**Titles**

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

**Or**

**Critical assessment of the “fetal microbiome” and the pitfalls of low-biomass microbial studies**

Katherine M. Kennedy\*1,2, Marcus C. de Goffau\*3,4, Maria Elisa Perez-Muñoz5, Marie-Claire Arrieta6, Fredrik Bӓckhed7,8,9, Peer Bork10, Thorsten Braun11, Frederic D. Bushman12, Joel Dore13**,** Willem M. de Vos14,15, Ashlee M. Earl16, Jonathan A. Eisen17,18,19, Michal A. Elovitz, MD20, Stephanie C. Ganal*-*Vonarburg21,22, Michael G. Gӓnzle5, Wendy S. Garrett23,24,25,26, Lindsay J. Hall27,28,29, Mathias W. Hornef30, Curtis Huttenhower23,26,31, Liza Konnikova32, Sarah Lebeer33, Andrew J. Macpherson22, Ruth C. Massey34,35, Alice Carolyn McHardy36,37,38, Omry Koren39, Trevor D. Lawley4, Ruth E. Ley40, Liam O’Mahony34,35,41, Paul W. O’Toole34,35, Eric G. Pamer42, Julian Parkhill43, Jeroen Raes44,45, Thomas Rattei46, Anne Salonen14, Eran Segal47, Nicola Segata48,49, Fergus Shanahan34,41, Deborah M. Sloboda1,2,50, Gordon C.S. Smith51,52, Harry Sokol53,54,55, Tim D. Spector56, Michael G. Surette1,2,57, Gerald W. Tannock58, Alan W. Walker59, Moran Yassour60,61, and Jens Walter34,35,41

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, EMBL, Meyerhofstr.1, 69117 Heidelberg
11. Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Department of Obstetrics and Experimental Obstetrics, Augustenburger Platz 1, 13353 Berlin, Germany
12. Department of Microbiology Perelman School of Medicine at the University of Pennsylvania, 425 Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104-6076
13. Université Paris-Saclay, INRAE, MetaGenoPolis, AgroParisTech, MICALIS, 78350, Jouy-en-Josas, France
14. Human Microbiome Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland
15. Laboratory of Microbiology Wageningen University, The Netherlands
16. Infectious Disease & Microbiome Program, Broad Institute of MIT & Harvard, Boston, MA 02142
17. Department of Evolution and Ecology, University of California, Davis
18. Department of Medical Microbiology and Immunology, University of California, Davis
19. Genome Center, University of California, Davis
20. Maternal and Child Health Research Center, Department of Obstetrics & Gynecology, University of Pennsylvania Perelman School of Medicine
21. Universitätsklinik für Viszerale Chirurgie und Medizin, Inselspital, Bern University Hospital, University of Bern, Switzerland
22. Department for BioMedical Research (DBMR), University of Bern, Bern, Switzerland
23. Department of Immunology and Infectious Diseases, Harvard T. H. Chan School of Public Health, Boston, Massachusetts
24. Harvard T. H. Chan Microbiome in Public Health Center, Boston, Massachusetts
25. Department of Medicine and Division of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts
26. Broad Institute of Harvard and MIT, Cambridge, Massachusetts
27. Quadram Institute Bioscience, Norwich Research Park, Norwich, NR4 7UQ, United Kingdom
28. Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, United Kingdom
29. Chair of Intestinal Microbiome, ZIEL - Institute for Food & Health, School of Life Sciences, Technical University of Munich, Freising, Germany
30. Institute of Medical Microbiology, RWTH University Hospital, 52074 Aachen, Germany
31. Department of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, MA, USA.
32. Departments of Pediatrics and Obstetrics, Gynecology and Reproductive Sciences, Yale School of Medicine, New Haven, CT, USA
33. University of Antwerp, Department of Bioscience Engineering, Groenenborgerlaan 171, 2020 Antwerp
34. APC Microbiome Ireland, University College Cork, Cork, Ireland
35. School of Microbiology, University College Cork, Cork, Ireland
36. Computational Biology of Infection Research, Helmholtz Centre for Infection Research, Braunschweig, Germany
37. German Center for Infection Research (DZIF), Hannover Braunschweig site
38. Braunschweig Integrated Centre of Systems Biology (BRICS),Technische Universität Braunschweig, Braunschweig, Germany
39. Azrieli Faculty of Medicine, Bar-Ilan University, Safed, Israel
40. Department of Microbiome Science, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany
41. Department of Medicine, University College Cork, Cork, Ireland
42. Duchossois Family Institute, University of Chicago, Chicago, Illinois
43. Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, CB3 0ES, UK
44. VIB Center for Microbiology, Herestraat 49, Leuven, Belgium
45. Department of Microbiology, Immunology and Transplantation, Rega Institute, KU Leuven, Herestraat 49, Leuven, Belgium
46. Centre for Microbiology and Environmental Systems Science, University of Vienna, Vienna, Austria
47. Weizmann Institute of Science, Israel
48. Department CIBIO, University of Trento, Trento, Italy
49. IEO, European Institute of Oncology IRCCS, Milan, Italy
50. Departments of Pediatrics, Obstetrics and Gynecology, McMaster University, Hamilton, Ontario, Canada
51. Department of Obstetrics and Gynaecology, University of Cambridge, UK
52. NIHR Cambridge Biomedical Research Centre, Cambridge, CB2 0SW, UK
53. Sorbonne Université, INSERM, Centre de Recherche Saint-Antoine, CRSA, AP-HP, Saint Antoine Hospital, Gastroenterology department, F-75012 Paris, France
54. Paris Center for Microbiome Medicine (PaCeMM) FHU, Paris, France
55. INRA, UMR1319 Micalis & AgroParisTech, Jouy en Josas, France
56. Dept of Twin Research, Kings College London, London SE1 7EH, UK
57. Department of Medicine, McMaster University, Hamilton, Ontario, Canada
58. Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand
59. Gut Health Group, Rowett Institute, University of Aberdeen, Aberdeen, Scotland, UK, AB25 2ZD.
60. School of Computer Science and Engineering, The Hebrew University of Jerusalem, Jerusalem 91904, Israel.
61. 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](mailto:jens.walter@ucc.ie)

**Preface**

Whether the human fetus and the prenatal intrauterine environment (amniotic fluid, placenta) are stably colonized by microbes in a healthy pregnancy remains the subject of a contentious scientific debate. Here, we evaluate recent studies that characterized microbial populations in human fetuses from the perspectives of reproductive biology, microbiology, bioinformatics and data science, immunology, clinical microbiology, and gnotobiology, and assess the likely mechanisms by which the fetus could interact with microbes. Our analysis indicates that the detected microbial signals are likely the result of contamination during the clinical procedures to obtain fetal samples, DNA extraction, and DNA sequencing. Further, the existence of live and replicating microbial populations in healthy fetal tissues is not compatible with fundamental concepts of immunology, clinical microbiology, and the derivation of germ-free mammals. These conclusions are not only important to our understanding of human immune development, but also illustrate common pitfalls in the microbial analyses of many other low-biomass environments. The pursuit of a “fetal microbiome” can serve as a cautionary example of the challenges of sequence-based microbiome studies when biomass is low or absent and emphasizes the critical 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 health1-4. Neonates born at term are not immunologically naïve and are specifically adapted to cope with abrupt exposure to microbial, dietary, and environmental stimuli and antigens5,6. Several research groups have characterized immune cell development in human fetal tissues7-9. However, our mechanistic understanding of how and when immune priming by microbes occurs, and the factors that drive it, is incomplete.

The long-held view that the prenatal intrauterine environment (placenta, amniotic fluid, fetus) is protected from live microbes has been challenged recently10-15, leading to the hypothesis that fetal immune development may be driven by the presence of live microbes or even entire microbiomes at intrauterine sites16-19. However, these results have been debated20-26 because several concurrent studies27-33 point to experimental contamination dominating low–microbial-biomass sequencing data34-36 as the source of microbial DNA apparently detected in the intrauterine environment. Since 2020, four studies have characterized the microbiology of the human fetus directly and resulted in opposing and irreconcilable conclusions. Two reports described viable low-density microbial populations in human fetal intestines37 and organs38, and linked these microbes to fetal immune development. In contrast, two other research groups, that included several of the authors of this perspective, reported no detectable microbes in fetal meconium and intestines28,39.

Such disagreement over a fundamental aspect of human biology poses a significant challenge for scientific progress. This is not simply a matter of controversy or a reluctance to relinquish established dogma; rather, the notion of a fetal microbiome, if proven correct, has implications for clinical medicine and would call for concepts and research not previously contemplated. It would require radical revision of our understanding of the development of the immune and other systems in early life and the anatomical and immunological mechanisms to facilitate symbiotic host-microbe interactions within fetal tissues. Failure to resolve the issue is a potential risk of diverting resources into research that ultimately results in no advancement for fetal and maternal health and misguided attempts to therapeutically modify a putative fetal microbiome. Moreover, the dilemma has immediate relevance to the characterization of all low-biomass samples.

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

**Claims and counterclaims**

Although the disagreement on the presence of microbes in prenatal intrauterine locations (placenta and amniotic fluid) spans dozens of studies with contradictory findings11,13,14,21,27,29-32,35,40-42, we focus our analysis on four recent studies since they provide a direct assessment of the fetus itself28,38,39,43. Collection of human fetal samples is difficult and restricted to either following pregnancy termination, or immediately prior to birth by C-section. Three of the studies used samples collected after vaginally delivered, elective, second trimester pregnancy terminations38,39,43, and one collected samples from breech C-section deliveries immediately at birth28.

Rackaityte *et al*.43 reported 18 bacterial taxa as enriched in intestinal contents of vaginally delivered fetuses from 2nd trimester terminations compared to negative controls using 16S rRNA gene amplicon sequencing (V4 region). To account for contamination, the authors removed Operational Taxonomic Units (OTUs) detected in >50% of procedural controls and then identified remaining contaminants *in silico* (using the decontam R package). They found that most fetal samples were microbiologically similar to negative controls (labelled as “other meconium”, n=25), but that some samples, dominated by *Lactobacillus* (6 samples) or *Micrococcaceae* (9 samples), had distinct bacterial profiles. The authors further detected low amounts of total bacteria by qPCR, Fluorescent *in situ* hybridization (FISH), Scanning Electron Microscopy (SEM), and culture (as discussed below).

Several of the study’s conclusions have been challenged by de Goffau *et al.*44, who re-analyzed the publicly available data and found no evidence for a distinct bacterial profile in the subset of samples with matched procedural controls, and concluded that the positive findings were caused by a sequencing batch effect and contamination during culture44. In addition, the authors’ suggestion that particles detected in SEM micrographs constitute micrococci43 was disputed as their size exceeded that of known *Micrococcaceae*44. Furthermore, the 16S rRNA gene sequence of the *Micrococcus luteus* cultured from the fetal samples differed from that detected by sequencing, suggesting contamination during culture (*Micrococcus luteus* is a common contaminant of clean rooms and surgical instruments45,46).

Mishra *et al.*38 detected a low but consistent microbial signal across tissues of vaginally delivered fetuses from 2nd trimester terminations by 16S rRNA gene amplicon sequencing (V4-V5 region), with 7 genera enriched in fetal samples (*Lactobacillus*, *Staphylococcus*, *Pseudomonas*, *Flavobacterium*, *Afipia*, *Bradyrhizobium*, and *Brevundimonas*). The 16S rRNA gene sequencing data were accompanied by SEM, RNA-*in situ* hybridization (RNA-ISH), and culture. In recognition of the high risk of contamination, all samples were processed in isolation with negative controls collected during sample processing. In contrast to Rackaityte *et al*., Mishra *et al*. found *Micrococcus* to be enriched in phosphate buffered saline (PBS) reagent controls and reported it as a contaminant, with the *M. luteus* cells detected by culture being consistent with the size and morphology of the coccoid structures found by SEM38.

Both the studies by Rackaityte *et al*. and Mishra *et al*. included assays to study immune development of the fetus and concluded that the microbes detected would contribute to immune maturation. Rackaityte *et al*.43 based this conclusion on differences in patterns of T cell composition and epithelial transcription between fetal intestines determined by whether *Micrococcaceae* were or were not the dominant species and suggested that bacterial antigens may contribute to T cell activation and immunological memory *in utero*. Mishra *et al*.38 employed flow cytometry to expand on previous findings of effector (TNF- /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.39*, who also investigated fetal intestinal tissue from second trimester terminations, did not detect bacterial DNA by PCR (V4 region of the 16S rRNA gene, 35 cycles) based on visual inspection of agarose gels in any of the 101 samples tested. The authors detected a diverse set of metabolites in fetal intestinal samples and hypothesized that maternal, microbiota-derived metabolites may pass through the placenta to ‘educate’ the fetal immune system. This conclusion is supported by research in mice that showed that fetal immune education can be driven in the absence of direct microbial exposure by trans-placental passage of microbial metabolites from the maternal gut47,48.

Kennedy *et al.28* used a different approach and collected samples using rectal swabs during elective C-section for breech presentation at term gestation28. 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 directly compare these recently published 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 second trimester vaginally delivered fetuses38,43, the bacterial taxa detected in fetuses derived by C-section28 were vastly different (Figure 1). The number of genera was much lower in C-section-derived fetuses, and entire groups of microbes, especially those generally found in the vagina, were absent. Most importantly, in the studies that claimed fetal microbial colonization38,43, every genus detected in fetal samples was also detected in most control samples. These findings indicate that the claimed microbiology of the human fetus is dependent on the methodology of sampling. Next, we apply perspectives from different disciplines to provide context and implications for the findings.

**Reproductive biology and obstetrics perspectives**

The embryo and fetus develop within the uterus but not in the uterine cavity, *per se*. The early embryo invades the maternal decidua and is completely embedded by 10 days post-fertilization. The fetus grows within the amniotic cavity, which originates between the trophoblast and inner cells mass in the second week post fertilization, surrounded by two layers of reproductive membranes as well as amniotic fluid. Hence, even if microbes were present in the uterine cavity49, they would have to pass through to the amniotic cavity and reside within amniotic fluid to colonize the fetus. Of note, amniotic fluid has antimicrobial properties, being enriched for example in Lysozyme50, Human beta-defensin 251, and Gp340/Dmbt152 (binds and agglutinates a broad spectrum of both 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, that showed some similarity with sequence data (Human Microbiome Project) of microbial communities of the oral cavity14. Contamination controls were not included in this early study, and subsequent evaluation of the work found that most genera detected are also common contaminants24,34,36,53. Several detected taxa, such as *Gloeobacter*, a genus of photosynthetic cyanobacteria, appeared biologically implausible as a component of a putative placental microbiome22,54. Irrespective of whether placental samples are collected by biopsy per vagina, clinically by chorionic villus sampling, or after delivery (most published studies to date have investigated the microbial communities in the placenta 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.*27 detected a range of species known to dominate the vaginal microbiota55, such as *Lactobacillus iners*, *L. jensenii*, *L. crispatus*, *L. gasseri*, and *Gardnerella vaginalis*. It is also noteworthy that when the presence of vaginal microbes and those in the laboratory reagents (the “kitome”) were accounted for, no placenta microbiome was detected in several recent studies21,27,29-32,35.

Infection of the placenta by viral or bacterial pathogens is a well-recognized clinical phenomenon that contributes to preterm birth and neonatal sepsis. As noted by de Goffau *et al*.27, *Streptococcus agalactiae* can be detected in around 5% of cases as the only verified bacterial signal in placentas obtained by C-section deliveries. The presence of this species is plausible as it colonizes the genital tract of about 20% of women and has invasive potential, being an important cause of both maternal and neonatal sepsis56. However, the ability of specific pathogens to colonize and/or infect the placenta is not tantamount to more widespread placental microbial colonization or even the presence of an indigenous microbiome (a prevalently occurring, stable, non-pathogenic, complex microbial community).

Research claiming the presence of viable low-density microbial communities in the fetal intestine43 and fetal organs38 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 prostaglandins38. This procedure typically involves the individual going through hours of labor and often leads to the rupture of the fetal membranes hours prior to vaginal delivery. Even with a standardized approach, labor may be prolonged and may be accompanied by infection and fever, which are common with 2nd trimester terminations57,58. Both Li *et al*.39 and Rackaityte *et al*.43 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*.37 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 delivering the fetus through the vaginal canal. As outlined later, the reported microbiology of these fetuses reflects the sources of microbes to which they are exposed.

**Microbiology and microbial ecology perspectives**

Host-microbe relationships range from benign mutualism (a prolonged symbiotic association from which both benefit) and commensalism (host is unaffected), to one in which the microbe harms the host (pathogen). Although claims for fetal microbial exposure38,43 have not established the nature of the host-microbe interaction, and the duration of exposure or colonization, they have suggested a beneficial role for live organisms in fetal immune development, thereby implying a symbiosis. The microbiological approaches applied by Rackaityte *et al.*43 and Mishra *et al.*38 are, in large part, robust, and well suited to study symbiotic microbial populations. The combination of 16S rRNA gene sequencing, quantitative PCR (qPCR), microscopy, FISH, and culture is laudable, as the approaches are complementary. Next-generation sequencing of 16S rRNA gene amplicons provides a broad community overview and can detect microbes that escape cultivation, while qPCR, microscopy, and bacterial cultures have a high dynamic range, very 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 do not preclude the introduction of contaminant species at the sample collection stage, and critical controls to determine if contamination occurred were missing.

In agreement with the unavoidable vaginal exposure of fetuses obtained by 2nd trimester abortions (see above), both Rackaityte *et al.*43 and Mishra *et al.*38 found the genera *Lactobacillus* and *Gardnerella*, which dominate the vaginal microbiota55, among their most consistent findings (Figure 1). The species cultured by Mishra *et al.*, *G. vaginalis*, *L.* *iners* and *L. jensenii*, are highly specific to the human vagina59. Other microbes detected such as *Staphylococcus* species and *Cutibacterium acnes*, are skin commensals. As shown in Figure 1, abundances of *Lactobacillus*, *Gardnerella*, and *Staphylococcus* found by Mishra *et al*. showed gradients with high population levels in fetal samples exposed to sources of contaminants (placenta and skin) and lower levels in internal samples (gut, lung, spleen, thymus). The omission of vaginal controls by both Rackaityte *et al*. and Mishra *et al*. to determine the microbiota of vaginally delivered fetuses is an unfortunate flaw that casts doubt on the authors’ conclusion that the microbes originate from the womb. Indeed, Li *et al*.39, who used samples from 2nd trimester surgical terminations performed with mechanical dilatation, which decreases the bacterial exposure of the fetus, did not report positive bacterial PCR results in their study, further raising suspicion that sampling contamination was a serious confounder in the work of Rackaityte *et al.* and Mishra *et al.*.

Although vaginal controls were not included by Rackaityte *et al.*43 and Mishra *et al.*38, direct comparisons of their findings with those by Kennedy *et al*.28 also provide clear evidence for vaginal contamination of terminated fetuses (Figure 1). The C-section derived fetal samples in Kennedy *et al.*, which were not exposed to the vagina, carried no *Gardnerella* or *Lactobacillus* but instead contained skin and reagent contaminants28,53. Despite attempts to reduce contamination, C-section derived fetal meconium had at least one positive culture28. Kennedy *et al*. did not consider these microbes of fetal origin, as they were skin commensals, and half of the samples as well as many culture replicates did not show growth. The authors concluded that such inconsistencies point to stochastic contamination and not colonization by a stable functional microbial community.

Despite vaginal contamination, the bacterial load found in terminated fetuses was extremely low38,43. 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 many fetuses and tissues being negative for the specific microbes (see Table S6 in the original publication38). Such inconsistent patterns are not logical based on ecological principles and do not resemble natural microbial populations, which should be consistently detectable, especially in sample replicates. Given that they are close to the detection limits of the technical approaches used, such findings should raise concerns of contamination rather than suggesting colonization.

Further indirect insights regarding the microbiological state of the fetus may be inferred from the infant gut microbiota very early in life. Neonatal meconium samples have been studied for a century by culture-based methods and more recently by DNA sequencing; this has also sometimes yielded contradictory findings10,41,42,60 due to contamination and because postnatal colonization may occur before a meconium is delivered24. However, when meconium appears early, culturable bacteria are seldom detected (as reviewed by Perez-Munoz *et al*.24). In agreement with this, an analysis of meconium samples collected from extremely premature infants61 showed that taxa identified as contaminants34,36 make up a large proportion of sequences in meconium collected within the first 3 days after delivery and then drop to almost zero in most samples at days 4-6 (Figure 2), suggesting that the genuine bacterial signal is low in early meconium.

Relatedly, members of a putative fetal microbiome should be, in theory, detectable independent of birth mode. There is indeed some overlap between the reported fetal microbial taxa38,43, e.g. staphylococci, enterococci, lactobacilli, and enterobacteria, and the microbiota detected in infant fecal samples in the first week62-64. 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 birth65, which should contain microbes reported *in utero* as the fetus swallows amniotic fluid. However, aspirates from vaginally-born infants contained the specific *Lactobacillus* species (*L. iners* and *L. crispatus*) that also dominate the microbiota of the vagina, while most samples from C-section deliveries clustered with negative controls65. 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 meconium61 were typical maternal skin representatives (*Staphylococcus* & *Corynebacterium*) and were strongly associated with C-section, or were maternal fecal microbiota representatives (*Escherichia* & *Bacteroides*) associated with vaginal delivery (Figure 2), indicating that these genuine signals were derived from microbes acquired *ex-utero*.

Research is beginning to determine the origin of post-partum neonatal microbial colonizers and has shown a delay in appearance of bacterial species presumed to originate from the mother’s gut (e.g. *Bifidobacterium* and *Bacteroides* species) in early fecal samples of infants born by C-sections62,63,66-68. A substantial proportion of strains acquired by infants postnatally can be traced back to their mothers68-70, and fecal microbiota transplant (FMT) restores the microbiome in C-section delivered infants71. Thus, the published evidence, although still incomplete, suggests that the early life microbiome in humans is acquired through the vertical and horizontal transfer of microbes whose origin is fecal or environmental (from outside) rather than fetal (from inside).

**Bioinformatic and data science perspectives**

Characterization of low-biomass samples by 16S rRNA gene amplicon sequencing is challenging as DNA contamination can occur from the microbial DNA present in reagents, tools, instruments, and DNA isolation kits34-36 and through cross-contamination between PCR tubes/wells, sequencing runs, or sequencing lanes35. 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, different sequencing runs), leading to batch effects which may be mistaken for genuine signals44. Overreliance on or under analysis of such negative controls in combination with the misuse of contamination removal programs like Decontam72, specifically by not having negative controls in all batches, frequently results in false retention of contaminants44. Even with appropriate controls, it is challenging to separate genuine signals from low abundance contaminants due to the law of small numbers, which means that contaminant signals may appear sporadically in samples and negative controls73. Thus, suboptimal handling 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 end up in negative controls through cross-contamination during PCR or sequencing (machine contamination)35.

Unfortunately, both Rackaityte *et al.*43 and Mishra *et al.*38 reported taxa as legitimate findings that are typical contaminants (Figure 1). The most obvious case is *Bradyrhizobium*, which is one of the most dominant and consistent contaminants found in sequencing studies36,74. Rackaityte *et al.* reported *Micrococcus* and *Lactobacillus* as genuine fetal inhabitants, but a re-analysis of the data revealed that this finding was driven by a batch effect44. Although the authors rejected this conclusion37, this batch effect is clearly visible if the findings of the two batches are plotted together (Figure 3). In addition, Mishra *et al.* considered their signal for *Micrococcus* to be derived from contamination38. *Afipia*, *Flavobacterium*, *Pseudomonas*, and *Brevundimonas* are genera reported by Mishra *et al.*38 that are commonly detected as kit or laboratory reagent contaminants34,36.

Mishra *et al*. and Rackaityte *et al.* also reported marginally higher total bacterial load in fetal samples as compared to controls, using qPCR38,43. However, eukaryotic DNA in tissue samples (which is absent in negative controls) might have a DNA carrier effect leading to a more efficient DNA precipitation of prokaryotic reagent contaminants. In addition, bacterial PCR primers also amplify mitochondrial DNA, which is evolutionarily of bacterial origin. Together these factors may explain why samples from low-biomass studies are often reported as having more bacterial DNA than controls and show that this cannot always be relied upon as evidence for the presence of microbes. Rackaityte *et al*. depleted human mitochondrial DNA (mtDNA) from their 16S rRNA gene sequence set that co-amplified in the PCR, but neither study accounted for mtDNA in their qPCR analysis, although their primers targeted the 16S rRNA gene and were therefore potentially susceptible to cross-reactivity38,43.

**Immunological perspective**

The enteric microbiota in general, and some microbial taxa in particular, undoubtedly act as potent drivers of adaptive mucosal immune maturation and priming in the adult host75-78. Besides their intrinsic immunogenic nature, microorganisms also generate metabolites that critically promote and shape immune maturation and priming79-81. Although the early fetal immune system is immature, recent research demonstrates migration of fetal dendritic cells (DCs) to the mesenteric lymph nodes; somatic hypermutation in fetal B cells; and increasing T cell receptor repertoire diversity, evenness and activation during late fetal development7,82,83.

The existence of metabolically active microbes in the fetus could, in principle, provide one possible explanation for these findings. Mishra *et al.*38 used an autologous T cell expansion assay to show that fetal DCs loaded with antigen from bacteria that had been isolated from fetal tissues stimulated proliferation of CD45RO+ and CD69+ T cells. T cell proliferation was reduced but still detectable in the absence of DC-derived cytokine release suggesting an activated memory response38. Demonstration that the fetal T cell memory response is specific for the bacteria present in one individual fetus would be necessary to strengthen the interpretation that specific immune responses are routinely driven by fetal bacterial colonization. There are alternative explanations for fetal immune responses apart from *bona fide* microbial colonization. Maternal antigen-IgG complexes have been detected in cord blood and transplacental immune priming of the fetal immune system in early gestation has been demonstrated84,85 Cross-reactivity, as observed for microbiota reactive enteric secretory immunoglobulin A, would support fetal priming by maternal microbial antigens80. Similarly, maternal microbiota-derived microbial molecules partly bound to IgG stimulated innate immune maturation of the murine fetal gut47, and maternal intestinal carriage of *Prevotella* protected the offspring from food allergy in humans86. Thus, maternal microbiota-derived microbial antigens and metabolites may pass the placental filter directly or bound to IgG and evoke the observed primary fetal immune response87.

If a significant biomass of microbes in fetal tissues is not rapidly cleared, it implies either overt infection and inflammation, or mechanisms of immune or microbial adaptation for symbiosis. At present, we have no clear evidence for such a symbiosis. Bacteria detected in fetal tissues from the genera *Staphylococcus, Escherichia, Enterococcus* or *Pseudomonas* represent important causative agents of infection in human preterm neonates (see section below on clinical microbiology). These can withstand the host’s innate defence system at least to some extent and provoke an inflammatory response88. Such bacteria are also capable of very rapid replication, as they expand several million-fold during microbiota assembly after birth89. Their presence in placental tissue in the absence of an inflammatory tissue response or colonization of fetal mucosal surfaces would require highly efficient host mechanisms of immune control and bacterial growth restriction, which are unlikely considering the immature state of the fetal immune system. On the other hand, bacteria such as *Micrococcus*, which were detected in fetal intestines by Rackaityte *et al.*37, rarely cause invasive infection in humans. Their prolonged presence within healthy tissues such as the placenta would require bacterial mechanisms of resistance against antimicrobial effector molecules of the host innate immune system such as complement. 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 contaminant45,46.

From an immunological perspective, the hypothesis of a fetal microbiome therefore requires the identification of mechanisms that control and tolerate bacterial populations and prevent overt inflammation and inflammation-driven tissue destruction in the presence of viable and metabolically active microorganisms, many of which are opportunistic pathogens (see below). Alongside this, mechanisms by which the commensal or symbiotic microbes survive the immune response 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. Given that such immunological and anatomical mechanisms have not been identified or even proposed26, the observed immune maturation and priming during fetal development is most likely not induced through colonization of the fetus with live microbes but rather through maternal immune components or microbial fragments and metabolites crossing the placental barrier.

**Clinical microbiology perspective**

No part of the human body is impregnable to bacterial invasion. Transient bloodstream bacteraemia is associated with something as innocuous as tooth brushing90, 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 treatment91. The claims for non-pathogenic fetal microbial exposure38,43 have not established whether host-microbe interactions reflect small scale translocation, asymptomatic infection, persistent symbiosis or mutualism, and how microbes might persist at low levels without immune elimination and without harming the host.

The ‘fetal-enriched taxa’ reported by Mishra *et al*. include *Flavobacterium, Lactobacillus, Staphylococcus, Afipia, Pseudomonas, Bradyrhizobium*, and *Brevundimonas*38*.* They also report successful culturing of lactobacilli and staphylococci from fetal tissue, but the lack of unambiguous species-level taxonomic identification of the cultured organisms is an unfortunate and significant technical limitation. Lactobacilli are usually of low pathogenic potential, they inhabit external mucosal surfaces of healthy humans, including the nose92 and vagina55, and they are often used as probiotics93. However, some strains and species lactobacilli do express potential virulence factors such as fibrinogen-binding, platelet-aggregation94 and inerolysin95 and have the ability to adhere to biotic surfaces with pili96. Furthermore, their ability to resist oxidative stress97 and grow in the absence of iron98, allows them to cause serious infections such as endocarditis when provided with the opportunity to access the bloodstream99,100. Such systemic infections can be life-threatening with mortality rates as high as 30%100. This casts doubt on the interpretation of lactobacilli being asymptomatic colonizers of fetal tissue rather than contaminants that are picked up during vaginal delivery.

A 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)38. These organisms can be long-term colonizers of external mucosal surfaces of humans 101,102, do not typically cause disease unless the mucosal barrier is breached. However, once they bypass mucosal barriers, they can deploy a more extensive repertoire of virulence factors to invade tissues by degrading connective tissues and, in the case of *S. aureus*, a repertoire of over a dozen cytolytic toxins genes that kill human cells103,104. CoNS, on the other hand, are ubiquitous skin colonizers, and their detection in clinical diagnostic laboratories (which is so common that it is considered a major diagnostic challenge105,106) is usually assumed to reflect contamination from the patient and occasionally the healthcare worker, in the absence of other reasons to suspect a CoNS infection77-79. 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, where they are the most common cause of late-onset neonatal sepsis107. Therefore, given that they are either comtaminants or overt pathogens, the detection of staphylococci, no matter whether *S. aureus* or CoNS, is difficult to accept as evidence for *in utero* colonization of a healthy fetus.

Other bacteria identified as part of a notional “fetal microbiome”, such as *Enterococcus faecalis* and *Klebsiella pneumoniae*, are equally problematic. These belong to a group known as “ESKAPE pathogens”, which include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. The lethality of tissue colonization with ESKAPE pathogens is well documented in mouse models, and these microbes are leading causes of healthcare-acquired infections worldwide with significant mortality and morbidity, even when treated with antibiotics108. Several ESKAPE pathogens readily survive in adverse conditions outside of vertebrate hosts, including drying, oxidative stress, and exposure to heat or sanitation chemicals109. They are likely to persist on inanimate surfaces including utensils or clinical fabrics110,111, thereby increasing their likelihood of being contaminants. While these microorganisms were not reported at the species level38, it is noteworthy that closely related organisms can also cause neonatal sepsis112-114 which makes them unlikely colonizers of a healthy fetus.

A consideration prompted by a notional fetal microbiome is the possibility that the fetus might cope better with nosocomial pathogens than neonates or even adults. However, there is ample evidence to show that amniotic fluid, the placenta and fetal tissues are highly susceptible to bacterial infection, and the outcomes of infections with *Streptococcus agalactiae* or *L. monocytogenes* are often catastrophic115,116. 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 placenta and fetus do not have greater resistance to infection than an adult human. Therefore, from a clinical perspective, most interpretations brought forward in recent publications38,43 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 mortality117-120.

**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 fetus121-123. 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 species24), which have long been used to study the biochemical, metabolic, and immunological influences of microbes on their mammalian hosts124-126. The primary consideration is whether germ-free animals are truly ‘free of all demonstrable forms of microbial life’127. If they lack microbial associates, there cannot be a fetal microbiome. Testing germ-free animals for contaminating microbes uses microscopic observation of stained fecal smears, culture of feces in nutrient media under various conditions of temperature and gaseous atmosphere122,127-129, PCR using ‘universal bacterial’ primers128,130, and serological assays for viral infections131. These tests consistently demonstrate an absence of microbial associates. Therefore, gnotobiology provides strong evidence that the fetus *in utero* is sterile.

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

In this perspective, we have applied a trans-disciplinary approach focused on scrutiny of existing evidence and mechanistic explanations and 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 typical human fetus, the available data does not support stable, functional, nontrivially abundant colonizers under normal, non-pathogenic circumstances. We are aware that our position conflicts with dozens of publications that claim evidence for *in utero* microbial populations, but we feel confident about the validity of our multi-layered approach. Our aim was to bring additional clarity to the debate and suggest re-focussing scientific effort towards other concepts that will provide solid scientific foundations, enable translation, and improve maternal-fetal and child health through appropriate research priorities and use of resources.

The processes by which the fetus matures and becomes immunologically equipped for life in a microbial world have life-long implications and is one of the most important areas in biology and medicine. This research calls for scientific minds that are open to fresh thinking and willing to change, and no dogma, no matter how well established, is exempt from scrutiny. Notwithstanding the caution and safeguards recommended in this perspective, scientists should not be dissuaded from exploring the microbial drivers of fetal immune development. Paradoxically, we contend that sterile tissues are both immunologically and microbiologically fascinating. 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 life87.

**Considerations for the critical evaluation of low- or no biomass samples**

Contamination has always been a confounder in microbiology but is of particular concern for those studying low- or no biomass samples.34,36. 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, to harbour diverse microbial communities. As with intrauterine studies described above, these microbial populations are generally discussed in light of their importance for human diseases and health. In instances of contamination, a tissue may be misjudged as non-sterile, whereas in others, a real microbiological signal may be obfuscated by contamination.

As Saffarian et al132 point out, one is faced in studies on low biomass samples with the difficult exercise of extracting relevant signals from among contaminating noise that cannot be rationally eliminated. 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 relevant biological signals. Post-sequencing contamination removal using software packages such as Decontam72 or other statistical approaches34 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.61 and Saffarian et al132, and we extend on these examples in Box 1. There is clearly a need for formal standardisation of best practices in the analysis of low and putative “no biomass” samples.

We draw attention to the distinction between “low biomass” and no biomass samples. This has practical significance; true “low (microbial) biomass” samples are amenable to contamination-removal approaches described above, but “no (microbial) biomass” samples require a different approach (Box 1). For credible proof of the presence of microbes, multiple layers of evidence are required, first with quantitative, sensitive (lower detection limit) approaches such as quantitative PCR 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, the flaws of the recent studies on fecal microbial populations demonstrates that 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 explanations26. When obligately photosynthetic, psychrophilic, thermophilic, halophilic, or chemolithoautotrophic bacteria are found in human tissues which do not provide the growth conditions for such organisms22,133, or if the detected genera are known contaminants of laboratory kits/reagents that should not have escaped decades of culture studies, such as Proteobacteria (*Pseudomonas and E.coli* for example)134-136, the authenticity of such signals must be questioned.

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

**High biomass samples**

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

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

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

**Low biomass**

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

***Impact of contamination*:** Ranges from low to high. Contaminated samples are progressively affected with reducing input microbial biomass36.

***Mitigations*:** Inclusion of multiple controls facilitate contamination recognition. When possible, samples should be concentrated prior to processing to increase input biomass. Advance consideration of potential sources of contamination during the sample acquisition stage is always recommended. After sample collection, processing should be carried out in a clean-room environment, preferably with all surfaces bleached and UV-treated. The extraction step may benefit from use of non-kit-based methods (e.g. phenol-chloroform extractions) where plasticware and individual reagents are UV-treated prior to use. Contamination from DNA isolation and PCR kits is usually identifiable, particularly if well-defined and controlled batch effects are created using different lot numbers of particular kits. Regardless of the DNA extraction method, the presence of contaminants should be monitored by including “blank” negative controls. The inclusion of controls generated by serial dilution of DNA of known composition (e.g. mock community) will indicate the biomass level at which contamination becomes a dominant feature of sequencing results. Contamination may also be estimated prior to sequencing by qPCR using serially diluted known quantities of spiked DNA. Post-sequencing analyses, using programs like Decontam, and analysis steps as described by de Goffau et al.34 and used by Heida et al.61 will usually identify contaminants. To elucidate the source of contaminants introduced during the sample collection stage, sufficient numbers of samples acquired with different methods should be included.

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

***Examples*:** Placental and fetal tissues, amniotic fluid, brain tissue and cerebrospinal fluid, blood, bone, and internal cancer tissues.

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

***Mitigations*:** Experimental design should be robust and directed specifically against contamination. An initial assessment using quantitative methods (e.g. qPCR) with low detection limit and microscopic visualisation (e.g. Gram staining/labelling by FISH) is required to determine if microbes are present, before embarking on a sequence-based approach. Note such approaches are still susceptible to sample contamination and other artefacts (e.g. non-specific staining or auto-fluorescence from mucins, can sometimes appear “microbe-like” in size and shape)44. All mitigations outlined for “Low biomass” samples above should be adopted. Furthermore, repeat sample analysis with different DNA extraction kits/methods30 and/or at different days137. These will track the presence of particular species in sequencing profiles associated with specific kits/reagents or environment. Species that are repeatedly detected regardless of technical approach used are more likely to be genuine signals, unless they were introduced during the sample collection. Binary statistics (absence/presence) are recommended. Ideally, the presence of microbes identified by sequencing should be verified with a different technique such as cultivation, another sequencing technique with sufficient taxonomic resolution, and a species-specific qPCR or FISH using high magnification to visualize the size and morphology of individual microbial cells.

**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 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).

11 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).

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

13 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).

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

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

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 Blaser, M. J. *et al.* Lessons learned from the prenatal microbiome controversy. *Microbiome* **9**, 8, doi:10.1186/s40168-020-00946-2 (2021).

21 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).

22 Editorial. Microbiome studies and "blue whales in the Himalayas". *Lancet Infect Dis* **18**, 925, doi:10.1016/S1473-3099(18)30503-6 (2018).

23 Hornef, M. & Penders, J. Does a prenatal bacterial microbiota exist? *Mucosal Immunol* **10**, 598-601, doi:10.1038/mi.2016.141 (2017).

24 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).

25 Segata, N. No bacteria found in healthy placentas. *Nature* **572**, 317-318, doi:10.1038/d41586-019-02262-8 (2019).

26 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).

27 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).

28 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).

29 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).

30 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).

31 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).

32 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).

33 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).

34 de Goffau, M. C. *et al.* Recognizing the reagent microbiome. *Nat Microbiol* **3**, 851-853, doi:10.1038/s41564-018-0202-y (2018).

35 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).

36 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).

37 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).

38 Mishra, A. *et al.* Microbial exposure during early human development primes fetal immune cells. *Cell*, doi:10.1016/j.cell.2021.04.039 (2021).

39 Li, Y. *et al.* In utero human intestine harbors unique metabolomic features including bacterial metabolites. *JCI Insight*, doi:10.1172/jci.insight.138751 (2020).

40 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).

41 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).

42 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).

43 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).

44 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).

45 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).

46 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).

47 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).

48 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).

49 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).

50 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).

51 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).

52 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).

53 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).

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

55 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).

56 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).

57 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).

58 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).

59 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).

60 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).

61 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).

62 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).

63 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).

64 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).

65 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).

66 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).

67 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).

68 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).

69 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).

70 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).

71 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).

72 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).

73 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).

74 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).

75 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).

76 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).

77 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).

78 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).

79 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).

80 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).

81 Arpaia, N. *et al.* Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* **504**, 451-455, doi:10.1038/nature12726 (2013).

82 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).

83 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).

84 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).

85 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).

86 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).

87 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).

88 Henneke, P., Kierdorf, K., Hall, L. J., Sperandio, M. & Hornef, M. Perinatal development of innate immune topology. *Elife* **10**, doi:10.7554/eLife.67793 (2021).

89 van Best, N. *et al.* Bile acids drive the newborn's gut microbiota maturation. *Nat Commun* **11**, 3692, doi:10.1038/s41467-020-17183-8 (2020).

90 Lockhart, P. B. *et al.* Bacteremia associated with toothbrushing and dental extraction. *Circulation* **117**, 3118-3125, doi:10.1161/CIRCULATIONAHA.107.758524 (2008).

91 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).

92 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).

93 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).

94 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).

95 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).

96 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).

97 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).

98 Weinberg, E. D. The Lactobacillus anomaly: total iron abstinence. *Perspect Biol Med* **40**, 578-583, doi:10.1353/pbm.1997.0072 (1997).

99 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).

100 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).

101 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).

102 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).

103 Otto, M. Staphylococcus aureus toxins. *Curr Opin Microbiol* **17**, 32-37, doi:10.1016/j.mib.2013.11.004 (2014).

104 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).

105 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).

106 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).

107 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).

108 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).

109 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).

110 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).

111 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).

112 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).

113 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).

114 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).

115 Nan, C. *et al.* Maternal group B Streptococcus-related stillbirth: a systematic review. *BJOG* **122**, 1437-1445, doi:10.1111/1471-0528.13527 (2015).

116 Vazquez-Boland, J. A., Krypotou, E. & Scortti, M. Listeria Placental Infection. *mBio* **8**, doi:10.1128/mBio.00949-17 (2017).

117 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).

118 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).

119 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).

120 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).

121 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).

122 Luckey, T. D. *Germfree Life and Gnotobiology*. (Academic Press, 1963).

123 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).

124 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).

125 Gordon, H. A. & Pesti, L. The gnotobiotic animal as a tool in the study of host microbial relationships. *Bacteriol Rev* **35**, 390-429 (1971).

126 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).

127 Wostman, B. S. *Germfree and Gnotobiotic Animal Models. Background and Applications*. (CRC Press, 1996).

128 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).

129 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).

130 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).

131 Schoeb, T. R. & Eaton, K. A. *Gnotobiotics.* . (Academic Press (Elsevier), 2017).

132 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).

133 Salzberg, S. in *Forbes* (2020).

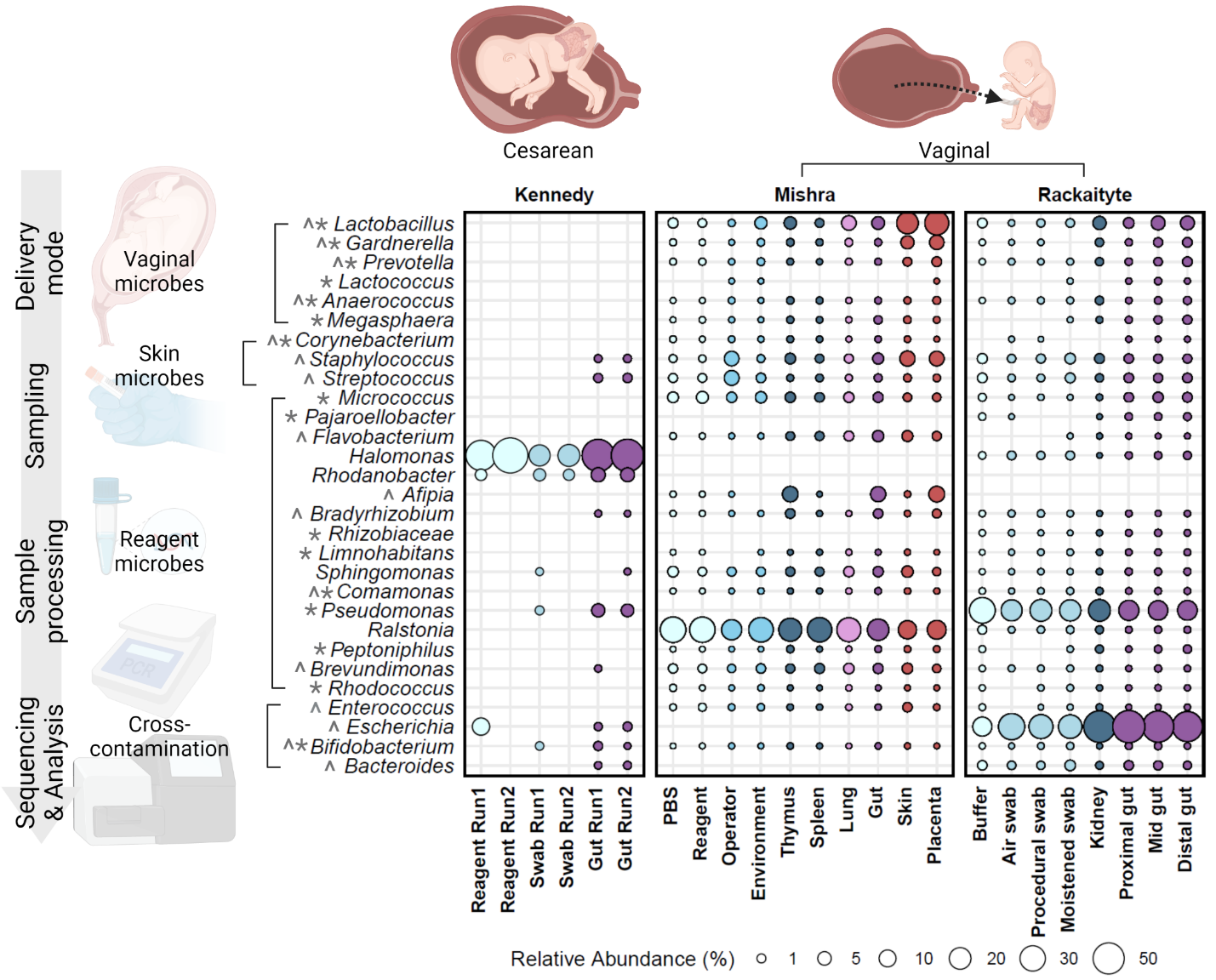
134 Jost, T., Lacroix, C., Braegger, C. & Chassard, C. Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. *Br J Nutr* **110**, 1253-1262, doi:10.1017/S0007114513000597 (2013).

135 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).

136 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).

137 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).

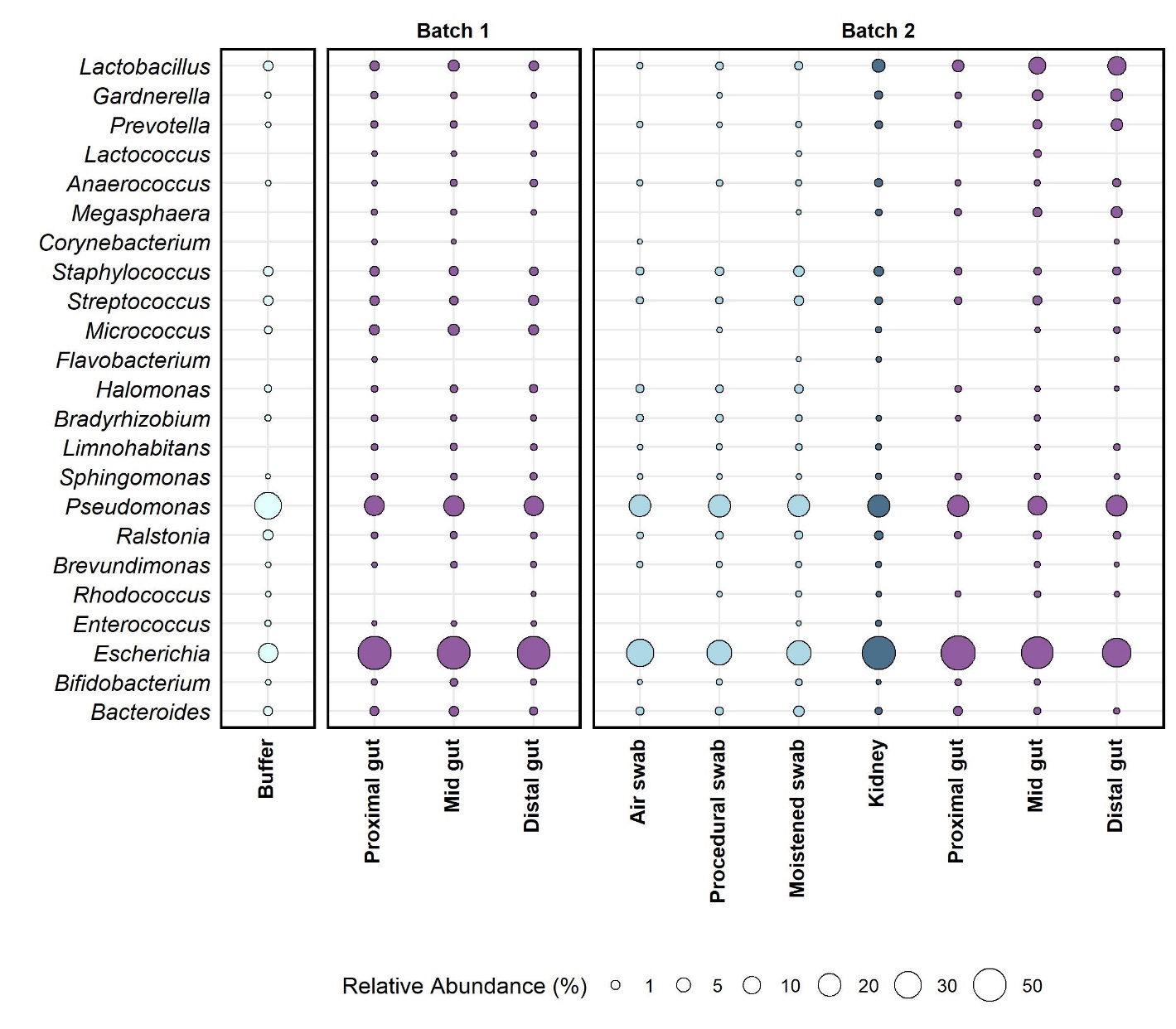
**Figures**



**Figure 1.** Distribution and mean relative abundance (%) of genera present in fetal samples from three recent studies28,38,43 investigating the fetal microbiome and their corresponding abundance in control samples. Taxa were selected based on the following criteria: Genera that were cultured from or enriched in fetal samples as described by Mishra *et al.*38 (indicated by ^) or by Rackaityte *et al.*43 (indicated by \*); all genera detected in fetal samples from Kennedy *et al.*28; and the PBS-enriched genus *Ralstonia*38. Taxa were grouped by potential source of contamination in agreement with the origin of genera (for skin microbes) and previous studies that characterized sources of contamination34-36. For taxonomic data from Rackaityte *et al.*, OTU10 (family *Micrococcaceae*) was manually assigned to the genus *Micrococcus* as in the original publication. Publicly available unfiltered relative abundance data associated with each publication were merged into a single phyloseq object (RRID:SCR\_01380). Amplicon Sequence Variants (ASVs) were grouped at the genus level. The mean relative abundance of each genus was calculated for each sample type within each study and plotted in R (tidyverse, ggplot2; RRID:SCR\_014601). Dot size corresponds to the mean relative abundance of each genus by sample type and study (mean relative abundances <0.0001% were excluded). Dots are colored by sample type: reagent controls in lightest blue (Mishra: PBS n=42, Reagent n=23; Rackaityte: Buffer n=11; Kennedy Reagent n=2); sampling negatives in light blue (Kennedy: Swab n=1; Rackaityte: Air swab n=19; Procedural swab n=16; Moistened swab n=17) and environmental negatives in sky blue (Mishra: Environment n=47, Operator n=12), internal controls in dark blue (Mishra: Thymus n=27, Spleen n=12; Rackaityte: Kidney n=16), fetal lung in pink (Mishra, n=25), fetal gut in purple (Kennedy: n=20; Mishra: n=44; Rackaityte: Proximal n=41, Mid n=45, Distal n=42), and external tissues in red (Mishra: Skin n=35, Placenta n=16).



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

****

**Figure 3.** Distribution and mean relative abundance (%) of genera present in fetal and control samples from Rackaityte *et al.*43 by batch as defined by *Rackaityte et al.*37. Dominant taxa were manually selected as described in Fig. 1. For taxonomic data OTU10 (family *Micrococcaceae*) was manually assigned to the genus *Micrococcus* as in the original publication43. Publicly available unfiltered relative abundance data associated with each publication were merged into a single phyloseq object (RRID:SCR\_01380). ASVs were grouped at the genus level. The mean relative abundance of each genus was calculated for each sample type within each batch and plotted in R (tidyverse, ggplot2; RRID:SCR\_014601). Dot size corresponds to the mean relative abundance of each Genus by sample type and batch. Dots are coloured by sample type: reagent controls in lightest blue (Buffer), sampling negatives in light blue, internal controls in dark blue (Kidney), and fetal gut in purple.

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

**Acknowledgements.** T.B receives funding from the Deutsche Forschungsgemeinschaft (German Research Foundation). J.D. acknowledges funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (Grant agreement ERC-2017-AdG No. 788191 - Homo.symbiosus). W.M.dV and A.S. are supported by the Academy of Finland (grants 1308255 and 1325103). A.M.E. is funded in part with Federal funds from the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health, Department of Health and Human Services, under Grant Number U19AI110818 to the Broad Institute. M.A.E is funded through grants R01HD102318, R01HD098867 and R01NR014784. S.C.G.V was funded through a Peter Hans Hofschneider Professorship provided by the Stiftung Molekulare Biomedizin. M.G.G is funded by the Canada Research Chairs Program. L.J.H. is supported by Wellcome Trust Investigator Awards 100974/C/13/Z and 220876/Z/20/Z; the Biotechnology and Biological Sciences Research Council (BBSRC), Institute Strategic Programme Gut Microbes and Health BB/R012490/1, and its constituent projects BBS/E/F/000PR10353 and BBS/E/F/000PR10356. M.W.H. has received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (Grant agreement No. 101019157). S.L. has received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (Grant agreement 852600 Lacto-Be). A.J.M receives funding from ERCAd HHMM-Neonates and Swiss National Science Sinergia. Work in P.W.O, L.O’M, and J.W. laboratories is supported by Science Foundation Ireland (SFI) through a Centre award (APC/SFI/12/RC/2273\_P2) to APC Microbiome Ireland. JW acknowledges support through an SFI Professorship (19/RP/6853) and thanks Victoria McMahon for coordinating the writing of this review and Ryan O'Callaghan for encouragement. JR 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.