The “fetal microbiome” and pitfalls of low-biomass microbial studies

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Preface

Whether the human fetus and the prenatal intrauterine environment (amniotic fluid, placenta) are stably colonized by microbes in a healthy pregnancy remains the subject of debate. Here, we

evaluate recent studies that characterized microbial populations in human fetuses from the perspectives of reproductive biology, microbiology, bioinformatics, immunology, clinical microbiology, and gnotobiology, and assess possible mechanisms by which the fetus might interact with microbes. Our analysis indicates that the detected microbial signals are likely the result of contamination during the clinical procedures to obtain fetal samples, DNA extraction, and DNA sequencing. Further, the existence of live and replicating microbial populations in healthy fetal tissues is not compatible with fundamental concepts of immunology, clinical microbiology, and the derivation of germ-free mammals. These conclusions are important to our understanding of human immune development and also illustrate common pitfalls in the microbial analyses of many other low-biomass environments. The pursuit of a “fetal microbiome” serves as a cautionary example of the challenges of sequence-based microbiome studies when biomass is low or absent and emphasizes the need for a trans-disciplinary approach that goes beyond contamination controls, also incorporating biological, ecological, and mechanistic concepts.

Introduction

Fetal immune development prepares the neonate for life in a microbial world and underpins lifelong health[1-4]. Neonates born at term are not immunologically naïve and are specifically

adapted to cope with abrupt exposure to microbial, dietary, and environmental stimuli[5,6]. Several research groups have characterized immune cell development in human fetal tissues[7-9]. However, our mechanistic understanding of how and when immune priming by microbes occurs, and the factors that drive it, is incomplete. The long-held view that the prenatal intrauterine environment (placenta, amniotic fluid, fetus) is protected from live microbes has been challenged recently[10-15], leading to the hypothesis that fetal immune development may be driven by the presence of live microbes or even entire microbiomes at intrauterine sites[16-20]. However, these results have been debated[21-27] because several concurrent studies[28-34] point to experimental contamination dominating low–microbial-biomass sequencing data[35-37] as the source of microbial DNA apparently detected in the intrauterine environment. Since 2020, four studies have characterized the microbiology of the human fetus directly and resulted in opposing and irreconcilable conclusions. Two reports described viable low-density microbial populations in human fetal intestines[38] and organs[39], and linked these microbes to fetal immune development. In contrast, two other research groups, that include several of the authors of this perspective, reported no detectable microbes in fetal meconium and intestines[29,40].

Such disagreement over a fundamental aspect of human biology poses a challenge for scientific progress. The notion of a fetal microbiome, if proven correct, has implications for clinical medicine and would call for a comprehensive reappraisal of previous concepts and research. It would require radical revision of our understanding of the development of the immune and other systems in early life and the anatomical and immunological mechanisms mediating host-microbe interactions within fetal tissues. Failure to resolve this issue has a potential risk of diverting finite resources into research that results in no advancement for fetal and maternal health, and misguided attempts to therapeutically modify a non-existent fetal microbiome. The dilemma has further relevance to the characterization of microbiota in all low-biomass samples. Therefore, we

assembled a trans-disciplinary group of scientists and clinician-scientists to clarify how and when the fetus becomes prepared for life with microbes, to identify research pitfalls and mitigation strategies, and to propose specific directions for future research. Diverse research perspectives were included:(i) reproductive biology and obstetrics; (ii) microbiology and microbial ecology; (iii), bioinformatics and data science; (iv) immunology; (v) clinical microbiology; and (vi) gnotobiology and the derivation of germ-free mammals.

Claims and counterclaims

Although the disagreement on the presence of microbes in prenatal intrauterine locations (placenta and amniotic fluid) spans dozens of studies with contradictory findings[11,13,14,22,28,30-33,36,41-43], we focused our analysis on four recent studies since they provide a direct assessment of the fetus itself[29,38-40]. Collection of human fetal samples is difficult and restricted to either following pregnancy termination, or immediately prior to birth by C-section. Three of the studies used samples collected after vaginally delivered, elective, second trimester pregnancy terminations[38-40], and one collected samples from breech C-section deliveries immediately at birth[29].

Rackaityte *et al*.[38] reported 18 bacterial taxa as enriched in intestinal contents of vaginally delivered fetuses from 2nd trimester terminations compared to negative controls using 16S rRNA gene amplicon sequencing (V4 region). To account for contamination, the authors removed Operational Taxonomic Units (OTUs) detected in >50% of procedural controls and then identified remaining contaminants *in silico* (using the decontam R package). They found that most fetal samples were microbiologically similar to negative controls (labelled as “other meconium”, n=25), but that some samples, dominated by *Lactobacillus* (6 samples) or Micrococcaceae (9 samples), had distinct bacterial profiles. The authors further detected low amounts of total bacteria by qPCR,

Fluorescent *in situ* hybridization (FISH), Scanning Electron Microscopy (SEM), and culture (as discussed below).

Several of the study's conclusions have been challenged by de Goffau *et al.*[44], who re-analyzed the publicly available data and found no evidence for a distinct bacterial profile in the subset of samples with matched procedural controls, and concluded that the positive findings were caused by a sequencing batch effect (indicative of contamination) and further contamination during culture[44]. In addition, the authors' suggestion that particles detected in SEM micrographs constitute micrococci[38] was disputed as their size exceeded that of known Micrococcaceae[44]. Furthermore, the 16S rRNA gene sequence of the *Micrococcus luteus* cultured from the fetal samples differed from that detected by sequencing, further supporting contamination during culture (*Micrococcus luteus* is a common contaminant of clean rooms and surgical instruments[45,46]).

Mishra *et al.*[39] detected a low but consistent microbial signal across tissues of vaginally delivered fetuses from 2nd trimester terminations by 16S rRNA gene amplicon sequencing (V4-V5 region), with 7 genera enriched in fetal samples (*Lactobacillus*, *Staphylococcus*, *Pseudomonas*, *Flavobacterium*, *Afipia*, *Bradyrhizobium*, and *Brevundimonas*). The 16S rRNA gene sequencing data were accompanied by SEM, RNA-*in situ* hybridization (RNA-ISH), and culture. In recognition of the high risk of contamination, all samples were processed in isolation with negative controls collected during sample processing. In contrast to Rackaityte *et al*., Mishra *et al*. found *Micrococcus* to be enriched in phosphate buffered saline (PBS) reagent controls and reported it as a contaminant, with the *M. luteus* cells detected by culture being consistent with the size and morphology of the coccoid structures found by SEM[39].

Both Rackaityte *et al*. and Mishra *et al*. included assays of fetal immune development and concluded that the microbes detected would contribute to immune maturation. Rackaityte *et al*.[38] based this conclusion on differences in patterns of T cell composition and epithelial transcription between fetal intestines determined by whether Micrococcaceae were or were not the dominant species and suggested that bacterial antigens may contribute to T cell activation and immunological memory *in utero*. Mishra *et al*.[39] employed flow cytometry to expand on previous findings of effector (TNF-α /IFN-γ producing) memory (CD45RO+) T cells in fetal tissues, including gut tissue and mesenteric lymph nodes. Bacterial isolates cultured from the fetal samples, including *Staphylococcus* and *Lactobacillus* strains, induced *in vitro* activation of memory T cells isolated from fetal mesenteric lymph nodes.

In contrast to these reports, Li *et al.*[40], who also investigated fetal intestinal tissue from second trimester terminations, did not detect bacterial DNA by PCR (V4 region of the 16S rRNA gene, 35 cycles) based on visual inspection of agarose gels in any of the 101 samples tested. The authors detected a diverse set of metabolites in fetal intestinal samples and hypothesized that maternal, microbiota-derived metabolites may pass through the placenta to 'educate' the fetal immune system. This conclusion is supported by research in mice that showed that fetal immune education can be driven in the absence of direct microbial exposure by trans-placental passage of microbial metabolites from the maternal gut[47,48].

Kennedy *et al.*[29] used a different approach and collected samples using rectal swabs during elective C-section for breech presentation at term gestation[29]. Comparisons with environmental and reagent-negative controls from two independent sequencing runs were included to account for contamination and stochastic noise. No microbial signal distinct from negative controls was detected, and aerobic and anaerobic bacteria (*Staphylococcus epidermidis* and *Cutibacterium*

acnes [formerly *Propionibacterium acnes*]) detected by culture of fetal samples were identified by the authors as skin contaminants.

To compare these reports, we re-analysed the publicly available unfiltered relative abundance data associated with the three publications that reported sequence data and determined the relative abundance of each detected genus. While there was good agreement between the two studies using 2nd trimester vaginally delivered fetuses[38,39], the bacterial taxa detected in fetuses derived by C-section[29] were significantly different (Figure 1). The number of genera was much lower in C-section-derived fetuses, and entire groups of microbes, especially those generally found in the vagina, were absent. Most importantly, in the studies that claimed fetal microbial colonization[38,39], every genus detected in fetal samples was also detected in most control samples.

Reproductive biology and obstetrics perspectives

The embryo and fetus develop within the uterus but not in the uterine cavity *per se*. The early embryo invades the maternal decidua and is completely embedded by 10 days post-fertilization. The fetus grows within the amniotic cavity, which originates between the trophoblast and inner cells mass in the second week post fertilization, surrounded by two layers of reproductive membranes and bathed in amniotic fluid. Hence, even if microbes were present in the uterine cavity[49], they would have to pass through to the amniotic cavity and enter the amniotic fluid to colonize the fetus. Amniotic fluid has antimicrobial properties, being enriched for example in lysozyme[50], human beta-defensin 2[51], and Gp340/Dmbt1[52],which binds and agglutinates diverse gram-negative and gram-positive bacteria.

The placenta mediates communication between the fetus and the mother and is a potent immune organ that protects the fetus. Historically, the placenta has been considered sterile (defined here as free from living microorganisms), but in 2014 a complex but low-biomass placental microbiome was detected by DNA sequencing. The proposed placenta microbiome showed some similarity with sequence data of microbial communities of the oral cavity[14]. Contamination controls were not included in this early study, and subsequent evaluation of the work found that most genera detected were also common contaminants[25,35,37,53]. Several detected taxa, such as *Gloeobacter*, a genus of photosynthetic cyanobacteria, appeared biologically implausible as a component of a putative placental microbiome[23,54]. Since this early report, dozens of studies have conducted a sequence-based microbial analysis of placenta tissues, with completely opposing conclusions (as reviewed by Bolte *et al.*[20]).

Regardless of whether placental samples are collected by biopsy per vagina, clinically by chorionic villus sampling, or after delivery, it is always necessary to control for contamination, particularly from the tissues through which a placenta must pass prior to sampling. Accordingly, de Goffau *et al.*[28] carried out a comprehensive study of the possible placental microbiome, and detected a range of species known to dominate the vaginal microbiota[55], such as *Lactobacillus iners*, *L. jensenii*, *L. crispatus*, *L. gasseri*, and *Gardnerella vaginalis*. When the presence of vaginal microbes and those in the laboratory reagents (the “kitome”) were accounted for, no placenta microbiome was detected in their studies or several further recent studies[22,28,30-33,36].

Pathogenic infection of the placenta by viral or bacterial pathogens is a well-recognized clinical phenomenon that contributes to preterm birth and neonatal sepsis[56]. de Goffau *et al*. detected *Streptococcus agalactiae* in around 5% of cases as the only verifiable bacterial signal in placentas obtained by C-section deliveries conducted prior to rupture of the fetal membranes and the onset

of labour[28]. The presence of this species is plausible as it colonizes the genital tract of about 20% of women and has invasive potential, being an important cause of maternal and neonatal sepsis[57]. However, the ability of specific pathogens to colonize and/or infect the placenta is distinct from the presence of an indigenous microbiota, that is, a prevalently stable, non-pathogenic, complex microbial community that is metabolically active[58].

Research claiming the presence of viable low-density microbial communities in the fetal intestine[38] and fetal organs[39] likewise calls for an evaluation of the sampling process. Mishra *et al*. obtained fetal tissues after medical termination of pregnancy in the 2nd trimester with prostaglandins[39]. This procedure typically involves the individual going through hours of labor and often leads to the rupture of the fetal membranes hours prior to vaginal delivery. Even with a standardized approach, labor may be prolonged and may be accompanied by infection and fever, which are common with 2nd trimester terminations[59,60]. Both Li *et al*.[40] and Rackaityte *et al*.[38] also used 2nd trimester terminations but obtained the fetal tissues from core facilities. The tissues used by Li *et al*. were from surgical terminations (14-23 weeks) performed with mechanical dilation. Unfortunately, Rackaityte *et al*.[61] did not provide sufficient information to determine if fetuses were obtained through surgical procedures or medical inductions. While the latter increases the risk of the fetus being exposed to vaginal microbes during labour, both procedures involve vaginal delivery of the fetus. As outlined below, the reported microbiology of these fetuses primarily reflects the sources of microbes to which they are exposed during these procedures.

Microbiology and microbial ecology perspectives

Host-microbe relationships range from mutualism (a prolonged symbiotic association from which both benefit) to commensalism (the host is unaffected), to pathogenesis where the microbe harms the host. Although claims for fetal microbial exposure[38,39] have not established the nature of the

host-microbe interaction, and the duration of exposure or colonization, they have suggested a beneficial role for live organisms in fetal immune development, thereby implying a symbiosis. The microbiological approaches applied by Rackaityte *et al.*[38] and Mishra *et al.*[39] are, in large part, robust, and well suited to study symbiotic microbial populations. The combination of 16S rRNA gene sequencing, quantitative PCR (qPCR), microscopy, FISH, and culture is laudable, as the approaches are complementary. Next-generation sequencing of 16S rRNA gene amplicons provides a broad community overview and can detect microbes that escape cultivation, while qPCR, microscopy, and bacterial cultures have a high dynamic range, low detection limits, and reasonable specificity. The DNA sequence-based microbiota composition data in both studies is quite consistent (Figure 1), suggesting that several of the bacterial taxa detected were present in the samples and not artifacts derived from laboratory reagents or DNA-isolation kit contamination. However, although the microbiological analyses of samples were sound, the sampling procedures allowed the introduction of contaminant species and critical controls to determine whether contamination occurred were missing.

In agreement with the unavoidable vaginal exposure of fetuses obtained by 2nd trimester abortions (see above), both Rackaityte *et al.*[38] and Mishra *et al.*[39] found the genera *Lactobacillus* and *Gardnerella*, which dominate the vaginal microbiota[55], among their most consistent findings (Figure 1). The species cultured by Mishra *et al.*, *G. vaginalis*, *L. iners* and *L. jensenii*, are highly specific to the human vagina[62]. Other microbes detected such as *Staphylococcus* species and *Cutibacterium acnes*, are skin commensals. As shown in Figure 1, abundances of *Lactobacillus*, *Gardnerella*, and *Staphylococcus* found by Mishra *et al*. showed gradients with high population levels in fetal samples exposed to sources of contaminants (placenta and skin) and lower levels in internal samples (gut, lung, spleen, thymus). The omission of vaginal controls by both Rackaityte *et al*. and Mishra *et al*. to determine the microbiota of vaginally delivered fetuses is an

unfortunate flaw that casts doubt on the authors' conclusion that the microbes originate from the womb. Indeed, Li *et al*.[40], who used samples from 2nd trimester surgical terminations performed with mechanical dilatation, which decreases the bacterial exposure of the fetus during sampling, did not report positive bacterial PCR results in their study, further raising suspicion that sampling contamination was a serious confounder in the work of Rackaityte *et al.* and Mishra *et al.*.

Although vaginal controls were not included by Rackaityte *et al.*[38] and Mishra *et al.*[39], direct comparisons of their findings with those by Kennedy *et al*.[29] also provide clear evidence for vaginal contamination of terminated fetuses (Figure 1). The C-section derived fetal samples in Kennedy *et al.*, which were not exposed to the vagina, carried no *Gardnerella* or *Lactobacillus,* but instead contained skin and reagent contaminants[29,53]. Despite attempts to reduce contamination, C-section derived fetal meconium had at least one positive culture[29]. Kennedy *et al*. did not consider these microbes of fetal origin, as they were skin commensals, and half of the samples, as well as many culture replicates, did not show growth. The authors concluded that such inconsistencies point to stochastic contamination and not colonization by a stable functional microbial community.

Despite vaginal contamination, the bacterial load found in terminated fetuses was extremely low[38,39]. Signals derived from qPCRs were only marginally higher than those of controls, with Mishra *et al*. reporting cycle thresholds (Ct) of >30 cycles, with Ct values for negative controls around 31-32 cycles. Cell counts as detected by both microscopy and culture were also low. Mishra *et al*. reported fewer than 100 colonies on average per entire fetus, with high inconsistencies among individual fetuses and tissues (see Table S6 in the original publication[39]). Such findings are readily explainable as contamination rather than mutualism/colonization.

Neonatal meconium samples have been studied for a century by culture-based methods and more recently by DNA sequencing; this has also sometimes yielded contradictory findings[10,42,43,63,64] due to contamination and because postnatal colonization may occur before the first passage of meconium[25]. However, when meconium is passed soon after birth, culturable bacteria are seldom detected (as reviewed by Perez-Munoz *et al*.[25]). In agreement with this, an analysis of meconium samples collected from extremely premature infants[65] showed that taxa regularly identified previously as contaminants[35,37] make up a large proportion of sequences collected within the first 3 days after delivery and then drop to almost zero in most samples at days 4-6 (Figure 2), indicating that the genuine bacterial signal is low in early meconium. This conclusion agrees with a recent study that applied strict controls for sequencing and culture and reported no meconium microbiota[64].

Members of an authentic fetal microbiota should be, in theory, detectable in early-life fecal samples independent of birth mode. There is indeed some overlap between the reported fetal microbial taxa in vaginal versus C-section deliveries[38,39], e.g. staphylococci, enterococci, lactobacilli, and enterobacteria, and the microbiota detected in infant fecal samples in the first week of life[66-68]. However, there have been few attempts to track species and strains to confirm fetal origin. One study investigated gastric aspirates of newborn infants collected immediately after birth[69], which should contain microbes reported *in utero,* as the fetus swallows amniotic fluid. However, aspirates from vaginally-born infants contained the specific *Lactobacillus* species (*L. iners* and *L. crispatus*) that also dominate the microbiota of the vagina, while most samples from C-section deliveries clustered with negative controls[69]. This finding is consistent with vaginal transfer of microbes to a sterile fetus during delivery. In addition, many of the genuine bacterial signals that were detected in early meconium[65] were typical maternal skin representatives (*Staphylococcus* & *Corynebacterium*) and were strongly associated with C-section, or were

maternal fecal microbiota representatives (*Escherichia* & *Bacteroides*) associated with vaginal delivery (Figure 2), indicating that these genuine signals were derived from microbes acquired *ex-utero*.

Research is beginning to determine the origin of post-partum neonatal microbial colonizers and has shown a delay in appearance of bacterial species presumed to originate from the mother's gut (e.g. *Bifidobacterium* and *Bacteroides* species) in early fecal samples of infants born by C-sections[66,67,70-72]. A substantial proportion of strains acquired by infants postnatally can be traced back to their mothers[72-74], and fecal microbiota transplant (FMT) restores the microbiome in C-section delivered infants[75]. Thus, the published evidence, although still incomplete, suggests that the early life microbiome in humans is acquired through the vertical and horizontal transfer of microbes whose origin is fecal or environmental (from outside) rather than fetal (from inside).

Bioinformatic and data science perspectives

Characterization of low-biomass samples by 16S rRNA gene amplicon sequencing is challenging as DNA contamination can occur from the microbial DNA present in reagents, tools, instruments, and DNA isolation kits,[35-37] and through cross-contamination between PCR tubes/wells, sequencing runs, or sequencing lanes[36]. A common misconception in the field of low microbial biomass samples is that the use of negative controls is sufficient to account for all kinds of contaminants. Commonly, imperfect negative controls are used that account only for a limited number of the sample processing steps or are not spread evenly amongst all batches (thus not accounting for processing days, reagent batches and different sequencing runs), leading to batch effects that may be mistaken for genuine signals[44]. Overreliance on or under-analysis of such negative controls, in combination with the misapplication of contamination removal programs like Decontam[76], specifically by not having negative controls in all batches, frequently results in false

409 retention of contaminants[44]. Even with appropriate controls, it is challenging to separate genuine
410 signals from low abundance contaminants due to the law of small numbers, which means that
411 contaminant signals may appear sporadically in samples and negative controls[77]. Thus,
412 suboptimal handling of sequencing control samples may not reveal the full spectrum of
413 contaminants because only the most abundant contaminant species are consistently
414 detected. On the other hand, potentially genuine sample-associated signals sometimes also
415 erroneously appear in negative control samples through cross-contamination during the PCR or
416 sequencing steps (machine contamination)[36].
417
418 Unfortunately, both Rackaityte *et al.*[38] and Mishra *et al.*[39] reported taxa as legitimate findings that
419 are very commonly reported contaminants (Figure 1). The most obvious case is *Bradyrhizobium*,
420 which is one of the most dominant and consistent contaminants found in sequencing studies[37,78].
421 Rackaityte *et al.* reported *Micrococcus* and *Lactobacillus* as genuine fetal inhabitants, but a re-
422 analysis of the data revealed that this finding was batch specific, indicative of contamination[44].
423 Although the authors rejected this conclusion[61], this batch effect is clearly visible if the findings of
424 the different batches are plotted together (Figure 3). In addition, Mishra *et al.* considered their
425 own signal for *Micrococcus* to be derived from contamination[39]. *Afipia*, *Flavobacterium*,
426 *Pseudomonas*, and *Brevundimonas* are further genera reported by Mishra *et al.*[39] that are
427 commonly detected as kit or laboratory reagent contaminants[35,37].
428
429 Mishra *et al*. and Rackaityte *et al.* also reported marginally higher total bacterial load in fetal
430 samples, as compared to controls, using qPCR[38,39]. However, eukaryotic DNA in tissue samples
431 (which is absent in negative controls) might have a DNA carrier effect leading to a more efficient
432 DNA precipitation of prokaryotic reagent contaminants. In addition, bacterial PCR primers may
433 also amplify mitochondrial DNA, which is evolutionarily of bacterial origin. Together these factors

may explain why samples from low-biomass studies are often reported as having more bacterial DNA than controls and show that this may not always be relied upon as evidence for the presence of microbes. Rackaityte *et al*. depleted human mitochondrial DNA (mtDNA) from their 16S rRNA gene sequence set that co-amplified in the PCR, but neither study accounted for mtDNA in their qPCR analysis, although their primers targeted the 16S rRNA gene and were therefore potentially susceptible to cross-reactivity[38,39].

Immunological perspective

The enteric microbiota acts as potent drivers of adaptive mucosal immune maturation and priming in the adult host[79-82]. Besides their intrinsic immunogenic nature, microorganisms also generate metabolites that critically promote and shape immune maturation and priming[83-85]. Although the early fetal immune system is immature, recent research demonstrates migration of fetal dendritic cells (DCs) to the mesenteric lymph nodes; somatic hypermutation in fetal B cells; and increasing T cell receptor repertoire diversity, evenness and activation during late fetal development[7,86,87].

The existence of metabolically active microbes in the fetus could, in principle, provide one possible explanation for these findings. Mishra *et al.*[39] used an autologous T cell expansion assay to show that fetal DCs loaded with antigen from bacteria that had been isolated from fetal tissues stimulated proliferation of CD45RO+ and CD69+ T cells. T cell proliferation was reduced but still detectable in the absence of DC-derived cytokine release suggesting an activated memory response[39]. Demonstration that the fetal T cell memory response is specific for the bacteria present in one individual fetus would be necessary to strengthen the interpretation that specific immune responses are routinely driven by fetal bacterial colonization.

There are alternative explanations for fetal immune responses apart from *bona fide* microbial colonization. Maternal antigen-IgG complexes have been detected in cord blood and transplacental immune priming of the fetal immune system in early gestation has been demonstrated[88,89] Cross-reactivity, as observed for microbiota reactive enteric secretory immunoglobulin A, would support fetal priming by maternal microbial antigens[84]. Similarly, maternal microbiota-derived microbial molecules partly bound to IgG stimulated innate immune maturation of the murine fetal gut[47], and maternal intestinal carriage of *Prevotella* protected the offspring from food allergy in humans[90]. Thus, maternal microbiota-derived microbial antigens and metabolites may pass the placental filter directly or bound to IgG and evoke the observed fetal immune responses[91].

The hypothesis of a low biomass fetal microbiome requires the identification of mechanisms that control and tolerate bacterial populations and prevent overt inflammation and tissue destruction in the presence of viable microorganisms, many of which are opportunistic pathogens (see below). Alongside this, mechanisms by which the commensal or symbiotic microbes survive the immune response and antimicrobial effector molecules would also have to be identified, and it is unclear how the fetal immune system would differentiate between pathogens and symbionts once protective barriers are breached[56]. Given that such immunological and anatomical mechanisms have not been identified or even proposed[27], the observed immune maturation and priming during fetal development is most likely not induced through colonization of the fetus with live microbes. Instead, fetal immune development might be driven through maternal immune components or microbial fragments and metabolites crossing the placenta, which protects the sterile fetus from live microbes through multiple layers of immunological defence[56].

Clinical microbiology perspective

No part of the human body is impregnable to bacterial invasion. Transient bloodstream bacteraemia can result from innocuous activities such as tooth brushing[92], and most host tissues can tolerate occasional ingress by microbes. However, to avoid serious pathology, bacteraemia must be rapidly cleared by innate immune mechanisms and inflammation. Some pathogens establish persistent infections that may be asymptomatic either by evading the immune system or by forming persister cells in response to antibiotic treatment[93]. The claims for non-pathogenic fetal microbial exposure[38,39] have not established whether host-microbe interactions reflect small scale translocation, asymptomatic infection, persistent symbiosis or mutualism.

The 'fetal-enriched taxa' reported include *Micrococcus*, *Lactobacillus, Flavobacterium, Staphylococcus, Escherichia, Enterococcus*, *Afipia, Pseudomonas, Bradyrhizobium*, and *Brevundimonas*[38,39]*.* Mishra *et al.* also report successful culturing of lactobacilli and staphylococci from fetal tissue[39], but the lack of unambiguous species-level taxonomic identification of the cultured organisms is an unfortunate and significant technical limitation. Bacteria such as *Micrococcus*, which were detected in fetal intestines by Rackaityte *et al.*[61], rarely cause invasive infection in humans. Their prolonged presence within healthy tissues and transmission through the placenta would require bacterial mechanisms of resistance against antimicrobial effector molecules of the host innate immune system[56]. Such mechanisms have not been described for the genus *Micrococcus*, which is an environmental organism found in water, dust, and soil, and is also a common contaminant[45,46]. Lactobacilli are usually of low pathogenic potential, they inhabit external mucosal surfaces of healthy humans, including the nose[94] and vagina[55], and they are often used as probiotics[95]. However, some strains and species of lactobacilli do express potential virulence factors[96-98], resist oxidative stress[99] and grow in the absence of iron[100], which allows them to cause serious infections such as endocarditis when provided with the opportunity to access the bloodstream[101,102]. This raises potential problems with the interpretation of

lactobacilli being asymptomatic colonizers of fetal tissue rather than contaminants that are picked up during vaginal delivery.

An even greater challenge arises when species of the genus *Staphylococcus* are considered, particularly strains that were cultured from fetal tissue and that exhibit high-level 16S rRNA gene sequence identity (99-100%) to *Staphylococcus aureus* and several closely related coagulase-negative *Staphylococcus* species (CoNS)[39]. These organisms can be long-term colonizers of external mucosal surfaces of humans [103,104] and do not typically cause disease unless the mucosal barrier is breached. However, once they bypass mucosal barriers, they can deploy a more extensive repertoire of virulence factors to invade tissues by degrading connective tissues and, in the case of *S. aureus*, a repertoire of over a dozen cytolytic toxins genes that kill human cells[105,106]. CoNS, on the other hand, are ubiquitous skin colonizers. Their detection in clinical diagnostic laboratories is so common that it is considered a major diagnostic challenge[107,108] and is usually assumed to reflect contamination from the patient and occasionally the healthcare worker, in the absence of other reasons to suspect a CoNS infection[77-79]. There are, however, distinct clinical scenarios where the presence of CoNS and their pathogenic capacity are considered critical: for example, in patients with indwelling devices and in preterm neonates, they are the most common cause of late-onset neonatal sepsis[109]. Therefore, given that they are either contaminants or overt pathogens, the detection of staphylococci, no matter whether *S. aureus* or CoNS, is difficult to reconciliate with *in utero* colonization of a healthy fetus.

Other bacteria identified as part of a notional “fetal microbiome”, such as *Enterococcus faecalis* and *Klebsiella pneumoniae*, are equally problematic. These belong to a group known as “ESKAPE pathogens”, which include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species.

The lethality of tissue colonization with ESKAPE pathogens is well documented, and these microbes are leading causes of healthcare-acquired infections worldwide with significant mortality and morbidity, even when treated with antibiotics[110]. Several ESKAPE pathogens readily survive in adverse conditions outside of vertebrate hosts, including drying, oxidative stress, and exposure to heat or sanitation chemicals[111]. They are likely to persist on inanimate surfaces including utensils or clinical fabrics[112,113], thereby increasing their likelihood of being contaminants. While these microorganisms were not reported at the species level[39], it is noteworthy that closely related organisms can also cause neonatal sepsis[114-116], which makes them unlikely colonizers of a healthy fetus.

A consideration prompted by a notional fetal microbiome is the possibility that the fetus might cope better with nosocomial pathogens than neonates or even adults. However, there is ample evidence to show that amniotic fluid, the placenta and fetal tissues are highly susceptible to bacterial infection, and the outcomes of infections with *Streptococcus agalactiae* or *Listeria monocytogenes* are often catastrophic[117,118]. Importantly, in *L. monocytogenes* infections that occur during the third trimester of pregnancy, fetal infection progresses while the mother's infection can be cleared, indicating that the fetus does not have greater resistance to infection than an adult human. Therefore, from a clinical perspective, most interpretations brought forward in recent publications[38,39] on the presence of microbes in fetuses seem to be biologically difficult to reconcile, as it is highly plausible that they would result in harm or death of the fetus. In agreement with this conclusion, in a series of well-controlled studies in various clinical settings, DiGiulio and co-workers found no evidence for microbes in amniotic fluid except when associated with neonatal morbidity and mortality[119-122].

Gnotobiology perspective

The traditional assumption that the human fetus is free from other life forms *in utero* is based primarily on the observation that, with few exceptions, bacterial and viral pathogens that infect the mother are incapable of crossing the placental barrier to infect the fetus[123-125]. Additionally, the amnio-chorionic membranes enclosing the fetus in the uterine cavity, as well as the cervical mucus plug, protect the fetus from external microbes. Sterility of the fetus is the basis for the derivation by hysterectomy of germ-free mammals (mainly mice and rats, but also pigs and other species[25]), which have long been used to study the biochemical, metabolic, and immunological influences of microbes on their mammalian hosts[126-128]. The primary consideration is whether germ-free animals are truly 'free of all demonstrable forms of microbial life'[129]. If they lack microbial associates, there cannot be a fetal microbiome. Testing germ-free animals for contaminating microbes uses microscopic observation of stained fecal smears, culture of feces in nutrient media under various conditions of temperature and gaseous atmosphere[124,129-131], PCR using 'universal bacterial' primers[130,132], and serological assays for viral infections[133]. These tests consistently demonstrate an absence of microbial associates. Therefore, gnotobiology provides strong evidence that the fetus *in utero* is sterile.

Summary - The experimental evidence indicates that the healthy human fetus is effectively sterile

Through multiple angles of explanatory considerations, we conclude that the evidence is strongly in favour of the sterile womb hypothesis. Although it is impossible to disprove the occasional presence of live microbes in a healthy human fetus, the available data does not support stable, abundant colonizers under normal, non-pathogenic circumstances. We are aware that our position conflicts with dozens of publications that claim evidence for *in utero* microbial populations[20], but we are confident about in the validity of our multi-layered approach.

583 The processes by which the fetus matures and becomes immunologically equipped for life in a
584 microbial have life-long implications. Aside from the caution and safeguards recommended in this
585 perspective, our aim here is not to dissuade scientists from exploring the microbial drivers of fetal
586 immune development. We agree with proposals that there is a need to better understand microbial
587 interactions at the maternal-fetal interface[20], but do not think that symbiotic microbial populations
588 in the placenta or fetus play a role in this. Paradoxically, we contend that sterile tissues are both
589 immunologically and microbiologically fascinating, but require an adjustment of the
590 methodological approaches used. How does the fetus mature and become immunologically
591 equipped for life in a microbial world in the absence of direct exposure to live microbes? Are
592 maternal-derived microbial metabolites sufficient for fetal immune education? Future research
593 could include exploration of how maternal microbial-derived metabolites and small molecules, as
594 well as maternal immune components, prepare the fetus for the microbial challenges of post-natal
595 life[91].
596
597 **Considerations for the critical evaluation of low- or no biomass samples**
598 Contamination is always a potential confounder in microbiology but is of particular concern for
599 those studying low- or no biomass samples.[35,37]. The issue has been highlighted by recent reports
600 of human tissues, such as blood, brain, and cancers (Box 1), previously thought to contain no, or
601 very little, bacterial biomass that apparently harbour diverse microbial communities. As with
602 intrauterine studies described above, these microbial populations are often discussed in light of
603 their perceived importance for human diseases and health.
604
605 In studies on low biomass samples, it is challenging to identify relevant signals from among
606 contaminating noise. In instances of contamination, a tissue may be misjudged as non-sterile,
607 whereas in others, a real microbiological signal may be obfuscated by contamination. The removal

608 of all sequences present in negative-control samples or that have been previously identified as
609 contaminants in the literature may result in loss of authentic signals. Post-sequencing
610 contamination removal using software packages such as Decontam[76] or other statistical
611 approaches[35,134] have been developed to remove the more abundant contaminants, leading to
612 microbiome profiles that are more likely to reflect the real community. Practical examples of
613 contamination removal in 16S rRNA gene sequence data is provided by Heida *et al*.[65], Saffarian
614 *et al.*[135], and Jorissen *et al*.[136] and we expand on these examples in Box 1.
615
616 We draw attention to the distinction between “low biomass” and no biomass samples. This has
617 practical significance; true “low (microbial) biomass” samples are amenable to contamination-
618 removal approaches but “no (microbial) biomass” samples require a different approach (Box 1).
619 For credible proposals of the presence of microbes, multiple layers of evidence are required, first
620 with quantitative, sensitive (lower detection limit) approaches, such as quantitative PCR with strict
621 controls before contamination-sensitive sequencing approaches are applied. Since contamination
622 removal will provide data regardless of whether microbes are present or absent, the starting
623 proposition should be the null hypothesis to avoid confirmation bias, particularly when results are
624 inconsistent and at the outer technical limits for detection, or if results defy mechanistic plausibility.
625
626 Given the limitation of sequencing approaches, confirmation by alternative methods, such as
627 FISH and culture, are required. However, as demonstrated with recent studies of fetal samples,
628 even a combination of approaches has the potential to produce false findings, as contamination
629 during sampling is a considerable challenge. We posit that studies on all low biomass samples
630 can benefit from a similar trans-disciplinary assessment, as applied above for fetal samples, to
631 interpret findings considering biological and mechanistic explanations[27]. When obligately
632 photosynthetic, psychrophilic, thermophilic, halophilic, or chemolithoautotrophic bacteria are

found in human tissues that do not provide the growth conditions for such organisms[23,137], or if the detected genera are known contaminants of laboratory kits/reagents (such as Proteobacteria readily culturable *Pseudomonas and E. coli* for example)[138-140], the authenticity of such signals should be questioned.

Box 1: Experimental considerations for biological samples containing different levels of biomass.

High biomass samples

***Examples*:** Faeces, dental plaque, wastewater treatment plant samples.

***Impact of contamination*:** Very low. The high microbial biomass derived from the sample dominates the signal derived from background contamination, meaning most observations are robust.

***Mitigations*:** Experimental design seldom needs to be significantly adjusted to account for contamination, beyond monitoring "blank" negative control samples that reveal which contaminating species are present and basic post sequencing analysis. Sequencing controls and removing samples with significant contamination levels is nevertheless prudent.

Low biomass samples

***Examples*:** Skin Swabs, nasal tract swabs, breastmilk, most respiratory tract samples, tissue biopsies & mucosal samples, including intestinal crypts.

***Impact of contamination*:** Ranges from low to high. Contaminated samples are progressively affected with reducing input microbial biomass[37].

***Mitigations*:** Inclusion of multiple controls to facilitate contamination recognition. When possible, samples should be concentrated prior to processing to increase input biomass. Advance consideration of potential sources of contamination during the sample acquisition stage is always recommended. After sample collection, processing should be carried out in a clean-room environment, preferably with all surfaces bleached and UV-treated. The extraction step may benefit from use of non-kit-based methods (e.g. phenol-chloroform extractions) where plasticware and individual reagents can be UV-treated prior to use. Contamination from DNA isolation and PCR kits is usually identifiable, particularly if well-defined batches are created[64] and controlled using different lot numbers of particular kits. Regardless of the DNA extraction method, the presence of contaminants should be monitored by including "blank" negative controls. The inclusion of controls generated by serial dilution of DNA of known composition (e.g. mock community) will indicate the biomass level at which contamination becomes a dominant feature of sequencing results. Contamination may also be estimated prior to sequencing by qPCR using serially diluted known quantities of spiked input DNA. Post-sequencing analyses, using programs like Decontam, and analysis steps as described by de Goffau et al.[35] and used by Heida et al.[65] will usually identify contaminants.

Samples in which the existence of microbes is not established (potential "No-biomass" samples)

***Examples*:** Placental and fetal tissues, amniotic fluid, meconium, brain tissue and cerebrospinal fluid, blood, bone, and internal cancer tissues, healthy middle ear samples.

***Impact of contamination*:** High and potentially up to 100%, unless infection/injury is present.

***Mitigations*:** Experimental design should be robust and directed specifically against contamination. An initial assessment using quantitative methods (e.g. qPCR) with low detection limit and microscopic visualisation (e.g. Gram staining/labelling by FISH) is required to determine

if microbes are present, before embarking on a sequence-based approach. Note such approaches are still susceptible to sample contamination and other artefacts (e.g. non-specific staining or auto-fluorescence from mucins, can sometimes appear "microbe-like" in size and shape)[44,141]. All mitigations outlined for "Low biomass" samples above should be adopted. Repeating sample processing with different DNA extraction kits/methods[31] and/or at different days can also be informative[142]. These will track the presence of particular species in sequencing profiles associated with specific kits/reagents or environment. Species that are repeatedly detected regardless of technical approach used are more likely to be genuine signals, unless they were introduced during the sample collection. Binary statistics (absence/presence) are recommended. Ideally, the presence of microbes identified by sequencing should be verified with a different technique such as cultivation, another sequencing technique with sufficient taxonomic resolution, and a species-specific qPCR or FISH using high magnification to visualize the size and morphology of individual microbial cells.

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Figures

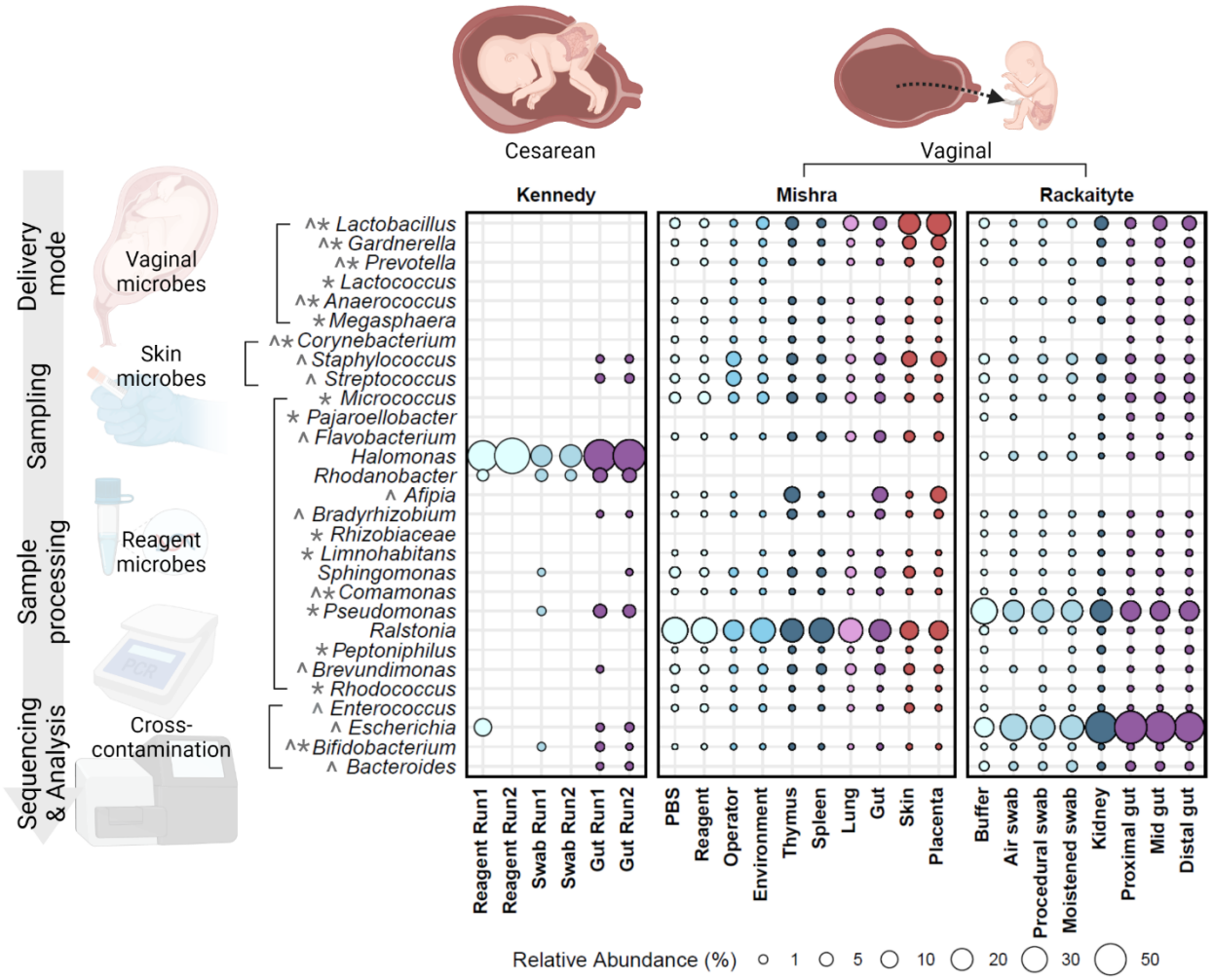

Figure 1. Distribution and mean relative abundance (%) of genera present in fetal samples from three recent studies[29,38,39] investigating the fetal microbiome and their corresponding abundance in control samples. Taxa were selected based on the following criteria: Genera that were cultured from or enriched in fetal samples as described by Mishra *et al.*[39] (indicated by ^) or by Rackaityte *et al.*[38] (indicated by *); all genera detected in fetal samples from Kennedy *et al.*[29]; and the PBS-enriched genus *Ralstonia*[39]. Taxa were grouped by potential source of contamination (see left-hand side illustrations) in agreement with the likely origin of genera (for skin microbes) and previous studies that characterized sources of contamination[35-37]. For taxonomic data from Rackaityte *et al.*, OTU10 (family *Micrococcaceae*) was manually assigned to the genus

Micrococcus as in the original publication. Publicly available unfiltered relative abundance data associated with each publication were merged into a single phyloseq object (RRID:SCR_01380). Amplicon Sequence Variants (ASVs) were grouped at the genus level. The mean relative abundance of each genus was calculated for each sample type within each study and plotted in R (tidyverse, ggplot2; RRID:SCR_014601). Dot size corresponds to the mean relative abundance of each genus by sample type and study (mean relative abundances <0.0001% were excluded). Dots are colored by sample type: reagent controls in lightest blue (Mishra: PBS n=42, Reagent n=23; Rackaityte: Buffer n=11; Kennedy Reagent n=2); sampling negatives in light blue (Kennedy: Swab n=1; Rackaityte: Air swab n=19; Procedural swab n=16; Moistened swab n=17) and environmental negatives in sky blue (Mishra: Environment n=47, Operator n=12), internal controls in dark blue (Mishra: Thymus n=27, Spleen n=12; Rackaityte: Kidney n=16), fetal lung in pink (Mishra, n=25), fetal gut in purple (Kennedy: n=20; Mishra: n=44; Rackaityte: Proximal n=41, Mid n=45, Distal n=42), and external tissues in red (Mishra: Skin n=35, Placenta n=16).

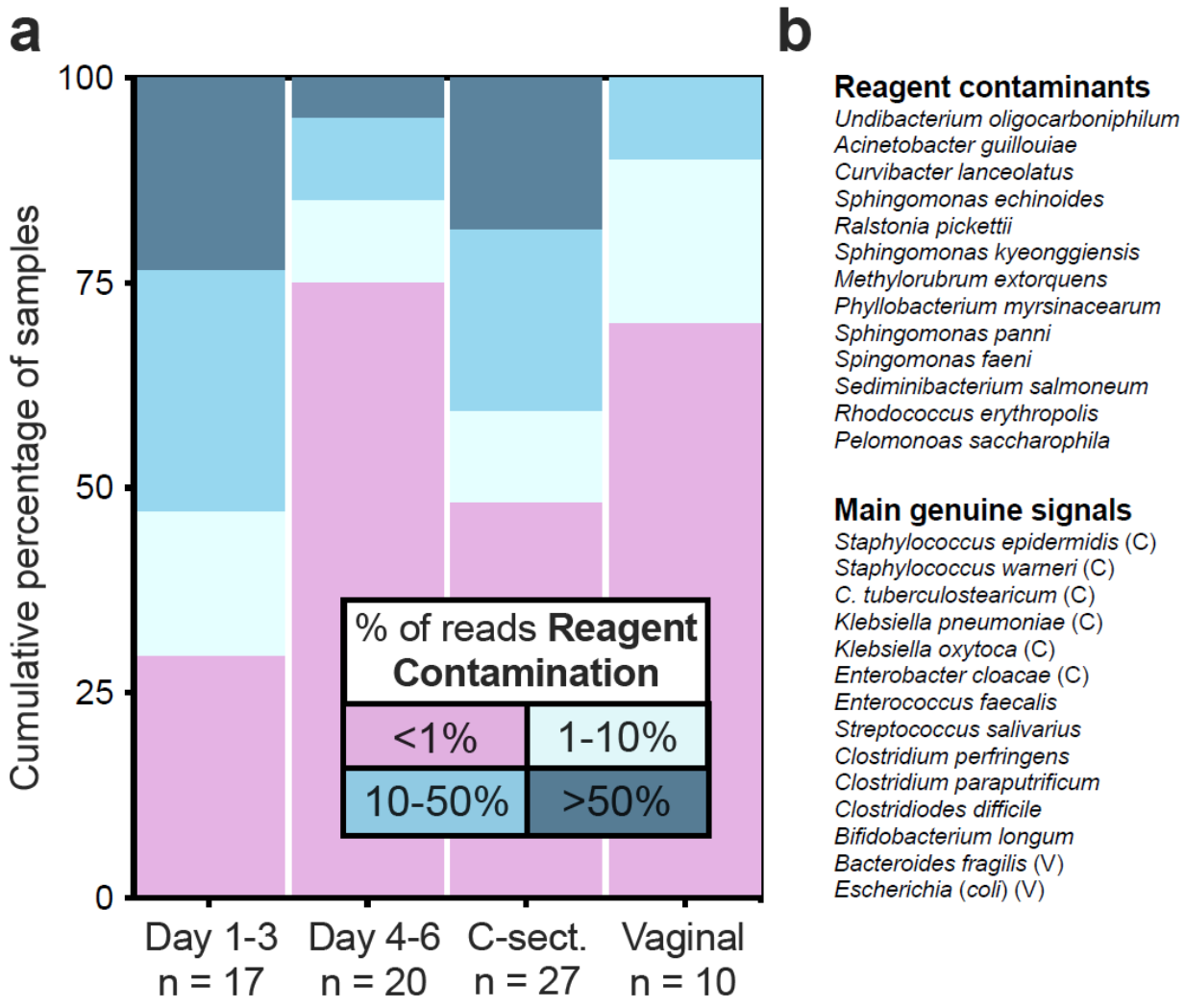

Figure 2. Reagent contamination in meconium samples of extremely premature infants. a) Representation of the % of reagent contamination in the first meconium of extremely premature infants in relation to the day of procurement of said samples (Day 1-3 or Day 4-6) or in regard to the mode of delivery (C-section or Vaginal). Colors indicate the percentage of reagent contamination reads (legend on top). The day of procurement is significantly correlated with the % of reagent contamination reads ($p = 0.005$ MW-U test or $p = 0.01$ Spearman rho test) and the mode of delivery shows a trend ($p = 0.07$ MW-U test). The number of samples is noted below each category (n). **b**) Lists of reagent contaminants shown together in **Figure 2a** (top) and of the

most abundant sample-associated-signals and their association (or lack thereof due to limited size of cohort) with vaginal (V) or C-section (C) delivery (bottom).

Figure 3. Distribution and mean relative abundance (%) of genera present in fetal and control samples from Rackaityte *et al.*[38] by batch as defined by *Rackaityte et al.*[61]. Dominant taxa were manually selected as described in Fig. 1. For taxonomic data OTU10 (family *Micrococcaceae*) was manually assigned to the genus *Micrococcus* as in the original publication[38]. Publicly available unfiltered relative abundance data associated with each publication were merged into a

single phyloseq object (RRID:SCR_01380). ASVs were grouped at the genus level. The mean relative abundance of each genus was calculated for each sample type within each batch and plotted in R (tidyverse, ggplot2; RRID:SCR_014601). Dot size corresponds to the mean relative abundance of each Genus by sample type and batch. Dots are coloured by sample type: reagent controls in lightest blue (Buffer), sampling negative controls in light blue, internal controls in dark blue (Kidney), and fetal gut samples in purple.

Author contributions. N.S. and J.W. conceived the project. K.M.K. and M.C.G. performed analyses and generated figures. K.M.K., M.C.G., M.E.P-M., F.D.B., M.A.E., S.C.G-V., M.G.G., M.W.H., A.J.M., R.C.M., E.G.P., J.P., F.S., D.M.S., G.C.S.S., G.W.T., A.W.W., and J.W. wrote the draft. All authors provided feedback, participated in discussions, and contributed to the final version of the manuscript.

Acknowledgements. T.B. receives funding from the Deutsche Forschungsgemeinschaft (German Research Foundation No. BR2925 10-1 & PL241 16-1). J.D. acknowledges funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (Grant agreement ERC-2017-AdG No. 788191 - Homo.symbiosus). W.M.dV. and A.S. are supported by the Academy of Finland (grants 1308255 and 1325103). A.M.E. is funded in part with Federal funds from the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health, Department of Health and Human Services, under Grant Number U19AI110818 to the Broad Institute. F.D.B. is funded by AI045008, AI120489, R33HL137063, CA219871, AI139240, and the PennCHOP Microbiome Program. M.A.E. is funded through grants R01HD102318, R01HD098867 and R01NR014784. S.C.G.V. was funded through a Peter Hans Hofschneider Professorship provided by the Stiftung Molekulare

Biomedizin. M.G.G. and D.M.S are funded by the Canada Research Chairs Program. L.J.H. is supported by Wellcome Trust Investigator Awards 100974/C/13/Z and 220876/Z/20/Z; the Biotechnology and Biological Sciences Research Council (BBSRC), Institute Strategic Programme Gut Microbes and Health BB/R012490/1, and its constituent projects BBS/E/F/000PR10353 and BBS/E/F/000PR10356. M.W.H. has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (Grant agreement No. 101019157). S.L. has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (Grant agreement 852600 Lacto-Be). A.J.M. receives funding from ERCAd HHMM-Neonates and Swiss National Science Sinergia. Work in P.W.O., L.O'M., and J.W. laboratories is supported by Science Foundation Ireland (SFI) through a Centre award (APC/SFI/12/RC/2273_P2) to APC Microbiome Ireland. J.W. acknowledges support through an SFI Professorship (19/RP/6853) and thanks Victoria McMahon for coordination of this review and Ryan O'Callaghan for encouragement. J.R. acknowledges funding from the Interuniversity Special Research Fund (iBOF) Flanders [FLEXIGUT R-11423], the Rega Institute, VIB and KU Leuven. N.S. receives funding from the European Research Council (ERC-STG project MetaPG-716575). F.S. is supported in part by Science Foundation Ireland. G.C.S.S. acknowledges funding from Medical Research Council (UK; MR/K021133/1) and the National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre (Women's Health theme). D.M.S. is funded by the Canadian Institute for Health Research and the Canada Research Chairs Program. A.W.W. receives core funding support from the Scottish Government's Rural and Environment Science and Analytical Services (RESAS). M.Y. is supported by the Azrieli Faculty Fellowship.