

Una destinatio, viae diversae

Does exposure to the vaginal microbiota confer health benefits to the infant, and does lack of exposure confer disease risk?

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There are two ways by which a baby can come into this world: vaginally or by Cesarean delivery. Although the developmental paths that result in either vaginal or Cesarean birth are highly diverse and very similar at the same time, the actual indication of whether a pregnant woman has to undergo surgery is usually clear. In most cases, Cesarean deliveries are performed for obstetric, fetal, or maternal indications, some of which are considered absolute. For example, if the placenta or its vessels obscure the cervix (complete placenta previa or vasa previa, respectively), vaginal delivery would be lethal to the mother and/or her newborn. What is less clear, however, is the child's developmental path after delivery. In particular, there is much debate on whether Cesarean delivery increases the risk of several diseases later in life, and whether any of these diseases result from a lack of exposure to the mother's vaginal microbiota during birth.

Risks of Cesarean delivery

Cesarean delivery is the most common abdominal surgery in the USA, accounting for more than 1 million operations annually. Four decades ago, 1 in 20 births occurred via Cesarean, now it is 1 in 3 to 1 in 4. The appropriate rate of Cesarean delivery is not easily determined, since it depends not only on multiple maternal, fetal, and obstetrical comorbidities and risk factors, but also on whether the mother had a Cesarean delivery before. The relative safety of Cesarean delivery—if performed by a competent obstetrical surgeon in a hospital—is

recognized by patients and their providers alike, and the attributable risk of morbidity and mortality for both mother and child is extremely low. However, Cesarean delivery as a procedure has come under increasing scrutiny in recent years, in part owing to its real or perceived risk of future disease or harm for both the child and the mother.

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On the maternal side, there is limited risk with the first Cesarean surgery. However, repeated surgeries in subsequent pregnancies may cause bowel and bladder trauma, surgical adhesions and scarring, uterine scar separation, and adherent placentation such as placenta accreta or percreta with a high risk of accompanying massive blood transfusions and surgical complications. These hazards are relatively rare but highly morbid and have inspired various approaches to reduce the rate of Cesarean deliveries, in particular primary surgeries.

What has been less appreciated is the potential harm to the fetus, but such concerns have garnered considerable

attention in recent years. Immediate threats for the baby include delayed delivery, owing to surgical adhesions or malpresentation, but these hazards are easily identified. In addition, there is emerging evidence that suggests an association between Cesarean delivery with increased rates of atopic allergic disorder and IgE-mediated sensitization to food allergens, as well as metabolic syndrome and obesity later in life. For instance, a recent large perspective study conducted over 16 years with more than 22,000 participants found that Cesarean-delivered infants had a 13% increased risk of obesity later in life [1]. However, how much of that risk can be truly attributed to the Cesarean procedure itself (rather than what led to the Cesarean, or the company it keeps) remains unclear. Investigators studying the human microbiome have attributed these observations to a lack of exposure to the mother's vaginal microbiota—or conversely overt exposure to skin microbiota—during delivery. But is that attribution supported by the available evidence? And how strong is the causal link between Cesarean delivery *per se* and subsequent atopy or obesity in the child?

Understanding the validity and robustness of these observations, and identifying alternative risk factors, is very important. Wrongly attributing an outcome to an incorrect cause will affect potentially beneficial or efficacious interventions. If the maternal medical indication for the Cesarean rather than the surgical procedure itself increases health risks for the child, then earlier interventions to reduce the maternal indication and thereby the prevalence of Cesarean

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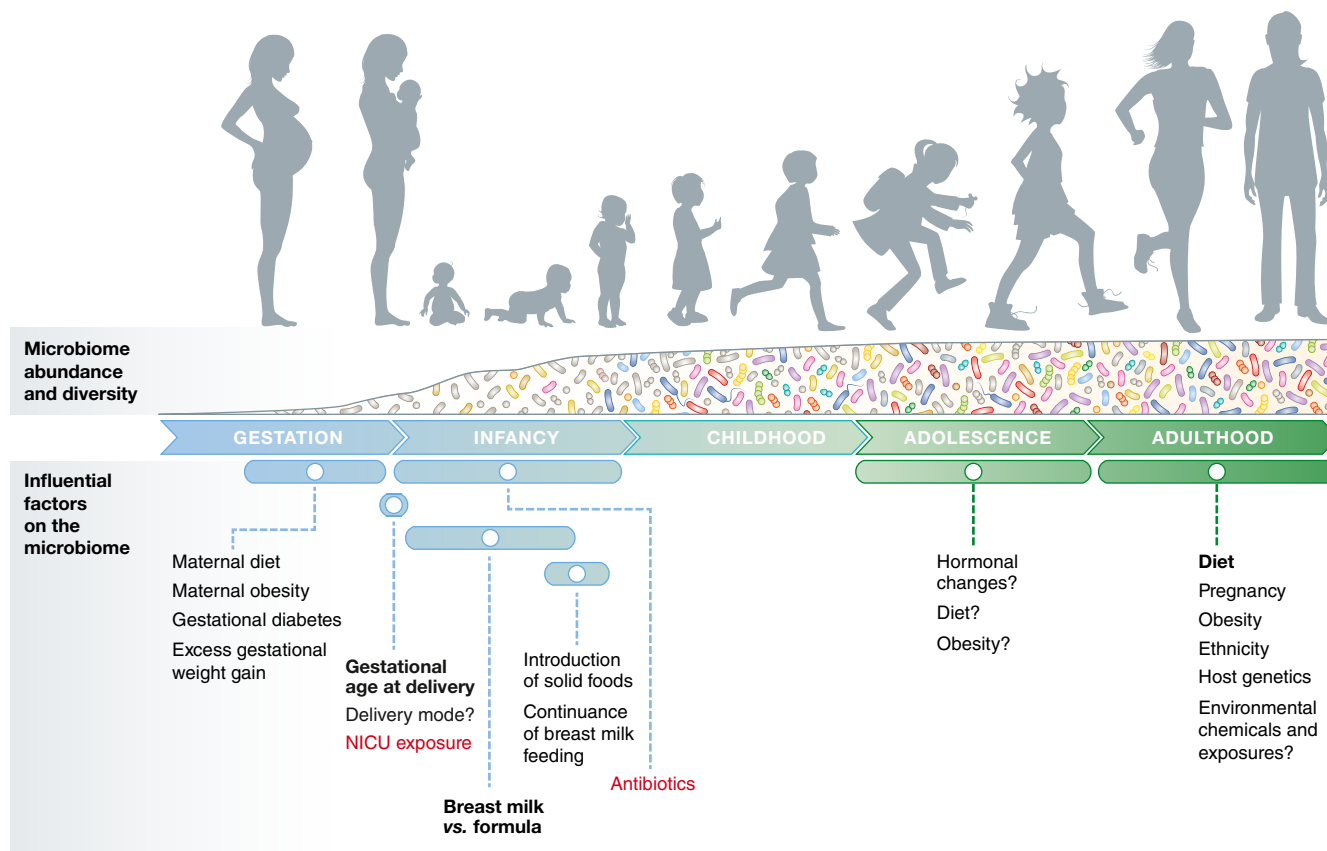


Figure 1. Important modifying factors thought to impact the microbiome throughout life.

The first contact with microbiota may begin during late gestation, with the largest exposure at the time of delivery. Over time, the abundance and diversity of the infant microbiome increase with life, stabilize around the time that the infant begins to eat solid foods, and persist throughout adulthood. A number of modifying factors are thought to have an influential role in shaping the identity and abundance of the infant microbiota throughout life. However, few studies have specifically examined major modifying factors within childhood and adolescence.

delivery would be both necessary and sufficient for preventing health risks for the child. For example, if maternal diet is overly rich in calories, which both disturbs the establishment of the infant's microbiome and renders a large baby that cannot readily nor safely fit through the birth canal, then any helpful mediations would need to address her diet. Conversely, if it is a postnatal event that happens to co-associate with Cesarean delivery, then the mode of delivery becomes irrelevant and effective modifiers would be needed after birth. Such postnatal factors would, for instance, include breast milk versus formula feeding: Obese women, who coincidentally have higher rates of Cesarean delivery, are more likely to feed formula. Hence, it is vitally important to understand whether it is the surgery, its indication, or postnatal factors that may be behind reported links between Cesarean delivery and infant atopic diseases or obesity risks later in life.

Unclear health risks

So, what do we know from the available literature about the association of Cesarean delivery on the gut microbiota and conversely on disease risk? Unfortunately, most studies to date do not report the indication for performing Cesarean delivery in the first place. In the landmark work by Dominguez-Bello *et al* [2], the authors describe differences in the neonatal microbiome immediately after birth and conclude that Cesarean delivery alters the early colonizers of the infant gut by denying initial exposure to the vaginal flora. It should be noted that their analysis is based on samples within 5 min of delivery and the baby's first stool collected within 24 h. The differences therefore largely describe the microbes transmitted from the mother and not true colonization *per se*.

Careful review of this study population also reveals other noteworthy details. The

study enrolled 9 Mestizo and Amerindian women in Venezuela at the time of delivery and their 10 neonates. Four women and their 4 infants make up the vaginal cohort; 5 women and 6 neonates represent the Cesarean cohort. Of these five women, one underwent surgery to deliver male twins. Except for the twins, the exact weight of each neonate was not given; the methods section of the manuscript states that “[a]ll mothers had healthy pregnancies and all babies were born at term, without complications. Babies weighed between 2 and 5.2 kg (the smallest baby was the twin in second order of birth, after his 3-kg brother)”.

However, this would suggest that a minimum of two of pregnancies—presumptively both Cesarean—would not have met standard definitions of “healthy and uncomplicated”. First, the twins—chorionicity was not provided and both were male, so mono-versus di-zygosity (in other words, identical

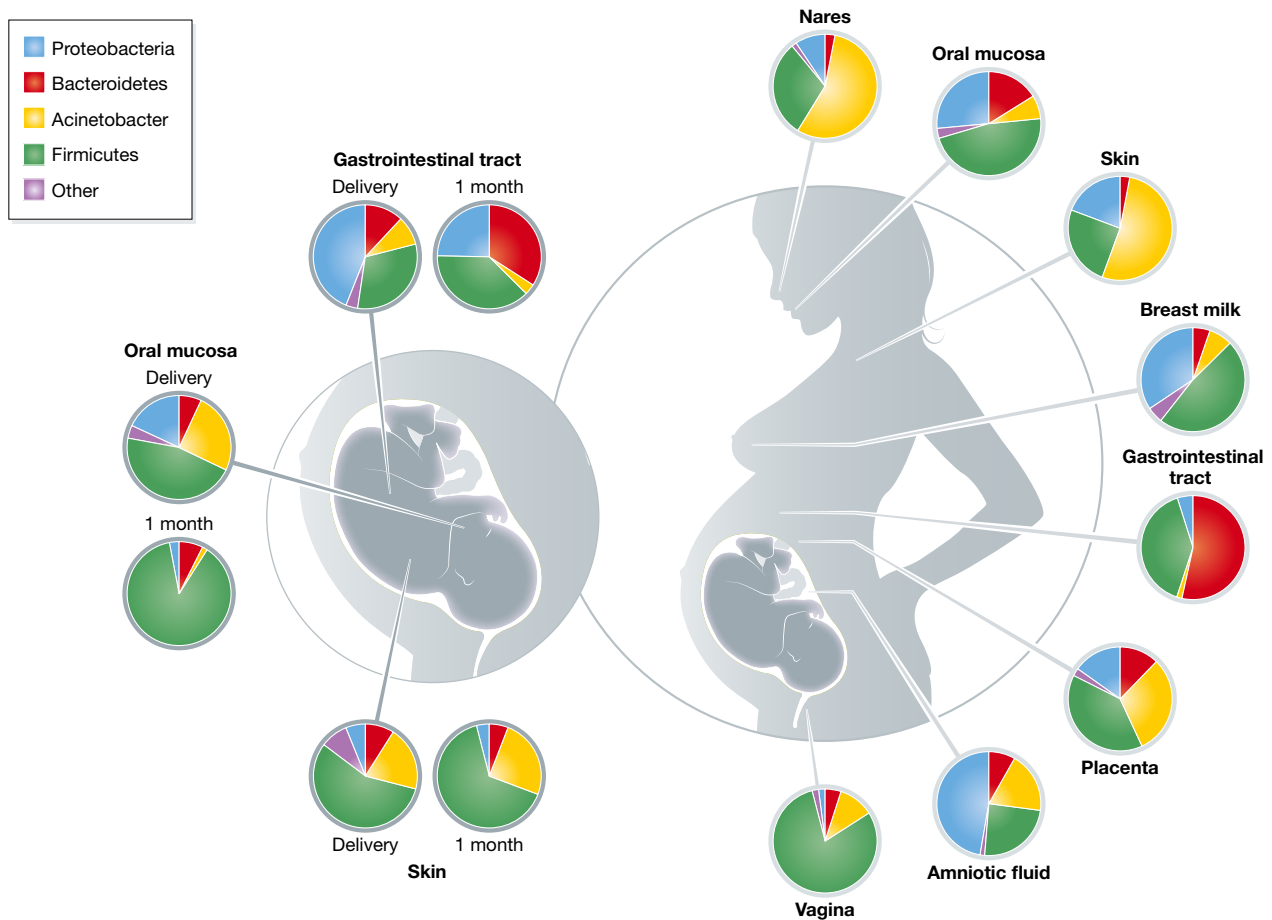


Figure 2. General composition of the maternal and infant microbiome in pregnancy and early life.

The composition of the microbiome is unique to each body niche. As demonstrated by the pie charts, the relative abundance of each major phyla is different between body sites, although considerable variation exists. Recent evidence has also demonstrated unique populations of bacteria in the intrauterine environment, including the placenta and amniotic fluid.

versus fraternal twins) may not be known—were 33% discordant in growth with reported weights of 2.0 and 3.0 kg. Discordance in twins is defined as the difference in birth weight between twins divided by the larger twin's birth weight. A discordance of more than 20% is associated with adverse perinatal and postnatal outcomes.

Second, at least one neonate was impressively larger than would be expected. This is called macrosomia, which is defined as a birth weight of more than 4 kg. In the USA, Cesarean is typically offered to diabetic mothers with a fetus estimated larger than 4.5 kg and a non-diabetic mother with a fetus estimated larger than 5 kg. A 5.2 kg fetus would be 0.8–1 kg heavier than more than 98% of the birth population according to WHO standards for male and female newborns, respectively (<http://www.cdc.gov/growthcharts/>). There are multiple

underlying causes of fetal macrosomia, but the most common one is poorly controlled maternal diabetes. Additional causes include genetic and epigenetic overgrowth disorders, chronic caloric excess, and maternal obesity. Unfortunately, this small but highly cited landmark study did not provide the underlying indication for the Cesarean deliveries, but at least three cases raise concerns about potential metabolic or fetal growth abnormalities as evident by the authors' brief description.

Confounding factors

Subsequent studies have further explored the impact of potential confounders in greater depth, though they still remain underreported (see Sidebar A) (Fig 1). For instance, a Canadian study of mothers and their infants categorized Cesarean deliveries

as either being elective or emergent, and found differences in bacterial richness and diversity. However, truly elective Cesarean deliveries and truly emergent Cesarean deliveries are extremely rare, and may not reflect varying degrees or whether the fetus had entered the vaginal canal. Further confounders that are scarcely accounted for in the literature include maternal intrapartum and neonatal antibiotic exposure, gestational age, diet, host genetics, and environment, among others. For instance, vaginal and placental microbiomes vary by gestational age throughout pregnancy, and thus infants delivered preterm have different *in utero* and intrapartum exposure. Maternal intrapartum antibiotics and infant diet have also been shown to significantly alter the infant microbiome, with reduced bacterial diversity most prominent in breast milk-fed infants whose mothers received intrapartum antibiotics.

However, all subjects in this study were delivered by vaginal delivery, and thus the influence of birth mode was not explored.

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Another study demonstrated that intrapartum antibiotics and Cesarean delivery significantly altered the neonatal gut microbiome, which was somewhat mitigated by breast milk feeding. Furthermore, intrapartum antibiotics and preterm birth result in significant alterations to the microbiome and inferred metabolic function, with significantly lower levels of health-associated short-chain fatty acids (SCFAs) and altered antibiotic resistance profiles. With so many variables known to perturb the developing microbiome, these latter studies demonstrate the importance of capturing multiple variables in microbiome analyses and not just attributing alterations to whether the infant passed through the vaginal canal.

Is there evidence of other potential factors that alter the microbiome and that are associated with a higher occurrence of Cesarean surgery? Perhaps the best-studied possible cause to date is maternal high-fat diet and obesity. The notion that weight gain, high-fat diet, and microbiome composition are intertwined is now well established, with excellent examples of causality in murine models. Transferring the gut microbiome from obese mice to germ-free mice promotes adiposity and other studies showed that bacterial functioning is strongly influenced by a high-fat diet. We have used a primate model and shown that maternal diet infers significant and persistent alterations to the juvenile microbiome, even when juveniles are co-housed and switched back to a healthful diet after weaning [3]. More recently, we confirmed these findings in a large human cohort of mothers and their infants, which showed that a maternal high-fat diet is associated with alterations in the infant microbiome at birth, and that

these changes persist for at least 4–6 weeks after birth [4]. Notably, this time frame encompasses the vast majority of studies that explore the role of birth mode on the developing gut microbiome in infants.

Impact on the gut microbiome

Given the lack of clear evidence that Cesarean delivery poses a health risk for newborns by way of changes in the gut microbiota, “What is the evidence that mode of delivery actually impacts on the baby’s microbiota?” In other words, are there microbial species or genera that are uniformly present in all vaginally delivered infants and uniformly absent in all Cesarean-born babies? A recent study of 39 Finnish infants, based on monthly samples from month 2 to year 3 of life, showed that 20% of vaginally delivered infants more closely resembled Cesarean-delivered infants in terms of the composition of their gut microbiome [5]. This was attributed to a low profile of *Bacteroides* species, which was not associated with any relevant clinical variables, such as maternal intrapartum antibiotics, gestational age, duration of delivery, time in hospital, and so on.

“... few studies to date have explored how a Cesarean birth-associated microbial profile in humans directly leads to alterations of host biology that eventually manifests as disease”

Previous studies had suggested that prolonged effects of birth mode on microbiota composition that co-occurred with Cesarean delivery result in reduced bacterial diversity throughout the initial years of life. However, Yassour *et al* [5] importantly demonstrate that this effect results from the low-*Bacteroides* group, which was independent of delivery mode. It was also notable that infants from the 16S taxonomic-profiled low-*Bacteroides* group—Cesarean birth and 20% of vaginally delivered infants—generally had higher abundances of *Bifidobacteria*. In accordance with this Finnish study, a separate study exploring the microbiome development in 43 American infants during the first 2 years of life showed reduced

Bacteroides abundances in Cesarean-delivered infants [6]. Although the US study did not specifically explore the low-*Bacteroides* profiles, as reported by Yassour *et al* to occur in 20% of vaginal deliveries, they found no difference in the abundance of *Bacteroides* among Cesarean and vaginally delivered infants after 1 year.

“Although parturition was traditionally assumed to be the first point at which neonates are exposed to bacteria, emerging evidence indicates that this may not be true”

Because *Bacteroides* species have been demonstrated to positively influence the immune system in animal models, it has been widely speculated that a low-*Bacteroides* profile associated with Cesarean delivery potentially puts the infant at risk for future disease. This may not be a correct assumption, however. For example, in addition to Cesarean delivery, a 16S-assigned low-*Bacteroides* profile is also associated with exclusive breast milk feeding. Clearly, exclusive breast milk feeding is uniformly recommended and has tremendous long-term health benefits and no known harms. Conversely, maternal high-fat diet and maternal obesity are also associated with both Cesarean delivery and lower *Bacteroides* profiles. Disentangling correlation and causation between such co-linear variables ultimately requires additional studies in relevant models. However, few studies to date have explored how a Cesarean birth-associated microbial profile in humans directly leads to alterations of host biology that eventually manifests as disease. In fact, in one of the few studies that sought to establish such as causal link, namely alterations to the regulatory component of the immune system associated with Cesarean delivery, the immune modulating affect was attributed to higher proportions of *Bacteroides* [7]. However, despite these changes, it reported no overt phenotype in adult mice. Certainly, additional work in experimental models is needed to further establish whether such a causal mechanism exists, but considering the multitude of factors that shape the early microbiome, demonstrating such a link may prove difficult.

Disentangling correlation and causation

Along these lines, Dominguez-Bello *et al* [8] took an innovative and provocative approach to establish causation by “restoring” the infant microbiome of Cesarean-delivered infants with maternal vaginal flora. Through incubating a gauze in the mothers vagina for 1 h before surgery and subsequently “wiping” the neonates’ mouth, face, and body at the time of delivery, the team described that they were able to “partially restore” bacterial members from the vaginal flora into the neonate microbiome when measured over 30 days. Although the study size was appropriate for a pilot study—18 women and their infants—only 4 of the total of 11 Cesarean-delivered infants were exposed to vaginal wiping. The authors report that, among Cesarean babies exposed to maternal vaginal fluids, the skin and oral sites were most comparable to vaginally delivered infants, whereas the anal site remained more comparable to Cesarean-delivered infants who did not undergo vaginal wiping. They conclude that Cesarean babies “lacked the vaginal bacteria that were restored by swabbing infants with gauze or that were present in vaginally delivered infants, particularly anal and skin *Lactobacillus* early in life”.

However, close inspection of the relative abundance of bacterial taxa at these sites revealed that this conclusion is based on limited repeat sampling data from a variable and small number of infants. Of the 4 Cesarean-delivered babies who were exposed to vaginal wipes, only one neonate yielded data at every time point. Similarly, of the 11 Cesarean-delivered neonates who did not undergo vaginal wiping, only two had anal and skin sample data and none had oral data from the initial day 1 reference time point. In fact, careful review of all body site samples reveals inconsistent sampling data among subjects at all six time points, making restorative analysis challenging.

Nevertheless, the authors conclude that *Lactobacillus* dominates anal samples in 25% of vaginally exposed infants at day 1—comparing a pool of four neonates to a pool of two—while samples collected on day 3 are more comparable to Cesarean-delivered newborns who were not vaginally wiped by comparing a pool of 2 neonates to a pool of 9 neonates. The authors comment that “In anal samples from exposed infants and

Sidebar A: Further reading

Studies that report other confounding factors for a possible association of Cesarean delivery with infant gut microbiota

Azad MB, Konya T, Maughan H, Guttman DS, Field CJ, Chari RS, Sears MR, Becker AB, Scott JA, Kozyrskyj AL *et al* (2013) Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. *CMAJ* **185**: 385–394

Mazzola G, Murphy K, Ross RP, Di Gioia D, Biavati B, Corvaglia LT, Faldella G, Stanton C (2016) Early gut microbiota perturbations following intrapartum antibiotic prophylaxis to prevent group B streptococcal disease. *PLoS One* **11**: e0157527

Azad MB, Konya T, Persaud RR, Guttman DS, Chari RS, Field CJ, Sears MR, Mandhane PJ, Turvey SE, Subbarao P *et al* (2016) Impact of maternal intrapartum antibiotics, method of birth and breastfeeding on gut microbiota during the first year of life: a prospective cohort study. *BJOG* **123**: 983–993

Arboleya S, Sánchez B, Solís G, Fernández N, Suárez M, Hernández-Barranco AM, Milani C, Margolles A, de Los Reyes-Gavilán CG, Ventura M *et al* (2016) Impact of prematurity and perinatal antibiotics on the developing intestinal microbiota: a functional inference study. *Int J Mol Sci* **17**: 649

Fallani M, Young D, Scott J, Norin E, Amarri S, Adam R, Aguilera M, Khanna S, Gil A, Edwards CA *et al* (2010) Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J Pediatr Gastroenterol Nutr* **51**: 77–84

Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE (2011) Succession of microbial consortia in the developing infant gut microbiome. *Proc Natl Acad Sci USA* **108**: 4578–4585

Cabrera-Rubio R, Collado MC, Laitinen K, Salminen S, Isolauri E, Mira A (2012) The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am J Clin Nutr* **96**: 544–551

Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, van den Brandt PA, Stobberingh EE (2006) Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* **118**: 511–521

Clausen TD, Bergholt T, Bouaziz O, Arpi M, Eriksson F, Rasmussen S, Keiding N, Løkkegaard EC (2016) Broad-spectrum antibiotic treatment and subsequent childhood type 1 diabetes: a nationwide Danish cohort study. *PLoS One* **11**: e0161654

Fallani M, Amarri S, Uusijarvi A, Adam R, Khanna S, Aguilera M, Gil A, Vieites JM, Norin E, Young D *et al* (2011) Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. *Microbiology* **157**: 1385–1392

Stewart C, Skeath T, Nelson A, Fernstad S, Marrs E, Perry J, Cummings S, Berrington J, Embleton N (2015) Preterm gut microbiota and metabolome following discharge from intensive care. *Sci Rep* **5**: 17141

vaginally delivered infants, there was an early enrichment of *Lactobacillus* followed by a bloom of *Bacteroides* from week 2, which was not observed in newborns that were not exposed to vaginal fluids”.

It is unclear, however, how robust these observations are, since only a single Cesarean-delivered infant was sampled at day 7 and day 14 and the referenced relative abundance plots do not appear to display *Bacteroides* [8, Fig S3 therein]. These observations may be further limited by the fact that the only exclusively breast milk-fed infants were the 3 who were delivered vaginally, and all Cesarean-delivered infants in the study received at least some formula. Nonetheless, this proof-of-principle study suggests one innovative potential method of partially restoring microbiota in neonates. It remains to be seen whether these findings, if are replicated in future studies with consistent longitudinal sampling over a longer time interval, will have a meaningful long-

term impact, or if efficacious interventions would need to be implemented long before the intrapartum interval and birth.

Pre- and postnatal risk factors

Regardless of the potential efficacy of such interventions, it remains unclear whether and how birth mode influences disease risk factors by modulating the microbiota, given that any initial differences in the infant microbiota between vaginal or Cesarean delivery become less profound or actually disappear over time. As noted by Bokulich *et al* [6], “we found that Cesarean- and vaginally delivered infants demonstrated similar degrees of microbiota maturation during the first 6 months of life. Subsequently, microbiota maturation stagnated in Cesarean-delivered infants, with relative maturation dropping compared to vaginally born infants for the remainder of the study period”. This same study also noted considerable

differences based on formula feeding and timing of the introduction of solid foods. Owing to the chaotic and stochastic development of the gut microbiota during the initial years of life, future studies need to implement large cohorts with robust longitudinal sampling and detailed consideration of potential prenatal, intrapartum at birth, and postnatal confounders.

Although parturition was traditionally assumed to be the first point at which neonates are exposed to bacteria, emerging evidence indicates that this may not be true. The presence of microbes and microbial communities within the intrauterine space—the placenta, amniotic fluid, and meconium—has been consistently documented in healthy term pregnancies, indicating that colonization of the fetal microbiome may occur in early gestation (Fig 2). However, how and from whence these microbiota arise is not yet fully understood. Nevertheless, these observations have tremendous implications for informing our understanding of the dynamics of the early microbiome in humans as it introduces the gestational period as a time during which the fetal microbiota may be initially established. As a result, exposures and events throughout pregnancy that may ultimately result in a Cesarean or follow thereafter—but not the surgery

itself—may be the true drivers of variation in the microbiome and risk of atopic disease, obesity, or metabolic disorders later in life.

Conflict of interest

The authors declare that they have no conflict of interest.

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