**Rediscovery of the Placental Microbiome**

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Arising from de Goff

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**Abstract/Summary Paragraph**

Paradigm shifts in science and medicine will, understandably, initially be met with resistance and reasonable skepticism. However, corroborating evidence acquired from replicative and orthogonal experiments result in adoption of new truths and key advancements in our knowledge and its application. Paramount to this process is an expectation that studies refuting paradigm-shifting science in support of the status quo will withstand equally rigorous scrutiny. In their study, de Goffau1 et al suggest that when they ‘*applied an experimental approach informed by the potential for false-positive results*’ they were unable to find evidence to support either (i) the existence of a placental microbiome, nor (ii) a significant relationship between placental infection with bacteria and the risk of preterm birth. In ascribing what they conclude to be five different patterns of contamination which would lead ourselves and many others to misclassify our prior findings reporting a low-biomass, low-abundance placental microbiome, de Goffau *et al* failed to (1) acknowledge key comparative differences and limitations in their experimental approach, and (2) recognize both their *a priori* and resultant errors and biases in their design, methodologies, computational analysis, and summative findings. Moreover, eventual attainment of the primary source data allowed us to clarify that Segata, in his accompanying opinion piece2, incidentally misrepresented not only the uniformity of use of negative controls by de Goffau1 *et al*, but the very notion that ‘*the study also sets a benchmark for investigations dealing with other human organs or tissues that, at most, carry a small number of bacteria, such as the lungs or blood*’. This thus calls into question Dr. Segata’s conclusion that, ‘*the results were clear: the placenta does not harbor microbes during healthy pregnancy, and contamination issues were a convincing explanation for the presence of any detected bacteria*.’ In contrast, by agnostically applying both established and novel computational pipelines to the de Goffau dataset, we rediscovered a placental microbiome significantly distinguishable from suspected contaminants.

**(Main text body)**

Advances in culture-dependent and culture-independent molecular methodologies reveal details of the human-microbe relationship and allow for robust identification of the membership and function of the human microbiome, including body-site niches with low-biomass, low-abundance communities. In their study1, de Goffau *et al* presume to have identified 5 different patterns of contamination from culture-independent metagenomics methods and concluded that there was no evidence of a placental microbiome. We offer the following concerns:

*Crucial mischaracterization and neglect of prior work in the field*. In setting the stage for their study and its conclusions {‘[several studies] *have concluded that the placenta is physiologically colonized by a diverse population of bacteria (the ‘placental microbiome’)* […] *This contrasts with the view in the pre-sequencing era that the placenta was normally sterile’*’}1, de Goffau and colleagues grossly miscite and neglect crucial aspects and objective findings of well over 50 studies pertaining to the presence of placental microbiota from both term and preterm deliveries. Acknowledgment and correction matters for two reasons. First, both our initial and the subsequent multitude of contemporary cultivation-independent studies explicitly and deliberately describe the placental microbiome as a sparse, low-biomass and low-abundance community of uncertain viability.3-10 Over a decade ago, Onderdonk and colleagues with the ELGAN study cultivated strains of overlapping generain their cohort of n=1083 placental parenchymal biopsies from both unlabored Cesarean and vaginal preterm (<28 week) deliveries, obtained under sterile conditions and analyzed in a double-blinded fashion.11,12 More recently, Collado cultured strains of *Enterococcus*, *Ralstonia, Staphyloccous* and enterobacteria from term placenta, amniotic fluid, and meconium.5 Second, ‘microbiome’ has an accepted definition (‘*all of the genetic material within a microbiota (the entire collection of microorganisms in a specific niche, such as the human gut). This can also be referred to as the metagenome of the microbiota.’*; <https://www.nature.com/subjects/microbiome>). While some might construe ‘microbiome’ to imply diversity, colonization, or abundance, the definition actually rests on pragmatic metagenomic characterization as part and parcel of current microbiome science. In fact, the unadulterated data of de Goffau *et al* documents an identifiable placental microbiome (distinct from contaminant controls) with rather remarkable taxonomic similarity to that reported by others via sequencing and/or cultivation approaches.3-12 As we detail further herein, once we received the primary metagenomics data from de Goffau *et al* under a required data use agreement, we were able to pinpoint key junctures where biased assumptions, methodologic failures, and data misclassifications led them astray.

*Potential for contamination at biopsy collection, with tissue “washing”, tissue processing, and prolonged sample storge and use.* Compared to ours and others work, there are three likely crucial methodologic perturbations in the study of de Goffau et al that led to their contamination with *D. geothermalis*, and *Rhodoccus*, *Sphingobium*, and *Methylobacterium*, taxa we have not previously detected in the placenta.3,4 First, their samples were collected over a decade earlier as part of their POP (Pregnancy Outcome Prediction) study and the integrity, sterility and total number of freeze/thaw cycles of their samples prior to the current study is undocumented.1 Second, their initial sample collection subarchitecture site and handling was highly atypical. Rather than uniformly sampling the parenchyma in a rapid and sterile manner3-12, after trimming away the decidua to collect only the ‘placental terminal villi’, the POP investigators vigorously rinsed the samples with PBS dissolved with ultrapure water.13 Third, they used a scant 25 mg of this washed terminal villous tissue (‘*combined weight obtained from fragments of all four biopsy collection points’*)1 for their metagenomic DNA extractions. All of these methodologies are in contrast to ours and others work in the field.

*Sequencing failure rather than sequencing contamination.* De Goffau and colleagues suggest that one source of contamination in low biomass samples is sequencing. However, that presumes that there is equivalent depth of sequencing and soundness of library preparation. As shown in Figure 1, that is not the case.

*Absence and underuse of “contaminant controls”*. Contrary to their study depiction diagram (Extended Data Fig. 1),

*Introduction of methodologic bias resulting from mapping to customized reference databases (WGS) and use of degenerate primers with oligotyping (16S).* Taxonomic overlap is not contamination.

*Rather, unbiased re-analysis with both validated and novel tools leads to a rediscovery of a low-biomass, low-abundance metagenomics (aka, a placental microbiome)*. We remain agnostic as to whether this community is viable or the placenta is “physiologically colonized”. Metagenomics cannot reveal those answers, either in support nor in denial.

*Unbiased analysis reveals no significant difference among Cesarean and vaginally delivered placental samples, but distinct and significant differences in term and preterm birth samples.*  De Goffau and colleagues built in several biased assumptions pertaining to vaginal contamination, which were not supported by their experimental evidence.

Although parturition was traditionally assumed the initial contact point during which neonates are exposed to their mother’s microbes, emerging evidence indicates that this is unlikely to be true. The presence of microbes and low biomass microbial communities within the intrauterine space – the uterine decidua, the placenta, and the amnion and chorionic membranes, and amniotic fluid– has now been consistently documented in a growing multitude of mammalian species [10–50]. Distinct microbial communities in the meconium among preterm and healthy-term neonates are detected and described within minutes to hours of birth, and these impressively expand in the first days to weeks of life to readily show discrete body niche communities long before that same infant will alter its diet or engage in meaningful contact with the outside world [51–54]. Moreover, although the notion of an “unsterile womb” has been challenged by a few [55–65], there are several rather consistent observations that collectively support a functional role for these sparse microbial communities [10–54]. First, it appears that the source and sink of these early developmental communities (i.e., breastmilk, placenta, amniotic fluid, meconium, newborn oral, and skin communities) are generally of low biomass and low abundance. We speculate that the presumptive active “pruning” efforts by the immune cells at the maternal-fetal interface are of paramount importance to both the health of the pregnancy and the offspring. Second, being truly “germ free” (or at least germ depleted) carries imminent risk of disease. Namely, gnotobiotic mice still carry pregnancies to term and can propagate relatively deplete or replete lineages, but are impressively prone to postnatal inflammation and sepsis from intestinal pathogens [66,67]. Thus, there appears to be a role for these microbes (or at least their antigens and DNA) in generating immune tolerance in the offspring, although the mechanisms remain poorly understood. Third, within a short span of time and devoid of extensive exposures that might seed horizontal transfer, the microbiome will remarkably expand and speciate by body niche [115]. The importance of who (versus what function their metagenome encodes for) is first present and may later mediate colonization permissiveness (or resistance) remains to be determined. Nonetheless, the consistency of these collective observations suggest that the underlying molecular events and processes that tailor the early microbiome very likely play some role in later health or disease risk, albeit imperfectly understood at present.

In this review, our goal is to provide an objective presentation of the evidence to date supporting the relationship between maternal exposures, her microbiome, and her offspring’s microbiome and metagenome. We will explore what has been learned about the impact of maternal exposures on the development of the offspring microbiome from Cesarean delivery and gnotobiotic animal studies. Recognizing the commonality (and controversy) of low biomass communities being present and of functional importance in early development, we will review the literature and strength of evidence behind a remarkably consistent set of observations. As an example, we will discuss the placental microbiome and the role it may play in contributing to the pregnancy, the offspring’s microbiome, and childhood health and disease.

***Pregnancy exposures are associated with alterations of the offspring microbiome, which precede later onset of disease in childhood and adult life.***

The combination of “nature” and “nurture” has been used over the years to explain the variability in human health, including both the range of susceptibility and severity of clinical disease from one individual to the next, or among different populations. For example, following World War II, undernourished women were observed to have increased risk of miscarriage, birth malformations, and low birth weight infants [68,69]. Kermack *et al.* showed that early life exposures lead to specific and predictable effects throughout the life of offspring [70]. More formal evidence continued to accrue following a series of sentinel observations by Barker and his colleagues, which collectively supported the theory that maternal pre-conception and pregnancy exposures can result in long-term health impacts on her offspring [71–77]. Now formally termed the Developmental Origins of Health and Disease (DOHaD) hypothesis, we appreciate today that fetal exposures persistently (and often permanently) alter human physiology and behavior in ways that last well into adult life [78]. We and others have described the effect of common pre-pregnancy and pregnancy exposures[[1]](#footnote-1) on the development of obesity, modulation of immunity, and dysfunction of metabolism in offspring via long-lasting perturbations at the level of the placenta, liver, pancreas, skeletal muscle, thyroid, and digestive tract [18,23,79–126]. The mechanisms driving these long-term effects are partially explained by the developing epigenetic code [79,81–98,100,102,103,105–112,116,117,119,122–126] and microbiome [18,23,104,113–115,120,121,127–129].

Correlative studies support the observation that pregnancy exposures can lead to alterations in the maternal and offspring microbiomes. On the maternal side, we have learned that exposure to potentially toxic environmental chemicals like polycyclic aromatic hydrocarbons (PAHs) can modulate the microbiome community membership and their function, but also that the microbiome contributes to the PAH transformation and metabolism [130]. These noxious organic substances are byproducts of carbon combustion (*i.e.,* vehicle emissions, petroleum processing) and cigarette smoking, and they are associated with contamination of soil/aquatic life and birth complications.[[2]](#footnote-2) Ingested PAHs are capable of disrupting gut microbial enzymes, leading to a state of induced dysbiosis within the gut community. But the relationship is not one-sided. Human commensal microbes can metabolize primary PAH products (that are inhaled or ingested) and secondary PAH substances (that are conjugated by the liver) [130]. For example, benzo[a]pyrene (BaP) is a PAH compound that cannot activate human estrogen receptor; however, the human gut microbiome transforms BaP into an estrogenic compound that can activate human estrogen receptor [131]. It is likely that extraneous estrogen receptor activation during pregnancy is dangerous to the short-term and long-term health of the offspring, and this is a subject of ongoing research for our laboratory and others.

On the offspring side, we have learned that maternal diet during pregnancy can alter the offspring microbiome and its encoded functions, even beyond weaning. In non-human primate juveniles fed a control diet for at least 6 months, the gut microbiome of offspring exposed to maternal high fat diet (mHFD) consumption during pregnancy and lactation is persistently altered compared to their peers who were not exposed to mHFD [6, 120, 121]. The difference seen in this mHFD-exposed gut microbiome occurs independent of juvenile obesity, is refractory to probiotic/prebiotic therapy, and is observed at least 6 months and up to 2-plus years after the cessation of mHFD consumption [6,120, 121].

Additionally, the maternal microbiome during pregnancy can impact development of the offspring immune system [8,45,99,132-134]. In mice, transient colonization of dams results in increased innate immune development in postnatal pups, enabling improved inflammatory response and intestinal symbiosis compared to their germ-free counterparts [132]. Another group administered antibiotics to pregnant and breastfeeding dams, resulting in increased adaptive immune cell counts in postnatal pups [133]. The maternal microbiome clearly impacts the offspring’s health in early life, but the infant microbiome can also shape later child development. Based on data from the Canadian Healthy Infant Longitudinal Development (CHILD) study, certain gut microbes present in the infant microbiome within the first 100 days of life are able to confer protection against development of asthma by 1-3 years old [134]. Collectively, these studies indicate that maternal exposures, including the maternal microbiome, can impact offspring health and development.

Collectively, these studies and their data show that pre-conception and pregnancy exposures can lead to both short-term and long-term alteration of the offspring’s gut microbes, and that some of these alterations are refractory to post-natal or post-weaning correction. When considered thoughtfully and with an open mind, we have been inspired to conclude that nurturing instructions for “how to form a symbiotic relationship with microbes” (*e.g.,* tolerance to commensal microbes) in placental mammals begins *in utero*, and precedes exposure to vaginal microbes during birth. Whether or not that process involves true microbial colonization of the fetus with more than a few sparse microbes remains to be determined [45]. However, we and others have been inspired to consider that possibility based (at least in part) by striking data arising from observations among offspring delivered via Cesarean and phenotypic analysis of gnotobiotic animal models.

***Cesarean delivery provides insight into the maternal-offspring microbiome relationship prior to birth.***

Although large cross-sectional and longitudinal studies fail to observe robust long-term negative outcomes in offspring born by Cesarean delivery [135,136], some groups report a moderate potential increased risk of childhood obesity and atopy/asthma, and some groups report potential increased risk of Celiac disease and diabetes in Cesarean-born offspring [137–143]; we have previously reviewed this data and provided commentary [144]. As awareness for the importance of the microbiome and its functional metagenome continues to grow, so has concern that failure to expose the emerging neonate to the “proper” community of microbes which presumptively prompt later onset adverse clinical outcomes which are more common among those born via Cesarean.

To investigate this claim, several years ago Dominguez-Bello and colleagues were the first to study the neonatal microbiome across body sites immediately following delivery in a small case-control study [145]. Across all body sites, vaginally-born neonates harbor mostly maternal vaginal microbes, such as *Lactobacillus*, *Prevotella*, or *Sneathia* spp., whereas cesarean-born neonates harbor mostly maternal skin microbes, such as *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. [145]. In our own study of this issue, variations in microbial community structure are observed immediately following delivery[[3]](#footnote-3) but not by 4 to 6 weeks of age [115]. By then, the infant microbiome has not only expanded and diversified by body site (i.e. stool microbiome is different from skin microbiome), but there is no discernable difference in microbiome structure or function between vaginally and Cesarean born infants after considering co-morbid or co-linear factors [e.g., "the company that Cesarean delivery often keeps", 115,144]. Furthermore, there is no observed difference between gut microbiome structure by 5 weeks old in exclusively preterm infants born by vaginal or Cesarean delivery [146]. These data have inspired us and others [115,144-147] to conclude that vaginal versus cesarean delivery does not in-and-of itself meaningful impair the long-term structure or function of the microbiome.

However, other groups continue to identify differences in the Cesarean-born gut microbiome beyond the neonatal period [148–149]. In order to consolidate these discordant conclusions, the causal versus correlative effect of delivery mode must carefully examined for confounders and company of Cesarean delivery [144]. Such variables include the underlying medical or obstetrical indication or pathology leading to the surgery, antibiotic exposure surrounding delivery, environmental exposure to the neonatal intensive care unit, human milk versus formula feeding, other maternal comorbidities, and yet-unidentified factors. For example, although delivery mode does not determine the proportion of microbes that can be traced to the mother’s microbiome, others have suggested that Cesarean delivery is associated with altered bacteria-bacteriophage interactions [150]. Bacteriophage (the viruses that infect bacteria) represent a largely-unexplored component of the microbiome that may play a role in early development of the infant microbiome. As we recently commented [151], identifying intrauterine transmission of bacteriophage long-prior to delivery is an exciting new avenue of research. Perhaps bacteriophage represent one of multiple unexplored variables with the potential for intrauterine transmission that may play a role in affecting the gut microbiome of offspring which are incidentally born via Cesarean. The maternal dietary and medical conditions which may modulate bacteriophage transmission remain as yet unexplored [151].

In summary, we can glean several important lessons from observations pertaining to studies attempting to discern causation from correlation with regards to delivery via Cesarean:

1. Although the microbiome immediately following delivery is different in vaginal versus cesarean-born neonates, multiple groups report the loss of this difference after 5-6 weeks [115,146, 147, 150].
2. Analysis of long-term effects of delivery mode on the microbiome absolutely requires careful incorporation of the clinical confounders which accompany Cesarean delivery, which in turn necessitates large, prospective studies naïve to eventual delivery mode.
3. In the absence clear, unequivocal or robust data demonstrating that birth by Cesarean in-and-of-itself is an independent risk of later-in-life disease, there is risk to continuing a narrative using low-level evidence that it does. Cesarean delivery is one of the most common and safest abdominal surgeries performed, and ready availability to medically indicated Cesarean with surgically competent providers is absolutely crucial in the reduction of maternal and neonatal mortality and decreasing social disparities worldwide [https://www.who.int/reproductivehealth/publications/maternal\_perinatal\_health/cs-statement/en/].
4. We must consider the implications that arise from the conclusion that Cesarean delivery itself is not associated with long-term differences in the microbiome. One such implication is this: if the moment of delivery does not direct the body’s future interactions with microbes, then when does the developing host receive these instructions? What can the DOHaD hypothesis contribute to this issue?

To further contemplate this latter question, we turn to observations arising from functional studies in gnotobiotic animal models.

***Gnotobiotic animal models provide insight into the maternal-offspring microbiome relationship pre and post-birth.***

One of the most powerful tools available to study host-microbial interactions is the development of germ-free (GF) animal models. Originally conceived of by Louis Pasteur in 1885 and first cultivated in guinea pigs [151] then rats [152] then mice [153], gnotobiotic animals are created and reared in environments “free” of (detectable) live bacteria. Their food, water, bedding, and any supplies are autoclaved or filter-sterilized, and sterility is continuously monitored by culture and molecular methods. While GF animals are viable and reproduce despite the lack of live microbes, they are by no means normal. Compared to conventionally-colonized animals, GF animals display morphological differences, biochemical abnormalities, atypical neurobehavior, and pronounced immunological changes.[[4]](#footnote-4) Based on these observations, we can propose that mammals have intimately coevolved with microbes and now require microbes (and the diverse functions that their metagenomes encode) for normal development.

To demonstrate the necessary role of microbes in development, GF animals are introduced to microbes through conventionalization. Many studies support their proof-of-principle that microbes impact development by comparing GF controls to the conventionalized animals [154,155]. However, a few groups have compared the conventionalized animals to conventional control animals (that have always been raised with microbes), with interesting results. For example, conventionalized mice display decreased transcription of UDP-glucuronosyltransferases 1a9 and 2a3 compared to conventional mice fed a probiotic [156]. In terms of development of immune competence, one group reports that conventionalized rats harbor altered levels of intestinal intraepithelial lymphocytes compared to both their GF and conventional control counterparts [157]. They also observe that the frequency of T cells co-expressing α4β7 in the intestinal lamina propria of conventionalized mice never normalizes to the base levels of conventional controls [158]. Another group demonstrates that conventionalized mice produce decreased IFNγ, phagocytotic ability, and reactive oxygen species production in response to fungal infection compared to GF and/or conventional controls [159]. Clearly, conventionalization does not restore all aspects of atypical development caused by a GF upbringing, particularly with respect to the immune system. Several groups identify the importance of “critical developmental windows,” where reconstitution of the microbiome after said window fails to restore normal development, even partially. [7,8]

Although the living environments of GF animals are free of live bacteria and yeast, they still contain detectable dead/killed microbes and microbial particles [160–162]. It is possible that these nonviable microbes and microbial components can serve as pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) for immune education. As such, even without the presence of living microbes to stimulate normal immune education, it may be possible that dead microbes and microbial particles partially contribute to the developing immune system of GF animals.

To summarize, observations in GF animals lead to three take-away messages about microbes and development.

1. GF littered animals are viable without exposure to live microbes, but they display abnormal development.
2. Even early-on conventionalization of GF animals does not fully correct this abnormal development.
3. Killed microbes and microbial components present in the living environments of GF animals can potentially contribute to immune education or other aspects of normal development.

When combined with the lessons learned from Cesarean delivery, we must consider the possibility that mammalian education for “how to form a symbiotic relationship with microbes” begins *in utero*, and these initial stages may not require live microbes nor a diverse or substantial abundance of metagenomics conent. To explore this theory, we will present the historical evidence of the placental microbiome, and how it may contribute to the development of the offspring microbiome and long-term health.

***A low-abundance, low-biomass intrauterine microbiome exists.***

Traditionally, it was largely assumed that the healthy intrauterine environment during pregnancy (including the uterine decidua, placenta, chorion/amnion, amniotic fluid, fetus, and meconium) was absolutely sterile until birth. Only during delivery was the neonate believed to first interact with microbes. This dogma was further supported by the negative clinical outcomes which may be associated with prolonged and untreated/undelivered cases of chorioamnionitis (the bacterial infection of the intraamniotic space that leads to inflammation of fetal membranes and is associated with serious morbidity and potential mortality of both the pregnant woman and her fetus or neonate). It has been argued that if bacteria were supposed to be present in the intrauterine environment, then why does chorioamnionitis occur? [[5]](#footnote-5)

The assumption of a sterile intrauterine environment remained largely unacknowledged, but was not unchallenged. Evidence accumulated supporting the existence of a sparse but consistently-present microbiome in the intrauterine environment of mammals during pregnancy [10–50] (see **Table 1**) and in the upper female reproductive tract (endometrium, fallopian tubes) both prior to and following successful and healthy pregnancies [152–157]. Although we presented the first large-scale metagenomic report of the presence of a human placental microbiome that is low-abundance, low-biomass, and metabolically-varied [18], we were inspired by the work of others in our interpretations both then and hence.

Briefly, in our initial work we utilized pyrosequencing of the 16S ribosomal DNA (rDNA) V1-3 hypervariable regions (HVRs) in 320 placentas, alongside comparative whole-genome shotgun (WGS) sequencing in a subset of 48. We used a nested cohort design, where we matched term to preterm, and women with and without a history of antenatal infection. We observed differences in the low biomass community membership and its metagenomics function by virtue of preterm versus term birth, and by history of antenatal infection (even as remote as the first trimester of pregnancy). We further ran comparative dissimilarity measures against the non-pregnant HMP datasets, which enabled us to show that the nearest “neighbor” microbiome composition was mainly nonpathogenic commensals, including *Proteobacteria* (such as *Escherichia coli* and *Neisseria spp.*) and common human oral microbes (such as *Fusobacterium*, *Streptococcus,* and *Prevotella spp.*) [18]. We failed to observe a difference based on Cesarean or vaginal birth. Based on these taxonomic and functional distinctions by disease (preterm birth and remote history of antenatal infection) classification and relative lack of overlap with either the HMP vaginal dataset or skin dataset, we concluded that the low biomass, low abundant metagenomic signal we were detecting was unlikely to represent contamination. However, computational tools designed to sort out potential contaminants in such low biomass environments had not yet been developed and thus were not deployed in our initial studies [18,23]. To both spatially localize any microbes in the placental microarchitecture and better distinguish the low biomass placental microbiome from contaminant controls with orthogonal methodologies, we thereafter performed signal-amplified 16S universal *in situ* hybridization (ISH) for bacterial rRNA with comparative 16S rDNA sequencing of the V4 HVR on the Illumina MiSeq sequencer employing computational “decontam” tools [34]. Through these endeavors and with ongoing consideration of others work, we stand-by our initial observations documenting a low abundance, low diversity, sparse, and low biomass collection of bacterial RNA (e.g., microbiome) in the placental villi and chorion that lends further credibility to the observations by our team and many others.

Consistent and creative experimental and observational evidence continues to emerge in support of the placental and other low biomass microbiomes in the intrauterine niche, yet (generally appropriate) skepticism remains. A limited number of groups have failed to identify or interpret their data as evidence of a placental microbiome [55–65,163], pointing to five main challenges in the field. We acknowledge these challenges, and appreciate the thoughtful criticisms that other investigators have instilled into the field which continues to inspire further hypothesis testing by our group and others.

First, environmental microbes and contamination can potentially be construed as or obscure the signal ascribed to the placental microbiome [55]. To control for this difficulty, strict sterile procedures are required, along with inclusion of environmental swabs from every possible source of contamination, including maternal body sites, surgical surfaces, DNA extraction equipment, and the sequencing site. When this precaution is taken, the microbiome of the placenta, amniotic fluid, and membranes is distinguishable from that of the robustly-collected environmental and contamination controls by 16S rDNA or WGS sequencing techniques [38]. However, it is important to note that the presence of the same microbe in both the intrauterine samples and the environment/contamination controls, does not automatically indicate that the microbe is a contaminant. DNA signatures of *Lactobacillus spp.* commonly found in the vagina (a potential source of contamination) are also found in endometrial biopsies [164], suggesting that *Lactobacillus spp.* DNA may be present from the beginning of placental development, rather than terminally introduced during labor and delivery. In the absence of concomitantly collected samples from the same subject with deep sequencing to species and strain level, definitive interpretations of overlapping genera as indicative of contamination should exercise caution.

Second, DNA extraction kit reagents contain a collection of low-abundance microbes that yield a “kit-ome”, which complicates detection of the placental-specific microbial signature [163]. To distinguish the low-abundance, low-biomass communities of the intrauterine environment, it is essential to include extraction control samples using just the kit reagents and no input sample. Kit negative samples are still distinguishable from microbiome of the intrauterine space in the hands of us and others (**Table 1**).

Third, different sequencing approaches (amount and location of starting material, primer choice for amplicons, sequencing platform, depth of sequencing, computational pipelines, mapping databases, etc) can lead to different interpretations of relatively similar findings [165]. The selection of primers for 16S rDNA sequencing is particularly important. Some primers, such as those used by Walker *et al.* to cover the V1-2 HVRs, are designed as degenerate and yield short amplicons [165]. In contrast, other primers, such as those we used to span the V1-3 HVRs, are nondegenerate and yield longer amplicons [notably on longer read platforms, such as the now defunction Roche 454; 18]. When analyzing these amplicon reads downstream, caution is warranted in making species-level calls from short amplicon data, due to significant similarity between members of the same genus within the 16S rDNA gene. This step represents an avenue for potential bias to enter the analysis, by accidentally calling one species over another when the read depth is insufficient to distinguish between two or more species. The requisite sequencing depth may be difficult to achieve (i.e., 2x coverage of a 2Mb microbial genome at 1-10% relative abundance requires 20-150 Gb of sequencing depth in a low biomass specimen).

Fourth, disagreement in the working definitions of “microbiota” and “microbiome” lend confusion to the field. Whereas “microbiota” refers to the community of microbes in an ecosystem, “microbiome” refers to the collective genomic material of the microbes in an ecosystem [2]. In other words, “microbiome” does not necessitate a community of live, colonizing microbes, such as is generally implied when we consider the “gut microbiome.” To date, we have detected the DNA signature of microbial constituents and visualized whole microbes within the placenta [18,23,25,34], but we have not cultured viable, colonizing microbes from the placenta. However, we recognize that cultivation of such organisms from the placenta has been documented for well-over a decade and by others hence [20,24,30,32,39,40,45,49]. As we have previously stated [18,23,25,34], we remain agnostic as to whether or not the placental microbiome at term contains live, viable microbes in appreciable abundance. We and others continue to explore the notion that progressive pruning of the sparse intrauterine metagenomic community may be important to maintenance of pregnancy, immune development, or resistance to pathogenic organisms. Perhaps one of the more important questions of our time is how sparse communities remain sparse, and do not increase in biomass. Ensuing from this observation is several others, such as why should the functional capacity of a low-abundance, low-biomass microbiome like the placenta matter? Can low numbers of microbes actually impact human physiology and development? Although more evidence from carefully-designed and well-powered studies is needed to clarify these questions, let us contemplate the hypothesis that the placental microbiome exists and contributes to the relationship between the offspring and its developing microbiome.

***Speculating as to whether a low biomass intrauterine microbiome may play a functional role during early development.***

Given that the membership of the placental and other characterized intrauterine niche microbiomes are largely human commensal microbes, these low abundant microbes may be a critical first step in ontogeny of the offspring immune system [39]. In this way, these microbial components could conceivably shape immune tolerance and pathogen colonization resistance to prepare the soon-to-be neonate for more fulminant colonization by commensal microbiota in post-natal life. Little is currently understood about microbial interactions at the maternal-fetal interface. It is known that macrophages and natural killer cells are present, but it is possible that the constituent placental trophoblast cells also play a role. Trophoblasts can recognize and respond to PAMPs/MAMPs and promote regulatory cytokine secretion; in this way, trophoblasts may contribute to tolerogenic education [169]. It is interesting to note that the 16S rRNA signal we detected by ISH was largely localized to the synctiotrophoblast [34]. Perhaps some basal level of microbial exposure is necessary *in utero* to prevent a massive immune reaction to the microbial assault that accompanies entrance to the *ex utero* realm.

We recognize that we are not alone in considering the evidence supporting a paradigm shift in our thinking regarding the relative sterility of the intrauterine environment. We remain inspired by many other forward-thinking scientists work in this regard, and take to heart the words of others to exercise great caution in over-interpreting data without strict functional correlation and limited orthogonal methods. Nevertheless, we the data arising from a multitude of mammalian species can be schematically depicted as shown in **Figure 1**. With inception of the microbiome’s development beginning *in utero*, we can better shed light on data showing that Cesarean delivery *per se* does not affect the trajectory of the infant microbiome but some conditions (which bear a higher risk of Cesarean) render greater risk of later onset obesity or atopic disease. If development has already begun, then colonization and maturation will continue down the path they were already programmed to prior to birth. For example, disruptions in this programmed development (and the intrauterine low-biomass community or its pruning) *in utero* may concomitantly contribute to women who experience preterm birth associated with excessive gestational weight gain [23,170]. As a second example and as noted previously, while we remain agnostic as to whether or not living bacteria are present in the placenta and/or true fetal colonization occurs, the data support the existence of bacterial morphologies and bacterial components (i.e. DNA) in the placental. Thus, in germ-free animals, the absence of live microbes renders the animals with profound immune abnormalities, yet they continue to be viable. Perhaps this viability is partially owing to the existence of residual dead microbes and microbial particles, which may participate in ontogeny of the immune system, as has been so eloquently demonstrated by other investigators [3,8].

In conclusion, there is a critical need to fund high-quality research to clarify the role of low-abundance, low-biomass communities (like the placental microbiome) given the evidence in support of its potential role in modulating development. Furthermore, understanding why these and other low biomass communities remain sparse, and what the potential role of host immunity is in that process, is both intriguing and likely impactful. Given that pregnancy exposures are known to have long-lasting, permanent influences on offspring health and predisposition to disease, the answers to these questions are vital to meaningful advances in scientific knowledge and public health.

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The authors declare that they have no competing interests.

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***Authors' contributions***

EB and KA equally contributed to the literature review and manuscript writing. Both authors read and approved the final manuscript.



**Figure 1. Potential roles of maternal exposures and the intrauterine microbiome working side-by-side to influence offspring development.** Many maternal exposures during pregnancy, including but not limited to diet, environmental chemical exposures, maternal antimicrobial consumption, endocrine disruption, and maternal co-morbidites, can impact the health of the pregnancy as well as the development and long-term outcomes of the offspring. The intrauterine microbiome may also play a role in these processes, and serve as a molecular mediator in maternal-fetal communication during gestation. The consistently observed sparse, low biomass and low abundance intrauterine microbiome has been detected at multiple sites, such as the uterine decidua, placenta, chorion/amnion, amniotic fluid, and meconium. Although the role of this microbiome in development of the fetus remains unknown, it is possible that the microbiome contributes to fetal immune education, immune system ontogeny, and colonization resistance of the neonate.

| **Table 1**: Historical evidence of the female intrauterine microbiome in pregnancy | | | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Year** | **Endometrium or Decidua** | **Placenta Parenchyma** | **Chorion** | **Amnion** | **Amniotic Fluid** | **Meconium or**  **Fetal Intestine** | **Culture-independent Detection** | | **Culture-dependent Detection a** | | **Ref.** |
| **Method** | **Conclusion** | **Method** | **Conclusion** |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **1927** |  |  |  |  |  | ✓ | n/a | n/a | Aerobic | 38% of meconium samples are culture-positive | [10] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **1934** |  |  |  |  |  | ✓ | Histology (Löffler’s methylene blue and Gram stains) | 6% of meconium samples contained recognized bacteria | Aerobic: EMB lactose, Blood, Dextrose broth, Deep iron brain | 38% of meconium samples are culture-positive | [11] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **1936** |  |  |  |  |  | ✓ | Histology (Cover slip and Gram stain) | No bacteria detected. | Aerobic: EMB, Blood, Brain or glucose broth. Anerobic: Blood slants | 10% of meconium samples collected within 30 minutes after delivery are culture-positive | [22] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **1982** |  |  | ✓ |  |  |  | n/a | n/a | Aerobic: Broth and blood agar | Aerobic bacteria have been cultured from the chorion in the absence of histologic chorioamnionitis | [33] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **1997** |  |  |  |  | ✓ |  | 16S rDNA polymerase chain reaction (PCR) of the V6-8 HVRs | 16S rDNA is detected in 94% of culture-positive and 36% of culture-negative amniotic fluid from cases of preterm labor with intact membranes | Traditional clinical culture | 20% of amniotic fluid samples are culture-positive and 80% are culture-negative | [44] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2004** |  |  |  |  | ✓ |  | 16S rDNA sequencing of the V6-8 HVRs using Sequenase or Cycle Sequencing | 16S rDNA identified in culture-negative amniotic fluid is mapped to *Leptotrichia sanguinegens, Fusobacterium nucleatum, Ureaplasma urealyticum* | Traditional clinical culture | 20% of amniotic fluid samples are culture-positive and 80% are culture-negative | [46] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2007** |  |  |  |  | ✓ |  | *Porphyromonas gingivalis* -specific PCR and 16S rDNA PCR of V5-8 HVRs | *P. gingivalis* DNA is detected in 30% of amniotic fluid from cases of threatened preterm labor | Anaerobic: non-selective Columbia blood | All amniotic fluid samples are culture-negative for *P. gingivalis* | [47] |
| **2007** |  | ✓ | ✓ | ✓ | ✓ |  | Gram stain of amniotic fluid samples | 10% of culture-negative amniotic fluid samples yield a positive Gram stain | Traditional clinical culture | Diverse microbes are recovered from term placentas without histological or clinical evidence of inflammation | [48] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2008** |  | ✓ | ✓ |  |  |  | 16S rDNA PCR of the V3-5 HVRs, histology (Gram stain), and gas-liquid chromatography of glucose fermentation products | There is a PCR inhibitor in the placenta. At higher concentrations of placental tissue, 16S rDNA PCR products are no longer detected from known culture-positive placental samples | Aerobic: Chocolate, TSA blood. Anaerobic: A-7, Brucella blood. | 51% of placental samples from Cesarean-delivered and 75% from vaginally delivered preterm births are culture-positive | [49] |
| **2008** |  |  |  |  | ✓ |  | 16S rDNA end-point PCR (V1-V4 and V3-5 HVRs) and 16S rDNA real-time PCR (V1-2 and V3-5 HVRs). Plus 18S fungal targets. | Microbial rDNA is detected from culture-negative amniotic fluid of preterm labor with intact membranes | Aerobic and anaerobic | 9.6% of amniotic fluid samples are culture-positive | [50] |
| **2008** |  |  |  |  |  | ✓ | PCR of genetically labeled *Enterococcus fecium* HA1 (that was orally inoculated into pregnant mice) | Meconium from term murine pups contains the genetic label of the *E. fecium* fed to their mothers during pregnancy | Aerobic: BHI, VRB, CNA. Anaerobic: Wilkins-Chalgren, MRS | *Enterococcus* and *Staphylococcus* are cultured from meconium | [12] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2009** |  | ✓ |  |  |  |  | *Bifidobacterium* and *Lactobacillus* -specific PCR | *Bifidobacterium* and *Lactobacillus spp.* DNA is detected in 91-97% of placental tissue | Anaerobic: Blood liver, LB | All samples are culture-negative for *Bifidobacterium spp.* and *Lactobacillus spp.* | [13] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2010** |  | ✓ |  |  |  |  | 16S rDNA PCR (primers A17F and 1512R) | After human oral bacteria are injected into murine tail vein, the 16S rDNA is detected in the murine placenta | n/a | n/a | [14] |
| **2010** |  |  |  |  | ✓ |  | 16S rDNA end-point PCR (V1-V4 and V3-5 HVRs) and 16S rDNA real-time PCR (V1-2 and V3-5 HVRs). Plus 18S fungal targets. | 16S rDNA is detected from culture-negative amniotic fluid in preterm pre-labor rupture of membranes | Aerobic and anaerobic | 34% of amniotic fluid samples were culture-positive | [15] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2013** | ✓ |  |  |  |  |  | Histology (H&E, Gram, Hema 3 modified Geimsa, Brown-Hopps modified Gram stains) | Intracellular bacteria are detected by histology in the non-inflamed maternal basal plate of the placenta | n/a | n/a | [16] |
| **2013** |  |  |  |  |  | ✓ | 16S rDNA PCR of the V6-8 HVRs analyzed by Denaturing Gradient Gel Electrophoresis and  16S rDNA Human Intestinal Tract Chip analysis of the V1-6 HVRs | Meconium microbiome is characterized by abundant Firmicutes and Proteobacteria, and is lower diversity than 3-week old infant stool | Aerobic: MRS, MacConkey, BP, SDC, BHI, CNA. Anaerobic: WC, MRS with L-cysteine | 78% of meconium samples are culture-positive | [17] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2014** |  | ✓ |  |  |  |  | 16S rDNA pyrosequencing of the V1-3 HVRs with comparative WGS metagenomic sequencing | Distinct placental microbiome composed of nonpathogenic commensals, most similar to the human oral microbiome. | n/a | n/a | [18] |
| **2014** |  |  | ✓ | ✓ |  |  | 16S rDNA pyrosequencing of the V1-2 and V5-6 HVRs | Common placental genera regardless of delivery mode. | n/a | n/a | [19] |
| **2014** | ✓ |  |  |  |  |  | Histology (Brown-Hopps modified Gram stain) | Bacteria are detected within extravillous trophoblasts of the placental basal plate | Inoculation of the placental basal plate *ex vivo* | Inoculated bacteria are detected within extravillous trophoblasts of the placental basal plate | [20] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2015** |  | ✓ |  |  |  |  | 16S rDNA sequencing of the V3-4 HVRs using Illumina MiSeq | Lower diversity and lower *Lactobacillus* percentage in placenta associated with low birth weight versus full term | n/a | n/a | [21] |
| **2015** |  | ✓ |  |  |  |  | 16S rDNA sequencing of the V3-4 HVRs using Illumina MiSeq | Lower diversity and lower *Lactobacillus* percentage in placenta associated with low birth weight versus full term | n/a | n/a | [21] |
| **2015** |  | ✓ |  |  |  |  | 16S rDNA pyrosequencing of the V1-3 HVRs with comparative WGS metagenomic sequencing | Distinct placental microbiome between women with and without excess gestational weight gain | n/a | n/a | [23] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2016** |  | ✓ |  |  | ✓ | ✓ | 16S rDNA pyrosequencing of the V1-3 HVRs with comparative quantitative PCR and denaturing gradient gel electrophoresis | Placental and amniotic fluid microbiomes both characterized by low richness, low diversity, and high Proteobacteria, which suggests microbial transfer at the fetal-maternal interface. | Anaerobic: Gifu, LB | 50% of taxa identified by culture-independent methods were recovered through culture | [24] |
| **2016** |  |  | ✓ |  |  |  | WGS metagenomic sequencing | Distinct placental microbiome in spontaneous preterm birth that further differentiates by severity of chorioamnionitis | Traditional clinical culture for *Ureaplasma* and *Mycoplasma* | Some chorioamnionitis-positive patients were negative for *Ureaplasma.* Some chorioamnionitis-negative patients were positive for *Ureaplasma.* | [25] |
| **2016** |  | ✓ |  |  |  |  | 16S rDNA sequencing of the V3-4 HVRs using Illumina MiSeq and TaqMan gene expression using RT-PCR | Distinct placental microbiome associated with gestational diabetes | n/a | n/a | [26] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2017** |  | ✓ | ✓ |  |  |  | 16S rDNA sequencing of the V6-8 HVRs using Illumina MiSeq | Fetal side of placenta harbors microbiome resembling the pregnant woman’s oral (not fecal) microbiome | n/a | n/a | [27] |
| **2017** |  | ✓ | ✓ | ✓ |  |  | 16S rDNA sequencing of the V5-7 HVRs using Illumina MiSeq with comparative qPCR | Only specific bacteria found in placental tissue is associated with chorioamnionitis and low-birth-weight neonates | n/a | n/a | [28] |
| **2017** |  | ✓ |  |  |  |  | 16S rDNA sequencing of the V3-4 HVRs using Illumina MiSeq | Distinct placental microbiome associated with fetal macrosomia | n/a | n/a | [29] |
| **2017** | ✓ | ✓ | ✓ | ✓ |  |  | 16S rDNA sequencing of all V1-9 HVRs using Illumina MiSeq with comparative qPCR (V4 region and *Ralstonia insidiosa*) | Composition of the placental microbiome differs between maternal, fetal-maternal, and fetal spaces, independent of delivery mode | n/a | n/a | [30] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2018** |  | ✓ |  |  | ✓ |  | 16S rDNA pyrosequencing of the V4 HVR | Amniotic fluid and placenta contain low diversity microbiomes dominated by Enterobacteriaceae phylotype | Anaerobic: BHI, Columbia blood | 0% of amniotic fluid samples and 20% of placenta samples are culture-positive | [31] |
| **2018** | ✓ | ✓ |  |  |  |  | 16S rDNA sequencing of the V3-4 HVR using Illumina MiSeq with comparative qPCR | 15S rDNA is detected in 49% of placental samples, with equal richness and diversity between HPV-positive and negative groups | n/a | n/a | [32] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2019** | ✓ | ✓ | ✓ | ✓ |  |  | 16S rRNA *ISH* with comparative  16S rDNA sequencing of the V4 HVR using Illumina MiSeq and histology (H&E, Warthin-Starry, and Gram stains) | Detection of low abundance bacterial RNA in the placental villi and chorion | Aerobic: Blood, MacConkey, Chocolate, A-7. Anaerobic: Brucella, PEA, K-V. | No detectable growth. | [34] |
| **2019** |  |  |  |  | ✓ | ✓ | 16S rDNA sequencing using PacBio SMRT cell | 100% of meconium and 84% of amniotic fluid samples contained bacterial 16S rDNA | n/a | n/a | [35] |
| **2019** |  | ✓ |  |  |  |  | GFP plasmid DNA PCR and immunohistochemical detection of fluorescent-tagged *Staphylococcus aureus* | Fluorescent-tagged *S. aureus* injected into the maternal bloodstream is recovered in the placenta | BHI, TSA | No detectable growth. | [36] |
| **2019** | ✓ |  |  |  |  |  | 16S rDNA sequencing of the V5-6 HVRs using Illumina MiSeq | Bacterial DNA detected in endometrial biopsies following elective caesarean delivery | n/a | n/a | [37] |
| **2019** |  | ✓ | ✓ | ✓ | ✓ | ✓ | 16S rDNA sequencing of the V4-5 HVRs using Illumina MiSeq | Bacterial DNA is detected in meconium, placenta, and fetal membranes independent of delivery method | n/a | n/a | [38] |
| **2019** | ✓ | ✓ |  | ✓ |  | ✓ | 16S rRNA *ISH* with comparative  16S rDNA sequencing of the V4 HVR using Illumina MiSeq | Bacterial DNA present in meconium partially aligns to DNA found in the *in utero* environment | Aerobic and Anaerobic: BHI, MRS, Chocolate, MacConkey | Early gestational samples are more likely to be culture-positive in murine fetus | [39] |
| **2019** |  | ✓ |  |  |  |  | 16S rDNA sequencing of the V3-4 HVR using Illumina MiSeq with comparative qPCR | Neonatal oral microbiota most resembles the 16S rDNA profile of the placenta | n/a | n/a | [40] |
|  |  |  |  |  |  |  |  |  |  |  |  |
| **2020** |  | ✓ |  |  |  | ✓ | n/a | n/a | Aerobic and Anerobic: Blood | 86.5% of meconium and 57% of placental samples are culture-positive | [41] |
| **2020** |  |  | ✓ | ✓ |  |  | 16S rDNA sequencing of the V4 HVR using Illumina MiSeq with comparative qPCR | Bacterial DNA profiles of placental samples without histological evidence of chorioamnionitis are distinctly different than negative controls | n/a | n/a | [42] |
| **2020** | ✓ | ✓ | ✓ |  |  |  | *ISH* and qPCR of the 16S rRNA inter-HVR segment between the V2-3 HVRs and Fusobacterium-specific 16S segment | Preeclampsia-exposed placenta has increased total bacterial and Fusobacterium DNA load | n/a | n/a | [43] |
| **2020** |  |  |  |  |  | ✓ | 16S rDNA sequencing of the V4 HVR using Illumina MiSeq with comparative V6 HVR qPCR, eubacterial fluorescence ISH, and scanning electron microscopy | Sparse but viable bacteria are visualized in human meconium and detected by 16S rDNA signal | Aerobic: *Micrococcus*-specific isolation in BHI | Guided by molecular identification, viable *Micrococcus spp.* isolates were cultured from meconium | [45] |
| **a Culture Media Abbreviations:** A-7: Shepard's Differential Agar Base, BHI: Brain Heart Infusion, BP: Baird Parker, CNA: Columbia Colistin-Nalidixic Acid, EMB: Eosin Methylene Blue, K-V: Kanamycin-Vancomycin, LB: Luria-Bertani, MRS: De Man, Rogosa, and Sharpe, PEA: Phenylethyl Alcohol Agar, PYG: Peptone Yeast Extract Glucose, SDC: Sabouraud Dextrose Agar with Chloramphenicol, TSA: Tryptic Soy Agar, VRB: Violet Red Bile, WC: Wilkins-Chalgren | | | | | | | | | | | |

While ‘microbiome’ has a clear definition (‘*all of the genetic material within a microbiota (the entire collection of microorganisms in a specific niche, such as the human gut). This can also be referred to as the metagenome of the microbiota.*’; <https://www.nature.com/subjects/microbiome>), there are divergent views on its meaning.

1. Specifically, we study the effect of (1) maternal high fat diet consumption during pre-conception, pregnancy, and lactation, and (2) inhalation/ingestion of polycyclic aromatic hydrocarbons (found in tobacco smoke and petroleum byproducts) during pregnancy. [↑](#footnote-ref-1)
2. The adverse pregnancy outcomes associated with PAH exposure include, but are not limited to, preterm delivery, low birthweight, neonatal bronchopulmonary dysplasia, child-onset asthma, low cognitive assessment scores in children, neurodevelopmental delay, DNA adduct formation, alteration of DNA methylation patterns, and persistent fetal reprogramming [171,172,181–183,173–180]. [↑](#footnote-ref-2)
3. We observed microbial community differences in neonatal mouth, nose, and skin, but not meconium/first stool [115]. It is important to note that the neonatal mouth, nose, and skin are first exposed to the *ex utero* environment. Meconium/first stool represents the amniotic fluid that was ingested *in utero*, starting in mid-gestation. [↑](#footnote-ref-3)
4. Germ-free animals are characterized by prolonged diestrus, small lymph nodes and spleen, thin intestinal villi and lamina propria, increased food/water intake, higher oxidation-reduction potential, altered mucosal enzyme patterns, and decreases in circulating leukocytes, immunoglobulin levels, Peyer’s patch size, intraepithelial T cells, inflammatory response, blood volume, regional blood flow, cardiac output, basal metabolic rate, motor activity, response to catecholamines, body fat, organ sizes, vitamin biosynthesis, enteric bile acid transformation, and intestinal-specific parameters (mass and surface area, peristalsis, epithelial cell renewal, pH levels) [184–195]. Additionally, germ-free animals display stress-resistant hypothalamic-pituitary-adrenal axis response [6] and reduced anxiety-like behavior [7,9,155,196]. [↑](#footnote-ref-4)
5. We previously used orthogonal methods to document a low-biomass, low abundant metagenome which can be discerned from appropriate controls in cases of term and preterm chorioamnionitis and term and preterm births without chorioamnionitis [18,23,25,34]. Of note, chorioamnionitis is a localized and not systemic infection which is most often “cured” by delivery and antibiotics are used to reduce the risk of post-partum endometritis. [↑](#footnote-ref-5)