately collected and analyzed microbial data cross-sectionally (Caporaso et al, 2010; Parks et al., 2014; Fukuyama et al., 2017). However, in order to arrive at a mechanistic understanding of microbial influence on outcome variables, it is of critical importance to understand how microbial patterns change in development and in response to certain events or interventions (Faust, et al., 2015), making longitudinal research designs the method of choice for fostering such an understanding (Morgan & Huttenhower, 2012). For example, an important unanswered question is how does the human microbiome change due to feeding and mode of delivery, and whether and how do these changes in the microbiome predict brain and cognitive development in children. New computational tools (software packages) have emerged to help visualize microbial time-series data, which can also be applied to longitudinal data. One such application is Temporal Insights into Microbial Ecology (TIME), a web-based software for longitudinal microbiome data analysis, offering a wide range of input data types and capability to identify potential taxonomic markers through analysis and visualization (Baksi, Kuntal, & Mande, 2018). Another web-based software tool is called BURRITO (https://github.com/borenstein-lab/burrito), which also offers time-series based visualization and analysis, coupled with taxonomic and functional profiling to elucidate the contribution of the microbiota to a biological function such as neurotransmitter transport, GABA-A receptor agonists/antagonists or systemic inflammatory responses (McNally, Eng, Noecker, Gagne-Maynard & Borenstein, 2018; Kanehisa et al, 2008). Applying these

methods to longitudinal data promises to innovatively capture and visualize the link between microbial and developmental changes.

Clustering. Clustering is a common technique to describe the proximity between subjects or samples (Cameron, 2012). Interestingly, centroid-based clustering algorithms, such as k-means using euclidean distance metrics, which group samples based on distance to the computed centroid, have shown to perform well on clinical and simulated microbial datasets (Cameron, 2012). Beyond distanced-based clustering algorithms, other data science methods are also able to account for complex biological data. For example, hierarchical clustering, which is a set of descriptive techniques used for grouping by similarity, has been particularly useful when applied to metagenomic data (McMurdie, 2016). Clustering algorithms may help researchers to profile similarity across microbiome samples and identify boundaries based on function, and thus help uncover clusters of microbes that best characterize any given developmental outcome.

Machine learning

For developmental, psychological, and clinical researchers, machine learning algorithms have been proposed to be effective in addressing questions concerning classification and prediction of biological and behavioral variables (Yarkoni & Westfall, 2017). Large datasets can be used to train models to answer classification problems or provide probabilities of an outcome. This section outlines some techniques for machine learning and areas for exploration in this new domain that focuses on prediction rather than description.

Reduction of Features. Feature reduction methods are extremely important in high-dimensional datasets. One widely used technique in microbial analysis to achieve a reduction in relevant features is Principal Component Analysis (PCA), which uses orthogonal transformation to reduce features and create a smaller set of components (Meng, Zeleznik, Thallinger, Kuster,

Gholami et al, 2016). PCA relies on linear methodologies which may not best describe the underlying truth. However, feature reduction can also be harnessed through neural networks using autoencoders, which provide a neural network structure for unsupervised learning of encoded nodes (Tan, Hammon, Hogan & Greene, 2015). The encoded nodes represent a component of the original data. Tools such as the Analysis Using Denoising Autoencoders of Gene Expression (ADAGE), allow researchers to train an autoencoder on a dataset to derive nodes thereby reduce features to highlight highly-active genes (Tan et al., 2015). Autoencoding is particularly relevant in datasets with a large number of participants and a wide range of behavioral and brain measurements. From a data science perspective, high dimensional problems arising from such data sets are thought to be best addressed using autoenconding methods.

Classification. In terms of classification, random forest models tend to be popular because they have been proven to be high in their prediction accuracy (Touw et al, 2012). Identifying conditional relations, such as the presence or absence of a certain microbes accounts for the presence or absence of certain outcomes, are prime purpose for using random forest modeling. Random forest models are supervised learning algorithms that generate decision trees allowing for classification on the basis of deterministic rather than random relations between a certain microbe and an outcome variable (Touw et al, 2012). Random forest models can be thus be used to better characterize which microbial species or Operational Taxonomic Units (OTUs) are most important for a particular classification task. This could include a classification problems in cognitive and brain development of clinical relevance such as the diagnosis of ASD. This can be implemented in the data processing stream through packages available in R such as ‘randomForest’ to be used in conjunction with ‘phyloseq’, which allows for the general analysis and visualization of microbial communities.

Deep Learning. Due to the known complexity of microbiome and developmental data, other data science methods are needed to further pinpoint health or disease-relevant outcomes. Deep learning is a collection of machine learning methods that are designed to carry out non-linear algorithms in an artificial neural network. Similar to autoencoders, alluded to above, deep learning methods make use of multiple connected layers in which output from the previous layer is employed to denoise and reconstruct the original data, while unveiling nodes, or representations of the data. Importantly, deep learning is generally considered as one of the most rigorous data science methods also due to its unbiased (and non-linear) nature of capturing patterns in complex data sets. Deep learning can be applied to both OTU or metagenomic data and is typically implemented through python-based software packages such as Keras and Tensorflow, but it can also be realized in R.

Taken together, this brief summary of some of the available data science practices is intended to provide a general guide for what analysis strategies might be useful in studying microbiome effects on brain and cognitive development. The review of the data science practices presented here is by no means exhaustive. Moreover, to date, there is no standardized procedure or platform available that integrates across these data science practices, and it is important to emphasize that the exact data science-based approach to be employed has to be specifically tailored to the particular research questions being addressed.

Conclusion

The growing body of research reviewed here provides first insights into how the gut microbiome influences early brain and cognitive development. We have seen that incorporating information regarding the gut microbiome into psychobiological research promises to further our understanding of how individual differences in brain and cognitive development emerge. While

the investigation of the gut-brain axis in development is still in its infancy, we have argued that an approach using data science methods has the potential to help us make progress in describing and predicting how the gut microbiome, as an eco-system containing millions of bacteria, influences brain and cognitive development. Applying data science methods including machine learning, data mining, and deep learning to mixed methods microbiome data sets will advance the study of the gut-brain axis in early human development. By summarizing some basic principles in microbiome analysis, data analytics and its application to brain and cognitive development, this review is meant to offer a brief introduction into this new frontier in developmental psychobiology. This is done in the hope that this review will help inspire the bold research efforts needed in the coming years to realize advances in our understanding of the microbiome's role in development.

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A call for mapping the development of the microbiota-gut-brain axis during human infancy

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Abstract

We argue for the importance of looking at the microbiota-gut-brain axis from a human developmental perspective. For this purpose, we first briefly highlight emerging research with infants attesting that the microbiome plays a role in early brain and cognitive development. We then discuss the use of developmentally informed humanized mouse models and implications of microbiome research that go beyond probiotic administration.

A call for mapping the development of the microbiota-gut-brain axis during human infancy

In the target article, Hooks et al. critically review the current state of microbiota-gut-brain axis research in animal models and make specific suggestions on how to improve research in this area. However, Hooks et al. appear to have overlooked what might be considered one of the most promising avenues for moving research in this emerging field forward. Specifically, we would like to argue that the time is ripe to explore the role of the human microbiota in brain and cognitive development, especially during infancy (Kelsey et al. 2018).

From birth to age 3, the gut microbiome changes from a relatively sterile environment to a diverse ecosystem with thousands of species of bacteria, suggesting that this might represent a formative, and possibly sensitive, period in microbiota-gut-brain axis development (Borre et al. 2014; Walker 2013). The target article highlights initial support from animal models (e.g., Sudo et al. 2004), showing that the timing of bacterial colonization plays an important role in the development of the gut-brain axis, yet it fails to acknowledge existing evidence from humans, which further supports the notion that early development during infancy may critically shape the microbiome-gut-brain axis. For example, both delivery and infant feeding methods, which have been shown to affect the gut microbiome composition in infants, have also been identified as risk factors for early emerging neurodevelopmental disorders such as autism spectrum disorder (Curran et al. 2015; Dominguez-Bello et al. 2010; Heikkilä & Saris 2003). These epidemiological findings provide indirect, correlational evidence for a microbiota-gut-brain axis link in early human development.

More direct evidence for such a link comes from a pioneering recent study by Carlson et al. (2018) in which fecal samples were collected from 89 typically developing infants and analyzed using 16S ribosomal RNA amplicon sequencing. In this study, the link between infant gut microbiome composition at 1 year of age and cognitive development (measured by the Mullen Scales of Early Learning) and brain development (measured using structural magnetic resonance imaging [MRI]), at both 1 and 2 years of age were assessed. Carlson et al.'s (2018) analysis revealed that cognitive development scores differed significantly between infants assigned into one of three gut microbiome taxonomic groups, as identified by cluster analysis. This study also reports some specific structural brain differences linked to the microbiome composition. However, it should be noted that the majority of structural brain measures, such as intracranial volume, total white or gray matter, total cerebrospinal fluid, or lateral ventricle volume, did not reveal any differences between infants for the three bacterial composition groups. Moreover, from these data it is still unclear how the small volume differences found in specific brain areas are related to infant brain and cognitive function. Contrary to what is known from adults where higher microbial diversity has typically been shown to be predictive of positive health outcomes (Abrahamsson et al. 2014; Kostic et al. 2015), Carlson et al. (2018) showed that increased microbial alpha diversity was associated with lower cognitive performance in infancy. Based on this discrepancy, Carlson et al. (2018) suggest that microbial diversity may affect cognitive functions differently in infancy than later in development. In any case, the study by Carlson et al. (2018) as a first of its kind sheds new light on how individual differences in brain and cognitive development during infancy emerge in the context of the developing microbiome-gut-brain axis. Collectively, this points to the importance of

developmental research, which systematically maps associations between microbial characteristics and brain and cognitive development across the entire human life span.

Related to taking a human developmental perspective, another potentially overlooked research approach is underscoring the use of developmentally informed humanized mouse models in order to create more translatable research. In the target article, authors make a poignant argument that there are inherent issues when one tries to make inferences about human mental health disorders from studies with animal models. The authors suggest that this area of research often uses language that overextends the implications of germ-free mouse models and rodent behavioral tests to human mental health. However, they fail to mention an alternative methodological strategy to addressing the issue of translatability, which is by creating humanized mouse models (for a review, see Walsh et al. 2017). Specifically, fecal samples from humans can be taken from clinically relevant populations (with or without mental health issues) at different points during development (from newborns to aging populations) and transplanted into animals – thus creating developmentally informed animal models that allow for a more mechanistic study of the microbiome-gut-brain axis.

Finally, we would like to argue that the implications for research on the early development of the microbiome-gut-brain axis in humans extend well beyond the somewhat overemphasized field of probiotic research. Specifically, in the context of infant development, research in this field has potentially major implications for delivery and neonatal care procedures. For example, medical facilities have recently started to examine the health benefits of "seeding" procedures, whereby infants delivered via C-section are wiped with maternal vaginal swabs, with the hope of colonizing infants with more diverse groups of bacteria. Moreover, the benefits of breastfeeding on infant brain and cognitive development have been

widely studied and documented (Krol & Grossmann 2018). However, the gut microbiome has been largely ignored as a potential contributor to the positive effects of breastfeeding on infant and child development. Therefore, recognizing the need for incorporating a microbiome perspective in delivery and breastfeeding research with infants might help inform clinical practice. Taken together, this commentary is intended to emphasize the importance of looking at the microbiota-gut-brain axis from a human developmental perspective with a specific focus on infancy. In addition, this commentary is meant to encourage the use of humanized animal models to tackle translatability issues and realize implications of this work, which extend well beyond probiotic administration. Overall, the hope is to complement the target article by inspiring the bold research programs needed to systematically examine the microbiome's role in early human brain and cognitive development.

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Resting-state functional brain connectivity is associated with differences in newborn behavioral temperament

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Abstract

Background: Brain connectivity at rest has been linked to behavioral traits and mental health in adults. Even though brain network connectivity can be detected from early in infancy, little is known about how functional connectivity in these networks is linked to infants' behavioral traits.

Method: The present study examined a large group of newborn infants (N = 75; 57.3% Male) in order to examine the link between brain connectivity patterns and behavioral temperament. Using a resting-state functional near-infrared spectroscopy (rs-fNIRS) procedure, we measured brain connectivity in three functional brain networks (fronto-parietal network, default mode network, homologous-interhemispheric network) and one (non-functional) control network. Infant temperament was assessed using parental report.

Results: Our results show that connectivity in the fronto-parietal network was positively associated with regulation and orienting behaviors, whereas connectivity in the default mode network showed the opposite effect on these behaviors. Our analysis also revealed a significant positive association between the homologous-interhemispheric network and behaviors associated with negative affect.

Conclusions: The current results suggest that variability in brain connectivity, previously linked to mental health in adults, is associated with specific differences in infant behavioral temperament. These findings shed new light on the brain origins of individual differences in early-emerging behavioral traits and provide the basis for future research examining the long-term consequences of this brain-behavior correlation.

Resting-state functional brain connectivity is associated with differences in newborn behavioral temperament

Psychiatric disorders have been systematically linked to variations in the functional connectivity of brain networks measured at rest. For example, depression has been characterized by: (1) hypoconnectivity within the fronto-parietal network (FPN) implicated in the cognitive control of attention and emotion (2) hyperconnectivity within the default mode network (DMN) involved in internally-oriented thought/mind-wandering, and (3) hypoconnectivity within the homologous-interhemispheric network (HIN) involved in emotion regulation (Banich & Karol, 1992; Kaiser, Andrews-Hanna, Wager, & Pizzagalli, 2015; Patashov, Goldstein, & Balberg, 2019; Wang et al., 2013). From a developmental perspective, resting-state brain networks can already be detected early in human infancy. However, to date, little is known about whether and how functional connectivity in these networks is linked to early behavioral traits, especially temperament, which is considered a precursor to adult personality linked to mental health outcomes (Chronis-Tuscano et al., 2009; Pérez-Edgar & Guyer, 2014).

Temperament and Mental Health Outcomes

Within the first few weeks of life, infants begin to display individual differences in their behavioral temperament as indexed by characteristic differences in their emotional and attentional responsivity to situations and people (Rothbart, 2007). Variability in infant and child behavioral temperament traits can be readily and reliably assessed through parental report and has been linked to long-term psychological development and mental health outcomes (Rothbart, 2007). For example, children showing lower levels of regulation and higher levels of negative emotionality are significantly more likely to develop internalizing disorders, such as anxiety and depression, in adolescence and adulthood (Frenkel et al., 2015; Kushnir, Gothelf, & Sadeh, 2014). There is also evidence to suggest that infants with high levels of behavioral inhibition –

the tendency to avoid new situations and people – measured at 14 months of age, had up to a four-fold increase of developing anxiety issues by mid-adolescence (Chronis-Tuscano et al., 2009). In fact, it has even been argued that displaying a strongly inhibited behavioral temperament may not simply be a risk factor for the later development of anxiety disorder but rather represent a mild form of an anxiety disorder in children (for an overview describing this debate see Pérez-Edgar & Guyer, 2014). Together, this suggests that individual differences in early behavioral temperament can be considered developmental precursors and perhaps markers of psychological functioning and mental health. However, little is known about which brain processes at the network level may account for differences in infant behavioral temperament (for a review of the existing evidence for the neural correlates of temperament see Fox, Calkins, & Bell, 2008; Rothbart, Sheese, & Posner, 2007)

Measuring Brain Network Connectivity with Functional Near-Infrared Spectroscopy

As noted above, research with adults shows that brain connectivity measured at rest through functional magnetic resonance imaging (fMRI) is linked to individual differences in behavioral traits and mental health outcomes (Kaiser et al., 2015; Wang et al., 2013). Even though brain network connectivity can be detected from early in infancy (Imai et al., 2014), it is unclear how functional connectivity in these networks is linked to infants' behavioral temperament traits. Functional Near-Infrared Spectroscopy (fNIRS) offers a non-invasive, infant-friendly, optical neuroimaging technique for assessing functional connectivity in cortical brain networks during infancy (Homae et al., 2010). In the current study, we used fNIRS to characterize individual differences in spontaneous brain network activity in networks previously linked to depression in adulthood, in order to test if these networks can be considered an early-emerging neural endophenotype (Kaiser et al., 2015; Wang et al., 2013). More specifically, we

examined if differences in connectivity may be linked to differences in behavioral temperament (phenotype) relevant to long-term psychological functioning and mental health.

The present study had two major goals. First, we aimed to identify a series of distinct functional networks (FPN, DMN, HIN). Given previous work which has examined functional networks using fNIRS, we expected that activity within the three functional networks will show higher levels of connectivity than in the (non-functional) control network (Sasai, Homae, Watanabe, & Taga, 2011). Second, we examined whether and how variability in network connectivity maps onto individual differences in behavioral temperament, focusing on three critical dimensions of infant behavioral temperament (regulation/orienting, negative emotionality, positive emotionality/surgency) which have been previously identified in a factor analysis (Gartstein & Rothbart, 2003). Based on prior work with adults with and without depression (Kaiser et al., 2015), we expected that reduced regulation/orienting behaviors in newborn infants will be associated with hypoconnectivity in the FPN (previously linked to cognitive control of attention and behavior in adults) and hyperconnectivity in the DMN (previously linked to stimulus-independent thought and mind-wandering in adults). We also expected that hypoconnectivity in the HIN will be linked to higher rates of negative emotionality, based on previous findings linking reduced cross-hemispheric connectivity to increased incidence of depression in adults (Patashov et al., 2019; Wang et al., 2013). Critically, we expected to see these associations only for the functional networks and not for the (non-functional) control network. Finally, considering that there is no prior work to inform how surgency/positive emotionality is linked to network connectivity, we did not have a specific hypothesis regarding this trait, but still included it in our analysis because surgency/positive

emotionality has been identified as an important factor in previous work and may be of interest for future meta-analytic work (Gartstein & Rothbart, 2003).

Method

Seventy-five newborns (*M* [age] = 25 days; *Median* [age] = 24 days; ranging from 9 days to 56 days; 32 females; 43 males) were included in the final sample used for the present analyses. Participants were recruited from a local hospital. The obtained sample of infants were representative of the surrounding Mid-Atlantic college town area such that the majority of infants were Caucasian (*n* = 49 Caucasian; *n* = 14 Black; *n* = 3 South Asian; *n* = 3 Pacific Islander; *n* = 2 Asian; *n* = 4 Other), from highly-educated parents (*n* = 31 obtained a Graduate Degree; *n* = 19 Bachelor's Degree; *n* = 12 some College/Associates Degree; *n* = 11 High School Diploma/GED; *n* = 2 some High School), and medium-income families (*n* = 21 $15-45,000; *n* = 18 $75-110,000; *n* = 11 $45-75,000; *n* = 11 $110-175,000; *n* = 8 $175,000+; *n* = 5 less than $15,000; *n* = 1 did not respond). All participants were born at term, with normal birth weight (>2,500g), and did not have any hearing or visual impairments. Thirty-three additional infants were tested but were excluded from the present analyses for the following reasons: *n* = 29 were excluded because they failed to reach our pre-determined inclusion criterion of having at least 100 seconds of continuous data during which the infant was not crying; *n* = 4 were excluded because more than 33% of the measured fNIRS channels had poor light intensity readings, more specifically, a signal-to-noise ratio of less than 1.5 (Bulgarelli et al., 2019; Xu et al., 2015). Note that the current attrition rate (30%) is lower than in previous infant fNIRS studies (Cristia et al., 2013). All parents gave informed consent for their infants to participate in accordance with the Declaration of Helsinki and families received a payment for their participation. All procedures

were approved by and carried out in accordance with The University of Virginia Institutional Review Board for Health Sciences (Protocol number 20381).

Infant Temperament

Infant temperament was assed using parental reports of the 91-item Infant Behavior Questionnaire Revised Short Form (IBQ-R; Gartstein & Rothbart, 2003). Parents filled out the questionnaire online using Qualtrics survey platform prior to their appointment. This measure has been widely used and shown to be reliable and valid at the newborn time point (see the following papers for examples of prior work using this measure with newborns Rigato, Stets, Bonneville-Roussy, & Holmboe, 2018; Stifter & Fox, 1990; Worobey & Blajda, 1989). The questionnaire asks parents to report their infant's behavior during the previous two weeks and rate the occurrence/frequency of the behavior on a 1 (Never) to 7 (Always) scale. Based on prior work using factor analysis (Gartstein & Rothbart, 2003), three general temperament dimensions were computed summarizing information from various sub-scales: (1) negative emotionality (contributing sub-scales: fear, distress to limitations, falling reactivity, sadness), (2) regulation/orienting (contributing sub-scales: low intensity pleasure, cuddliness, duration of orienting, soothability), and (3) surgency/positive emotionality (contributing sub-scales: activity level, smiling and laughing, high intensity pleasure, perceptual sensitivity, approach, vocal reactivity) (Gartstein & Rothbart, 2003). If parents reported the behavior was not applicable at the current time then this item was given a value of 0. Chronbach's alpha coefficients were calculated to determine reliability of the temperament measures and all values were in acceptable ranges for each of the three dimensions: surgency/positive emotionality $\alpha = .78$, regulation/orienting $\alpha = .78$, and negative emotionality $\alpha = .91$.

Procedure

The resting state (rs)-fNIRS task took place in a quiet, dimly-lit testing area. Infants were seated on their parents' lap and placed approximately 60 cm from the screen (23-inch monitor). The newborns were fitted with a fNIRS fabric cap (EasyCap, Germany) which was secured in place using infant overalls and outside netting. The experimental paradigm was presented using the Presentation software package (Neurobehavioral Systems, USA). A non-social stimulus was created by selecting non-social clips from a popular infant video (Baby Einstein) that featured videos of toys, stuffed animals, and still images of everyday objects. This video was played for a total of seven minutes while fNIRS data were being recorded. The clips were segmented into 30 second intervals and the order of presentation was randomized for each infant. Parents were asked to remain quiet throughout the fNIRS recording session. Sessions were video-recorded using a camera mounted above the screen. This allowed for later offline coding of infants' alertness.

Data acquisition

Infants' fNIRS data were recorded using a NIRx Nirscout system and NirStar acquisition software. The fNIRS method quantifies concentration changes of oxygenated hemoglobin (oxyHb) and deoxygenated hemoglobin (deoxyHb) in the cerebral cortex through shining specific frequencies of light that are selectively absorbed by these chromophores (for more information regarding this technique see Lloyd-Fox, Széplaki-Köllőd, Yin, & Csibra, 2015). The fNIRS system used contains 16 source-detector pairs (approximately 2.5 cm apart) resulting in a total of 49 channels positioned over frontal and temporal-parietal regions (see Altvater-Mackensen & Grossmann, 2016; Grossmann, Missana, & Krol, 2018; Kelsey, Krol, Kret, & Grossmann, 2019; Krol, Puglia, Morris, Connelly, & Grossmann, 2019 for infant work using the identical channel positioning/layout). The system emits two wavelengths of light in the Near-

Infrared spectrum, 780 nm and 830 nm, to capture deoxyHb and oxyHb, respectively. The diodes have a power of 20 mW/wavelength and data were recorded at a sampling rate of 3.91 Hz.

Behavioral Coding

Infants' behavior during the fNIRS recording session was coded by a trained research assistant using video recordings of the experimental session. In line with previous studies, infants were only included in the present analysis if they had at least 100 seconds of continuous data during which the infant was not crying (Bulgarelli et al., 2019). On average, infants contributed 317.59 seconds of data (*SD* = 115.46 seconds).

Data Analysis

The fNIRS data were analyzed using the functional connectivity program, FC-NIRS (Xu et al., 2015). First, channels were assessed for light intensity quality and channels were removed if the signal-to-noise ratio was less than 1.5 (Xu et al., 2015). In order to be included in the present analyses, infants needed to have at least 66% of their channels passing this threshold (Bulgarelli et al., 2019). Next, data were band-pass filtered (using a .08 Hz low-pass filter, to remove fast fluctuations related to heart rate, and a high-pass filter of .01 Hz, to remove changes that were too slow and related to drift; Bulgarelli et al., 2019; Lu et al., 2009). This range of .01 to .08 Hz was chosen on the basis of prior work (Bulgarelli et al., 2019; Sasai et al., 2011). This range was also selected because it falls well below the reported range for cardiac fluctuations (greater than 1 Hz), providing us with greater confidence that the measured changes reflect hemodynamic events tied to cortical activity rather than (systemic) cardiovascular system activity (e.g., heart rate Elwell, Springett, Hillman, & Delpy, 1999; Obrig et al., 2000). Finally, concentration changes were calculated using the modified Beer-Lambert law (Villringer & Chance, 1997).

For each infant, we obtained a 49 by 49 correlation matrix corresponding to all of the relations between all of the channels measured. Considering that negative values are difficult to interpret in terms of their neurobiological basis, and based on prior work, we replaced all negative correlation values with zeros (Fox, Zhang, Snyder, & Raichle, 2009; Murphy, Birn, Handwerker, Jones, & Bandettini, 2009). In order to standardize the values, Fisher Z-transformations were performed on all correlation matrices. Networks of interest were created by selecting channels that corresponded to specific regions of interest. Brain networks were composed based on the anatomical information available in Kabdebon et al. (2014), a meta-analysis of resting state fMRI (Kaiser et al., 2015), and prior work using rs-fNIRS (Patashov et al., 2019; Sasai et al., 2011). Based on this information four networks were created: (1) The FPN was created by averaging all correlations between three channels in the dorsolateral prefrontal cortex (corresponding with the F3, F4, F5, F6 electrodes) and two channels in the parietal area (corresponding with CP3 and CP4 electrodes); (2) The DMN was created by averaging all correlations between three channels in the medial prefrontal cortex (corresponding with the Fpz electrode) and four channels in the superior temporal cortex (corresponding with FT7, T7, FT8, T8 electrodes); (3) The HIN was created by averaging all correlations between the 21 channels in the left hemisphere (including frontal, temporal and parietal cortical regions) with their corresponding (homologous) channels in the right hemisphere; and, (4) a (non-functional) control network was created by averaging all correlations between three channels in the left frontal area (corresponding with the F7 electrode) with three channels in the right temporal area (corresponding with the T8 electrode) and three channels in the right frontal area (corresponding with F8 electrode) with three channels in the left temporal area (corresponding with the T7 electrode; see Figure 1 for schematic of network configurations). All analyses were conducted

for both oxyHb and deoxyHb (for deoxyHb results please see supplemental materials). Moreover, statistical outliers – values that were more than 3 SD above the mean – were removed for the subsequent analyses (FPN $n = 2$, negative emotionality $n = 1$).

Results

Functional connectivity across networks.

To analyze differences in overall connectivity levels across networks an omnibus repeated measures ANOVA with network type (HIN, DMN, FPN, control) as a within-subjects factor was conducted. This analysis revealed a significant within-subjects effect across network types, $F(3, 216) = 18.78$, $p < .001$, $\eta^2 = .207$. Post-hoc analyses with Bonferroni adjustments for multiple comparisons were conducted to assess which networks significantly differed from one another. Importantly, all functional networks of interest had significantly higher connectivity than the (non-functional) control network ($M = .05$; $SD = .12$; range: -.20–.44), all p's < .001. In addition, we found that there was significantly greater connectivity in the FPN ($M = .21$; $SD = .20$; range: -.16–.72) compared to both the HIN ($M = .13$; $SD = .12$; range: -.12–.48), $p = .003$, and the DMN ($M = .13$; $SD = .16$; range: -.28–.73), $p = .010$. However, there was no significant difference found between the level of connectivity for the HIN from the DMN, $p = 1.00$ (see Figure 2).

Functional connectivity and temperament.

In order to assess how functional connectivity patterns differentially predicted temperament characteristics, three separate regressions with all four network types (HIN, DMN, FPN, control) predicting each of the three domains of temperament (negative emotionality, regulation/orienting, surgency/positive emotionality) were conducted.

Regulation/Orienting. A linear regression was conducted with the four network types (HIN, DMN, FPN, control) predicting regulation/orienting using the entry method. The regression model significantly predicted regulation/orienting, $F(4, 68) = 4.45$, $p = .003$, $R^2 = .21$. More specifically, connectivity in the DMN was negatively associated with regulation/orienting ($\beta = -1.09$, $SE = .43$, $p = .015$); whereas, connectivity in the FPN was positively associated with regulation/orienting ($\beta = .91$, $SE = .35$, $p = .012$). There was a marginal negative association between the HIN and regulation/orienting, $p = .07$. However, there was no significant association found between the (non-functional) control network and regulation/orienting, $p = .69$ (see Figure 3).

Negative Emotionality. A multiple linear regression was conducted with the four network types (HIN, DMN, FPN, control) predicting negative emotionality using the entry method. Here, the model did not significantly predict negative emotionality $F(4, 67) = 1.73$, $p = .15$. However, when all non-significant factors were removed, and only the significant predictor from the previous model, HIN connectivity, was included as a factor, the model significantly predicted negative emotionality, $F(1, 72) = 4.74$, $p = .033$, $R^2 = .062$. More specifically, we found a significant positive relation between HIN connectivity and negative emotionality, ($\beta = 1.47$, $SE = .68$, $p = .033$; See Figure 4).

Surgency/Positive Emotionality. A linear regression was conducted with the four network types (HIN, DMN, FPN, control) predicting surgency/positive emotionality using the entry method. Here, the regression model did not significantly predict surgency/positive emotionality, $p = .39$. Moreover, none of the network types were significantly associated with surgency/positive emotionality (all p's $> .14$).

Discussion

The current study examined brain network connectivity using rs-fNIRS and behavioral temperament using parental report in newborn infants. We observed that, spontaneous activity in functional brain networks, (a) was significantly greater than in a (non-functional) control network and (b) varied considerably among infants. This supports the suitability of using rs-fNIRS to map individual differences in resting-state brain function among newborn infants. Importantly, our results also show that variability in functional brain network connectivity systematically maps onto individual differences in newborn behavioral temperament. Overall, the current findings provide novel insights into the brain origins of individual differences in behavioral temperament with potential implications for long-term psychological functioning and mental health.

With respect to our analysis of the rs-fNIRS data, our results show that connectivity within all three functional networks (FPM, DMN, HIN) is significantly greater than in the (non-functional) control network. This provides further evidence that functional brain networks exist from early in ontogeny and are detectable in newborn infants (Graham et al., 2016; Imai et al., 2014; Thomas et al., 2019). In addition to the general difference in connectivity between the functional and the (non-functional) control network, we also found that activity in the FPN was significantly greater than in the DMN and HIN (whereas there was no difference in connectivity level found between the DMN and HIN). One possible interpretation of this finding is that activity in the FPN might have been enhanced when compared to the other functional networks, because newborn infants were watching an infant-friendly video during the measurement of rs-fNIRS as is often done during resting state brain activity measurements in young infants (Bulgarelli et al., 2019). In other words, the FPN might have been more engaged because infants

were attending to an external video stimulus (note that all infants were watching the same video stimulus). Overall, our analysis support the notion that spontaneous functional network activity and variability can be measured using rs-fNIRS already within the first few weeks of life.

Having established rs-fNIRS connectivity in these functional brain networks as variable and distinct from a (non-functional) control network allowed for the examination of specific associations between brain network connectivity and infant behavioral temperament. Our results show that infants with greater connectivity (hyperconnectivity) in the DMN and decreased connectivity (hypoconnectivity) in the FPN exhibited lower levels of regulatory and orienting behaviors. This result confirms our hypothesis and is directly in line with prior work with adults showing that hyperconnectivity in the DMN and hypoconnectivity in the FPN is associated with depression (Kaiser et al., 2015). In addition, we examined the associations between activity in the HIN and behavioral temperament in infants, considering that prior work has implicated this network in emotion processing and depression. Indeed, our data from newborn infants showed an association between HIN connectivity and negative emotionality. However, contrary to our hypothesis based on previous work with adults showing that hypoconnectivity is associated with depression (Patashov et al., 2019; Wang et al., 2013), the current newborn infant data index that greater connectivity (hyperconnectivity) was associated with greater negative affect. It is unclear why the direction of the association (positive versus negative) would differ as a function of age (newborn infant in the current study and adults in previous work), but it is worth noting that the experience and display of negative emotions only gradually emerges during infancy and may not be fully present in newborn infants (Stifter & Fox, 1990). For example, fear responding, as one element of negative emotionality is not thought to come online until 7 months of age and older (Grossmann & Jessen, 2017; Grossmann et al., 2018; Jessen & Grossmann, 2016). In any case,

future work will have to systematically track the development of negative emotionality and its link to brain connectivity measures. Taken together, these current findings demonstrate specific associations between brain network connectivity and behavioral temperament in newborn infants which suggests a remarkably early emergence of functional networks with behavioral relevance and highlights the importance of evaluating individual differences reflected in resting-state brain connectivity.

Although there are many advantages in the current approach of using rs-fNIRS to examine brain connectivity, including its easy and infant-friendly application, there are some limitations that need to be mentioned. First, because fNIRS is limited in monitoring activity from (superficial) cortical structures (Lloyd-Fox et al., 2010), our rs-fNIRS approach did not allow us to measure activity from deeper cortical and subcortical regions and include those in our network analyses. Second, from a developmental perspective, it should be noted that our analysis is limited to only one age group and comprised of very young (newborn) infants. It is thus important to further assess the development of variability in these brain networks and their associations with behavioral temperament over developmental time to determine its long-term effects and the robustness of these associations (Imai et al., 2014).

In summary, the current study provides novel insights into the use of rs-fNIRS in identifying neural endophenotypes (variability in brain network connectivity) linked to behavioral temperament traits in early human development. The present findings support the notion that functionally distinct neural networks are implicated in regulatory and emotional behaviors already in newborn infants. These findings shed new light on the brain origins of individual differences in early-emerging behavioral traits and provide the basis for future

research examining the long-term consequences of this brain-behavior correlation for mental health outcomes.

Figures

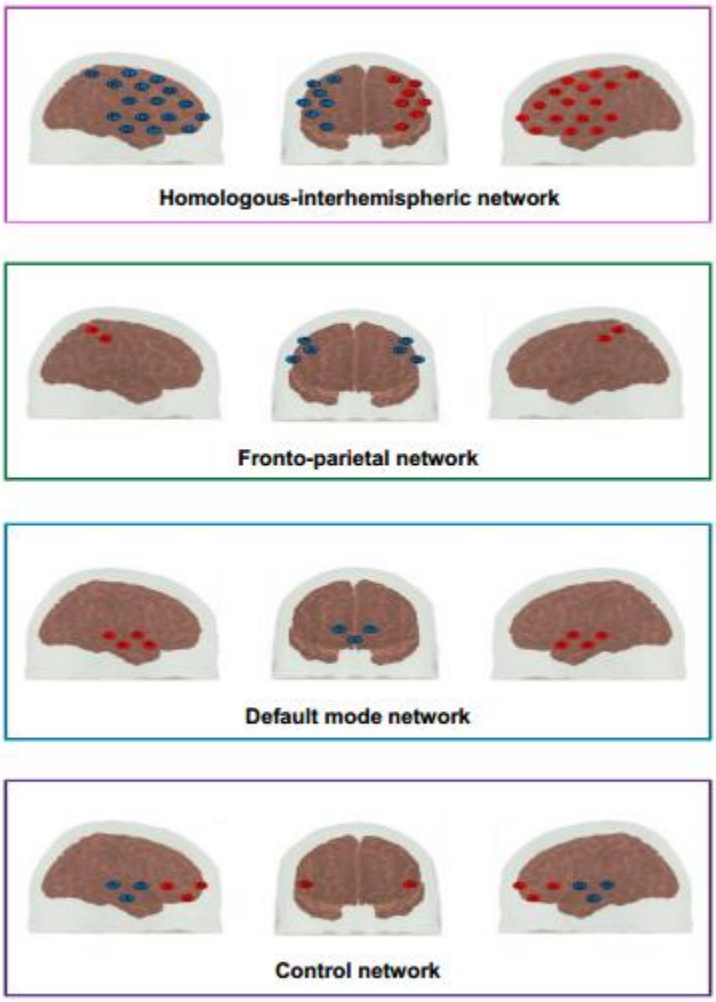

Figure 1. Shows the configurations for each of the network patterns. Note, each network consists of the average of all of the connections between red and blue channels of the same letter.

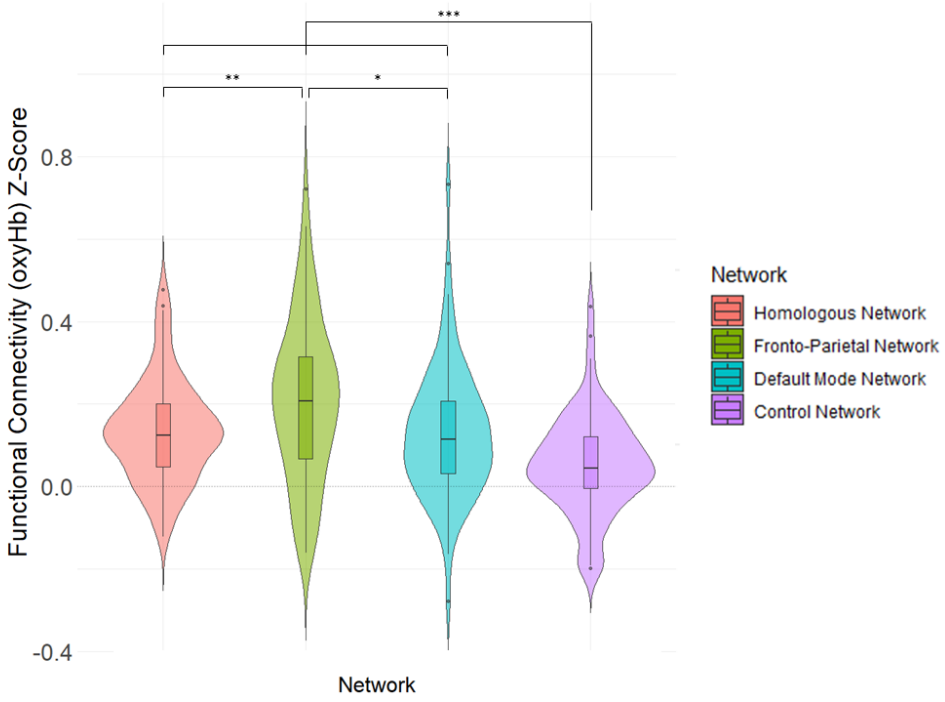

Figure 2. Shows the average levels of functional connectivity (oxyHb) and range of variability for each network. The boxplot horizontal lines from bottom to top reflect values for the lower quartile, median, and upper quartile respectively. Note, * $p < .05$, ** $p < .01$, *** $p < .001$.

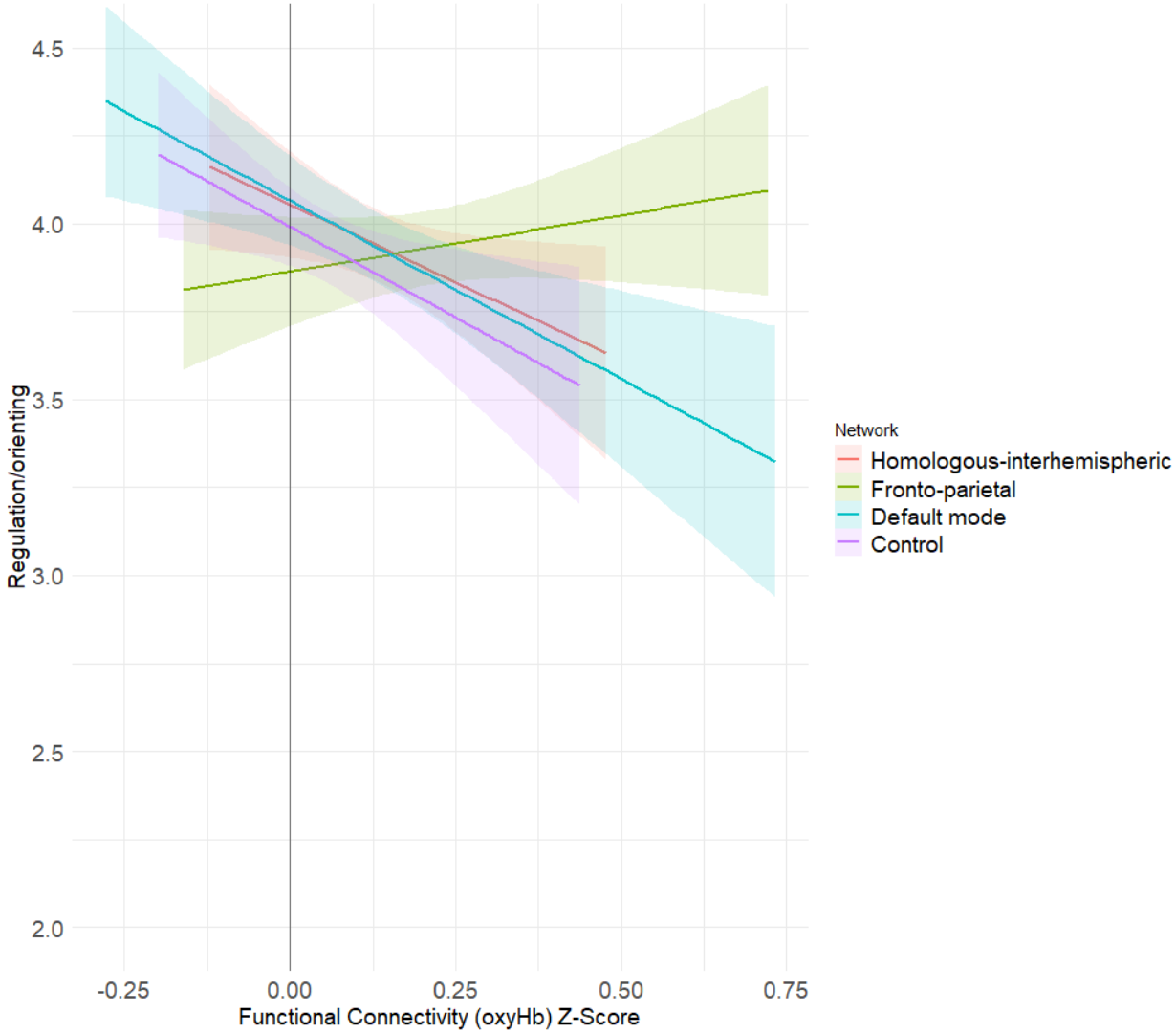

Figure 3. Shows the unadjusted relation between functional connectivity (oxyHb) Z-score and regulation/orienting. Here, we found that connectivity in the FPN was positively associated with regulation/orienting (p = .012) whereas, connectivity in the DMN was negatively associated with regulation/orienting (p = .015). Note, shaded regions represent 90% confidence intervals.

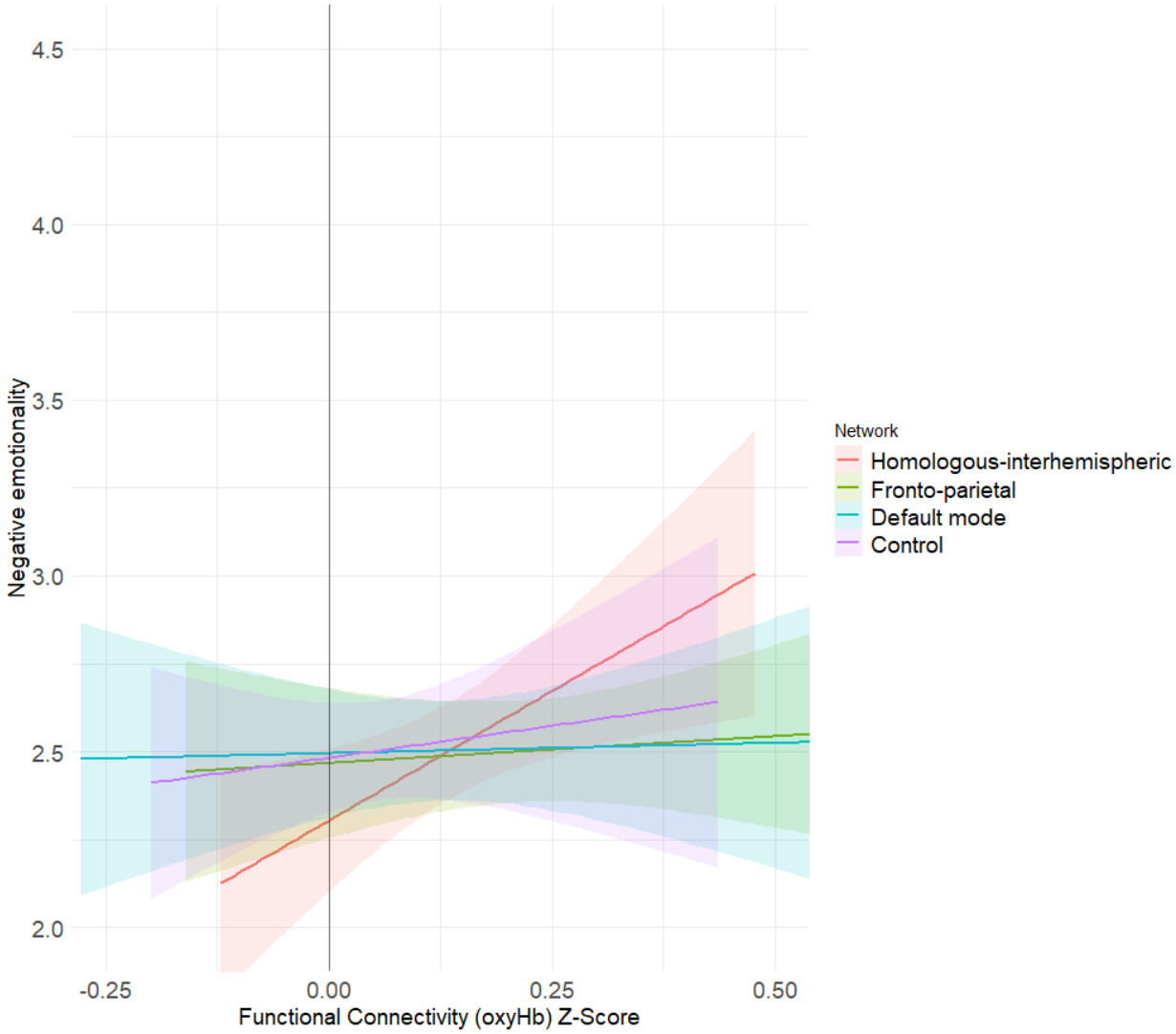

Figure 4. Shows the unadjusted relation between functional connectivity (oxyHb) Z-score and negative emotionality. Here, we found a significant positive relation between the HIN and negative emotionality (p = .013). Note, shaded regions represent 90% confidence intervals.

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Gut microbiota composition is linked to newborn functional brain connectivity and behavioral temperament

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Abstract

There is a new burgeoning body of work suggesting that the gut microbiome plays an important role in early infant development. To further understand this association, the current study examined the link between gut microbiome, brain and behavior in newborn infants (N = 63). Infant gut microbiome diversity was measured from stool samples using metagenomic sequencing, infant functional brain network connectivity was assessed using a resting state functional near infrared spectroscopy (rs-fNIRS) procedure and infant behavioral temperament was assessed using parental report. Our results show that gut microbiota composition was linked to individual variability in brain network connectivity, which in turn mediated individual differences in behavioral temperament, specifically negative emotionality, among infants. Furthermore, our results provide evidence for an association between virulence factors, possibly indexing pathogenic activity and brain network connectivity linked to negative emotionality. These findings provide novel insights into the early developmental origins of the gut microbiome-brain axis and its association with variability in important behavioral traits, providing the basis for future research examining brain and behavioral development long-term.

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The human gut microbiome is a complex ecosystem comprised of the microorganisms lining the intestinal tract, including bacteria, viruses, fungi, and archaea. Outnumbering our own human body cells by an astonishing margin (currently estimated at 150:1), the gut microbiome is crucial to normal physiological, metabolic, and immune function (Qin et al., 2010). Infancy represents a sensitive period in gut microbiome formation as the gut microbiome changes from a relatively sterile environment to a diverse ecosystem with over 3×10^{13} species of microorganisms (Cryan & Dinan, 2012; Sender, Fuchs, & Milo, 2016). Importantly, the gut microbiome is thought to impact psychological functioning and mental health through the microbiota-gut-brain axis (Borre et al., 2014; Cryan & Dinan, 2012; Spichak et al., 2018). Yet, little is known about how the gut microbiome impacts developing brain function and psychological health during this sensitive period of early human development (Cowan, Dinan, & Cryan, 2019; Kelsey, Dreisbach, Alhusen, & Grossmann, 2018).

Previous correlational studies in adults have shown that gut dysbiosis – referring to a general imbalance (but not a specific measure) of microorganisms in the gut – is linked to heightened negative affect and internalizing disorders such as anxiety and depression (Evrensel & Ceylan, 2015). Research more specifically assessing gut microbiome diversity in adulthood, however, has produced mixed results. For example, individuals with Major Depressive Disorder are reported as having increased, decreased, and no significant difference in alpha diversity (within-sample species diversity; Bastiaanssen et al., 2020). Moreover, due to the correlational nature of these existing findings and its limitation to adult samples, the specific mechanisms and developmental history through which an association between the gut and psychological functioning are established remains elusive.

The majority of our understanding of the mechanisms by which the microbiome impacts mental health outcomes comes from research conducted with animal models. Specifically, there has been a focus in animal work to characterize how the gut signals to the brain. To date, a number of potential pathways have emerged, including activation of the vagus nerve, the production of metabolites, and immuno-signaling (Sherwin, Bordenstein, Quinn, Dinan, & Cryan, 2019). In addition, germ-free mice, delivered by C-section and housed in a sterile environment, have been used for a sledge hammer approach to facilitate discoveries pertaining to how the gut microbiota broadly impacts brain and behavioral development (Heijtz et al., 2011). For example, germ free mice exhibit increased myelination in the prefrontal cortex, immature microglia development, aberrant neurogenesis, differing grey matter volumes in social brain areas (e.g., neocortex and amygdala), and increased blood-brain barrier permeability, indexing specific differences in brain structure and physiology (Hoban et al., 2016; Sharon, Sampson, Geschwind, & Mazmanian, 2016; Spichak et al., 2018). Furthermore, germ-free mice exhibit differences in their internalizing behaviors, such as aberrant fear conditioning (reduced freezing to the conditioned fear stimulus) and a decrease in species-typical anxiety behaviors (assessed through open field tests and elevated plus mazes; Chu et al., 2019; De Palma et al., 2015; Hsiao et al., 2013). In particular, it has been theorized the initial commensal microbiome, or the founding microbial population, has an exceedingly large and lasting influence over the lifetime composition of the microbiota (Litvak & Bäumler, 2019). In line with this hypothesis, studies have shown social deficits and aberrant stress responses in germ-free mice were reversed when recolonization of the gut microbiome occurred prior to but not after sexual maturity (Buffington et al., 2016; Heijtz et al., 2011; Sudo et al., 2004). Given the emerging evidence from animal models suggesting the existence of sensitive periods in the development of the gut microbiome-

brain-behavior relations, research elucidating these links in early human development is much needed.

There now are a few developmental studies investigating the role of the gut microbiota in brain and behavioral development through direct assessment of the gut microbiota in humans (Kelsey et al., 2018). Across the four existing infant studies there lacks a conclusive and unifying link between gut microbiota alpha diversity and behavioral traits Specifically, greater diversity of the gut microbiota has been associated with heighted surgency/extraversion, decreased negative emotionality, increased internalizing symptoms, and decreased cognitive performance in human infants (Aatsinki et al., 2019; Carlson et al., 2018; Christian et al., 2015; Loughman et al., 2020). Making any conclusions between diversity and positive infant mental health outcomes difficult to parse. Notably, these studies also relied upon on taxa diversity as the main characterization of the gut microbiota; this over simplification likely contributed to their mixed findings (Cowan et al., 2019). Therefore, there is a need to go beyond the typically reported taxa diversity measures and assess the functionality of the microbes, or the genes expressed in the microbiota, allowing insights into not only the microorganisms present but also the biological processes they are functionally involved in (Hooks, Konsman, & O'Malley, 2019; Knight et al., 2018).

The existing infant studies have relied on 16s rRNA gene sequencing which only affords insight into the taxonomic composition of the bacterial species in the gut microbiota and does not provide transcriptional information on the functional state of the microbiome. Therefore, any functional information provided is inferred from the present bacteria and not directly assessed (Aatsinki et al., 2019; Carlson et al., 2018; Christian et al., 2015; Gao et al., 2019). Alternatively, Shotgun metagenomics sequences all genomic DNA within a given sample, characterizing the

full contents of the microbial microorganisms (e.g., bacteria, viruses, and fungi) and their underlying functional pathways (e.g., gene products, virulence factors, and antibiotic resistance) (Kelsey et al., 2018). The direct assessment of functional pathways is an improvement on 16s rRNA sequencing and provides us with a more powerful tool to better characterize and understand the potential link with brain and psychological development.

Functionality can be characterized in multiple ways, and, for the current study, we focused on three aspects of microbial function: 1) GO Terms (Gene Ontology Terms), characterizing how individual genes contribute to the biology of an organism at the molecular, cellular, and organism levels, 2) virulence factors, characterizing the molecules created by microorganisms to aid in their ability to colonize, suppress immunity, and divert nutrients away from the host, and 3) antibiograms, characterizing overall antimicrobial susceptibility. In addition, it is important to more directly examine the potential effect the gut microbiome has on brain function in human infants, further contributing to individual differences in behavioral traits. Two published studies to date have investigated the role of the gut microbiota in infant brain structure and function (Carlson et al., 2018; Gao et al., 2019). Across both studies, limited evidence points to some links between alpha diversity of taxa and brain structure and function (see Table 1 for a summary). Specifically, increased alpha diversity was found to be associated with increased cortical volume in the parietal cortex and increased connectivity between the parietal cortex and supplemental motor area (Carlson et al., 2018; Gao et al., 2019). Given the limited current evidence suggesting the gut microbiome might be involved in the brain development and brain connectivity, more systematic research investigating this link is needed. Therefore, the first goal of the study was to examine if and how taxa diversity and functional diversity are linked to cortical connectivity. In order to test if gut microbiota composition is

linked to brain connectivity in cortical networks, we used functional Near Infrared Spectroscopy (fNIRS) to characterize individual differences in spontaneous brain network activity in prefrontal and parietal cortical networks previously linked to internalizing symptoms in adulthood and behavioral temperament in infancy (Kaiser, Andrews-Hanna, Wager, & Pizzagalli, 2015; Wang et al., 2013). The second goal of the present study was to examine if and how both taxa diversity and functional diversity are linked to behavioral temperament in the newborn period. Temperament refers to individual differences in one's emotional and attentional responses to the everyday situations (Rothbart, 2007). Specifically, the present study focused on the following, previously identified, dimensions of behavioral temperament: regulation/orienting, negative emotionality, and surgency/positive emotionality (Gartstein & Rothbart, 2003).

The present study examined the link between gut microbiome composition and brain and behavioral traits in newborn infants. This is the first study to use state-of-the art metagenomic sequencing, allowing us not only insights into full taxonomic make-up but also the functionality of the microbes. To this end, the present study took a multifaceted approach to characterizing the gut microbiota to assess if individual differences in behavioral temperament and cortical connectivity measured using fNIRS can be captured by (1) alpha diversity of taxa, (2) alpha diversity of functional terms, and/or (3) specific taxa biomarkers. Based on the prior work linking alpha diversity of taxa with mental health outcomes in adults (Bastiaanssen et al., 2020) and the work with infants assessing the link between taxa diversity and behavioral temperament (Aatsinki et al., 2019; Bastiaanssen et al., 2020; Carlson et al., 2018), we predicted alpha diversity of taxa, would be associated with decreased negative emotionality, and increased regulation/orienting behaviors. Moreover, we hypothesized that alpha diversity of taxa would be associated with brain connectivity in resting-state networks previously linked to internalizing

disorders in adults (Kaiser et al., 2015; Patashov, Goldstein, & Balberg, 2019). More specifically, we hypothesized taxa diversity would be associated with hyperconnectivity in the Fronto-parietal network (previously linked to cognitive control of attention and behavior in adults), hypoconnectivity in the Default mode network (previously linked to stimulus-independent thought and mind-wandering in adults), and hypoconnectivity in the Homologous-interhemispheric network (previously linked to emotional integration in adults; Patashov et al., 2019; Wang et al., 2013). Critically, we expected to see these associations only for the functional resting-state brain networks and not for the (non-functional) control network (see Methods). As a third goal, we were interested in exploring potential pathways by which the gut microbiome may influence behavioral temperament. Based on prior work linking gut microbiota to brain structure and function, and functional connectivity to behavioral temperament, we hypothesized that functional connectivity may be a significant mediator for the gut microbiota-behavioral temperament relation (Aatsinki et al., 2019; Carlson et al., 2018; Graham et al., 2019).

Moreover, we predicted specific functional profiles of the gut microbiome, decreased GO Terms (indicative of a diverse microbiome), increased virulence (indicative of potential sickness), and increased antibiotic resistance (indicative of prior medical intervention) would be linked to negative behavioral traits, including reduced behavioral regulation and enhanced negative emotionality (Firestein et al., 2019; Slykerman et al., 2019). As a fourth, and final goal, we were interested in utilizing exploratory, unsupervised machine learning algorithms in order to identify potential taxa biomarkers of functional connectivity and behavioral temperament. The current study aimed to expound upon the influence of the gut microbiota on early-emerging individual differences in brain and behavioral, providing foundational insights into gut microbiome-brain-behavior relations.

Method

Sixty-three newborns (*M* [age] = 25 days; *Median* [age] = 24 days; ranging from 9 days to 56 days; 26 females; 37 males) were included in the final sample used in the present analyses. Participants were recruited from a local hospital and are a representative sample of the surrounding Mid-Atlantic college town (for socio-demographic information see Table 2). All participants were born at term, with normal birth weight (>2,500g), and did not have any hearing or visual impairments. Twenty-three additional infants were tested and subsequently excluded from the present analyses for the following reasons: *n* = 17 were excluded because they failed to reach our pre-determined inclusion criterion of having at least 100 seconds of continuous data during which the infant was not crying; *n* = 4 were excluded because more than 33% of the measured fNIRS channels had poor light intensity readings, more specifically, a signal-to-noise ratio of less than 1.5 (Bulgarelli et al., 2019; Xu et al., 2015); *n* = 2 were excluded because their stool samples did not meet quality control thresholds for DNA sequencing. Note that the current attrition rate (36.5%) is lower than in previous infant fNIRS studies (Cristia et al., 2013). All parents gave informed consent for their infants to participate in accordance with the Declaration of Helsinki, and families received a payment for their participation. All procedures were approved by and carried out in accordance with The University of Virginia Institutional Review Board for Health Sciences (Protocol number 20381).

Stool collection and processing.

Infant stool samples were collected, aliquoted into cryovials containing a glycerol solution, and stored at -80°C. Biospecimens were processed and sequenced at the National Cancer Institute (NCI). Automated DNA extraction was performed with the MagAttract PowerMicrobiome DNA/RNA kit (Qiagen, Cat No./ID: 27500-4-EP,) with Qubit™

quantification following manufacturer's instructions. Samples that did not meet quality control thresholds for DNA concentration were removed from further analyses (n = 2). Library preparation and sequencing was completed using the Illumina Nextera DNA Flex Library Prep and Illumina NovaSeq 6000 sequencing platform, respectively.

Read processing pipeline and quality control.

The paired-end sequencing FASTQ files generated from the Illumina NovaSeq platform were entered into the Just Another Microbiology System (JAMS) pipeline, Version 1.39 (McCulloch, 2019). The JAMSalpha pipeline was used to assess quantity and quality of both taxonomic and functional sequences within a biospecimen (McCulloch, 2019). In JAMSalpha, paired-end sequencing reads were (1) quality trimmed using Trimmomatic (Bolger et al. 2014), (2) aligned to the human genome using Bowtie2 and host DNA was subsequently removed (Langmead and Salzberg 2012), (3) were assembled into contigs, overlapping sets of DNA fragments, and (4) referenced to the microbial genome using Megahit (Li, Liu, Luo, Sadakane, & Lam, 2015). If reads were not mapped to contigs, they were taxonomically classified using k-mer analysis using kraken (Wood & Salzberg, 2014). All mapped reads were then assigned to a last known taxon (LKT), which represents the most specific taxonomic classification available. This is the information which was used to calculate alpha diversity scores and biomarker discovery.

Functional terms.

Microbial genes were mapped to functional terms using JAMSalpha pipeline. Microbial function, in this study, was defined as the relative abundance of genes contributing to a specific GO (gene ontology) terms, antibiotic resistance, and virulence factors. The GO terms were sourced from the Gene Ontology Consortium Resource website (Ashburner et al., 2000;

Consortium, 2019). The antibiograms were sourced from JAMS. The virulence factors were sourced from the Virulence factors database website (Liu, Zheng, Jin, Chen, & Yang, 2018)

Infant temperament.

Infant behavioral temperament was assessed using parental reports of the widely used and reliable newborn measure, the 91-item Infant Behavior Questionnaire Revised Short Form (IBQ-R; Gartstein & Rothbart, 2003; Rigato, Stets, Bonneville-Roussy, & Holmboe, 2018; Stifter & Fox, 1990; Worobey & Blajda, 1989. Parents completed the questionnaire online prior to their appointment. Three general temperament dimensions were computed summarizing information from various sub-scales: (1) negative emotionality (contributing sub-scales: fear, distress to limitations, falling reactivity, sadness), (2) regulation/orienting (contributing sub-scales: low intensity pleasure, cuddliness, duration of orienting, soothability), and (3) surgency/positive emotionality (contributing sub-scales: activity level, smiling and laughing, high intensity pleasure, perceptual sensitivity, approach, and vocal reactivity; Gartstein & Rothbart, 2003). If parents reported the behavior was not applicable at the current time then this item was given a value of 0.

Resting state fNIRS.

Procedure. The resting state (rs)-fNIRS task took place in a small, quiet testing area. Infants were seated on their parent's lap and placed approximately 60 cm from the screen (23-inch monitor). Parents were asked to remain quiet throughout the testing session. A fNIRS fabric cap (EasyCap, Germany) was fitted to each newborn and secured in place using a waist-band and outside netting. The presentation software package (Neurobehavioral Systems, USA) was used for the design and viewing of the experimental paradigm. A non-social stimulus was created by selecting non-social clips from a popular infant video (Baby Einstein) that featured videos of

toys, stuffed animals, and still images of everyday objects. These clips were shown in 30 second intervals, and the order of presentation was randomized for each infant. The full recording session took place over a 7 minute time period. Sessions were video-recorded using a camera mounted above the screen. This allowed for later offline coding of the infants' behavior, fussiness, and cap placement.

Data acquisition. Infants' fNIRS data were recorded using a NIRx Nirscout system and NirStar acquisition software. Concentration changes of oxygenated hemoglobin (oxyHb) and deoxygenated hemoglobin (deoxyHb) in the cerebral cortex are measured using fNIRS through the quantification of refracted light, (for more information regarding this technique see Lloyd-Fox, Széplaki-Köllőd, Yin, & Csibra, 2015). The fNIRS system used contains 49 channels positioned over frontal and temporal-parietal regions and recorded measurements (as previously described in Altvater-Mackensen & Grossmann, 2016; Grossmann, Missana, & Krol, 2018; Kelsey, Krol, Kret, & Grossmann, 2019; Krol, Puglia, Morris, Connelly, & Grossmann). The system emits two wavelengths of light in the Near-Infrared spectrum, 780 nm and 830 nm, to capture deoxyHb and oxyHb, respectively. The diodes have a power of 20 mW/wavelength and data were recorded at a sampling rate of 3.91 Hz.

Behavioral Coding. Infants' behavior during the fNIRS recording session was coded by a trained research assistant using video recordings of the experimental session. Similarly to previous studies, infants were only included in the present analysis if they had at least 100 seconds of continuous data during which the infant was not crying (Bulgarelli et al., 2019). On average, infants contributed 331.29 seconds of data (*SD* = 115.75 seconds).

Functional Networks. The fNIRS data were analyzed using the functional connectivity program, FC-NIRS (Xu et al., 2015). First, channels were removed on the basis of poor light

intensity (signal-to-noise ratio was less than 1.5) (Xu et al., 2015). In order to be included in the present analyses, infants needed to have at least 66% of their channels passing this pre-defined threshold (Bulgarelli et al., 2019). Next, data were band-pass filtered using a previously validated low frequency filter (.01-.08 Hz; Bulgarelli et al., 2019; Lu et al., 2009). Finally, concentration changes were calculated using the modified Beer-Lambert law (Villringer & Chance, 1997).

For each infant, we obtained a 49 by 49 correlation matrix corresponding to all of the relations between all of the channels measured. Considering that negative values are difficult to interpret in terms of their neurobiological basis (and based on prior work) we replaced all negative correlation values with zeros (Fox, Zhang, Snyder, & Raichle, 2009; Murphy, Birn, Handwerker, Jones, & Bandettini, 2009). Next, Fisher Z-transformations were performed on all correlation matrices. Networks of interest were created by selecting channels that corresponded to specific regions of interest. Brain networks were composed based on the anatomical information available in Kabdebon et al. (2014), a meta-analysis of resting state fMRI (Kaiser et al., 2015), and prior work using rs-fNIRS (Patashov et al., 2019; Sasai, Homae, Watanabe, & Taga, 2011). Based on this information, four networks were created: (1) The Fronto-parietal network, the average of all correlations between three channels in the dorsolateral prefrontal cortex (corresponding with the F3, F4, F5, F6 electrodes) and two channels in the parietal area (corresponding with CP3 and CP4 electrodes); (2) The Default mode network, the average of all correlations between three channels in the medial prefrontal cortex (corresponding with the Fpz electrode) and four channels in the superior temporal cortex (corresponding with FT7, T7, FT8, T8 electrodes); (3) The Homologous-interhemispheric network, the average of all correlations between the 21 channels in the left hemisphere (including frontal, temporal and parietal cortical

regions) with their corresponding (homologous) channels in the right hemisphere; and, (4) a (non-functional) control network, the average of all correlations between three channels in the left frontal area (corresponding with the F7 electrode) with three channels in the right temporal area (corresponding with the T8 electrode) and three channels in the right frontal area (corresponding with F8 electrode) with three channels in the left temporal area (corresponding with the T7 electrode; see Figure 1 for schematic of network configurations). Based on prior infant work, which has found laterality differences, networks were separated into left and right hemispheres (Carlson et al., 2018). Moreover, statistical outliers – values that were more than 3 SD above or below the mean or based on multivariate mahalanobis' distance – were removed for the subsequent analyses (functional connectivity data $n = 1$, negative emotionality $n = 1$).

Results

Analysis Plan

Alpha diversity values (Shannon Diversity Index and Chao1) for both the taxa and functional terms were calculated using the vegan R-package. Associations between the covariates and the variables of interest were investigated using Wilcoxon's rank-sum test and Kruskal–Wallis H- test. We included covariates in the model based on previous identification in prior work and significant associations found in the present sample. For the covariate analyses, we used the less stringent p-value < 0.05 cutoffs in order to be conservative in our later assessments. To account for the use of multiple comparisons across our models, we adjusted our *p*-values against the False Discovery Rate (FDR). We considered results with FDR <0.25 as significant (see Aatsinki et al., 2019 for another example of a paper using this threshold). FDR was estimated using the Benjamini & Hochberg method with the R function *p.adjust*.

Linear discriminant analysis of effect size (LefSE) was used to identify potential microbial biomarkers of functional connectivity and behavioral temperament using the Galaxy tool (http://huttenhower.sph.harvard.edu/galaxy/). High and Low groupings were created for the outcome variable by applying a Median Split. The LefSE tool identifies the taxa and functional terms that are differentially abundant between groups by applying 1) non-parametric factorial Kruskal-Wallis (KW) test, 2) pairwise (unpaired) Wilcoxon rank-sum test and 3) Linear Discriminant analysis to estimate effect size of each differentially abundant feature (Segata et al., 2011). Per-sample normalization and an alpha value of 0.05 for the Kruskal-Wallis and Wilcoxin rank-sum test was used. The logarithmic LDA score for discriminative features was set at an absolute value of Log 3 fold change.

Associations with clinical covariates

A series of Wilcoxon's rank-sum test and Kruskal-Wallis H-tests were used to identify significant relationships between taxa diversity and potential clinical covariates (for a schematic representation for all associations see Figure 2). We found significant associations between the Shannon-Taxa and birthweight (Spearman's rank correlation $r_s = –.40$, $p = .001$), income (Spearman's rank correlation $r_s = –.25$, $p = .049$), breastfeeding (Kruskal-Wallis H $X^2 = 9.14$, $p = .002$), gestational age (Spearman's rank correlation $r_s = –.31$, $p = .016$), and head circumference (Spearman's rank correlation $r_s = –.37$, $p = .004$). However, there were no significant associations found between the Chao1-Taxa diversity measure and any of the covariates.

Next, we assessed the relationship between functional term diversity (Chao1 index for antibiograms, virulence terms, and GO Terms) and clinical covariates. Here, we found that antibiogram diversity was significantly associated with both income, (Spearman's rank correlation $r_s = –.31$, $p = .016$), and gestational age, (Spearman's rank correlation $r_s = –.36$, $p =$

.004). Similarly, virulence factor diversity was associated with income (Spearman's rank correlation r_s = .33, p = .008). Furthermore, GO Term diversity was associated with sex (Kruskal-Wallis H X^2 = 5.37, p = .02) and head circumference (Spearman's rank correlation r_s = –.37, p = .004).

Finally, we assessed the relation between clinical covariates and psychological outcome measures (behavioral temperament and functional connectivity). Here, we found significant associations between negative emotionality, infant age (Spearman's rank correlation r_s = .43, p = .001), and income (Spearman's rank correlation r_s = .36, p = .005). However, there were no other significant associations found between clinical covariates and psychological outcome measures.

Alpha diversity of last known taxa and functional connectivity.

A series of univariate regressions with alpha diversity of last known taxa (either Shannon Diversity Index or Chao1, separately) as the predictor variables and functional connectivity network patterns (Fronto-Parietal [Left and Right], Default Mode [Left and Right], Homologous-Interhemispheric, and Control Network) as the outcome variables were conducted. There was a significant positive association between alpha diversity and the left Fronto-parietal network (Chao1-Taxa standardized β = 0.71, FDR = 0.08, adjusted R^2 = .13, Shannon-Taxa β = 0.14, FDR = 0.03, adjusted R^2 = .17), as well as alpha diversity of taxa and Homologous-interhemispheric network connectivity (Chao1-Taxa standardized β = 0.16, FDR = 0.10, adjusted R^2 = .07; Shannon-Taxa β = 0.05, FDR = 0.23, adjusted R^2 = .09; See Figure 3). When the models were adjusted for significant covariate associations, only the relation between Shannon-Taxa and Left Fronto-parietal network connectivity remained significant (Shannon-Taxa β = 0.17, FDR = 0.10, partial R^2 = .16; covariates included: antibiotics, delivery method, breastfeeding, infant age, infant weight at birth and at study visit, gestational age, maternal

education, sex, and head circumference at birth). Importantly, there was no association between alpha diversity and connectivity in the Control network (Chao1-Taxa FDR = 0.92; Shannon-Taxa FDR = 0.87).

Alpha diversity of functional terms and functional connectivity.

In order to examine how the particular functions of the microorganisms may be contributing to the functional connectivity differences, a series of univariate entry-method linear regressions were conducted with each of the Chao1 functional terms (Virulence factors, Antibiograms, and GO terms) entered together in the model predicting each of the previously identified functional connectivity networks (Left Fronto-Parietal and Homologous-Interhemispheric) in addition to the Control network seperately. We discovered Chao1 functional terms predicted Homologous-Interhemispheric network connectivity. Specifically, Virulence factor diversity was positively associated with the Homologous-interhemispheric network connectivity (standardized $\beta = 0.22$, FDR = 0.13, partial $R^2 = .14$; See Figure 4). Moreover, when the model was adjusted for significant covariate associations, the relation between Virulence factor diversity and Homologous-interhemispheric network connectivity remained (standardized $\beta = 0.24$, FDR = 0.10, partial $R^2 = .19$; covariates included: antibiotics, delivery method, breastfeeding, infant age, infant weight at study visit, gestational age, maternal education, sex, number of siblings, and head circumference at birth). However, none of the other functional terms significantly predicted Homologous-interhemispheric network connectivity. Moreover, there were no significant associations found between Chao1 functional terms and the Left Frontal-parietal network (FDR > .26) or the Control network (FDR >.35) for the unadjusted models.

Alpha diversity of last known taxa, alpha diversity of functional terms, and behavioral temperament.

A series of multivariate regressions with alpha diversity of taxa (Chao1-Taxa, Shannon-Taxa) and Chao1 functional terms (Virulence factors, Antibiograms, and GO terms) as the predictors and behavioral temperament (Surgency, Regulation, Negative Emotionality) as the outcome variables were conducted. We did not find a significant association between either of the alpha diversity metrics for taxa and behavioral temperament. Similarly, we did not find an association between any of the alpha diversity indices for the functional terms and behavioral temperament.

Assessment of indirect effects

Simple mediation analyses were conducted in order to test the hypothesis that the gut microbiota indirectly influences behavioral temperament (negative emotionality and regulation/orienting) through its effect on functional connectivity (for a schematic representation and relevant statistics see Figure 5). Specifically, we were interested in the possible mediation effects of Homologous-interhemispheric connectivity based on its significant association with negative emotionality ($\beta = 0.30$, FDR = 0.22, adjusted $R^2 = .08$) and regulation/orienting ($\beta = -0.26$, FDR = 0.23, adjusted $R^2 = .07$).

To do this, we used ordinary least squares path analysis and boot strapped confidence intervals based on 5,000 bootstrap samples. First we tested possible mediation effects for the relation between alpha diversity of taxa and behavioral temperament. In line with previous findings, increased alpha diversity (Chao1-Taxa $\beta = .29$; Shannon-Taxa $\beta = .31$) was associated with increased Homologous-interhemispheric connectivity. Additionally, Homologous-interhemispheric connectivity was associated with increased negative emotionality ($\beta = .31$-$.39$).

There was a significant indirect effect found, suggesting the relationship between alpha diversity and negative emotionality may be mediated by Homologous-interhemispheric connectivity (Chao1-Taxa β = .09, CI = [.002, .211]; Shannon-Taxa β = .12, CI = [.020, .273]). There were, however, no significant indirect effects found for the relations between taxa diversity (Shannon and Chao1) and regulation/orienting.

We then assessed if virulence factors influence behavioral temperament through its effect on Homologous-interhemispheric connectivity. Mirroring previous findings, increased virulence diversity (β = .47) was associated with increased Homologous-interhemispheric connectivity. In addition, Homologous-interhemispheric connectivity was associated with increased negative emotionality (β = .27). There was a significant indirect effect found, suggesting the relation between virulence diversity and negative emotionality may be mediated by Homologous-interhemispheric connectivity (β = .13; CI = [.005-.271]). Similarly, we found evidence for a significant indirect effect, suggesting the relation between virulence factor diversity and regulation/orienting may also be mediated by Homologous-interhemispheric connectivity (β = -.19, CI = [-.412, -.023]).

Taxa biomarker identification

Functional connectivity. The LefSE analysis identified fourteen total potential microbial biomarkers for the functional connectivity networks (LDA Log fold change cut-off = 3) and are described in Table 3. The Left Fronto-parietal network was marked by an overall enrichment of *Clostridium* taxa in the high connectivity group. In particular, the species *C. perfringens* was a shared feature of both high connectivity group for the left Fronto-parietal network (Log fold change = 3.41) and low connectivity group for the left Default mode network (Log fold change = 3.56). For the high connectivity Homologous-interhemispheric network,

there was an increased enrichment of *E. coli* (Log fold change = 4.36) whereas the low connectivity Homologous-interhemispheric network group had an increased enrichment of *B. dentium* (Log fold change = 4.01).

Temperament. The LefSE identified a total of five microorganisms as potential biomarkers for temperament and are described in Table 4. Both negative emotionality and regulation/orienting were marked by an enrichment of *Bifidobacterium*. In particular, *B. pseudocatenulatum* was enriched in high negative emotionality group (Log fold change = 4.09) and the high regulation/orienting group (Log fold change = 4.48).

Discussion

The current study examined the relations between gut microbiota composition, functional brain network connectivity, and behavioral temperament in newborn infants. Our results show gut microbiota composition is linked to individual variability in brain network connectivity, which in turn, mediates individual differences in behavioral temperament among infants. Furthermore, using metagenomics shotgun sequencing, our results provide new evidence for an association between virulence factors and brain network connectivity. These findings provide novel insights into the early developmental origins of the gut microbiome-brain axis and its association with variability in important behavioral traits, potentially affecting long-term development.

Our results demonstrate gut microbiota taxa diversity is positively associated with functional connectivity in two resting-state brain networks in newborn infants. In concordance with our hypotheses, increased taxa diversity was linked to fronto-parietal connectivity, a brain network previously associated with positive mental health outcomes in adults and positive behavioral traits in infants (Kaiser et al., 2015; Kelsey, Farris, & Grossmann, Under Review;

Rothbart, Sheese, & Posner, 2007). Specifically, greater connectivity in the frontal-parietal network has been linked to decreased incidence of internalizing disorder in adulthood and increased regulation and orienting behaviors in infancy (Kaiser et al., 2015). Our findings, in addition, corroborate data from previous infant studies, showing a positive association between taxa diversity and parietal cortex structure and function (Carlson et al., 2018; Gao et al., 2019). This points to a consistent pattern of association between the gut microbiome diversity and the developing brain. It is important to consider potential mechanisms by which such an association may arise. In previous studies with mice, antibiotic administration during pregnancy induced a dysregulated state of microglia localized to the prefrontal and parietal cortices (Lebovitz et al., 2019), suggesting one potential mechanism by which chemical intervention affecting the microbiome composition may impact brain development *in utero*.

Contrary to our hypothesis, in our study, taxa diversity was positively associated with connectivity in infants' homologous-interhemispheric network, consequently linked to heighted negative emotionality and decreased regulatory behaviors. Even though this was in opposition to our hypothesis partly based on prior work with adults, our finding is similar to prior work with infants. In particular, a study by Carlson et al. (2018) provided evidence to suggest alpha diversity assessed at 1 year of age was associated with decreased cognitive performance at two years of age. In addition, a Christian et al. (2015) study reported that alpha diversity was associated with decreased regulatory behaviors both of which were assessed at same time (18-27 months). Taken together, our findings are in line with previous results from studies performed with infants.

To further examine the association between gut microbial composition and homologous-interhemispheric connectivity, we assessed the functional term diversity of the samples

(Consortium, 2019; Li et al., 2015). Using this approach, we found that increased virulence factor diversity was linked to increased homologous-interhemispheric connectivity among infants. Taken together with the taxa diversity findings, it appears the aforementioned increase in taxa diversity may be driven, at least partly, by an increase in pathogenic microorganisms. This result further highlights the limitations of relying on the assessment of taxa diversity (Cowan et al., 2019), and how this can be addressed through the analysis of function terms. It is also interesting to consider the possibility that the increase in virulence factors may be seen in infants who are more susceptible to, or might even be currently experiencing, an infection. In this context, only a few studies with adults have reported associations between somatic symptoms (e.g., stomach ache and irritable bowel syndrome) and mental health outcomes (Callaghan et al., 2020; Lee et al., 2009); however, little is known about the directionality or causality of such associations. It is important to mention the stool samples and temperament measurements were taken at the same time point in the current study. As a result, we are not able to address questions concerning potential directionality. Nonetheless, the current findings with newborn infants point to a remarkably early emergence of the association markers of sickness (virulence factors) and brain function. Longitudinal studies would be required to unpack more fully the association between gut microbiota composition, infection status, and brain and behavioral traits during infancy.

Contrary to prior work with infants (Aatsinki et al., 2019; Christian et al., 2015), we did not find evidence for a direct association between taxa diversity and infant behavioral temperament. There are several possible differences potentially accounting for the discrepancies between the current and previous studies. For example, though prior work has examined the gut microbiota within the first few months of life (Aatsinki et al., 2019; Loughman et al., 2020), our

study examined the youngest sample of newborns. It is therefore possible the predicted association between gut microbiota and behavioral traits only emerges later in infant development. In conjunction, certain components of behavioral temperament, such as fear behaviors, do not emerge until later during the first year of life (Grossmann & Jessen, 2017). In line with this potential explanation, Aatsinki et al. (2019) reported a significant association between taxa diversity (assessed at 2.5 months) and fear behaviors (assessed at 6 months). This suggests gut microbiota influences on brain network connectivity may precede the direct associations with behavioral traits.

We also explored the possibility of a link between taxa diversity and behavioral temperament and found this link to be mediated by functional brain network connectivity. Indeed, the current results demonstrate that infants' taxa diversity and virulence factor diversity are mediated by the homologous-interhemispheric brain network connectivity and indirectly associated with negative emotionality (Figure 4). Gao et al. (2019) obtained a similar pattern in infants that was suggestive of a mediation but they did not test this directly. They found that alpha diversity was linked to increased connectivity between the parietal lobe and supplemental motor area, and functional connectivity in this network was associated with behavioral (cognitive) performance. In conjunction with prior work, our findings support the notion that the gut microbiome may be more directly linked to or impact the brain through the gut-brain axis, whereas links between the gut microbiome and overt behavior will be harder to establish and detect in humans. More generally, in order to arrive at a more complete understanding of linking the gut microbiome and behavioral traits, it is critical to include measures of brain function.

To identify candidate biomarkers for behavioral temperament and brain connectivity, we took an unbiased approach using LefSE. We identified 14 microbial species associated with early functional brain connectivity, including several microbes from the orders Clostridiales (including Lachnospircea, and Bacteriodes) which have been previously identified as a microbe of interest due to its role in serotonin modulation (Yano et al., 2015). To this end, microbes from the order Clostridiales have previously been associated with global brain connectivity metric in both cortical and subcortical areas in adults (Labus et al., 2019). Our analysis showed that Lachnospircea and Bacteriodes were associated with infants' fronto-parietal brain network connectivity. Interestingly, the same microbes have been shown to be associated with brain development in adolescents in a previous study (Callaghan et al., 2020). In the Callaghan et al. (2020) study , Clostridiales was significantly lower among adolescents that had experienced early adversity (institutionalization during infancy) compared to a control group. Furthermore, this study also identified Lachnospircea and Bacteriodes as being linked to heightened mPFC responses to fearful faces assessed using fMRI. This prior study with adolescents shows early life experiences may shape the colonization of these microbes, and this may have downstream consequences for brain development. Our study adds important evidence directly from infants to further support the role these microbes play in early human brain function. Our analysis also identified a particular species of bacteria, *C. perfringens,* linked to both hyperconnectivity in the left fronto-parietal network and hypoconnectivity in the left default mode network, suggesting this microbe may disrupt early brain network formation. This is of particular interest as *C. perfringens* is one of the most common causes of food poisoning in the United States (CDC, 2020), and preliminary work suggests strains of *C. perfringens* may cause brain lesions similar to what is seen in multiple sclerosis (Rumah, Linden, Fischetti, & Vartanian, 2013).

With respect to negative emotionality and regulation/orienting temperamental domains, our analysis identified five associated microbes, with three belonging to the genus *Bifidobacterium*. Specifically, *Bifidobacterium* was enriched for high levels of negative emotionality (*Bifidobacterium pseudocatenulatum)* and regulation/orienting (*Bifidobacterium pseudocatenulatum and Bifidobacterium catenulatum*). Prior work with infants has also identified *Bifidobacterium* as a potential biomarker for behavioral temperament linked to decreased regulation/orienting and increased surgency/positive emotionality (Aatsinki et al., 2019). In addition, this genera of microbes is thought to play an important role in fighting infections. Many *Bifidobacterium* species are involved in the conversion of lactose, found in breastmilk, to lactic acid. The accumulation of lactic acid, lowers the overall ph and makes it a less hospitable environment for other pathogens (Lievien et al. 2000). Overall, our current findings, together with the prior work, hint at the involvement of *Clostridiales* and *Bifidobacterium* in brain and behavioral development; however, more careful experimental work is required to fully characterize and understand the associations revealed in these preliminary findings using LefSE.

Our current study may have a number of strengths and include novel methods, such as the use of shotgun-metagenomic sequencing and rs-fNIRS to index functional brain network connectivity, but there are some limitations that merit acknowledgement. First, our analysis is limited to one time point in early development and limited to newborn infants. It will be important for future studies to assess the development and variability in the gut microbiota composition and its association with brain network connectivity and behavioral temperament over time to determine its long-term effects (Kelsey et al., 2018). Second, although we selected the current approach of rs-fNIRS to examine brain connectivity because of its infant-friendly

application, fNIRS is limited in monitoring activity from (superficial) cortical structures (Lloyd-Fox et al., 2010) and prevents us from gleaning insights into networks including deeper cortical and subcortical structures. Third, by adjusting our analytical models for potential confounds (covariates), some association effects are no longer statistically significant. Accordingly, it is unclear if the absence of significant effects when making these adjustments in the current analysis is due to reduced power or the covariate adjustment itself, as it is known that power can be reduced with an increase in the number of variables in a model. To address these and other potential statistical limitations, the field needs to move beyond single time point, low sample size studies, and take an unbiased data science approach utilizing machine learning techniques to better characterize the nuances and complexities of the gut microbiota-brain interactions (Kelsey et al., 2018).

In summary, the current study provides novel insights into the early emergence of the gut-brain axis and support the connection between the gut microbial composition and functional brain connectivity already present in newborn infants. These findings shed new light on the microbial origins of individual differences in early-emerging functional brain networks and behavioral traits and provide the basis for future research examining the long-term consequences of this gut-brain-behavioral correlation on mental health outcomes.

Tables

Table 1. Study characteristics and findings for work that has directly assessed gut microbiota in relation to cortical brain development and cognitive/behavioral outcomes.

Study	Sample size	Age at gut microbiota assessment	Age at psychological assessment	Covariates
Carlson et al. (2018)	N = 27	1 year old	2 years old MRI	Older siblings, paternal ethnicity, and total intracranial volume
	N = 69	1 year old	2 years old Cognitive Development	Older siblings, paternal ethnicity, sex, maternal education, paternal age, twin status, and income
Gao et al. (2019)	N = 39	1 year old	1 year old	Older sibling, paternal ethnicity, birth weight, postnatal age at scan, sex, twin status, maternal/paternal education, and residual Frame-wise Displacement
Christian et al. (2015)	N = 77	1-2 years old	1-2 years old	Separated by sex and remained after controlling for age.
Aatsinki et al. (2019)	N = 301	2.5 months	6 months	*Cluster analysis:* sex and delivery method *Gut microbiome phenotype analysis*: infant age at the time of sample collection, infant sex and mode of delivery *Alpha Diversity*: gestational age, infant age, sex, mode of delivery, breastfeeding and antibiotics intake
Loughman et al. (2020)	N = 201	1 month, 6 months, and 12 months*	2 years old	Storage in a freezer and duration of time stored in a freezer

	Cortical Brain Areas		
Gut Microbiota Measure	Left Posterior Frontal Lobe Volume	Right Parietal Cortex Volume	Left Parietal Cortex – Supplemental Motor Area Connectivity
Alpha diversity	+	+	+
	Behavioral Temperament and Cognitive Development		
	Negative Emotionality & Internalizing Symptoms	Regulation & Cognitive Development	Surgency/Positive Emotionality
Alpha diversity	– +	– –	+
Genus Level Identification			
Bifiobacterium/Enterobacteriaceae		–	+
Veillonella		–	
Atopobium			–
Streptococcus			+
Erwinia	+	+	
Rothia	+		
Serratia	+		
Prevotella	–		
Lachnospiraceae (family level identification)	–		

*Note, only the 12 month fecal samples were significantly associated with psychological outcomes.

Table 2. Socio-demographic information for the present study sample.

Socio-demographic information		Mean/Count (SD/%)
Antibiotic Treatments, n		28 (45%)
Apgar Score at 1st Minute		8.19 (0.94)
Apgar Score at 5th Minute		8.94 (0.44)
Birth Length, inches		19.75 (0.82)
Birthweight, grams		3445.42 (466.24)
Breastfeeding, n		56 (90%)
Epidural, n		37 (60%)
Gestational Age, weeks		39.43 (1.18)
Girls, n		25 (40%)
Head Circumference, cm		34.74 (1.17)
Income, n		
	Less than $15,000	5 (8%)
	$15,001 to $30,000	5 (8%)
	$30,001 to $45,000	3 (5%)
	$45,001 to $60,000	1 (2%)
	$60,001 to $75,000	2 (3%)
	$75,001 to $90,000	9 (15%)
	$90,001 to $110,000	7 (11%)
	$110,001 to $125,000	7 (11%)
	$125,001 to $175,000	2 (3%)
	$175,001 to $225,000	8 (13%)
	$225,001 to $275,000	8 (13%)
	$275,001+	3 (5%)
Infant Age at data collection, days		24.92 (10.68)
Maternal Education		
	Some High School	2 (3%)
	High School Diploma/GED	11 (18%)
	Some College/Associates	7 (11%)
	Bachelor's Degree	16 (26%)
	Graduate Degree	26 (42%)
Number of Siblings		2.13 (1.11)
Lived with pet(s), n		37 (60%)
Pitocin, n		31 (50%)
Race white, n		45 (73%)
Vaginal Delivery, n		47 (76%)

Note: There were two points of missing data for birth length and head circumference, and one point missing for Pitocin use. Children whose parent reported breastfeeding at any amount were considered breastfed.

Table 3. LefSE identified taxa biomarkers of functional connectivity networks.

Phylum	Family	Genus	Species	Log fold change	Group with the highest Median
Left Default mode network					
Firmicutes	Clostridiaceae	Clostridium	*perfringens*	3.559	Low
Left Fronto-parietal network					
Firmicutes	Enterococcaceae	Enterococcus	*faecalis*	3.765	High
Actinobacteria	Coriobacteriaceae	Collinsella	*Unclassified*	3.665	High
Firmicutes	Clostridiaceae	Clostridium	*disporicum*	3.548	High
Bacteroidetes	Prevotellaceae	Prevotella	*copri*	3.523	High
Firmicutes	Clostridiaceae	Clostridium	*perfringens*	3.415	High
Firmicutes	Clostridiaceae	Clostridium	*tertium*	3.367	High
Firmicutes	Lachnospiraceae	Robinsoniella	*peoriensis*	3.265	High
Firmicutes	Clostridiaceae	Clostridium	*Unclassified*	3.167	High
Bacteroidetes	Bacteroidaceae	Bacteroides	*caccae*	3.164	High
Firmicutes	Streptococcaceae	Streptococcus	*salivarius*	3.397	Low
Firmicutes	Enterococcaceae	Enterococcus	*Unclassified*	3.042	Low
Homologous-interhemispheric network					
Proteobacteria	Enterobacteriaceae	Escherichia	*coli*	4.357	High
Actinobacteria	Bifidobacteriaceae	Bifidobacterium	*dentium*	4.012	Low

Table 4. LefSE identified taxa biomarkers of behavioral temperament.

Phylum	**Family**	**Genus**	**Species**	**Log fold change**	**Group with the highest Median abundance**
Negative emotionality					
Actinobacteria	Bifidobacteriaceae	Bifidobacterium	*pseudocatenulatum*	4.085	High
Firmicutes	Streptococcaceae	Streptococcus	*vestibularis*	3.120	Low
Actinobacteria	Actinomycetaceae	Schaalia	*radingae*	3.385	Low
Regulation/orienting					
Actinobacteria	Bifidobacteriaceae	Bifidobacterium	*catenulatum*	4.177	High
Actinobacteria	Bifidobacteriaceae	Bifidobacterium	*pseudocatenulatum*	4.047	High

Figures

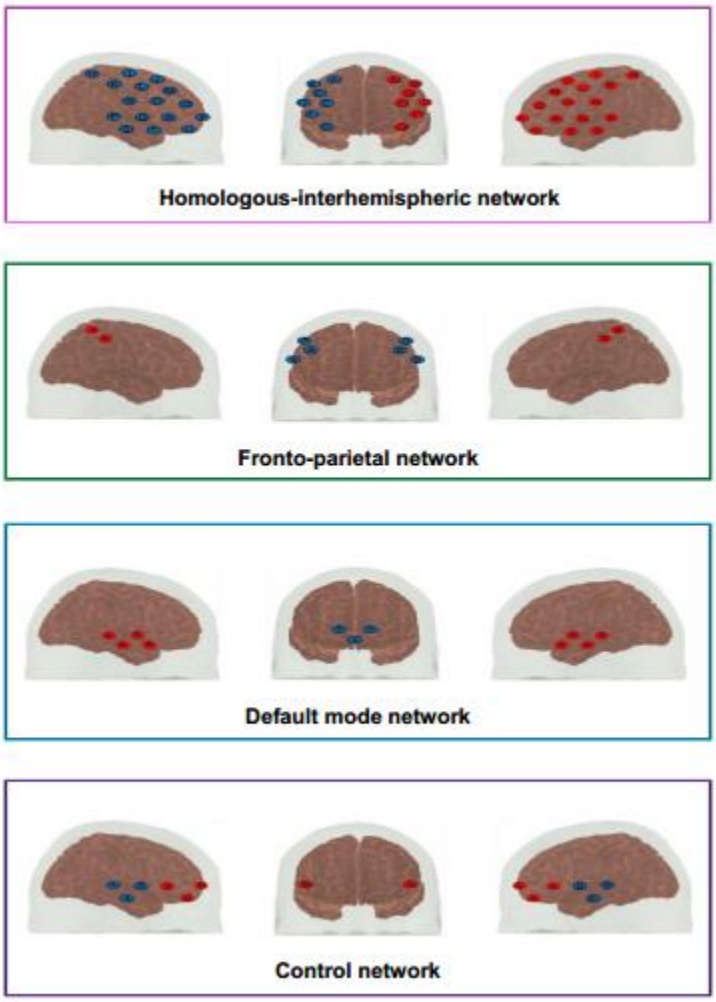


Figure 1. Shows the configurations for each of the network patterns. Note, each network consists of the average of all of the connections between red and blue channels of the same letter.

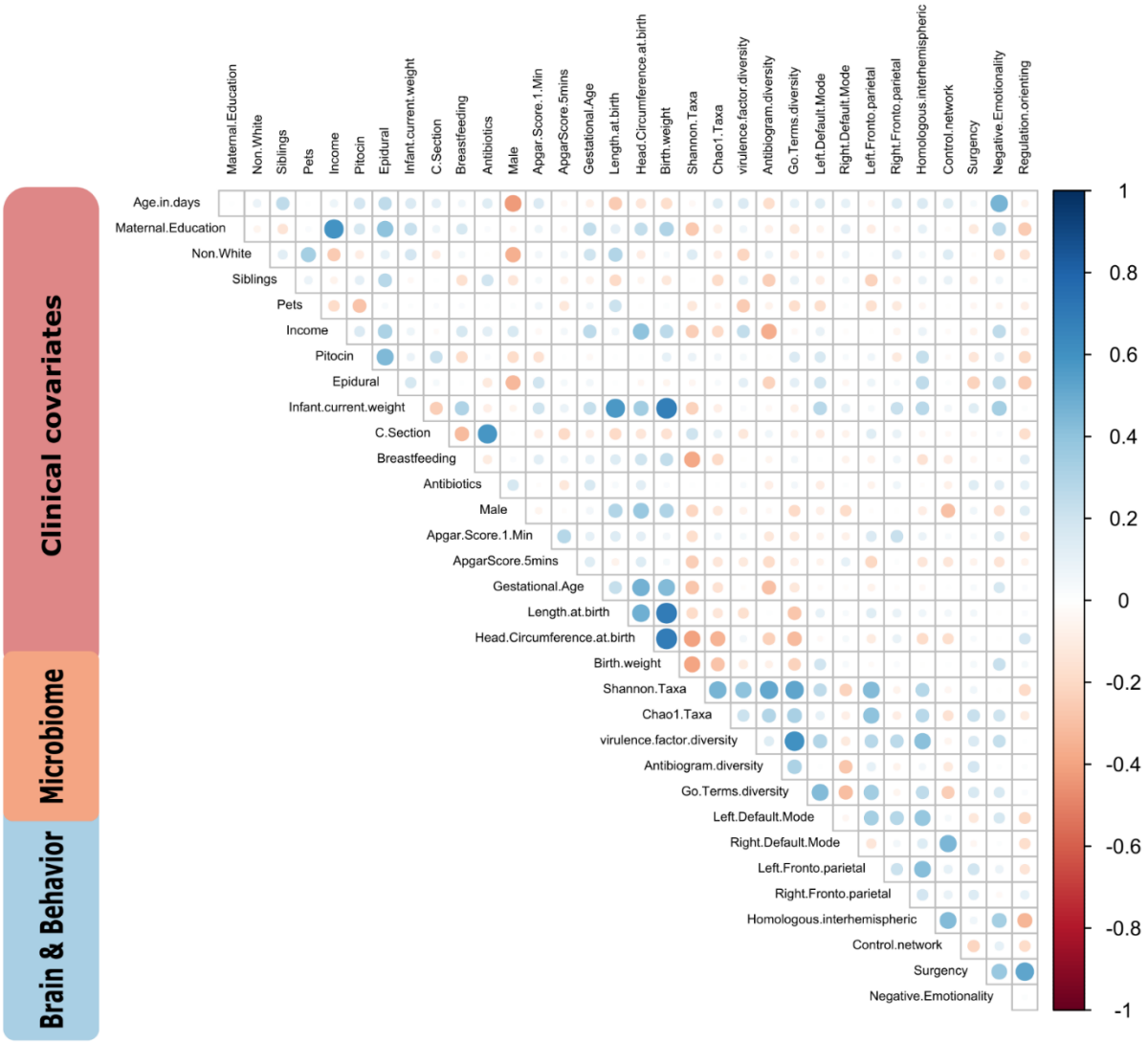


Figure 2. Schematic representation of correlations between all clinical covariates and study variables. Note, larger circles represent higher correlation values.

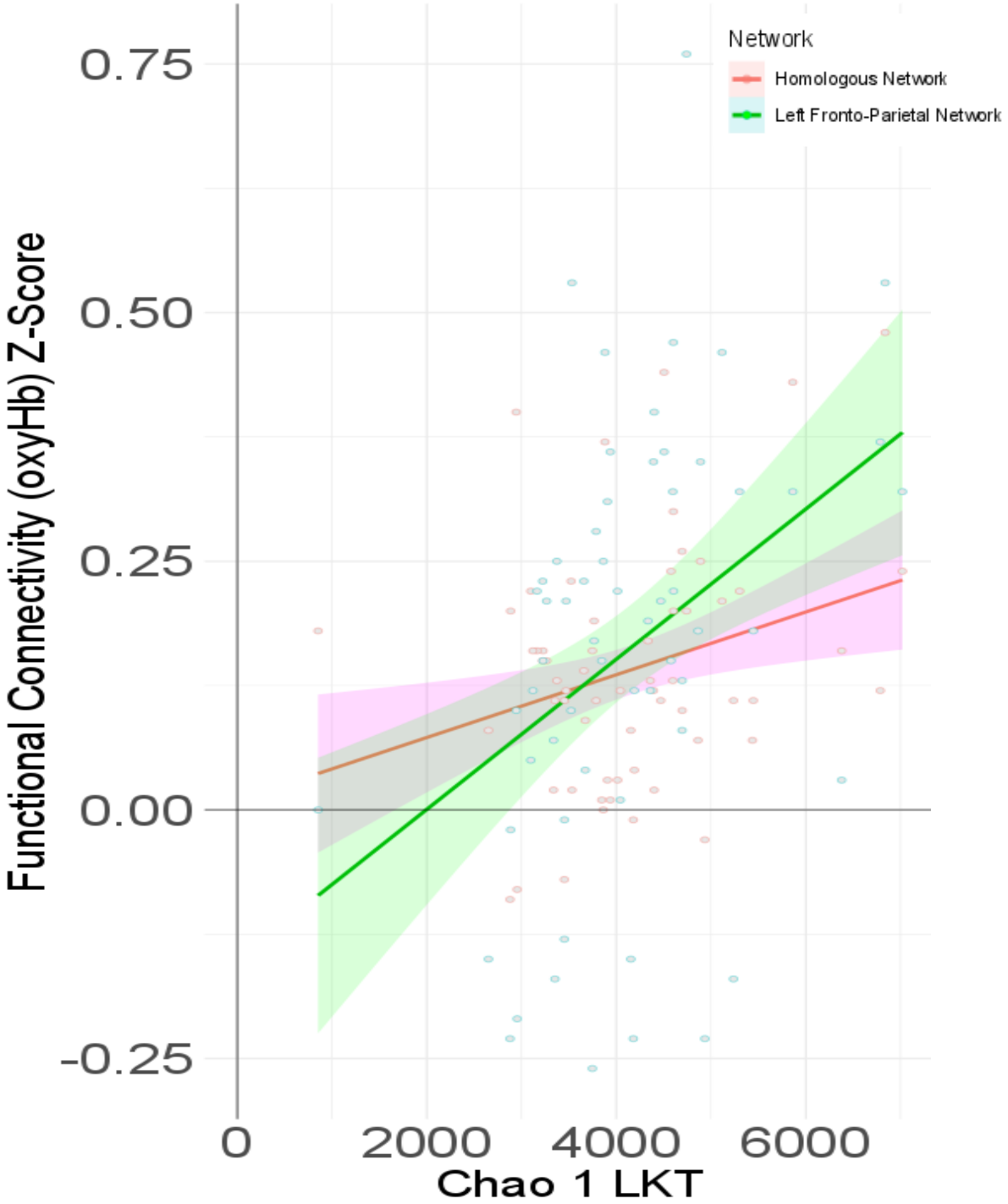


Figure 3. Shows the unadjusted relation between Chao1-Taxa and functional connectivity (oxyHb) Z-score for the Homologous-interhemispheric network and Left Fronto-parietal network. Note, shaded regions represent 90% confidence intervals.

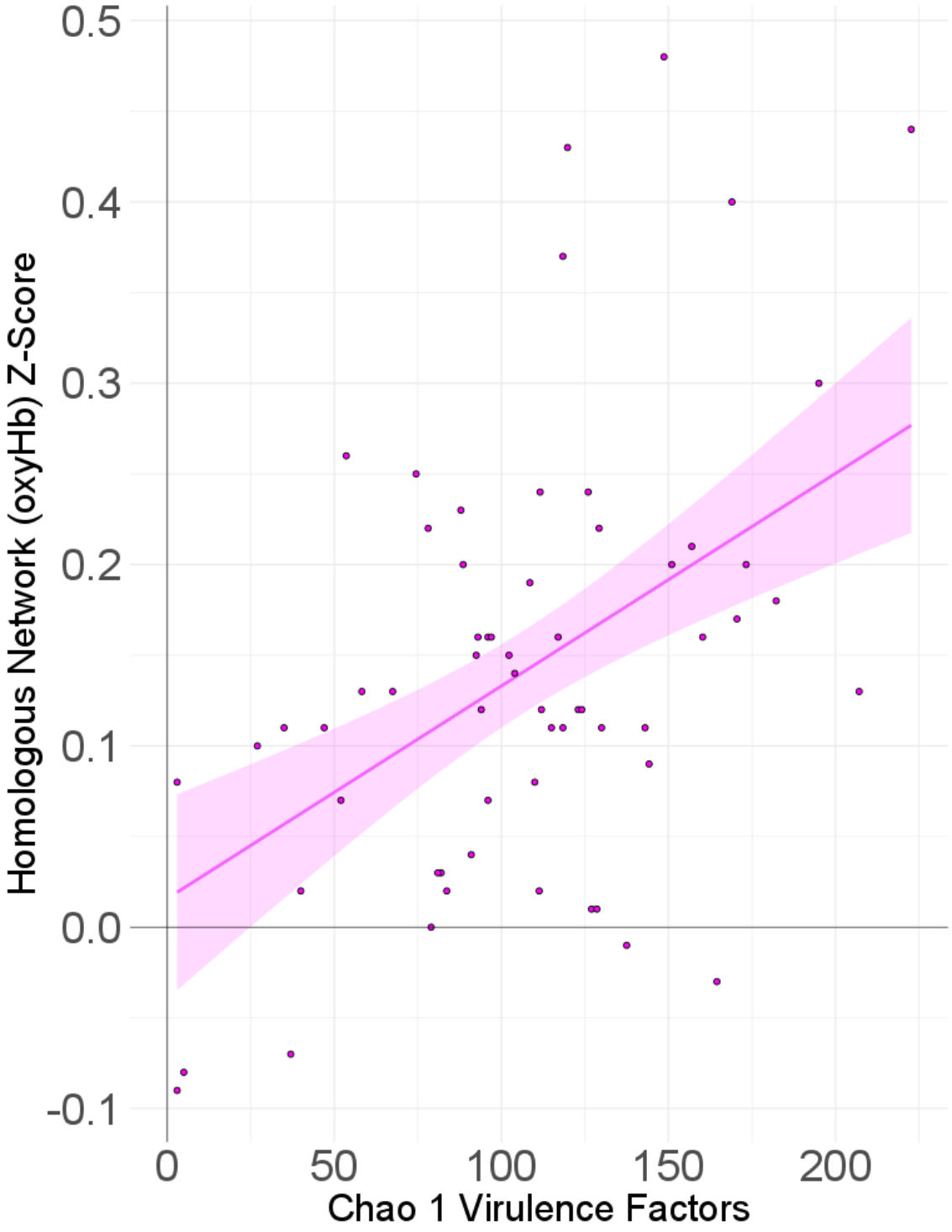
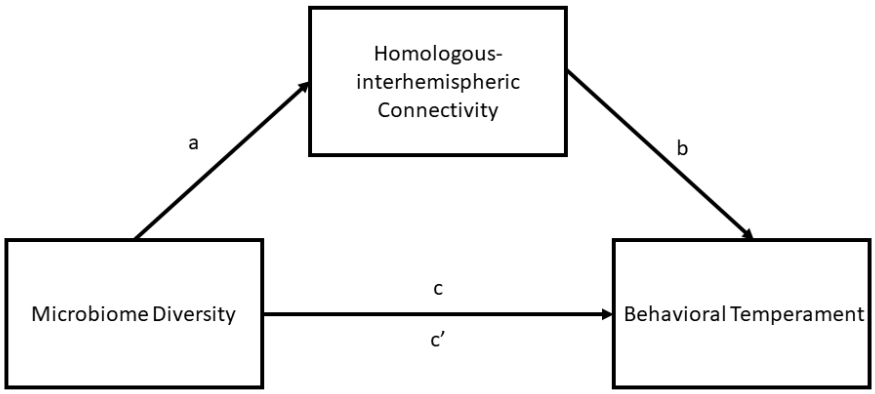


Figure 4. Shows the unadjusted relation between Virulence Factor Diversity and Homologous-interhemispheric network connectivity. Note, shaded regions represent 90% confidence intervals.

A



B

Variables	a	b	c	c'
Behavioral Temperament: Negative Emotionality				
Shannon-Taxa	β = .31,	β = .39,	β = .-.26,	β = .12
	p = .015	*p* = .004	*p* = .051	CI: **[.020, .273]**
Chao1-Taxa	β = .29,	β = .31,	β = .00,	β = .09
	p = .023	*p* = .022	*p* = 1.0	CI: **[.002, .211]**
Virulence Factors	β = .47,	β = .27,	β = .09,	β = .13
	p < .001	*p* = .064	*p* = .55	CI: **[.005, .271]**
Behavioral Temperament: Regulation/Orienting				
Shannon-Taxa	β = .32,	β = -.28,	β = -.054,	β = -.088
	p = .013	*p* = .038	*p* = .68	CI: [-.224, .000]
Chao1-Taxa	β = .29,	β = -.32,	β = .071,	β = -.091
	p = .025	*p* = .018	*p* = .59	CI: [-.252, .000]
Virulence Factors	β = .47,	β = -.39,	β = .20,	β = -.19
	p < .001	*p* = .007	*p* = .15	**CI: [-.412, -.023]**

Note: significant indirect effects are in bold.

Figure 5. (A) The theorized mediation model where gut microbial diversity indirectly impacts behavioral temperament through its influence on functional brain connectivity, (B) Shows the corresponding statistical values for paths outlined in the mediation model.

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