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Gut microbiota composition is associated with newborn functional brain connectivity and behavioral temperament

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ABSTRACT

The gut microbiome appears to play an important role in human health and disease. However, only little is known about how variability in the gut microbiome contributes to individual differences during early and sensitive stages of brain and behavioral development. The current study examined the link between gut microbiome, brain, and behavior in newborn infants (N = 63; *M* [age] = 25 days). 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 is linked to individual variability in brain network connectivity, which in turn mediated individual differences in behavioral temperament, specifically negative emotionality, among infants. Furthermore, virulence factors, possibly indexing pathogenic activity, were associated with differences in 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. This suggests that the gut microbiome is an important biological factor to consider when studying human development and health.

1. Introduction

The human gut microbiome is a complex ecosystem comprised of the microorganisms lining the intestinal tract, including bacteria, viruses, fungi, and archaea. The gut microbiome is crucial to normal physiological, metabolic, and immune function (for an example of another paper using this method see (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 and Dinan, 2012; Sender et al., 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 and 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 (Kelsey and Grossmann, 2019; Cowan et al., 2019; Kelsey et al., 2018).

Previous correlational studies in adults have shown that changes in the gut microbiome – 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 and 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.,

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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 et al., 2019). In addition, germ-free mice, which are reared in an entirely sterile environment, have been used for a sledgehammer 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 et al., 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 that the initial commensal microbiome, or the founding microbial population, has an exceedingly large and lasting influence over the lifetime composition of the microbiota (Litvak and Bäumler, 2019). In line with this hypothesis, studies have shown that 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.

The prenatal and early postnatal life represent sensitive periods marked by tremendous growth in brain, behavioral, and gut microbiota development (Cowan et al., 2019). Specifically, new evidence has emerged that functional brain networks, or brain regions with high temporal correlations for low frequency oscillations, come online earlier than previously thought (Damoiseaux et al., 2006; Kaiser et al., 2015). Specifically, short range and interhemispheric (homologous) networks are detectable even before birth whereas long range networks (such as the default mode and fronto-parietal network) show a more protracted development across the first year of postnatal life (Gao et al., 2015; van den Heuvel and Thomason, 2016). Similar patterns are seen at the level of newborn behavior. Already within the first few hours of development, infants orient to the sight, sounds, and smells of other humans and their mothers (DeCasper and Fifer, 1980; Farroni et al., 2013; Farroni, Csibra, Simion, & Johnson, 2002; Farroni et al., 2005; Rattaz et al., 2005). These behavioral and regulatory capacities continue to grow and develop in step with improved sensory functions (Feldman, 2009; Sheese et al., 2008). During the same period and in a similar manner to brain and behavioral development, the gut microbiota also go from a sparse environment marked by high levels of *Bifidobacteria* (microbes involved in digestion of human milk oligosaccharides) to a diverse flora rich in *Bacteroides* (microbes involved in the digestion of complex starches) coinciding with the introduction of solid foods (Moore and Townsend, 2019).

Although the brain and microbiome share similar windows of rapid development, limited research has investigated this connection through direct assessment of the gut microbiota in humans (Kelsey et al., 2018). Across the five existing infant studies with four cohorts of infants, 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., 2019a; Carlson et al., 2018; Christian et al., 2015; Loughman et al., 2020), making any clear conclusions regarding the link between diversity and positive infant mental health outcomes difficult to parse. Notably, these studies also relied upon taxa diversity as the main characterization of the gut microbiota; this limitation may have contributed to the mixed findings (Cowan et al., 2019). To advance our understanding of these relationships, there is a need to assess the functionality of the microbes, or the genes expressed in the microbiota, allowing insights into not only the taxonomy of microorganisms present, but also the relevant biological processes in which they are functionally contributing (Hooks et al., 2019; Knight et al., 2018).

The existing infant studies have relied on 16S rRNA gene sequencing which affords insight into the taxonomic composition of the bacterial species in the gut microbiota yet 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., 2019a; Carlson et al., 2018; Christian et al., 2015; Gao et al., 2019). Alternatively, shotgun metagenomics encompasses all DNA sequences 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 microbial functional pathways provides a more powerful tool to better characterize and understand the potential link with brain and psychological development.

The predicted functionality of protein coding genes found in a (meta) genome can be characterized at multiple levels, from simple annotation by homology to protein databases, to further grouping of functionally related genes into signatures. 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, genes coding for molecules created by microorganisms to aid in their ability to colonize, suppress immunity, and divert nutrients away from the host, and 3) resistome, genes coding for products which are predicted to yield resistance to antibiotics 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 (using the same cohort of infants) 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 supplemental materials 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 brain development and brain connectivity, more systematic research investigating this link is needed. Therefore, the first goal of this study was to examine whether and how taxa diversity and functional diversity are linked to functional brain connectivity. In order to test whether gut microbiota composition is linked to functional 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 et al., 2015; Wang et al., 2013). The second goal was to examine whether and how both taxa diversity and functional diversity are linked to behavioral temperament in the newborn period. Temperament refers to individual differences in an infant's emotional and attentional responses to everyday situations (Rothbart, 2007).

Specifically, the present investigation focused on the following, previously identified, dimensions of behavioral temperament: regulation/orienting, negative emotionality, and surgency/positive emotionality (Gartstein and 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 of its kind to use state-of-the art metagenomic sequencing, allowing 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 whether individual differences in behavioral temperament and functional brain connectivity measured using fNIRS could 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., 2019a; Bastiaanssen et al., 2020; Carlson et al., 2018), we predicted alpha diversity of taxa would be associated with negative emotionality, and regulation/orienting behaviors. Moreover, we hypothesized that alpha diversity of taxa would be associated with brain connectivity in resting-state brain networks previously linked to internalizing disorders in adults (Kaiser et al., 2015; Patashov et al., 2019). Specifically, we hypothesized that 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 microbiota 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., 2016).

Moreover, we predicted specific functional profiles of the gut microbiome such as decreased GO Terms (indicative of genes that function together as part of a network), increased number of virulence factors genes (indicative of potential pathogenicity of the microbes present), and increased number of antibiotic resistance genes 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 microbial species as 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 behavior, providing foundational insights into gut microbiome-brain-behavior relations.

2. Materials and methods

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 academic medical center and are a representative sample of the surrounding Mid-Atlantic college town (for socio-demographic information see Table 1). All participants were born at term, with normal birth weight (>2,500 g) 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* = 13 were excluded because they failed to reach our pre-determined inclusion criterion of having at least 100 s of continuous data during which the infant was not crying; *n* = 4 were excluded because>30% of the measured fNIRS channels had poor light intensity readings, more specifically, a signal-to-noise ratio of<1.5 (Bulgarelli et al., 2019; Xu et al., 2015) ; *n* = 4 were excluded because of bad capping; *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). Furthermore, in order to test that the infants were excluded at random and that the criteria for inclusion were not related to outcomes of interest, we compared the temperament profiles (negative emotionality, regulation/orienting, and surgency/positive emotionality) of infants that were included to those that were excluded using independent

Table 1
Socio-demographic information for the present study sample (N = 63).

Socio-demographic information		Mean/Count (SD/%)
Any antibiotic Treatments, n		30 (48%)
	Prenatal antibiotics	8 (12%)
	During labor and delivery	26 (41%)
	Postpartum administration to mother	3 (5%)
	Administered directly to the infant between delivery and study appointment.	4 (6%)
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)
Bristol Stool Scale Score		6.41 (0.61)
Breastfeeding, n		56 (90%)
Epidural, n		37 (60%)
Gestational Age, weeks		39.43 (1.18)
Female, 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 Depression		10.92 (3.22)
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)
Hours between stool sample collection and freezing		7.96 (8.57)
Lived with pet(s), n		37 (60%)
Pitocin, n		31 (50%)
Race white, n		45 (73%)
Vaginal Delivery, n		47 (76%)

Note: Maternal depression was assessed using the Edinburgh postnatal depression scale (Cox et al., 1987). There were two points of missing data for birth length and head circumference, one point missing for Pitocin use, and one point of missing data for the Bristol Stool Scale. Infants whose parent reported breastfeeding at any amount were considered breastfed. Any antibiotic treatment included infants potentially exposed to antibiotics during labor and delivery and through administration of antibiotics directly to the infant.

samples t-tests; However, no significant differences were found between the groups (all p-values > 0.30). 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).

2.1. Stool collection and processing.

Parents were instructed to collect infant stool samples at home using infant diapers within 24 h of the study visit. This instruction was based on previous work showing that microbial communities are stable at room temperature for up to 24 h (Cardona et al., 2012; Guo et al., 2016; Liang et al., 2020). The average time between infant reported defecation and freezing the samples was 7.96 h, which is well below the 24-hour recommendation. The average Bristol Stool Scale score was 6.41. Once received by the investigators, stool samples were immediately aliquoted into cryovials containing a 20% Glycerol and 80% Phosphate-Buffered Saline solution (this solution was used in order to preserve microbial species for future studies interested in reconstituting microbiome in animal models), and stored at −80 °C. Note, efforts were made by investigators to isolate the innermost portion of the stool sample, as it is least likely to be contaminated by urine. Bio-specimens 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.

2.2. Shotgun metagenomic Analysis.

Shotgun sequencing was analyzed using a series of pipelines and functions in the R language developed *in-house* and publicly available on Github under the package name JAMS, found at https://github.com/johnmcculloch/JAMS_BW (For an example of another paper using this method see (Rosshart et al., 2019). This package includes a pipeline (JAMSalpha) for obtaining taxonomic and functional relative abundance of features within each sample using FASTQ files as input and a series of functions in the R language for comparison between samples (beta analysis).

For each metagenomic shotgun sequencing sample in this study, the paired-end sequencing FASTQ files generated from the Illumina NovaSeq platform were used as input for JAMSalpha, Version 1.39 in order to gauge counts for taxonomic and functional features (McCulloch, 2019). Briefly, 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), using Megahit (Li et al., 2015). Contigs were taxonomically classified using k-mer analysis with kraken2 (Wood and Salzberg, 2014), using a custom-built database containing all draft and complete genomes of all Bacteria, Archaea, Fungi, Viruses and Protozoa deposited in NCBI GenBank. Contigs were also annotated, *ab initio,* using Prokka (Seemann, 2014), yielding the predicted proteome for the metagenomic sample. Sequencing reads were then aligned back to assembled contigs in order to gauge base pair counts for each contig, and thus, each predicted gene. The total basepair count for each last known taxon (LKT) – deepest taxonomic level up to species confidently classified using kraken2, was computed as the number of bases covering all contigs classified for each LKT. As of note, for other taxonomic classification methods, if there is no classification at the species level, sequences are simply deemed "unclassified". However, k-mer based classification of contigs rather than short reads or alignment to a reference genome, allows for a more granular assessment of these sequences by allowing them to be classified into their most appropriate taxomonic level even if it is above the species level.

The predicted proteome of each metagenomic sample (translated genes found within contigs) was further functionally classified using InterproScan (https://github.com/ebi-pf-team/interproscan) in order to attribute Gene Ontology Terms to each predicted protein. In parallel, the predicted proteome was also used as query against local instances of the VFDB database (Chen et al., 2016) and the ResFinder database (Zankari et al., 2012) using BLASTp. Hits with $< 75\%$ identity and/or $< 75\%$ query coverage were discarded.

For beta-diversity analyses, the relative abundance, in parts per million (PPM) of each feature was used. This is obtained by dividing the number of bases covering a feature by the total number of bases sequenced for that analysis in a sample multiplied by 10^6.

For alpha-diversity analyses, alpha-diversity measures were obtained using the Vegan package in R (https://cran.r-project.org/web/packages/vegan/index.html).

2.3. Infant temperament.

Infant behavioral temperament was assessed using parental reports the 91-item Infant Behavior Questionnaire Revised Short Form (IBQ-R; (Gartstein and Rothbart, 2003); Rigato, Stets, Bonneville-Roussy, & Holmboe, 2018; (Stifter and Fox, 1990; Worobey and Blajda, 1989). This measure has shown to be reliable and valid during the newborn period (for examples of other studies using this measure with newborn populations see: Rigato et al., 2018, (Stifter and Fox, 1990; Worobey and Blajda, 1989). Parents completed the questionnaire online using the Qualtrics platform 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 and Rothbart, 2003). If parents reported the behavior was not applicable at the current time, then this item was given a value of 0.

2.4. 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. This stimulus was selected based on prior work that has shown that presenting a non-social video increases compliance and decreases movement for young infants, recent recommendations for the design of connectivity tasks for pediatric populations, and adult work suggesting that the presentation of non-social videos does not influence functional connectivity (Bulgarelli et al., 2020; Camacho et al., 2020; Vanderwal et al., 2015). Specifically, Vanderwal and colleagues (2015) showed children (ages 3–7) three sets of stimuli (a fixation cross, low-level movie, and a popular musical cartoon) during a resting state fMRI task. The authors concluded that a non-social movie was the best choice as it provided a functional connectivity metric that more closely matched the fixation cross while also providing higher quality (more

artifact free) data. These clips were shown in 30 s 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 et al., 2010). The fNIRS system used contains 49 channels positioned over frontal and temporal-parietal regions and recorded measurements (as previously described in Altvater-Mackensen and Grossmann, 2016; Altvater-Mackensen and Grossmann, 2015; Grossmann et al., 2018; Kelsey et al., 2019; Krol et al., 2019). The system emits two wavelengths of light in the Near-Infrared spectrum, 760 nm and 850 nm, and captures both deoxyHb and oxyHb. The diodes have a power of 25 mW/wavelength and data were recorded at a preset default 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. Specifically, researchers coded for behavioral signs of fussiness/irritability and alertness displayed by infants during the testing session. To assess the reliability of the attentional coding done by the primary coder, an additional trained coder also coded infant behavior from selected subsample of infants ($n = 19$). This analysis showed that inter-rater reliability amount of data included was excellent (Cronbach's $\alpha = 0.94$). In line with previous studies, infants were only included in the present analysis if they had at least 100 s of data during which the infant was not crying (Bulgarelli et al., 2019). Moreover, as it takes a minimum of 8 s for the Hemodynamic response function to return to baseline after a stimulus-evoked event, the onset of useable data was delayed for 8 s (Bulgarelli et al., 2019, 2020). However, unlike Bulgarelli and colleagues (2019, 2020) the time series was continuous. On average, infants contributed 331.29 s of data ($SD = 115.75$ s). Note, this amount of data included is comparable to other fNIRS functional connectivity papers with young infant (see 11-month time point in (Bulgarelli et al., 2020). Finally, infants were rated on their levels of alertness during the task with 1 reflecting deep sleep and 6 reflecting crying (Brazelton et al., 1987). On average, infants were rated as being midway between an active light sleep to a drowsy state ($M = 2.55$; $SD = 1.26$).

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<1.5) (please note, this value was selected by the author's in order to optimize the number and quality of channels being retained; Xu et al., 2015). In order to be included in the present analyses, infants needed to have at least 70% of their channels passing this predefined threshold (Bulgarelli et al., 2020). Next, data were band-pass filtered using a previously validated low frequency filter (0.01-0.08 Hz; Bulgarelli et al., 2019; Lu et al., 2009). Finally, concentration changes were calculated using the modified Beer-Lambert law [partial path length factor: 6.0] (Villringer and 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 et al., 2009; Murphy et al., 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. (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 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 Fig. 1 for schematic of network configurations; for more details on network configuration see Kelsey et al., 2020). Cortical projections were created using NIRSite by using 10–20 system references from the cap layout. The present study focused on oxyHb based on previous work by the authors that has found brain-behavior correlation for this chromophore (Kelsey et al., 2020). 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 > 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 $n = 1$).

2.5. 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), or expected proportion of type I errors (false positives). We considered results with FDR < 0.25 as significant (see Aatsinki et al., 2019a for another example of a paper using this threshold and Aatsinki et al., 2019b for a discussion of the use of a 0.25 FDR cutoff). FDR was estimated using the Benjamini & Hochberg method with the R function *p.adjust*.

Linear discriminant analysis of effect size (LefSE) and Microbiome Multivariate Association with Linear Models (Maaslin2) were used to identify potential microbial biomarkers of functional connectivity and behavioral temperament using the Galaxy tool (http://huttenhower.sph.harvard.edu/galaxy/) and R respectively (Mallick et al., In Submission). For LefSE, 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. For analysis with Maaslin2 the following default options were used: minimum abundance = 0, minimum prevalence = 10%, normalization = TSS, transformation = Log, q-value threshold = 0.25.

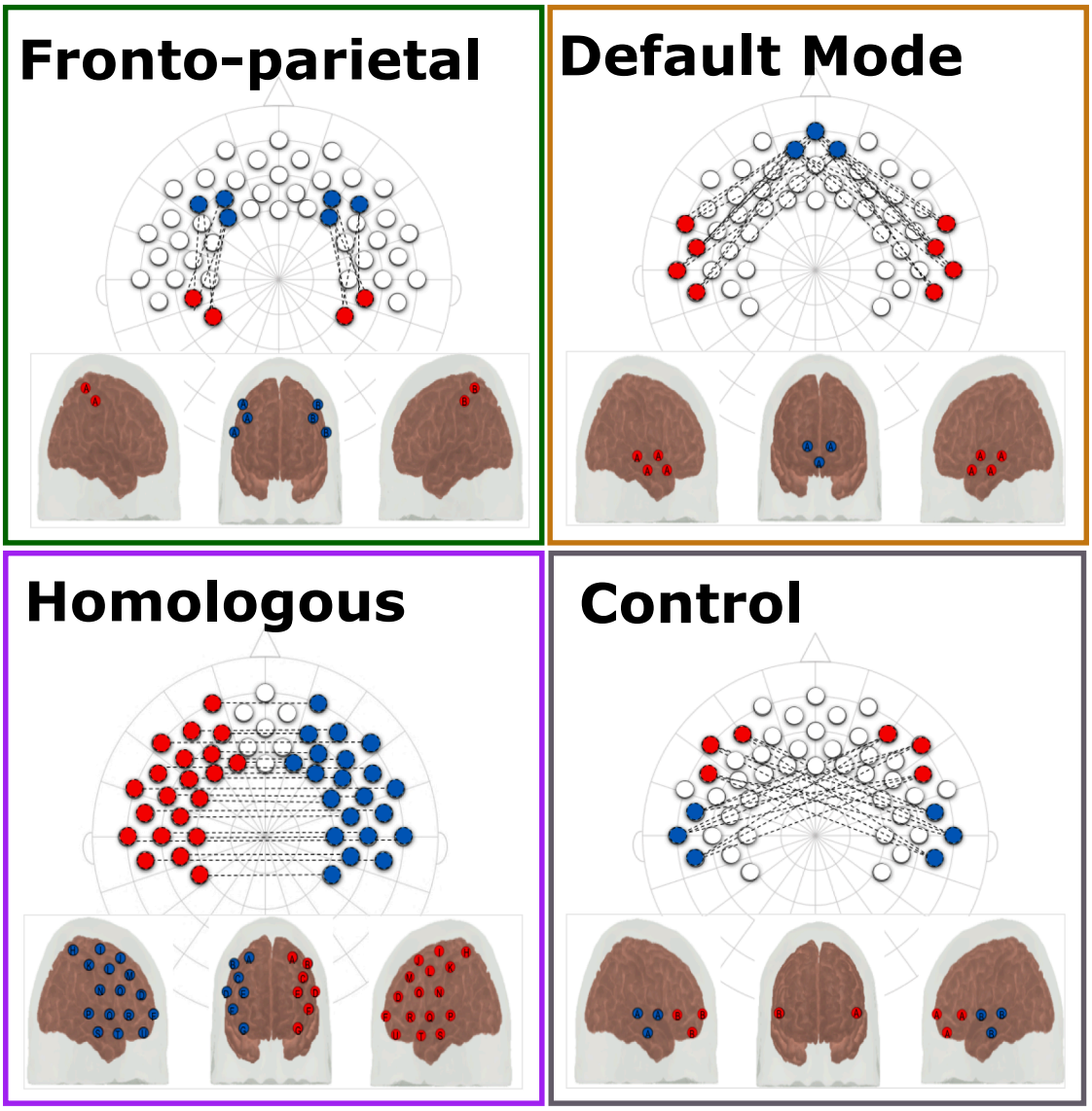


Fig. 1. Shows the configurations for each of the network patterns. Note, each network consists of the average of all of the connections between red and blue channels of the same letter. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. 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 Fig. 2). Any clinical variables found to be significantly associated with a study variable of interest were then included in the subsequent models assessing differences in said study variable as a covariate. We found significant associations between the Shannon-Taxa and birthweight (Spearman's rank correlation $r_s = -0.40$, $p = .001$), income (Spearman's rank correlation $r_s = -0.25$, $p = .049$), breastfeeding (Kruskal-Wallis H $X^2 = 9.14$, $p = .002$), gestational age (Spearman's rank correlation $r_s = -0.31$, $p = .016$), and head circumference (Spearman's rank correlation $r_s = -0.37$, $p = .004$). 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 resistome, virulence terms, and GO Terms) and clinical covariates. Here, we found that resistome diversity was significantly associated with income (Spearman's rank correlation $r_s = -0.31$, $p = .016$), gestational age,(Spearman's rank correlation $r_s = -0.36$, $p = .004$), and maternal depression scores (Spearman's rank correlation $r_s = 0.26$, $p = .044$). Similarly, virulence factor diversity was associated with income (Spearman's rank correlation $r_s = 0.33$, $p = .008$) and antibiotics administered at the hospital after birth (Spearman's rank correlation $r_s = 0.38$, $p = .002$). Furthermore, GO Term diversity was associated with sex (Kruskal-Wallis H $X^2 = 5.37$, $p = .02$) and head circumference (Spearman's rank correlation $r_s = -0.37$, $p = .004$).

Finally, we assessed the relation between clinical covariates and psychological outcome measures (behavioral temperament and functional brain connectivity). Here, we found significant associations between negative emotionality, infant age (Spearman's rank correlation $r_s = 0.43$, $p = .001$), and income (Spearman's rank correlation $r_s = 0.36$, $p = .005$). However, there were no other significant associations found between clinical covariates and psychological outcome measures.

3.2. 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

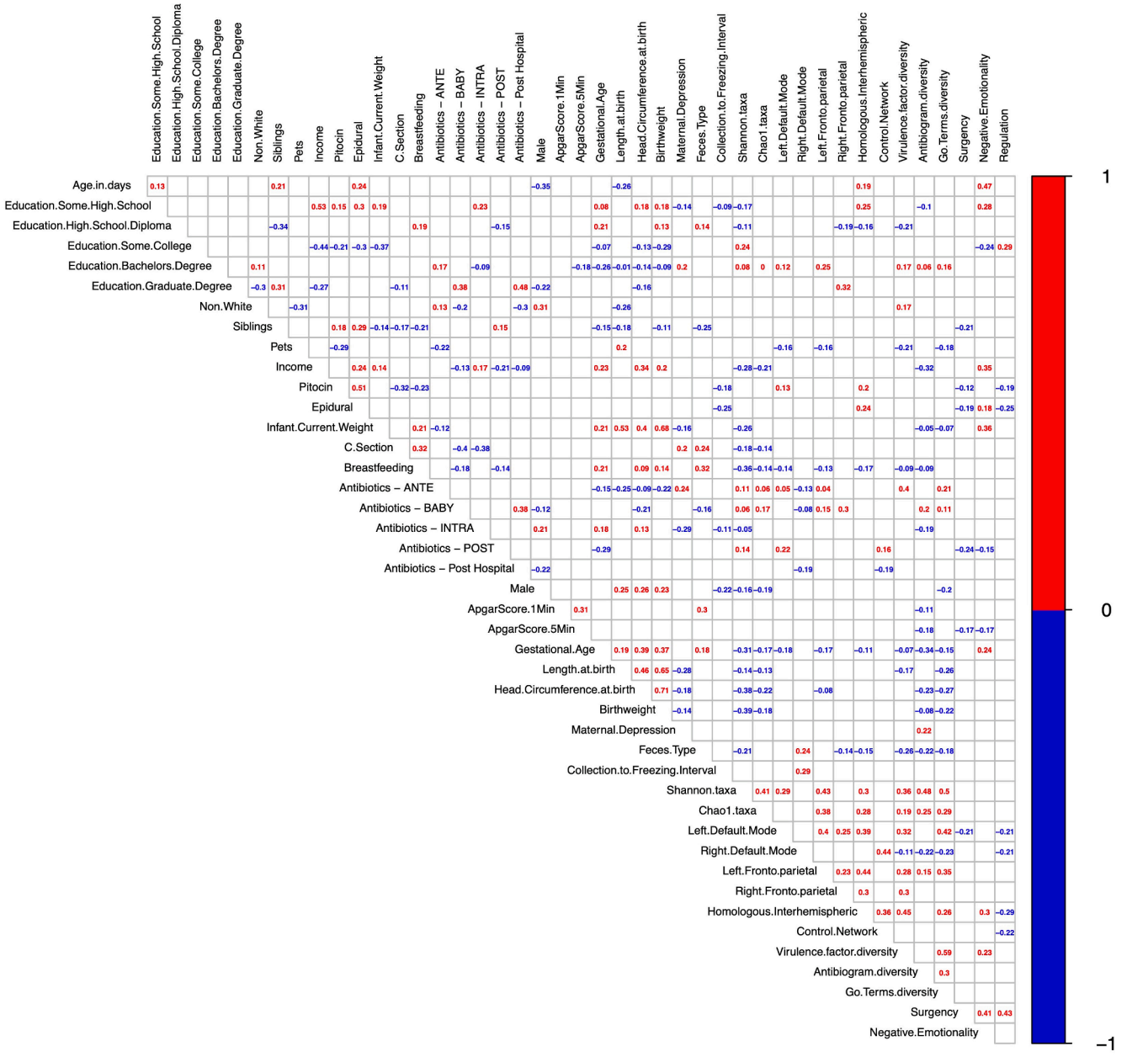


Fig. 2. Schematic representation of correlations between all clinical covariates and study variables. Note, blue text represents significant positive associations, red text represents significant negative associations, and blank cells represent nonsignificant associations. Note, statistical significance is defined here as $p < .05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 $\beta = 0.71$, FDR $= 0.07$, adjusted $R^2 = 0.13$, Shannon-Taxa $\beta = 0.14$, FDR $= 0.03$, adjusted $R^2 = 0.17$), as well as alpha diversity of taxa and homologous-interhemispheric network connectivity (Chao1-taxa standardized $\beta = 0.16$, FDR $= 0.10$, adjusted $R^2 = 0.07$; Shannon-Taxa $\beta = 0.06$, FDR $= 0.20$, adjusted $R^2 = 0.09$; See Fig. 3). When the models were adjusted for significant covariate associations, both the relations between Shannon-taxa and Chao1-taxa with the left fronto-parietal network connectivity remained significant (Chao1-taxa standardized $\beta = 0.46$, FDR $= 0.20$, partial $R^2 = 0.12$; Shannon-taxa $\beta = 0.18$, FDR $= 0.07$, partial $R^2 = 0.17$; covariates included: antibiotics, delivery method, breastfeeding, infant age, infant weight at birth and at study visit, gestational age, income, 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 $= 0.88$).

3.3. *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, resistome, 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 separately. Note, Chao1 (and not Shannon) index was used to test for how the number of functional terms (as opposed to the evenness)

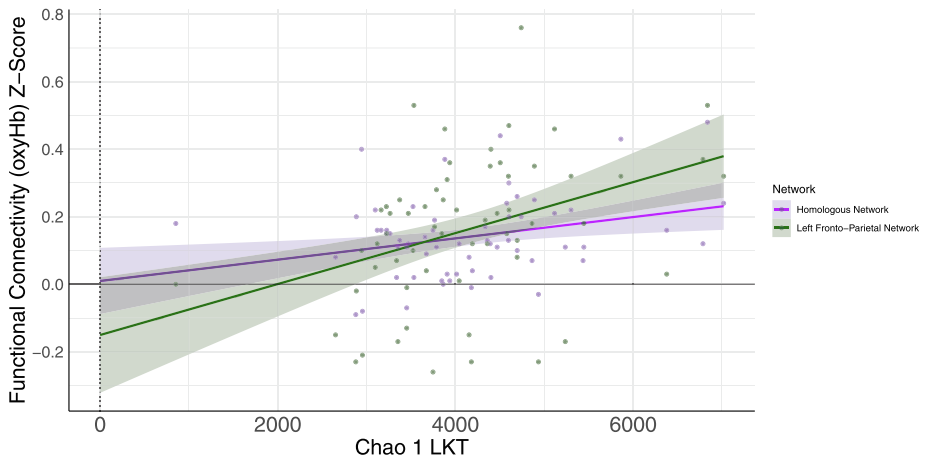


Fig. 3. Shows the unadjusted relation between Chao1-Taxa and functional connectivity (oxyHb) Z-score for the homologous-interhemispheric network and left Fronto-parietal network. Note, shaded regions represent 90% confidence intervals.

is related to the outcomes of interest. We observed that 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 = 0.12, partial $R^2 = 0.14$; See Fig. 4). Moreover, when the model was adjusted for significant covariate associations, the relation between Virulence factor diversity and homologous-interhemispheric network connectivity remained (standardized $\beta = 0.23$, FDR = 0.13, partial $R^2 = 0.16$; covariates included: antibiotics, delivery method, breastfeeding, infant age, infant weight at study visit, gestational age, income, sex, maternal depression, 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 > 0.26) or the control network (FDR > 0.31) for the unadjusted models.

3.4. 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, resistome, and GO terms) as the predictors and behavioral temperament (negative emotionality, regulation/orienting, surgency/positive emotionality) as the outcome variables were conducted. We did not find any significant associations between either of the alpha diversity metrics for taxa and behavioral temperament. Similarly, we did not find any associations between the alpha diversity indices for the functional terms and behavioral temperament.

3.5. 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 Fig. 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 = 0.19, adjusted $R^2 = 0.08$) and regulation/orienting ($\beta = -0.26$, FDR = 0.20, adjusted $R^2 = 0.07$).

To do this, we used ordinary least squares path analysis and bootstrapped 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 from the present study, increased alpha diversity (Chao1-Taxa $\beta = 0.29$; Shannon-Taxa $\beta = 0.31$) was associated with increased homologous-interhemispheric connectivity. Additionally, homologous-

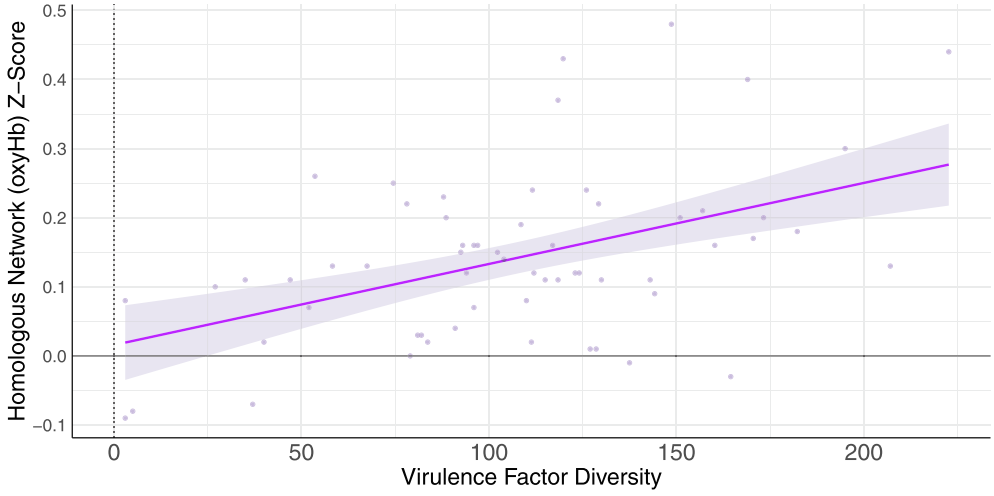
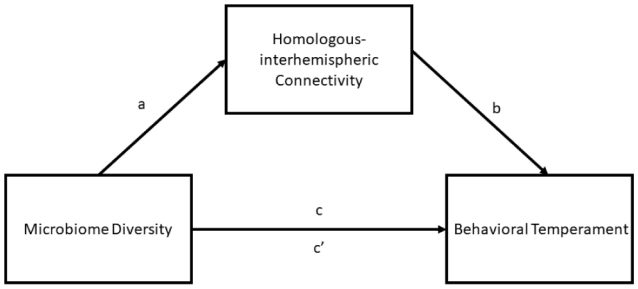


Fig. 4. Shows the unadjusted relation between virulence factor diversity and homologous-interhemispheric network connectivity. Note: shaded regions represent 90% confidence intervals.

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.

Fig. 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.

interhemispheric connectivity was associated with increased negative emotionality (β = 0.31-0.39). We also observed a significant indirect effect, suggesting the relationship between increased alpha diversity and negative emotionality may be mediated by homologous-interhemispheric connectivity (Chao1-taxa β = 0.09, CI = [0.002, 0.211]; Shannon-taxa β = 0.12, CI = [0.020, 0.273]). There were, however, no significant indirect effects found for the relations between taxa diversity (Chao1-taxa and Shannon-taxa) and regulation/orienting.

We then assessed if virulence factors influence behavioral temperament through its effect on homologous-interhemispheric connectivity. Increased virulence factor diversity (β = 0.47) was associated with increased homologous-interhemispheric connectivity. In addition, homologous-interhemispheric connectivity was associated with increased negative emotionality (β = 0.27). There was a significant indirect effect found, suggesting the relation between virulence factor diversity and negative emotionality may be mediated by homologous-interhemispheric connectivity (β = 0.13, CI = [0.005-0.271]). 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 through a negative association (β = -0.19, CI = [-0.412, -0.023]).

3.6. Taxa biomarker identification

Functional connectivity. LefSE identified fourteen total potential microbial biomarkers for the functional connectivity networks (LDA log fold change cut-off = 3) and are described in Table 2. 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).

An alternative biomarker discover technique, MaAslin2, was performed to validate findings. More specifically, a linear model with the five functional networks (fronto-parietal [left and right], default mode [left and right], homologous-interhemispheric) included as fixed effects was conducted (an additional model with covariates included as random effects was run and included in supplementary materials). For the unadjusted model, Maaslin2 identified 479 total potential microbial biomarkers for the functional connectivity networks (q-value < 0.25) and the top fifty hits are summarized in Fig. 6. Again, we see the left fronto-

Table 2
LefSE identified taxa biomarkers of functional connectivity networks.

Phylum	Family	Genus	Species	Log fold change	Group with thehighest Median Connectivity
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

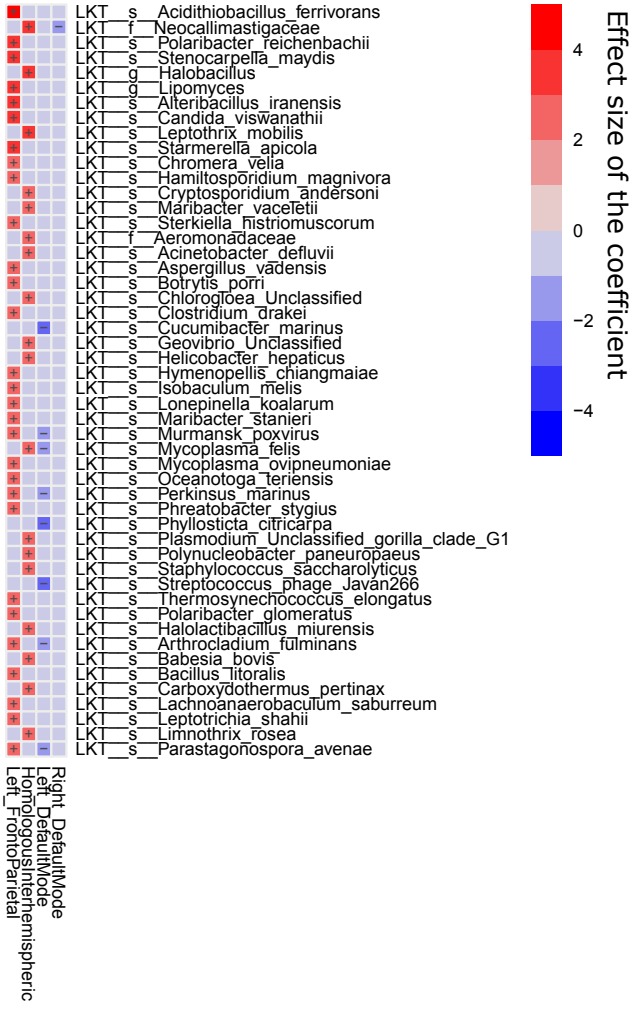


Fig. 6. MaAslin2 top fifty taxa biomarkers of functional connectivity identified in the unadjusted model. Taxa with the lowest q-values are at the top.

parietal network was marked by an overall enrichment of *Clostridium* taxa (21 taxa identified; β = 1.76–2.99, q-values = 0.06-0.25) and the left default mode network was associated with a diminution of *Clostridium* (2 taxa identified, β = -2.59 to −2.68, q-values = 0.19-0.25).

Temperament. LefSE identified a total of five microorganisms as potential biomarkers for temperament and are described in Table 3. Both negative emotionality and regulation/orienting were marked by an enrichment of *Bifidobacterium*. In particular, *B. pseudocatenulatum* was enriched in the high negative emotionality group (Log fold change = 4.09) and the high regulation/orienting group (Log fold change = 4.48).

Similarly, MaAslin2 was performed as an additional biomarker discovery tool for behavioral temperament. More specifically, a linear model with the three temperament domains (regulation/orienting, negative emotionality, and surgency) included as fixed effects was conducted (an additional model with covariates included as random effects was run and included in supplementary materials). For the unadjusted model, Maaslin2 identified one biomarker for Negative emotionality, *Thermovibrio guaymasensis* (β = 0.37, q-value = 0.087).

4. 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 shotgun metagenomic sequencing, our results provide new evidence for an association between genes coding for microbial 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; Rothbart, 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 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

Table 3
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

affecting the microbiota composition may impact brain development *in utero*.

Contrary to our hypothesis, in the current study, taxa diversity was positively associated with connectivity in infants' homologous-interhemispheric network, consequently linked to heightened negative emotionality and decreased regulatory behaviors. While this finding was in opposition to our hypothesis partly based on prior work with adults, it is similar to prior work with infants. In particular, a study by Carlson et al. (Carlson et al., 2018) found that increased 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. (Christian et al., 2015) study reported that increased alpha diversity was associated with decreased regulatory behaviors both of which were assessed at same time (18–27 months). It is interesting to consider the possibility that these results, like the present results, may be attributed to an increase in microorganisms that are high in virulence factors. Given that certain external factors such as cesarean section and cessation of breastfeeding contribute to a premature progression of the gut microbiota, it is possible that lower taxa diversity is developmentally appropriate at this stage of life (Bäckhed et al., 2015). Taken together, our findings are thus 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 diversity of genes coding for various functions within the microbiota (The Gene Ontology Consortium, 2019; Li et al., 2015). Using this approach, we found that an increase in microbial genes coding for virulence factors was linked to increased homologous-interhemispheric connectivity among infants. Taken together with the taxa diversity findings, it appears that the aforementioned increase in taxa diversity may be driven, at least partly, by an increase in virulence factors. This result further highlights the limitations of 16S rDNA sequencing methods (Cowan et al., 2019), and how this can be addressed through shotgun metagenomics. 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 currently experiencing subacute 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 that 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 between microbial genes for virulence and brain function. Longitudinal studies will be required to unpack more fully the association between gut microbiota composition, infection status, and the influence of microbial virulence on the brain and behavioral traits during infancy. Furthermore, as evidence is accumulating in support of the gut-brain axis being mediated by microbiota-immune signaling, future work should consider collecting pre-inflammatory cytokines and other inflammatory markers (Fung, 2020). This would allow for a better understanding of these associations and may identify possible points of intervention (Fung, 2020). Moreover, while we do see a positive association between virulence factor diversity and taxa diversity, we are not currently able to determine whether this is driven by an increase of pathogenic microbes or just an increase in genetic machinery and potential to be pathogenic under the right circumstances. Therefore, future work should carefully characterize the microbial profile of high and low diversity microbiomes in infants.

Contrary to prior work with infants (Aatsinki, Lahti, Uusitupa, 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 accounting for the mixed findings. For example, though prior work has examined the gut microbiota within the first few months of life (Aatsinki, Lahti, Uusitupa, et al., 2019; (Loughman et al., 2020), our study examined the behavioral temperament in the youngest sample of infants studied to date. It is therefore possible that the predicted association between gut microbiota and behavioral traits only emerges later in infant development. Related, certain components of behavioral temperament, such as fear behaviors, do not emerge until later during the first year of life (Grossmann and Jessen, 2017). In line with this potential explanation, Aatsinki et al. (Aatsinki, Lahti, Uusitupa, 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 associated with homologous-interhemispheric brain network connectivity and indirectly associated with negative emotionality (Fig. 5). Gao et al. (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. Therefore, in conjunction with prior work, our findings support the notion that the gut microbiome may be more directly linked to or impacting the brain through the gut-brain axis, whereas links between the gut microbiome and overt behaviors are possibly harder to detect or emerge only later in development. More generally, in order to better characterize the link between the gut microbiome and behavioral traits, the current data indicate that it is critical to include measures of brain function.

To identify candidate biomarkers for behavioral temperament and brain connectivity, we took an unbiased and thorough approach through using both LefSE and MaAslin2. Across both analyses we identified multiple microbial species associated with early functional brain connectivity, including several microbes from the orders *Clostridiales* (including *Lachnospiraceae*, and *Bacteroides*) 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 metrics in both cortical and subcortical areas in adults (Labus et al., 2019). Our analysis showed that *Lachnospiraceae* and *Bacteroides* 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 this study by Callaghan et al. (2020) *Clostridiales* was significantly lower among adolescents that had experienced early adversity (institutionalization during infancy) compared to a control group. Furthermore, this study also identified *Lachnospiraceae* and *Bacteroides* as being linked to heightened medial Prefrontal Cortex responses to fearful faces assessed using fMRI. This prior study with adolescents shows early life experiences may shape the colonization of the gut with these microbes, and that this may affect brain development. Our study adds important evidence directly from infants to further support the role that 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 hypo-connectivity 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 et al., 2013).

With respect to negative emotionality and regulation/orienting temperamental domains, our analysis identified five associated microbes using unadjusted analyses, with two 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., 2019a). In addition, this genus 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 pathogens (Liévin et al., 2000). Overall, our current findings, together with 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 here using LefSE and MaAslin2.

Furthermore, while our results show some agreement between the two analysis methods, there were also significant differences in the biomarkers identified depending on the analysis method used. These differences can likely be attributed to specific difference across analytical methods. Specifically, LefSE first screens out candidates using nonparametric tests and then uses linear discriminant analysis to maximize the differences between groups (created in the present study by using a median split). On the other hand, MaAslin uses a form of generalized linear model and examines associations based on continuous outcome measures. Future work should continue to use both methods in conjunction to identify biomarkers more reliably.

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. 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 and better understand directionality of the associations seen in the current study (Kelsey et al., 2020). Relatedly, another limitation of the present study is the assessment of a single stool sample per infant which was collected in the home (and not in the laboratory) environment. In this context is important to note that prior work has highlighted significant temporal variation in the adult gut microbiome (Davenport et al., 2014; Riddle and Connor, 2016). However, the present study elected to collect a single sample in order to decrease burden on participants and reduce study drop-out. Moreover, other evidence has emerged suggesting intraindividual variability in key groups of microorganisms remains relatively low during infancy (Raman et al., 2019; Subramanian et al., 2014). Second, although we selected the current approach of rs-fNIRS to examine brain connectivity because it is relatively non-confining, allows the infant to remain with their mother, and it is a relatively affordable tool, 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, Dreisbach, et al., 2018). Fourth, it is important to note that even though parents were instructed to collect stool samples at home within 24 h before bringing their infants into the laboratory, it was not possible to freeze stool samples immediately after collection. This was done because, as for all developmental studies, the goal is to develop reliable and high-quality collection and storage methods that place the lowest possible burden on the participating families. Prior work has obtained mixed results in regards to room temperature stool storage. For example, Shaw et al. (2016) found significant differences in microbiome communities after storage at room temperature for 48 h. However, other work suggests that stool samples stored at room temperature from anywhere between 2 h to 52 h have microbiome communities that remain relatively stable (Cardona et al., 2012; Guo et al., 2016; Liang et al., 2020). In the present study samples had on average been stored at room temperature for only about 8 h. Moreover, in line with the latter set of studies (Cardona et al., 2012; Guo et al., 2016; Liang et al., 2020), we found no associations between amount of time passed between stool collection and freezing and microbial composition measures (both taxa and functional group diversity). Future research should continue efforts to better understand and monitor how stool collection and storage procedures account for inter- and intra-study variability in microbiome analysis. Finally, it is important to acknowledge that our use of stool samples provides an incomplete view of the entire intestinal tract (Donaldson et al., 2016). Stool samples are most reflective of the lumen and outer mucosa layers of the gastrointestinal tract due to the continuous secretion of the outer layers shedding into the stool sample (Donaldson et al., 2016). Thus, while stool samples might be limited in their ability to characterize gut microbiota across the entire intestinal tract, relying on stool samples is still the preferred method in developmental work given the low burden on infants and families and minimally invasive collection compared to other more comprehensive methodologies (e.g., colonoscopies or biopsies of the mucosa or small intestine tissue).

In summary, the current study provides novel insights into the early emergence of the gut-brain axis and supports that the connection between the gut microbial composition and functional brain connectivity is 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.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.11.003.

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