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Early-life gut microbiome is associated with behavioral disorders in the Rio birth cohort

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Emerging evidence has been linking changes in the early-life gut microbiome and neurodevelopmental outcomes. The founder bacteria that first colonize the infant's gut determine the microbial succession that signals host tissues and impact development including the brain. Here we investigated the association between the meconium microbiome and neurobehavior. To this end, we surveyed the 16S rRNA gene on meconium samples and assessed behavioral outcomes at six-months of age by the Denver Developmental Screening Test II (DDST-II). Among the four behavioral domains investigated, the personal-social domain was associated with significant differences in meconium bacterial beta diversity (unweighted UniFrac; R^2 0.078, p = 0.021) and reduced alpha diversity (β = −2.290, 95% CI = −4.212; CI = −0.368), after adjustment for gestational antibiotics, preterm delivery, and delivery mode. Besides, this altered neurobehavior (failing to meet the milestone) was associated with overrepresented Ruminococcaceae, Christensenellaceae, and *Eubacterium*, *Treponema*, *Senegalimassilia*, *Ruminiclostridium*, *Roseburia*, *Romboutsia*, *Prevotella*, and *Veillonella seminalis*. Predicted functional genes showed reduced abundance in association with altered neurobehavior (all q < 0.15). Fine and gross motor skills presented no associations with the microbiome. This pilot study shows associations between the first gut microbiome and behavioral outcomes that deserve further studies in different neonate populations.

Keywords Early-life microbiome, Founder microbes, Ruminococcaceae, Infant neurodevelopment, Neurodevelopmental screening

A growing body of epidemiological evidence has linked differences in the early-life gut microbiome structure with neurodevelopmental changes. Genera *Bacteroides* and *Bifidobacterium* have been associated with non-social fear behavior, duration of orientation, and cognitive and motricity development[1]. Microbial associations with neurodevelopment are poorly understood and need to be confirmed in different human populations, causality demonstrated, and underlying mechanisms clarified.

The neonatal microbiome starts assembling during partum in babies vaginally born or right after birth in babies born by C-section. Founder bacterial populations, which naturally come from the maternal vagina-perineum arrive to initiate an ecological succession that matures in a stable adult-like microbiome, which for the gut takes at least 3 years[2]. The microbiome maturation occurs in tandem with the development of the host, including the endocrine, mucosal immune, and central nervous system[3]. Alterations to the normal microbiome maturation affect this critical developmental process and increase the risk of metabolic[4], immune[5], and behavioral outcomes[1]. Specifically, C-section birth and antibiotics have been epidemiologically associated with increased risk of autism spectrum disorder (ADS).

A few studies have explored specifically the associated effects of primordial gut microbiome in the neonate. We previously reported that maternal exposure to environmental pollutants was associated with changes in meconium and developmental feces[6]. The health consequences of gut microbiome alterations are to be elucidated. Here, we characterize the meconium microbiome structure in neonates with the Denver Developmental Screening Test II (DDST-II) score assessed at 6 months of age.

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Results

Characteristics of the study population

In this study, we characterized the meconium microbiome and associated it with four Denver developmental domains at 6 months of age. The domains investigated included the personal-social, language, gross and fine motor and, only the first was associated with changes in meconium microbiome. Thus, our analyses included 36 infants, 14 female (38.9%), 19 (55.6%) were born by C-section, three (8.3%) were exposed to antibiotics during gestation, the mean (sd) birth weight was 3309.73 g (512.92 g) and, two (5.6%) were pre-term, 26 were born to non-white mothers (72.2%). The mean (sd) family income was U$ 578.04 (300.45), mothers had a mean (sd) of 12.53 (2.55) years of education and a mean (sd) of 29.81 (7.10) years old. Infants within the failure group of the personal-social domain had higher exposure to antibiotics during pregnancy (n = 2; 40.0%; p = 0.006). No other difference in sociodemographic and clinical characteristics was found between infants' groups (Table 1).

Associations between meconium microbiome and behavioral outcomes

We observed significant differences between infants' groups for beta diversity, based on both weighted and unweighted UniFrac metrics (R^2: 0.078, p = 0.021, PERMANOVA) (Fig. 1A). Infants who meet milestones in the personal-social domain (p = 0.001, Wilcoxon test; $n = 31$) showed higher meconium alpha diversity (Fig. 1B) compared to infants who did not meet milestones in the personal-social domain ($n = 5$). Differential taxa within the Ruminococcaceae family (UCG010, UCG005, UCG002, UCG014) (all q < 0.15) was associated with infants' groups after adjustment for gestational antibiotics, preterm delivery, and delivery mode (Fig. 1C).

Infants who did not meet milestones in the test showed significantly enriched OTUs in the Ruminococcaceae family (UCG010, UCG005, UCG002, UCG014), and genera *Eubacterium*, *Veillonella seminalis*, *Treponema*, Senegalimassilia, *Ruminiclostridium*, *Roseburia*, *Romboutsia sp.*, *Prevotella*, Micrococcales, and Christensenellaceae (all q < 0.15) while those were depleted in infants who meet milestones in the test, after adjusting for gestational antibiotics, delivery mode and preterm delivery (Table 2). Furthermore, multivariable linear modeling reveals that meconium alpha diversity was inversely associated with failing in the personal-social domain at 6 months of age (β: − 2.290; 95% CI − 4.212; − 0.368) after adjustment for gestational antibiotics, preterm delivery, and delivery mode (Table 3).

Meconium microbiome functional prediction

To investigate the differences in functional composition between the meconium microbiome associated with the personal-social domain groups, we applied the PICRUSt software package to predict metagenome functional content from 16S rRNA survived on the meconium samples. Figure 2 shows the potential gene content changes by infants' groups. In infants who did not meet milestones in the personal-social domain, a total of 34 pathways were depleted. These included pathways for the biosynthesis of heme, cysteine, preQ0, vitamins K and B12, amino acids, thiamine, and pyrimidine. Additionally, pathways involved in building block components (such as colanic acid, O-antigen, and peptidoglycan), fatty acids and lipids, sugar nucleotides, and polyamines were affected. Pathways related to sulfate reduction, energy metabolism, and glycine betaine degradation were also depleted. In the same infant group, a total of 13 pathways were enriched. These included five pathways associated with energy metabolism (such as sucrose and starch degradation, glycogen biosynthesis and degradation, and the pentose phosphate pathway), four pathways involved in nucleoside and nucleotide biosynthesis (including adenosine, guanosine, and ribonucleotides), as well as pathways for fermentation, amino acid biosynthesis, and aminoacyl-tRNA biosynthesis.

Discussion

The relationship between the gut microbiome and neurodevelopment has gained increasing acceptance largely as a result of epidemiological evidence[1] and mechanistic studies in animal models[7]. In this work we found increase in the abundance of Ruminococcaceae (UCG002, UCG005, UCG010, UCG014), Christensenellaceae,

	Personal-social domain groups			
	Passed (N = 31)	Failed (N = 5)	Total	p-value
Female, *N.* (%)	13 (41.9%)	1 (20.0%)	14 (38.9%)	0.350
Male, *N.* (%)	18 (58.1%)	4 (80.0%)	22 (61.1%)	
C-section delivery, *N.* (%)	18 (58.1%)	2 (40.0%)	20 (55.6%)	0.451
Gestational antibiotics, *N.* (%)	1 (3.2%)	2 (40.0%)	3 (8.3%)	**0.006**
Birth weight (g), mean (sd)	3389.39 (521.17)	3393.00 (373.72)	3389.89 (498.78)	0.988
Preterm delivery	2 (6.5%)	0 (0.0%)	2 (5.6%)	0.559
Maternal ethnicity (non-white), *N.* (%)	22 (71.0%)	4 (80.0%)	26 (72.2%)	0.676
Family income (US dollar), mean (sd)	591.81 (386.95)	485.10 (242.91)	578.04 (300.45)	0.517
Maternal education (years), mean (sd)	12.64 (2.66)	11.80 (1.64)	12.53 (2.55)	0.499
Maternal age, mean (sd)	30.55 (6.91)	25.20 (7.19)	29.81 (7.10)	0.119
Shannon Index	1.99 (0.85)	0.62 (0.54)	1.80 (0.94)	**0.002**

Table 1. Characteristics of the participants stratified by neurodevelopmental outcome at 6-month of age. Significant values are in [bold].

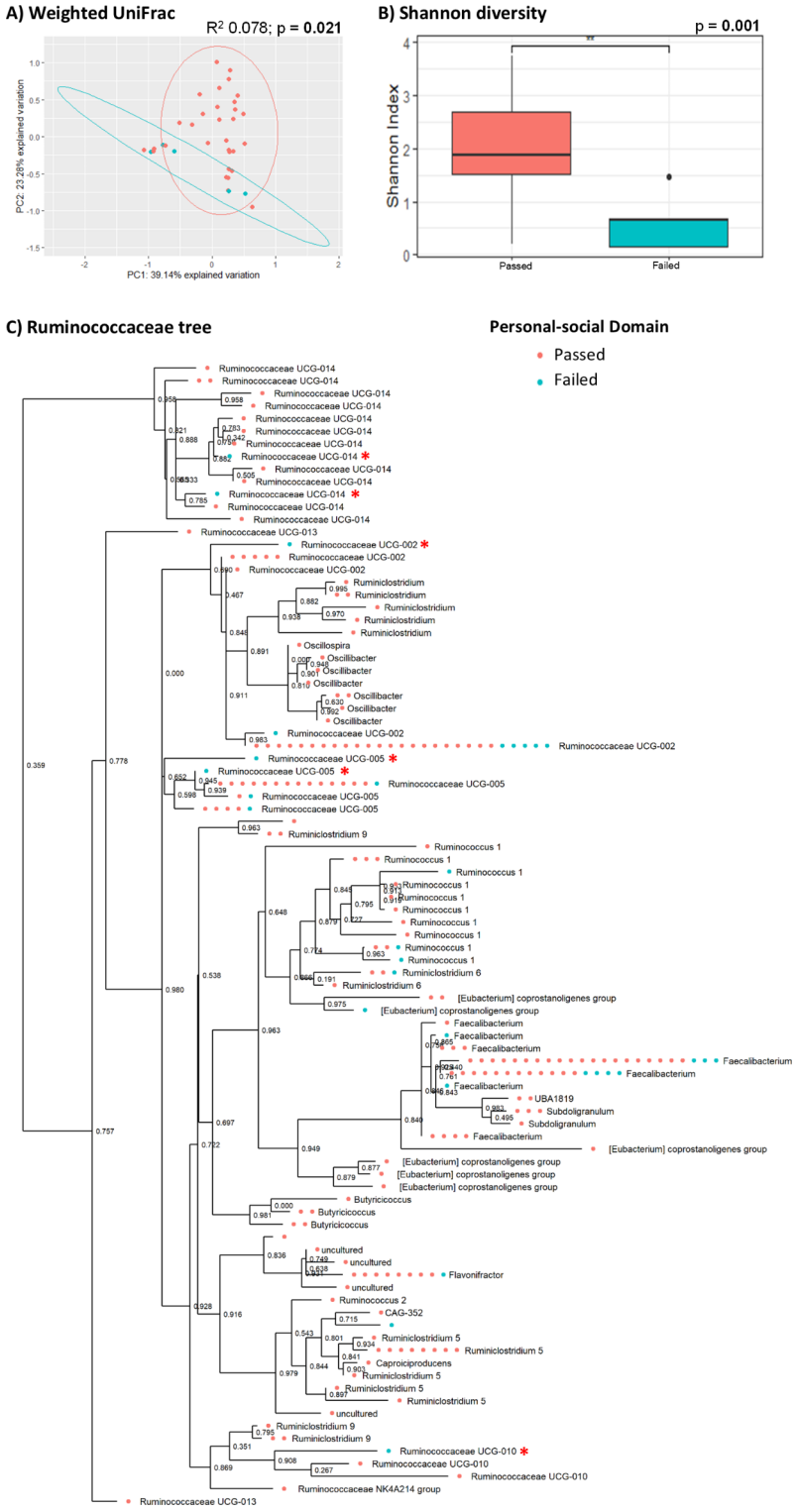

Fig. 1. Associations between meconium microbiome and the Denver personal-social domain at 6 months of age. (**A**) Principal Coordinate Analysis (PCoA) using the weighted UniFrac distance metric, the ellipses and colors indicate Denver' test milestones (passed/failed). (**B**) Alpha diversity differences between the two groups (passed [N = 31]/failed [N = 5]) from Wilcoxon test. All meconium models were adjusted for gestational antibiotics, delivery mode, and preterm delivery. (**C**) Differential taxa within the Ruminococcaceae tree by Denver groups (passed/failed) in the personal-social domain obtained from linear regression models. *p-value < 0.001 & q-value < 0.15.

Taxon	Coefficient	SE	p-value	q-value
Christensenellaceae.R.7.group.s_.otu_985	0.264	0.082	0.003	0.146
Eubacterium.coprostanoligenes.group.otu_353	0.264	0.082	0.003	0.146
Eubacterium.hallii.group.s_.otu_1735	0.274	0.080	0.002	0.102
Firmicutes.c_.otu_2081	0.264	0.082	0.003	0.146
Micrococcales.f_.otu_895	0.264	0.082	0.003	0.146
Prevotella.2.otu_1144	0.274	0.080	0.002	0.102
Romboutsia.sp.otu_205	0.264	0.082	0.003	0.146
Roseburia.s_.otu_1798	0.264	0.082	0.003	0.146
Ruminiclostridium.6.otu_361	0.274	0.080	0.002	0.102
Ruminiclostridium.otu_427	0.274	0.080	0.002	0.102
Ruminococcaceae.UCG.002.otu_439	0.264	0.082	0.003	0.146
Ruminococcaceae.UCG.002.otu_440	0.264	0.082	0.003	0.146
Ruminococcaceae.UCG.005.otu_403	0.264	0.082	0.003	0.146
Ruminococcaceae.UCG.005.s_.otu_399	0.274	0.080	0.002	0.102
Ruminococcaceae.UCG.010.otu_289	0.264	0.082	0.003	0.146
Ruminococcaceae.UCG.014.otu_1025	0.264	0.082	0.003	0.146
Senegalimassilia.otu_800	0.274	0.080	0.002	0.102
Treponema.2.otu_832	0.274	0.080	0.002	0.102
Veillonella.seminalis.otu_947	0.264	0.082	0.003	0.146

Table 2. Gut microbiome taxa significantly associated with failing in the personal-social domain at 6 months of age.

Personal-social domain				
	Crude model		Adjusted model*	
	β	95% CI	β	95% CI
Shannon	− 2.297	**− 4.140; − 0.453**	− 2.290	**− 4.212; − 0.368**

Table 3. The association between the meconium alpha diversity at birth and failing in the personal-social domain at 6 months of age. *Adjusted for gestational antibiotics, preterm delivery, and delivery mode. Significant values are in [bold].

Eubacterium, *Treponema*, *Senegalimassilia*, *Ruminiclostridium*, *Roseburia*, *Romboutsia*, *Prevotella*, *Veillonella seminalis* and *Eubacterium coprostanoligenes.* and reduced meconium alpha diversity and functional capacity associated with failing in the personal-social skills test at 6 months of age.

The results from previous studies point to *Bacteroides* and *Bifidobacterium* as benefic genera impacting behavior, cognition and motricity development at 1–12 months of age. Conversely, the effects related to Lachnospiraceae, *Streptococcus* and *Faecalibacterium* have a variable influence on behavior and brain development[1]. Additionally, the Ruminococcaceae family was associated with surgency in 2.5 months-old infants[10], smaller volume of superior occipital gyrus at 1 year of age[11], and consistently with depressive symptoms in adults[12]. Notably, a large study involving 2593 adults associated Ruminococcaceae (UCG002, UCG003, UCG005) and Lachnospiraceae families (*Sellimonas*, *Lachnoclostridium*, *Hungatella*, *Eubacterium ventriosum*, LachnospiraceaeUCG001, and *Ruminococcus Gauvreau Group*) with depressive symptoms[13], which aligns with the findings of our study.

In our study, increased abundance of *Roseburia*, *Eubacterium hallii,* and the family Ruminococcaceae was associated with failing in the personal-social domain. These genera belong to the Lachnospiraceae family, in which the influence on human health is related to the production of short chain fatty acids (SCFAs). In particular, *Roseburia* and the Ruminococcaceae family are among the primary bacteria responsible for butyrate production[14,15]. Previous studies have reported associations between *Roseburia* abundance and brain functional connectivity, with this relationship mediated by the pro-inflammatory cytokine interleukin-6 and tumor necrosis factor alpha. This suggests that immune signaling pathways, potentially influenced by *Roseburia*'s production of butyrate, play a role in microbiota-brain communication[16]. In murine model, a low dose of penicillin led to an increase in Lachnospiracea, which in turn increased cytokine expression in the frontal cortex, modified blood–brain barrier integrity and altered behavior. The antibiotic-treated mice exhibit impaired anxiety-like and aggressive behavior[17].

Together with *Roseburia*, *Eubacterium hallii* was also associated with personal-social skills. This genus contributes to the production of propionate. While butyrate is involved in neurotransmitter release mechanisms[18], as a facilitator of synaptic plasticity and processes related to cognition, learning and memory[19], propionate can influence gap junction gating in the blood brain barrier[20].

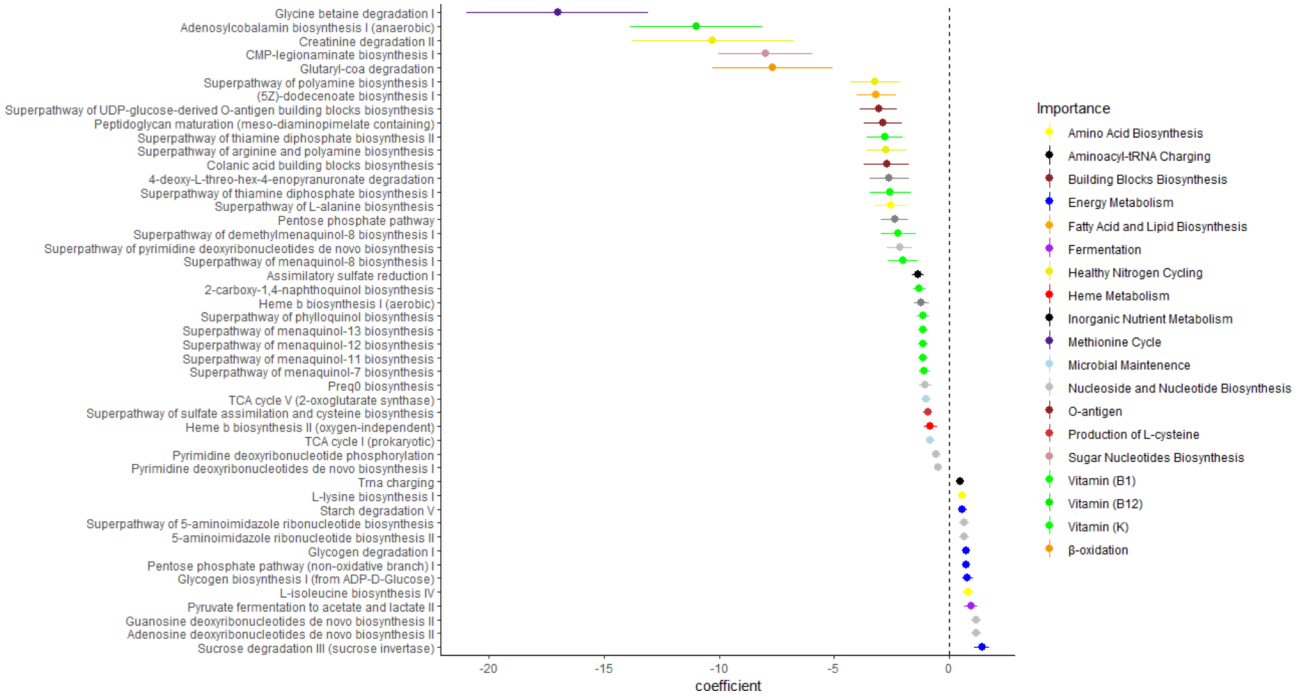

Fig. 2. Altered meconium microbiome gene content prediction in infants who did not meet milestones in the personal-social domain at 6 months of age. The point indicates the β estimate of the associations between infants' group on microbiome functional profile adjusted for gestational antibiotics, delivery mode, and preterm delivery. The horizontal band represents the standard error. All q-value < 0.15. Colors indicate either superclass2 or pathways' importance.

In addition to the interaction with the immune system, a lower abundance of Lachnospiraceae has been associated with greater hypothalamic–pituitary–adrenal (HPA) reactivity, as assessed by measuring salivary cortisol levels in one-month-old infants. Changes in the HPA axis have been related to psychiatric disorders in adults[21]. Findings regarding the Lachnospiraceae family and the impact on brain development are very divergent across the studies, probably due to the different genera described by them. And possibly owing to the methodologies for taxonomy alignment that do not always identify at the genus level within this family.

Increases in *Treponema* and *Ruminiclostridium* after early-life stress have been associated with elevated levels of corticosterone, adrenocorticotrophic hormone, and glucocorticoid receptors in the hippocampus[22]. These microbiome changes, along with altered glutamate receptor expression, show a positive correlation with depressive phenotypes in mice, suggesting a link between early-life stress, gut microbiome shifts, and mood disorders[22].

A clinical trial in patients with ASD found a reduction in *Eubacterium coprostanoligenes* abundance after fecal microbiota transplantation and this was positively correlated with decreased gastrointestinal and autism-like symptoms. *Eubacterium coprostanoligenes* was negatively correlated with serum GABA levels, suggesting it may influence neurotransmitters involved in mood and behavior. Additionally, children in ASD group showed a relative higher abundance of Christensenellaceae R-7 group[23]. In our study, both *Eubacterium coprostanoligenes* and Christensenellaceae R-7 group were found elevated in infant's group who did not meet milestones in the personal-social domain, suggesting a possible link between these genera and social-emotional aspect of development.

Beyond shifts in the taxonomic profile, functional gene prediction revealed several microbial pathways to be underrepresented in infants who did not meet milestones in the personal-social domain. The lower microbiome diversity observed in these infants' meconium samples may be linked to a reduced metabolic capacity. Typically, a diverse microbiome supports a wider array of metabolic functions, which are critical for producing essential metabolites that may influence host development, including neurological and social-behavioral outcomes[24,25]. Additionally, this group of infants showed an increased relative abundance of OTUs within the same family, which may explain the reduced functional capacity observed compared to infants who meet milestones in the personal-social domain.

Notably, we observed a significant reduction in the glycine betaine degradation pathway, which may influence the methionine cycle. Predicted genes related to vitamin B12 biosynthesis were also reduced, along with pathways for glutaryl-CoA and creatinine degradation. These findings suggest potential disruptions in key metabolic pathways: methionine, a precursor to homocysteine, relies on B12 for its reconversion, and glutaryl-CoA is a product of lysine and tryptophan catabolism. Altered homocysteine metabolism and reduced levels of glutathione and amino acids like tryptophan and tyrosine have been associated with mood disorders, including depression[26].

We recently showed in 106 infants and their mothers that gestational exposure to environmental pollutants is associated with alterations in the infant's microbiome with a compounded effect of stressors such as antibiotics, C-section, and preterm birth, which aggravate the pollutants' effect[6]. Here we show that changes in meconium microbiome are associated with personal-social skills at 6 months of age.

The personal-social domain of the Denver test encompasses the social-emotional aspect of development which, up to one year of age, includes smiling spontaneously, discriminating between familiar people and strangers, responding to own name, imitating simple actions of others, among others[27]. Later, stronger elements of a child's socialization are screened by this domain[28]. It is interesting that similar taxa found in infants who did not meet milestones in the personal-social domain were previously found in adults with depression symptoms[13]. Suggesting that differences or interventions in early life can have outsized and longer-term consequences.

Potential limitations of our study should be considered. The small and imbalanced sample size may lead to variability in effect size estimates, potentially introducing bias and affecting the power to detect true associations. Besides, the findings should be interpreted and generalized with caution, and further research with larger and more balanced groups will be valuable in confirming these results and enhancing the robustness of our conclusions. Finally, while our study design could demonstrate temporality, it was not intended to establish causality. However, although preliminarily, this study deserves appreciation for being innovative in assessing the primordial gut microbiome and behavioral outcomes in infants. Once the abundance of Lachnospiraceae and Ruminococcaceae have been previously linked to depression in adults and, in this study, with social-emotional skills in infants, we highlight that interventions in early life can have significant and lasting consequences due to the dynamic and plastic nature of both the gut microbiome and the brain. Further studies in larger cohorts, and in different neonate populations, surveying microbial functional pathways and metabolites are needed to provide mechanistic insights.

In conclusion, this study found associations between meconium alpha and beta diversity with personal-social skills at 6 months of age. Several taxa were associated with these changes including enrichment in the Ruminococcaceae family, and genera *Eubacterium*, *Veillonella seminalis, Treponema*, Senegalimassilia, *Ruminiclostridium*, *Roseburia*, *Romboutsia sp.*, *Prevotella*, Micrococcales and, Christensenellaceae. Predicted functional genes showed reduced abundance in association with altered neurobehavior. Further studies, with a larger population size and assessing microbial function and metabolites are needed to confirm the role of these bacteria in neurobehavior.

Methods

Study population

A longitudinal analysis was caried out on the PIPA study, a birth cohort study conducted in a public-School Maternity located in Rio de Janeiro, Brazil, between October 2017 and August 2018. Briefly, a cohort of infants (n = 131) delivered at the School Maternity was enrolled to investigate the effects of environmental pollutants on maternal-child health. Details on the study design and cohort profile can be found elsewhere[29]. Individuals with microbiome sequencing and Denver assessment (n = 36) were included in the analysis.

Sample collection

Meconium samples were collected at birth by nurses, and the 16S rRNA gene was surveyed as previously described[6]. In short, DNA from the meconium samples was extracted using the DNeasy PowerSoil HTP Kit according to the manufacturer's instructions. The V4 region of the 16S rRNA gene of the DNA extracted was amplified with a set of primers and molecular markers. PCR products with the expected fragment size were cleaned using the UltraClean-htp 96-well PCR Cleanup kit (QIAGEN), and the 16S amplicons were sequenced on an Illumina HiSeq platform.

Trained interviewers collected data on demographic characteristics (per-capita family income, ethnicity [self-reported], age, and educational attainment) at enrollment and clinical information concerning the newborn was filled up by copying birth health records from the hospital charts at the time of delivery.

Microbiome analysis and statistics

Sequences were demultiplexed and denoised and the operational taxonomic units (OTUs) were assigned using SILVA database. Alpha and beta diversity were calculated using QIIME pipeline[30]. We applied the PICRUSt software package[31] to predict metagenome functional content based on the abundance data at the OTU level. The statistical significance of beta diversity differences was tested with Permutational Multivariate Analysis of Variance (PERMANOVA) using the R package *vegan*[32], which tests significance with 999 permutations. We performed visualizations with principal coordinates analysis (PCoA). Differences in alpha diversity between the Denver groups were tested using Multiple linear regression models. Discordant bacterial taxa and predicted functional composition between the Denver groups were determined using *Maaslin2* R package (Microbiome Multivariable Association with Linear Models)[33], at a significance threshold of q-value < 0.15. The adjusted models included delivery mode (C-section/Vaginal), preterm delivery (< 37 weeks gestation), and antibiotics exposure during pregnancy (yes/no) as covariates. Data on antibiotic types was unavailable, so it was not included in the analysis.

The Denver development screening test II (DDST-II) assessment

Infant neurodevelopment was assessed using the Denver Developmental Screening Test II (DDST-II)[34] by a trained pediatrician who was unaware of the child's developmental history. The purpose of this tool is to screen neurodevelopmental assessment, from birth to 6 years of age, regarding their ability to perform tasks organized in four domains: "Personal-Social", "Fine Adaptive Motor", "Gross Motor", and "Language". Each task is represented by a bar that indicates the age at which task compliance is performed by 25%, 50%, 75%, and 90% of children. A score is given to each task evaluated, as follows: "pass, fail, refuse, no opportunity". For the analyses, the infants

were divided into "fail group" and "not fail group". Following the guidance of the DDST II manual, "fail" and "refuse" scores were considered as "fail", and "pass" scores were considered as "not fail".

Data availability

The datasets generated and/or analysed during the current study are available in the NCBI sequence read archive (SRA) repository, PRJNA1128585.

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Author contributions
Conceptualization: NFN, AM, MGDB, JCM, Data analysis and interpretation: NFN, AM, MGDB, Data collection: APN, Literature search: NFN, Project coordination and funding acquisition: CIFA, Supervision: AM, JCM, MGDB, Writing—original draft: NFN, Writing—review & editing: NFN, AM, MGDB. All authors reviewed the manuscript.

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Declarations

Competing interests
All authors declare no conflict of interest.

Ethics approval and consent to participate
Ethics approvals were obtained from the Ethics Committee of the School Maternity Hospital (ref. number 2.092.440) and the Oswaldo Cruz Foundation Ethics Committee (ref. number 2.121.397). Following the Declaration of Helsinki, all caregivers provide written informed consent before completing any study measure. Cases of developmental, health, and mental health issues are being referred to specialized health services.

Additional information
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