Questioning the fetal microbiome and pitfalls of low biomass microbial studies

Katherine M. Kennedy*[1,2], Marcus C. de Goffau*[3,4], Maria Elisa Perez-Muñoz[5], Marie-Claire Arrieta[6], Fredrik Bäckhed[7,8,9], Peer Bork[10, 11,12,13], Thorsten Braun[14], Frederic D. Bushman[15], Joel Dore[16], Willem M. de Vos[17,18], Ashlee M. Earl[19], Jonathan A. Eisen[20,21,22], Michal A. Elovitz, MD[23], Stephanie C. Ganal-Vonarburg[24,25], Michael G. Gänzle[5], Wendy S. Garrett[26,27,28,29], Lindsay J. Hall[30,31,32], Mathias W. Hornef[33], Curtis Huttenhower[26,29,34], Liza Konnikova[35], Sarah Lebeer[36], Andrew J. Macpherson[25], Ruth C. Massey[37,38], Alice Carolyn McHardy[39,40,41], Omry Koren[42], Trevor D. Lawley[4], Ruth E. Ley[43], Liam O'Mahony[37,38,44], Paul W. O'Toole[37,38], Eric G. Pamer[45], Julian Parkhill[46], Jeroen Raes[47,48], Thomas Rattei[49], Anne Salonen[17], Eran Segal[50], Nicola Segata[51,52], Fergus Shanahan[37,44], Deborah M. Sloboda[1,2,53], Gordon C.S. Smith[54,55], Harry Sokol[56,57,58], Tim D. Spector[59], Michael G. Surette[1,2,60], Gerald W. Tannock[61], Alan W. Walker[62], Moran Yassour[63,64], and Jens Walter[37,38,44]

1. Department of Biochemistry and Biomedical Sciences, McMaster University, Ontario, Hamilton, Canada
2. Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, Ontario, Canada
3. Department of Vascular Medicine, Academic Medical Centre, University of Amsterdam, 1105 AZ Amsterdam, the Netherlands
4. Wellcome Sanger Institute, Cambridge, UK
5. Department of Agriculture, Food and Nutrition Sciences, University of Alberta, Edmonton, AB, Canada
6. International Microbiome Center, University of Calgary
7. The Wallenberg Laboratory, Department of Molecular and Clinical Medicine, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
8. Region Västra Götaland, Sahlgrenska University Hospital, Department of Clinical Physiology, Gothenburg, Sweden
9. Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark
10. European Molecular Biology Laboratory, Structural and Computational Biology Unit, 69117 Heidelberg, Germany
11. Max Delbrück Centre for Molecular Medicine, 13125 Berlin, Germany
12. Yonsei Frontier Lab (YFL), Yonsei University, Seoul 03722, South Korea
13. Department of Bioinformatics, Biocenter, University of Würzburg, 97074 Würzburg, Germany
14. Department of Obstetrics and Experimental Obstetrics, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany
15. Department of Microbiology Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
16. Université Paris-Saclay, INRAE, MetaGenoPolis, AgroParisTech, MICALIS, 78350, Jouy-en-Josas, France

17. Human Microbiome Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland
18. Laboratory of Microbiology Wageningen University, The Netherlands
19. Infectious Disease & Microbiome Program, Broad Institute of MIT & Harvard, Boston, MA 02142
20. Department of Evolution and Ecology, University of California, Davis
21. Department of Medical Microbiology and Immunology, University of California, Davis
22. UC Davis Genome Center, University of California, Davis
23. Maternal and Child Health Research Center, Department of Obstetrics & Gynecology, University of Pennsylvania Perelman School of Medicine
24. Universitätsklinik für Viszerale Chirurgie und Medizin, Inselspital, Bern University Hospital, University of Bern, Switzerland
25. Department for Biomedical Research (DBMR), University of Bern, Bern, Switzerland
26. Department of Immunology and Infectious Diseases, Harvard T. H. Chan School of Public Health, Boston, Massachusetts
27. Harvard T. H. Chan Microbiome in Public Health Center, Boston, Massachusetts
28. Department of Medicine and Division of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts
29. Broad Institute of Harvard and MIT, Cambridge, Massachusetts
30. Quadram Institute Bioscience, Norwich Research Park, Norwich, NR4 7UQ, United Kingdom
31. Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, United Kingdom
32. Chair of Intestinal Microbiome, ZIEL - Institute for Food & Health, School of Life Sciences, Technical University of Munich, Freising, Germany
33. Institute of Medical Microbiology, RWTH University Hospital, 52074 Aachen, Germany
34. Department of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, MA, USA.
35. Departments of Pediatrics and Obstetrics, Gynecology and Reproductive Sciences, Yale School of Medicine, New Haven, CT, USA
36. University of Antwerp, Department of Bioscience Engineering, Groenenborgerlaan 171, 2020 Antwerp, Belgium
37. APC Microbiome Ireland, University College Cork, Cork, Ireland
38. School of Microbiology, University College Cork, Cork, Ireland
39. Computational Biology of Infection Research, Helmholtz Centre for Infection Research, Braunschweig, Germany
40. German Center for Infection Research (DZIF), Hannover Braunschweig site
41. Braunschweig Integrated Centre of Systems Biology (BRICS), Technische Universität Braunschweig, Braunschweig, Germany
42. Azrieli Faculty of Medicine, Bar-Ilan University, Safed, Israel
43. Department of Microbiome Science, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany
44. Department of Medicine, University College Cork, Cork, Ireland
45. Duchossois Family Institute, University of Chicago, Chicago, Illinois, USA
46. Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, CB3 0ES, UK
47. VIB Center for Microbiology, Herestraat 49, Leuven, Belgium
48. Department of Microbiology, Immunology and Transplantation, Rega Institute, KU Leuven, Herestraat 49, Leuven, Belgium
49. Centre for Microbiology and Environmental Systems Science, University of Vienna, Vienna, Austria
50. Weizmann Institute of Science, Israel
51. Department CIBIO, University of Trento, Trento, Italy
52. IEO, European Institute of Oncology IRCCS, Milan, Italy
53. Departments of Pediatrics, and Obstetrics and Gynecology, McMaster University, Hamilton, Ontario, Canada
54. Department of Obstetrics and Gynaecology, University of Cambridge, UK
55. NIHR Cambridge Biomedical Research Centre, Cambridge, CB2 0SW, UK
56. Sorbonne Université, INSERM, Centre de Recherche Saint-Antoine, CRSA, AP-HP, Saint Antoine Hospital, Gastroenterology department, F-75012 Paris, France

57. Paris Center for Microbiome Medicine (PaCeMM) FHU, Paris, France
58. INRA, UMR1319 Micalis & AgroParisTech, Jouy en Josas, France
59. Department of Twin Research, Kings College London, London SE1 7EH, UK
60. Department of Medicine, McMaster University, Hamilton, Ontario, Canada
61. Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand
62. Gut Health Group, Rowett Institute, University of Aberdeen, Aberdeen, Scotland, UK, AB25 2ZD.
63. School of Computer Science and Engineering, The Hebrew University of Jerusalem, Jerusalem 91904, Israel.
64. Department of Microbiology and Molecular Genetics, IMRIC, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91121, Israel.

*These authors contributed equally

Correspondence and requests for materials should be addressed to Jens Walter, Professor of Ecology, Food, and the Microbiome, APC Microbiome Ireland, School of Microbiology, and Department of Medicine, 4.05 Biosciences Building, University College Cork – National University of Ireland, Cork, T12 YT20, Ireland. Phone: +353 (0)21 490 1773; Email: jenswalter@ucc.ie

Preface

Whether the human fetus and the prenatal intrauterine environment (amniotic fluid, placenta) are stably colonised by microbial communities 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, microbial ecology, 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, or during 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 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[10] has been challenged recently[11-15], leading to the hypothesis that fetal immune development may be driven by the presence of live microbes at intrauterine sites[16-20]. Some groups have reported the presence of a microbiota[13], defined as a community of microorganisms in a defined habitat, or a microbiome[15], referring to a microbiota as well as their constituent genes and metabolites, which form a dynamic and interactive micro-ecosystem that is integrated within environments including eukaryotic hosts[21]. However, these interpretations have been debated[22-28] because several concurrent studies[29-35] point to contaminating microbial DNA in sequencing data from sites of low microbial biomass[36-38] as likely the only source of microbial DNA 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[39] and organs[40], 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[30,41].

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 system and other systems in early life and the anatomical and immunological mechanisms mediating host-microbe interactions within fetal tissues. Failure to resolve this issue risks 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 for the characterization of the microbiota in other low biomass samples, such as those derived from blood, the brain, other internal organs, and cancer tissues. Therefore, we assembled a trans-disciplinary group of scientists and clinician-scientists to examine experimental evidence relating to 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.

Claims and counterclaims

Although disagreement over the presence of microbes in prenatal intrauterine locations (placenta and amniotic fluid) spans dozens of studies with contradictory findings[12,14,15,23,29,31-34,37,42-44], we focused our analysis on four recent studies since they provide a direct assessment of the fetus itself[30,39-41]. Collection of human fetal samples is difficult and can only occur 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[39-41], and one collected samples from breech C-section deliveries immediately at birth[30].

Rackaityte *et al*.[39] 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* (six samples) or Micrococcaceae (nine samples), had distinct bacterial profiles. The authors further detected low amounts of total bacteria by quantitative polymerase chain reaction (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.*[45], 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[45]. In addition, the authors' suggestion that particles detected in SEM micrographs constitute micrococci[39] was disputed as their size exceeded that of known Micrococcaceae[45]. 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[46,47]).

Mishra *et al.*[40] 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 seven 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[40].

Both Rackaityte *et al*. and Mishra *et al*. included assays of fetal immune development and concluded that the microbes detected could contribute to immune maturation. In Rackaityte *et al*.[39] this conclusion was based on differences in T cell composition and epithelial transcription between fetal intestines in which Micrococcaceae was observed to be dominant and those in which this taxa was absent, leading to the suggestion that bacterial antigens may contribute to T cell activation and immunological memory *in utero*. Mishra *et al*.[40] 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.*[41], 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 microbially-derived metabolites present and enriched in the fetal intestinal samples and hypothesized that these microbiota-derived metabolites are passed via the mother's blood 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 originating from the maternal gut[48,49].

Kennedy *et al.*[30] used a different approach and collected samples using rectal swabs during elective C-section for breech presentation at term gestation[30]. 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[39,40], the bacterial taxa detected in fetuses derived by C-section[30] were significantly different (Figure 1). The number of genera was much lower in C-section-derived fetuses, and entire groups of microbes, especially those usually found in the vagina, were absent. Most importantly, in the studies that claimed fetal microbial colonisation[39,40], every genus detected in fetal samples was also detected in a majority of 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

cell 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[50], they would have to pass through to the amniotic cavity and enter the amniotic fluid to colonise the fetus. Amniotic fluid has antimicrobial properties, being enriched for example in lysozyme[51], human beta-defensin 2[52], and Gp340/Dmbt1[53], 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 placental microbiome showed some similarity with sequencing data of microbial communities of the oral cavity[15]. Contamination controls were not included in this early study, and subsequent evaluation of the work found that most genera detected were also common contaminants[26,36,38,54]. Several detected taxa, such as *Gloeobacter*, a genus of photosynthetic cyanobacteria, appeared biologically implausible as a component of a putative placental microbiome[24,55]. Since this early report, dozens of studies have conducted a sequence-based microbial analysis of placenta tissues, with opposing conclusions (as reviewed by Bolte *et al.*[20]).

Regardless of whether placental samples are collected by biopsy via the 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.*[29] carried out a comprehensive study of the possible placental microbiome, using samples from uncomplicated and complicated (pre-eclampsia and small for gestational age) pregnancies that were delivered both at term and pre-term either vaginally or via c-section.

Sampling was confined to the placental terminal villi (fetal tissue), as this represents the site of exchange (across the vasculosyncytial membrane) between the fetus and the mother's blood and tissues. The authors detected a range of species known to dominate the vaginal microbiota[56], 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, there was no evidence for a placental microbiome, which is in agreement with several additional recent studies[23,29,31-34,37].

Pathogenic infection of the placenta by viral or bacterial pathogens is a well-recognized clinical phenomenon that contributes to preterm birth and neonatal sepsis[57]. 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 labor[29]. The presence of this species is plausible as it colonises the genital tract of about 20% of women and has invasive potential, being an important cause of maternal and neonatal sepsis[58]. However, the ability of specific pathogens to colonise 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[21].

Research claiming the presence of viable low-density microbial communities in the fetal intestine[39] and fetal organs[40] 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[40]. This procedure typically involves the individual going through hours of labour and often leads to the rupture of the fetal membranes hours prior to vaginal delivery. Even with a standardized approach, labour may be prolonged and may be accompanied by infection and fever, which are common with 2nd trimester terminations[59,60]. Both Li *et al*.[41] and Rackaityte *et al*.[39] 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.

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[39,40] have not established the nature of the host-microbe interaction, and the duration of exposure or colonisation, 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.*[39] and Mishra *et al.*[40] are, in large part, robust, and well suited to study symbiotic microbial populations. The combination of 16S rRNA gene sequencing, 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.*[39] and Mishra *et al.*[40] found the genera *Lactobacillus* and *Gardnerella*, which dominate the vaginal microbiota[56], among their most consistent findings (Figure 1). The species cultured by Mishra *et al.*, *G. vaginalis*, *L. iners* and *L. jensenii*, are largely restricted 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 a considerable limitation that casts doubt on the authors' conclusion that the microbes originate from the womb. Indeed, Li *et al*.[41], obtained samples from 2nd trimester surgical terminations using mechanical dilatation, which reduces the risk of bacterial exposure to the fetus during sampling. In this study, positive bacterial PCR results were not reported, which raises the possibility that sampling contamination may be a serious confounder in both of the other studies claiming the presence of microbes at these sites.

Although vaginal controls were not included by Rackaityte *et al.*[39] and Mishra *et al.*[40], direct comparisons of their findings with those by Kennedy *et al*.[30] also provide 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[30,54]. Despite attempts to reduce contamination, C-

section derived fetal meconium had at least one positive culture[30]. 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 colonisation by a stable functional microbial community.

In addition to the potential of contaminant detection, the bacterial load found in terminated fetuses was extremely low[39,40]. 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[40]). Such findings are more likely a result of contamination rather than colonisation.

Neonatal meconium samples have been studied for a century by culture-based methods, and more recently by DNA sequencing. Evaluations of such samples are also associated with contradictory findings[11,43,44,63], likely due to contamination[64] and because postnatal colonisation may occur before the first passage of meconium[26]. However, when meconium is passed soon after birth, culturable bacteria are seldom detected (as reviewed by Perez-Munoz *et al*.[26]). In agreement with this, an analysis of meconium samples collected from extremely premature infants[65] showed that taxa regularly identified as contaminants[36,38] make up a large proportion of sequences collected within the first 3 days after delivery and which drop to levels below 1% of the total microbiota profile in most samples at days 4-6 (Figure 2). This indicated that bacterial sequences that cannot be assigned to contamination are initially rare in early meconium, which is consistent with a recent study that applied strict controls for sequencing and culture and did not detect a meconium microbiota[64].

Members of an authentic fetal microbiota should be, in theory, detectable in early life faecal samples independent of birth mode. There is indeed some overlap between the reported fetal microbial taxa in vaginal versus C-section deliveries[39,40], e.g. staphylococci, enterococci, lactobacilli, and enterobacteria, and the microbiota detected in infant faecal 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 in theory detect *in utero* bacterial exposure as the fetus swallows amniotic fluid (as demonstrated by the detection of pathogenic *Ureaplasma* species[70]). 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 contained low microbial loads near the detection limit and 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* spp. and *Corynebacterium* spp.) and were strongly associated with C-section, or in the case of vaginal deliveries, species that are common in the maternal faecal microbiota (*Escherichia coli* & *Bacteroides fragilis*) (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 colonisers 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 faecal samples of infants born by C-sections[66,67,71-73]. A substantial proportion of strains acquired by infants postnatally can be traced back to their mother's faecal samples[73-75], and faecal microbiota transplant (FMT) from the mother restores the microbiome in C-section delivered infants[76]. Thus, the published evidence, although

incomplete, suggests that the early life microbiota in humans is acquired through the vertical and horizontal transfer of microbes whose origin is faecal 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, labware, tools, instruments, and DNA isolation kits,[36-38] and through cross-contamination between PCR tubes/wells, sequencing runs, or sequencing lanes[37]. 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[45]. Overreliance on or under-analysis of such negative controls, in combination with the misapplication of contamination removal programs like Decontam[77], specifically by not having negative controls in all batches, frequently results in false positive signals owing to the detection of contaminants[45]. Even with appropriate controls, it is challenging to separate genuine signals from low abundance contaminants as signals may appear sporadically in samples and negative controls[78]. Thus, suboptimal processing of sequencing control samples may not reveal the full spectrum of contaminants because only the most abundant contaminant species are consistently detected. On the other hand, potentially genuine sample-associated signals sometimes also erroneously appear in negative control samples through cross-contamination during the PCR or sequencing steps (machine contamination)[37].

In the case of both Rackaityte *et al.*[39] and Mishra *et al.*[40] many of the taxa reported are common contaminants (Figure 1). The most obvious case is *Bradyrhizobium*, which is one of the most dominant and consistent contaminants found in sequencing studies[38,79]. Rackaityte *et al.* interpreted the presence of *Micrococcus* and *Lactobacillus* as genuine fetal inhabitants, but a re-analysis of the data suggested that these findings were a result of batch effects (indicative of contamination[45]). Although the authors rejected this interpretation[AU: ok?][61], this batch effect is clearly visible if the findings of the different batches are plotted together (Figure 3). Furthermore, in the study by Mishra *et al*., the authors concluded that *Micrococcus* was a likely [40]contaminant[40], while the genera *Afipia*, *Flavobacterium*, *Pseudomonas* and *Brevundimonas* were reported as part of the fetal microbiota.[40], although these taxa are also commonly detected as kit or laboratory reagent contaminants[36,38].

Mishra *et al*. and Rackaityte *et al.* also reported marginally higher total bacterial load in fetal samples, as compared to controls, using qPCR[39,40]. However, nucleic acids (DNA, RNA, and tRNA) in tissue samples (which is absent in negative controls) might have a DNA carrier effect[80], leading to a more efficient DNA precipitation of prokaryotic material**.** In addition, bacterial PCR primers that target the 16S rRNA gene can also amplify mitochondrial DNA[81], which is evolutionarily of bacterial origin. Together these factors offer alternative explanations for a higher microbial burden in samples from low biomass sites compared to controls. Rackaityte *et al*. removed human mitochondrial DNA (mtDNA) from their 16S rRNA gene sequence-based results that co-amplified in the PCR, but neither study accounted for mtDNA in their qPCR analysis, although their qPCR primers targeted the 16S rRNA gene and were therefore potentially susceptible to cross-reactivity[39,40].

Immunological perspective

458 The enteric microbiota is a potent driver of adaptive mucosal immune maturation and priming in
459 the adult host[82-85]. Besides their intrinsic immunogenic nature, microorganisms also generate
460 metabolites that promote and shape immune maturation and priming[86-88]. Although the early fetal
461 immune system is immature, recent research demonstrates migration of fetal dendritic cells (DCs)
462 to the mesenteric lymph nodes; somatic hypermutation in fetal B cells; and an expansion of T cell
463 receptor repertoire diversity, evenness and activation during late fetal development[7,89,90].
464
465 The existence of metabolically active microbes in the fetus could, in principle, provide one
466 possible explanation for these findings. Mishra *et al.*[40] used an autologous T cell expansion assay
467 to show that fetal DCs loaded with antigen from bacteria that had been isolated from fetal tissues
468 stimulated proliferation of CD45RO+ and CD69+ T cells. T cell proliferation was reduced but still
469 detectable in the absence of DC-derived cytokine release suggesting an activated memory
470 response[40]. Demonstration that the fetal T cell memory response is specific for the bacteria
471 present in one individual fetus would be necessary to strengthen the interpretation that specific
472 immune responses are routinely driven by fetal bacterial colonisation.
473
474 There are alternative explanations for fetal immune responses apart from *bona fide* microbial
475 colonisation. Maternal antigen-IgG complexes have been detected in cord blood, and
476 transplacental immune priming of the fetal immune system in early gestation has been
477 demonstrated[91,92] Cross-reactivity, as observed for microbiota reactive enteric secretory
478 immunoglobulin A, would support fetal priming by maternal microbial antigens[87]. Similarly,
479 maternal microbiota-derived molecules partly bound to IgG stimulated innate immune maturation
480 of the murine fetal gut[48], and maternal intestinal carriage of *Prevotella* has been reported to protect
481 the offspring from food allergy in humans[93]. Thus, maternal microbiota-derived antigens and

metabolites can pass the placental filter directly or bound to IgG and offer an alternative explanation for the observed fetal immune responses[94].

The hypothesis of a low biomass fetal microbiome requires the identification of host 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[57]. Given that such immunological and anatomical mechanisms have not been identified or even proposed[28], the observed immune maturation and priming during fetal development is most likely not induced through colonisation 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[57].

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[95], 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[96]. The claims for non-pathogenic fetal microbial exposure[39,40] 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*[39,40]. Mishra *et al.* also report successful culturing of lactobacilli and staphylococci from fetal tissue[40], 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[57]. 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[46,47]. Lactobacilli are usually of low pathogenic potential, they inhabit external mucosal surfaces of healthy humans, including the nose[97] and vagina[56], and they are often used as probiotics[98]. However, some strains and species of lactobacilli do express potential virulence factors[99-101], resist oxidative stress[102] and grow in the absence of iron[103], which allows them to cause serious infections such as endocarditis when provided with the opportunity to access the bloodstream[104,105]. This raises potential problems with the interpretation of lactobacilli being asymptomatic colonisers 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)[40]. These organisms can be long-term colonisers of external mucosal surfaces of humans [106,107] 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 that kill human cells[108,109]. CoNS, on the other hand, are ubiquitous skin colonisers. Their detection in clinical diagnostic laboratories is so common that it is considered a major diagnostic challenge[110,111] 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[112]. Therefore, given that they are either contaminants or overt pathogens, the detection of staphylococci, no matter whether *S. aureus* or CoNS, is difficult to reconcile with *in utero* colonisation 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 colonisation 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[113]. Several ESKAPE pathogens readily survive in adverse conditions outside of vertebrate hosts, including drying, oxidative stress, and exposure to heat or sanitation chemicals[114]. They are likely to persist on inanimate surfaces including utensils or clinical fabrics[115,116], thereby increasing their likelihood of being contaminants. While these microorganisms were not reported at the species level[40], it is noteworthy that closely related

organisms can also cause neonatal sepsis[117-119], which makes them unlikely colonisers 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[120,121]. 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[39,40] 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[122-125].

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[126-128]. 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[26]), which have long been used to study the biochemical, metabolic, and immunological

influences of microbes on their mammalian hosts[129-131]. The primary consideration is whether germ-free animals are truly 'free of all demonstrable forms of microbial life'[132]. If they lack microbial associates, there cannot be a fetal microbiome. Testing germ-free animals for contaminating microbes uses microscopic observation of stained faecal smears, culture of faeces in nutrient media under various conditions of temperature and gaseous atmosphere[127,132-134], PCR using 'universal bacterial' primers[133,135], and serological assays for viral infections[136]. These tests consistently demonstrate an absence of microbial associates. Therefore, gnotobiology provides strong evidence that the fetus *in utero* is sterile.

A healthy human fetus is 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 colonisers 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 in the validity of our multi-layered approach.

The processes by which the fetus matures and becomes immunologically equipped for life in a microbial world have life-long implications. Aside from the caution and safeguards recommended in this perspective, our aim here is not to dissuade scientists from exploring the microbial drivers of fetal immune development. We agree with proposals that there is a need to better understand microbial interactions at the maternal-fetal interface[20], but do not think that symbiotic microbial populations in the placenta or fetus play a role in this. Paradoxically, we contend that sterile tissues are both immunologically and microbiologically fascinating but require an adjustment of the methodological approaches used. How does the fetus mature and become immunologically

equipped for life in a microbial world in the absence of direct exposure to live microbes? Are maternal-derived microbial metabolites sufficient for fetal immune education? Future research could include exploration of how maternal microbial-derived metabolites and small molecules, as well as maternal immune components, prepare the fetus for the microbial challenges of post-natal life[94].

Lessons for low biomass research

Contamination is always a potential confounder in microbiology but is of particular concern for those studying low- or no biomass samples[36,38]. The issue has been highlighted by recent reports of human tissues, such as blood, brain, and cancers (Box 1), previously thought to contain no, or very little, bacterial biomass that apparently harbour diverse microbial communities. As with intrauterine studies described above, these microbial populations are often discussed considering their perceived importance for human diseases and health.

In studies on low biomass samples, it is challenging to identify relevant signals from among contaminating noise. In instances of contamination, a tissue may be misjudged as non-sterile, whereas in others, a real microbiological signal may be obfuscated by contamination. The removal of all sequences present in negative-control samples or that have been previously identified as contaminants in the literature may result in loss of authentic signals. Post-sequencing contamination removal using software packages such as Decontam[77] or other statistical approaches[36,137] have been developed to remove the more abundant contaminants, leading to microbiome profiles that are more likely to reflect the real community. Practical examples of contamination removal in 16S rRNA gene sequence data is provided by Heida *et al*.[65], Saffarian *et al.*[138], and Jorissen *et al*.[139] and we expand on these examples in Box 1.

We draw attention to the distinction between “low biomass” and “no (zero) biomass” samples. This has practical significance; true “low (microbial) biomass” samples are amenable to contamination-removal approaches but “no (microbial) biomass” samples require a different approach (Box 1). For credible proposals of the presence of microbes, multiple layers of evidence are required, first with quantitative, sensitive (lower detection limit) approaches, such as qPCR with strict controls before contamination-sensitive sequencing approaches are applied. Since contamination removal will provide data regardless of whether microbes are present or absent, the starting proposition should be the null hypothesis to avoid confirmation bias, particularly when results are inconsistent and at the outer technical limits for detection, or if results defy mechanistic plausibility.

Given the limitation of sequencing approaches, confirmation by alternative methods, such as FISH and culture, are required. However, as demonstrated with recent studies of fetal samples, even a combination of approaches has the potential to produce false findings, as contamination during sampling is a considerable challenge. We posit that studies on all low biomass samples can benefit from a similar trans-disciplinary assessment, as applied above for fetal samples, to interpret findings considering biological and mechanistic explanations[28]. When obligately photosynthetic, psychrophilic, thermophilic, halophilic, or chemolithoautotrophic bacteria are found in human tissues that do not provide the growth conditions for such organisms[24,140], or if the detected genera are known contaminants of laboratory kits/reagents (such as readily culturable Proteobacteria like *Pseudomonas and E. coli* for example)[141-143], the authenticity of such signals should be questioned.

Figure legends

Figure 1. Relative abundance of bacterial taxa from three recent fetal studies. Distribution and mean relative abundance (%) of taxa present in fetal samples from three recent studies[30,39,40] 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 detected as enriched in fetal samples as described by Mishra *et al.*[40] (indicated by ^) or by Rackaityte *et al.*[39] (indicated by *, including the family Micrococcaceae); all genera detected in fetal samples from Kennedy *et al.*[30]; and the PBS-enriched genus *Ralstonia*[40]. 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[36-38]. 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 or family level (for Micrococcaceae). The mean relative abundance of each taxon 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 by sample type and study (mean relative abundances <0.0001% were excluded). Dots are coloured 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). Parts of this figure were created with BioRender.com.

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 collected in a previous study[65] 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). Colours 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. Relative abundance of bacterial taxa in samples from *Rackaityte et al.*[39]. Distribution and mean relative abundance (%) of taxa present in fetal and control samples from Rackaityte *et al.*[39] by batch as defined by *Rackaityte et al.*[61]. Dominant taxa were selected as described in Fig. 1. 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 or family (for Micrococcaceae) level. The mean relative abundance of each taxon 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 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.

Box 1: Experimental considerations for low biomass research

High biomass samples

***Examples*:** Faeces, dental plaque, wastewater, soil.

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

***Mitigations*:** Experimental design seldom needs to be significantly adjusted to account for contamination. Inclusion of "blank" negative sequencing controls and removing samples with significant contamination levels using basic post sequencing analysis 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*:** Low to High: Contaminated samples are progressively affected with reducing input microbial biomass[38].

***Mitigations*:** Inclusion of multiple controls for contamination recognition. Ideally, samples should be concentrated before processing to increase input biomass. 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. DNA extraction may benefit from use of non-kit-based methods (e.g., phenol-chloroform extractions) where plasticware andreagents 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 kits. Regardless of the DNA extraction method, the presence of contaminants should be monitored by including "blank" negative controls. 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 described by de Goffau et al.[36] and used by Heida et al.[65] will usually identify contaminants.

Samples in which the existence of microbes is not established (potential "No (zero) 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 directed specifically against contamination. 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 sequencing approaches. Such techniques 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)[45,144]. All mitigations outlined for "Low biomass" samples above should be adopted. Repeating sample processing with different DNA extraction kits/methods[32] and/or at different days can be informative[145]. These will track the presence of species in sequencing profiles associated with specific kits/reagents or environment. Species that are repeatedly detected regardless of technical approach are more likely to be genuine signals, unless they were introduced during sample collection. Binary statistics (absence/presence) are recommended. The presence of microbes identified by sequencing should be verified with a different technique such as cultivation, another sequencing technique

753 with sufficient taxonomic resolution, and/or species-specific qPCR or FISH using high
754 magnification to visualize the size and morphology of individual microbial cells.
755
756 **Author contributions.** N.S. and J.W. conceived the project. K.M.K. and M.C.G. performed
757 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.,
758 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
759 the draft. All authors provided feedback, participated in discussions, and contributed to the final
760 version of the manuscript.
761
762 **Competing Interests**
763 The authors declare no competing interests in relation to this manuscript.
764
765 **Acknowledgements.** T.B. receives funding from the Deutsche Forschungsgemeinschaft
766 (German Research Foundation No. BR2925 10-1 & PL241 16-1). F.D.B. is funded by AI045008,
767 AI120489, R33HL137063, CA219871, AI139240, and the PennCHOP Microbiome Program. J.D.
768 acknowledges funding from the European Research Council (ERC) under the European Union's
769 Horizon 2020 research and innovation programme (Grant agreement ERC-2017-AdG No. 788191
770 - Homo.symbiosus). W.M.dV. and A.S. are supported by the Academy of Finland (grants 1308255
771 and 1325103). A.M.E. is funded in part with Federal funds from the National Institute of Allergy
772 and Infectious Diseases (NIAID), National Institutes of Health, Department of Health and Human
773 Services, under Grant Number U19AI110818 to the Broad Institute.. M.A.E. is funded through
774 grants R01HD102318, R01HD098867 and R01NR014784. S.C.G.V. was funded through a Peter
775 Hans Hofschneider Professorship provided by the Stiftung Molekulare Biomedizin. M.G.G. and
776 D.M.S are funded by the Canada Research Chairs Program. L.J.H. is supported by Wellcome
777 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. O.K. is supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (Grant agreement ERC-2020-COG No. 101001355). 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.

References

1 Macpherson, A. J., de Aguero, M. G. & Ganal-Vonarburg, S. C. How nutrition and the maternal microbiota shape the neonatal immune system. *Nat Rev Immunol* **17**, 508-517, doi:10.1038/nri.2017.58 (2017).

2 Kalbermatter, C., Fernandez Trigo, N., Christensen, S. & Ganal-Vonarburg, S. C. Maternal Microbiota, Early Life Colonization and Breast Milk Drive Immune Development in the Newborn. *Front Immunol* **12**, 683022, doi:10.3389/fimmu.2021.683022 (2021).

3 Gensollen, T., Iyer, S. S., Kasper, D. L. & Blumberg, R. S. How colonization by microbiota in early life shapes the immune system. *Science* **352**, 539-544, doi:10.1126/science.aad9378 (2016).

4 Jain, N. The early life education of the immune system: Moms, microbes and (missed) opportunities. *Gut Microbes* **12**, 1824564, doi:10.1080/19490976.2020.1824564 (2020).

5 Hornef, M. W. & Torow, N. 'Layered immunity' and the 'neonatal window of opportunity' - timed succession of non-redundant phases to establish mucosal host-microbial homeostasis after birth. *Immunology* **159**, 15-25, doi:10.1111/imm.13149 (2020).

6 Torow, N., Marsland, B. J., Hornef, M. W. & Gollwitzer, E. S. Neonatal mucosal immunology. *Mucosal Immunol* **10**, 5-17, doi:10.1038/mi.2016.81 (2017).

7 Schreurs, R. *et al.* Human Fetal TNF-alpha-Cytokine-Producing CD4(+) Effector Memory T Cells Promote Intestinal Development and Mediate Inflammation Early in Life. *Immunity* **50**, 462-476 e468, doi:10.1016/j.immuni.2018.12.010 (2019).

8 Stras, S. F. *et al.* Maturation of the Human Intestinal Immune System Occurs Early in Fetal Development. *Dev Cell* **51**, 357-373 e355, doi:10.1016/j.devcel.2019.09.008 (2019).

9 Zhang, X. *et al.* CD4 T cells with effector memory phenotype and function develop in the sterile environment of the fetus. *Sci Transl Med* **6**, 238ra272, doi:10.1126/scitranslmed.3008748 (2014).

10 Tissier, H. *Recherches sur la flore intestinale des nourrissons (état normal et pathologique)*. (1900).

11 He, Q. *et al.* The meconium microbiota shares more features with the amniotic fluid microbiota than the maternal fecal and vaginal microbiota. *Gut Microbes* **12**, 1794266, doi:10.1080/19490976.2020.1794266 (2020).

12 Stinson, L. *et al.* Comparison of Bacterial DNA Profiles in Mid-Trimester Amniotic Fluid Samples From Preterm and Term Deliveries. *Front Microbiol* **11**, 415, doi:10.3389/fmicb.2020.00415 (2020).

13 Younge, N. *et al.* Fetal exposure to the maternal microbiota in humans and mice. *JCI Insight* **4**, doi:10.1172/jci.insight.127806 (2019).

14 Stinson, L. F., Boyce, M. C., Payne, M. S. & Keelan, J. A. The Not-so-Sterile Womb: Evidence That the Human Fetus Is Exposed to Bacteria Prior to Birth. *Front Microbiol* **10**, 1124, doi:10.3389/fmicb.2019.01124 (2019).

15 Aagaard, K. *et al.* The placenta harbors a unique microbiome. *Sci Transl Med* **6**, 237ra265, doi:10.1126/scitranslmed.3008599 (2014).

16 D'Argenio, V. The Prenatal Microbiome: A New Player for Human Health. *High Throughput* **7**, doi:10.3390/ht7040038 (2018).

17 Funkhouser, L. J. & Bordenstein, S. R. Mom knows best: the universality of maternal microbial transmission. *PLoS Biol* **11**, e1001631, doi:10.1371/journal.pbio.1001631 (2013).

18 Stinson, L. F., Payne, M. S. & Keelan, J. A. Planting the seed: Origins, composition, and postnatal health significance of the fetal gastrointestinal microbiota. *Crit Rev Microbiol* **43**, 352-369, doi:10.1080/1040841X.2016.1211088 (2017).

19 Walker, R. W., Clemente, J. C., Peter, I. & Loos, R. J. F. The prenatal gut microbiome: are we colonized with bacteria in utero? *Pediatr Obes* **12 Suppl 1**, 3-17, doi:10.1111/ijpo.12217 (2017).

20 Bolte, E. E., Moorshead, D. & Aagaard, K. M. Maternal and early life exposures and their potential to influence development of the microbiome. *Genome Med* **14**, 4, doi:10.1186/s13073-021-01005-7 (2022).
21 Berg, G. *et al.* Microbiome definition re-visited: old concepts and new challenges. *Microbiome* **8**, 103, doi:10.1186/s40168-020-00875-0 (2020).
22 Blaser, M. J. *et al.* Lessons learned from the prenatal microbiome controversy. *Microbiome* **9**, 8, doi:10.1186/s40168-020-00946-2 (2021).
Discussion about the prenatal microbiome controversy by several experts of the microbiome field.
23 Bushman, F. D. De-Discovery of the Placenta Microbiome. *Am J Obstet Gynecol* **220**, 213-214, doi:10.1016/j.ajog.2018.11.1093 (2019).
24 Editorial. Microbiome studies and "blue whales in the Himalayas". *Lancet Infect Dis* **18**, 925, doi:10.1016/S1473-3099(18)30503-6 (2018).
25 Hornef, M. & Penders, J. Does a prenatal bacterial microbiota exist? *Mucosal Immunol* **10**, 598-601, doi:10.1038/mi.2016.141 (2017).
26 Perez-Munoz, M. E., Arrieta, M. C., Ramer-Tait, A. E. & Walter, J. A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome. *Microbiome* **5**, 48, doi:10.1186/s40168-017-0268-4 (2017).
27 Segata, N. No bacteria found in healthy placentas. *Nature* **572**, 317-318, doi:10.1038/d41586-019-02262-8 (2019).
28 Walter, J. & Hornef, M. W. A philosophical perspective on the prenatal in utero microbiome debate. *Microbiome* **9**, 5, doi:10.1186/s40168-020-00979-7 (2021).
29 de Goffau, M. C. *et al.* Human placenta has no microbiome but can contain potential pathogens. *Nature* **572**, 329-334, doi:10.1038/s41586-019-1451-5 (2019).
Sequencing study using robust controls concluding that there is no evidence for a placental microbiome.
30 Kennedy, K. M. *et al.* Fetal meconium does not have a detectable microbiota before birth. *Nat Microbiol*, doi:10.1038/s41564-021-00904-0 (2021).
The only sequencing study to date that characterized the microbial populations in human fetuses using meconium samples obtained after c-section, concluding no evidence for a microbiota.
31 Kuperman, A. A. *et al.* Deep microbial analysis of multiple placentas shows no evidence for a placental microbiome. *BJOG* **127**, 159-169, doi:10.1111/1471-0528.15896 (2020).
32 Lauder, A. P. *et al.* Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. *Microbiome* **4**, 29, doi:10.1186/s40168-016-0172-3 (2016).
33 Leiby, J. S. *et al.* Lack of detection of a human placenta microbiome in samples from preterm and term deliveries. *Microbiome* **6**, 196, doi:10.1186/s40168-018-0575-4 (2018).
34 Theis, K. R. *et al.* Does the human placenta delivered at term have a microbiota? Results of cultivation, quantitative real-time PCR, 16S rRNA gene sequencing, and metagenomics. *Am J Obstet Gynecol* **220**, 267 e261-267 e239, doi:10.1016/j.ajog.2018.10.018 (2019).
35 Sterpu, I. *et al.* No evidence for a placental microbiome in human pregnancies at term. *Am J Obstet Gynecol* **224**, 296 e291-296 e223, doi:10.1016/j.ajog.2020.08.103 (2021).
36 de Goffau, M. C. *et al.* Recognizing the reagent microbiome. *Nat Microbiol* **3**, 851-853, doi:10.1038/s41564-018-0202-y (2018).

37 Olomu, I. N. *et al.* Elimination of "kitome" and "splashome" contamination results in lack of detection of a unique placental microbiome. *BMC Microbiol* **20**, 157, doi:10.1186/s12866-020-01839-y (2020).
38 Salter, S. J. *et al.* Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol* **12**, 87, doi:10.1186/s12915-014-0087-z (2014).
39 Rackaityte, E. *et al.* Viable bacterial colonization is highly limited in the human intestine in utero. *Nat Med* **26**, 599-607, doi:10.1038/s41591-020-0761-3 (2020).
Microbial characterisation of fetal samples obtained after vaginal delivery reporting highly limited bacterial colonization.
40 Mishra, A. *et al.* Microbial exposure during early human development primes fetal immune cells. *Cell*, doi:10.1016/j.cell.2021.04.039 (2021).
Analysis of fetal tissues obtained after medical termination of pregnancy in the 2nd trimester and vaginal delivery reporting microbial colonization of the fetus and bacterial priming of fetal immune cells.
41 Li, Y. *et al.* In utero human intestine harbors unique metabolomic features including bacterial metabolites. *JCI Insight*, doi:10.1172/jci.insight.138751 (2020).
Characterization of the microbiota in fetuses obtained by vaginal delivery reporting no evidence for bacterial colonization.
42 Lim, E. S., Rodriguez, C. & Holtz, L. R. Amniotic fluid from healthy term pregnancies does not harbor a detectable microbial community. *Microbiome* **6**, 87, doi:10.1186/s40168-018-0475-7 (2018).
43 Liu, Y. *et al.* Midtrimester amniotic fluid from healthy pregnancies has no microorganisms using multiple methods of microbiologic inquiry. *Am J Obstet Gynecol* **223**, 248 e241-248 e221, doi:10.1016/j.ajog.2020.01.056 (2020).
44 Rehbinder, E. M. *et al.* Is amniotic fluid of women with uncomplicated term pregnancies free of bacteria? *Am J Obstet Gynecol* **219**, 289 e281-289 e212, doi:10.1016/j.ajog.2018.05.028 (2018).
45 de Goffau, M. C., Charnock-Jones, D. S., Smith, G. C. S. & Parkhill, J. Batch effects account for the main findings of an in utero human intestinal bacterial colonization study. *Microbiome* **9**, 6, doi:10.1186/s40168-020-00949-z (2021).
46 Powell, S., Perry, J. & Meikle, D. Microbial contamination of non-disposable instruments in otolaryngology out-patients. *J Laryngol Otol* **117**, 122-125, doi:10.1258/002221503762624567 (2003).
47 Wistrand, C., Soderquist, B. & Sundqvist, A. S. Time-dependent bacterial air contamination of sterile fields in a controlled operating room environment: an experimental intervention study. *J Hosp Infect* **110**, 97-102, doi:10.1016/j.jhin.2021.01.016 (2021).
48 Gomez de Aguero, M. *et al.* The maternal microbiota drives early postnatal innate immune development. *Science* **351**, 1296-1302, doi:10.1126/science.aad2571 (2016).
Study demonstrating that aspects of prenatal immune development induced by maternal microbial compounds can occur in the absence of live microbes in the fetus.
49 Vuong, H. E. *et al.* The maternal microbiome modulates fetal neurodevelopment in mice. *Nature* **586**, 281-286, doi:10.1038/s41586-020-2745-3 (2020).
50 Baker, J. M., Chase, D. M. & Herbst-Kralovetz, M. M. Uterine Microbiota: Residents, Tourists, or Invaders? *Front Immunol* **9**, 208, doi:10.3389/fimmu.2018.00208 (2018).
51 Cherry, S. H., Filler, M. & Harvey, H. Lysozyme content of amniotic fluid. *Am J Obstet Gynecol* **116**, 639-642, doi:10.1016/s0002-9378(15)33127-6 (1973).

52 Soto, E. *et al.* Human beta-defensin-2: a natural antimicrobial peptide present in amniotic fluid participates in the host response to microbial invasion of the amniotic cavity. *J Matern Fetal Neonatal Med* **20**, 15-22, doi:10.1080/14767050601036212 (2007).

53 Reichhardt, M. P. *et al.* The salivary scavenger and agglutinin in early life: diverse roles in amniotic fluid and in the infant intestine. *J Immunol* **193**, 5240-5248, doi:10.4049/jimmunol.1401631 (2014).

54 Sinha, R. *et al.* Assessment of variation in microbial community amplicon sequencing by the Microbiome Quality Control (MBQC) project consortium. *Nat Biotechnol* **35**, 1077-1086, doi:10.1038/nbt.3981 (2017).

55 Grettenberger, C. L. Novel Gloeobacterales spp. from Diverse Environments across the Globe. *mSphere* **6**, e0006121, doi:10.1128/mSphere.00061-21 (2021).

56 Ravel, J. *et al.* Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A* **108 Suppl 1**, 4680-4687, doi:10.1073/pnas.1002611107 (2011).

57 Megli, C. J. & Coyne, C. B. Infections at the maternal-fetal interface: an overview of pathogenesis and defence. *Nat Rev Microbiol* **20**, 67-82, doi:10.1038/s41579-021-00610-y (2022).

58 Armistead, B., Oler, E., Adams Waldorf, K. & Rajagopal, L. The Double Life of Group B Streptococcus: Asymptomatic Colonizer and Potent Pathogen. *J Mol Biol* **431**, 2914-2931, doi:10.1016/j.jmb.2019.01.035 (2019).

59 Dodd, J. M. & Crowther, C. A. Misoprostol for induction of labour to terminate pregnancy in the second or third trimester for women with a fetal anomaly or after intrauterine fetal death. *Cochrane Database Syst Rev*, CD004901, doi:10.1002/14651858.CD004901.pub2 (2010).

60 Nijman, T. A. *et al.* Association between infection and fever in terminations of pregnancy using misoprostol: a retrospective cohort study. *BMC Pregnancy Childbirth* **17**, 7, doi:10.1186/s12884-016-1188-1 (2017).

61 Rackaityte, E. *et al.* Corroborating evidence refutes batch effect as explanation for fetal bacteria. *Microbiome* **9**, 10, doi:10.1186/s40168-020-00948-0 (2021).

62 Duar, R. M. *et al.* Lifestyles in transition: evolution and natural history of the genus Lactobacillus. *FEMS Microbiol Rev* **41**, S27-S48, doi:10.1093/femsre/fux030 (2017).

63 Dominguez-Bello, M. G. *et al.* Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci U S A* **107**, 11971-11975, doi:10.1073/pnas.1002601107 (2010).

64 Dos Santos, S. J. *et al.* Early Neonatal Meconium Does Not Have a Demonstrable Microbiota Determined through Use of Robust Negative Controls with cpn60-Based Microbiome Profiling. *Microbiol Spectr* **9**, e0006721, doi:10.1128/Spectrum.00067-21 (2021).

65 Heida, F. H. *et al.* Weight shapes the intestinal microbiome in preterm infants: results of a prospective observational study. *BMC Microbiol* **21**, 219, doi:10.1186/s12866-021-02279-y (2021).

66 Backhed, F. *et al.* Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host Microbe* **17**, 690-703, doi:10.1016/j.chom.2015.04.004 (2015).

67 Shao, Y. *et al.* Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature* **574**, 117-121, doi:10.1038/s41586-019-1560-1 (2019).

68 Podlesny, D. & Fricke, W. F. Strain inheritance and neonatal gut microbiota development: A meta-analysis. *Int J Med Microbiol* **311**, 151483, doi:10.1016/j.ijmm.2021.151483 (2021).

69 Bajorek, S. *et al.* Initial microbial community of the neonatal stomach immediately after birth. *Gut Microbes* **10**, 289-297, doi:10.1080/19490976.2018.1520578 (2019).

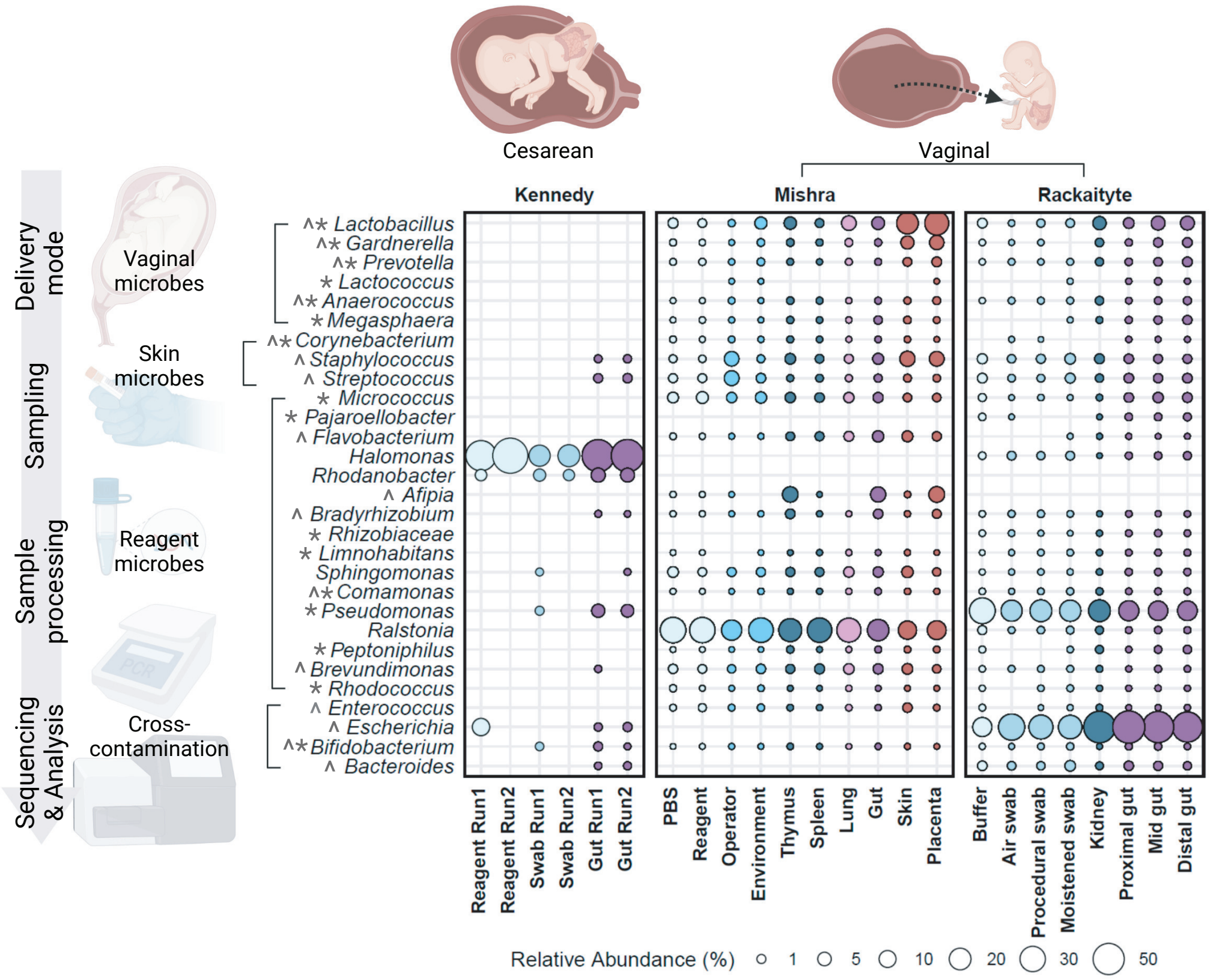
70 Kim, S. M. *et al.* Gastric fluid versus amniotic fluid analysis for the identification of intra-amniotic infection due to Ureaplasma species. *J Matern Fetal Neonatal Med* **29**, 2579-2587, doi:10.3109/14767058.2015.1098614 (2016).
71 Martin, R. *et al.* Early-Life Events, Including Mode of Delivery and Type of Feeding, Siblings and Gender, Shape the Developing Gut Microbiota. *PLoS One* **11**, e0158498, doi:10.1371/journal.pone.0158498 (2016).
72 Yassour, M. *et al.* Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. *Sci Transl Med* **8**, 343ra381, doi:10.1126/scitranslmed.aad0917 (2016).
73 Mitchell, C. M. *et al.* Delivery Mode Affects Stability of Early Infant Gut Microbiota. *Cell Rep Med* **1**, 100156, doi:10.1016/j.xcrm.2020.100156 (2020).
74 Ferretti, P. *et al.* Mother-to-Infant Microbial Transmission from Different Body Sites Shapes the Developing Infant Gut Microbiome. *Cell Host Microbe* **24**, 133-145 e135, doi:10.1016/j.chom.2018.06.005 (2018).
75 Yassour, M. *et al.* Strain-Level Analysis of Mother-to-Child Bacterial Transmission during the First Few Months of Life. *Cell Host Microbe* **24**, 146-154 e144, doi:10.1016/j.chom.2018.06.007 (2018).
76 Korpela, K. *et al.* Maternal Fecal Microbiota Transplantation in Cesarean-Born Infants Rapidly Restores Normal Gut Microbial Development: A Proof-of-Concept Study. *Cell* **183**, 324-334 e325, doi:10.1016/j.cell.2020.08.047 (2020).
77 Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A. & Callahan, B. J. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* **6**, 226, doi:10.1186/s40168-018-0605-2 (2018).
78 Dyrhovden, R. *et al.* Managing Contamination and Diverse Bacterial Loads in 16S rRNA Deep Sequencing of Clinical Samples: Implications of the Law of Small Numbers. *mBio* **12**, e0059821, doi:10.1128/mBio.00598-21 (2021).
79 Laurence, M., Hatzis, C. & Brash, D. E. Common contaminants in next-generation sequencing that hinder discovery of low-abundance microbes. *PLoS One* **9**, e97876, doi:10.1371/journal.pone.0097876 (2014).
80 Read, S. J. Recovery efficiences on nucleic acid extraction kits as measured by quantitative LightCycler PCR. *Mol Pathol* **54**, 86-90, doi:10.1136/mp.54.2.86 (2001).
81 Walker, S. P. *et al.* Non-specific amplification of human DNA is a major challenge for 16S rRNA gene sequence analysis. *Sci Rep* **10**, 16356, doi:10.1038/s41598-020-73403-7 (2020).
82 Cebra, J. J., Periwal, S. B., Lee, G., Lee, F. & Shroff, K. E. Development and maintenance of the gut-associated lymphoid tissue (GALT): the roles of enteric bacteria and viruses. *Dev Immunol* **6**, 13-18, doi:10.1155/1998/68382 (1998).
83 Gaboriau-Routhiau, V. *et al.* The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity* **31**, 677-689, doi:10.1016/j.immuni.2009.08.020 (2009).
84 Wesemann, D. R. *et al.* Microbial colonization influences early B-lineage development in the gut lamina propria. *Nature* **501**, 112-115, doi:10.1038/nature12496 (2013).
85 Li, H. *et al.* Mucosal or systemic microbiota exposures shape the B cell repertoire. *Nature* **584**, 274-278, doi:10.1038/s41586-020-2564-6 (2020).
86 Bacher, P. *et al.* Human Anti-fungal Th17 Immunity and Pathology Rely on Cross-Reactivity against Candida albicans. *Cell* **176**, 1340-1355 e1315, doi:10.1016/j.cell.2019.01.041 (2019).
87 Kabbert, J. *et al.* High microbiota reactivity of adult human intestinal IgA requires somatic mutations. *J Exp Med* **217**, doi:10.1084/jem.20200275 (2020).

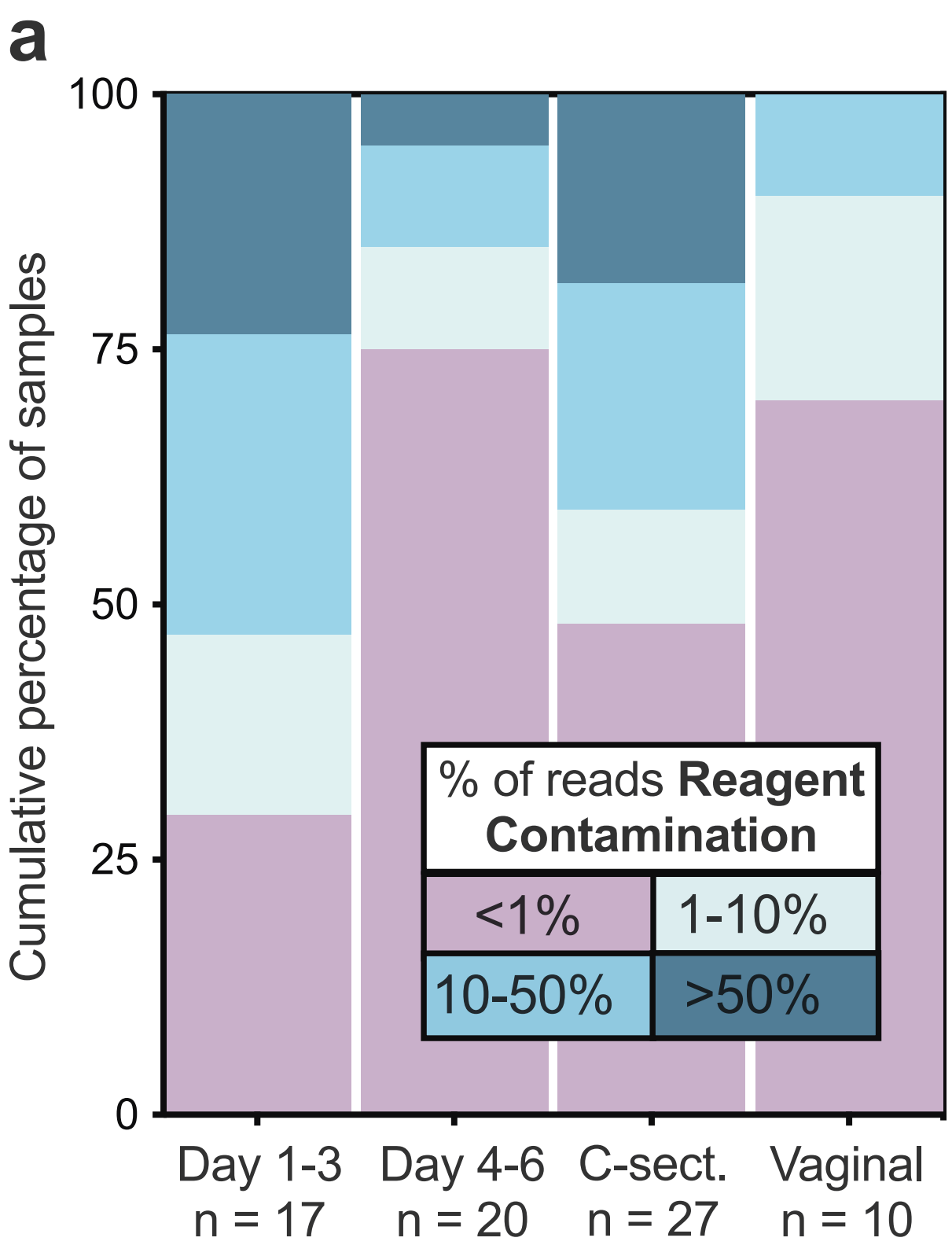
88 Arpaia, N. *et al.* Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* **504**, 451-455, doi:10.1038/nature12726 (2013).
89 McGovern, N. *et al.* Human fetal dendritic cells promote prenatal T-cell immune suppression through arginase-2. *Nature* **546**, 662-666, doi:10.1038/nature22795 (2017).
90 Rechavi, E. *et al.* Timely and spatially regulated maturation of B and T cell repertoire during human fetal development. *Sci Transl Med* **7**, 276ra225, doi:10.1126/scitranslmed.aaa0072 (2015).
91 Casas, R. & Bjorksten, B. Detection of Fel d 1-immunoglobulin G immune complexes in cord blood and sera from allergic and non-allergic mothers. *Pediatr Allergy Immunol* **12**, 59-64, doi:10.1034/j.1399-3038.2001.012002059.x (2001).
92 Szepfalusi, Z. *et al.* Transplacental priming of the human immune system with environmental allergens can occur early in gestation. *J Allergy Clin Immunol* **106**, 530-536, doi:10.1067/mai.2000.108710 (2000).
93 Vuillermin, P. J. *et al.* Maternal carriage of Prevotella during pregnancy associates with protection against food allergy in the offspring. *Nat Commun* **11**, 1452, doi:10.1038/s41467-020-14552-1 (2020).
94 Ganal-Vonarburg, S. C., Hornef, M. W. & Macpherson, A. J. Microbial-host molecular exchange and its functional consequences in early mammalian life. *Science* **368**, 604-607, doi:10.1126/science.aba0478 (2020).
95 Lockhart, P. B. *et al.* Bacteremia associated with toothbrushing and dental extraction. *Circulation* **117**, 3118-3125, doi:10.1161/CIRCULATIONAHA.107.758524 (2008).
96 Fisher, R. A., Gollan, B. & Helaine, S. Persistent bacterial infections and persister cells. *Nat Rev Microbiol* **15**, 453-464, doi:10.1038/nrmicro.2017.42 (2017).
97 De Boeck, I. *et al.* Lactobacilli Have a Niche in the Human Nose. *Cell Rep* **31**, 107674, doi:10.1016/j.celrep.2020.107674 (2020).
98 Lebeer, S., Vanderleyden, J. & De Keersmaecker, S. C. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nat Rev Microbiol* **8**, 171-184, doi:10.1038/nrmicro2297 (2010).
99 Collins, J. *et al.* Fibrinogen-binding and platelet-aggregation activities of a Lactobacillus salivarius septicaemia isolate are mediated by a novel fibrinogen-binding protein. *Mol Microbiol* **85**, 862-877, doi:10.1111/j.1365-2958.2012.08148.x (2012).
100 Kankainen, M. *et al.* Comparative genomic analysis of Lactobacillus rhamnosus GG reveals pili containing a human- mucus binding protein. *Proc Natl Acad Sci U S A* **106**, 17193-17198, doi:10.1073/pnas.0908876106 (2009).
101 Rampersaud, R. *et al.* Inerolysin, a cholesterol-dependent cytolysin produced by Lactobacillus iners. *J Bacteriol* **193**, 1034-1041, doi:10.1128/JB.00694-10 (2011).
102 Wuyts, S. *et al.* Large-Scale Phylogenomics of the Lactobacillus casei Group Highlights Taxonomic Inconsistencies and Reveals Novel Clade-Associated Features. *mSystems* **2**, doi:10.1128/mSystems.00061-17 (2017).
103 Weinberg, E. D. The Lactobacillus anomaly: total iron abstinence. *Perspect Biol Med* **40**, 578-583, doi:10.1353/pbm.1997.0072 (1997).
104 Hazards, E. P. o. B. *et al.* Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 7: suitability of taxonomic units notified to EFSA until September 2017. *EFSA J* **16**, e05131, doi:10.2903/j.efsa.2018.5131 (2018).
105 Cannon, J. P., Lee, T. A., Bolanos, J. T. & Danziger, L. H. Pathogenic relevance of Lactobacillus: a retrospective review of over 200 cases. *Eur J Clin Microbiol Infect Dis* **24**, 31-40, doi:10.1007/s10096-004-1253-y (2005).

106 Richardson, E. J. *et al.* Gene exchange drives the ecological success of a multi-host bacterial pathogen. *Nat Ecol Evol* **2**, 1468-1478, doi:10.1038/s41559-018-0617-0 (2018).
107 Gordon, R. J. & Lowy, F. D. Pathogenesis of methicillin-resistant Staphylococcus aureus infection. *Clin Infect Dis* **46 Suppl 5**, S350-359, doi:10.1086/533591 (2008).
108 Otto, M. Staphylococcus aureus toxins. *Curr Opin Microbiol* **17**, 32-37, doi:10.1016/j.mib.2013.11.004 (2014).
109 Powers, M. E. & Bubeck Wardenburg, J. Igniting the fire: Staphylococcus aureus virulence factors in the pathogenesis of sepsis. *PLoS Pathog* **10**, e1003871, doi:10.1371/journal.ppat.1003871 (2014).
110 Healy, C. M., Baker, C. J., Palazzi, D. L., Campbell, J. R. & Edwards, M. S. Distinguishing true coagulase-negative Staphylococcus infections from contaminants in the neonatal intensive care unit. *J Perinatol* **33**, 52-58, doi:10.1038/jp.2012.36 (2013).
111 Michels, R., Last, K., Becker, S. L. & Papan, C. Update on Coagulase-Negative Staphylococci-What the Clinician Should Know. *Microorganisms* **9**, doi:10.3390/microorganisms9040830 (2021).
112 Marchant, E. A., Boyce, G. K., Sadarangani, M. & Lavoie, P. M. Neonatal sepsis due to coagulase-negative staphylococci. *Clin Dev Immunol* **2013**, 586076, doi:10.1155/2013/586076 (2013).
113 Zhen, X., Lundborg, C. S., Sun, X., Hu, X. & Dong, H. Economic burden of antibiotic resistance in ESKAPE organisms: a systematic review. *Antimicrob Resist Infect Control* **8**, 137, doi:10.1186/s13756-019-0590-7 (2019).
114 Kamal, S. M., Simpson, D. J., Wang, Z., Ganzle, M. & Romling, U. Horizontal Transmission of Stress Resistance Genes Shape the Ecology of Beta- and Gamma-Proteobacteria. *Front Microbiol* **12**, 696522, doi:10.3389/fmicb.2021.696522 (2021).
115 Kramer, A., Schwebke, I. & Kampf, G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect Dis* **6**, 130, doi:10.1186/1471-2334-6-130 (2006).
116 Neely, A. N. & Maley, M. P. Survival of enterococci and staphylococci on hospital fabrics and plastic. *J Clin Microbiol* **38**, 724-726, doi:10.1128/JCM.38.2.724-726.2000 (2000).
117 Bizzarro, M. J. *et al.* Neonatal sepsis 2004-2013: the rise and fall of coagulase-negative staphylococci. *J Pediatr* **166**, 1193-1199, doi:10.1016/j.jpeds.2015.02.009 (2015).
118 Dong, Y., Speer, C. P. & Glaser, K. Beyond sepsis: Staphylococcus epidermidis is an underestimated but significant contributor to neonatal morbidity. *Virulence* **9**, 621-633, doi:10.1080/21505594.2017.1419117 (2018).
119 Glaser, M. A., Hughes, L. M., Jnah, A. & Newberry, D. Neonatal Sepsis: A Review of Pathophysiology and Current Management Strategies. *Adv Neonatal Care* **21**, 49-60, doi:10.1097/ANC.0000000000000769 (2021).
120 Nan, C. *et al.* Maternal group B Streptococcus-related stillbirth: a systematic review. *BJOG* **122**, 1437-1445, doi:10.1111/1471-0528.13527 (2015).
121 Vazquez-Boland, J. A., Krypotou, E. & Scortti, M. Listeria Placental Infection. *mBio* **8**, doi:10.1128/mBio.00949-17 (2017).
122 DiGiulio, D. B. *et al.* Microbial invasion of the amniotic cavity in preeclampsia as assessed by cultivation and sequence-based methods. *J Perinat Med* **38**, 503-513, doi:10.1515/jpm.2010.078 (2010).
123 DiGiulio, D. B. *et al.* Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS One* **3**, e3056, doi:10.1371/journal.pone.0003056 (2008).
Sequencing study of amniotic fluid of 166 women in preterm labor with PCR and culture that showed near-complete positive correlation of bacterial detection with neonatal morbidity and mortality.

124 DiGiulio, D. B. *et al.* Prevalence and diversity of microbes in the amniotic fluid, the fetal inflammatory response, and pregnancy outcome in women with preterm pre-labor rupture of membranes. *Am J Reprod Immunol* **64**, 38-57, doi:10.1111/j.1600-0897.2010.00830.x (2010).
125 DiGiulio, D. B. *et al.* Microbial invasion of the amniotic cavity in pregnancies with small-for-gestational-age fetuses. *J Perinat Med* **38**, 495-502, doi:10.1515/jpm.2010.076 (2010).
126 Enders, G., Daiminger, A., Bader, U., Exler, S. & Enders, M. Intrauterine transmission and clinical outcome of 248 pregnancies with primary cytomegalovirus infection in relation to gestational age. *J Clin Virol* **52**, 244-246, doi:10.1016/j.jcv.2011.07.005 (2011).
127 Luckey, T. D. *Germfree Life and Gnotobiology*. (Academic Press, 1963).
128 Rasmussen, S. A., Jamieson, D. J., Honein, M. A. & Petersen, L. R. Zika Virus and Birth Defects--Reviewing the Evidence for Causality. *N Engl J Med* **374**, 1981-1987, doi:10.1056/NEJMsr1604338 (2016).
129 Falk, P. G., Hooper, L. V., Midtvedt, T. & Gordon, J. I. Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol Mol Biol Rev* **62**, 1157-1170, doi:10.1128/MMBR.62.4.1157-1170.1998 (1998).
130 Gordon, H. A. & Pesti, L. The gnotobiotic animal as a tool in the study of host microbial relationships. *Bacteriol Rev* **35**, 390-429 (1971).
131 Hooper, L. V. *et al.* Molecular analysis of commensal host-microbial relationships in the intestine. *Science* **291**, 881-884, doi:10.1126/science.291.5505.881 (2001).
132 Wostman, B. S. *Germfree and Gnotobiotic Animal Models. Background and Applications*. (CRC Press, 1996).
133 Arvidsson, C., Hallen, A. & Backhed, F. Generating and Analyzing Germ-Free Mice. *Curr Protoc Mouse Biol* **2**, 307-316, doi:10.1002/9780470942390.mo120064 (2012).
134 Carter, P. B., Norin, E. & Swennes, A. G. Gnotobiotics and the Microbiome. *The Laboratory Rat*, 827–848, doi:10.1016/B978-0-12-814338-4.00021-0 (2020).
135 Qv, L. *et al.* Methods for Establishment and Maintenance of Germ-Free Rat Models. *Front Microbiol* **11**, 1148, doi:10.3389/fmicb.2020.01148 (2020).
136 Schoeb, T. R. & Eaton, K. A. *Gnotobiotics.* . (Academic Press (Elsevier), 2017).
137 Jervis-Bardy, J. *et al.* Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. *Microbiome* **3**, 19, doi:10.1186/s40168-015-0083-8 (2015).
138 Saffarian, A. *et al.* Crypt- and Mucosa-Associated Core Microbiotas in Humans and Their Alteration in Colon Cancer Patients. *mBio* **10**, doi:10.1128/mBio.01315-19 (2019).
139 Jorissen, J. *et al.* Case-Control Microbiome Study of Chronic Otitis Media with Effusion in Children Points at Streptococcus salivarius as a Pathobiont-Inhibiting Species. *mSystems* **6**, doi:10.1128/mSystems.00056-21 (2021).
140 Salzberg, S. in *Forbes* (2020).
141 Jost, T., Lacroix, C., Braegger, C. & Chassard, C. Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. *Br J Nutr* **110**, 1253-1262, doi:10.1017/S0007114513000597 (2013).
142 Treven, P. *et al.* Evaluation of Human Milk Microbiota by 16S rRNA Gene Next-Generation Sequencing (NGS) and Cultivation/MALDI-TOF Mass Spectrometry Identification. *Front Microbiol* **10**, 2612, doi:10.3389/fmicb.2019.02612 (2019).
143 Bihl, S. *et al.* When to suspect contamination rather than colonization - lessons from a putative fetal sheep microbiome. *Gut Microbes* **14**, 2005751, doi:10.1080/19490976.2021.2005751 (2022).

144 Kennedy, K. M. *et al.* Over-celling fetal microbial exposure. *Cell* **184**, 5839-5841, doi:10.1016/j.cell.2021.10.026 (2021).
145 Eisenhofer, R. *et al.* Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. *Trends Microbiol* **27**, 105-117, doi:10.1016/j.tim.2018.11.003 (2019).


Cesarean
Vaginal
Kennedy
Mishra
Rackaityte
Delivery mode
Sampling
Sample processing
Sequencing & Analysis
Vaginal microbes
Skin microbes
Reagent microbes
Cross-contamination
^* Lactobacillus
^* Gardnerella
^* Prevotella
* Lactococcus
^* Anaerococcus
* Megasphaera
^* Corynebacterium
^ Staphylococcus
^ Streptococcus
* Micrococcus
* Pajaroellobacter
^ Flavobacterium
Halomonas
Rhodanobacter
^ Afipia
^ Bradyrhizobium
* Rhizobiaceae
* Limnohabitans
Sphingomonas
^* Comamonas
* Pseudomonas
Ralstonia
* Peptoniphilus
^ Brevundimonas
* Rhodococcus
^ Enterococcus
^ Escherichia
^* Bifidobacterium
^ Bacteroides
Reagent Run1
Reagent Run2
Swab Run1
Swab Run2
Gut Run1
Gut Run2
PBS
Reagent
Operator
Environment
Thymus
Spleen
Lung
Gut
Skin
Placenta
Buffer
Air swab
Procedural swab
Moistened swab
Kidney
Proximal gut
Mid gut
Distal gut
Relative Abundance (%)
1
5
10
20
30
50



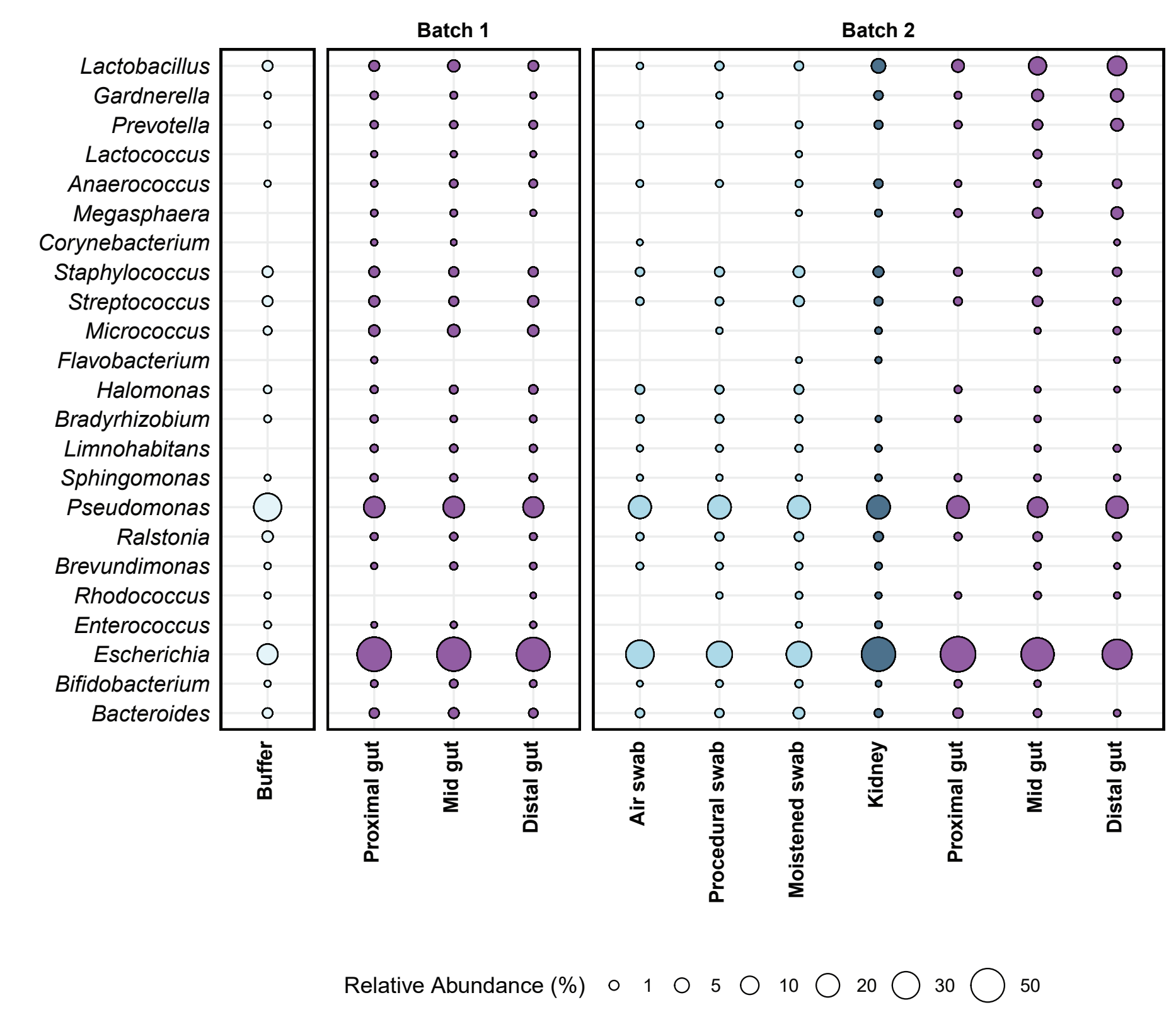
b

Reagent contaminants

Undibacterium oligocarboniphilum
Acinetobacter guillouiae
Curvibacter lanceolatus
Sphingomonas echinoides
Ralstonia pickettii
Sphingomonas kyeonggiensis
Methylorubrum extorquens
Phyllobacterium myrsinacearum
Sphingomonas panni
Sphingomonas faeni
Sediminibacterium salmoneum
Rhodococcus erythropolis
Pelomonas saccharophila

Main genuine signals

Staphylococcus epidermidis (C)
Staphylococcus warneri (C)
C. tuberculostearicum (C)
Klebsiella pneumoniae (C)
Klebsiella oxytoca (C)
Enterobacter cloacae (C)
Enterococcus faecalis
Streptococcus salivarius
Clostridium perfringens
Clostridium paraputrificum
Clostridioides difficile
Bifidobacterium longum
Bacteroides fragilis (V)
Escherichia (*coli*) (V)


Batch 1
Batch 2
Lactobacillus
Gardnerella
Prevotella
Lactococcus
Anaerococcus
Megasphaera
Corynebacterium
Staphylococcus
Streptococcus
Micrococcus
Flavobacterium
Halomonas
Bradyrhizobium
Limnohabitans
Sphingomonas
Pseudomonas
Ralstonia
Brevundimonas
Rhodococcus
Enterococcus
Escherichia
Bifidobacterium
Bacteroides
Buffer
Proximal gut
Mid gut
Distal gut
Air swab
Procedural swab
Moistened swab
Kidney
Proximal gut
Mid gut
Distal gut
Relative Abundance (%)
1
5
10
20
30
50