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Cesarean delivery is associated with decreased beneficial gut microbes and increased pathobionts and butyrate excretion: a longitudinal study over the first year of life.

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Abstract

Objective: C-section interrupts mother-to-newborn microbial transfer at birth. Beyond the neonatal period, the impact of C-section on offspring gut microbiota and their short chain fatty acids (SCFAs) remains unclear. Here we examine delivery mode (C-section vs. vaginal) with the infant gut microbiota and fecal SCFAs measured 3 and 12 months after birth.

Design: Longitudinal study

Setting: North Carolina

Population: In 2013–2015 we enrolled pregnant women and followed up their offspring for 12 months. We asked a subset of participants, enrolled over a 3-month period, to provide fecal samples at the 3-month and 12-month follow-up visits.

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Author contributions: N.T.M. developed the research question, facilitated analysis of the samples, designed the study and analytic plan, interpreted the data, and drafted the manuscript. M.K.D. analyzed the data and contributed to writing the manuscript. All authors edited, read and approved the final manuscript. S.B.N, T.O, C.H. were involved with the conception and creation of the Nurture study.

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Details of ethics approval: Nurture was conducted according to guidelines in the Declaration of Helsinki and all procedures involving human subjects were approved by Duke University Medical Center IRB (human subjects committee) (Pro0036242). The most recent IRB approval for Nurture was April 18th, 2020. Although an observational study, Nurture is registered at clinicaltrials.gov (NCT01788644). We obtained written informed consent at recruitment in pregnancy and women confirmed their willingness to participate again after delivery.

Methods/Main Outcomes: We sequenced the 16S rRNA V4 region with Illumina MiSeq and quantified SCFA concentrations using gas chromatography. We examined delivery mode with differential abundance of microbiota amplicon sequence variants (ASVs) using beta-binomial regression and fecal SCFAs using linear regression. We adjusted models for confounders.

Results: Of the 70 infants in our analytic sample, 25 (36%) were C-section delivered. C-section (vs. vaginal) delivery was associated with differential abundance of 14 infant bacterial ASVs at 3 months and 13 ASVs at 12 months. C-section infants had a higher abundance of the potential pathobionts *C. neonatale* ($p = 0.04$) and *C. perfringens* ($p = 0.04$), and a lower abundance of potentially beneficial *Bifidobacterium* and *Bacteroides spp.* C-section infants also had higher fecal butyrate at 3 months ($p < 0.005$).

Conclusions: C-section infants were more likely to be colonized by pathobionts in lieu of beneficial microbes, and they excreted more butyrate at 3 months of life.

Tweetable abstract: C-section delivery was associated with increased butyrate excretion, decreased Bifidobacterium, & colonization of the infant gut by pathobionts at 3mo. of age.

Keywords

delivery mode; infant gut microbiome; short-chain fatty acids

Introduction

C-section delivery (vs. vaginal delivery) has been associated with higher risk of chronic immune disorders (e.g. asthma and allergies),¹ neurodevelopmental outcomes (i.e. attention deficit conditions),² as well as infectious morbidity.³ We have also found that C-section delivered infants have accelerated adiposity gain over the first year of life⁴ and are more likely to develop overweight or obesity later in life.⁵ It is postulated that these myriad health associations are explained by C-section interrupting the natural mother-to-newborn sharing of microbiota at birth. Yet uncertainty remains about how long delivery-mode differences in the microbiota persist and if they are associated with changes in production of microbiota-derived short chain fatty acids (SCFAs) such as butyrate, which are involved in the education of the immune system and metabolism.⁶

In this study we sought to address these gaps in the literature by prospectively examining the association between delivery mode and (a) the composition and diversity of the infant gut microbiota and (b) fecal SCFAs at 3 and 12 months of life. We hypothesized that C-section delivered infants have lower relative abundances of *Bifidobacterium* and *Bacteroides*, the bacterial genera most commonly depleted in C-section infants in previous studies,⁷ and higher relative abundances of potential pathobiontic Clostridia species found in previous studies.⁷ Moreover, given that prior literature has linked fecal butyrate with excess weight gain⁸ and obesity,^{9, 10} we explored whether C-section delivery is associated with the concentration of butyrate and other SCFAs in the stool.

Methods

Study population

This study was conducted in the prospective Nurture study, which has been previously described.¹¹ Briefly, Nurture is prospective birth cohort study designed to assess longitudinal associations of early-life factors with infant adiposity and weight in the first year of life. Grants from the National Institutes of Health (R01DK094841) and the Mid-Atlantic Nutrition Obesity Research Center, under NIH award number P30DK0372488, supported the research reported in this publication. Although it is an observational study and a core outcome set was not used, Nurture was registered at [clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01788644) (NCT01788644).

Nurture was conducted according to guidelines in the Declaration of Helsinki. Duke University Medical Center IRB (human subjects committee) (Pro0036242) approved all procedures involving human subjects. The most recent IRB approval for Nurture was April 18th, 2020. At study recruitment, while women were pregnant, we obtained written informed consent. Women confirmed their willingness to participate again after delivery.

Between 2013 and 2015, Nurture investigators and staff recruited women between 20 and 36 weeks of pregnancy from a private prenatal clinic and a county health department in Durham, North Carolina. To be considered for inclusion in the study women had to be > 18 years of age, be able to speak and read English, and be having a singleton birth. They also had to confirm their intention to remain local for home visits for at least one year after delivery. Infants were excluded if they were born < 28 weeks into gestation, were born with a congenital abnormality, were not able to take food by mouth at discharge, or if they required > 3 weeks of hospitalization after birth. Home visits were conducted when infants were 3, 6, 9, and 12 months of age. In addition, there were monthly telephone calls in between visits to collect data on breastfeeding, formula feeding, and timing of solid food introduction.

In total, 666 mother-infant pairs were followed from pregnancy to the first year of life. We conducted a substudy in which we invited all mothers that were enrolled over a 3 month time period to collect stool samples from their infants at the 3-month and 12-month follow-up visits. In total, 70 mother-infant pairs provided at least one stool sample that was used for microbiota and SCFA analyses. The mother-infant pairs included in the microbiome substudy had a distribution of sociodemographic and clinical characteristics similar to the entire cohort.

Exposure variables

We obtained data on mode of delivery (C-section delivery and vaginal delivery), infant sex, birth weight (kg), gestational age (weeks), and antibiotic use from medical records. Mothers' reported (via questionnaire) their age (years), race and ethnicity, pre-pregnancy weight (kg) and height (m), level of educational achievement, household income, and cigarette smoking status in pregnancy. We used pre-pregnancy weight and height to calculate mothers' pre-pregnancy body mass index (BMI; kg/m²) and we defined overweight or obese as BMI ≥ 25. During monthly phone calls and the home visits we had mothers prospectively report if they

were still breastfeeding, if had introduced formula, and if they had introduced solid foods and if so the timing of solid food introduction.

Stool Collection and Storage

Stool samples were taken from diapers at the 3-month and the 12-month home visits. We promptly transferred collected stool to 2 ml cryogenic vials (ThermoFisher) and froze them at -80°C .

16S rRNA-based bacterial community sequencing using Illumina MiSeq platform

We sent frozen fecal samples on dry ice to Microbiome Insights. Their lab technicians used standardized methods to extract microbial DNA, to amplify the V4 region of the 16S ribosomal RNA (rRNA) gene using PCR, and to sequence the prepared libraries using the Illumina MiSeq platform. The full methodologic details for the 16S rRNA gene sequencing can be found in our prior publication using this cohort.¹²

16S rRNA sequence pre-processing

We controlled the quality of reads and resolved amplicon sequence variants (ASVs) in the statistical programming environment R, using version 1.8 of the DADA2 package, and following author recommendations.¹³ First we examined sequence quality scores for forward reads and reverse reads (Figure S1). Then we trimmed all forward reads before nucleotide position 10 and after position 240, and all reverse reads before position 10 and after position 150. We required each read to have less than two expected errors given based on their quality scores, and we filtered out reads mapping to the phiX genome and reads with ambiguous base assignments (using the overlapping paired reads “filterandtrim” function). We dereplicated the reads (using the “derep” function) and denoised the reads (using the “dada” function for pooled reads) with estimated error models. To estimate our error models we used the “learnErrors” function, randomly selecting 106,431,120 bases from 462,744 reads to estimate the forward-read error model, and 102,199,160 bases from 729,994 reads to estimate the reverse-read error model (Figure S2). We merged overlapping paired reads (using the “mergePairs” function) and removed chimeric ASVs (using the “removeBimeraDenovo” function with the pooled sample setting). Our final sequence pool retained 74% of the original raw reads (Figure S3). We assigned taxonomy using the HITdb v.1.00 16S rRNA sequence database of human intestinal microbiome taxa and the DADA2 function “assignTaxonomy”.¹⁴

Phylogenetic tree generation

Next, we followed the recommendations of a recent Bioconductor microbiome data analysis workflow to generate a phylogenetic tree from the denoised ASVs.¹⁵ In brief, we first used the DECIPHER Bioconductor package to align ASVs and construct a neighbor-joining tree.¹⁶ Then we used the phangorn package to generate a Generalized time-reversible (with Gamma rate variation) maximum likelihood phylogenetic tree, which we rooted at the midpoint.¹⁷ And finally we used the phyloseq package to merge the phylogenetic tree and the microbial ASVs with the other study variables.¹⁸

Short chain fatty acid quantification

Short chain fatty acid (SCFA) concentrations were quantified using gas chromatography (Thermo Trace 1310) coupled to a flame ionization detector as previously described by Zhao et al.¹⁹ in our prior publication in this cohort.¹²

Statistical Analysis

Microbial community composition analysis

Our primary hypothesis was that vaginal-born infants have fecal microbiota with higher relative abundances of species in *Bifidobacterium* and *Bacteroides* genera and lower relative abundances of opportunistic pathobionts within the genus *Clostridium* at 3 months of age.

To test for differential relative abundance of ASVs in C-section-born vs. vaginally-born infants we used the R package *corncob* to employ beta-binomial regression models that account for within-sample taxa correlation and variable sequencing depth from.²⁰ One of the strengths of these models is that they allow for multivariable adjustment; the variables included in our multivariable model are described below. To reduce the possibility of false discovery of rare ASVs, given our relatively small sample size, we removed microbial ASVs that did not have a mean count at or above the 25th percentile in at least 10% of samples. A list of all ASVs included in the study with their assigned taxonomy can be found in Appendix S1.

Beta diversity analysis

We used the *phyloseq* package¹⁸ to estimate weighted UniFrac distances, a measure of pairwise community composition. We then used permutational multivariate analysis of variance, using the function “*adonis*” in the *vegan* package²¹ with 9,999 permutations, to test for differences in weighted UniFrac distances before and after multivariable adjustment.

Alpha diversity analysis

We also used the *phyloseq* package¹⁸ to estimate the Shannon diversity index, a measure that combines bacterial richness (the number of unique ASVs observed within each sample) with the evenness of the relative abundance within each sample. We then tested for differences in Shannon diversity index using unadjusted and multivariable-adjusted generalized linear regression models.

SCFA analysis

Our secondary outcome was fecal concentration of the SCFA butyrate at 3 and 12 months of age. We performed a $\log_{10} + 1$ transformation on butyrate, propionate, and total SCFA concentrations to normalize the distributions at both timepoints. We fitted multivariable generalized linear regression models to examine the association of delivery mode (C-section vs. vaginal delivery) with SCFA concentrations in infant fecal samples collected at 3 and 12 months of age. We then selected microbial ASVs and genera that were statistically significantly associated with delivery mode and estimated their (Spearman) correlation with SCFA concentrations.

Multivariable adjustment and statistical significance

We adjusted multivariable regression models for maternal pre-pregnancy BMI and breastfeeding (never vs. ever). We adjusted for these factors based on prior literature that indicated they were potentially associated with both our exposure and outcomes.

We considered $p < 0.05$ significant for models with Shannon diversity index and Weighted Unifrac as the outcomes. For the microbial ASV differential abundance analyses, we used a two-sided false discovery rate (FDR) adjusted $p < 0.05$ to denote statistical significance.

Results

Participant characteristics

Among the 70 mother-infant dyads included in our analysis sample, 63% of mothers identified their infants as Black, 46% reported education below the high school level, and 63% had an annual household income of \leq \$20,000. The range of gestational age was 34.7 weeks to 41.3 weeks. A total of 25 (36%) infants were C-section delivered, similar to the prevalence of this exposure in the United States.²² C-section mother-infant pairs were similar to vaginal-delivery mother-infant pairs with respect to socio-demographic and clinical factors, except for maternal pre-pregnancy BMI, which was higher among C-section pairs (32.9 vs. 27.6 kg/m²), as shown in Table 1. Of the 25 children born via C-section, 18 had data on type of C-section. Of these, 6 were scheduled C-sections (31.6%), 8 were unscheduled C-sections (42.1%), and 4 progressed from labor to C-section (21.1%). Of the 6 scheduled C-section deliveries, 2 had membrane rupture (1 with labor). Of the 8 unscheduled C-section deliveries, 6 had membrane rupture (4 with labor). Of the 4 labor to C-section deliveries, 4 had membrane rupture.

Delivery mode and gut microbiome diversity

C-section delivery explained 3.2% variation in Weighted Unifrac at 3 months, the most among the covariates included in the model, but only 0.5% of the variation at 12 months (Figure S4). Association of delivery mode with measures of gut microbiota alpha diversity (Shannon diversity index) at 3 and 12 months of age are summarized in Figure S6. C-section delivered infants trended toward lower Shannon diversity index at 3 months of age (beta = -0.18, 95% CI: -0.41, 0.05), but not at 12 months (beta = -0.03, 95% CI: -0.36, 0.30).

Delivery mode and Gut microbiome composition

Figure S5 shows the unadjusted relative abundances of the top bacterial families and genera in our sample of infants according to delivery mode and time of stool collection. After multivariable adjustment, delivery mode was associated with 14 bacterial ASVs at 3 months (Figure 1; Table S1) and 13 ASV at 12 months (Figure 1; Table S2). Of note, at 3 months of age, C-section delivery (vs. vaginal delivery) was significantly associated with lower relative abundances of *Bacteroides* (sp. unknown), *Bifidobacterium* (sp. unknown), and *Bifidobacterium bifidum*, and higher relative abundances of several opportunistic pathobionts including *Clostridium perfringens* and *Clostridium neonatale*. At 12 months, *Akkermansia muciniphila* and *Ruminococcus* (sp. unknown) were significantly lower among

C-section-born infants. In Figure 2, we further show the relative abundances of the opportunistic pathobionts, *C. neonatale* and *C. perfringens*.

SCFA concentrations

Association of delivery mode with SCFAs at 3 and 12 months is shown in Figure 3. C-section delivery was associated with higher concentration of fecal butyrate at 3 months of age (beta = 0.60, 95% CI: 0.21, 0.99), but this association was no longer significant at 12 months of age. There were no differences in concentrations of fecal propionate, fecal acetic acid, or total SCFAs measured (Figure 3; all $p > 0.05$).

After FDR-correction, the only significant Spearman correlation between ASVs, that were significantly associated with delivery mode, and SCFAs was a *Veillonella* ASV and isovaleric acid measured at 3 months of age. Furthermore, at 3 months the relative abundance of the genera *Lachnoclostridium* was positively associated with butyric acid (rho=0.38, FDR $p=0.05$), *Bacteroides* was positively correlated with propionic acid (rho=0.39, FDR $p=0.05$), and *Bifidobacterium* was positively correlated with acetic acid, although this did not reach statistical significant after FDR correction (rho=0.21, FDR $p=0.28$). The full correlation matrices between gut microbiota and SCFAs at each time point can be found in Figures S7-S10.

Discussion

Main findings

In our racially diverse, predominantly low-income cohort of mother-child dyads from North Carolina, C-section delivery was associated with lower relative abundance of several beneficial bacteria, e.g. *Bacteroides* and *Bifidobacterium*, and higher relative abundances of the pathobiontic *C. neonatale* and *C. perfringens* bacteria. C-section was also associated with higher concentration of fecal butyrate at 3 months of age. Associations with butyrate were no longer significant at 12 months, however delivery mode was still associated with the differential abundance of 13 bacterial taxa at 12 months.

Strengths and limitations

Despite a number of strengths, including the longitudinal design, use of multivariable regression models to adjust for confounders, and measurement of both microbial communities and short chain fatty acids, there are limitations to our study. First, although we controlled for pre-pregnancy BMI and breastfeeding duration, and other participant characteristics were similar by delivery mode (Table 1), it is still possible that residual confounding by factors for which we did not have complete data (e.g. intrapartum antibiotics, chorioamnionitis, admission to NICU) could have influenced our findings. Also, future studies are needed to determine whether type of C-section has an effect on the infant microbiome, as our study was too small to rigorously conduct such an analysis. Second, while our study sample was larger than many previous studies on this topic, we cannot rule out the possibility that non-significant results could be due to not having power to detect differences. Our sample size also precluded us from stratifying on potential effect measure modifiers such as sex, prenatal antibiotic use, presence of labor, or membrane status at the

time of delivery. Third, we cannot rule out the chance of false discovery; however, our results are corroborated by evidence from many other cohort studies. Finally, a limitation of the 16S rRNA sequencing is that we are only able to examine relative abundances of prokaryotic taxa. As such, culture-based studies are still needed to quantify the absolute differences in the bacteria that were found to have differential relative abundance by delivery mode in our study.

Interpretation in light of other evidence

Our results extend previous findings that delivery-mode differences in the infant microbiome emerge after meconium, when the subsequent transitional stool is passed,²³ and a differential abundance of key bacterial taxa persist until 3 and 12 months of age. Our findings are largely consistent with at least 26 independent birth cohorts that have now shown that C-section delivered infants have differential abundances of gut microbiota, including delayed colonization by *Bacteroides*.^{7, 23–48} Although our study only followed infants to 12 months, a recent longitudinal study indicates that delivery-mode associated gut microbiota differences persist out to 4 years of age.⁴⁰ While our study shows evidence that delivery mode affects the microbiome composition at 12 months, even if impacts to the gut microbiome converged after the first several months of life, altering the microbiome during this critical early-life window when the immune system is being formed has been shown experimentally to have long-term health consequences.⁴⁹

An important finding of our analysis is the confirmation that C-section delivered infants had higher relative abundances of the potential pathobionts *C. neonatale* and *C. perfringens*. A recent study⁷ found that the intestinal microbiota of C-section infants (vs. vaginally delivered infants) was enriched with the pathobionts *Clostridium perfringens* in addition to *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus epidermis*, *Streptococcus parasanguinis*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*. We were able to replicate the *C. perfringens* result, but we did not identify the other taxa at the species level (we did, however, observe associations for unclassified higher order taxa in the same direction). Future studies are needed to examine whether these bacteria mediate or modify an association between delivery mode and infant infection and diarrhea.

We also found that C-section delivered infants had a lower abundance of the potentially beneficial *Bifidobacterium* and a lower abundance of *Akkermansia*. *Bifidobacterium* spp. are considered to be important constituents of a healthy infant gut microbiota until the age of weaning.^{50, 51} They play a critical role in fermentation of non-digestible carbohydrates, such as human milk oligosaccharides found in breastmilk, and producing acetic acid and B vitamins.^{52–55} A lower abundance of the genera *Bifidobacterium* in the gut microbiota has been associated with higher levels of inflammation.⁵³ Furthermore, *Akkermansia* is a mucolytic bacteria^{56, 57} associated with lower adiposity and improved insulin sensitivity in adults.^{58–61}

The observation that C-section delivery is associated with higher fecal butyrate at 3 months of age is novel and provides insight into the association of delivery mode with infant health. Butyrate is an important SCFA that serves as the main energy source for the colonocytes⁶² and that has a complex relationships with immunometabolism.⁶ A higher concentration of

butyrate in the feces may indicate that bacteria enriched in the gut of C-section delivered infants uniquely produce excess butyrate, not other SCFAs. This postulate is supported by the finding that several butyrate-producing bacteria, including notably the genera *Lachnospirillum*,⁶³ were not only higher in C-section infants but also positively correlated with fecal butyrate at 3 months. Alternatively, C-section delivery may decrease absorption of butyrate across the apical membrane of colonocytes, resulting in excess excretion of butyrate in the stool. Regardless, higher fecal butyrate has been associated with excess weight gain in mice⁸ and obesity¹⁰ and poor cardiometabolic health in humans.⁹ Longitudinal studies with longer follow-up are needed to shed light on higher infant fecal butyrate, among other short chain fatty acids, and its association with future health outcomes in infants and children.

Conclusions

In conclusion, after careful control for maternal pre-pregnancy BMI and breastfeeding, C-section delivery is associated with differences in gut microbiota composition and butyrate production at 3 months. Although it appears that the association of delivery mode on microbiome structure and SCFAs diminishes after 3 months of age, we still found delivery mode was significantly associated with differences in the relative abundance of 13 bacterial taxa at 12 months. Our results are complemented by the finding that C-section delivery accelerates gains in body weight until 12 months of life in this same cohort of infants.⁴ Future clinical trials are needed to test whether introduction of maternal microbiota to C-section delivered neonates can restore the microbiome of vaginal delivery and mitigate C-section-disease associations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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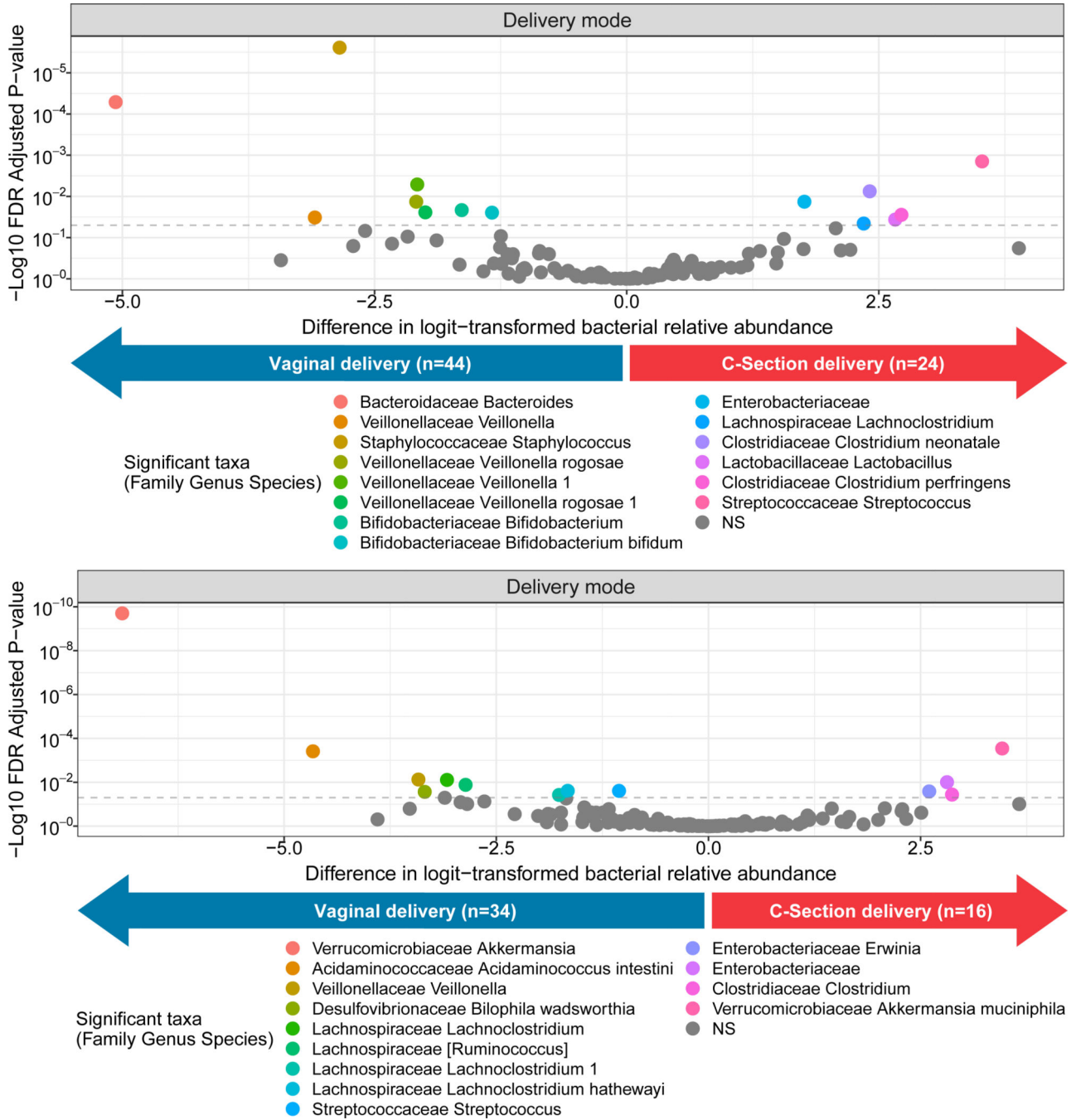


Figure 1. Adjusted* associations of delivery mode (C-section and vaginal delivery) with the logit-transformed relative abundances of infant gut microbial taxa at **a)** 3 months of age and **b)** 12 months of age. *Beta-binomial regression models were adjusted for maternal pre-pregnancy body mass index and breastfeeding. NS = Not significant.

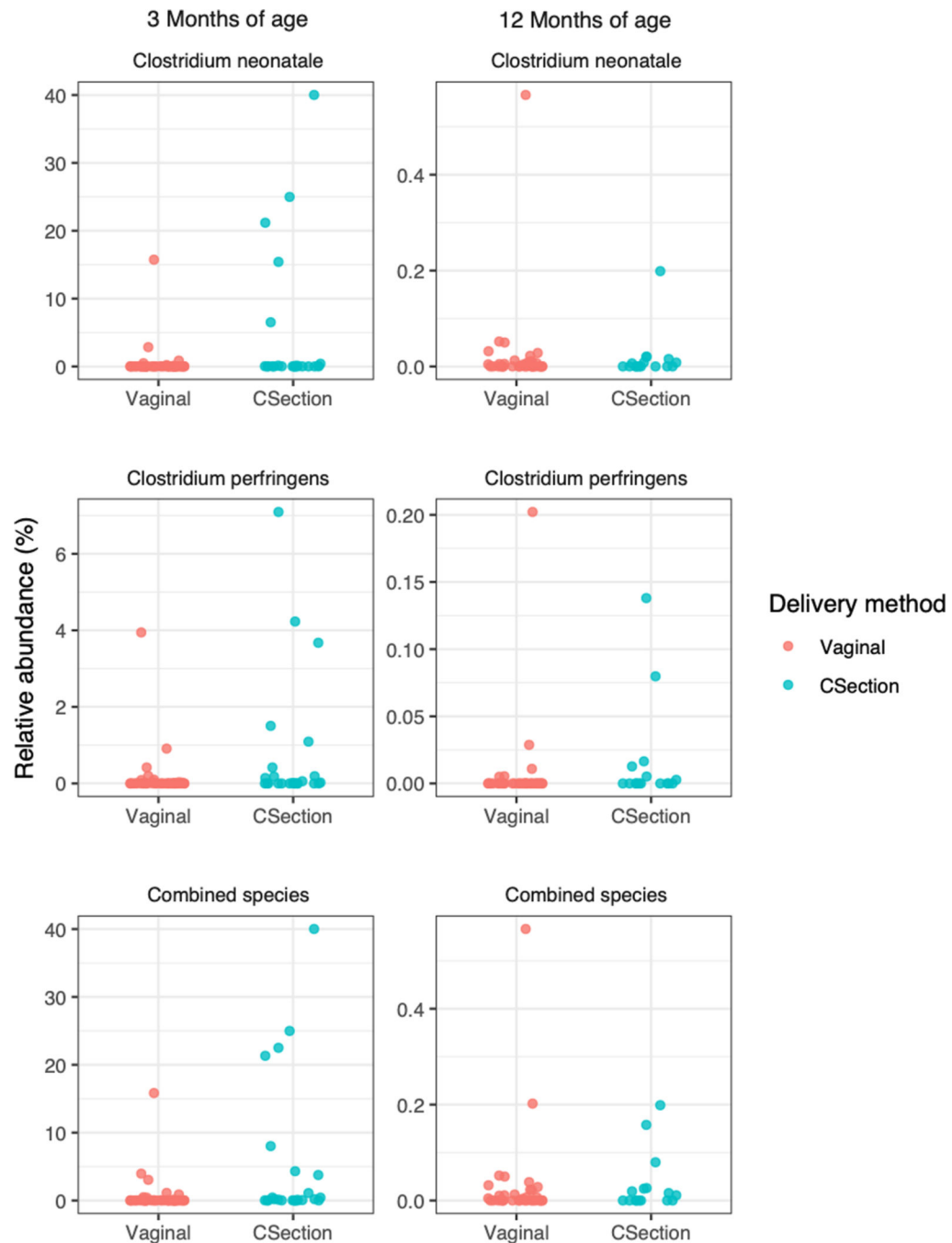


Figure 2. Relative abundance of potential pathobionts in the infant gut microbiota according to delivery mode (C-section and vaginal delivery) and time of follow up. According to Mann-Whitney U test: $p = 0.04$ for difference in *C. neonatale* at 3 months and $p = 0.94$ at 12 months; $p = 0.04$ for difference in *C. perfringens* at 3 months and $p = 0.08$ at 12 months; and $p = 0.002$ for difference in combined species abundance of both *C. neonatale* and *C. perfringens* at 3 months and $p = 0.59$ at 12 months.

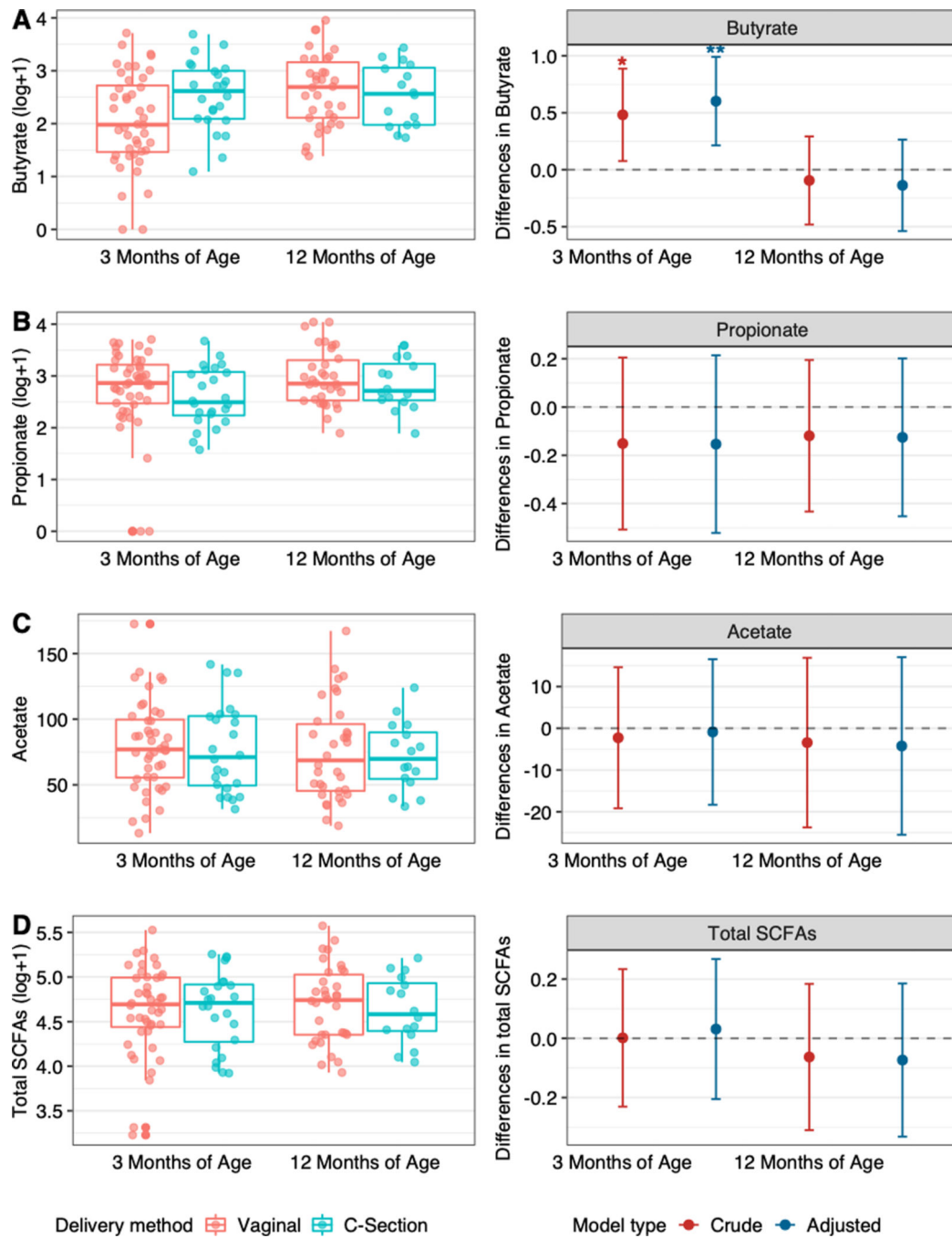


Figure 3. Distributions (left side) and differences (right side) in the fecal concentrations of **A**) butyrate, **B**) propionate, **C**) acetate, and **D**) total SCFAs according to delivery mode (C-section infants vs. vaginal infants [reference]). Differences on the right side of figure are presented before adjustment (crude) and after adjustment for maternal pre-pregnancy body mass index and breastfeeding. * = $p < 0.05$, ** = $p < 0.01$.

Table 1.

Participant characteristics by delivery mode.

	Delivery mode		p-value
	Vaginal (N=45)	C-Section (N=25)	
Pre-pregnancy BMI < 25 kg/m ² (%)	30 (66.7%)	18 (72.0%)	0.65
Pre-pregnancy BMI, kg/m ² (median [IQR])	27.58 [22.96, 35.18]	32.92 [24.96, 40.77]	0.04
Maternal age (median [IQR])	25.94 [22.75, 29.41]	26.26 [24.79, 30.93]	0.24
Female sex	18 (40.0%)	14 (56.0%)	0.20
Black or African-American infant race	30 (66.7%)	14 (56.0%)	0.46
Breastfed ever (%)	37 (82.2%)	19 (76.0%)	0.53
Breastfeeding duration, weeks (median[IQR])	4.50 [1.00, 13.01]	4.00 [0.50, 7.00]	0.69
Introduction to solids before 3 months of age	12 (25.0%)	9 (30.4%)	0.63
Maternal education below high school level (%)	23 (51.1%)	9 (36.0%)	0.22
Household income (%)			0.53
\$20,000	26 (60.5%)	17 (68.0%)	
> \$20,000	17 (39.5%)	8 (32.0%)	
No infant antibiotics at/before 3 months (%)	37 (82.2%)	20 (80.0%)	0.82
No current maternal smoking (%)	33 (82.5%)	15 (75.0%)	0.49
Birth weight, kg (median [IQR])	3.19 [2.89, 3.49]	3.19 [2.82, 3.64]	0.79
Gestational age, weeks (median [IQR])	39.57 [38.71, 40.43]	39.00 [38.29, 39.29]	0.07