

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

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## 107 **Preface**

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

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

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### 133 **Introduction**

134 Fetal immune development prepares the neonate for life in a microbial world and underpins  
135 lifelong health<sup>1-4</sup>. Neonates born at term are not immunologically naïve and are specifically

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

151  
152 Such disagreement over a fundamental aspect of human biology poses a challenge for scientific  
153 progress. The notion of a fetal microbiome, if proven correct, has implications for clinical medicine  
154 and would call for a comprehensive reappraisal of previous concepts and research. It would  
155 require radical revision of our understanding of the development of the immune and other systems  
156 in early life and the anatomical and immunological mechanisms mediating host-microbe  
157 interactions within fetal tissues. Failure to resolve this issue has a potential risk of diverting finite  
158 resources into research that results in no advancement for fetal and maternal health, and  
159 misguided attempts to therapeutically modify a non-existent fetal microbiome. The dilemma has  
160 further relevance to the characterization of microbiota in all low-biomass samples. Therefore, we

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

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### 168 **Claims and counterclaims**

169 Although the disagreement on the presence of microbes in prenatal intrauterine locations  
170 (placenta and amniotic fluid) spans dozens of studies with contradictory findings<sup>11,13,14,22,28,30-  
171 33,36,41-43</sup>, we focused our analysis on four recent studies since they provide a direct assessment  
172 of the fetus itself<sup>29,38-40</sup>. Collection of human fetal samples is difficult and restricted to either  
173 following pregnancy termination, or immediately prior to birth by C-section. Three of the studies  
174 used samples collected after vaginally delivered, elective, second trimester pregnancy  
175 terminations<sup>38-40</sup>, and one collected samples from breech C-section deliveries immediately at  
176 birth<sup>29</sup>.

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

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

188  
189 Several of the study's conclusions have been challenged by de Goffau *et al.*<sup>44</sup>, who re-analyzed  
190 the publicly available data and found no evidence for a distinct bacterial profile in the subset of  
191 samples with matched procedural controls, and concluded that the positive findings were caused  
192 by a sequencing batch effect (indicative of contamination) and further contamination during  
193 culture<sup>44</sup>. In addition, the authors' suggestion that particles detected in SEM micrographs  
194 constitute micrococci<sup>38</sup> was disputed as their size exceeded that of known Micrococcaceae<sup>44</sup>.  
195 Furthermore, the 16S rRNA gene sequence of the *Micrococcus luteus* cultured from the fetal  
196 samples differed from that detected by sequencing, further supporting contamination during  
197 culture (*Micrococcus luteus* is a common contaminant of clean rooms and surgical  
198 instruments<sup>45,46</sup>).

199  
200 Mishra *et al.*<sup>39</sup> detected a low but consistent microbial signal across tissues of vaginally delivered  
201 fetuses from 2<sup>nd</sup> trimester terminations by 16S rRNA gene amplicon sequencing (V4-V5 region),  
202 with 7 genera enriched in fetal samples (*Lactobacillus*, *Staphylococcus*, *Pseudomonas*,  
203 *Flavobacterium*, *Afipia*, *Bradyrhizobium*, and *Brevundimonas*). The 16S rRNA gene sequencing  
204 data were accompanied by SEM, RNA-*in situ* hybridization (RNA-ISH), and culture. In recognition  
205 of the high risk of contamination, all samples were processed in isolation with negative controls  
206 collected during sample processing. In contrast to Rackaityte *et al.*, Mishra *et al.* found  
207 *Micrococcus* to be enriched in phosphate buffered saline (PBS) reagent controls and reported it  
208 as a contaminant, with the *M. luteus* cells detected by culture being consistent with the size and  
209 morphology of the coccoid structures found by SEM<sup>39</sup>.

210

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

221  
222 In contrast to these reports, Li *et al.*<sup>40</sup>, who also investigated fetal intestinal tissue from second  
223 trimester terminations, did not detect bacterial DNA by PCR (V4 region of the 16S rRNA gene, 35  
224 cycles) based on visual inspection of agarose gels in any of the 101 samples tested. The authors  
225 detected a diverse set of metabolites in fetal intestinal samples and hypothesized that maternal,  
226 microbiota-derived metabolites may pass through the placenta to 'educate' the fetal immune  
227 system. This conclusion is supported by research in mice that showed that fetal immune  
228 education can be driven in the absence of direct microbial exposure by trans-placental passage  
229 of microbial metabolites from the maternal gut<sup>47,48</sup>.

230  
231 Kennedy *et al.*<sup>29</sup> used a different approach and collected samples using rectal swabs during  
232 elective C-section for breech presentation at term gestation<sup>29</sup>. Comparisons with environmental  
233 and reagent-negative controls from two independent sequencing runs were included to account  
234 for contamination and stochastic noise. No microbial signal distinct from negative controls was  
235 detected, and aerobic and anaerobic bacteria (*Staphylococcus epidermidis* and *Cutibacterium*



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

238

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

248

### 249 **Reproductive biology and obstetrics perspectives**

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

259

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

271  
272 Regardless of whether placental samples are collected by biopsy per vagina, clinically by  
273 chorionic villus sampling, or after delivery, it is always necessary to control for contamination,  
274 particularly from the tissues through which a placenta must pass prior to sampling. Accordingly,  
275 de Goffau *et al.*<sup>28</sup> carried out a comprehensive study of the possible placental microbiome, and  
276 detected a range of species known to dominate the vaginal microbiota<sup>55</sup>, such as *Lactobacillus*  
277 *iners*, *L. jensenii*, *L. crispatus*, *L. gasseri*, and *Gardnerella vaginalis*. When the presence of vaginal  
278 microbes and those in the laboratory reagents (the “kitome”) were accounted for, no placenta  
279 microbiome was detected in their studies or several further recent studies<sup>22,28,30-33,36</sup>.

280  
281 Pathogenic infection of the placenta by viral or bacterial pathogens is a well-recognized clinical  
282 phenomenon that contributes to preterm birth and neonatal sepsis<sup>56</sup>. de Goffau *et al.* detected  
283 *Streptococcus agalactiae* in around 5% of cases as the only verifiable bacterial signal in placentas  
284 obtained by C-section deliveries conducted prior to rupture of the fetal membranes and the onset

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

290  
291 Research claiming the presence of viable low-density microbial communities in the fetal intestine<sup>38</sup>  
292 and fetal organs<sup>39</sup> likewise calls for an evaluation of the sampling process. Mishra *et al.* obtained  
293 fetal tissues after medical termination of pregnancy in the 2<sup>nd</sup> trimester with prostaglandins<sup>39</sup>. This  
294 procedure typically involves the individual going through hours of labor and often leads to the  
295 rupture of the fetal membranes hours prior to vaginal delivery. Even with a standardized approach,  
296 labor may be prolonged and may be accompanied by infection and fever, which are common with  
297 2<sup>nd</sup> trimester terminations<sup>59,60</sup>. Both Li *et al.*<sup>40</sup> and Rackaityte *et al.*<sup>38</sup> also used 2<sup>nd</sup> trimester  
298 terminations but obtained the fetal tissues from core facilities. The tissues used by Li *et al.* were  
299 from surgical terminations (14-23 weeks) performed with mechanical dilation. Unfortunately,  
300 Rackaityte *et al.*<sup>61</sup> did not provide sufficient information to determine if fetuses were obtained  
301 through surgical procedures or medical inductions. While the latter increases the risk of the fetus  
302 being exposed to vaginal microbes during labour, both procedures involve vaginal delivery of the  
303 fetus. As outlined below, the reported microbiology of these fetuses primarily reflects the sources  
304 of microbes to which they are exposed during these procedures.

305  
306 **Microbiology and microbial ecology perspectives**

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

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

324  
325 In agreement with the unavoidable vaginal exposure of fetuses obtained by 2<sup>nd</sup> trimester abortions  
326 (see above), both Rackaityte *et al.*<sup>38</sup> and Mishra *et al.*<sup>39</sup> found the genera *Lactobacillus* and  
327 *Gardnerella*, which dominate the vaginal microbiota<sup>55</sup>, among their most consistent findings  
328 (Figure 1). The species cultured by Mishra *et al.*, *G. vaginalis*, *L. iners* and *L. jensenii*, are highly  
329 specific to the human vagina<sup>62</sup>. Other microbes detected such as *Staphylococcus* species and  
330 *Cutibacterium acnes*, are skin commensals. As shown in Figure 1, abundances of *Lactobacillus*,  
331 *Gardnerella*, and *Staphylococcus* found by Mishra *et al.* showed gradients with high population  
332 levels in fetal samples exposed to sources of contaminants (placenta and skin) and lower levels  
333 in internal samples (gut, lung, spleen, thymus). The omission of vaginal controls by both  
334 Rackaityte *et al.* and Mishra *et al.* to determine the microbiota of vaginally delivered fetuses is an

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

340

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

350

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

358

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

370

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

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

387  
388 Research is beginning to determine the origin of post-partum neonatal microbial colonizers and  
389 has shown a delay in appearance of bacterial species presumed to originate from the mother's  
390 gut (e.g. *Bifidobacterium* and *Bacteroides* species) in early fecal samples of infants born by C-  
391 sections<sup>66,67,70-72</sup>. A substantial proportion of strains acquired by infants postnatally can be traced  
392 back to their mothers<sup>72-74</sup>, and fecal microbiota transplant (FMT) restores the microbiome in C-  
393 section delivered infants<sup>75</sup>. Thus, the published evidence, although still incomplete, suggests that  
394 the early life microbiome in humans is acquired through the vertical and horizontal transfer of  
395 microbes whose origin is fecal or environmental (from outside) rather than fetal (from inside).

### 396 397 **Bioinformatic and data science perspectives**

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

409 retention of contaminants<sup>44</sup>. Even with appropriate controls, it is challenging to separate genuine  
410 signals from low abundance contaminants due to the law of small numbers, which means that  
411 contaminant signals may appear sporadically in samples and negative controls<sup>77</sup>. Thus,  
412 suboptimal handling of sequencing control samples may not reveal the full spectrum of  
413 contaminants because only the most abundant contaminant species are consistently  
414 detected. On the other hand, potentially genuine sample-associated signals sometimes also  
415 erroneously appear in negative control samples through cross-contamination during the PCR or  
416 sequencing steps (machine contamination)<sup>36</sup>.

417  
418 Unfortunately, both Rackaityte *et al.*<sup>38</sup> and Mishra *et al.*<sup>39</sup> reported taxa as legitimate findings that  
419 are very commonly reported contaminants (Figure 1). The most obvious case is *Bradyrhizobium*,  
420 which is one of the most dominant and consistent contaminants found in sequencing studies<sup>37,78</sup>.  
421 Rackaityte *et al.* reported *Micrococcus* and *Lactobacillus* as genuine fetal inhabitants, but a re-  
422 analysis of the data revealed that this finding was batch specific, indicative of contamination<sup>44</sup>.  
423 Although the authors rejected this conclusion<sup>61</sup>, this batch effect is clearly visible if the findings of  
424 the different batches are plotted together (Figure 3). In addition, Mishra *et al.* considered their  
425 own signal for *Micrococcus* to be derived from contamination<sup>39</sup>. *Afipia*, *Flavobacterium*,  
426 *Pseudomonas*, and *Brevundimonas* are further genera reported by Mishra *et al.*<sup>39</sup> that are  
427 commonly detected as kit or laboratory reagent contaminants<sup>35,37</sup>.

428  
429 Mishra *et al.* and Rackaityte *et al.* also reported marginally higher total bacterial load in fetal  
430 samples, as compared to controls, using qPCR<sup>38,39</sup>. However, eukaryotic DNA in tissue samples  
431 (which is absent in negative controls) might have a DNA carrier effect leading to a more efficient  
432 DNA precipitation of prokaryotic reagent contaminants. In addition, bacterial PCR primers may  
433 also amplify mitochondrial DNA, which is evolutionarily of bacterial origin. Together these factors



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

440

#### 441 **Immunological perspective**

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

448

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

457

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

468

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

481

482 **Clinical microbiology perspective**

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

491  
492 The 'fetal-enriched taxa' reported include *Micrococcus*, *Lactobacillus*, *Flavobacterium*,  
493 *Staphylococcus*, *Escherichia*, *Enterococcus*, *Afipia*, *Pseudomonas*, *Bradyrhizobium*, and  
494 *Brevundimonas*<sup>38,39</sup>. Mishra *et al.* also report successful culturing of lactobacilli and staphylococci  
495 from fetal tissue<sup>39</sup>, but the lack of unambiguous species-level taxonomic identification of the  
496 cultured organisms is an unfortunate and significant technical limitation. Bacteria such as  
497 *Micrococcus*, which were detected in fetal intestines by Rackaityte *et al.*<sup>61</sup>, rarely cause invasive  
498 infection in humans. Their prolonged presence within healthy tissues and transmission through  
499 the placenta would require bacterial mechanisms of resistance against antimicrobial effector  
500 molecules of the host innate immune system<sup>56</sup>. Such mechanisms have not been described for  
501 the genus *Micrococcus*, which is an environmental organism found in water, dust, and soil, and  
502 is also a common contaminant<sup>45,46</sup>. Lactobacilli are usually of low pathogenic potential, they  
503 inhabit external mucosal surfaces of healthy humans, including the nose<sup>94</sup> and vagina<sup>55</sup>, and they  
504 are often used as probiotics<sup>95</sup>. However, some strains and species of lactobacilli do express  
505 potential virulence factors<sup>96-98</sup>, resist oxidative stress<sup>99</sup> and grow in the absence of iron<sup>100</sup>, which  
506 allows them to cause serious infections such as endocarditis when provided with the opportunity  
507 to access the bloodstream<sup>101,102</sup>. This raises potential problems with the interpretation of

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

510

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

528

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

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

542  
543 A consideration prompted by a notional fetal microbiome is the possibility that the fetus might  
544 cope better with nosocomial pathogens than neonates or even adults. However, there is ample  
545 evidence to show that amniotic fluid, the placenta and fetal tissues are highly susceptible to  
546 bacterial infection, and the outcomes of infections with *Streptococcus agalactiae* or *Listeria*  
547 *monocytogenes* are often catastrophic<sup>117,118</sup>. Importantly, in *L. monocytogenes* infections that  
548 occur during the third trimester of pregnancy, fetal infection progresses while the mother's  
549 infection can be cleared, indicating that the fetus does not have greater resistance to infection  
550 than an adult human. Therefore, from a clinical perspective, most interpretations brought forward  
551 in recent publications<sup>38,39</sup> on the presence of microbes in fetuses seem to be biologically difficult  
552 to reconcile, as it is highly plausible that they would result in harm or death of the fetus. In  
553 agreement with this conclusion, in a series of well-controlled studies in various clinical settings,  
554 DiGiulio and co-workers found no evidence for microbes in amniotic fluid except when associated  
555 with neonatal morbidity and mortality<sup>119-122</sup>.

556

557 **Gnotobiology perspective**

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

573

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

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

582

583 The processes by which the fetus matures and becomes immunologically equipped for life in a  
584 microbial have life-long implications. Aside from the caution and safeguards recommended in this  
585 perspective, our aim here is not to dissuade scientists from exploring the microbial drivers of fetal  
586 immune development. We agree with proposals that there is a need to better understand microbial  
587 interactions at the maternal-fetal interface<sup>20</sup>, but do not think that symbiotic microbial populations  
588 in the placenta or fetus play a role in this. Paradoxically, we contend that sterile tissues are both  
589 immunologically and microbiologically fascinating, but require an adjustment of the  
590 methodological approaches used. How does the fetus mature and become immunologically  
591 equipped for life in a microbial world in the absence of direct exposure to live microbes? Are  
592 maternal-derived microbial metabolites sufficient for fetal immune education? Future research  
593 could include exploration of how maternal microbial-derived metabolites and small molecules, as  
594 well as maternal immune components, prepare the fetus for the microbial challenges of post-natal  
595 life<sup>91</sup>.

596

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

598 Contamination is always a potential confounder in microbiology but is of particular concern for  
599 those studying low- or no biomass samples.<sup>35,37</sup> The issue has been highlighted by recent reports  
600 of human tissues, such as blood, brain, and cancers (Box 1), previously thought to contain no, or  
601 very little, bacterial biomass that apparently harbour diverse microbial communities. As with  
602 intrauterine studies described above, these microbial populations are often discussed in light of  
603 their perceived importance for human diseases and health.

604

605 In studies on low biomass samples, it is challenging to identify relevant signals from among  
606 contaminating noise. In instances of contamination, a tissue may be misjudged as non-sterile,  
607 whereas in others, a real microbiological signal may be obfuscated by contamination. The removal

608 of all sequences present in negative-control samples or that have been previously identified as  
609 contaminants in the literature may result in loss of authentic signals. Post-sequencing  
610 contamination removal using software packages such as Decontam<sup>76</sup> or other statistical  
611 approaches<sup>35,134</sup> have been developed to remove the more abundant contaminants, leading to  
612 microbiome profiles that are more likely to reflect the real community. Practical examples of  
613 contamination removal in 16S rRNA gene sequence data is provided by Heida *et al.*<sup>65</sup>, Saffarian  
614 *et al.*<sup>135</sup>, and Jorissen *et al.*<sup>136</sup> and we expand on these examples in Box 1.

615  
616 We draw attention to the distinction between “low biomass” and no biomass samples. This has  
617 practical significance; true “low (microbial) biomass” samples are amenable to contamination-  
618 removal approaches but “no (microbial) biomass” samples require a different approach (Box 1).  
619 For credible proposals of the presence of microbes, multiple layers of evidence are required, first  
620 with quantitative, sensitive (lower detection limit) approaches, such as quantitative PCR with strict  
621 controls before contamination-sensitive sequencing approaches are applied. Since contamination  
622 removal will provide data regardless of whether microbes are present or absent, the starting  
623 proposition should be the null hypothesis to avoid confirmation bias, particularly when results are  
624 inconsistent and at the outer technical limits for detection, or if results defy mechanistic plausibility.

625  
626 Given the limitation of sequencing approaches, confirmation by alternative methods, such as  
627 FISH and culture, are required. However, as demonstrated with recent studies of fetal samples,  
628 even a combination of approaches has the potential to produce false findings, as contamination  
629 during sampling is a considerable challenge. We posit that studies on all low biomass samples  
630 can benefit from a similar trans-disciplinary assessment, as applied above for fetal samples, to  
631 interpret findings considering biological and mechanistic explanations<sup>27</sup>. When obligately  
632 photosynthetic, psychrophilic, thermophilic, halophilic, or chemolithoautotrophic bacteria are



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

637

638

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

641

642 **High biomass samples**

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

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

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

651

652 **Low biomass samples**

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

655 **Impact of contamination:** Ranges from low to high. Contaminated samples are progressively  
656 affected with reducing input microbial biomass<sup>37</sup>.

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

673

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

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

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

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

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

695

696

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1018 **mortality.**

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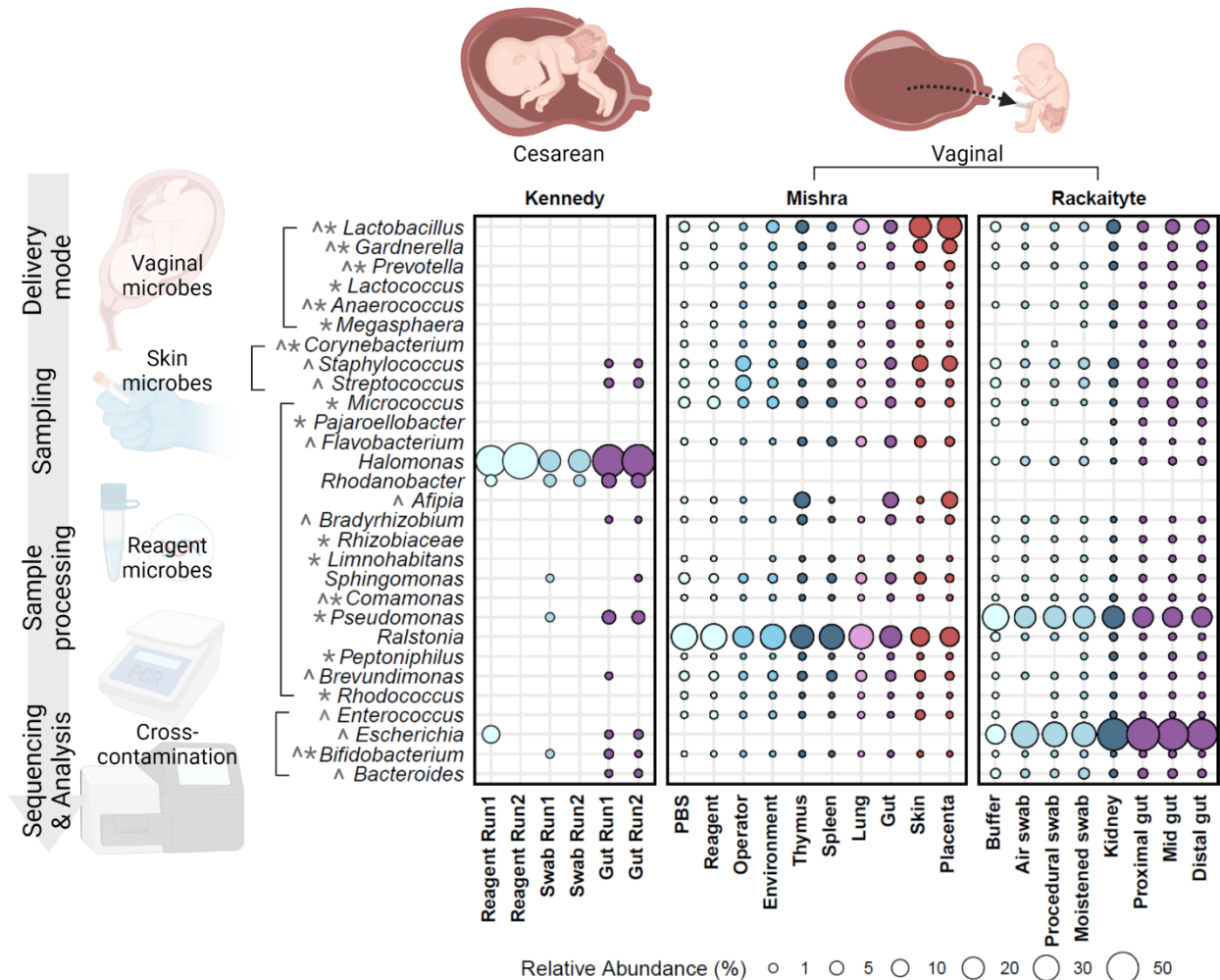
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1074 **Figures**



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1077 **Figure 1.** Distribution and mean relative abundance (%) of genera present in fetal samples from  
 1078 three recent studies<sup>29,38,39</sup> investigating the fetal microbiome and their corresponding abundance  
 1079 in control samples. Taxa were selected based on the following criteria: Genera that were cultured  
 1080 from or enriched in fetal samples as described by Mishra *et al.*<sup>39</sup> (indicated by ^) or by Rackaityte  
 1081 *et al.*<sup>38</sup> (indicated by \*); all genera detected in fetal samples from Kennedy *et al.*<sup>29</sup>; and the PBS-  
 1082 enriched genus *Ralstonia*<sup>39</sup>. Taxa were grouped by potential source of contamination (see left-  
 1083 hand side illustrations) in agreement with the likely origin of genera (for skin microbes) and  
 1084 previous studies that characterized sources of contamination<sup>35-37</sup>. For taxonomic data from  
 1085 Rackaityte *et al.*, OTU10 (family *Micrococcaceae*) was manually assigned to the genus

1086 *Micrococcus* as in the original publication. Publicly available unfiltered relative abundance data  
1087 associated with each publication were merged into a single phyloseq object (RRID:SCR\_01380).  
1088 Amplicon Sequence Variants (ASVs) were grouped at the genus level. The mean relative  
1089 abundance of each genus was calculated for each sample type within each study and plotted in  
1090 R (tidyverse, ggplot2; RRID:SCR\_014601). Dot size corresponds to the mean relative abundance  
1091 of each genus by sample type and study (mean relative abundances <0.0001% were excluded).  
1092 Dots are colored by sample type: reagent controls in lightest blue (Mishra: PBS n=42, Reagent  
1093 n=23; Rackaityte: Buffer n=11; Kennedy Reagent n=2); sampling negatives in light blue  
1094 (Kennedy: Swab n=1; Rackaityte: Air swab n=19; Procedural swab n=16; Moistened swab n=17)  
1095 and environmental negatives in sky blue (Mishra: Environment n=47, Operator n=12), internal  
1096 controls in dark blue (Mishra: Thymus n=27, Spleen n=12; Rackaityte: Kidney n=16), fetal lung in  
1097 pink (Mishra, n=25), fetal gut in purple (Kennedy: n=20; Mishra: n=44; Rackaityte: Proximal n=41,  
1098 Mid n=45, Distal n=42), and external tissues in red (Mishra: Skin n=35, Placenta n=16).

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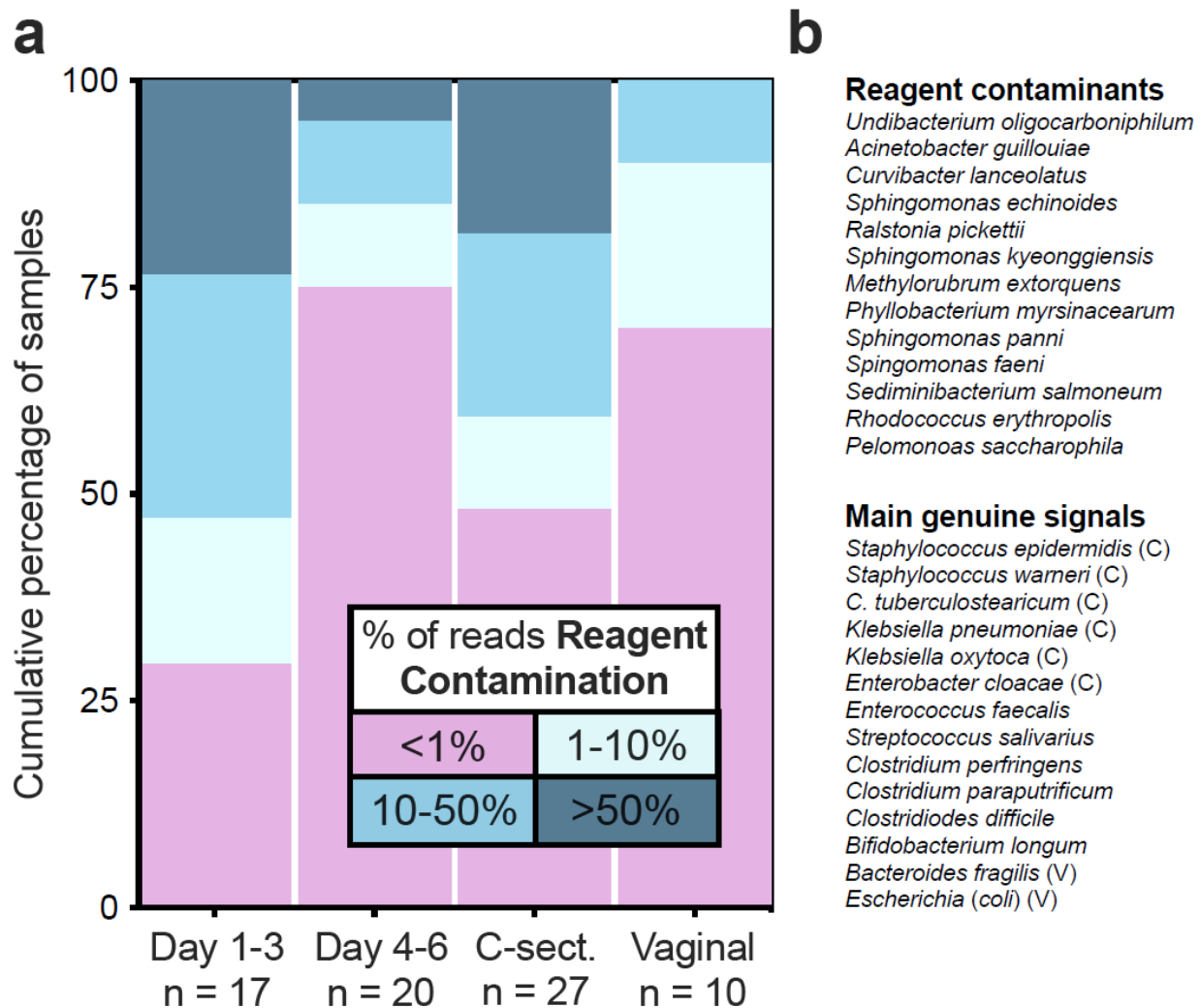
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1109 **Figure 2. Reagent contamination in meconium samples of extremely premature infants. a)**

1110 Representation of the % of reagent contamination in the first meconium of extremely premature

1111 infants in relation to the day of procurement of said samples (Day 1-3 or Day 4-6) or in regard to

1112 the mode of delivery (C-section or Vaginal). Colors indicate the percentage of reagent

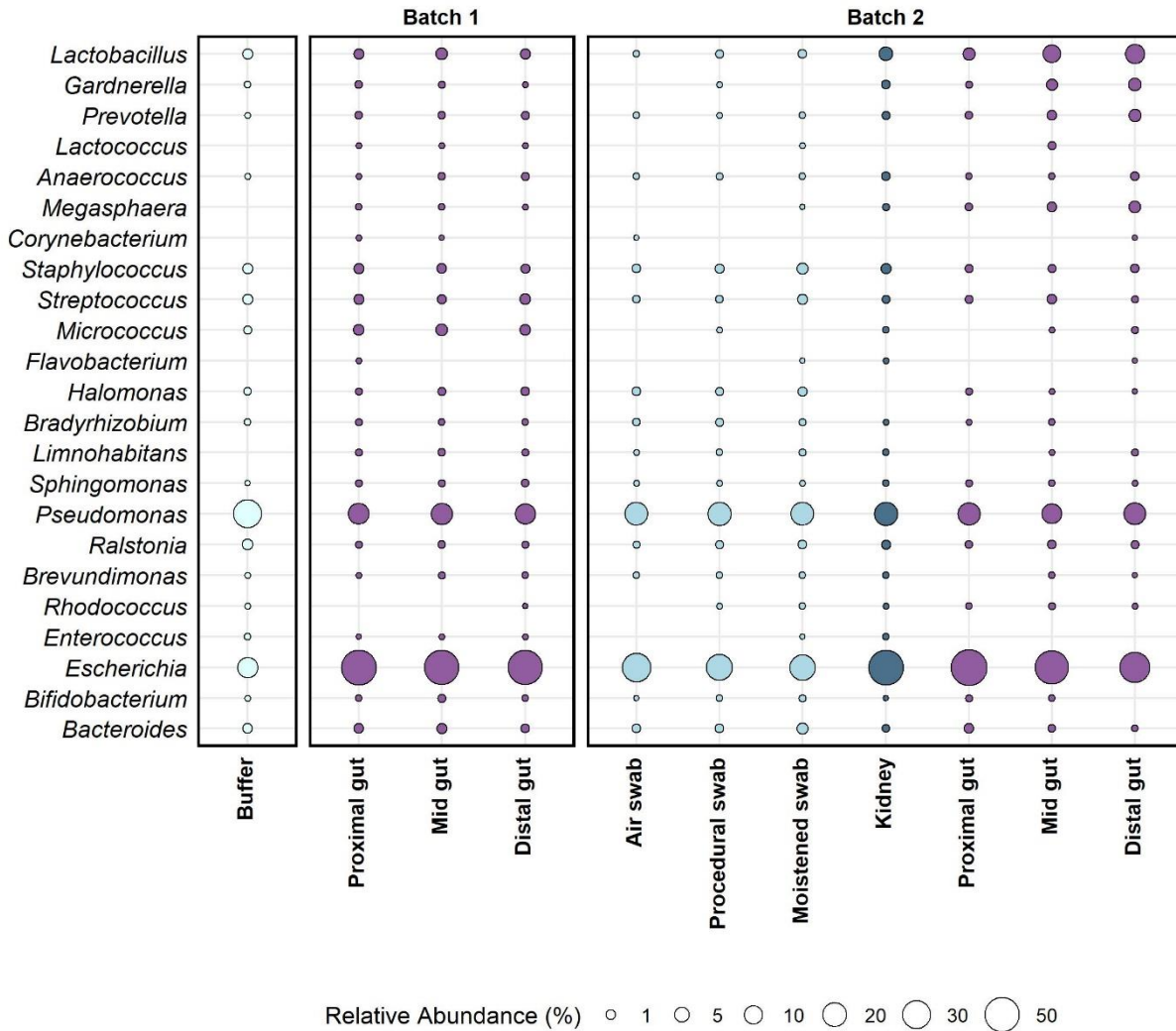
1113 contamination reads (legend on top). The day of procurement is significantly correlated with the

1114 % of reagent contamination reads ( $p = 0.005$  MW-U test or  $p = 0.01$  Spearman rho test) and the

1115 mode of delivery shows a trend ( $p = 0.07$  MW-U test). The number of samples is noted below

1116 each category (n). **b**) Lists of reagent contaminants shown together in **Figure 2a** (top) and of the

1117 most abundant sample-associated-signals and their association (or lack thereof due to limited  
 1118 size of cohort) with vaginal (V) or C-section (C) delivery (bottom).  
 1119



1120  
 1121  
 1122 **Figure 3.** Distribution and mean relative abundance (%) of genera present in fetal and control  
 1123 samples from Rackaityte *et al.*<sup>38</sup> by batch as defined by Rackaityte *et al.*<sup>61</sup>. Dominant taxa were  
 1124 manually selected as described in Fig. 1. For taxonomic data OTU10 (family *Micrococcaceae*)  
 1125 was manually assigned to the genus *Micrococcus* as in the original publication<sup>38</sup>. Publicly  
 1126 available unfiltered relative abundance data associated with each publication were merged into a

1127 single phyloseq object (RRID:SCR\_01380). ASVs were grouped at the genus level. The mean  
1128 relative abundance of each genus was calculated for each sample type within each batch and  
1129 plotted in R (tidyverse, ggplot2; RRID:SCR\_014601). Dot size corresponds to the mean relative  
1130 abundance of each Genus by sample type and batch. Dots are coloured by sample type: reagent  
1131 controls in lightest blue (Buffer), sampling negative controls in light blue, internal controls in dark  
1132 blue (Kidney), and fetal gut samples in purple.

1133

1134

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1137 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  
1138 the draft. All authors provided feedback, participated in discussions, and contributed to the final  
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1140

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