HHS Public Access

Author manuscript

JAMA Pediatr. Author manuscript; available in PMC 2017 March 01.

Published in final edited form as:

JAMA Pediatr. 2016 March 1; 170(3): 212–219. doi:10.1001/jamapediatrics.2015.3732.

Effects of Cesarean delivery and formula supplementation on the intestinal microbiome of six-week old infants

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Abstract

Importance—The intestinal microbiome plays a critical role in infant development, and delivery mode and feeding method (breastmilk vs. formula) are determinants of its composition. However,

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Author Contributions: Design and conduct of research performed by KLC, SFF, JCM and MRK; collection, management, analysis, and interpretation of data were conducted by AGH, JHM, HM, MS, JCM, KLC, and HL ; preparation, review, or approval of the manuscript were performed by AGH, MRK and JCM; all authors reviewed, revised, and participated in the decision to submit the manuscript for publication. JCM and AGH had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest Disclosures: None reported.

Role of the Funder/Sponsor: The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Additional Contributions: Microbiology laboratory expertise and direction of Molly Housman, MS, of Geisel School of Medicine was invaluable. The authors are grateful to the children and families that made this study possible and to the staff of the New Hampshire Birth Cohort Study.

the importance of delivery mode beyond the first days of life is unknown, and studies of associations between infant feeding and microbiome composition have been generally limited to comparisons between exclusively breastfed and formula fed infants, with little consideration given to combination feeding of both breastmilk and formula.

Objectives—To examine the relative effects of delivery mode and feeding method on infant intestinal microbiome composition at approximately six weeks of life.

Design, Setting and Participants—Prospective observational study of 102 infants followed as part of a US pregnancy cohort study.

Exposures—Delivery mode was abstracted from delivery medical records and feeding method prior to the time of stool collection was ascertained through detailed questionnaires.

Main Outcomes and Measures—Stool microbiome composition was characterized using next-generation sequencing of the 16S rRNA gene.

Results—We identified independent associations between microbial community composition and both delivery mode and feeding method. Differences in microbial community composition between vaginally and infants delivered by Cesarean section were equivalent to or significantly larger than those between feeding groups. Bacterial communities associated with combination feeding were more similar to those associated with exclusive formula feeding than exclusive breastfeeding. We identified individual bacterial genera that were differentially abundant between delivery mode and feeding groups.

Conclusions and Relevance—The infant intestinal microbiome at approximately six weeks of age is significantly associated with both delivery mode and feeding method, and the supplementation of breastmilk feeding with formula is associated with a microbiome composition that resembles that of infants who are exclusively formula fed. These results may inform feeding choices and shed light on the mechanisms behind the lifelong health consequences of delivery and infant feeding modalities.

Keywords

microbiome; early childhood; breastfeeding; cesarean section

Introduction

Following birth and the initiation of feeding, the human gastrointestinal tract is colonized by a large diversity of bacterial life. An emerging body of literature in adults has begun to establish clear relationships between gut microbiome composition and a wide range of health outcomes [1–6]. In contrast, comparatively little is known about the gut microbiome in infants and children, the exposures that shape it, and its lifelong health impacts [7]. Although limited in their size and scope, a number of studies have established relationships between intestinal microbiome profiles in infants, delivery mode and/or breastmilk exposure [8–15]. These factors both have long-term health consequences. Cesarean delivery has been associated with an increased risk of obesity, asthma, celiac disease and type 1 diabetes [16–19], whereas breastfeeding has been related to decreased risks of asthma, obesity, infection, metabolic syndrome, and diabetes, among other illnesses compared with formula feeding

(reviewed in [20]). The underlying mechanisms are not well understood, but there is growing evidence linking exposure to microflora that is present during vaginal delivery with the patterns of the microbiome that become established in infants [21]. In addition, following delivery, the feeding of human milk primes and matures the infant gastrointestinal system, and is believed to promote a unique microbial colonization profile that has yet to be clearly defined in healthy populations [22]. The acquisition of specific microbes in succession, as the core microbiome of the gut is created, may be permanently affected by exposure to maternal vaginal microflora and/or to breastmilk and could represent a key mechanism underlying differences in immune development that influence lifelong disease risk.

The contribution of bacteria through vaginal delivery followed by exclusive breast feeding promotes specific microbial profiles that facilitate optimal nutrient metabolism and early systemic immune training [23]. The potential short and long term effects of perturbations of the gut microbiome of infancy as influenced by operative delivery or formula feeding are beginning to be examined. The contribution of mode of delivery to the infant microbiome has been evaluated [13,15,24]. However, no study has examined the effects of delivery mode and breastfeeding following adjustment for the other, and there is little data on the effects of combination feeding—feeding breastmilk and formula together. Determining the associations between mode of delivery and breastmilk versus formula feeding and microbiome development in infants is critical to informing delivery and feeding decisions or interventions to alter the microbiome for improved health. Our objective was to evaluate the relative effects of delivery and feeding modes on the composition of the intestinal microbiota at approximately six weeks of age in 102 infants from a US pregnancy cohort study. The observed differences due to delivery and feeding modes highlight their importance in shaping the early intestinal microbiome and point to possible explanations for some of the risks and benefits associated with infant delivery and feeding practices.

Methods

Ethical approval, informed consent and privacy

Institutional review board approval was obtained from the Center for the Protection of Human Subjects at Dartmouth with yearly renewal of approval, and subjects provided written informed consent to participate and to permit their children to participate.

The New Hampshire Birth Cohort Study

Pregnant women ages 18–45 were recruited from prenatal clinics beginning at approximately 24 to 28 weeks gestation as described previously [25,26]. We performed microbiome characterizations of stool samples collected at approximately six weeks of age from full term infants (>37 weeks gestational age at delivery, and appropriate growth for gestational age). Six weeks was chosen as it is likely that exclusive breastmilk or formula feeding would be well established at this age, and six weeks corresponded to routine maternal postpartum visits, a time that allowed for optimal sample collection with minimal particpant burden. We evaluated infant diet from birth until the time of stool collection by telephone questionnaires that included questions regarding the duration of breastfeeding and the timing of formula introduction, if any. Infants who were fed breastmilk and who had

never been given formula prior to the time of stool collection were given the status of exclusive breastmilk feeding; infants who had not been breastfed and who had been fed formula only prior to their stool collection were assigned the status exclusively formula-fed; and infants who had received both breastmilk and formula prior to their stool collection were identified as having a diet of both breastmilk and formula. When possible, we confirmed exclusive breastmilk and exclusive formula feeding status using a feeding diary kept by subjects' mothers during the 48-hour period prior to stool collection.

Delivery mode (Cesarean vs. vaginal delivery) was abstracted from maternal delivery records. Data about infant exposures to medication were derived from questions asked during the telephone questionnaires described above. Mothers were asked whether their infant had received a prescription medication in the first four months of life. A free text field was used to record the medication name. If the exact name could not be recalled, as much detail as could be recalled was recorded. Topical medications including those given for conjunctivitis and antifungals such as those given for thrush were not considered. Because antibiotic exposure has both been shown to influence the intestinal microbiome [7] we excluded infants who had received a prescription antibiotic.

Sample collection, DNA extraction and sequencing

Study participants provided infant stool samples collected at regularly scheduled maternal postnatal follow-up visits (six weeks post partum). Stool was aliquoted in sterile tubes and frozen at −80°C within 24 hours of receipt. Samples were thawed and DNA was extracted using the Zymo DNA extraction kit (Zymo Research). Quantity and purity of the DNA were determined by OD260/280 nanodrop measurement. Reliability and stability of these methods were described by Wu et al [27]. Illumina tag sequencing of the 16S rRNA gene V4–V5 hypervariable region was performed at the Marine Biological Laboratories (MBL) in Woods Hole, MA using established methods [28,29]. Details of sequencing methods, quality control and filtering, and statistical modeling are presented in the Supplement (see eMethods).

Results

Study subject characteristics and variability and diversity of the early neonatal microbiome

We evaluated the relationships between the composition of the six-week intestinal microbiome and both delivery mode and feeding method in 102 full term, appropriately grown infants enrolled in the New Hampshire Birth Cohort Study. Delivery medical records, telephone surveys and feeding diaries were used to assess study participant characteristics including delivery mode and feeding method at the time of stool sample collection (table 1). We found no significant relationship between delivery mode and feeding method (see eTable in the Supplement; Fisher exact test: p=0.66).

We sequenced the V4–V5 regions of the bacterial 16S rDNA to characterize the microbial communities present in a stool sample from each study subject at six weeks of age. Sequencing yielded a total of 14,362,739 (mean: 140,811, range: 27,897 – 260,579)

bacterial DNA reads, of which, 8,210,402 (mean: 80,494, range: 12,244 – 178,802) passed quality filters (see eMethods in the Supplement). These were assigned to 241 bacterial genera. Over 90% of reads were represented by 10 genera (table 2). Stool samples were dominated by *Bacteroides* and *Bifidobacterium* comprising half of sequence reads, with *Streptococcus, Clostridium, Enterococcus, Blautia, Veillonella, Lactobacillus, Staphylococcus, Planococcus*, and others representing the remainder (table 2).

Relationships between delivery mode, feeding method and microbial community composition

Overall stool microbiome community composition was characterized using generalized UniFrac analysis [30]. Controlling for the effects of feeding method, delivery mode was strongly associated with infant gut microbiome composition ($p<0.0001$, $q=0.0005$) (figure 1a). Likewise, controlling for the effects of delivery mode, the overall association between feeding method and stool microbiome community composition was also statistically significant ($p=0.01$, $q=0.02$) (figure 1c). In pairwise comparisons of the three feeding methods, exclusive breastfeeding was associated with a microbiome community distinct from that of infants who were either exclusively formula-fed ($p=0.04$, $q=0.05$) or fed a combination of breastmilk and formula prior to stool collection ($p=0.02$, $q=0.04$). There was no statistically significant difference between infants fed a combination of breastmilk and formula and those fed exclusively formula in terms of microbiome composition. There was no significant interaction observed between delivery mode and feeding method ($p=0.49$). The lack of an interaction between delivery mode and feeding method remained even after combining exclusively formula fed and combination fed infants into single group ($p=0.53$).

We calculated within- and between-group average UniFrac distances in order to assess the group-specific phylogenetic diversity of the microbial communities we observed in our subjects. Within-group distances between infants within specific delivery mode and feeding method groups revealed similar average phylogenetic distances among infants who were born vaginally compared with those born by Cesarean section (figure 1b). The greatest within-group average pairwise phylogenetic distance was observed among those infants who were fed breastmilk supplemented with formula; pairs within this group were on average significantly less similar than pairs within the exclusively breastfed group ($p=0.02$, $p=0.04$), while other comparisons did not reach statistical significance (figure 1d).

Between-group pairwise UniFrac distances were, on average, as large or larger between vaginal and Cesarean section-delivered infants as they were between infants from different feeding groups (figure 1e). For feeding method, average bacterial community phylogenetic distance was greatest between infants who were exclusively breastfed as compared to those exclusively formula fed and between infants who were exclusively breastfed as compared to those who were fed breastmilk supplemented with formula. In contrast, the average distance was smallest between infants who were fed a mix of breastmilk and formula and those fed exclusively formula.

We were concerned that some of the subjects in the combination fed group may have been offered the breast in the first few days following delivery but were otherwise effectively exclusively formula fed, which may have driven the difference we observed between

exclusively breast fed and combination fed infants in terms of between group differences. In fact, of subjects who were combination fed in our study, all but two were fed breastmilk for at least the first two weeks of life. To test the robustness of this finding in light of the possible effect of these two subjects, we repeated the UniFrac analysis after reassigning those two infants from the mixed feeding group to the exclusively formula fed group, and no qualitative differences in the results were observed (data not shown).

Individual taxon abundance by delivery mode and feeding method

Vaginal delivery (vs. Cesarean section delivery) was associated with increased abundance of *Bacteroides* (p=0.0007, q=0.02) and *Pectobacterium* (p=0.001, q=0.02) and with decreased abundance of *Staphylococcus* (p=0.001, q=0.02), *Rothia* (p=0.006, q=0.07) and *Propionibacterium* (p=0.01, q=0.099), in infant stool, after adjustment for feeding method (figure 2a). Feeding was associated with differential abundance in *Lactococcus* (p<0.0001, q=0.002) which was depleted in exclusively breastfed infants compared with those who were exclusively formula fed (figure 2b). No taxa were significantly differentially abundant between subjects who were combination fed vs. exclusively formula fed or exclusively breastfed (figures 2c and 2d).

Discussion

We characterized the intestinal microbiome of 102 six-week old infants and observed independent associations between stool microbial community composition, mode of delivery and feeding method. In healthy infants, the process of delivery is the initial encounter with microorganisms capable of colonizing the intestinal tract. In a previous study of 24 healthy women, vaginal microbiome composition became less diverse between the second and third trimesters of pregnancy, and just before delivery was enriched with *Lactobacillus* species, likely contributing to vertical transmission of these bacteria during vaginal birth [21]. In a study of 10 newborns in Venezuela, within hours of delivery, the intestinal tracts of infants born vaginally were colonized by *Lactobacillus* and *Prevotella*, whereas infants delivered operatively acquired bacteria present on the mother's skin and the hospital environment, such as *Staphylococcus*, *Proprionibacterium* and *Corynebacterium* [15]. Our findings, based on a large group of six week old infants, indicate that *Lactobacillus* also contributes to the microbial environment of the gut, but to a lesser extent than *Bifidobacteria*, *Bacteroides* and *Streptococcus*.

Other studies have observed differences in older infants according to delivery mode. A study of 24 infants aged 3–4 months in Canada found that 2 out of 26 taxa evaluated were differentially abundant between vaginally and operatively delivered babies, including *Bacteroides*, which was depleted in Cesarean section delivered infants relative to those who were vaginally delivered [9]. This result was also observed in a longitudinal study of 24 infants in Sweden, which reported that the depletion of *Bacteroides* in Cesarean section-delivered infants persisted until 12 months of age [13]. Another longitudinal study of 75 infants in Singapore found that the acquisition of "normal" gut flora was delayed in infants born by Cesarean section [31].

Our study is the first to examine the contribution of delivery mode to infant intestinal microbiome composition in relationship to that of another important predictor of microbiome composition, infant diet. We found that delivery mode was more strongly associated with infant microbiome composition than was diet at six weeks. We observed differences in microbial community composition between vaginal and Cesarean section delivered infants that were comparable or slightly greater than the largest differences associated with feeding.

We observed an association between feeding method and microbiome composition that remained statistically significant even after adjusting for delivery mode. Though a few previous studies have found associations between infant feeding and intestinal microbiome composition [9–12,14], none has examined the relative contribution of combination feeding (breastmilk and formula) alongside exclusive formula or breastfeeding to overall microbial community composition. This is an important group to consider because combination feeding is common, for example, in the first few days in the hospital when lactogenesis II is delayed while mother's breastmilk is becoming established, among mothers who have difficulty producing adequate milk and supplement their own milk with infant formula, or among mothers who are unable or choose not to pump breastmilk when separated from their babies. We found that the distinction between the microbial communities according to feeding method was largest between infants fed exclusively breastmilk and those fed either combination diets or exclusively formula. Infants fed both breastmilk and formula had intestinal microbial communities that were similar to those fed exclusively formula, and relatively distinct from those fed exclusively breastmilk. This finding offers new evidence to support the tenets of the World Health Organization's Baby Friendly Hospital Initiative, which promotes exclusive breastmilk feeding beginning at birth in hospitals and birthing centers and the avoidance of formula supplementation unless deemed medically necessary (http://www.who.int/nutrition/topics/bfhi/en/). The findings in this study also provide new evidence for pediatricians as they provide guidance to breastfeeding mothers who may be considering incorporating formula into their infant's diet, and may have implications for decisions around the use of donor human milk in cases when supplementation is needed.

There have been no long-term longitudinal studies of the effects of early feeding method on the microbiome, but early feeding has the potential for lasting effects on microbial community structure [32], and these impacts may be one mechanism for the health benefits of breastfeeding on childhood and lifelong health. Digestion and metabolism of nutrients are likely influenced by the intestinal microbiome [33], and there is a well-established connection between breastfeeding and lower risk of childhood and adult onset obesity likely mediated in part by the microbiome in early life (reviewed in [34]). Oligosaccharides in breastmilk are thought to promote *Bifidobacterium* growth [35], and decreased *Bifidobacterium* in infancy has been related to an increased risk of being overweight at age 10 [36]. Many formulas are supplemented with prebiotics such as short chain galacto-oligosaccharides and long chain fructo-oligosaccharides that increase the overall representation of *Bifidobacterium* in the microbiome of formula fed infants, and similar to breastmilk, promote lactate and short chain fatty acid prevalence in the infant gut (reviewed in [37]). Although we did not observe a significant association between increased abundance of *Bifidobacterium* and breastfeeding

in our study, *Bifidobacterium* was present at greater abundance in exclusively breastfed infants compared with others; compared with combination fed subjects, this enrichment approached statistical significance before correction for multiple comparisons.

Our conclusions are limited by our study population, which was selected from a single cohort from the US and sampled at a single time point, and thus our findings may not be entirely generalizable to populations elsewhere or to different points in infant development. While ours is one of the largest studies examining the factors that shape the infant microbiome, our sample size of 102 subjects limited our statistical power which precluded stratified analyses for identifying any interactions between delivery mode and feeding method. In addition, while we were able to categorize feeding practices, the exact proportion of the diet that was made up of either breastmilk or formula and the exact timing of formula supplementation (e.g. in hospital after delivery versus beginning just prior to six weeks) was not considered. It is possible that infants who received formula supplementation only at birth were able to recover a microbiome that resembles that of an exclusively breast fed infant. A recent study highlighted infant nutrition as a major contributor to the early microbiota composition and function, with cessation of breastfeeding contributing the most fundamental shift in the composition of bacteria [8]. A longitudinal study with more subjects would allow us to determine the temporal dynamics of the effects of feeding practices and changes therein, as well as the persistence of the effects of both feeding and delivery mode later in infancy. Additionally, exposures such as postnatal antibiotics were rare in this cohort and therefore subjects with antibiotic exposure were eliminated from analysis; in the future the evaluation of prenatal, peripartum, and postpartum antibiotic exposure and their role in the trajectory of microbiome development, and interrelationship with delivery mode and dietary exposures, will be important. Thus, our results will need to be replicated in larger, multi-center studies and in prospective analyses. While the UniFrac analyses we performed suggest independent associations between microbiome composition and both delivery mode and feeding method, the substantial overlap between the communities defined by both factors suggests that there are other important drivers of microbiome community composition that remain to be identified in future analyses.

In conclusion, understanding the patterns of microbial colonization of the intestinal tract of healthy infants is critical for determining the health impacts of specific, alterable early life risk factors and exposures. To this end, we have identified measurable differences in microbial communities in the intestinal tracts of infants according to their delivery mode and diet, with possible consequences for both short and long term health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding/Support: This work was supported in part by grants from the National Institutes of Health (NIEHS P01ES022832, NIEHS P20ES018175, NIGMS P20GM104416, and NLM K01LM011985) and the US Environmental Protection Agency (RD83459901 and RD83544201).

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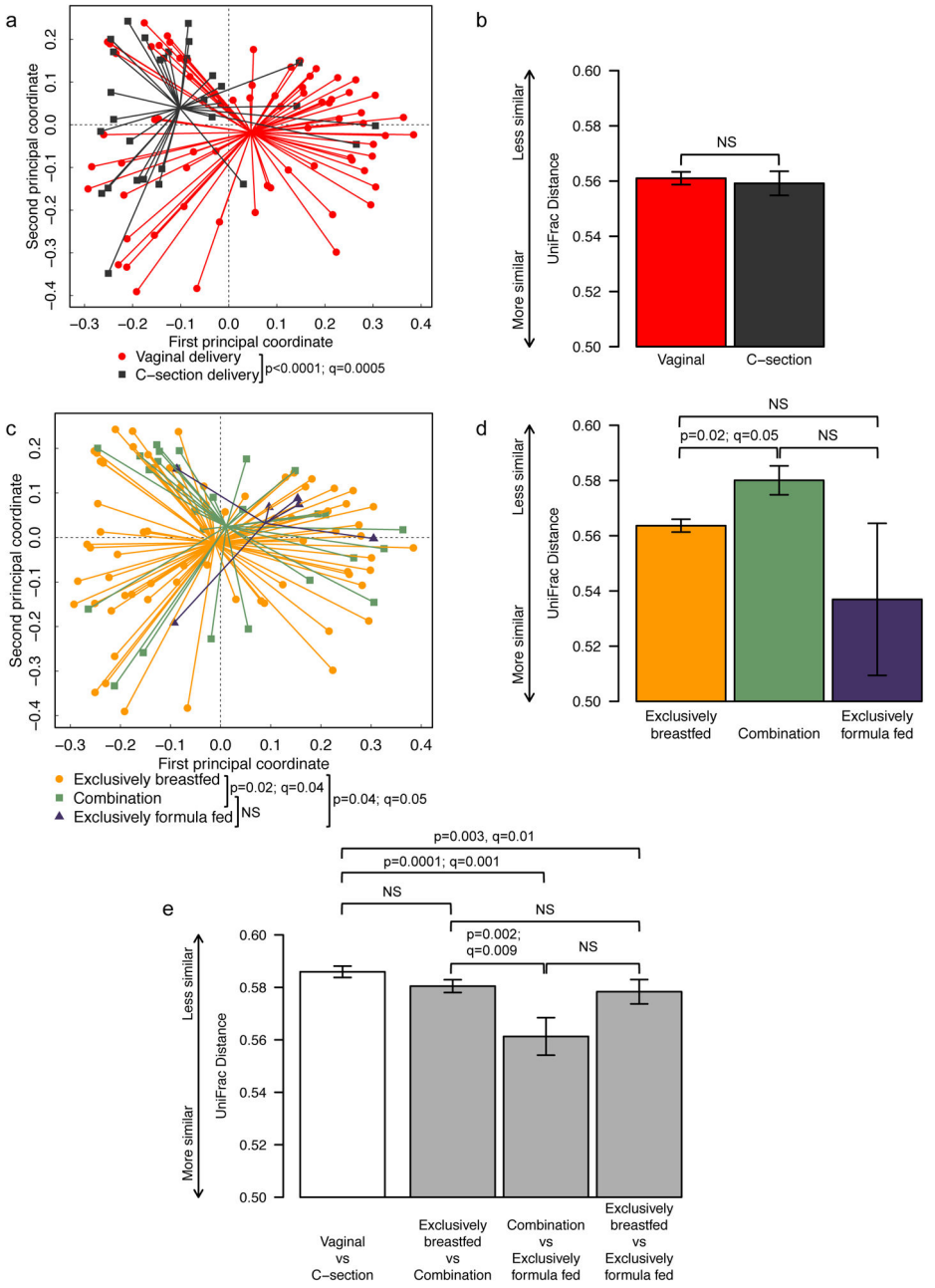

Fig. 1. Comparison of microbial community composition between delivery mode and feeding method groups

Principal coordinate plots (a, c) and mean pairwise UniFrac distances (b, d, e) within groups for delivery mode (a, b) and feeding method (c, d) and between groups (e). **UniFrac is a distance metric used for comparing biological communities that incorporates information on the phylogenetic relatedness of community members.** For (a) and (c), individual subjects are represented by points marked according to delivery mode (a) or feeding method (c) and are plotted on the first two principal coordinates with ADONIS p-values indicated. Lines are drawn from each point to its group centroid. For (b), (d) and (e), bar height is proportional to mean pairwise UniFrac distance within (b) and (d) or between

(e) groups with error bars indicating standard error of the mean. In (e), white bars indicate delivery mode groups while gray bars indicate feeding method groups. Throughout, Q-values indicate significance of differences after adjusting for multiple comparisons by controlling the false discovery rate for selected comparisons.

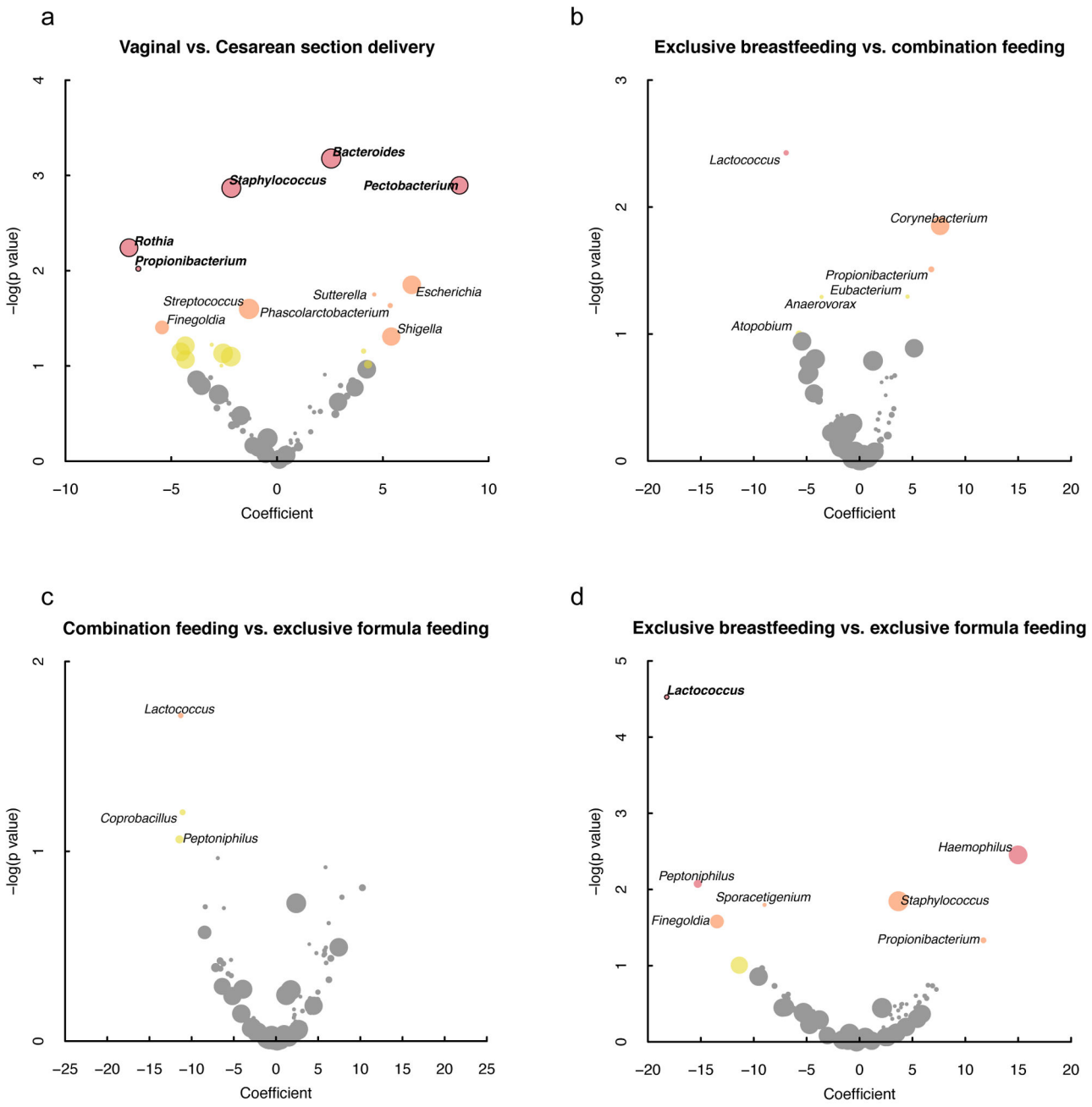

Fig. 2. Relationships between individual genus abundance and (a) delivery mode and (b–d) feeding method

Positive coefficients indicate independent associations with vaginal delivery (a) or breastmilk exposure (b–d) after controlling for the other. Point colors correspond to adjusted p values of >0.1 (gray), ≤0.1 and >0.05 (yellow), ≤0.05 and >0.01 (orange), and ≤0.01 (red). Circles are sized according to relative log-ratio transformed abundance. Black borders/bold labels indicate genera that were significantly differentially abundant after controlling for the false discovery rate at a significance level of q=0.1. Note differences in axis scales.

Table 1

Subject characteristics (n=102)

Variable	Mean (range) or %
Gestational age (weeks)	39.7 (37.1–41.9)
Delivery mode	
Vaginal	69%
Cesarean section	31%
Infant sex	
Male	54%
Female	46%
Infant birth weight (g)	3530 (2700–4710)
Feeding at six weeks	
Exclusively breastfed	69%
Combination feeding	25%
Exclusively formula fed	6%
Age at formula introduction among combination fed subjects (weeks)	3.1 (0.1, 8.7)

Table 2

Relative abundance of the 10 most abundant bacterial genera identified, for all subjects overall and for individual delivery mode and feeding groups.

Genus	Overall (n=102) (%)	Vaginally delivered (n=70) (%)	Delivered by C-section (n=32) (%)	Exclusively breastfed (n=70) (%)	Combination fed (n=26) (%)	Exclusively formula fed (n=6) (%)
Bacteroides	26.4	34.6	20.7	27.9	22.1	28.8
Bifidobacterium	22.5	23.3	17.4	25.5	16.8	11.4
Streptococcus	13.8	12.1	14	11.7	18.7	16.9
Clostridium	7.9	5.1	8.8	6.8	11.9	2.4
Enterococcus	5.7	4.3	8.7	4.8	6.1	14.6
Blautia	3.6	2.7	5.5	1.8	7.1	9.4
Veillonella	3.4	3.6	4.6	3.5	3.2	2.9
Lactobacillus	3	2.5	4.2	3.4	2.8	0
Staphylococcus	2.6	1.6	3.4	3.3	1.2	0.1
Planococcus	2	1.4	2.9	1.5	3.3	2.6
Other genera	9.1	8.8	9.8	9.8	6.8	10.9