

1 **The vaginal microbiota of pregnant women varies with gestational age, maternal age, and**
2 **parity**

3

4 **Short title: Vaginal microbiota across term pregnancy**

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43 **ABSTRACT**

44 The composition of the vaginal microbiota is heavily influenced by pregnancy and may factor into
45 pregnancy complications, including spontaneous preterm birth. However, results among studies
46 have been inconsistent, due in part to variation in sample sizes and ethnicity. Thus an association
47 between the vaginal microbiota and preterm labor continues to be debated. Yet, before assessing
48 associations between the composition of the vaginal microbiota and preterm labor, a robust and
49 in-depth characterization of the vaginal microbiota throughout pregnancy in the specific study
50 population under investigation is required. Herein, we report a large longitudinal study (N = 474
51 women, 1862 vaginal samples) of a primarily African-American cohort– which experiences a
52 relatively high rate of pregnancy complications – evaluating associations between individual
53 identity, gestational age, and other maternal characteristics with the composition of the vaginal
54 microbiota throughout gestation resulting in term delivery. The primary factors influencing the
55 composition of the vaginal microbiota in pregnancy are individual identity and gestational age at
56 sampling. Secondary factors are maternal age, parity, obesity, and self-reported *Cannabis* use. The
57 principal pattern across gestation is for the vaginal microbiota to remain or transition to a state of
58 *Lactobacillus* dominance. This pattern can be mitigated by maternal parity and obesity.
59 Regardless, network analyses reveal dynamic associations among specific bacterial taxa within the
60 vaginal ecosystem, which shift throughout the course of pregnancy. This study provides a robust
61 foundational understanding of the vaginal microbiota in pregnancy among African-Americans, in
62 particular, and sets the stage for further investigation of this microbiota in obstetrical disease.

63

64

65 **IMPORTANCE**

66 There is debate regarding links between the vaginal microbiota and pregnancy complications,
67 especially spontaneous preterm birth. Inconsistencies in results among studies are likely due to
68 differences in sample sizes and cohort ethnicity. Ethnicity is a complicating factor because,
69 although all bacterial taxa commonly inhabiting the vagina are present among all ethnicities, the
70 frequencies of these taxa vary among ethnicities. Therefore, an in-depth characterization of the
71 vaginal microbiota throughout pregnancy in the specific study population under investigation is
72 required prior to evaluating associations between the vaginal microbiota and obstetrical disease.
73 This initial investigation is a large longitudinal study of the vaginal microbiota throughout
74 gestation resulting in a term delivery in a primarily African-American cohort, a population that
75 experiences disproportionately negative maternal-fetal health outcomes. It establishes the
76 magnitude of associations between maternal characteristics, such as age, parity, BMI, and self-
77 reported *Cannabis* use, on the vaginal microbiota in pregnancy.

78

79 **KEYWORDS**

80 *Cannabis*, *Gardnerella*, gestation, *Lactobacillus*, microbiome, obesity, term gestation

81 INTRODUCTION

82 The composition of the vaginal microbiota is broadly consistent across populations of reproductive
83 age women ([1-5](#)). In general, the vaginal microbiota can be categorized into five primary
84 community state types (CSTs) that are defined by a predominance, or a lack thereof, of
85 *Lactobacillus* spp. ([1-9](#)). Four of these CSTs are dominated by *Lactobacillus* spp. (*L. crispatus* -
86 CST I, *L. gasseri* - CST II, *L. iners* - CST III, *L. jensenii* - CST V) and the other CST (CST IV) is
87 typically not dominated by any one bacterium, but rather is comprised of a diverse array of
88 microorganisms ([4-6](#), [8-10](#)). CST IV has been further subcategorized as CST IV-A or CST IV-B
89 ([11](#)). CST IV-A is characterized by high relative abundances of *Candidatus Lachnocurva vaginae*
90 (formerly Bacterial Vaginosis-Associated Bacterium 1, or BVAB1 ([12](#))) *Gardnerella vaginalis*,
91 and *L. iners*, whereas CST IV-B has high relative abundances of *Atopobium vaginae*, *G. vaginalis*,
92 and *L. iners* ([5](#), [11](#)). Importantly, the *Lactobacillus*-dominated CSTs (I, II, III, V), and especially
93 CST I, which is dominated by *L. crispatus*, are associated with optimal vaginal health ([13-19](#)) and
94 positive reproductive outcomes ([20-29](#)). In contrast, CST IV-A and CST IV-B have been
95 associated with bacterial vaginosis ([7](#), [13](#), [30-35](#)) and, among pregnant women, CST IV ([23](#), [25](#),
96 [36-38](#)), CST IV-associated bacteria ([23](#), [25-29](#), [38-41](#)), and/or a greater vaginal microbiota
97 diversity in general ([22](#)), have been associated with an increase in the risk of spontaneous preterm
98 birth (sPTB) – the leading cause of neonatal mortality and morbidity worldwide ([42](#), [43](#)).
99 Nevertheless, non-pregnant and pregnant women alike with vaginal microbiotas classified as CST
100 IV can be asymptomatic ([4](#), [44](#)), and their reproductive health and pregnancy outcomes are
101 generally normal. Therefore, the strength and clinical relevance of associations between vaginal
102 CSTs and female reproductive health and pregnancy outcomes remains unclear ([45](#)).

103 Ethnicity is a complicating factor in such studies as it is associated with the structure of the
104 vaginal microbiota – all CSTs are present among all ethnicities, yet the frequencies of the CSTs
105 among ethnicities vary ([2-4](#), [22](#), [46-48](#)). For example, African American and Hispanic women are
106 more likely to exhibit CST IV vaginal communities, whereas Caucasian and Asian women tend to
107 more frequently display *Lactobacillus*-dominated CSTs ([2-4](#)). Overall, regardless ethnicity, the
108 composition of the vaginal microbiota can be highly labile and some of the factors influencing this
109 lability include the menstrual cycle ([5](#), [49-54](#)), sexual activity ([55-57](#)), and pregnancy ([58](#)). The
110 menstrual cycle appears to have a stabilizing effect on the composition of the vaginal microbiota,
111 an affect that has been attributed to high estrogen levels, which favor the proliferation of
112 *Lactobacillus* spp. ([5](#), [49-54](#)). Conversely, sexual activity increases the likelihood of CST IV
113 vaginal communities ([57](#)) and decreases the presence of potentially protective *L. crispatus* ([56](#)).
114 Pregnancy, a vulnerable period accommodating the growth and development of the fetus, and that
115 includes a drastic rise in steroid hormones (e.g. progesterone and estrogen) ([59](#), [60](#)), also favors
116 the presence of *Lactobacillus*-dominated CSTs in the vagina ([44](#), [58](#)). Indeed, we previously
117 reported that the vaginal microbiota in pregnancy differs from that in non-pregnant women ([58](#)).
118 Specifically, pregnant women have higher relative abundances of *L. vaginalis*, *L. crispatus*, *L.*
119 *gasseri* and *L. jensenii*, and lower abundances of 22 other non-*Lactobacillus* phylotypes ([58](#)). In
120 addition, the vaginal microbiota of pregnant women is typically more stable (i.e., consistent across
121 time) than that of non-pregnant women ([58](#)). These general findings have been replicated by other
122 investigators ([20](#), [21](#), [48](#)). Therefore, it has been proposed that increased stability of the vaginal
123 microbiota and *Lactobacillus*-dominance during pregnancy play a protective role and reduce the
124 likelihood of pregnancy complications, especially sPTB ([47](#), [61](#)). However, the association
125 between variation in the composition of the vaginal microbiota and preterm birth continues to be

126 debated ([45](#), [62-64](#)). Potential explanations for the inconsistencies in results among published
127 studies include differences in sample sizes and cohort ethnicity. Therefore, before evaluating
128 associations between the composition of the vaginal microbiota and obstetrical disease, including
129 sPTB, a robust and in-depth characterization of the vaginal microbiota throughout pregnancy in
130 the specific study population under investigation is required.

131 This initial investigation focuses on an urban population that experiences a high risk of
132 pregnancy complications ([65-75](#)). It uses 16S rRNA gene amplicon sequencing to assess the
133 trajectory of the composition of the vaginal microbiota throughout gestation ending in term
134 delivery. Leveraging longitudinal samples from a large set of patients with well-characterized
135 demographic and clinical data, this study establishes the magnitude of associations between
136 maternal characteristics, such as age, parity, and ethnicity on the vaginal microbiota. Such
137 knowledge is important for the assessment of previous reports and for informing future analyses
138 of the vaginal microbiota in relationship to obstetrical complications, especially sPTB. Moreover,
139 this study provides information on a primarily African American population, for which available
140 data are overall sparse despite this population experiencing disproportionately negative maternal-
141 fetal health outcomes ([65-75](#)).

142

143

144 **RESULTS AND DISCUSSION**

145 The demographic characteristics of the 474 patients with term delivery included in this study (the
146 largest cohort sampled to date) are presented in **Table 1**. This cohort is primarily African-
147 American [94.5% (448/474)] with a body mass index (BMI) above 25 kg/m² [65% (306/472)].
148 The distribution of the gestational ages at which the 1862 vaginal fluids were collected from these
149 patients is depicted in **Figure 1**. Each woman had 3 to 4 samples (median of 4) collected between
150 8 and 38⁺⁶ weeks of gestation.

151

152 *Effect of patient/subject identity*

153 The structure of the vaginal microbiota during pregnancy has been reported to vary with
154 gestational and maternal age ([47](#), [48](#), [58](#)). However, the scope and strength of all factors potentially
155 influencing the structure and fluidity of the vaginal microbiota during pregnancy remain to be
156 elucidated. In the current study, beta diversity, or the shared diversity of the microbiota between
157 samples, was characterized using the Jaccard (i.e., microbiota composition) and Bray-Curtis (i.e.,
158 microbiota structure) indices. The variation in vaginal microbiota composition and structure was
159 primarily explained by patient identity (SubjectID: composition - R²=58%-61%; structure -
160 R²=65%-68%) and by the patient-specific variation with gestational age (interaction between
161 SubjectID and Gestational age: composition - R²=16%-18%; structure - R²=14%-16%). Only
162 relatively modest percentages of the variance in the composition and structure of the vaginal
163 microbiota were explained by maternal characteristics, such as age (0.2%-1.4%), parity (0.3%-
164 1.9%), and self-reported *Cannabis* use (0.3%-1.8%). Overall, these findings illustrate the large
165 influence that patient (i.e., individual) identity has on the composition and structure of the vaginal
166 microbiota, which is consistent with general observations of microbiotas at other body sites, such

167 as the oral cavity, gut, and skin ([76-81](#)). Furthermore, this highlights the importance of robust
168 longitudinal, as opposed to cross-sectional, studies to account for inter- and intra-individual
169 variability in the microbiota ([80](#), [82-84](#)).

170

171 *Effect of gestational age*

172 Alpha diversity, which is the diversity of the microbiota within individual samples, was
173 characterized using Chao1 (i.e., richness) and Shannon and Simpson (i.e., evenness) indices. Both
174 richness (**Figure 2A,C**) and evenness (**Figure 2B,D**) of the vaginal microbiota decreased with
175 advancing gestational age from the first to the third trimester ($p < 0.0001$ for all). This is consistent
176 with previous reports of alpha diversity in cohorts of primarily African-Americans ([28](#), [48](#), [58](#)). In
177 contrast, previous reports of largely Caucasian cohorts indicated that alpha diversity is generally
178 low and consistent throughout the entirety of gestation ([23](#), [47](#), [48](#), [85](#)). Nevertheless, in the current
179 study, there was substantial heterogeneity in the rate of decrease in vaginal microbiota alpha
180 diversity among patients – the decrease was steeper for women who had higher baseline diversity
181 early in pregnancy (correlation between random intercepts and random slopes: Shannon -0.79,
182 Simpson -0.67, Chao1 = -0.67) (see for example **Figure 3**). The decrease in alpha diversity with
183 advancing gestational age remained significant after adjusting for potential confounding variables,
184 including maternal age, parity, and BMI (**Table 2**). These alterations in the overall structure of the
185 vaginal microbiota likely reflect physiological alterations (e.g., glycogen levels ([5](#), [86](#), [87](#))) in the
186 vaginal microenvironment across gestation that favor the predominance of a few bacterial taxa that
187 can thrive in these conditions (e.g. *Lactobacillus* spp.).

188 The vaginal microbiota is consistently categorized into CSTs that are defined by a
189 dominance or lack thereof of *Lactobacillus* spp. ([4](#), [11](#), [23](#), [44](#)). Using a previously established

190 protocol for assigning CSTs to vaginal samples based on 16S rRNA gene sequence data ([11](#)), we
191 identified seven CSTs among the 1862 samples included in this study (**Figure 4A**). These CSTs
192 included four dominated by *L. crispatus* (I), *L. gasseri* (II), *L. iners* (III), or *L. jensenii* (V), and
193 three more diverse CSTs (CST IV) comprised of *L. iners*, *Gardnerella* sp., and *Megasphaera* sp.,
194 with *Ca. Lachnocurva vaginae*, *Atopobium vaginae*, and *Bifidobacterium* sp. being relatively
195 abundant in CST IV-A, -B, and -C, respectively. These CSTs are consistent with previous
196 investigations of the vaginal microbiota in smaller cohorts ([27](#), [29](#), [36](#), [39-41](#), [44](#), [47](#), [58](#), [85](#), [88-](#)
197 [94](#)), further illustrating the depth of complexity of CST IV-designated communities.

198 In this study, CST prevalence was a function of gestational age (**Figure 4B**). Except for
199 the two least abundant CSTs (II and IV-C), for which statistical power was inherently limited, the
200 membership probability to any CST displayed dynamic changes with gestational age ($p < 0.05$ for
201 all; **Figure 4B**). While *Lactobacillus*-dominated CSTs I, III, and V tended to be more abundant
202 with advancing gestational age, the abundance of the more diverse CSTs IV-A and -B declined
203 steadily as term gestation approached (**Figure 4B**). Notably, in a secondary analysis of women (N
204 = 309) for which samples were available from each of four discrete time points across gestation,
205 there was a pronounced shift in CST composition with advancing gestational age; specifically,
206 there was an increase in CSTs I and III at the end of pregnancy, derived primarily from patients
207 with an initial CST IV-A or IV-B (**Figure 4C**). These findings are in line with prior cross-sectional
208 ([21](#), [24](#), [29](#), [36](#), [39](#), [40](#), [48](#), [88](#), [89](#), [91](#), [93](#)) and longitudinal ([22](#), [23](#), [25-28](#), [41](#), [44](#), [47](#), [48](#), [58](#), [85](#),
209 [90](#), [92](#), [94](#), [95](#)) studies which included characterization of the structure and dynamics of the vaginal
210 microbiota in term pregnancies. At a community level, pregnancy has been shown to create
211 favorable conditions for a *Lactobacillus*-dominated vaginal microbiota, particularly CSTs I and
212 III, and a shift away from the more diverse CSTs IV-A and IV-B, as gestation progresses to term

213 ([20](#), [23](#), [48](#), [58](#)). Although shifts in the vaginal microbiota occur in both gravid and non-gravid
214 women, an increased prevalence of specifically *Lactobacillus*-dominated CSTs in pregnant
215 women is intriguing because it could protect against ascending infection ([96](#)), which could
216 culminate in sPTB, through the competitive exclusion of opportunistic pathogens within the
217 vaginal microenvironment ([47](#), [97](#)). Furthermore, *Lactobacillus* species produce lactic acid, which
218 has anti-inflammatory properties ([98-100](#)). Additional research exploring the functional role of the
219 vaginal microbiota on the host in large longitudinal cohorts is warranted to address these
220 hypotheses.

221 After describing the changes in the composite measures of the vaginal microbiota alpha
222 and beta diversity and CSTs, we utilized linear mixed-effects (LME) modeling to analyze the
223 relationships between gestational age and maternal characteristics with the relative abundances of
224 individual bacterial taxa denoted as amplicon sequence variants, or ASVs (**Supplemental Table**
225 **1**). Increased gestational age was positively correlated with 33 exclusively *Lactobacillus* ASVs
226 ($q < 0.1$), and negatively correlated with ASVs that are typical members of vaginal CST IV
227 (**Supplemental Table 1, Figure 5**).

228 To supplement the LME models at the ASV level, we further implemented Analysis of
229 Composition of Microbiomes, or ANCOM ([101](#)), to identify ASVs changing in abundance
230 throughout gestation. Gestational age was treated as a main fixed effect, patient identity as a
231 random effect, and maternal age, parity, *Cannabis* use, ethnicity, and race were included as
232 covariates. Seventy-five ASVs were positively or negatively associated with gestational age
233 (**Supplemental Table 2, Figure 6**). Fifty-six of these ASVs overlapped with those identified as
234 being significantly associated with gestational age in the LME analysis (**Supplemental Figure 1**).
235 As in the LME analysis, many *Lactobacillus* ASVs were positively associated with gestational age

236 while many bacteria typically associated with CST IV were negatively associated with gestational
237 age. The direction of these associations is intriguing given prior reports that *Lactobacillus*-
238 dominated vaginal CSTs are linked with positive reproductive outcomes (23, 26, 28) while,
239 conversely, CST IV and CST IV-typical bacteria have been associated with an increased risk of
240 sPTB (25, 28, 38). Notably, however, the ANCOM analyses additionally indicated that multiple
241 ASVs classified as *Ca. Lachnocurva vaginae* also increased in abundance with advancing
242 gestation. *Ca. Lachnocurva vaginae*, previously referred to as *Shuttleworthia* spp. (12), is an
243 established resident bacterium of the vaginal ecosystem (102, 103), and it is typically a component
244 of CST IV. Its increase in abundance throughout pregnancy is a novel finding, the potential clinical
245 significance of which warrants further investigation.

246 Overall, the results were largely congruent between the linear mixed-effects models and
247 ANCOM analyses, although there were some notable differences (e.g. *Ca. Lachnocurva vaginae*).
248 The fundamental difference between LME and ANCOM is that ANCOM is inferring about
249 abundances whereas LME is inferring about relative abundances. Specifically, LME evaluates
250 whether the abundance of a particular taxon, in a unit volume of an ecosystem, relative to all other
251 taxa, has changed between two ecosystems. On the other hand, ANCOM evaluates whether the
252 abundance of a particular taxon, in a unit volume of an ecosystem, has changed between two
253 ecosystems. This explains the differences in the results obtained using the two approaches.
254 Regardless, LME and ANCOM identified ecologically plausible variation in microbiota
255 membership across gestational age, and a large proportion of the bacterial ASVs changing in
256 composition and abundance across pregnancy were discovered using both approaches.

257

258 *Effect of maternal parity*

259 In addition to the changes in alpha and beta diversity observed with increasing gestational age,
260 there was also a significant effect of parity. Specifically, parity was positively correlated with alpha
261 diversity (**Table 2**). One potential explanation for the association between parity and increased
262 alpha diversity is that there is a marked increase in the alpha diversity of the vaginal microbiota
263 after live birth ([85](#), [104](#)), and this phenomenon may be cumulative across multiple pregnancies,
264 mirroring maternal-fetal immunological memory ([105-108](#)). Notably, this phenomenon cannot be
265 explained simply by advancing maternal age, since this covariate was not positively correlated
266 with alpha diversity of the vaginal microbiota (**Table 2**). This finding indicates that, while there is
267 a consistent reduction in the richness and evenness of the vaginal microbiota throughout
268 pregnancy, at least among women who have a diverse microbiota at pregnancy onset, this effect
269 may be mitigated by parity.

270 With respect to beta diversity, only modest percentages of the variance in the composition
271 and structure of the vaginal microbiota were explained by parity (0.3%-1.9%). Nevertheless,
272 differences in CST membership based on parity, while adjusting for gestational age, were found
273 (**Table 3**), with parity (OR=1.46 for each additional previous delivery) being associated with a
274 decrease in CST III. At an ASV-level, higher maternal parity was significantly associated with an
275 increase in 58 ASVs classified as typical vaginal CST IV bacteria (e.g., *Gardnerella*,
276 *Megasphaera*, *Prevotella*, and *Sneathia*), while lower maternal parity was exclusively correlated
277 with 7 *Lactobacillus* ASVs, 6 of which were classified as *L. crispatus* ($q < 0.1$) (**Supplemental**
278 **Table 1**). This finding is consistent with a recent report of increased vaginal microbiota diversity
279 with higher parity during subsequent gestations ([109](#)). The ecological and clinical implications of
280 the correlation between increased parity and vaginal microbiota diversity warrant further
281 investigation.

282

283 *Effect of maternal age*

284 Only modest percentages of the variance in the composition and structure of the vaginal microbiota
285 were explained by maternal age (0.2%-1.4%). Nevertheless, differences in CST membership based
286 on maternal age, while adjusting for gestational age, were found (**Table 3**), with higher maternal
287 age (OR=0.64 for each additional 5 years) being associated with a decrease in CST III. Similarly,
288 at the ASV level, there were significant negative correlations between maternal age and 18 ASVs,
289 16 of which were classified as *L. iners*, while only 4 ASVs, classified as *L. crispatus*, were
290 positively correlated with maternal age (**Supplemental Table 1**). While these correlations contrast
291 with a previous report ([47](#)), the differences in ethnic makeup and sample size between the two
292 cohorts could account for this discrepancy.

293

294 *Effect of obesity*

295 There was a significant effect of obesity, defined as having a BMI greater than 28 kg/m², on alpha
296 diversity of the vaginal microbiota (**Table 2**). Specifically, there was a positive correlation between
297 obesity and richness of the vaginal microbiota across gestation. This is in contrast with the
298 intestinal microbiota, for which there tends to be a negative correlation between obesity and
299 richness across gestation ([110](#)). Similar patterns are evident outside pregnancy as well. Obesity is
300 associated with high alpha diversity of the vaginal microbiota ([111](#)) and, in general, low alpha
301 diversity of the gut microbiota ([112-115](#)). Thus, for both of these body sites, obesity is associated
302 with levels of microbiota alpha diversity that are widely viewed as non-optimal. As obesity is
303 characterized by a low-grade systemic inflammatory response ([116-119](#)), these data highlight
304 potential dynamic interactions between systemic inflammation and microbiota alpha diversity

305 throughout the human body that can influence health and disease, including pregnancy outcomes
306 ([120](#), [121](#)).

307

308 *Effect of Cannabis use*

309 Only modest percentages of the variance in the composition and structure of the vaginal microbiota
310 were explained by self-reported *Cannabis* use (0.3%-1.8%). Nevertheless, *Cannabis* use was
311 associated with an increase in ASVs classified as *L. iners* (16 ASVs) and a decrease in those
312 classified as *L. crispatus* (6 ASVs) (**Supplemental Table 1**). Given that *L. crispatus* has been
313 associated with female reproductive health and positive pregnancy outcomes ([4](#), [13](#), [15](#), [16](#), [19](#),
314 [20](#), [25](#), [27](#), [28](#), [36](#), [40](#), [44](#), [89](#), [90](#), [92](#), [97](#), [122-127](#)), these findings, among other potential general
315 concerns ([128](#), [129](#)), caution against *Cannabis* use during pregnancy.

316

317 *Bacterial taxa are highly correlated with one another during normal pregnancy*

318 **Supplemental Table 3** shows some of the strongest associations (LME adjusted $q < 0.05$ and
319 absolute spearman correlation coefficient > 0.5) between pairs of bacterial taxa during pregnancy.
320 In this analysis, each genus-level taxon (or family, if genus-level designation was not available)
321 was represented by one ASV retained based on the strongest association with gestational age. A
322 subset of these significant correlations (involving the most relatively abundant ASVs) is shown in
323 **Figure 7**. *Atopobium* (ASV10) and *Gardnerella* (ASV3) ($r=0.74$), Eggerthellaceae (ASV39) and
324 *Parvimonas* (ASV21) ($r=0.83$), *Dialister* (ASV25) and Eggerthellaceae (ASV39) ($r=0.83$), and
325 *Sneathia* (ASV11) and *Parvimonas* (ASV 21) ($r=0.74$) were among the most highly correlated
326 pairs of bacterial taxa in pregnancy (**Supplemental Table 3**). These data suggest potential

327 synergistic relationships among these typical members of CST IV in pregnancy and potentially
328 beyond.

329

330 *Network analysis reveals further changes in microbiota structure throughout pregnancy*

331 The results from LME modeling were followed up with network analyses throughout
332 gestation. Network analyses of the 25 most relatively abundant ASVs revealed that *Lactobacillus*
333 ASVs were consistently network hubs, defined as ASVs closest to the center of the network, across
334 term gestation (**Figure 8A-D**). It is worth mentioning that there was limited resolution to
335 differentiate *Lactobacillus* spp., given that the V4 hypervariable region of the 16S rRNA gene was
336 targeted for sequencing ([130](#)). Nevertheless, *Lactobacillus* ASVs were clearly split between a
337 primary group (ASVs 2, 7, 12, 13, 15, and 20) that included a mixture of *L. crispatus*, *L. jensenii*,
338 and *L. gasseri*, and a secondary group (ASVs 1 and 6) comprised exclusively of *L. iners* (**Figure**
339 **8A-D**). These positive associations were interesting, given the exclusionary nature of the CST-
340 defining *Lactobacillus* spp. in the former group. In addition, this group maintained strong negative
341 associations with *Gardnerella* (ASVs 3, 8, and 9), *Atopobium* (ASV 10), and *Megasphaera* (ASV
342 4) throughout gestation. By contrast, *L. iners* ASVs had very few associations with other ASVs,
343 either positive or negative (**Figure 8A-D**).

344 This dichotomy may be due to the species-specific ability of *Lactobacillus* to produce lactic
345 acid (both L- and D- isomers) and - to a lesser extent - hydrogen peroxide, each of which can create
346 hostile conditions for other bacteria ([131](#), [132](#)). While *L. iners* can produce L-lactic acid, it lacks
347 key genes to produce D-lactic acid ([133](#)). Conversely, *Lactobacillus crispatus* produces both
348 isomers ([127](#)). Given that these two isomers of lactic acid differentially affect the biochemistry of
349 vaginal fluid ([127](#)), they may also differentially influence the composition of the broader

350 microbiota. Furthermore, unlike *L. crispatus*, *L. iners* lacks the ability to produce hydrogen
351 peroxide ([20](#), [134](#)). Hydrogen peroxide is an established antimicrobial compound, yet it may only
352 play a minor role within the vaginal ecosystem, as its production may be limited in this typically
353 hypoxic environment ([135](#)). Regardless, the inability to produce both inhibitory metabolites may
354 explain the lack of strong negative associations, and therefore, the permissive nature of *L. iners*,
355 towards other ASVs when it predominates in the vagina ([20](#)).

356 We further analyzed the networks by defining clusters, formed by optimal grouping
357 of ASVs based on strengths of association, which revealed differences in the degree of association
358 between *Lactobacillus* spp. and CST IV-typical bacteria (**Figure 8A-D**). In particular, CST IV
359 bacteria were split between two groups. The first, which contained *Gardnerella* (ASVs 3, 8, 9),
360 *Atopobium* (ASV 10), and *Megasphaera*, exhibited strong negative associations with
361 *Lactobacillus* ASVs (**Figure 8A-D**). The second, which contained *Sneathia*, *Dialister*,
362 *Fastidiosipila*, *Shuttleworthia*, *Parvimonas*, and *Atopobium* (ASV 24), had only weak negative
363 associations with *Lactobacillus* ASVs (**Figure 8A-D**). Interestingly, bacteria within the latter
364 group formed increasingly positive associations amongst themselves as gestation progressed
365 (**Figure 8A-D**). These contrasting patterns among non-*Lactobacillus* ASVs are in concordance
366 with a prior report ([104](#)) that identified strong exclusionary associations between *L. crispatus* and
367 *G. vaginalis* but only moderate negative associations between *L. crispatus* and other CST IV
368 bacteria.

369 These clustering profiles mirror previously proposed splits within CST IV, with the green
370 cluster comprised of *Gardnerella*, *Atopobium*, and *Megasphaera* representing CST IV-B and the
371 orange cluster, formed by diverse bacteria (*Atopobium*, *Dialister*, Ca. *Lachnocurva*, *Parvimonas*,
372 *Prevotella*, *Sneathia*) representing CST IV-C (**Figure 8A-D**). While CST IV-B is defined

373 by *Gardnerella* predominance, CST IV-C lacks a predominance of both *Lactobacillus*
374 and *Gardnerella*. Instead, CST IV-C is formed by a multitude of diverse bacteria (11). The
375 increase in positive associations amongst these particular CST IV-C members suggests that CST
376 IV-C bacteria can co-exist, leading to more species-rich and diverse vaginal microbiotas than the
377 more exclusionary CSTs. These findings are echoed in **Supplemental Table 3**, which reveals that
378 the strongest associations among ASVs, as determined by LME modeling, exist among CST IV
379 bacteria. For example, *Sneathia* ASV 11 and *Parvimonas* ASV 21 were highly correlated ($r=0.74$)
380 and were consistently positively associated throughout gestation in the network analyses (**Figure**
381 **8A-D**).

382 Intriguingly, *Gardnerella* ASVs 18 and 23 exhibited a distinct *Gardnerella*-correlative
383 phenotype from the other *Gardnerella* ASVs (3, 8, and 9), which were in a separate cluster
384 throughout most of gestation (**Figure 8 A,C-D**). Instead of exhibiting the strong *Lactobacillus*-
385 negative associations of *Gardnerella* ASVs 3, 8, and 9, *Gardnerella* ASVs 18 and 23 were often
386 positively correlated with other ASVs throughout gestation, including those classified as
387 *Lactobacillus* (**Figure 8B-D**). Notably, *Gardnerella* ASV G2, which was an ASV associated with
388 sPTB in a prior study (25), shared 100% identity with *G. vaginalis* ASV 9 and, in our study, it
389 displayed strong negative associations with the *L. crispatus* cluster (**Figure 8A-D**). ASVs 18 and
390 23 did not match any current *Gardnerella* type strain with 100% identity using BLAST
391 (**Supplemental Table 4**); they may represent unique *Gardnerella* strains. This is important
392 because *Gardnerella* is associated with bacterial vaginosis (136) and sPTB (137), yet, seemingly
393 in a strain-dependent manner (25). Therefore, these network analyses highlight the need for strain-
394 level resolution of the vaginal microbiota to fully understand its complex dynamics and ecology
395 in health and disease.

396 Lastly, each of the four networks from different time points in gestation were compared
397 with each other to identify global and ASV-specific network changes. Globally, natural
398 connectivity (i.e., robustness of the network) significantly decreased and positive edge percentage
399 (i.e., proportion of positive associations) significantly increased (**Figure 8E**) as pregnancy
400 progressed, both with strong linear trends across gestation ($R^2= 0.895$ and 0.985 , respectively).
401 The increase in positive edge percentage was a combination of a decrease in negative
402 *Lactobacillus*-CST IV-B associations, and an increase in the strength of positive associations
403 among CST IV-C-associated ASVs. These gestational changes observed in positive edge
404 percentage were associated with a significant decrease in closeness (i.e., the sum of the shortest
405 paths between a node and all other nodes) for *Gardnerella* ASVs 3, 8, and 9, since many of their
406 strong negative *Lactobacillus* spp. associations were lost or diminished as pregnancy progressed
407 (**Figure 8B-D**). Conversely, positive associations for CST IV-associated ASVs increased in
408 general, likely resulting from an increase in available niches within the vaginal ecosystem as term
409 approaches. Such changes may be due to the shift towards *Lactobacillus*-dominated CSTs
410 observed in **Figures 4B and 4C**.

411 Collectively, these network analyses demonstrate the complex interactions between
412 members of the vaginal microbiota. Not only were we able to confirm classical associations of
413 *Lactobacillus* species with members of other genera, but through longitudinal collection of vaginal
414 samples, we identified shifts in network connectivity as gestation progressed. Furthermore,
415 different ASVs attributed to the same genus (e.g., *Gardnerella*) demonstrated distinct ecological
416 dynamics, suggesting that strain-level variation is indeed driving community phenotypes
417 commonly denoted as CSTs. Therefore, this study highlights the need for strain-level

418 investigations utilizing metagenomic data to further characterize these shifts in vaginal microbiota
419 ecology throughout gestation and determine their underlying causes and consequences.

420

421 *Conclusions and Future Directions*

422 The composition of the vaginal microbiota is broadly consistent across populations of
423 reproductive age women worldwide ([1-5](#)), yet, the relative abundances of CSTs can vary within
424 populations, including by ethnicity ([2-5](#)), and within individuals over time. It is increasingly
425 hypothesized that variation in the vaginal microbiota is contributing to obstetrical complications,
426 especially sPTB ([22, 23, 25, 27, 28](#)). Here, we have provided a longitudinal study of a primarily
427 African-American population with a large sample size, and extensive demographic and clinical
428 data, which allowed for the simultaneous evaluation of a broad range of maternal characteristics
429 on the vaginal microbiota. Indeed, this study represents the largest and most comprehensive
430 longitudinal survey of the vaginal microbiota throughout gestation resulting in a term delivery and
431 thereby provides foundational understanding. The focus on African-American women is a clear
432 strength of the study because they constitute a high-risk population that experiences a relatively
433 high rate of pregnancy complications ([65-75](#)).

434 In the current study, we report that the principal factors influencing the composition and
435 structure of the vaginal microbiota in pregnancy are individual patient identity and gestational age
436 at sampling. The pronounced effect of individual identity highlights the need for longitudinal
437 studies to account for inter- and intra-individual variability when evaluating the strengths of
438 potential relationships between the composition of the vaginal microbiota and obstetrical
439 complications. Furthermore, the richness and evenness of the vaginal microbiota decreased
440 throughout pregnancy, with the microbiota becoming increasingly predominated by *Lactobacillus*

441 species with advancing gestation. Such typical changes can be partially mitigated by maternal
442 parity and obesity. Importantly, *Lactobacillus* species, especially *L. crispatus*, are generally
443 perceived to promote the vaginal and reproductive health of women ([4](#), [13](#), [15](#), [16](#), [19](#), [20](#), [25](#), [27](#),
444 [28](#), [36](#), [40](#), [44](#), [89](#), [90](#), [92](#), [97](#), [122-127](#)); therefore, any factors that could potentially reduce the
445 likelihood of the transition of the vaginal microbiota to a *Lactobacillus*-dominant community
446 during pregnancy need to be identified. Lastly, network analyses revealed dynamic interactions
447 among individual bacterial strains within the vaginal microbiota during pregnancy and the number
448 and structure of these interactions change with advancing gestation. A critical consideration
449 moving forward will be to assess whether patterns in these strain-level interactions within the
450 vaginal microbiota differ between women delivering at term or those who ultimately experience
451 sPTB. Ideally, this should be done using metagenomics ([138](#)), in addition to 16S rRNA gene
452 sequencing, so that strain-level designation of bacterial taxa can be more readily achieved and
453 information on the functional and virulence potential of various strains within individual
454 microbiotas can be gleaned ([28](#), [104](#)). Furthermore, a key element missing from most studies is
455 the characterization of host-immune microbiome interactions, which can be readily assessed by
456 evaluating the immunoproteome (cytokines, chemokines, defensins, etc.) within the vaginal
457 ecosystem. The local immune responses of women to their vaginal microbiota may be as or more
458 variable than the compositions of their microbiota themselves. Therefore, elucidating the dynamics
459 of host immune-microbiome interactions and their potential influence on obstetrical outcomes,
460 especially sPTB, is a critical future direction ([27](#), [28](#), [139](#), [140](#)).

461

462 **MATERIALS AND METHODS**

463 *Vaginal fluid specimens*

464 Vaginal fluid samples were obtained at the Perinatology Research Branch, an intramural program
465 of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development,
466 National Institutes of Health, U.S. Department of Health and Human Services, Wayne State
467 University (Detroit, MI), and the Detroit Medical Center (Detroit, MI). The collection and use of
468 human materials for research purposes were approved by the Institutional Review Boards of the
469 National Institute of Child Health and Human Development and Wayne State University
470 (#110605MP2F(RCR)). All participating women provided written informed consent prior to
471 sample collection.

472

473 *Study design*

474 This was a retrospective longitudinal cohort study to characterize variation in the vaginal
475 microbiota across gestation in pregnancies ending in normal term delivery. A normal pregnancy
476 was defined as a woman with no obstetrical, medical or surgical complications, who agreed to
477 participate in this study, provided written signed informed consent, and delivered at term (38 to 42
478 weeks) without complications. Three or four samples of vaginal fluid were collected longitudinally
479 across pregnancy from each woman under direct visualization from the posterior vaginal fornix
480 using a Dacron swab (Medical Packaging Corp., Camarillo, CA). Vaginal swabs were stored at
481 -80°C until time of DNA extraction.

482

483 *DNA extraction from vaginal swabs*

484 Genomic DNA was extracted from vaginal swabs (N=1,862) alongside non-template negative
485 controls addressing any potential background DNA contamination (N=73). All vaginal swabs were
486 randomized across extraction runs. Extractions were conducted using a Qiagen MagAttract
487 PowerMicrobiome DNA/RNA EP extraction kit (Qiagen, Germantown, MD), with minor
488 modifications to the manufacturer's protocols. Briefly, swabs were transferred to clean, labeled
489 Corning cryovials (Corning, Corning, NY) and immersed in 750 μ L solution MBL pre-heated to
490 60°C. Swabs were then vortexed for 10 min. A provided empty PowerBead plate was then
491 centrifuged for 1 min at 4,400 x g, and vaginal swab lysates were added to corresponding wells of
492 the PowerBead plate. Plates containing lysates were centrifuged for 1 min at 4,400 x g. The plates
493 were then loaded onto a TissueLyser II plate shaker (Qiagen, Germantown, MD), firmly secured,
494 and shaken at 17 Hz for 20 min. Plates were then removed from the shaker and immediately
495 centrifuged at 4,400 x g for 6 min. The supernatant was then carefully transferred 185 μ L at a time
496 to a provided collection plate. Following transfer, 150 μ L of solution IRS was added to each well
497 and plates were incubated at 4°C for 10 min. Plates were centrifuged for 15 min at 4,400 x g and
498 supernatant was transferred to a new collection plate. The plate was centrifuged for 2 min at 4,400
499 x g, and 850 μ L of the supernatants were transferred to a clean collection plate. The collection
500 plate was loaded onto the epMotion 5075 liquid handler (Eppendorf, Enfield, CT, USA) for further
501 processing following the default onboard protocols. The above procedure yielded between 0.13
502 and 550 ng/ μ L purified DNA from the vaginal swabs as measured by a Qubit 3.0 fluorimeter and
503 Qubit dsDNA assay kit (Life Technologies, Carlsbad, CA) following the manufacturer's protocol.
504 The purified DNA was transferred to the provided 96-well microplates and stored at -20°C.

505

506 *16S rRNA gene amplicon sequencing and bioinformatic processing*

507 The V4 region of the 16S rRNA gene was amplified from vaginal swab DNA extracts and
508 sequenced at Michigan State University's Research Technology Support Facility
509 (<https://rtsf.natsci.msu.edu/>) using the dual indexing sequencing strategy developed by Kozich et
510 al. (141). The forward primer was 515F: 5'-GTGCCAGCMGCCGCGGTAA-3' and the reverse
511 primer was 806R: 5'-GGACTACHVGGGTWTCTAAT-3'. Each PCR reaction contained 0.5 μ M
512 of each primer, 1.0 μ l template DNA, 7.5 μ l of 2X DreamTaq™ Hot Start PCR Master Mix (Life
513 Technologies, Carlsbad, CA), and nuclease-free water to produce a final volume of 15 μ l.
514 Reactions were performed using the following conditions: 95 °C for 3 minutes, followed by 30
515 cycles of 95 °C for 45 seconds, 50 °C for 60 seconds, and 72 °C for 90 seconds, with an additional
516 elongation at 72 °C for 10 minutes.

517

518 16S rRNA gene amplicon sequences were clustered into amplicon sequence variants (ASVs)
519 defined by 100% sequence similarity using DADA2 version 1.12 (142) in R version 3.6.1 (143)
520 according to the online MiSeq protocol (<https://benjjneb.github.io/dada2/tutorial.html>) with minor
521 modifications, as previously described (144). These modifications included allowing truncation
522 lengths of 250 and 150 bases, and a maximum number of expected errors of 2 and 7 bases, for
523 forward and reverse reads, respectively. Reads were truncated at the first instance of a quality score
524 less than or equal to 2. Any reads containing ambiguous nucleotides were removed from the
525 dataset. To increase power for detecting rare variants, sample inference allowed for pooling of
526 samples. Additionally, samples in the resulting sequence table were pooled prior to removal of
527 chimeric sequences. Sequences were then classified using the *silva_nr_v132_train_set* database
528 with a minimum bootstrap value of 80%, and sequences that were derived from Archaea,
529 chloroplast, or Eukaryota were removed. Per Holm et al. (12), ASVs classified as *Shuttleworthia*

530 were manually reclassified as *Ca. Lachnocurva vaginae*.

531

532 The R package decontam version 1.6.0 ([145](#)) was used to identify ASVs that were likely potential
533 background DNA contaminants based on their distribution among biological samples and negative
534 controls using the “IsContaminant” method. An ASV was identified as a contaminant and
535 subsequently removed from the dataset if it had a decontam P score ≤ 0.5 , was present in at least
536 15% of negative controls with an overall average relative abundance of at least 1.0%, and had a
537 greater average relative abundance in controls than biological samples. Based on these criteria, a
538 total of four ASVs classified as *Escherichia*, *Pelomonas*, *Pseudomonas*, and Micrococcaceae were
539 identified as contaminants.

540

541 For assigning community state types (CSTs) to the bacterial community profiles, ASVs were first
542 taxonomically classified using the V4_trimmed_noEuks_nr_Complete.fa reference library
543 supplied with the speciateIT classifier code (<http://ravel-lab.org/speciateit/>) and the classify.seqs
544 command in mothur ([146](#)) with a bootstrap cutoff value of 80. Read counts for ASVs that were
545 assigned to the same taxon were then combined, and CSTs were assigned using VALENCIA, a
546 nearest centroid-based classifier ([11](#)).

547

548 ***Statistical analysis***

549 *Analysis of vaginal microbiota composition and structure*

550 To determine the percentage of variance explained (R^2) in the composition (Jaccard index) or
551 structure (Bray-Curtis index) of the vaginal microbiota, PERMANOVA analyses ([147](#)) were
552 performed using the interaction terms between SubjectID and gestational age at sampling within

553 the “adonis2” function in the R package *vegan* version 2.5-6 ([148](#)). The confidence intervals of R^2
554 statistics were obtained by bootstrap sampling of patients and all their associated longitudinal
555 measurements. Confidence intervals for R^2 statistics for additional patient specific covariates (i.e.,
556 maternal age, parity, obesity, race, ethnicity, *cannabis* use) while accounting for gestational age
557 were obtained in a separate analysis in which PERMANOVA analysis was performed on bootstrap
558 samples of subjects. For each subject only one random longitudinal observation was selected,
559 hence generating cross-sectional datasets in which observations were independent and hence
560 PERMANOVA could be applied. Empirical 95% confidence intervals of R^2 statistics were
561 obtained using 1000 bootstrap iterations.

562

563 *Changes in alpha diversity with gestational age and maternal characteristics*

564 Relative abundance for each amplicon sequence variant (ASV) was determined as the ratio of the
565 count of each ASV divided by the total number of ASVs in each sample. Starting with the relative
566 abundance data, for each sample, we calculated the Shannon and Simpson diversity using the
567 *diversity* function in the *vegan* package and the Chao1 diversity using the *chao1* function in the
568 *fossil* package. Each measure of diversity was then correlated with the continuous variable
569 gestational age at sampling using linear mixed-effects models implemented in the *lme4* package
570 in R. In these models a random intercept and a random slope with gestational age was allowed for
571 each subject to account for the repeated and potentially correlated observations from the same
572 subject. Gestational age values were centered at 8 weeks and then scaled by 4 to facilitate
573 interpretation of random intercepts and convergence of model fitting algorithms. The complexity
574 of the gestational age dependence was assessed by comparing the model fit between a linear and
575 quadratic trend using a likelihood ratio test for linear mixed-effects models. The same test was

576 used to determine the need for subject specific gestational age slopes. To further inspect nonlinear
577 trends in alpha diversity as a function of gestational age, Generalized Additive Models (GAM) for
578 repeated observations were also fit based on spline transformation of gestational age. Such models
579 were available from the *mgcv* package in R. The effects of maternal characteristics (maternal age,
580 obesity, parity, race, ethnicity, smoking, and *cannabis* use) were assessed by including these as
581 co-variates in linear mixed-effects models. A p-value <0.05 was used to infer significance in these
582 analyses.

583

584 *Changes in vaginal community state types (CSTs) with gestational age and maternal*
585 *characteristics*

586 The log-odds of membership in a given community state type (CST) were modeled using binomial
587 linear mixed-effects models using the *glmer* function in R. Fixed effects in these models included
588 gestational age at sampling (linear and quadratic terms, as needed) and maternal characteristics.
589 Of note, due to the sparse responses in these models (membership to a given CST), it was not
590 feasible to test whether there were subject-specific departures in the CST membership probability
591 trends versus gestational age (random slopes for gestational age), yet subject specific shifts in
592 membership probabilities were allowed via random intercepts in the mixed effects models.

593

594 *Changes in the relative abundance of individual amplicon sequence variants (ASVs) with*
595 *gestational age and maternal characteristics*

596 The analysis of the relative abundance of each amplicon sequence variant (ASV) in association
597 with gestational age at sampling was performed using linear mixed-effects models based on ASV
598 count data while assuming a negative binomial distribution of the counts. Such models were

599 implemented in the *glmmTMB* package in R and included an offset term of the total number of
600 reads per sample, so that changes in relative abundance with gestational age were being estimated
601 as opposed to differences in absolute counts. These models included gestational age and maternal
602 characteristics as fixed effects and random intercept and random gestational age slope for each
603 subject. All analyses involved control of the false discovery rate at a 10% level ($q < 0.1$).

604 Additionally, we implemented Analysis of Composition of Microbiomes, or ANCOM
605 ([101](#)), for further differential abundance analysis of ASVs. After adding a pseudo-count (1) to all
606 observed abundances, ANCOM accounts for the compositionality issue of the microbiome data by
607 performing the additive log ratio (ALR) transformation. For each taxon, ANCOM uses all other
608 taxa, one at a time, as the reference in forming the ALR transformation. The transformed data were
609 treated as the response of the LME model which includes gestational age as the main fixed effect,
610 maternal age, parity, marijuana use, ethnicity, and race as covariates while allowing a random
611 intercept and a random slope for each subject. For a given taxon, the output W statistic represents
612 the number of ALR transformed models where the taxon is differentially abundant with regard to
613 the main fixed effect, after adjusting for multiple testing correction for the number of ALR models
614 corresponding to each taxon. The larger the value of W, the more likely the taxon is differentially
615 abundant between compared sample groups.

616

617 *Bacterial taxa with the most highly associated relative abundances during normal pregnancy*

618 To assess the association between pairs of ASVs, we modeled their log-transformed relative
619 abundance data using linear mixed-effects models. In these models one of the two ASVs was
620 treated as a response variable while the other was treated as an explanatory variable. A random
621 effect was allowed for each subject. Naïve Spearman correlation coefficients were also calculated

622 for each pair. Significance of correlations was based on an adjusted p-value <0.05.

623

624 *Associations of ASVs across term pregnancy through network analysis*

625 The R packages NetCoMi ([149](#))1.0.2, SpiecEasi 1.1.2, and seqtime 0.1.1 were used in R version
626 4.0.3 to create correlation networks between ASVs from vaginal samples across four time points
627 in gestation resulting in term delivery. Only one sample per subject was included for each time
628 point to control for subject ID. Networks were generated using Spearman's correlation since the
629 data were not normally distributed and nonparametric. Only the top 25 predominant ASVs in the
630 entire dataset were considered for each network. A topological overlap matrix generated from the
631 network adjacency matrix was utilized as a dissimilarity measure after transforming the data
632 through multiplicative simple replacement and a centered log-ratio transformation to account for
633 the zero-inflated data and to normalize the data, respectively. Community structure was
634 determined by implementing the fast greedy modularity optimization algorithm ([150](#)). The layout
635 of the network for the first time period was used as the layout for all subsequent time periods.
636 Edges displayed in the network exceeded a threshold of 0.3 and edge thickness was tied to the
637 strength of the correlation between two given nodes. Networks of the four time periods were
638 compared using the NetCoMi package "netCompare" function with 1000 permutations.

639

640

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652

653 **CONFLICT OF INTEREST STATEMENT**

654 The authors declare no conflicts of interest.

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1129 **TABLES**

1130

1131 **Table 1.** Clinical and demographic characteristics of the study population.

1132

1133

	Term Birth (n = 474)	
Maternal age (years; median [IQR])	24 (21-27)	1136
Body mass index (kg/m ² ; median [IQR])	27.5 (22.7-33.7) ^a	1137 1138
Primiparity	20.9% (99/474)	1139
Race/Ethnicity		1140 1141
African-American	94.5% (448/474)	1142
White	1.7% (8/474)	1143 1144
Asian	0.2% (1/474)	1145
Hispanic	0.2% (1/474)	1146
Other	3.4% (16/474)	1147 1148
Gestational age at delivery (weeks; median [IQR])	39.6 (39-40.4)	1149 1150
Cesarean Section	25.9% (123/474)	1151
Fetal sex		1152 1153
Female	48.7% (231/474)	1154
Male	51.3% (243/474)	1155 1156
Birthweight (grams; median [IQR])	3286 (3091-3580)	1157 1158

1159

1160 Data are given as median (interquartile range, IQR) and percentage (n/N). ^aTwo missing data

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1162 **Table 2.** Differences in alpha diversity values (Chao1 richness, Shannon, and Simpson diversity)
1163 of the vaginal microbiota profiles of women who delivered at term.

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Diversity	Covariate	Estimate	p
Chao1	Gestational age*	-2.41	0.000
Chao1	Obese	4.19	0.033
Shannon	Gestational age*	-0.08	0.000
Shannon	Parity	0.09	0.000
Shannon	Ethnicity Hispanic/Latino	-0.40	0.024
Simpson	Gestational age*	0.56	0.000
Simpson	Age**	-0.02	0.000
Simpson	Parity	0.03	0.000
Simpson	Ethnicity Hispanic/Latino	-0.23	0.003
Simpson	Race other than African-American	0.14	0.014

1181

1182

1183 *Gestational age was centered at 8 weeks and then scaled by 4, therefore the change in diversity
1184 corresponds to a four week interval

1185 **Maternal age was scaled by 5, therefore the diversity corresponds to a 5 year increase in maternal
1186 age

1187

1188 **Table 3.** Factors associated with variation in vaginal community state type (CST) membership
 1189 among women who delivered at term.

CST	Covariate	Odds Ratio	p
I	Gestational age*	1.77	0.000
I	Marijuana use	0.41	0.278
I	Sex male	0.73	0.650
I	Age**	1.81	0.211
I	Parity	0.71	0.264
II	Gestational age*	0.88	0.479
II	Marijuana use	1.24	0.880
II	Sex male	2.12	0.605
II	Age**	2.07	0.362
II	Parity	0.72	0.593
III	Gestational age*	1.20	0.000
III	Marijuana use	1.83	0.006
III	Sex male	1.08	0.714
III	Age**	0.64	0.001
III	Parity	1.04	0.684
IV-A	Gestational age*	0.69	0.000
IV-A	Marijuana use	1.56	0.292
IV-A	Sex male	0.61	0.194
IV-A	Age**	1.18	0.532
IV-A	Parity	1.16	0.364
IV-B	Gestational age*	0.75	0.000
IV-B	Marijuana use	0.86	0.541
IV-B	Sex male	1.60	0.039
IV-B	Age**	0.77	0.102
IV-B	Parity	1.46	0.000
V	Gestational age*	1.33	0.006
V	Marijuana use	1.16	0.849
V	Sex male	0.90	0.890
V	Age**	0.99	0.987
V	Parity	0.93	0.815

1227 *Gestational age was scaled by 4, therefore the Odds Ratio corresponds to a four week interval

1228 **Maternal age was scaled by 5, therefore the Odds Ratio corresponds to 5 year increase in
 1229 maternal age

1230

1231 **FIGURE LEGENDS**

1232

1233 **Figure 1. Gestational ages at the time of vaginal fluid sample collection in a cohort of women**
1234 **ultimately delivering at term.** (A) 1,862 vaginal fluid samples were collected from 474 pregnant
1235 women between 8 and 38⁺⁶ weeks of gestation. The vaginal microbiota was profiled using 16S
1236 rRNA gene sequencing. (B) Each line corresponds to one patient and each dot to a sample for
1237 which the vaginal microbiota was characterized. Gestational ages at delivery are shown using red
1238 triangles.

1239

1240 **Figure 2. Decrease in alpha diversity of the vaginal microbiota with gestational age in women**
1241 **ultimately delivering at term.** Graphical representation of low and high bacterial community
1242 richness (A) and evenness (B). Linear mixed-effects models illustrating decreases of bacterial
1243 community richness (C) and evenness (D) over the course of gestation. Each dot corresponds to
1244 one sample. The red line represents the linear fit using linear mixed-effects models. The dark blue
1245 line represents the model fit and light blue areas define the 95% confidence intervals derived from
1246 generalized additive models with splines transformation of gestational age.

1247

1248 **Figure 3. Rate of decrease in alpha diversity (Shannon diversity index) of the vaginal**
1249 **microbiota with gestational age is steeper in women with higher baseline diversity.** The left
1250 panel shows the baseline diversity for each patient (blue dots) and corresponding 95% confidence
1251 intervals (black lines). The right panel shows the rate of change in diversity (blue dots) and
1252 confidence intervals (black lines). Women who had higher baseline diversity had steeper decrease

1253 in diversity with advancing gestation (correlation between random intercepts and random slopes
1254 of -0.79).

1255

1256 **Figure 4. Variation in the community state type (CST) of the vaginal microbiota throughout**
1257 **gestation among women who ultimately delivered at term.** (A) Heatmap illustrating the relative
1258 abundances of the 30 most abundant amplicon sequence variants (ASVs) among the vaginal 16S
1259 rRNA gene profiles. The bar on top indicates vaginal CSTs assigned using the program
1260 VALENCIA ([11](#)). (B) Dynamics of vaginal CST prevalence as a function of gestational age among
1261 women ultimately delivering at term. The log-odds of membership for each CST were modeled
1262 using binomial linear-mixed effects models. Fixed effects in these models included gestational age
1263 (linear and quadratic terms, as needed) and maternal characteristics, while one random intercept
1264 was allowed for each subject. (C) Alluvial plot illustrating the temporal dynamics of vaginal CST
1265 prevalence and transitions among 309 women who delivered at term and contributed one sample
1266 per each of the four discrete time points (10 to 37 weeks).

1267

1268 **Figure 5. Changes in the relative abundance of amplicon sequence variants (ASVs) in vaginal**
1269 **16S rRNA gene profiles across gestational age in women who ultimately delivered at term.**
1270 Only the first ASV for each microbial taxon with a significant corrected p-value ($q < 0.05$) presented
1271 in Supplemental Table 1 is shown. Panels with positive correlations are ordered before those with
1272 negative correlations. Each dot within an individual panel corresponds to one sample. The red lines
1273 represent linear fits through relative abundance data using linear mixed-effects models. The blue
1274 lines and grey bands represent the model fits and 95% confidence intervals derived from

1275 generalized additive models, respectively. The green lines represent the estimates from negative
1276 binomial mixed-effects generalized additive models.

1277

1278 **Figure 6. Amplicon sequence variants (ASVs) classified at the genus level which were**
1279 **identified as being less or more abundant in the vaginal microbiota with advancing**
1280 **gestational age.** As gestation advances, *Lactobacillus* , and to a lesser extent Ca. *Lachnocurva*,
1281 ASVs become more abundant and many members of community state type (CST) IV become less
1282 abundant.

1283

1284 **Figure 7. Positive correlations of relative abundances of vaginal microbial taxa.** Alluvial plot
1285 shows pairs of vaginal bacterial taxa with highly correlated relative abundances throughout
1286 gestation. Relative abundances of amplicon sequence variants (ASVs) were compared using linear
1287 mixed-effects models.

1288

1289 **Figure 8. Network analysis illustrating changes in associations between amplicon sequence**
1290 **variants (ASVs) throughout pregnancy.** Networks at (A) 10-24 weeks, (B) 24-28 weeks, (C) 28-
1291 32 weeks, and (D) 32-37 weeks of gestation were generated using the NetCoMi package ([149](#)).
1292 Nodes, which represented individual amplicon sequence variants (ASVs) were color coded
1293 according to their respective genus-level classification. Edges were weighted by strength using
1294 fitness and color coded by interaction type with positive (blue) and negative (red) interactions.
1295 Nodes that represent hubs, defined as an ASV with an eigenvector above the 95% quantile of the
1296 empirical distribution, are outlined in black and are in bold font. Clusters are represented by
1297 background coloration and darker borders. (E) A matrix of comparative network statistics with

1298 positive edge percentage above the diagonal and natural connectivity below the diagonal. Cells
1299 shaded red and cells shaded blue represent statistically significant differences in the respective
1300 time periods for positive edge percentage and natural connectivity, respectively.
1301

1302 **SUPPLEMENTAL TABLE LEGENDS**

1303 **Supplemental Table 1. Multivariate analysis of relative abundances of amplicon sequence**
1304 **variants (ASVs) in vaginal 16S rRNA gene data as a function of gestational age, maternal**
1305 **age, parity, ethnicity, and marijuana use among women who ultimately delivered at term.**

1306 The p-value and false discovery rate adjusted p-value (q-value) are provided for each covariate.
1307 The coefficients represent changes in log (base e) relative abundance with: (A) one additional
1308 month of gestational age, (B) 5 additional years of maternal age, (C) use of marijuana, (D) ethnicity
1309 Hispanic or Latino, (E) race other than African American, and (F) one additional previous delivery.
1310 ASVs classified at the genus level as *Lactobacillus* were secondarily classified at the species level,
1311 if possible, using the National Center for Biotechnology Information's BLAST (Basic Local
1312 Alignment Search Tool). These ASVs are highlighted in blue.

1313

1314 **Supplemental Table 2. Comparison of analyses evaluating the relationship between**
1315 **gestational age and the vaginal microbiota using absolute abundance analysis (Analysis of**
1316 **Composition of Microbiomes; ANCOM) and relative abundance analysis (Linear mixed-**
1317 **effects models; LME).**

1318

1319 **Supplemental Table 3. Vaginal bacterial taxa with highly correlated relative abundances**
1320 **based on longitudinal sampling in women who ultimately delivered at term.** Significance of
1321 correlations between log-transformed relative abundances was assessed using linear mixed-
1322 effects models. In these models one of the two amplicon sequence variants (ASVs) was treated
1323 as a response while the other as an explanatory variable. A random effect was allowed for each

1324 subject. Naïve spearman correlation coefficients were calculated for each pair of taxa. The p-
1325 value and false discovery rate-adjusted p-value (q-value) are provided.

1326

1327 **Supplemental Table 4. Top 25 amplicon sequence variants (ASVs) by relative abundance**
1328 **compared to NCBI BLAST bacterial type strains with percent identity.** ASVs were
1329 compared to type strains in the NCBI BLAST database. Highest percentage identity type strains
1330 were listed if not 100%.

1331

1332

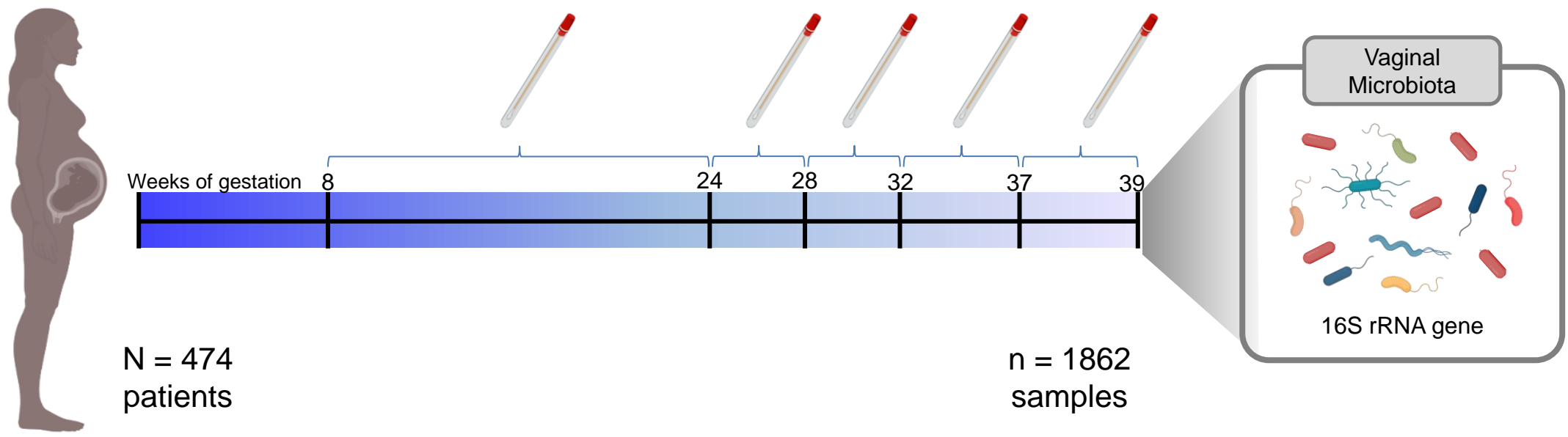
1333 **SUPPLEMENTAL FIGURE LEGENDS**

1334 **Supplemental Figure 1. Venn diagram showing the relationship between absolute abundance**
1335 **analysis (Analysis of Composition of Microbiomes; ANCOM) and relative abundance**
1336 **analysis (Linear mixed-effects models; LME) in evaluating changes in the structure of the**
1337 **vaginal microbiota throughout gestation.** The two analyses identified some common significant
1338 bacterial taxa (i.e., amplicon sequence variants, or ASVs), yet the ANCOM approach tends to be
1339 more conservative than LME.

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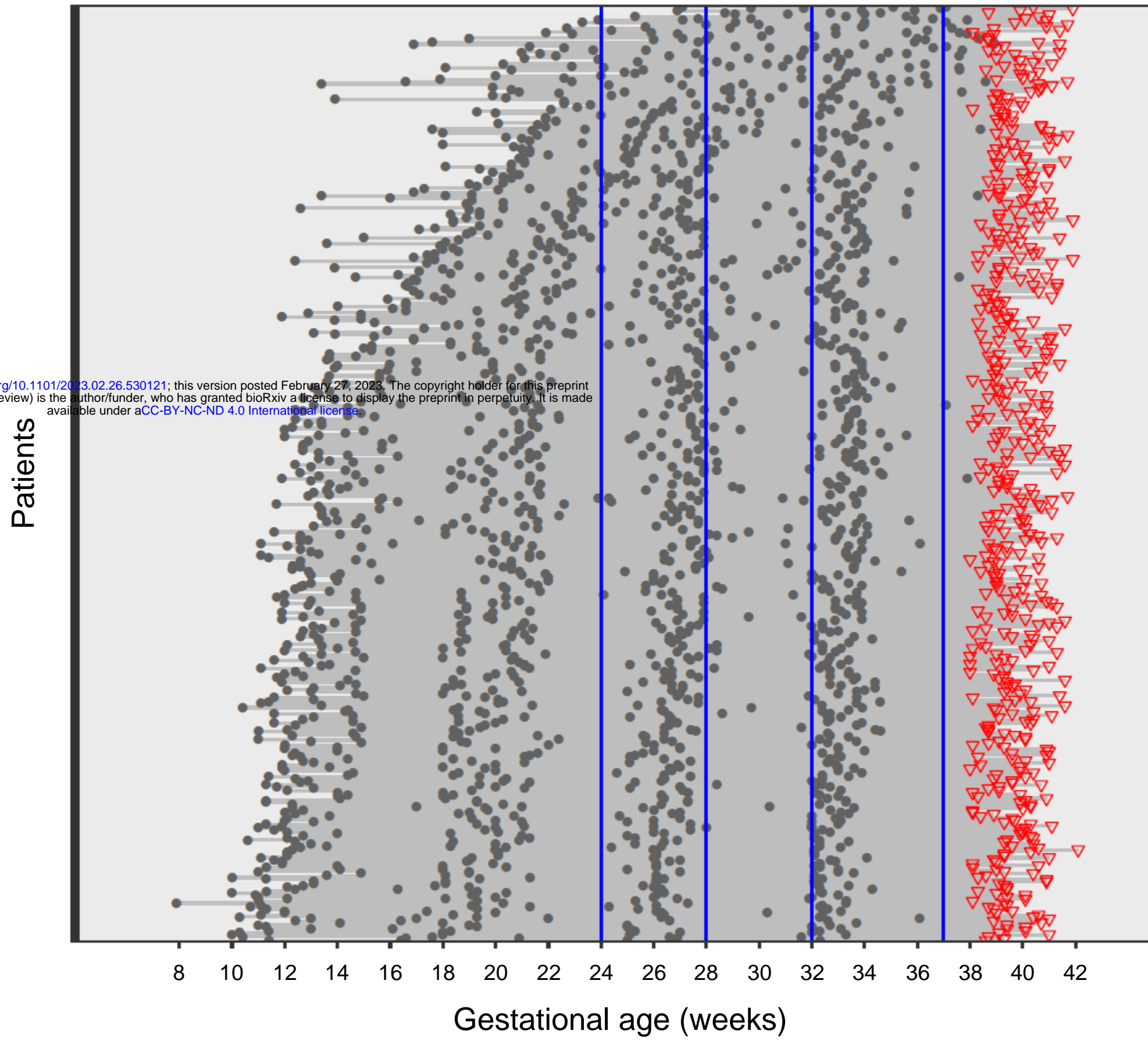
Figure 1

A



● Gestational age at sample ▼ Gestational age at delivery

B



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Figure 2

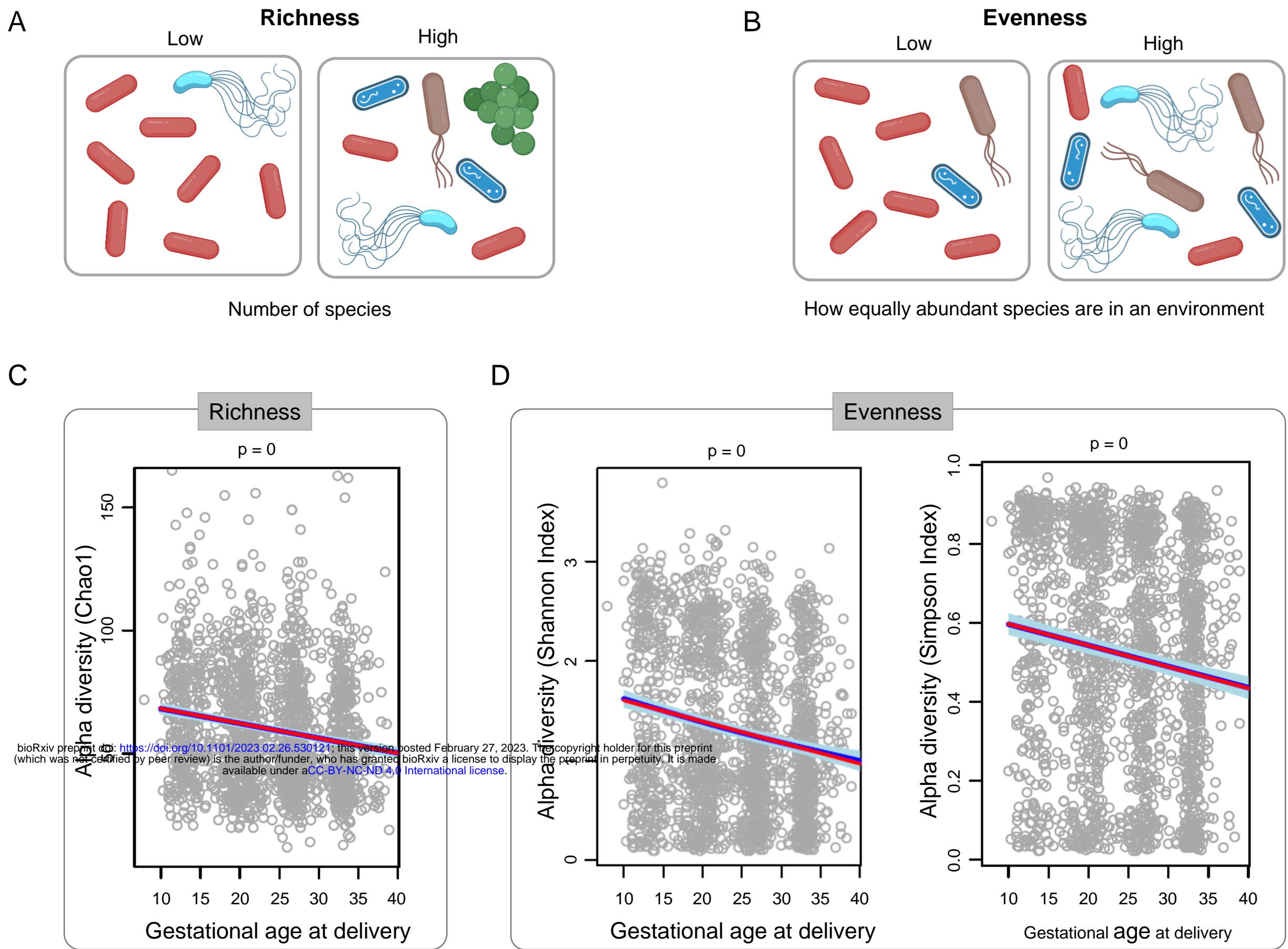


Figure 3

Shannon Diversity

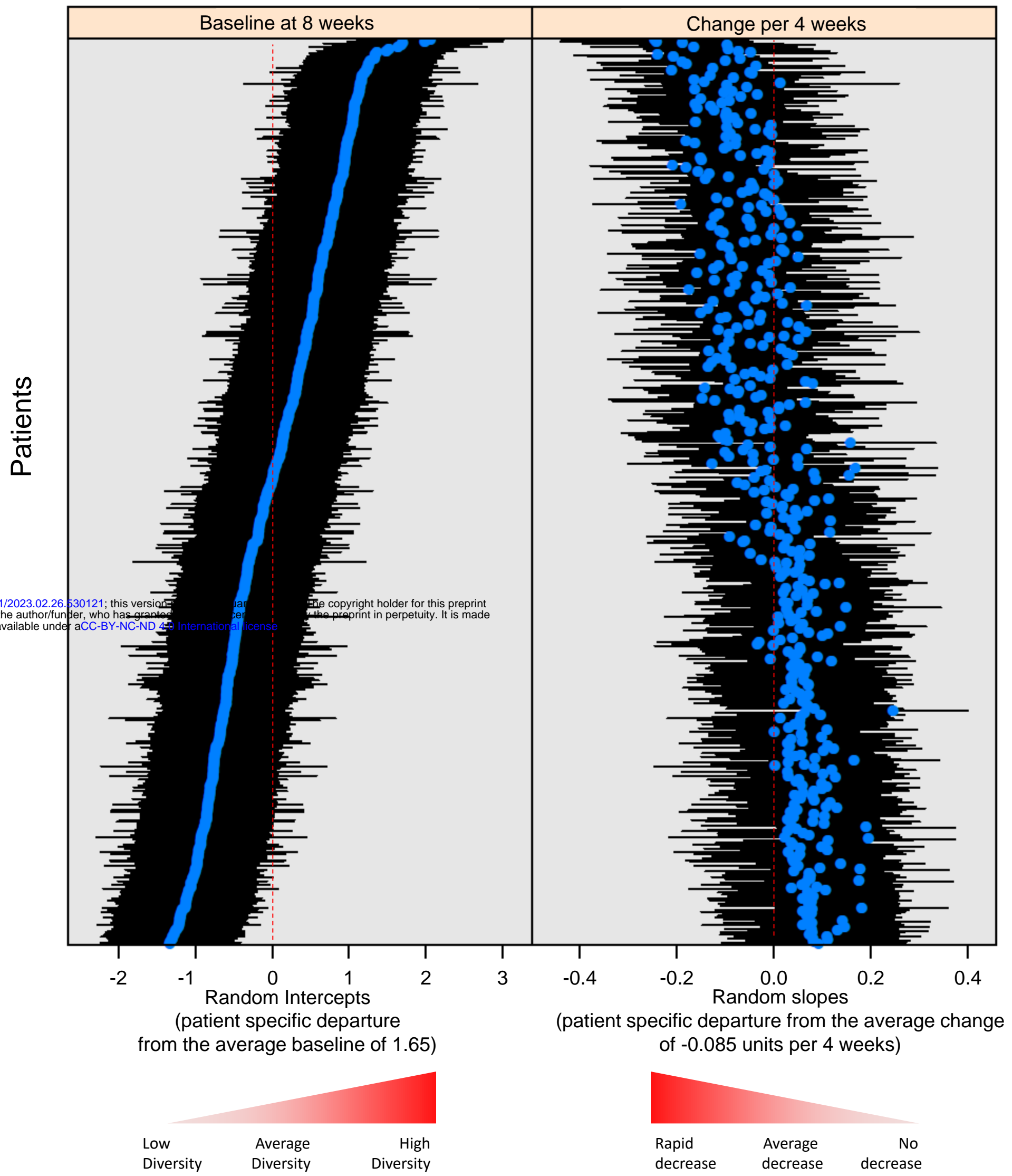


Figure 4

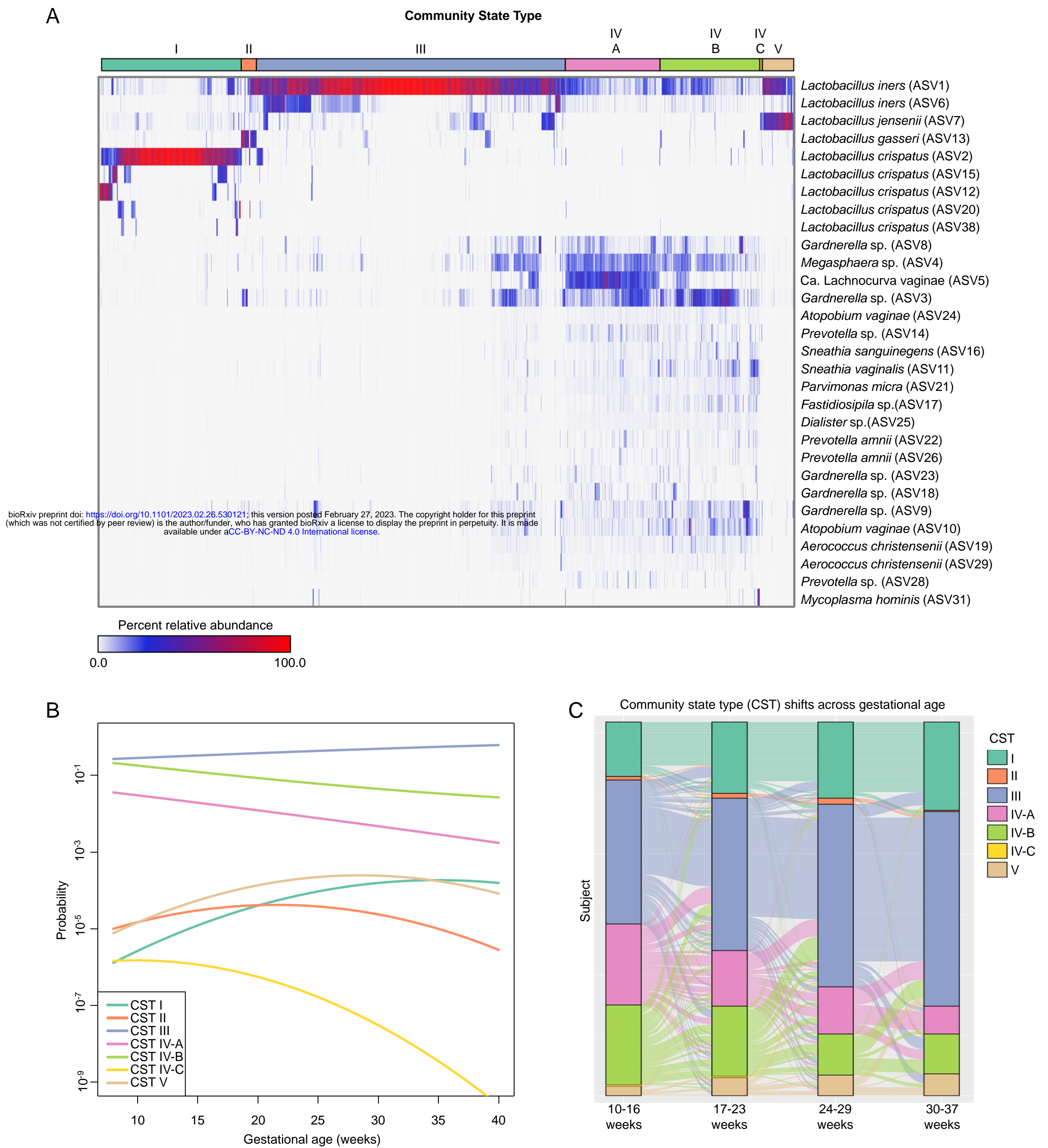


Figure 5

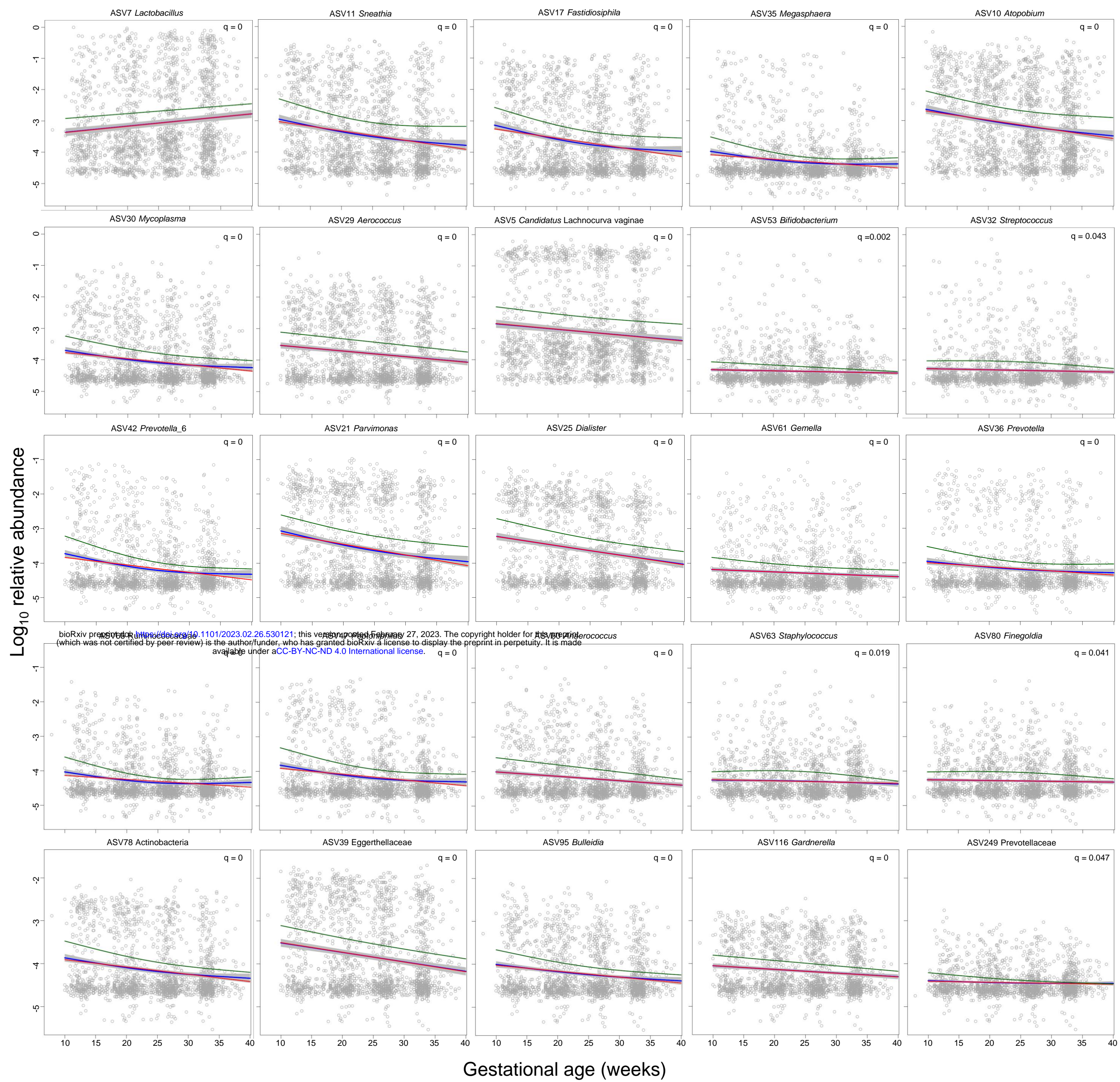


Figure 6

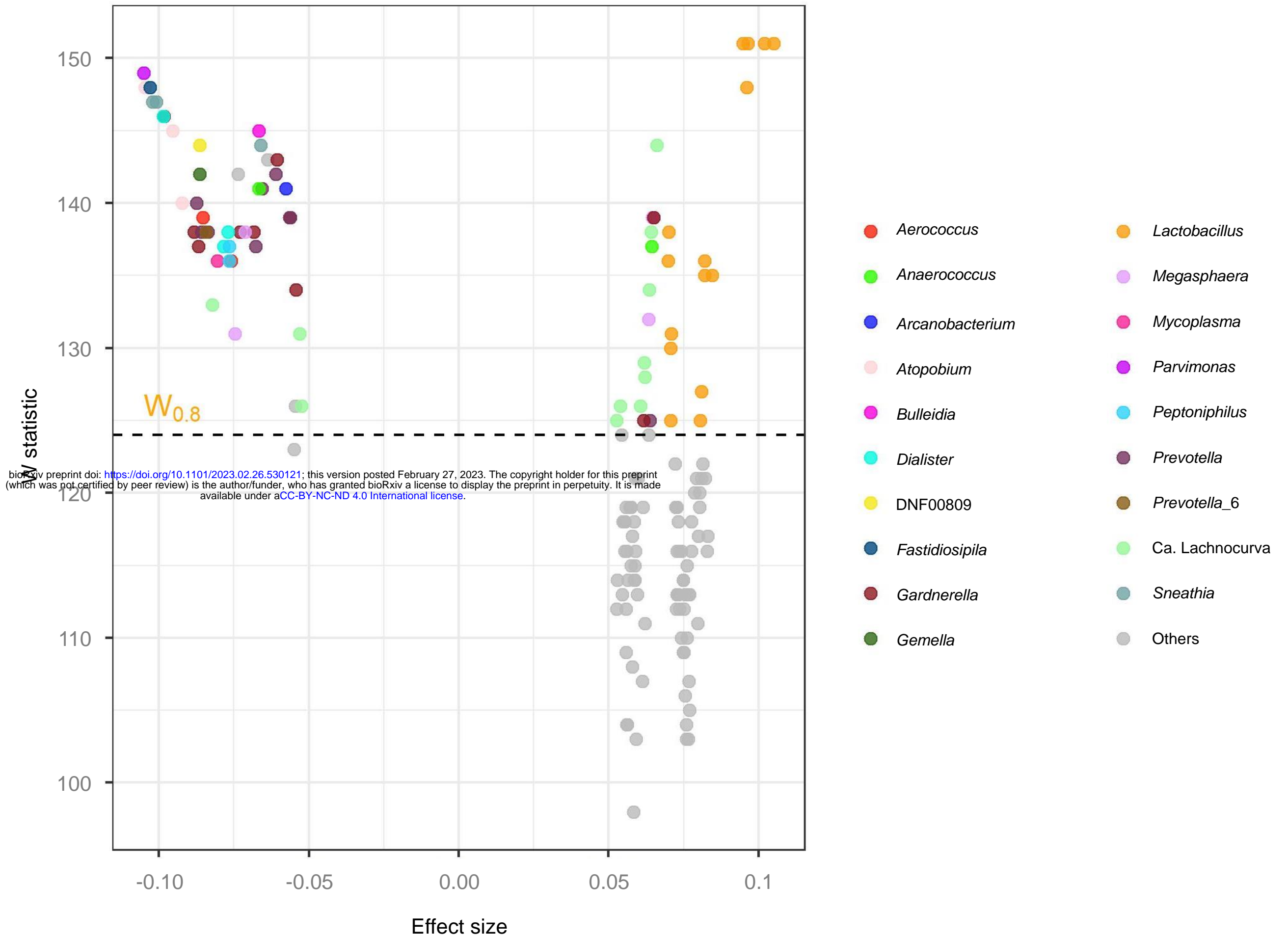


Figure 7

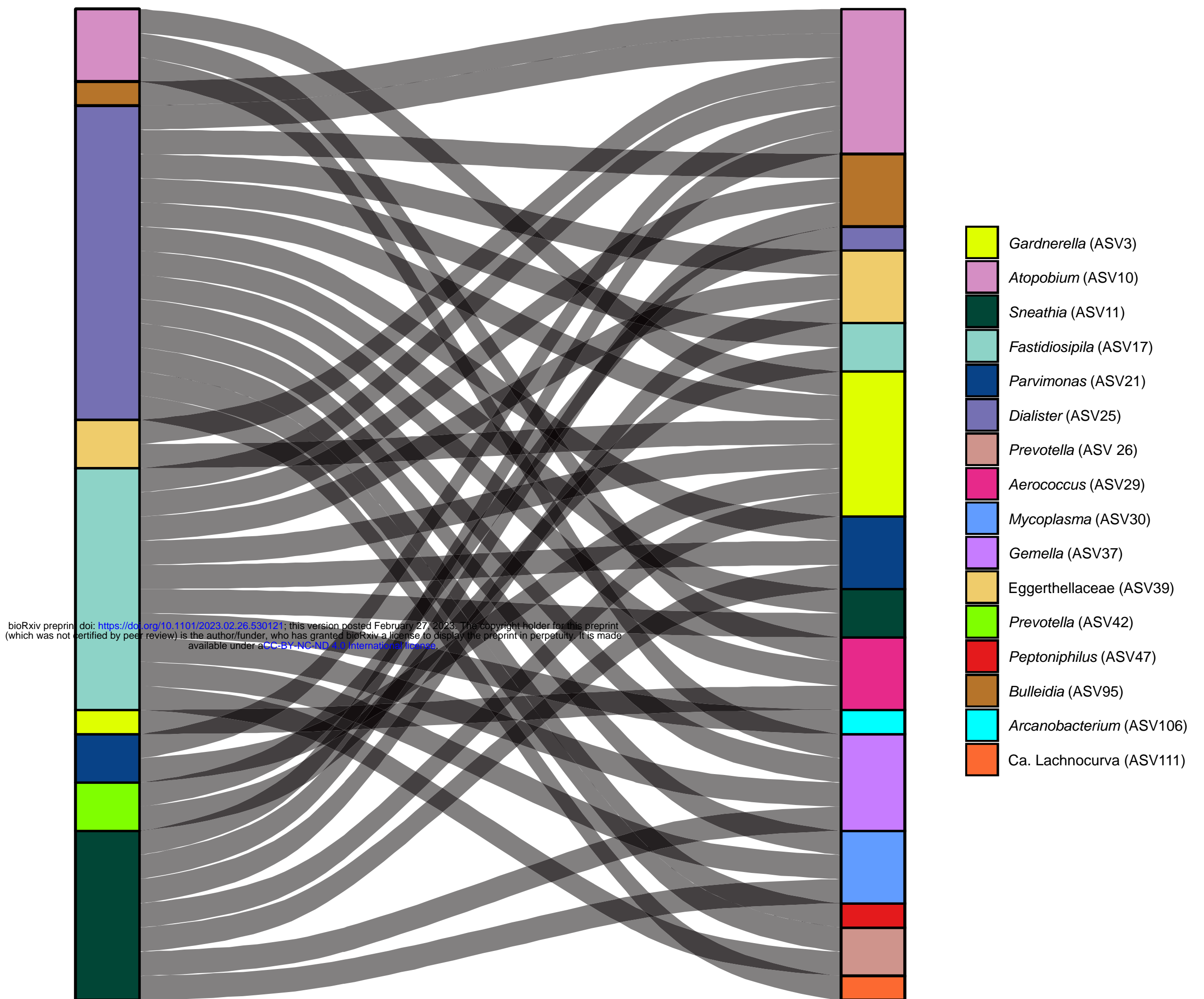
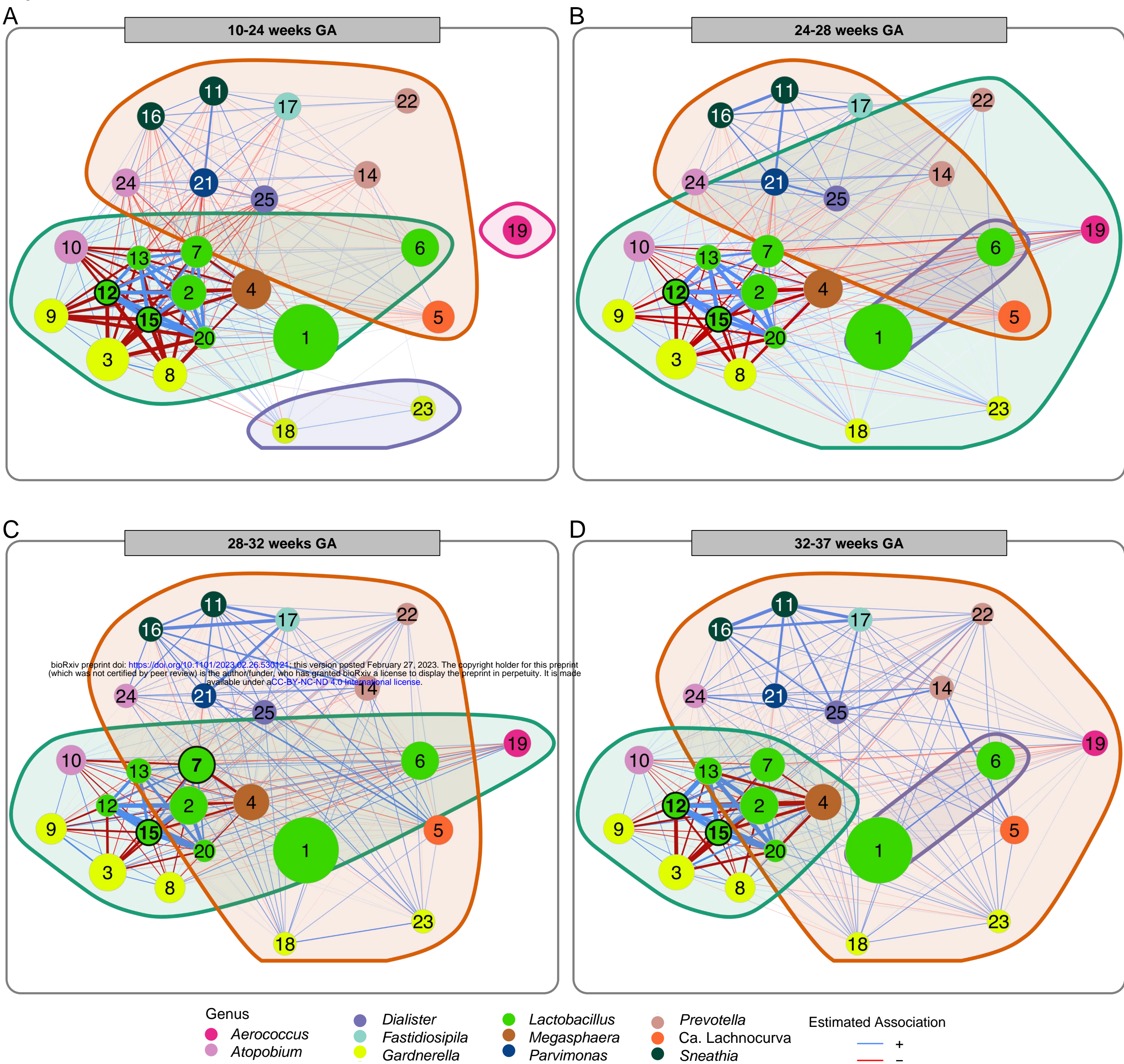


Figure 8



E

	Positive Edge Percentage			
	10-24 weeks	24-28 weeks	28-32 weeks	32-37 weeks
10-24 weeks		8.25697	16.423	21.04605
24-28 weeks	-0.05114		8.16603	12.78908
28-32 weeks	-0.06905	-0.01791		4.62305
32-36 ⁺⁶ weeks	-0.08177	-0.03063	-0.01272	

Natural Connectivity