

1 **IS THERE A PLACENTAL MICROBIOTA? A CRITICAL REVIEW AND**  
2 **RE-ANALYSIS OF PUBLISHED PLACENTAL MICROBIOTA DATASETS**  
3 **RUNNING TITLE: IS THERE A PLACENTAL MICROBIOTA?**

4 Jonathan Panzer<sup>1,2</sup>, Roberto Romero<sup>1,3-6,\*</sup>, Jonathan M. Greenberg<sup>1,7</sup>, Andrew D.  
5 Winters<sup>1,2</sup>, Jose Galaz<sup>1,7,8</sup>, Nardhy Gomez-Lopez<sup>1,2,7</sup>, Kevin R. Theis<sup>1,2,7,\*</sup>

6 **AUTHOR AFFILIATIONS**

7 <sup>1</sup> *Perinatology Research Branch, Division of Obstetrics and Maternal-Fetal Medicine, Division*  
8 *of Intramural Research, Eunice Kennedy Shriver National Institute of Child Health and Human*  
9 *Development, National Institutes of Health, U.S. Department of Health and Human Services,*  
10 *Bethesda, MD, and Detroit, MI, United States*

11 <sup>2</sup> *Department of Biochemistry, Microbiology, and Immunology, Wayne State University School of*  
12 *Medicine, Detroit, MI, United States*

13 <sup>3</sup> *Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, MI, United*  
14 *States*

15 <sup>4</sup> *Department of Epidemiology and Biostatistics, Michigan State University, East Lansing, MI,*  
16 *United States*

17 <sup>5</sup> *Center for Molecular Medicine and Genetics, Wayne State University, Detroit, MI, United*  
18 *States*

19 <sup>6</sup> *Detroit Medical Center, Detroit, MI, United States*

20 <sup>7</sup> *Department of Obstetrics and Gynecology, Wayne State University School of Medicine,*  
21 *Detroit, MI, United States*

22 <sup>8</sup> *Division of Obstetrics and Gynecology, School of Medicine, Faculty of Medicine, Pontificia*  
23 *Universidad Catolica de Chile, Santiago, Chile*

24 **CORRESPONDING AUTHORS**

25 \* Kevin R. Theis ([ktheis@med.wayne.edu](mailto:ktheis@med.wayne.edu))

26 \* Roberto Romero ([prbchiefstaff@med.wayne.edu](mailto:prbchiefstaff@med.wayne.edu))

27 **ABSTRACT – 200 words**

28 The existence of a placental microbiota is under debate. The human placenta has  
29 historically been considered sterile and microbial colonization has been associated with  
30 adverse pregnancy outcomes. Yet, recent investigations using DNA sequencing reported a  
31 microbiota in human placentas from typical term pregnancies. However, this detected  
32 microbiota could represent background DNA contamination. Using fifteen publicly  
33 available 16S rRNA gene datasets, existing data were uniformly re-analyzed. 16S rRNA  
34 gene Amplicon Sequence Variants (ASVs) identified as *Lactobacillus* were highly  
35 abundant in eight of fifteen studies. However, the prevalence of *Lactobacillus*, a typical  
36 vaginal bacterium, was clearly driven by bacterial contamination from vaginal delivery and  
37 background DNA. After removal of likely DNA contaminants, *Lactobacillus* ASVs were  
38 highly abundant in only one of five studies for which data analysis could be restricted to  
39 placentas from term cesarean deliveries. A six study sub-analysis targeting the 16S rRNA  
40 gene V4 hypervariable region demonstrated that bacterial profiles of placental samples and  
41 technical controls share principal bacterial ASVs and that placental samples clustered  
42 primarily by study origin and mode of delivery. Across studies, placentas from typical term  
43 pregnancies did not share a consistent bacterial taxonomic signal. Contemporary DNA-  
44 based evidence does not support the existence of a placental microbiota.

45 **IMPORTANCE – 150 words**

46 Early-gestational microbial influences on human development are unclear. By applying DNA  
47 sequencing technologies to placental tissue, bacterial DNA signals were observed, leading some  
48 to conclude that a live bacterial placental microbiome exists in typical term pregnancy. However,  
49 the low-biomass nature of the proposed microbiome and high sensitivity of current DNA  
50 sequencing technologies indicate that the signal may alternatively derive from environmental or  
51 delivery-associated bacterial DNA contamination. Here we address these alternatives with a re-  
52 analysis of 16S rRNA gene sequencing data from 15 publicly available placental datasets. After  
53 identical DADA2 pipeline processing of the raw data, subanalyses were performed to control for  
54 mode of delivery and environmental DNA contamination. Both environment and mode of  
55 delivery profoundly influenced the bacterial DNA signal from term-delivered placentas. Aside  
56 from these contamination-associated signals, consistency was lacking across studies. Thus,  
57 placentas delivered at term are unlikely to be the original source of observed bacterial DNA  
58 signals.

59 **KEYWORDS**

60 16S rRNA gene sequencing, low microbial biomass sampling, re-analysis, microbiota,  
61 placenta

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72

### 73 **DISCLOSURE OF INTEREST**

74 The authors report there are no competing interests or conflicts of interest to declare.

### 75 **DATA DEPOSITION**

76 Raw data for each of the studies included in analyses can be accessed through the following  
77 accession numbers:

78 De Goffau et al. PMID: 31367035 /Accession no. ERP109246;

79 Dinsdale et al. PMID: 33194782 /Accession no. PRJEB39698;

80 Gomez-Arango et al. PMID: 28240736 /Accession no. PRJNA357524;

81 Lauder et al. PMID: 27338728 / Accession no. PRJNA309332;

82 Leiby et al. PMID: 30376898 / Accession no. PRJNA451186;

83 Leon et al. PMID: 29776928 / Accession no. PRJEB25986;

84 Liu et al. PMID: 31685443 / Accession no. PRJNA559967;

85 Olomu et al. PMID: 32527226 / Accession no. PRJNA577959;

86 Parnell et al. PMID: 28894161 / Accession no. PRJNA395716;

87 Seferovic et al. PMID: 31055031 / Accession no. PRJNA511648;

88 Sterpu et al. PMID: 32871131 / Accession no. PRJEB38528;

89 Tang et al. PMID: 33193081 / Accession no. PRJNA564455;

90 Theis et al. PMID: 30832984 / Accession no. PRJNA397876;

91 Theis, Winters et al. bioRxiv MS: 497119 / Accession no. PRJNA692425;

92 Younge et al. PMID: 31479427 / Accession no. PRJNA557826

### 93 **DATA AVAILABILITY STATEMENT**

94 All DADA2 processed sequence data and metadata from the studies included in this critical  
95 review, as well as an R markdown file with the code to produce each of the figures and tables,  
96 are available online at [https://github.com/jp589/Placental\\_Microbiota\\_Reanalysis](https://github.com/jp589/Placental_Microbiota_Reanalysis). In addition, an  
97 R package ‘dada2tools’ with functions for efficient analysis of the data, is available at  
98 <https://github.com/jp589/dada2tools>.

99 **WORD COUNT**

100 9512 words exclusive of bibliography and with numbered in-text citations.

## 101 INTRODUCTION

102 The womb has historically been considered sterile throughout typical pregnancy (1-3);  
103 yet, the detection of microorganisms, especially bacteria, in some placentas from complicated  
104 pregnancies is an established phenomenon (4-7). For instance, there are demonstrated  
105 associations between bacterial colonization of the placenta and preterm labor (5, 8-15), preterm  
106 prelabor rupture of membranes (PPROM) (11, 12), histological chorioamnionitis (8, 14, 16), and  
107 clinical chorioamnionitis (12-14, 16). Therefore, research has largely focused on the presence (9,  
108 17-20) and types of bacteria (21-26) associated with the human placenta in the context of  
109 spontaneous preterm birth and other pregnancy complications.

110 However, in 2014, bacterial DNA-based evidence was presented for a universal low-  
111 biomass placental microbiota even among placentas from term pregnancies (27). Since placental  
112 colonization by bacteria suggests that fetal colonization is also feasible, this study revitalized the  
113 *in utero* colonization hypothesis, which maintains that commensal bacteria residing in the  
114 placenta and/or amniotic fluid colonize the developing fetus during gestation (3, 28-34). The *in*  
115 *utero* colonization hypothesis stands in stark contrast to the traditional sterile womb hypothesis,  
116 which posits fetal sterility until colonization at delivery or following rupture of the amniotic  
117 membranes (1-3, 35-41). Since publication of this seminal study in 2014, many other studies  
118 have similarly utilized DNA sequencing techniques to investigate the existence of a placental  
119 microbiota in term pregnancies (28, 29, 34, 36-65). Yet, the existence of a placental microbiota  
120 remains under debate eight years later (66-69).

121 The debate over the existence of a placental microbiota is fueled by several issues. First,  
122 the placenta cannot be readily sampled *in utero*. Thus, attempts at characterizing a placental  
123 microbiota have entailed collection of placental samples following either a vaginal or cesarean

124 delivery. While both delivery methods can introduce bacterial contamination (36, 38, 40, 42, 51,  
125 70), in the form of vaginal and skin bacteria, respectively, vaginal delivery likely exposes the  
126 placenta to bacterial contamination to an extent that would overwhelm any weak bacterial DNA  
127 signal legitimately present in placental tissue *in utero* (40, 42, 56). Thus, to establish that a  
128 placental microbiota exists, it must be documented in placentas from term cesarean deliveries to  
129 minimize misinterpretation of potential delivery associated contamination (3, 29, 71).

130         Second, a lack of robust technical controls has made it difficult to determine if reagent or  
131 environmental DNA contamination might be the source of bacterial DNA signals attributed to  
132 placentas rather than a resident placental microbiota (27, 29, 46, 53, 54, 57-61, 63-65), given that  
133 such a theoretically sparse bacterial community could easily be obfuscated by background DNA  
134 contamination in laboratories, kits, and reagents (39, 72-75). Technical controls and sterile  
135 collection conditions are therefore essential for the verification of a placental microbiota. Indeed,  
136 several recent studies have shown that the bacterial loads (41) and profiles of placentas from  
137 term cesarean deliveries do not exceed or differ from those of technical controls (41, 42).

138         Finally, a lack of consistency in the analytical pipelines used to process the DNA  
139 sequence data has resulted in additional debate, including how sequences should be grouped or  
140 split into taxonomic units (73, 76, 77). Specifically, too coarse or too fine a taxonomic resolution  
141 could either potentially reveal a shared bacterial DNA signal between placental tissues and  
142 technical controls or a signal unique to the placenta, respectively.

143         Ultimately, if there is a placental microbiota it should exist in a majority of, if not all,  
144 placentas from women delivering at term without complications, and there should be some  
145 degree of consistency in the bacterial taxa residing in placentas across studies. For example, the  
146 human vaginal microbiota worldwide is consistently predominated by various species of



147 *Lactobacillus* and, in a smaller proportion of women, higher diversity bacterial communities  
148 exist, which consist of nevertheless predictable genera such as *Prevotella*, *Sneathia*,  
149 *Megasphaera*, *Atopobium*, *Mobiluncus*, *Streptococcus*, and *Gardnerella* (78, 79). Yet, among  
150 investigators proposing the existence of a placental microbiota, there are conflicting reports  
151 regarding its predominant bacterial members (27-29, 44, 49, 54, 58, 62-65) and, when  
152 complementary culture results are available, placental samples are often culture negative or the  
153 bacteria recovered are discrepant with the DNA sequencing results (19, 28, 40, 41, 44, 56, 80-  
154 85).

155         Given these current conflicting conclusions regarding the existence of a placental  
156 microbiota, here we performed a critical review and re-analysis of fifteen publicly available 16S  
157 rRNA gene sequencing datasets from human placental microbiota studies for which sample  
158 distinguishing metadata were available (29, 36-44, 50, 53, 57, 86). In this re-analysis we  
159 standardized the analytical process to enable assessment of taxonomic consistency in placental  
160 bacterial profiles across studies conducted by different laboratories across the world (**Figure S1**).  
161 Briefly, raw sequencing data from each study were processed using the same analytical pipeline,  
162 the Divisive Amplicon Denoising Algorithm (DADA2), to provide consistent sequence filtering  
163 and clustering methods (87). Additionally, eight of the fifteen studies included sequence data for  
164 at least six technical controls to account for potential background DNA contamination (88). For  
165 these studies, the R package DECONTAM (88) was used to identify and remove likely DNA  
166 contaminants and we report the DNA signal from the bacterial taxa that remained.

167         Three primary analyses were performed. The first analysis was a comparison of the  
168 bacterial profiles of placental samples to technical controls for studies which included at least six  
169 controls for background DNA contamination (88) since this environmental contamination could

170 be a source of the purported placental bacterial DNA signal. Ideally, a valid placental microbiota  
171 would be expected to exhibit a bacterial DNA signal distinct from that of kit reagents or  
172 surrounding laboratory environments. The first analysis revealed no consistent differentiation  
173 between the bacterial DNA signal from placental samples and technical controls, or vaginal  
174 swabs when available.

175         The second analysis was restricted to placentas from term cesarean deliveries so as to  
176 avoid potential bacterial contamination of placentas that could occur during vaginal delivery (36,  
177 42, 51, 89). If there were a placental microbiota, the bacterial DNA signals should be clear and  
178 consistent across placentas from term cesarean deliveries. This analysis was therefore performed  
179 using data from the six studies for which placental samples could be restricted to those from term  
180 cesarean deliveries. Of the studies which included at least six background technical controls,  
181 after likely contaminant removal, there was no consistent bacterial DNA signal among placental  
182 samples.

183         The third and final analysis was restricted to studies that targeted the V4 hypervariable  
184 region of the 16S rRNA gene to control for any variation which might arise due to variation in  
185 targeted 16S rRNA gene hypervariable regions across studies or the DNA sequence processing  
186 algorithms used. A valid placental microbiota would be expected to be independent of study  
187 identity and mode of delivery; however, both of these factors largely contributed to the structure  
188 of placental bacterial profiles. Indeed, in these studies, there was a large degree of similarity in  
189 the bacterial profiles of placental samples and technical controls from the same study, and there  
190 were no bacterial taxa that were consistently identified across studies whose presence could not  
191 be explained by background DNA contamination.

192           Collectively, these analyses do not support the presence of a placental microbiota in  
193 typical term pregnancies. Observed bacterial signals were products of mode of delivery and  
194 background DNA contamination. Although there may be a true, consistent, extremely low  
195 biomass bacterial signal beyond the limits of detection by contemporary 16S rRNA gene  
196 sequencing, it remains to be demonstrated that the placenta harbors a legitimate bacterial DNA  
197 signal or a viable microbiota in typical human pregnancy.

## 198 RESULTS

### 199 Overview of studies included in this re-analysis

200 Fifteen studies (**Table 1**) were included in this re-analysis of investigations of the existence of a  
201 placental microbiota. Seven included sequence data from the V4 hypervariable region of the 16S  
202 rRNA gene (29, 39, 41, 43, 44, 50, 86), allowing for direct comparisons of sequence data across  
203 six of those studies (29, 39, 41, 44, 50, 86); one study could not be included in the direct  
204 comparison due to short read lengths of sequences in the publicly available dataset (43). Three of  
205 the remaining studies included sequence data from the V1-V2 16S rRNA gene hypervariable  
206 region (36-38), two studies sequenced the V6-V8 region (40, 53), and one study each sequenced  
207 the V3-V4 (58), V4-V5 (57), and V5-V7 (42) regions. All fifteen studies included at least one  
208 placental sample from a term cesarean delivery, but only eight included more than one term  
209 cesarean delivered placenta and sufficient background technical controls [i.e., N = 6 (88)] to  
210 infer likely DNA contaminants using the R package DECONTAM (36, 39-43, 50, 86) (**Figure**  
211 **2**). Two of these studies lacked available metadata to discriminate placental samples by  
212 gestational age at delivery (42, 50), leaving a total of six studies (36, 39-41, 43, 86) for assessing  
213 uniformity of bacterial profiles among term cesarean delivered placentas across studies while  
214 accounting for potential background DNA contamination (**Figure 2**). Notably, five of these six  
215 studies concluded that there was no evidence for a placental microbiota in uncomplicated term  
216 pregnancies (36, 39-41, 86) (**Figure 2**). In contrast, the four studies which did not include  
217 sequence data from background technical controls concluded that a placental microbiota does  
218 exist (29, 53, 57, 58) (**Figure 2**).

219

220 ***Lactobacillus* ASVs are the most consistently identified ASVs across placental microbiota**  
221 **studies**

222 After processing the raw 16S rRNA gene sequence data from placental samples from all 15  
223 studies through the same DADA2 analytical pipeline, the most prominent bacterial ASVs, as  
224 defined by mean relative abundance, across studies were classified as *Lactobacillus*,  
225 *Escherichia/Shigella*, *Staphylococcus*, *Streptococcus*, and *Pseudomonas* (**Table S1**).  
226 *Lactobacillus* ASVs were among the top five ranked ASVs in eight of the 15 studies (37-39, 42,  
227 50, 53, 57, 86), making *Lactobacillus* the most consistently detected genus in placental samples  
228 across studies with publicly available 16S rRNA gene sequencing data.

229 The detection of *Lactobacillus* ASVs was not exclusive to the targeted sequencing of  
230 specifically any one 16S rRNA gene hypervariable region; *Lactobacillus* ASVs were found  
231 among the top five ASVs in the dataset of at least one study targeting the V1-V2, V4, V4-V5,  
232 V5-V7, or V6-V8 hypervariable region(s) of the 16S rRNA gene (**Table S1**). Other genera which  
233 were not 16S rRNA gene hypervariable region specific and were detected in the top five ranked  
234 ASVs in more than one dataset, but in no more than four, included *Staphylococcus* (40, 44, 54,  
235 86), *Streptococcus* (38, 40, 42), and *Pseudomonas* (50, 54, 57). In contrast, *Escherichia/Shigella*  
236 ASVs were exclusively among the top five ranked ASVs in datasets of studies that targeted the  
237 V4 hypervariable region of the 16S rRNA gene for sequencing (3/7 such datasets) (29, 39, 86)  
238 (**Table S1**).

239

240 ***Lactobacillus* ASVs in placental samples are typically contaminants introduced through**  
241 **vaginal delivery and/or background DNA contamination**

242 While it can be difficult to identify the definitive source of a particular ASV in placental  
243 samples, the difference in *Lactobacillus* predominance between vaginally delivered placentas  
244 and cesarean delivered placentas is striking. *Lactobacillus* ASVs were among the top five ASVs  
245 in five of seven datasets which included placentas from vaginal deliveries before running the R  
246 package DECONTAM, and three of four datasets which included placentas from vaginal  
247 deliveries after running DECONTAM (**Table 2, Table S1**). Consider, for instance, the Lauder et  
248 al. (37) and Leiby et al. (38) datasets. While all samples in the Lauder et al. dataset (37) had  
249 *Lactobacillus* ASVs, the percentage of *Lactobacillus* normalized reads in cesarean delivered  
250 placental samples was 23% compared to 46% in vaginally delivered placental samples. In the  
251 Leiby et al. dataset (38) only four of 23 (17%) cesarean delivered placentas had any  
252 *Lactobacillus* ASVs, and they made up only 2% of the total reads from their respective samples.  
253 In contrast, 35 of 116 (30%) placentas from vaginal deliveries had *Lactobacillus* ASVs, and they  
254 made up 22% of the total reads from those 35 samples.

255 *Lactobacillus* ASVs were among the top five ranked ASVs in three (39, 57, 86) of the six  
256 datasets which could be restricted to placental samples from cesarean term deliveries (**Table 2**).  
257 Yet, after removing potential background DNA contaminants using DECONTAM, only the  
258 Olomu et al. (39) dataset still retained a *Lactobacillus* ASV in the top five ranked ASVs (**Table**  
259 **2**). Notably, the authors of that study identified the origin of *Lactobacillus* in placental samples  
260 as well-to-well DNA contamination from vaginal to placental samples during 16S rRNA gene  
261 sequence library generation.

262 Furthermore, *Lactobacillus* ASVs were also more prominent in samples of placental  
263 tissues of maternal origin, such as the decidua or basal plate, than placental tissues of fetal origin,  
264 such as the amnion, chorion, or villous tree. After separating placental sample data from non-

265 labor term cesarean deliveries by fetal and maternal origin, with the exception of the Olomu et al.  
266 (39) study, *Lactobacillus* ASVs were absent from placental samples of fetal origin (**Table S2**). In  
267 contrast, among samples of maternal origin from the Theis, Winters et al. dataset (86),  
268 *Lactobacillus* was the most relatively abundant ASV even after removal of likely DNA  
269 contaminants with DECONTAM, and in the Lauder et al. (37) study, only the maternal side of  
270 the single cesarean delivered placenta had a high predominance of *Lactobacillus*.

271

### 272 **The bacterial ASV profiles of placental samples and background technical controls cluster** 273 **based on study origin**

274 Beta diversity between placental samples and technical controls was visualized through Principal  
275 Coordinates Analysis for each study in the re-analysis to assess the extent of influence of  
276 background DNA contamination on the bacterial ASV profiles of placental samples (**Figure 3**).  
277 A majority of placental samples cluster with their respective technical controls across the studies.  
278 Specifically, in five of eleven studies, technical controls covered the entire bacterial profile  
279 spectrum of placental samples (**Figure 3A-E**), and in the remaining six studies which included  
280 technical controls, the bacterial profiles of most placental samples largely clustered with those of  
281 technical controls (**Figure 3F-K**). Placental samples in the latter group which did not cluster  
282 with technical controls were characterized by a predominance of *Lactobacillus* (**3F-H**),  
283 *Cutibacterium* (**3I,K**), *Gardnerella* (**3F**), *Pseudomonas* (**3F**), *Ureaplasma* (**3G**), *Lactobacillus*  
284 (**3G-H**), *Mesorhizobium* (**3I**), *Prevotella* (**3J**), *Actinomyces* (**3J**), *Streptococcus* (**3J**), *Veillonella*  
285 (**3J**), and *Staphylococcus* (**3K**). Notably, the bacterial profiles of most placental samples from  
286 term cesarean deliveries were not significantly different from those of technical controls in either  
287 dispersion or structure (**Table S3**). In cases where the structure of the bacterial profiles differed

288 between placental samples and technical controls, but the dispersion of the bacterial profiles did  
289 not, it was only the bacterial profiles of the exterior surfaces of the placenta which differed from  
290 those of controls. In these cases, the bacterial profiles of the exterior surfaces of placental  
291 samples were characterized by *Cupriavidus*, *Serratia*, *Corynebacterium*, and *Staphylococcus*  
292 (**Table S3**), the latter two of which are well-known commensal bacteria of the human skin (90).

293

294 **The bacterial ASV profiles of vaginally delivered placental samples also cluster with their**  
295 **respective vaginal samples across studies**

296 Six studies (29, 37-39, 50, 57) in the re-analysis included vaginal or vaginal-rectal swab samples  
297 as a complement to placental samples; four of these studies also included technical controls (37-  
298 39, 50). While most technical controls did not cluster with vaginal samples, placental samples  
299 typically clustered with vaginal samples and/or technical controls (**Figure 4A-D**), or if technical  
300 controls were not included in the study, with vaginal samples (**Figure 4E-F**). Notably, nine  
301 *Lactobacillus* ASVs were shared between the top ranked ASVs of placental and vaginal swab  
302 samples across five studies (37-39, 50, 57) (**Figure 4**).

303

304 **Placental and technical control samples co-cluster by study and placental samples**  
305 **additionally cluster by mode of delivery**

306 In order to fully utilize the capacity for ASVs to be directly compared across placental  
307 microbiota studies, taxonomy and ASV count tables were merged based on the exact ASV  
308 sequence data for six (29, 39, 41, 44, 50, 86) of seven studies (29, 39, 41, 43, 44, 86) which  
309 sequenced the V4 hypervariable region of the 16S rRNA gene using the PCR primers 515F and  
310 805R. Principal Coordinates Analysis (PCoA) illustrated that placental and technical control



311 samples formed distinct clusters based on study origin (**Figure 5A**; NPMANOVA using Bray-  
312 Curtis; placental samples:  $F=16.0$ ,  $P=0.001$ ; technical controls:  $F=4.64$ ,  $P=0.001$ ). The only  
313 exception was the Theis, Winters et al. dataset (86), which encompassed the bacterial profiles of  
314 placental samples from the other studies. This was likely due to the inclusion of samples in  
315 Theis, Winters et al. (86) from multiple regions of the placenta (i.e., amnion, amnion-chorion  
316 interface, subchorion, villous tree, and basal plate) as well as placentas from term and preterm  
317 vaginal and cesarean deliveries (**Figure 5A**). When stratifying by study and thereby taking study  
318 origin into account, placental and technical control samples did exhibit distinct bacterial DNA  
319 profiles (**Figure 5A**;  $F=6.66$ ;  $P=0.512$ ). When technical controls were excluded from the PCoA,  
320 discrete clustering of placental samples by study origin was still apparent (**Figure 5B**).  
321 Furthermore, the bacterial DNA profiles of placental samples were clearly affected by mode of  
322 delivery across studies (**Figure 5C**;  $F=21.6$ ,  $P=0.001$ ). Unsurprisingly, common vaginal bacteria  
323 such as *Lactobacillus*, *Ureaplasma*, and *Gardnerella* were predominant in the profiles of  
324 placental samples from vaginal deliveries (**Figure 5C**).

325

#### 326 **Bacterial profiles of placental and technical control samples characterized using the V4** 327 **hypervariable region of the 16S rRNA gene share prominent ASVs**

328 While placental samples from each study exhibited characteristic patterns of predominant ASVs,  
329 some ASVs such as ASV2533-*Escherichia/Shigella*, ASV6218-*Lactobacillus*, and ASV6216-  
330 *Lactobacillus* were predominant in the datasets of several studies (**Figure 6A-B, E**). However,  
331 across studies, nearly every ASV that was consistently predominant in the bacterial DNA  
332 profiles of placental samples, was also consistently predominant in the profiles of the technical  
333 control samples from the same dataset (**Figure 6**). For instance, in two studies, all ASVs with a

334 mean relative abundance greater than 1% in placental samples were also greater than 2% mean  
335 relative abundance in technical control samples (**Figure 6B-C**). In a third study, all ASVs other  
336 than ASV5229-*Cutibacterium* were also greater than 2% mean relative abundance across  
337 technical control samples (**Figure 6D**). These data collectively indicate that prominent placental  
338 ASVs were likely derived from background DNA contamination captured by the technical  
339 control samples.

## 340 **DISCUSSION**

### 341 **Principal findings of the study**

342 In this re-analysis of fifteen placental microbiota studies, of the ASVs which were ranked  
343 in the top five ASVs for relative abundance in any one study, *Lactobacillus* ASVs were clearly  
344 the most prevalent across studies. Yet, *Lactobacillus* ASV prevalence was explained by  
345 background DNA contamination, contamination from the birth canal during vaginal delivery, or  
346 well-to-well contamination from vaginal samples during the sequence library build process.

347 Overall, the bacterial DNA profiles of placental samples were highly similar to those of technical  
348 controls in their respective studies. Indeed, a secondary analysis of the six studies which targeted  
349 the V4 hypervariable region of the 16S rRNA gene for sequencing, showed that the bacterial  
350 DNA signal of both placental and technical control samples clustered by study of origin rather  
351 than by sample type. In addition, the top two ASVs in placental samples from each of the six  
352 studies in the secondary analysis were also the top ranked ASVs in technical controls from the  
353 corresponding study. Considered in isolation, placental samples clustered by mode of delivery,  
354 suggesting that the process of delivery greatly affected the bacterial DNA profiles of placentas.  
355 Therefore, placental samples included in this re-analysis do not provide evidence of a consistent  
356 bacterial DNA signal in typical term pregnancy independent of mode of delivery. Instead, the  
357 modest consistency in bacterial DNA signals identified across studies was associated with  
358 general background DNA contamination or contamination introduced during vaginal delivery.

359

### 360 **The findings of this study in the context of prior reports**

361 Currently, the extent of bacterial presence within the placenta is under debate. There have been  
362 numerous reviews, commentaries, and editorials, which have sought to synthesize and resolve

363 conflicting results regarding the existence of a placental microbiota (3, 30, 32, 33, 66-68, 73, 91-  
364 135). Although there has been disagreement about the existence of a placental microbiota in  
365 typical human pregnancy, there is a consensus that any given body site, including the placenta,  
366 can be at least transiently infected by microorganisms. Several reviews have emphasized that  
367 microorganisms in placental tissue would not be able to survive for long durations given the  
368 structure of the placenta and the immunobiological response of the host (3, 101). In contrast,  
369 some have proposed that microorganisms could survive intracellularly within the basal plate of  
370 the placenta and thus effectively evade the host immune system (68, 136). Many reviews  
371 addressing prior microbiota datasets have been challenged to draw conclusions given the  
372 multiple confounding factors which could significantly influence results: the specific 16S rRNA  
373 gene hypervariable region targeted for sequencing, brand and lot number of the DNA extraction  
374 kits, gestational age at delivery and sampling, mode of delivery of the placenta, inadequate  
375 metadata for deposited sequence data, and a general lack of technical controls to account for  
376 background DNA contamination. Regardless, many have viewed the current evidence for  
377 placental and/or *in utero* colonization as theoretically tenuous given the existence of germ-free  
378 mammals and the strong potential for background bacterial DNA to influence DNA sequencing  
379 surveys of low microbial biomass samples (36-41, 81, 103). Finally, similar to the results  
380 presented here in this re-analysis, the prevalence of *Lactobacillus* across placental samples in  
381 prior studies has been acknowledged, yet so too has been the high variability in the bacterial taxa  
382 reported within placental tissues across studies. Indeed, variability is high even across studies of  
383 similar cohorts from the same research groups (27, 41, 44, 63-65, 86). The current study sought  
384 to remedy the lack of consensus in the literature regarding the existence of a placental microbiota  
385 in typical term pregnancy through a re-analysis of the current publicly available data on placental

386 microbiotas while controlling for targeted region of the 16S rRNA gene for sequencing,  
387 background DNA contamination, and mode of delivery.

388

389 *Mode of delivery must be taken into account when investigating the existence and structure of a*  
390 *placental bacterial DNA signal*

391 Eleven studies (27, 38, 43, 44, 52, 54, 57, 59) concluded that the bacterial DNA signals in  
392 placentas from cesarean deliveries were not significantly different from those in placentas  
393 delivered vaginally. Yet, six other studies (36, 38, 42, 51, 86, 89) have reported that the bacterial  
394 DNA signals in placentas from vaginal and cesarean deliveries significantly differ. The latter  
395 studies have reported increased prevalence and relative abundance of *Lactobacillus* and other  
396 vaginally associated taxa in placentas from vaginal deliveries. Additionally, even the rupture of  
397 membranes, a prerequisite for labor and vaginal delivery, provides microorganisms access to the  
398 amniotic cavity (137) and thus the placenta, with prolonged access leading to microbial invasion  
399 and infection (138, 139). Notably, bacterial culture of placentas from vaginal deliveries have  
400 significantly higher positivity rates (18, 86), higher total colony counts (40), and a higher  
401 prevalence of bacterial colonies from *Lactobacillus* and *Gardnerella*, both of which are typical  
402 residents of the human vagina (78). In contrast, placentas from cesarean deliveries consistently  
403 yield bacteria which typically predominate on the skin, such as *Propionibacterium*,  
404 *Staphylococcus*, and *Streptococcus* (40, 90).

405       Importantly, through robust analysis of the entire bacterial DNA signal from hundreds of  
406 placental samples, this re-analysis clearly highlights the influence of mode of delivery on the  
407 bacterial DNA signal from placental samples by demonstrating mode of delivery-associated  
408 clustering across six studies. Furthermore, it is apparent that removing the exterior layers (i.e.,

409 amnion, chorion, and basal plate) of a placenta delivered vaginally is not sufficient to eliminate  
410 delivery associated DNA contamination of the sample since the diversity and structure of  
411 bacterial DNA profiles from the inner layers (i.e., subchorion, villous tree) of the placenta  
412 differed significantly between cesarean and vaginal deliveries. Evidence in the literature  
413 combined with this re-analysis warrants careful consideration of mode of delivery and even time  
414 since rupture of membranes (52, 138, 139) when investigating the bacterial DNA signal from  
415 placental samples.

416  
417 *Background bacterial DNA limits analysis of bacterial 16S rRNA gene signal from the placenta*

418 Theoretically, a low bacterial biomass community is detectable using 16S rRNA gene  
419 sequencing when its concentration is at least 10-100CFU/mL (140). However, discerning a true  
420 tissue-derived low bacterial DNA signal from other potential sources is exceedingly difficult.  
421 This re-analysis, along with eight other studies (36-41, 81, 86), found that placental samples and  
422 technical controls share highly abundant bacterial taxa when 16S rRNA gene sequencing is used.  
423 Since technical controls represent the environment and reagents to which the placenta is exposed  
424 post-delivery, it follows that a majority of the bacterial DNA signal from placental samples is  
425 also acquired from those environments and reagents. While a placental tissue limit of bacterial  
426 detection through DNA sequencing is yet to be determined, other low-bacterial-biomass sample  
427 types such as oral rinse, bronchoalveolar lavage fluid, and exhaled breath condensate were  
428 predominated by stochastic noise below  $10^4$  16S rRNA gene copies per sample (141). Even the  
429 bacterial DNA signal from a pure culture of *Salmonella bongori* serially diluted to a final  
430 concentration of  $10^3$  CFU/mL was mostly contamination (74). If these limits are comparable to  
431 those in placental tissue, then stochastic noise and background DNA contamination would

432 predominate the bacterial DNA signal from placental tissue leaving any true DNA signal well  
433 beyond the detection limits of 16S rRNA gene sequencing. Therefore it follows that 16S rRNA  
434 gene sequencing is inadequate to make a clear assessment of the existence of a placental  
435 microbiota.

436

#### 437 **Prior reports of 16S rRNA gene sequencing on placentas from term pregnancies**

438 With the prior considerations in mind, out of the 40 studies which performed 16S rRNA gene  
439 sequencing on placental samples, 32 included at least some term deliveries. However, only 16  
440 focused exclusively on placentas from term deliveries (28, 37, 39-41, 43, 49, 53, 54, 56-58, 62-  
441 65). Additionally, only nine of these studies focused exclusively on placentas from cesarean  
442 deliveries (28, 39, 41, 49, 56, 58, 62, 64, 65) and only three included technical controls and their  
443 DNA sequencing data thus accounting for gestational age, mode of delivery, and background  
444 DNA contamination (39, 41, 49). Two of three concluded that there was no evidence for a  
445 placental microbiota in the context of term cesarean delivery (39, 41).

446 Many studies have reported evidence for a low biomass placental microbiota (27, 29, 30,  
447 43-47, 49, 50, 52-54, 57, 58, 60, 61, 63-65, 82, 83, 136, 142) but only nine of these studies  
448 exclusively sampled placentas from term deliveries (43, 49, 53, 54, 57, 58, 63-65). Predominant  
449 bacterial taxa reported in these studies included *Pseudomonas* (54, 64, 65), *Lactobacillus* (49,  
450 54), Bacteroidales (64), *Enterococcus* (63), *Mesorhizobium* (43), *Prevotella* (58), unclassified  
451 Proteobacteria (57), *Ralstonia* (43), and *Streptococcus* (54). Two studies from this term delivery  
452 subset, which sampled multiple regions of the placenta, observed gradients of *Lactobacillus*  
453 relative abundance across levels of the placenta, but in opposite directions (43, 49).

454 In contrast, five studies did not find evidence for a microbiota in placentas from term  
455 deliveries since neither the placental bacterial DNA signal from 16S rRNA gene sequencing (37,  
456 39-41) nor the bacterial load as determined by quantitative real-time PCR (37, 39-41, 56) were  
457 significantly different from technical controls. One study even noted that no operational  
458 taxonomic units greater than 1% relative abundance in placental samples, were less than 1% in  
459 technical control samples, emphasizing the overlap between the two sample types (37). Three of  
460 these studies (40, 41, 56) also attempted to culture viable bacteria from placental tissue, but were  
461 rarely successful. In cases where culture was successful, viable bacteria often conflicted with the  
462 DNA sequencing results suggesting that cultured bacteria were likely contaminants (40, 41).

463

#### 464 **Clinical significance**

465 *Non-viable or viable bacterial DNA could feasibly be filtered from maternal blood by the*  
466 *placenta leading to a placental bacterial DNA signal*

467 The placenta is a transient internal organ with functions that include promotion of gas exchange,  
468 nutrient and waste transport, maternal immunoglobulin transport, and secretion of hormones  
469 critical for fetal growth and development (143). These exchanges and transfers occur due to  
470 diffusion gradients between fetal and maternal blood, the latter of which bathes the chorionic  
471 villi in the intervillous space of the placenta (99). This maternal blood, which cannot be fully  
472 drained from the placenta before biopsy or sampling, can undoubtedly contain bacterial particles  
473 or even the remnants of a low-grade bacterial infection (56, 103, 144). Because of its structure,  
474 the placenta functions as a filter and retains these particles or bacteria, dead or alive. A bacterial  
475 DNA signal due to this filtering process would be extremely weak and transient. In addition, the



476 bacterial taxa identified would be highly variable since they do not correspond to a specific  
477 niche, which is consistent with the findings of this re-analysis.

478

479 *Infection is a potential source for the placental bacterial DNA signal*

480 Instead of *in-utero* colonization, it is more likely that the bacterial DNA signal coming from a  
481 subset of placental samples is caused by infection. It is curious to note that specific bacteria are  
482 associated with stronger bacterial DNA signals and inflammation in placental tissue resulting in  
483 adverse pregnancy outcomes including preterm birth and/or preterm prelabor rupture of  
484 membranes (PPROM) (52, 55, 89). Spontaneous preterm birth has been shown to increase  
485 bacterial load (55) and the relative abundances of several taxa in placental samples including but  
486 not limited to *Ureaplasma* (26, 36, 38, 42, 51, 52, 145, 146), *Fusobacterium* (51, 52),  
487 *Mycoplasma* (42, 51, 52), *Streptococcus* (36, 51), *Burkholderia* (27), *Escherichia/Shigella* (55),  
488 *Gardnerella* (51), *Gemella* (52), and *Pseudomonas* (50). *Ureaplasma urealyticum*, *Mycoplasma*  
489 *hominis*, *Bacteroides* spp., *Gardenerella* spp, *Mobiluncus* spp., various enterococci, and  
490 *Streptococcus agalactiae* (also known as Group B Streptococcus or GBS) are frequently  
491 associated with histologic acute chorioamnionitis as well as uterine infection (16, 26, 99, 146).  
492 GBS is also a major cause of early onset neonatal sepsis and has been commonly isolated at  
493 autopsy in addition to *E. coli*, and *Enterococcus* (16, 147). While strain level variation could  
494 conclusively determine the pathogenicity of bacterial DNA in the placenta, 16S rRNA gene  
495 sequencing is not capable of such resolution. Nevertheless, the DNA of the notoriously  
496 pathogenic bacterial genera detailed above were all found in placental tissue, suggesting an  
497 invasive phenotype rather than commensal colonization.

498

499 **Recommendations for future studies**

500 In order to establish the existence of a viable placental microbiota several criteria need to be met,  
501 which have been detailed previously (36, 41). Studies which aim to assess the viability of a  
502 bacterial DNA signal in a purported low biomass sample type should start with the null  
503 hypothesis that the entire DNA signal results from contamination and subsequently attempt to  
504 reject it with experimental evidence (148). Therefore, any study evaluating a potential microbiota  
505 of the placenta should attempt to demonstrate viability through both culture and DNA  
506 sequencing. Placentas should come from term cesarean deliveries without labor to obviate  
507 contamination during vaginal delivery and subjects should be screened to ensure that only  
508 healthy women are sampled (i.e., no history of antenatal infection, pre-eclampsia, recent  
509 antibiotic use, signs of infection or inflammation). Additionally, future studies should include  
510 ample sequenced technical controls in order to identify and account for sources of contamination,  
511 which will inevitably exist no matter how rigorous and/or sterile the protocol (71). Further,  
512 biological replicates from the same placenta should also be included to ascertain the consistency  
513 of any bacterial DNA signal. Since 16S rRNA gene sequencing limits of detection have not yet  
514 been thoroughly interrogated in placental tissue, serial dilutions of spiked-in live bacteria or cell-  
515 free DNA should be included in a portion of tissue samples to demonstrate the feasibility of  
516 recovering the bacterial DNA signal from placental tissue. When multiplexing samples, unique  
517 dual index primer sets should be used to eliminate the possibility of barcode hopping which is  
518 another source of sample “contamination” (149, 150), and before sequencing, low biomass  
519 samples should be segregated from higher biomass samples to avoid well-to-well contamination  
520 (39, 151). Finally, in conjunction with publishing, all sequence data and detailed metadata should  
521 be submitted to a public database so that others can replicate the work and verify the results.

522

523 **Strengths of this study**

524 Broad searches of the available literature were utilized to ensure that all publicly available 16S  
525 rRNA gene sequencing data of placental samples (with associated metadata to partition pooled  
526 sample data) were incorporated into the re-analysis, which re-examined the data with in-depth  
527 comparisons of term placental samples to technical controls. This allowed for the detection of  
528 background DNA contamination in the bacterial DNA signal from placental tissue. In addition,  
529 potential confounding variables such as mode of delivery, gestational age at delivery, and 16S  
530 rRNA gene target hypervariable region were controlled for whenever possible. By utilizing  
531 DADA2 to process the sequence data, variation and biases due to post-sequencing processing  
532 were eliminated. This enabled ASV-to-ASV comparisons for six studies which targeted the same  
533 16S rRNA gene hypervariable region using the same PCR primers, a first in the placental  
534 microbiota field.

535

536 **Limitations of this study**

537 The quality and public availability of data and metadata were the primary limiting factors of this  
538 re-analysis. Unfortunately, the availability of metadata or even the data themselves is a pervasive  
539 issue in the microbiome field (152-154). While study cohort statistics were well reported overall,  
540 detailed metadata for each subject are required in order to perform a proper re-analysis. Ideally,  
541 any study investigating the existence of a viable placental microbiota would, at a minimum,  
542 include associated metadata by subject for potential confounders (e.g., gestational age at  
543 delivery, and mode of delivery).

544 An additional limitation of the current study was that the impacts of individual low  
545 abundance ASVs (i.e., less than 1% mean relative abundance) were not evaluated, but these  
546 ASVs were likely stochastic environmental DNA contamination. Finally, while the R package  
547 DECONTAM was used to remove likely contaminants by comparing the prevalence of ASVs in  
548 biological samples and technical controls, this tool is not appropriate for identifying  
549 contaminants introduced during sampling or delivery. In addition, the contaminant identification  
550 accuracy of DECONTAM also diminishes when used on low biomass samples such as placental  
551 samples where the majority of the sequences are likely contaminants (71, 155).

552

### 553 **Conclusion**

554 The initial premise of this critical review and re-analysis was to determine if a true consistent  
555 bacterial DNA signal could be identified in placental samples from women delivering at term  
556 across studies despite various differences in sampling methodologies and sequencing analyses.  
557 16S rRNA gene sequencing data from fifteen studies were processed and analysed in the same  
558 manner to control for as much post-sequencing variation as possible. By doing so, *Lactobacillus*  
559 ASVs were identified as the most prevalent top-ranked ASVs by relative abundance across  
560 studies; however, their prevalence in placentas from term cesarean deliveries was attributable to  
561 some form of contamination in every case. While bacterial DNA signals were observed in  
562 placental samples, they were largely similar to those seen in technical control samples.  
563 Furthermore, the bacterial DNA signal from placental samples clustered by mode of delivery,  
564 indicating placental delivery-associated contamination. This observation, in combination with  
565 the existence of germ-free mammals (156, 157), has yet to be reconciled with *in-utero*  
566 colonization. Even if the placenta has a bacterial DNA signal apart from that of background

567 DNA or delivery-associated contamination, alternative sources for the bacterial DNA signal such  
568 as extracellular DNA or dead bacteria circulating within maternal blood still need to be ruled out.

569 As we push the boundaries of DNA sequencing technologies we need to tread carefully,  
570 especially in purported low-biomass sites such as the placenta. The limitations of current DNA  
571 sequencing technology make detection of a legitimate signal or determination of viability  
572 unattainable at such low levels (72, 74). Regardless, a bacterial DNA signal can indeed be  
573 detected even in placentas from term cesarean deliveries, but the placenta is unlikely to be the  
574 source. Only after demonstrating a valid, viable bacterial DNA signal from term cesarean  
575 deliveries, through sterile protocol, with technical controls, and associated culture positive data,  
576 can we evaluate the degree to which the maternal immune system tolerates these bacteria and  
577 whether their presence resembles commensal existence or infection. Finally, the placental  
578 microbiota may or may not exist, but it is quite clear that attempts to maintain sterility and avoid  
579 contamination have not been successful since the vast majority of sequencing reads from  
580 placental samples can be attributed to multiple modes of contamination. Therefore, sequencing  
581 methodologies require significant improvement before a placental microbiota can be established  
582 as 16S rRNA gene sequencing appears to lack the ability to discriminate between a markedly  
583 low biomass microbiota and background DNA contamination at present.

## 584 MATERIALS AND METHODS

### 585 *Study inclusion criteria*

586 Searches for “human placental microbiome”, “placenta microbiota”, “placental microbiomes”,  
587 and “placenta 16S” were queried on PubMed with a cutoff date of 6/16/21 to identify studies  
588 addressing the existence of a placental microbiota or lack thereof. Additionally we included our  
589 recent preprint (86) in this pool of studies. Of the 387 unique studies identified, 58 performed  
590 primary research and 41 implemented 16S rRNA gene sequencing on placental samples (Error!  
591 Reference source not found.). Therefore we focused on 16S rRNA gene sequencing data. 16S  
592 rRNA gene sequencing is a well-characterized way of detecting and classifying bacterial  
593 communities within biological samples (158-160), and it is potentially sensitive enough to detect  
594 the typically low number of 16S rRNA bacterial gene copies hypothesized to be in the placenta  
595 (34, 161). 33 of the 41 studies which implemented 16S rRNA gene sequencing included at least  
596 one placental sample from an uncomplicated delivery at term (27-29, 36-45, 47, 49-65, 86, 142).  
597 However, only 15 of these 33 studies included publicly available 16S rRNA gene sequence data  
598 (i.e., sequencing files uploaded to a public database with a published and accurate accession  
599 number with sufficient metadata to partition pooled sample data) (29, 36-44, 50, 53, 57, 58, 86).  
600 Thus, the re-analysis ultimately included 15 studies.

### 601 *Processing of 16S rRNA gene sequence data using DADA2*

602 Fastq files of the 16S rRNA gene sequence data from samples included in each study were  
603 downloaded from publicly accessible databases. If a study included fastq files that contained  
604 sequence data from multiple samples, the data were demultiplexed using QIIME2 (version  
605 2020.2) (162) and SED (GNU Sed 4.7), a stream editor for text processing (163).

606           Sequence datasets from each study were individually processed using the Differential  
607 Abundance Denoising Algorithm (DADA2), which is an R package designed to partition 16S  
608 rRNA gene sequences into distinct Amplicon Sequence Variants (ASVs) and to taxonomically  
609 classify the resultant ASVs (87). R version 3.6.1 (164) was used for DADA2 processing and all  
610 downstream analyses. Processing followed the 1.16 DADA2 guidelines  
611 (<https://benjjneb.github.io/dada2/tutorial.html>), except when stated otherwise. Samples that had  
612 an average sequence quality score which dipped below 30 before the expected trim length cutoffs  
613 were removed from the dataset. Trim length cutoffs were set to maximize the read length and  
614 number of passing samples while still removing poor quality base calls from the ends of reads.  
615 Reads were then filtered using the `filterAndTrim()` function with *multithread* set to TRUE to  
616 enable parallel computation and decrease real time spent computing. Error rates of base calling  
617 in the filtered sequences were inferred from the data using the `learnErrors()` function with  
618 *multithread* set to TRUE. Using the inferred error rates, sequences were partitioned into ASVs  
619 with *pool* and *multithread* set to TRUE. If the `dada()` function failed to complete partitioning  
620 after 7 days for a particular study's dataset, which occurred for only one study (36), *pool* was set  
621 to FALSE for sample independent sequence partitioning.

622           If forward and reverse sequences were not yet merged, they were merged using the  
623 `mergePairs()` function. In cases where the forward and reverse reads were already merged in  
624 publicly available data files, the DADA2 merging step was omitted and the code adjusted for  
625 merged input sequences. Merged sequences with lengths greater or less than 20 nucleotides from  
626 the expected amplified region were removed from the data set since they were most likely due to  
627 non-specific merging of forward and reverse reads resulting in extra-long or extra-short reads.  
628 Chimeric sequences were detected and removed using the `removeBimeraDenovo()` function with

629 *multithread* set to FALSE. This employs the default consensus method instead of the pooled  
630 method. The consensus method determines chimeric sequences in each sample and then  
631 compares detected chimeric sequences across samples for a consensus. Taxonomy was assigned  
632 to sequences using the Silva 16S rRNA gene bacterial database (v 138) (159, 165). Species  
633 assignments were added, when possible.

634 For each study, merged datasets of ASV counts and taxonomic classifications were  
635 filtered using functions from the R package dplyr (166) to remove ASVs that were classified as  
636 mitochondrial, chloroplast, or not of bacterial origin. ASVs not classified at the phylum level and  
637 samples which did not have at least 100 sequence reads after filtering were removed from the  
638 data set.

#### 639 ***Removal of likely DNA contaminants through the R package DECONTAM***

640 To control for background DNA contamination, the R package DECONTAM was used to  
641 identify and remove sequences which were more prevalent in technical controls than in placental  
642 samples. For likely sequence contaminant removal, studies which included at least six technical  
643 controls (36-43, 50, 86) were included based on the recommendation of the authors of  
644 DECONTAM (88). Technical controls included air swabs, blank DNA extraction kits, and  
645 template-free PCR reactions. The DECONTAM function `isNotContaminant()` was used to  
646 remove ASVs which were more prevalent in technical controls than in biological samples.  
647 Thresholds were study specific with the goal of excluding most of the low prevalence ASVs  
648 while retaining high prevalence ASVs not likely to be contaminants. Despite using these  
649 stringent study specific thresholds, the results were unchanged if the default threshold of 0.5 was  
650 used instead.



651 ***Normalization of 16S rRNA gene sequence datasets within and across studies***

652 All datasets were normalized using the function `rarefy_even_depth()` from the R package  
653 `phyloseq` (1.30.0) (167). Following the normalization process, samples whose sequence libraries  
654 did not have at least 100 reads were excluded. The remaining samples were subsampled without  
655 replacement (i.e., the same sequence was never reselected when subsampling) to the minimum  
656 number of sequences per sample within a study. `RNGseed` was set to 1 to fix the seed for  
657 reproducible random number generation. This normalization approach was utilized since 16S  
658 rRNA gene read counts can vary by five orders of magnitude among samples in a single study.  
659 Given this degree of variability, normalization to the same sequence depth is justified and  
660 required for accurate comparisons of 16S rRNA gene profiles among samples (168).

661 ***Data Visualization***

662 Heatmaps illustrating the relative abundances of ASVs were prepared using the  
663 `ComplexHeatmap` R package (version 2.2.0) (169). Samples were grouped by sample type and  
664 ASVs were ordered based on ASV mean relative abundances within samples.

665 The function `vegdist()` from the R package `vegan` (version 2.5-6) (170) was used to create  
666 Bray-Curtis dissimilarity matrices which were then used as the basis for Principal Coordinates  
667 Analysis (PCoA) plots that were generated using the `pco()` function from the R package `ecodist`  
668 (version 2.0.7) (171). The Bray-Curtis index was used because it takes into account both the  
669 composition and structure of 16S rRNA gene sequence bacterial profiles (172). The Lingoes  
670 method was used to correct for negative eigenvalues so that dissimilarity between samples could  
671 be completely explained in Euclidean space (173).

672 All code to produce the published figures from the raw data is included in the  
673 supplementary materials in an R markdown file available at  
674 [https://github.com/jp589/Placental\\_Microbiota\\_Reanalysis](https://github.com/jp589/Placental_Microbiota_Reanalysis).

675 ***Statistical analysis***

676 Homogeneity of 16S rRNA gene sequence profiles between placental samples and technical  
677 controls was tested using `betadisper()` from the R package `vegan` (version 2.5-6) (170).  
678 Differences in 16S rRNA gene profile structure between placental samples by sampling level and  
679 technical controls were evaluated using the function `pairwise.adonis()` from the R package  
680 `pairwiseAdonis` (version 0.4) (174).

681 All code to recapitulate these analyses are included in an R markdown file available at  
682 [https://github.com/jp589/Placental\\_Microbiota\\_Reanalysis](https://github.com/jp589/Placental_Microbiota_Reanalysis).

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688 delivery for the Leon et al. study, which used samples taken from the Baby Bio Bank (175). We  
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1142

1143 **TABLES –**

1144 **Table 1. Overview of placental microbiota studies that were based on 16S rRNA gene**  
1145 **sequencing data and that were included in this critical review and re-analysis**

1146 The presented study characteristics include the: name of the first author(s); geographical location  
1147 at which subjects were sampled; specific 16S rRNA gene hypervariable region that was targeted  
1148 for sequencing; number of placentas sampled by mode of delivery and whether delivery was  
1149 before (i.e., preterm) or after (i.e. term) 37 weeks; number of technical controls included to  
1150 address potential background DNA contamination; and whether we were able to categorize  
1151 placental samples based on mode of delivery, gestational age at delivery (i.e., before or after 37  
1152 weeks), and whether DECONTAM analysis could be performed to identify background DNA  
1153 contaminants (i.e.,  $N \geq 6$  technical controls included in the study) (88). Square brackets indicate  
1154 that available sample metadata did not allow for placentas to be grouped by gestational age at  
1155 delivery.



Study	Geographic Location	16S rRNA gene hypervariable region	Cesarean		Vaginal		Technical Controls	Ability to group by		Ability to run DECONTAM
			Term	Preterm	Term	Preterm		Delivery	Gestational Age	
de Goffau Part I <sup>b</sup>	Cambridge, UK	V1-V2	80	0	0	0	47	X	X	X
Lauder	Philadelphia, PA, USA	V1-V2	1	0	5	0	39	X	X	X
Leiby	Philadelphia, PA, USA	V1-V2	1	5	19	15	103	X	X	X
Olomu	Lansing, MI, USA	V4	47 <sup>a</sup>	0	0	0	131	X	X	X
Parnell	St. Louis, MO, USA	V4	34	0	23	0	21	X	X	X
Theis <sup>c</sup>	Detroit, MI, USA	V4	29 <sup>a</sup>	0	0	0	43	X	X	X
Theis, Winters	Detroit, MI, USA	V4	28	14	21	6	12	X	X	X
Sterpu	Stockholm, Sweden	V6-V8	50 <sup>a</sup>	0	26	0	6	X	X	X
Dinsdale	South Hedland, Australia	V4	[19]		[31]		8	X		X
Tang	Shanghai, China	V3-V4	15 <sup>a</sup>	0	0	0	0	X	X	
Liu	Kunming, China	V4-V5	42	0	36	0	0	X	X	
Gomez-Arango	Brisbane, Australia	V6-V8	16 <sup>a</sup>	0	20	0	0	X	X	
Younge	Durham, NC, USA	V4	5 <sup>a</sup>	5	0	0	0	X		
Leon	London, England, UK	V5-V7	[136]		[120]		21			X
Seferovic	Houston, TX, USA	V4	26	8	0	18	2			

<sup>a</sup> Indicates that placental samples were delivered without labor.

<sup>b</sup> Analyzed data are from the Cohort 1 component of the study.

<sup>c</sup> Analyzed data are from the nested PCR component of the study.

1157 **Table 2. Summary of prominent bacterial ASVs in term cesarean delivered placental**  
1158 **samples before and after removal of background DNA contaminants using the R package**  
1159 **DECONTAM**

1160 The top five ASVs as determined by mean relative abundance across placental samples after  
1161 DADA2 processing are provided for studies which could be restricted to cesarean delivered  
1162 placental samples. Asterisks indicate ASV sequence genus level classifications which were  
1163 assigned by NCBI BLAST with the highest percent identity in excess of 95%. The Liu et al. [1]  
1164 dataset could not be assessed post-DECONTAM since no technical controls were included in the  
1165 study.



Study	16S rRNA gene hypervariable region	ASV1	ASV2	ASV3	ASV4	ASV5
<b>Before DECONTAM</b>						
Olomu	V4	<i>Escherichia/Shigella</i>	<b>Lactobacillus</b>	<b>Lactobacillus</b>	<i>Finegoldia</i>	<i>Fenollaria</i>
Parnell	V4	<i>Leptospira</i>	<i>Leptospira</i>	<i>Leptospira</i>	<i>Cutibacterium</i>	<i>Leptospira</i>
Theis	V4	<i>Achromobacter</i>	<i>Delftia</i>	<i>Phyllobacterium</i>	<i>Clostridium sensu stricto 5</i>	<i>Stenotrophomonas</i>
Theis, Winters	V4	<b>Lactobacillus</b>	<i>Staphylococcus</i>	<i>Escherichia/Shigella</i>	<i>Serratia</i> *	<i>Streptococcus</i>
Liu	V4-V5	<i>Pseudomonas</i>	<i>Pantoea</i> *	<i>Pantoea</i> *	<b>Lactobacillus</b>	<i>Pantoea</i> *
Sterpu	V6-V8	<i>Cutibacterium</i>	<i>Staphylococcus</i>	<i>Streptococcus</i>	<i>Streptococcus</i>	<i>Streptococcus</i>
<b>After DECONTAM</b>						
Olomu	V4	<i>Fenollaria</i>	<i>Acinetobacter</i>	<b>Lactobacillus</b>	<i>Campylobacter</i>	<i>Peptoniphilus</i>
Parnell	V4	<i>Leptospira</i>	<i>Leptospira</i>	<i>Leptospira</i>	<i>Cutibacterium</i>	<i>Leptospira</i>
Theis	V4	<i>Achromobacter</i>	Alcaligenaceae	<i>Escherichia/Shigella</i>	<i>Achromobacter</i>	Alcaligenaceae
Theis, Winters	V4	<i>Corynebacterium</i>	<i>Serratia</i> *	<i>Mycoplasma</i>	<i>Corynebacterium</i>	<i>Fusobacterium</i>
Sterpu	V6-V8	<i>Staphylococcus</i>	<i>Gardnerella</i>	<i>Staphylococcus</i>	<i>Staphylococcus</i>	<i>Streptococcus</i>

ASV1-5 are rank designations based on percent relative abundance

## 1166 **FIGURE LEGENDS**

### 1167 **Figure 1. Study inclusion flowchart**

1168 Four searches were performed on PubMed to identify studies for inclusion in the re-analysis.

1169 Filtering criteria were: primary research article, 16S rRNA gene sequencing data, placentas

1170 obtained from term deliveries, sequencing data accessible with published accession number, and

1171 sufficient metadata available to parse sequencing data into individual samples.

### 1172 **Figure 2. Conclusions of thirteen studies evaluating the existence of a placental microbiota,** 1173 **which included data from multiple placentas delivered via cesarean section at term**

1174 The studies are principally separated and contrasted depending upon whether they included

1175 technical controls to account for potential background DNA contamination.

### 1176 **Figure 3. Principal Coordinates Analyses of the beta diversity of bacterial DNA profiles**

#### 1177 **between placental samples and technical controls in published placental microbiota studies**

1178 Studies were included if technical controls were sequenced and made publicly available to

1179 account for background DNA contamination. Beta diversity between placental (red open circles)

1180 and technical control (black open circles) samples is illustrated by study in PCoA plots based on

1181 the Bray-Curtis dissimilarity index. Genus level classifications of the top ten ASVs in placental

1182 samples and technical controls by total reads are plotted at their weighted average positions (grey

1183 diamonds). Asterisks indicate ASV sequence genus level classifications which were assigned by

1184 NCBI BLAST with the highest percent identity in excess of 95%.

### 1185 **Figure 4. Principal Coordinates Analyses of the beta diversity of the bacterial DNA profiles** 1186 **of placental and vaginal/vaginal-rectal samples in placental microbiota studies**

1187 Prior published studies were included if vaginal or vaginal-rectal samples were sequenced and

1188 made publicly available alongside placental samples. The top ten ASVs shared between placental

1189 samples and technical controls, and the top ten ASVs in vaginal samples are plotted at their  
1190 weighted average positions in the ordination space (grey diamonds) and their genus level  
1191 classifications are noted. Agglomerated genus level classifications were plotted for the Liu  
1192 dataset instead of ASVs since no ASVs were greater than 1% mean relative abundance across  
1193 placental samples. Asterisks indicate ASV sequence genus level classifications which were  
1194 assigned by NCBI BLAST with the highest percent identity in excess of 95%.

1195 **Figure 5. Placental and technical control samples cluster by study origin, mode of delivery,**  
1196 **and gestational age at delivery**

1197 A) Beta diversity between placental (open circles) and technical control samples (open triangles)  
1198 from studies which sequenced the V4 hypervariable region of the 16S rRNA gene is visualized  
1199 through principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity index. B)  
1200 Beta diversity of placental samples without technical control samples from each study. C)  
1201 Placental samples from the same six studies were characterized by mode of delivery and  
1202 gestational age at delivery. Weighted average positions of ASVs greater than 1% were plotted as  
1203 grey diamonds and labelled with genus level classifications. Asterisks indicate ASV sequence  
1204 genus level classifications which were assigned by NCBI BLAST with the highest percent  
1205 identity in excess of 95%.

1206 **Figure 6. Heatmaps of the bacterial DNA profiles of placental and technical control**  
1207 **samples from studies which sequenced the V4 hypervariable region of the 16S rRNA gene**  
1208 **demonstrating a high degree of overlap between these two sample types**

1209 ASVs are listed by study if they had a mean relative abundance greater than 1% across placental  
1210 samples (green bar). Red asterisks indicate ASVs which had a mean relative abundance greater  
1211 than 2% across all technical control samples (purple bar) from that study. Regular asterisks

1212 indicate ASV sequence genus level classifications which were assigned by NCBI BLAST with  
1213 the highest percent identity in excess of 95%.  
1214

387 unique articles returned from searches for "human placental microbiome", "placental microbiota", "placental microbiomes", and "placenta 16S"



58 were primary research articles



41 performed 16S rRNA gene sequencing on placental samples



33 included term delivered samples (>37 weeks gestation)



17 were publicly available (i.e., data was uploaded to a public database with an accurate accession number)



15 included complete data which were able to be parsed into individual samples

Publicly available 16S rRNA gene sequencing datasets which included multiple placentas delivered via cesarean section at term (N=13)

Yes

Included at least six technical controls to remove likely contaminants with the R package DECONTAM [88]

No

Yes

(N=5)

(N=8)

Included any technical controls

Included metadata to distinguish samples by gestational age at delivery

No

Yes

No

Yes

(N=4)  
[29,54,57-58]

(N=1)  
[44]

(N=2)  
[36,42]

(N=6)  
[39-41,43,50,86]

Concluded that there was evidence for a placental microbiota in typical term placental samples

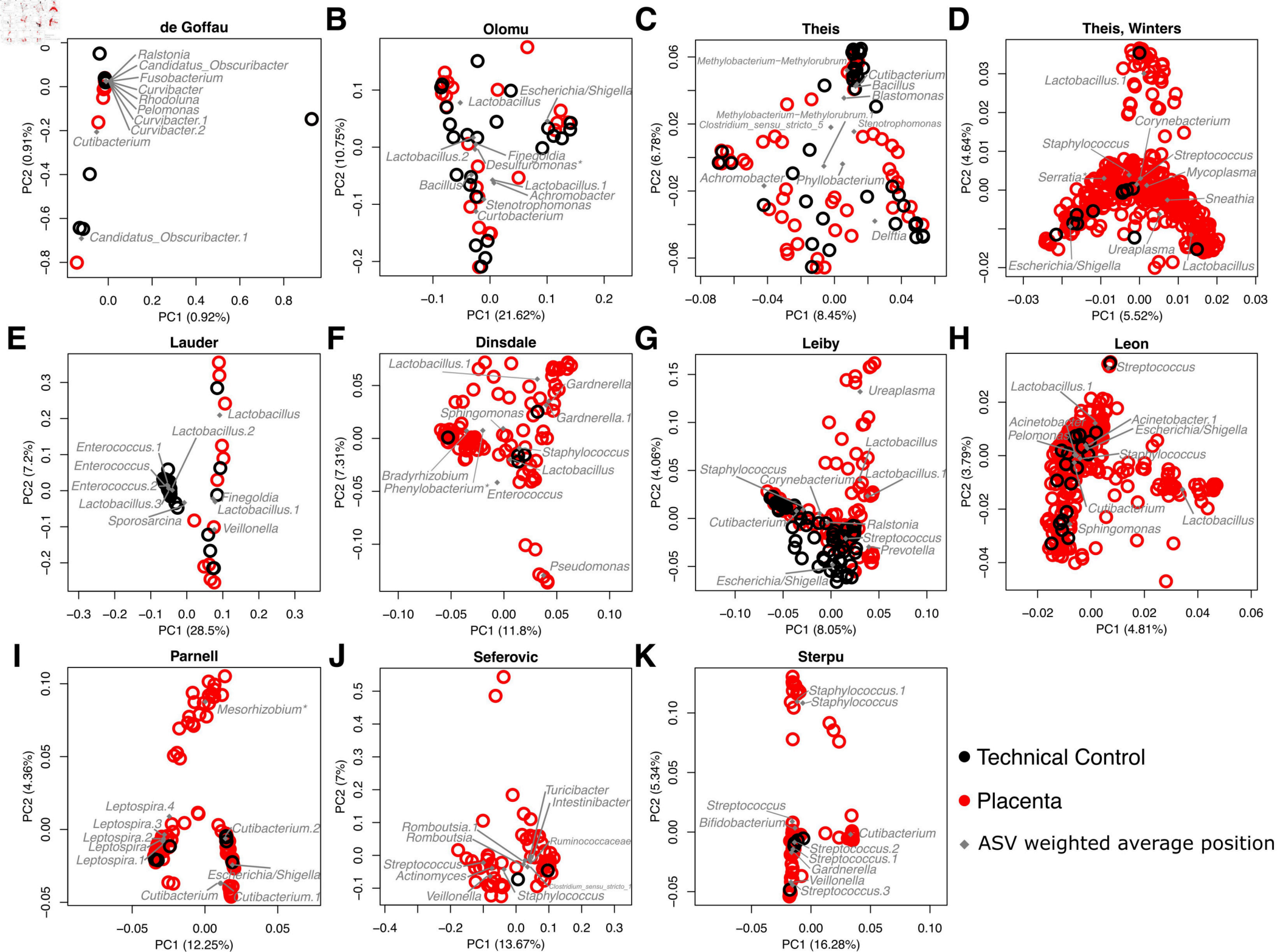
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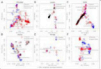
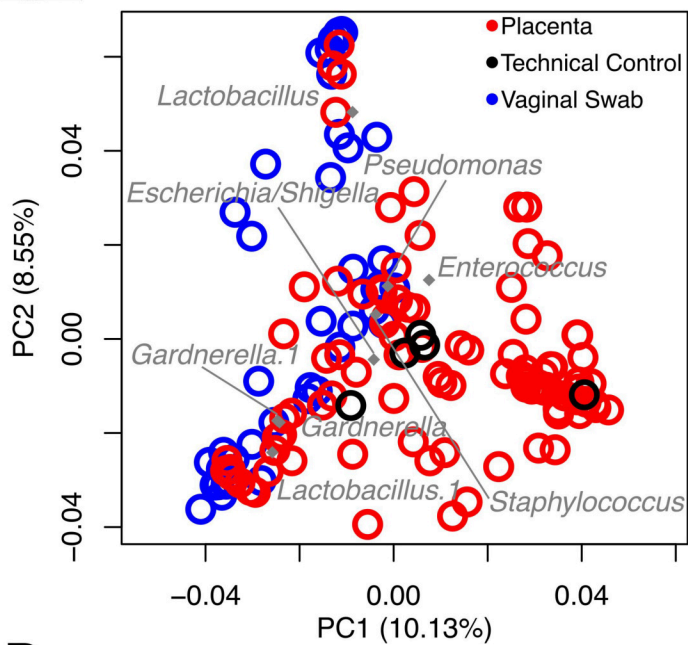
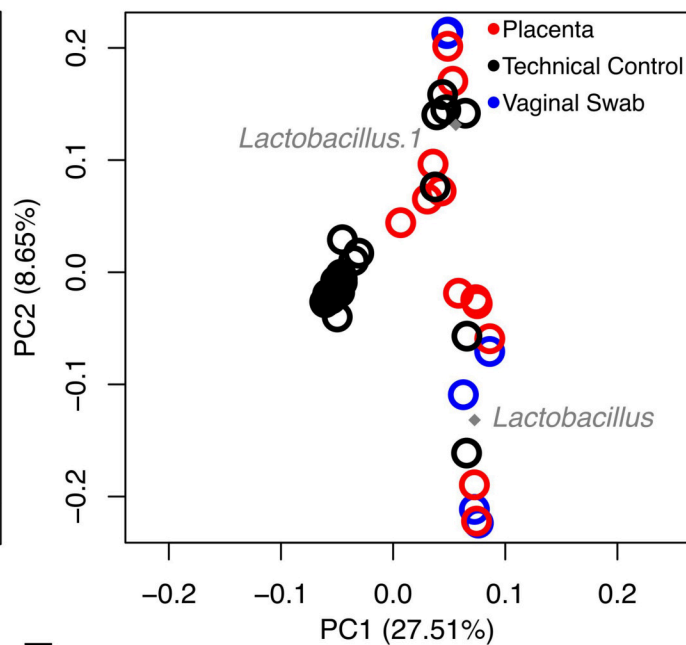
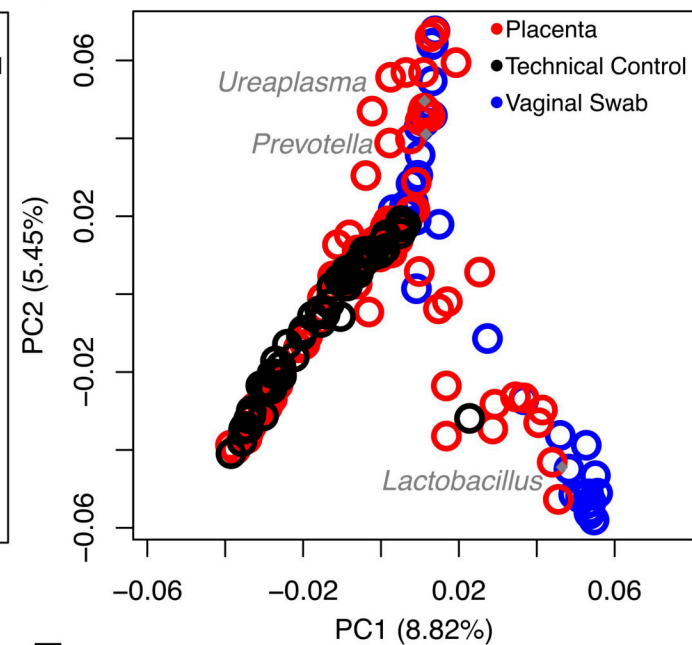
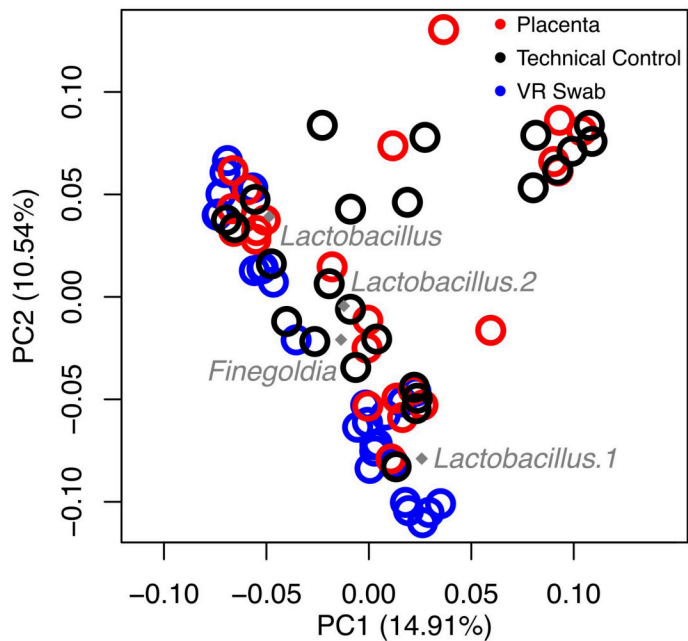
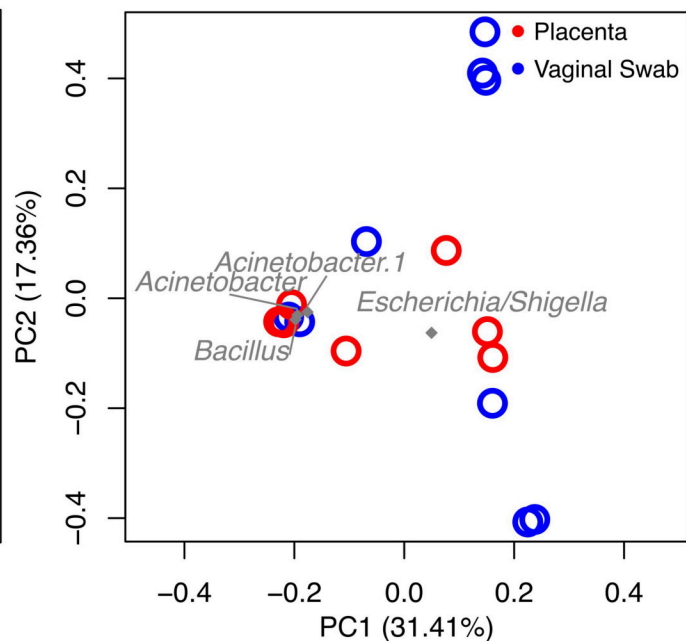
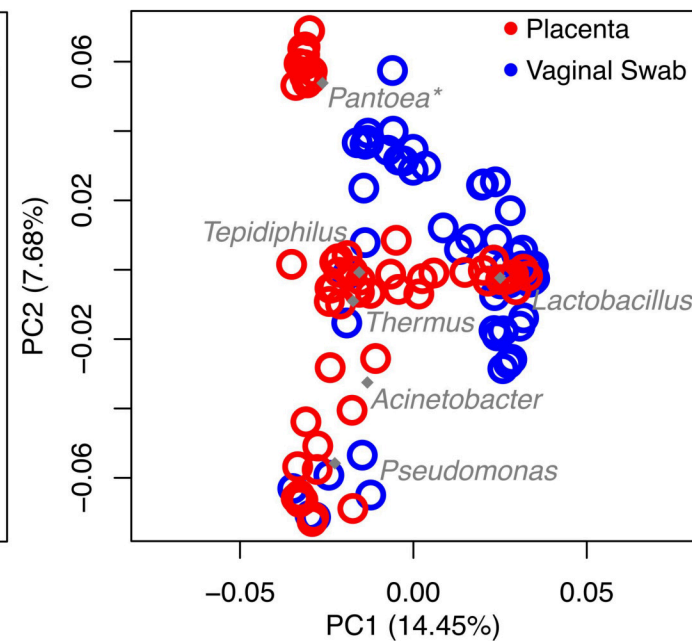
Yes

(N=5)  
[39-41,50,86]

(N=1) (N=5)  
[43] [29,44,54,57-58]





**Dinsdale****B****Lauder****C****Leiby****D****Olomu****E****Younger****F****Liu**

◆ ASV weighted average position



