Lactobacillus iners dominates the vaginal microbiota of healthy Italian women of reproductive age.

Authors

E. Vinerbi[1, †], F. Chillotti[1, †], A. Maschio[1, †], S. Lenarduzzi[2, †], S. Camarda[3], F. Crobu[1], D. V. Zhernakova[1], V. Lo Faro[1], G. Beltrame Vriz[3], S. Incollu[1], J. Spreckels[4], N. Kuzub[4], A. Kadric[5], R. Gacesa[6], A. Zhernakova[4], F. De Seta[2,3, *], D. Mazzà[2], F. Busonero[1], M. L. Ferrando[1], G. Girotto[2,3, #], S. Sanna[1,4, #]

Affiliations

[1] *Institute of Genetic and Biomedical Research (IRGB), National Research Council (CNR), Monserrato, Italy*

[2] *Institute for Maternal and Child Health—IRCCS "Burlo Garofolo", Trieste, Italy*

[3] *Department of Medicine, Surgery and Health Sciences, University of Trieste, Trieste, Italy*

[4] *Department of Genetics, University Medical Center Groningen, Groningen, The Netherlands*

[5] *Department of Life Science, University of Trieste, Trieste, Italy*

[6] *Department of Gastroenterology and Hepatology, University Medical Center Groningen, Groningen, The Netherlands*

Running Head: *L. iners is predominant in healthy Italian women*

[†] These authors contributed equally; authors order was determined in order of increasing seniority

#Address Correspondence to Serena Sanna, serena.sanna@cnr.it and Giorgia Girotto, giorgia.girotto@burlo.trieste.it

*Present address: Department of Obstetrics and Gynecology, IRCCS San Raffaele Scientific Institute, University Vita and Salute, Milan, Italy.

Abstract words count: 209

Text words count: 7057

ABSTRACT

Large sex hormonal fluctuations are thought to influence vaginal microbiota, but little is known about the impact of small, physiological variations. Here we tracked changes in vaginal microbiota during four key menstrual cycle phases in 61 healthy, naturally menstruating Italian women from the Women4Health cohort. The microbiota, characterized using a high depth 16S rRNA amplicon sequencing approach covering four hypervariable regions, was primarily composed of *Lactobacillus species*, with *Lactobacillus iners* being the most abundant (average relative abundance 40%) and the most prevalent (prevalence 98%). Individual microbiota were generally stable, but beta diversity was higher during the follicular phase (p=0.007). Only 11 women exhibited compositional shifts, mostly occurring between follicular and ovulatory phases. Finally, using linear mixed models we assessed the association between taxa relative abundance and five sex-hormones along the menstrual cycle. Among these, 17-beta estradiol showed the largest number of significant associations, linking its increase to a decrease in relative abundance of taxa that are more common after menopause. Our study highlights specific features of the Italian population and points to the resilience of the vaginal microbiota to physiological hormonal changes. Noteworthy, the observed high abundance of *L. iners* contrasts with previous studies in European populations, challenging its proposed pathogenic role and suggesting distinct microbiota profiles within Europe.

IMPORTANCE

The vaginal microbiota plays an important role in women's health, yet we know little about how it responds to normal hormonal fluctuations. In this study, we followed 61 healthy Italian women over a natural menstrual cycle to explore microbiota changes across different hormonal phases. We found that *Lactobacillus iners* was the most common species, unlike previous findings in Northern Europe, suggesting population-specific patterns. The common hypothesis that *L. iners* is invariably linked

with poor health is called into question by our findings. They emphasize the importance of considering population context and hormonal status when assessing vaginal health.

The vaginal microbiota was generally stable, with only a few changes observed between the follicular and ovulatory phases. When evaluating association between five sex hormones and taxa abundances, we found that 17-beta estradiol levels had the largest number of significant associations. These highlight an association between increase levels of 17-beta estradiol and increased relative abundance of rare bacterial taxa rather than dominant species like *Lactobacillus*. Our findings help define what constitutes a “healthy microbiota” in generally healthy Italian women of reproductive age and may inform future strategies for diagnosing or preventing women’s health conditions.

INTRODUCTION

The vaginal microbiota refers to the community of microorganisms that colonize the vagina. Unlike microbiota from other mucosal sites (i.e., the gastrointestinal tract), it has lower diversity, and it is dominated by a few species, primarily *Lactobacillus spp.*, which play a key role in regulating genital health. Producing lactic acid and antimicrobial molecules, these bacteria reduce the risk of colonization by other microorganisms, including potential pathogens involved in infections and diseases of the vaginal tract (1); (2); (3); (4); (5); (6); (7); (8). In general, the prevalence of the *Lactobacillus* genus in healthy women increases with puberty and remains stable until menopause, except during vaginal infections (9).

The most common species of *Lactobacillus* are *Lactobacillus iners*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii*. Earliest studies showed that a vaginal microbiota dominated by *L. crispatus* was associated with a healthy status, whereas *L. iners* was often isolated during infections and therefore a microbiota dominated by it is frequently considered unhealthy. However, this hypothesis originated from studies with small sample sizes and a direct pathogenic role for *L. iners* has not been proved (2); (10); (11). Likewise, a vaginal microbiota dominated by other anaerobic bacteria, such as *Gardnerella vaginalis* (classified as *Bifidobacterium vaginale* and other *Bifidobacterium spp.* in Genome Taxonomy data base (GTDB) nomenclature (12)*), Prevotella*, and *Fannyehessea*, has been associated with clinical conditions of the female genital tract, such as bacterial vaginosis (BV) and vulvovaginal atrophy (VVA), due to the virulence potential of some strains (13); (14).

The pathogenetic role of *L. iners* remains under debate since its prevalence can vary substantially among different populations (3); (15); (16); (17); (18). The vaginal microbiota of African and Hispanic women during the reproductive age is dominated by *L. iners*, while is much rarer in Northern European women with *L. crispatus* being the most abundant and prevalent *Lactobacillus* species (15); (2). The reasons for this north-to-south gradient in the prevalence of *L. crispatus* and *L. iners* are still unknown. It is worth noting that these two species can co-exist in the same microbiota, therefore it is unlikely they are antagonists (15).

The vaginal microbiota has been considered an overall stable ecosystem, although two recent studies conducted on short-term (42 days) and medium-term (18 months) longitudinal follow-up have shown that variations may occur in a small fraction of women (2); (19). These studies evaluated changes in the so-called Community State Types (CST), a method that classifies the vaginal microbiota into 5 groups depending on the composition and the most abundant species (3); (20). In the shorter 42-days study, only microbiotas classified as CST-I (dominated by *L. crispatus*) and CST-

101 IV (dominated by anaerobic bacteria) remained stable, while variations were observed in other CST
102 types. In the longer 18 months longitudinal study, vaginal microbiota of CST-I and CST-III (dominated
103 by *L. iners*) remained stable, while CST-IV microbiota displayed lower temporal stability
104 (approximately 6 months). The underlying causes of these variations, how they occur in populations
105 with different *Lactobacillus spp.* prevalences, and which species may be responsible for these
106 changes in CSTs are not yet known.

107 It is worth noting that none of these studies have characterized the association between
108 compositional changes in the microbiota and specific fluctuations in sex hormones.

109 Here we present vaginal microbiome composition and its dynamics in 61 healthy women from the
110 Italian population cohort Women4Health (W4H), an observational longitudinal study centered on
111 one menstrual cycle, set up to explore the interactions between sex hormones, vaginal and intestinal
112 microbiome, and lipid and glucose metabolism in women (21). We characterized vaginal microbiome
113 composition and evaluated dynamics at four time points pivotal for the menstrual cycle: follicular
114 (F), ovulatory (O), early luteal (EL) and late luteal (LL) phases (**Figure 1)**. We also evaluated host
115 factors associated with diversity and identified sex-hormones that are mostly associated with the
116 observed dynamic changes.

117

118 MATERIAL AND METHODS

119

120 **The Women4Health cohort**

121 In this study, we analyzed the vaginal swabs, metadata, and sex hormone levels for the first 61
122 women enrolled in the Women4Health (W4H) cohort, an ongoing short-term longitudinal study

aiming to recruit up to 300 healthy women and to follow them up through a natural menstrual cycle. The W4H study design has been previously described (21). Briefly, women of European origin aged 18 to 45 years old, who were not using hormonal contraceptives and who have not a diagnosis of gynecological, metabolic, or gastrointestinal diseases, and reported absence of urogenital symptoms, were enrolled in the study at the Institute for Maternal and Child Health – IRCCS Burlo Garofolo in Trieste, Italy. Upon inclusion and signature of an informed consent form, electronic questionnaires were used to gather data on family history, clinical and anthropometric measurements, lifestyle and diet. They were then scheduled for 4 weekly follow-up appointments to hand in self-collected biological samples, including a vaginal swab, and to undergo a blood withdrawal. Moreover, they were given an additional questionnaire to collect information related to their health status and lifestyle in days preceding the appointment. Weekly follow-up visits were scheduled according to pivotal phases of the menstrual cycle: the 1st at the follicular phase (between days 6 and 9), the 2nd at the ovulatory phase (between days 12 and 16), the 3rd at the early luteal phase (between days 18 and 22), and the 4th at late luteal phase (between days 24 and 27) (**Figure 1**). Blood samples were processed within 1 hour after collection. On serum, we measured 5 sex hormones (17-beta estradiol, luteinizing hormone, follicle stimulating hormone, progesterone and prolactin). During the first visit, we also measured testosterone and thyroid parameters (thyroid stimulating hormone and thyroxine (T_4)) to exclude endocrine disorders. Hormones were measured using 1.5 ml serum on the ADVIA Centaur CP Immunoassay System instrument from Siemens Healthineers.

The study protocol - including informed consent forms - was approved by the Friuli-Venezia Giulia Ethical Committee on 09/2021 with prot. N. 0034184/P/GEN/ARCS and modifications amended on 09/2023 with prot. N. 0033477/P/GEN/ARCS. Descriptive statistics for the 61 women are presented in **Table S1.**

147 **Vaginal swabs collection, processing, and microbial DNA extraction**

148 Vaginal swabs were self-collected by volunteers before each follow-up visit (the same day or the day
149 before) using the OMNIgene-VAGINAL kits (OMR-130, DNA Genotek), stored at room temperature
150 and handed at IRCCS Burlo Garofolo within 24 hours, where kits were promptly stored, as
151 recommended by the manufacturer, and shipped to the Institute for Genetic and Biomedical
152 Research - National Research Council (IRGB-CNR) within 21 days. DNA was extracted from swabs
153 with QIAamp PowerFecal Pro DNA kits (QIAgen) using a custom semiautomated protocol on a
154 QIAcube platform. Prior to DNA extraction, a bead-beating step on a TissueLyser II (QIAgen) at 7.5
155 Hz for 5 min was performed to improve lysis. The composition of vaginal microbiota was established
156 by sequencing amplicons of the V3-V4 and V7-V9 regions of the 16S rRNA gene, along with ITS1 gene
157 of fungi. Library construction was performed with a 2-step PCR protocol and dual-barcoding strategy
158 (QIAseq 16S/ITS Region Panel kits, QIAgen). Sequencing was carried out with the MiSeq sequencing
159 system (Illumina) targeting an average of 100K 250-bp paired-end reads per sample. Samples were
160 processed in three different batches, each including one QIAseq 16S/ITS Smart Control (QIAgen), a
161 synthetic DNA construct used both as positive control for library preparation and as control for the
162 identification of contaminants, and a PCR negative control (distilled sterile water). Data obtained
163 were demultiplexed using Qiagen's GeneGlobe software.

164

165 **Quality Control (QC) process**

166 All demultiplexed *FastQ* underwent quality control screening before bioinformatic analysis. *FastQC*
167 v.0.12.1 (see **URLs**) and *MultiQC* v.1.22.2 (22) (see **URLs**) tools were used to detect outliers based on
168 read quality. The *trimmomatic* tool v.0.39 (23) (see **URLs**) was used for trimming low quality paired-
169 end reads. We used the options *SLIDINGWINDOW:4:25* to evaluate whether the average quality
170 score was at least 25 per four bases; if not, this part of the read was trimmed. The option MILEN:50

171 was used to remove reads shorter than 50 bp. After the trimming process, samples were re-analyzed
172 with *FastQC* and *MultiQC* to evaluate their post-process quality.
173 We first processed 185 samples, 4 smart controls and 3 negative controls in two plates and decided
174 to remove **i)** all samples that had less than 80K reads as input before the trimming process and/or
175 less than 60K reads survived post-trimming (N=10 samples), **ii)** one sample with a very high number
176 of ITS reads mapping on *Sporobolomyces spp.*, a known environmental contaminant, and **iii)** samples
177 whose libraries were prepared together with a negative control that showed contamination (>500
178 reads) (N=10) (Table S2). These 21 samples were repeated on a third plate, containing additional 28
179 samples, 1 smart control and 1 negative control. The same quality control filters were applied and
180 only 1 sample was removed from plate 3. Finally, one sample that showed 1.5 million reads and 1.3
181 million post-trimming reads as input, due to an error in library equimolar pooling, was subjected to
182 the subsampling process by randomly selecting 187K reads (the average value of reads survived post-
183 trimming for Plate 3), through the R package *ShortRead* v1.62.0 (see **URLs**).
184 After all these QC steps, a total of 212 samples were left for downstream analyses with the median
185 number of QCed reads per sample being 138,531 (127,689, 141,450 and 190,975 for plates 1, 2 and
186 3, respectively), across all sequenced regions. The average number of QCed reads was 148,660.0 for
187 V3V4V7V9 regions, and 195.2 for the ITS region.
188

189 **Characterization of the vaginal microbiota**

190 To characterize the bacterial composition of the samples, the *DADA2* v3.19.0 package (24) (see **URLs**)
191 was used in the R (v 4.4.1) environment. Using the *"filterAndTrim()"* function, only sequences with a
192 minimum length of 160 bp were selected. With the "*removeBimeraDenovo()*" function, all chimeras
193 were removed.

194 The microorganisms were classified up to the taxonomic level of species with the
195 *"assignTaxonomy()"* and *"addSpecies()"* function Genome Taxonomy DataBase (GTDB) v.r95 (25) was
196 used to profile a phylogenetically consistent microbial taxonomy (see **URLs**). Hypervariable regions
197 V3V4 and V7V9 were processed together. Rare amplicon sequence variants (ASVs) resulting from
198 DADA2 were removed; only ASVs that had at least 5 reads in at least 2 samples were maintained for
199 subsequent analyses. To increase taxonomic resolution for the genus *Lactobacillus,* we followed a
200 previously described protocol (15). Using their code and data files provided (see **URLs**), the genus
201 *Lactobacillus* was reclassified into different subgenera, allowing the identification of more
202 *Lactobacillus* species. Finally, *Lactobacillus* species names were replaced by the respective subgenus
203 identified (**Table S3**). Next, we selected ambiguous ASVs – i.e. those ASVs that were assigned by the
204 algorithm as *Lactobacillus* species but belonged to other genera - and uploaded the corresponding
205 sequences on BLAST to identify the most similar species. Since ambiguity remained, we decided to
206 assign these ASVs the label "unclassified" (**Table S4).**
207 To characterize the fungi composition of the samples, the ITS fastQ files were processed with the
208 *DADA2* v3.19.0 (24) (see **URLs**) package on R (v.4.4.1). To this end, "*filterAndTrim()*" function was set
209 as "*minLen = 50*" and "*maxLen = 200*". We profiled samples with the Unite database v.10.0 (26) (see
210 **URLs).**
211 We derived global metrics of bacterial microbiome composition such as alpha and beta diversity
212 using the *vegan* package v.2.6-4 (27) (see **URLs**). Alpha diversity was calculated based on taxa counts
213 according to the Shannon-Weiner (H) and Simpson (D) indices. Beta diversity was calculated
214 according to the Bray-Curtis dissimilarity index using taxa relative abundances and according to
215 Euclidean distance using taxa relative abundances normalized using the Centered log ratio (CLR)
216 transformation. The Euclidean distance was used in addition to Bray-Curtis because it allowed us to
217 use normalized and corrected data (**Table S5**). To perform CLR transformation we used our own

218 function that calculates the transformation in parallel among taxa, using the *Parallel* package v.4.4.1
219 on R (see **URLs**).
220 In addition, we classified our samples according to the Community State Types (CSTs) definition of
221 the VALENCIA algorithm (20) (see **URLs**). Toward this end, it was necessary to manually modify the
222 taxonomy names according to those required by VALENCIA (**Table S6)**. To identify which ASV could
223 be attributed to *Gardnerella vaginalis*, given the different nomenclature in GTDB, we run again
224 DADA2 with the SILVA v.138.1 database where the nomenclature of species aligns to that used in the
225 VALENCIA algorithm (**Figure S1)**. Specifically, we identified 46 ASVs assigned to the genus
226 *Bifidobacterium* using GTDB v r.95, among these, 15 ASVs classified as *B. vaginalis* (6 ASV) and *B.*
227 *unclassified* (9 ASV) corresponded to *Gardnerella vaginalis* when annotated using the SILVA
228 database. These 15 ASVs were renamed accordingly and used in the VALENCIA algorithm, while the
229 remaining ASVs were discarded.
230 Finally, we used the t-SNE algorithm (28) to visualize the vaginal microbiome composition in a two-
231 dimensional space and used color-coding labels to describe the observed variation in terms of taxa
232 abundance as well as CSTs (**Figure S2**). For this analysis, we employed the t-SNE algorithm to CLR-
233 transformed data using the R package *Rtsne* v.0.17 *(29)* (see **URLs**).
234 Finally, we profiled microbiotas using the same pipeline but subsampling 30K (and 60K) survived
235 reads per sample using the R package *ShortRead* (see **URLs**).
236

237 Statistical analyses

238 Identification of factors associated with beta diversity

239 We evaluated the impact on beta diversity of technical confounders, characteristics of volunteers (as
240 recorded in questionnaires) and of sample collection. Using the *vegan* package (27) (see **URLs**), we

first investigated whether the first five Bray-Curtis beta diversity principal coordinate analyses (PCoA) axes were correlated with technical variables, such as microbial DNA concentration, library concentration, and total reads counts (survived reads). Each PCoA axis was linearly adjusted using only the covariates with which it showed significant correlation. As the dataset included longitudinal measurements, independence tests were conducted separately for each visit to ensure independence between observations. Namely, we used the Kruskal-Wallis independence test in R through the function *kruskal.test()* of *stats* package v.4.4.1 (30); (see **URLs**).

Variables from the questionnaires encompassed both categorical and continuous data related to demographics (e.g., age), anthropometric measures (e.g., body mass index [BMI], waist-to-hip ratio [WHR]), lifestyle factors (e.g., smoking, hormonal contraceptive use), clinical history related to women's health (e.g., number of pregnancies), and conditions surrounding vaginal swab collection (e.g., medication or supplement use prior to collection, sexual intercourse within two days of collection, swab timing, and stool consistency assessed using the Bristol stool scale). Continuous variables were categorized to allow the application of the independence test. All tested variables are summarized in **Table S7**.

We also run PERMANOVA analyses for the significant associations resulting from independence test, using *adonis2()* function from *vegan* R package (27) (see **URLs**).

Evaluation of vaginal microbiota dynamics

To evaluate changes in alpha diversity, beta diversity and taxa relative abundance across the four phases of the menstrual cycle, we used the non-parametric Friedman test. To evaluate changes between two phases (pairwise comparisons) we used the non-parametric Wilcoxon pairwise test for the untransformed data, and the parametric pairwise t-test for the CLR-transformed data. All these

comparative tests were run using the *stat* package v.4.4.1 (30) (see **URLs**). All pairwise comparisons were corrected using the Bonferroni approach.

To evaluate beta diversity changes (both Bray-Curtis and Euclidean distance, adjusted for technical covariates), we used two approaches. First, we calculated mean and median beta diversity across all women within each menstrual cycle phase and compared these across the different phases, using the paired Wilcoxon test (**Table S8**).

In a second step, we calculated mean and median beta diversity for each woman among her different collections (up to 4), and compared the distribution obtained when comparing her first collection to 3 collections from different women from the other 3 phases, using again the unpaired Wilcoxon test. All comparisons were corrected using Bonferroni.

We instead used linear models to investigate dynamics of specific taxa and association with sex hormones. Specifically, we fitted three groups of linear models to: **i)** investigate whether bacterial abundances vary across the menstrual cycle; **ii)** explore the relationship between hormones and bacterial abundance variations between two consecutive phases and **iii)** explore the overall relationship between hormones and bacteria relative abundances.

For all linear models, taxa were filtered using a prevalence > 20% and a mean relative abundance threshold of 0.10 % **(Table S9).**

In the first group of models, we fitted the following Gaussian mixed models:

$$\text{Taxa_adj} \sim \text{Visit_number} + (1|\text{Woman}) \qquad \textbf{(1)}$$

where visit_number is a numeric variable coded as 1, 2, 3, 4 according to the four phases of the menstrual cycle, and Taxa_adj are the residuals of the linear models:

$$\text{Taxa_CLR-transformed} \sim \text{covariates}$$

fitted using the function "*LinearRegression()*" of *sklearn* package v.1.5.2 in Python v.3.10.12 (31) (see **URLs**). The placeholder "covariates" here means that we systematically considered several sets

288 of covariates based on the results of independence tests and technical variables to evaluate if these
289 changes could be confounded by a specific covariate. We opted for stepwise models with an
290 increasing number of covariates, rather than running a model with the full set of covariates, given
291 the limited sample size of the study. A total of 10 models were considered **(Table S10).**
292 From model (1) we used the regression coefficients and the p-values for the variable Visit_number
293 to identify taxa with significant changes. We used permutations to derive an empirical p-value and
294 evaluate robustness of significant results ($p<0.05$). We permuted the column containing taxa
295 abundances and re-run the model 1000 times. The proportion of "fake" p-values less than or equal
296 to the original p-value was calculated to provide the empirical p-value. In addition, false discovery
297 rate (FDR) was instead used to adjust p-values for multiple testing (original pvalues, not empirical
298 pvalues), with the function *"p.adjust()"* of *stats* package and method 'BH' (32).
299 In addition, we calculated the interclass correlation coefficient (ICC) for the model where bacteria
300 were adjusted for the technical covariates (model **i)** above). This parameter quantifies the
301 proportion of total variance attributable to between-subject variability, reflecting the stability of
302 bacterial abundance across different time points within subjects. The ICC was calculated using the
303 function "*icc()"* from the package *performance v 0.12.4 (32)* (see **URLs**).
304 In the second group of models, we again used linear mixed models to investigate if changes in
305 bacteria relative abundance between two phases were associated with changes in sex hormones.
306 Firstly, we removed outlier values in the sex hormone variables exceeding 4 standard deviations from
307 the mean. Secondly, we calculated the differences between two consecutive phases of both sex
308 hormones and bacteria relative abundances and then we normalized these differences using the
309 function "*ordernorm()*" of the R package *bestNormalize v 1.9.1 (33)*(see **URLs**).
310 The following Gaussian mixed models were fitted:

311 $$(\text{Hormone}_{t+1} - \text{Hormone}_{t}) \sim (\text{Taxa_adj}_{t+1} - \text{Taxa_adj}_{t}) \qquad \textbf{(2)}$$

312 where t represents the menstrual cycle phase, and taxa_adj is the CLR-transformed abundance
313 adjusted by technical covariates, calculated as the residuals of the models:

314 Taxa_CLR_transformed ~ Qubit_DNA + Qubit_Library + Total_counts **(3)**

315 where Qubit_DNA is the DNA concentration, Qubit_Library is the library concentration, and
316 Total_counts is the total number of survived reads.

317 In the third group of models we investigate relationships between taxa relative abundances and sex
318 hormones changes across the entire menstrual cycle by fitting the following:

319 Hormone ~ Visit_number + Taxa_adj + (1|Woman) + Pregnancy_category **(4)**

320 In both models **(2)** and **(4)** we used the same strategies described above for models (1) to derive
321 empirical p-values and to correct p-values for multiple testing. To fit all linear mixed models in (1),
322 (2) and (4) we used the *lme4 v 1.1.35.5* (34) *(see* **URLs***)* and the *lmerTest v.3.1.3 (32)* R packages
323 (see **URLs**).

324

325 **Shotgun metagenomics sequencing**

326 A total of 10 samples with different microbiome composition were selected for whole-genome
327 sequencing (WGS). We used the beta diversity matrix to maximize dissimilarity among selected
328 samples, as follows. The first three selected samples were those showing a high relative abundance
329 of *L. iners, L. crispatus* and *L. gasseri,* respectively, and had the maximum beta diversity between
330 them. Then, we selected seven samples showing maximum distance from the first three samples.
331 Sequencing was performed at Prebiomics srl on the Illumina NovaSeq 6000 sequencing system, and
332 microbiome profiling was carried out using *MetaPhlan 4* (35). An average of 3 million bacterial reads
333 per sample was obtained **(Table S11a)**. Relative abundances from the two data sets (16S and WGS)
334 were compared for taxa with abundances > 0 in at least one of the samples. Spearman's correlation

335 test was used to check the correlations between relative abundances of taxa detected by both
336 approaches and for which at least 3 samples had no-zero value **(Table S11b).**

337 RESULTS

338

339 **Vaginal Microbiome Composition**

340 A total of 61 women contributed 213 vaginal swabs to this study, of which 212 passed quality control
341 (QC) as described in **Methods**. Bacterial composition was profiled using previously described
342 protocols (see **Methods**). At the genus level, *Lactobacillus* was the most common genus, followed
343 by *Bifidobacterium* and *Streptococcus*. The two most abundant *Lactobacillus spp.* were *L. iners* and
344 *L. crispatus* (**Figure S2**) and were identified in almost all samples (prevalence > 90%). When
345 restricting to samples with a relative abundance > 60%, *L. iners* was detected in 43.4% of the samples
346 (corresponding to 62.2% of women), whereas *L. crispatus* was detected in 34.9% (55.7% of women).
347 In addition to *Lactobacillus spp.*, we identified other species, such as *Bifidobacterium spp.* and
348 *Fannyhessea vaginae* (**Figure S3** and **Table S9**). Of note, some of the ASVs classified as
349 *Bifidobacterium spp.* by the GTDB used in this study, would be classified as *Gardnerella vaginalis*
350 according to other nomenclatures (see **Methods** and **Supplementary Figure 1**).
351 Overall, vaginal microbiota remained stable throughout the four menstrual cycle phases here
352 investigated. There were no significant differences in alpha diversity across the phases, as measured
353 by the Shannon (H) and Simpson (D) indices (**Figure S4** and **Table S12**). The average beta diversity
354 was instead higher in the follicular phase compared to the ovulatory phase (O) ($p_{wilcoxon}$ = 0.009 and
355 $p_{t\text{-}test}$ = 0.007) and early luteal phase (EL) ($p_{wilcoxon}$ = 0.04 and $p_{t\text{-}test}$ = 0.03) (**Figure 2A** and **Table S8).**
356 Similar results were obtained when using median instead of average (**Figure S5** and **Table S8**), or the

357 overall distribution **(Figure S6** and **Table S13)**. Nevertheless, these changes were not significant and
358 did not lead women's microbiota to align to a similar composition during a particular phase. In fact,
359 the average beta diversity of a woman's microbiota across all her samples was lower than the
360 average obtained when using random samples from other women (p value << 0.05 for all
361 comparisons), indicating that microbiota is largely determined by individual factors, rather than by
362 menstrual phase (**Figure 2B**). We investigated the impact of host factors and described these in the
363 next paragraph.
364 Analyses of dynamic changes could not be performed for the mycobiome, due to the limited number
365 of samples for which mycobiome could be profiled. Although we sequenced the ribosomal internal
366 transcribed spacer (ITS) region in all 213 samples, only 25 samples had at least 200 reads, cutoff
367 deemed necessary to obtain fungi classification (**Figure S7** and **Table S14)**. Twenty-five fungal species
368 were identified, the most prevalent being *Candida albicans*, a fungus associated with vaginal
369 infections (candidiasis) but also frequently found as part of the normal vaginal microbiota. The
370 average number of reads on the 8 women with presence of *Candida albicans* and at least 200 fungal
371 reads was low (749 compared to 92832 reads on *Lactobacillus spp*. on the same women) (**Table S14**)
372 and thus the presence of this fungus is unlikely to be a sign of infection. Of note, we excluded at
373 enrollment all women who reported to currently have vaginal infections or urogenital symptoms.
374 The relatively low number of reads overall detected in the ITS region may be due to the use of a
375 protocol and kit optimized for bacterial rather than fungal DNA extraction, as reported in previous
376 studies (36).
377

378 **The impact of host phenotypes**

379 We then investigated the impact on vaginal microbiota of host phenotypes, lifestyle and information
380 on sample collection, by correlating genus-based and species-based beta diversity PCoA axes with

available metadata. Among the 12 variables tested, the variable “having had at least one pregnancy” had the higher impact at both genus and species level, followed by “having used oral hormonal contraceptives in the past” (**Table S15**). Other variables that were significant albeit with less impact include age, waist-to-hip ratio, smoking habits, intercourse in the days preceding the swab, time of collection, and use of medication or supplements **(Figure S8)**. Intriguingly, using a PERMANOVA a test and projecting values in the PCoA space, we observed that the microbiota of women who had at least one pregnancy before participating in the study had a lower likelihood to be dominated by *L. crispatus* (p=0.005), in line with observations from the ISALA study (15), suggesting the existence of a potential lifelong pregnancy signature (**Figure S9)**. Likewise, using PERMANOVA we observed that the microbiota of older women is less likely dominated by *Lactobacillus spp.* (p=0.003), in line with the observed decline in their abundance during menopause (**Figure S10)** (9)**;** (37). Similarly, dominance of *Lactobacillus spp*. is less frequent in women who reported to have collected vaginal swabs after feces collection compared to those who collected them prior feces (p=0.02) **(Figure S11),** and in those who collected vaginal swabs in the morning compared to those who collected hem during the day (p=0.003) **(Figure S12).**

Frequency of *L. iners* and *L. crispatus* along the menstrual cycle

In contrast to findings from cohort studies on Northern European women (Belgium, Denmark, Sweden) (15); (2), our study found a greater abundance of *L. iners* than *L. crispatus*, a microbiome composition typically observed instead in Hispanic and African populations (16); (18); (3) (**Table 1**). Notably, all volunteers have reported absence of urogenital symptoms and or diagnosis for sexually transmitted diseases or infection, so we can rule out the abundance of *L. iners* in our samples due to either vaginal pathologies or infections. We also ruled out the possibility that our result is driven by a potential bias from the 16S rRNA gene profiling method, since comparison of the relative

405 abundances of these two *Lactobacillus spp.*in 10 samples showed very high concordance with
406 profiles obtained using the shotgun whole-genome sequencing (WGS) method (**Figure S13** and **Table**
407 **S11b**). Likewise, we also excluded the possibility that this result is driven by the higher number of
408 reads compared to protocols used in previous studies. In fact, taxa relative abundances were almost
409 identical when using a subsampling approach on our 16S rRNA data to restrict analyses to maximum
410 30K or 60K reads per sample (**Figure S14**).
411 In our cohort, *L. iners* and *L. crispatus* have a similar prevalence in the follicular phase (98% and 95%,
412 respectively), but the average relative abundance was higher for *L. iners* (average relative abundance
413 40% and 25% for *L. iners* and *L. crispatus*, respectively) (**Table 1**). The difference in average relative
414 abundance between these two species was negligible in the other 3 phases (absolute difference
415 between percentages: 2.03, -1.27, 2.21 in the ovulatory, early and late luteal phases, respectively).
416 Interestingly, all microbiota dominated by *L. crispatus* in the follicular phase were stable during the
417 menstrual cycle, while a small fraction (20%) of those dominated by *L. iners* at the follicular phase
418 switched to *L. crispatus*-dominant - or to other species - in the ovulatory or in the late luteal phase
419 (**Figure 3**).
420 In addition, the microbiota of two women dominated by low prevalent species in the follicular phase
421 became *L. crispatus*-dominant in the ovulatory phase. Inter-class correlation (ICC) analyses indicated
422 that these two species explained the largest variance of total variability between women (**Table S16**).
423 While these results suggest a trend for women's microbiota to switch to *L. crispatus* during the
424 ovulatory phase, *L. iners* still remains the most abundant species in this cohort across all time points.
425

426 **Vaginal microbiota dynamics**

427 Given these clear-cut changes observed in main *Lactobacillus spp*., we sought to thoroughly
428 investigate microbiota dynamics along the menstrual cycle. First, we assessed the composition and

429 variations along the menstrual cycle using the CST classification (see **Methods**). Overall, we found
430 that CST-V (*L. jensenii* dominated) was the rarest among all samples and phases (3.3%), followed by
431 CST-II (*L. gasseri* dominated) (12.7%) and CST-IV (defined by moderate to high abundance of
432 different anaerobic species, such as *Prevotella spp.* and *G. vaginalis,* and low abundance of
433 *Lactobacillus spp.* (14.1%) (**Figure S15A**). In line with relative abundances, the CST-III (*L. iners*
434 dominated) was the most common (38.2%) and with higher prevalence in the follicular phase, while
435 the second most common was CST-I (31.6%) (*L. crispatus* dominated), whose prevalence increased
436 during ovulatory phase (**Figure S15B**)**.** Most women maintained a stable CST throughout the
437 menstrual cycle: only 11 out of 61 women changed CST at any time point (18 if we consider changes
438 in subCST) (**Figure 4A**). Interestingly, 72% (N=8) of these changes occurred from follicular to
439 ovulatory phases (**Figure 4B**). Among them, 6 were transitions from CST-III or CST-IV (N=2) to CST-I
440 (N=4). The remaining changes occurred from early to luteal phase, with 3 women's microbiota
441 switching to CST-IV.
442 None of the women's microbiota classified as CST-I exhibited CST shifts, indicating the high stability
443 of communities dominated by *L. crispatus*. Of the women who undergo CST shifts, only one changed
444 CST types more than once (**Figure S16**). We were unable to detect association with CST/subCST
445 changes and presence of fungi, given the limited amount of information (only 4 out of 11 women
446 exhibiting shits in CST had fungi information at the relevant time points). Likewise, we did not detect
447 association with CST/subCST changes with having had sex intercourse in the two days prior sample
448 collection (p>0.05).
449 Secondly, we examined the longitudinal changes of taxa relative abundances using linear mixed
450 models and controlling for potential confounding variables, as described in **Methods**. We found that
451 the relative abundance of a few bacteria species varies significantly across phases, with the majority
452 remaining stable when different covariates were adjusted. For the genus *Lactobacillus*, *L. iners, L.*

453 *gasseri* and *L. jensenii* showed significant variations across mostly all corrections applied, while *L.*
454 *crispatus* was not significant in any of the models (**Figure 5**).
455 Likewise, for the genus *Streptococcus,* two taxa, namely *S. unclassified* and *S. agalactiae,* varied
456 significantly across phases regardless of covariates used. Finally, another bacterial species, *Dialister*
457 *B micraerophilus* showed significant variation in 3 of the 10 models (**Figure 5).**

458

459 **Longitudinal changes of bacteria abundance associate with sex hormone levels**
460 We investigated whether these observed significant changes in *Lactobacillus* and *Streptococcus*
461 species between two consecutive phases were associated with changes in sex hormone levels. We
462 found that an increase in abundance *L. crispatus* between early luteal and late luteal phases was
463 associated with an increase in progesterone (PROG) levels (p=0.03) (**Table S17** and **Figure 6A**).
464 However, these results were only nominally significant, therefore larger samples size and/or
465 additional cohorts are needed to confirm this finding.
466 Furthermore, among all taxa with prevalence >20%, we identified several bacteria whose
467 longitudinal fluctuations along the entire menstrual cycle were significantly associated with sex
468 hormones levels, resulting in a total of 14 significant associations (**Figure 6B** and **Figure S17** and
469 **Table S18)**. Most of the associations involved 17-beta estradiol (BES17), with some shared between
470 BES17 and LH, including *Prevotella* genus and species *P. timonensis_A*, and genera *Finegoldia* and
471 *Anaerococcus.* Only one association was detected with prolactin (PRL), namely with the abundance
472 of *Bifidobacterium vaginale*. All these associations were negative, indicating that an increase in sex
473 hormones levels is associated with a decrease in the relative abundance of these bacteria (**Figure 6B**
474 and **Figure S17**). The number of significant associations decreased to 9 out of 14, when considering
475 permuted p-values to evaluate robustness of results to outliers. Notably, 4 of these 9 associations,

476 all linked to BES17, also meet a stricter significance threshold applied after correcting multiple
477 testing, suggesting that these are likely genuine.
478

479 DISCUSSION

480 We carried out the first short-term longitudinal study on the vaginal microbiota of non-pregnant,
481 healthy women from an Italian population. In the W4H cohort we observed a higher abundance of
482 *L. iners* compared to *L. crispatus*, in contrast to earlier studies on cohorts from Northern Europe (2);
483 (15) (**Table 1**). Of note, a recent study on young French women (19) reported as well a higher
484 prevalence of *L. crispatus*-dominated CST (CST-I) over the *L. iners*-dominated *CST* (CST-III), although
485 the difference in frequency between the two CSTs was less pronounced (40.5 vs 38.1%, while our
486 study has obtained 32% for CST-I and 38% for CST-III) (**Figure S15**) compared to observations from
487 cohorts based in Belgium (ISALA study), Danmark and Sweden (MiMens study) (15); (2). In the
488 French cohort, 70% of women were enrolled in Bordeaux (south of France). Hence, considered these
489 results and the higher prevalence of *L. iners* in African and Hispanic cohorts (16); (3); (17), the results
490 from our study of the Italian population, we provide evidence for a north-to-south gradient of
491 increased *L. iners* abundance even within Europe.
492 The reasons for these geographical differences in microbiota composition remain unclear, but results
493 from our study and those from the ISALA study offer insights for speculation. For example, in our
494 study we found that women who previously had children were less likely to have a microbiota
495 dominated by *L. crispatus*. Likewise, in the ISALA study, having children was negatively correlated
496 with *L. crispatus* and positively with *L. iners*; this variable was the strongest host factor associated -
497 with opposite direction - with the abundance of these two species (15). Geographic differences may
498 thus be related to the delivery mode, or to post-pregnancy health care practices. Another
499 contributing factor might be related to different cultural and hygiene practices, such as the soap type

500 used, the frequency of washing, the use of intimate wipes and even the type of pads, tampons or
501 other periods supplies (38); (5). While we do not have this information in our cohort, results from
502 the ISALA study indicate that the use of menstrual pads is negatively correlated with *L. crispatus*
503 abundance, while the use menstrual cups was positively correlated (15). Intriguingly, the ISALA study
504 also reported a positive correlation with *L. crispatus* abundance with use of hormonal contraceptive
505 methods (combination pill, vaginal ring or patch). Previously, also Tuddenham S. (39) showed that
506 the proportion of CST I (*L. crispatus*-dominated) was higher among users of oral hormonal
507 contraceptive than the proportion of CST I among non-users, albeit this result was significant only in
508 White and not in African American. No association was instead seen in these two studies between
509 hormonal contraceptives use and *L. iners*.
510 All our volunteers had a natural menstrual cycle and did not use hormonal contraceptives for at least
511 the past 30 days. This, together with the overall limited use of hormonal contraceptive in the Italian
512 population (average 19.1% of females during reproductive age versus ~30% in Northern and Western
513 European countries, according to WHO (40)) could explain the observed low abundance of *L.*
514 *crispatus*. Prolonged and/or early use of hormonal contraceptives may also be relevant. In Italy, free
515 access to hormonal contraceptives was given only in 2023, while in some North European countries
516 has been available since decades (41), facilitating its use in the young generation and for longer
517 periods. Finally, differences in abundance of the two *Lactobacillus* species may be attributed to
518 variations in the host-immune system, in the amount and quality of vaginal secretion, in genetic
519 variants of gene encoding receptors expressed on the surface of the vaginal epithelial cells, and
520 other genetically determined factors specific to each host and bacteria (7).
521 The very high prevalence and abundance of *L. iners* in our cohort of healthy women is striking and
522 challenges the idea that *L. iners* dominated microbiotas are 'unhealthy' or ‘dysbiotic’. In fact, W4H
523 volunteers have reported no current urogenital symptoms or diagnosed sexually transmitted

diseases or vaginal infections, so we can rule out the abundance of *L. iners* in our samples due to either vaginal pathologies or infections. We speculate that the presence of different strains could be on the most plausible explanation for geographic and clinical differences. Unfortunately, with the 16S sequencing approach we (and others) are unable to identify which strains of *L. iners* are present in the studied vaginal microbiota. However, given its high genetic variability, is reasonable to assume that certain strains of *L. iners* may promote healthy conditions, while others may increase the risk of infection, thus explaining its presence as species abundance in both healthy and dysbiotic vaginal conditions (42); (11). In addition, a woman can be colonized by multiple coexisting strains with different genetic characteristics and metabolic functions, enabling the survival of this species in a variety of vaginal microenvironments (43). A deep genetic study on *L. iners* strains coupled with human phenotypes is needed to better understand the ecology of this bacterial species and their differential impact on infections and other gynecological diseases.

Our results on dynamic changes along menstrual cycle phases agree with previous studies reporting that vaginal microbiota is generally stable over the menstrual cycle in healthy women and support current knowledge on the strong stability of microbiomes dominated by *L. crispatus* (4); (19); (2). Furthermore, we were able to pinpoint to species that show dynamic changes in a minority of women, namely *L. iners*, *L. gasseri, L. jensenii* and *Streptococcus spp*. The decreased stability conferred by these species could be explained by the production of two distinct isoforms of lactic acid by these vaginal microorganisms. Unlike *L. crispatus, L. iners*, *Bifidobacterium spp*. and *Streptoccosus spp*. produce only the L- enantiomer of lactic acid that allows easier colonization by other microbes than species also producing the D- enantiomer (44); (5). Furthermore, *L. iners* possesses additional genes compared to *L. crispatus*, encoding stress-tolerance proteins, iron–sulfur proteins, exogenous L-cysteine transport, and exhibits superior metabolic adaptation to the changing carbohydrate sources in the vaginal environment (45); (1).

548 Intriguingly, we observed that even though only a few women's microbiota changed along the
549 menstrual cycle, most changes occurred from follicular to ovulatory phase, with most of these
550 aligning to an *L. crispatus* profile. Beta diversity was also lower during the ovulatory and early luteal
551 phases compared to follicular phase, which could be due to a protective mechanism to support a
552 potentially fertilized ovum. It is possible that the fluctuation of sex hormones plays a key role in
553 vaginal microbiota changes. Our analyses showed that 17-beta estradiol has the major impact on
554 longitudinal variation in taxa abundances, although none of the hormone-taxa association involved
555 *Lactobacillus* species and thus they cannot fully explain changes we observed during ovulatory
556 phase. Nonetheless, these associations with 17-beta estradiol are fascinating. In fact, we detected
557 significant negative relationships with *Prevotella spp*., *Finegoldia* and *Corynebacterium* genera, taxa
558 that are often found at high abundances in women after menopause with menopause-associated
559 symptoms, such as vulvo-vaginal atrophy and genito-urinary symptoms (46). The decline in estrogen
560 levels during menopause may play a prominent role in driving microbiota change, as reduced
561 estrogen leads to a decrease in glycogen by vaginal cells. This causes an elevated vaginal pH, creating
562 conditions that favor the colonization of multiple microbial species adapted to a less acidic
563 environment such as *Bifidobacterium spp*. and *Prevotella spp*., as well as fungi of the genus *Candida*.

564 Our study has several strengths and novelties, including the high depth 16S rRNA sequencing, the
565 enrollment of naturally menstruating women, the direct measurement of sex hormones, and the
566 investigation of a south European healthy population. While our results have not a direct clinical
567 implication, knowledge of vaginal microbiota dynamics under physiological sex hormones variations
568 in all populations is an essential route for investigating the potential role of microbes also in
569 diagnosis and prevention of diseases conditions such infertility and endometriosis.

We also recognize the limitations of the study. First, we did not collect samples during menses, a stage where important changes in bacterial composition have been reported to occur, leading in some women to an excess of anaerobic bacteria (2). The compositional shifts observed for 8 women between the follicular phase and ovulatory phase, five of which occuring from anaerobic to lactobacilli dominated CSTs, may be a signature of a perturbation occurred during menses. Still, there was no evidence in our data that these compositional shifts were due to women collecting vaginal swabs on those days right after menses (day 5 and 6) compared to women who did not experienced shifts (Fisher test p=0.71). Moreover, we have focused on a single menstrual cycle and therefore we were unable to assess if observed changes were cyclic, i.e. the microbiota of that minority of women experiencing shift returns to the original profile after menstruation. Furthermore, we have no measurement of vaginal pH, which is a crucial factor in the regulation of the microbiota. Finally, while we have employed a very deep 16S sequencing approach, we are limited to the relative abundance of bacteria and do not have information on bacterial function or strains, features that metagenomic sequencing could provide.

In conclusion, our study highlights the importance to extend investigations on vaginal microbiota in healthy women from diverse populations, also from the same ethnicity, and the need to assess stability and dynamics with sex hormone changes, including those occurring during a natural menstrual cycle.

FIGURE LEGENDS

Figure 1. Schematic representation of data and biological samples collection.

Schematic representation of data collected and analyzed in this work. For a description of additional data and samples being collected in the Women4Health study see Busonero F et al EHJ Open 2024. Created in BioRender. Sanna, S. (2025) https://BioRender.com/rcl99h9

Figure 2. Average beta diversity across menstrual cycle phases.

In **(A)** Average Beta diversity across the four menstrual cycle phases: Follicular phase (F), Ovulatory phase (O), Early Luteal phase (EL), and Late Luteal phase (LL). The black line inside the boxplot represents the median, with the lowest and highest values within the 1.5 interquartile range represented by the whiskers. The black lines between violins indicate significant differences (*p< 0.01). P-values were obtained using paired T-test. In **(B)** Average beta diversity within the same woman across phases (Self) and average of beta diversity among different women (Random 1 to Random 10). In both panels, beta diversity was calculated using Euclidean distances on transformed taxa adjusted for technical covariates (see **Material and methods)**.

Figure 3: Distribution of the most abundant taxa across menstrual cycle phases.

Relative abundance of the 10 most abundant species in the four different phases. In **(A)** samples are sorted according to the abundance of *L. iners*, *L. crispatus*, *L. gasseri*, respectively. Samples in **(B)**, **(C)**, and **(D)** are ordered as in **(A)**. The grey bars show missing information for those women in the specific week. In the x-axis title we indicate how many samples had the microbiome profile available. Of note, some of the ASVs classified as *Bifidobacterium spp.* by the GTDB used in this study, would be classified as *Gardnerella vaginalis* according to other nomenclatures (see **Methods** and **Supplementary Figure 1**).

Figure 4: Community State Type (CST) across menstrual cycle phases

In **(A)** t-SNE representation of the vaginal microbiome composition, samples are colored according to their CST classification. Arrows highlight instances where a woman changes CST between consecutive phases, with the notation start_phase → final_phase indicated with color-coding of the arrow as in the pie chart. In the bottom-left corner, a pie chart shows the proportions of phases where CST changes occur. The legend provides color coding for points according to the assigned CST. In **(B)** Alluvial plot showing Community State Type (subCST) changes between phases. The four columns represent the Follicular (F), Ovulatory (O), Early Luteal (EL), and Late Luteal (LL) phases, respectively. In legend of the plot: CH: a change occurred in at least one phase, complete data available (15/61); CH + NA: a change occurred in at least one phase, missing samples in some phases (3/61); NO CH: No changes occurred, complete data available (26/61); NO CH + NA: No changes occurred, but missing samples in some phases (17/61).

Figure 5: Longitudinal changes in taxa relative abundance across phases

This figure shows the results of a linear model investigating whether bacterial abundances change across menstrual cycle phases. In each panel, the model coefficients for each taxon are shown with points representing the beta coefficient of the variable time and the bars at the 95% confidence interval of the estimate. The actual p-values are shown above each point. Colour coding indicates the significance of the associations (p-values< 0.05 yellow, black otherwise).

Each panel corresponds to the same model using different corrections for the taxa, with the addition of an increasing number of covariates. In particular: model 1 is the basic model with CLR transformed bacteria, model 2 adds to previous model the technical covariates, model 3 adds Age, model 4 adds sex_less_than_two_days, model 5 adds Pregnancy_category, model 6 adds Pill_use, model 7 adds Swab_after_feces, model 8 adds Bristol_stool_scale, model adds Swab_morning_or_not and model 10 adds WHR_ranges. Of note, some of the ASVs classified as *Bifidobacterium spp.* by the GTDB used in this study, would be classified as *Gardnerella vaginalis* according to other nomenclatures (see **Methods** and **Supplementary Figure 1**).

Figure 6: Relationship between sex hormones and bacteria

These forest plots display the significant associations between sex hormones and bacterial taxa when considering two consecutive phases **(A)** or in the overall menustral cycle **(B)**. The x-axis represents the model coefficients, indicating the strength and direction of each association, while the y-axis lists bacterial taxa. Each line illustrates the 95% confidence interval for the coefficient, with colors representing the sex hormone associated with each taxon. The p-value for each model is shown above the corresponding point. Associations that remain significant after FDR correction, with a threshold of 0.2, are highlighted with an asterisk on the y-axis. In the legend, the color coding for the hormones is as follows: BES17=17-beta estradiol, FSH=follicle-stimulating hormone, LH=luteinizing hormone, PRL=prolactin, and PROG=progesterone. Of note, some of the ASVs classified as *Bifidobacterium spp.* by the GTDB used in this study, would be classified as *Gardnerella vaginalis* according to other nomenclatures (see **Methods** and **Supplementary Figure 1**).

DATA AVAILABILITY

All our code used to analyze the data and instructions are available at: https://github.com/Sanna-s-LAB/Women4Health

The human-depleted 16S rRNA and ITS sequences are deposited at the Sequence Read Archive (SRA) repository under accession number PRJNA1222832 [which will be released upon acceptance of the manuscript]. Participant-level personally identifiable data cannot be shared in respect to participants' informed consent and are protected under the Italian Personal Data Protection Code and European Regulation 2016/679 of the European Parliament and of the Council (GDPR) that prohibit distribution even in pseudo-anonymized form.

All data necessary to support the conclusions drawn in this study are available in the Supplementary Tables.

URLs

Code for the reclassification of *Lactobacillus spp.:*

https://github.com/LebeerLab/Citizen-science-map-of-the-vaginal-microbiome

Code and files for VALENCIA algorithm

https://github.com/ravel-lab/VALENCIA

Tools

FastQC: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Trimmomatic: https://github.com/timflutre/trimmomatic?tab=readme-ov-file

MultiQC: https://github.com/MultiQC/MultiQC

R packages:

vegan: https://github.com/vegandevs/vegan/

lme4: 10.32614/CRAN.package.lme4

lmerTest: 10.32614/CRAN.package.lmerTest

Rtsne: 10.32614/CRAN.package.Rtsne

Parallel: https://www.rdocumentation.org/packages/parallel/versions/3.6.2

STAT: https://doi.org/10.32614/CRAN.package.STAT

DADA2: https://www.bioconductor.org/packages/release/bioc/html/dada2.html

ShortRead: https://kasperdanielhansen.github.io/genbioconductor/html/ShortRead.html

Performance: 10.32614/CRAN.package.performance

sklearn: https://jmlr.csail.mit.edu/papers/v12/pedregosa11a.html

Databases:

GTDB: https://gtdb.ecogenomic.org/stats/r95

UNITE DB: https://doi.org/10.15156/BIO/2959330

ACKNOWLEDGMENTS

We thank all the volunteers who participated in the study for their time and commitment. We thank the physicians of the University of Trieste for their contribution to the recruitment phase, including but not limited to, Francesco Cracco, Roberta Maria Gentile, Elena Stefani and Michele Stracquadaini, the Directors of I.R.C.C.S. Burlo-Garofolo and of IRGB-CNR for logistic support, the Agenzia Regionale Sardegna Ricerche for providing access to laboratories, technologists Davide Murrau, Marco Masala and Michele Marongiu from the IRGB-CNR for IT support. Finally, we thank Dr. Alessandra Meloni and Dr. Ferdinando Coghe from the Azienda Ospedaliero Universitaria di Cagliari and Prof. Stefano Guerriero from the University of Cagliari for having joined the

Women4Health project and initiating a second recruiting center in Cagliari starting in October 2024. Likewise, we thank Prof. Maria Raffaella Barbaro and Prof. Giovanni Barbara from the IRCCS Azienda Ospedaliero Universitaria di Bologna, Policlinico Sant' Orsola, for having joined the Women4Health project and initiating a second recruiting center in Bologna starting in April 2025.

AUTHOR'S CONTRIBUTION

Conceptualization: F.D.S., G.G., S.S.

Writing - original draft preparation: E.V., F.C., A.M., F.B., M.L.F., S.S.,

Project supervision: S.S.

Funding acquisition: G.G., S.S.

Statistical and Bioinformatic analyses: E.V., F.C., S.S.

Critical support for bioinformatic analyses: D.V.Z., V. LF., R.G., J.S., N.K., A.Z., S.S.

Samples and data collection: S.L., S.C., G.V.B., A.K., F.D.S., D.M, G.G.

Vaginal samples processing: A.M., F.Cr., S.I., F.B., M.L.F.

All authors read and revised the manuscript and approved its final version. Furthermore, all authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This study was co-funded by the Italian Ministry of Health through the contribution given to the Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste - Italy (SD 02/21 to G.G.), by the European Union (ERC Stg 2022 to S.S., acronym SEMICYCLE, GA n.101075624), by the Next

Generation EU, in the context of the National Recovery and Resilience Plan, Investment PE8 – Project Age-It: “Ageing Well in an Ageing Society, ” [DM 1557 11.10.2022 to S.S.] and by NutrAGE grant (CNR Project FOE-2021 DBA.AD005.225). Views and opinions expressed are, however, those of the author(s) only and do not necessarily reflect those of the European Union or the European Research Council. Neither the European Union nor the granting authority can be held responsible for them. In addition, S.S. also received a PRIN2022 grant from the Next Generation EU funds (DSB.PN004.021 2022PMZKEC_LS2_PRIN2022 SANNA, CUP B53D23008300006).

USE OF AI STATEMENT

The authors declare that they have used generative artificial intelligence, specifically Quillbot, ChatGPT and Reverso online platforms only to check spelling and grammar. The authors declare that ChatGPT was also used to improve R scripts for handling data formatting and for the generation of figures.

Table 1. Prevalence and relative abundance of *L. iners* and *L. crispatus* in our study and other European cohorts.

The table reports the statistics from previously published studies on European cohorts and those from our study, for *L. iners* and *L. crispatus*. The column Time point indicates weather statistics refer to samples collected in a specific menstrual cycle phase or at any time during menstrual cycle (or menopause).

							Frequency (%)		Prevalence (%)		Average Relative Abundance (%)	
Study	**Country**	**Sequencing Method**	**N reads (K)**	**N samples**	**Age range**	**Time point**	***CST-I***	***CST-III***	***L.iners***	***L.crispatus***	***L.iners***	***L.crispatus***
ISALA	Belgium	16S (V4)	25	3000	18-73	any	43	28	72	90	24	38
MiMens	Denmark, Sweden	16S (V3V4) WGS	--	49	20-28	Follicular phase	--	--	63	76	12	59
						Ovulatory phase	--	--	66	83	14	67
i-Predict	France	16S (V3V4)	34	241	18-25	any	41	38	--	--	38	45
W4H	Italy	16S (V3V4, V7V9)	155	61	18-45	Follicular phase	25	42	98	95	40	25
						Ovulatory phase	36	38	93	93	37	35
						Early Luteal phase	35	37	100	98	34	35
						Late Luteal phase	30	35	100	100	33	31

REFERENCE

1. France MT, Mendes-Soares H, Forney LJ. Genomic Comparisons of Lactobacillus crispatus and Lactobacillus iners Reveal Potential Ecological Drivers of Community Composition in the Vagina. Appl Environ Microbiol. 2016 Dec 15;82(24):7063–73.

2. Hugerth LW, Krog MC, Vomstein K, Du J, Bashir Z, Kaldhusdal V, Fransson E, Engstrand L, Nielsen HS, Schuppe-Koistinen I. Defining Vaginal Community Dynamics: daily microbiome transitions, the role of menstruation, bacteriophages, and bacterial genes. Microbiome. 2024 Aug 19;12(1):153.

3. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci. 2011 Mar 15;108(supplement_1):4680–7.

4. Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UME, Zhong X, Koenig SSK, Fu L, Ma Z (Sam), Zhou X, Abdo Z, Forney LJ, Ravel J. Temporal Dynamics of the Human Vaginal Microbiota. Sci Transl Med. 2012 May 2;4(132):132ra52-132ra52.

5. Holm JB, France MT, Gajer P, Ma B, Brotman RM, Shardell M, Forney L, Ravel J. Integrating compositional and functional content to describe vaginal microbiomes in health and disease. Microbiome. 2023 Nov 30;11(1):259.

6. Petrova MI, Lievens E, Malik S, Imholz N, Lebeer S. Lactobacillus species as biomarkers and agents that can promote various aspects of vaginal health. Front Physiol [Internet]. 2015 Mar 25 [cited 2024 Oct 22];6. Available from: https://www.frontiersin.org/journals/physiology/articles/10.3389/fphys.2015.00081/full

7. Nori SRC, Walsh CJ, McAuliffe FM, Moore RL, Van Sinderen D, Feehily C, Cotter PD. Strain-level variation among vaginal Lactobacillus crispatus and Lactobacillus iners as identified by comparative metagenomics. Npj Biofilms Microbiomes. 2025 Mar 23;11(1):1–11.

8. Scillato M, Spitale A, Mongelli G, Privitera GF, Mangano K, Cianci A, Stefani S, Santagati M. Antimicrobial properties of Lactobacillus cell-free supernatants against multidrug-resistant urogenital pathogens. MicrobiologyOpen. 2021;10(2):e1173.

9. Łaniewski P, Herbst-Kralovetz MM. Connecting microbiome and menopause for healthy ageing. Nat Microbiol. 2022 Mar;7(3):354–8.

10. Ravel J, Brotman RM, Gajer P, Ma B, Nandy M, Fadrosh DW, Sakamoto J, Koenig SS, Fu L, Zhou X, Hickey RJ, Schwebke JR, Forney LJ. Daily temporal dynamics of vaginal microbiota before, during and after episodes of bacterial vaginosis. Microbiome. 2013 Dec 2;1(1):29.

11. Petrova MI, Reid G, Vaneechoutte M, Lebeer S. *Lactobacillus iners*: Friend or Foe? Trends Microbiol. 2017 Mar 1;25(3):182–91.

12. Cornejo OE, Hickey RJ, Suzuki H, Forney LJ. Focusing the diversity of Gardnerella vaginalis through the lens of ecotypes. Evol Appl. 2018;11(3):312–24.

13. Muzny CA, Łaniewski P, Schwebke JR, Herbst-Kralovetz MM. Host–vaginal microbiota interactions in the pathogenesis of bacterial vaginosis. Curr Opin Infect Dis. 2020 Feb;33(1):59.

14. Brotman RM, Shardell MD, Gajer P, Fadrosh D, Chang K, Silver MI, Viscidi RP, Burke AE, Ravel J, Gravitt PE. Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy. Menopause. 2014 May;21(5):450.

15. Lebeer S, Ahannach S, Gehrmann T, Wittouck S, Eilers T, Oerlemans E, Condori S, Dillen J, Spacova I, Vander Donck L, Masquillier C, Allonsius CN, Bron PA, Van Beeck W, De Backer C, Donders G, Verhoeven V. A citizen-science-enabled catalogue of the vaginal microbiome and associated factors. Nat Microbiol. 2023 Nov;8(11):2183–95.

16. Serrano MG, Parikh HI, Brooks JP, Edwards DJ, Arodz TJ, Edupuganti L, Huang B, Girerd PH, Bokhari YA, Bradley SP, Brooks JL, Dickinson MR, Drake JI, Duckworth RA, Fong SS, Glascock AL, Jean S, Jimenez NR, Khoury J, Koparde VN, Lara AM, Lee V, Matveyev AV, Milton SH, Mistry SD, Rozycki SK, Sheth NU, Smirnova E, Vivadelli SC, Wijesooriya NR, Xu J, Xu P, Chaffin DO, Sexton AL, Gravett MG, Rubens CE, Hendricks-Muñoz KD, Jefferson KK, Strauss JF, Fettweis JM, Buck GA. Racioethnic diversity in the dynamics of the vaginal microbiome during pregnancy. Nat Med. 2019 Jun;25(6):1001–11.

17. Wei X, Tsai MS, Liang L, Jiang L, Hung CJ, Jelliffe-Pawlowski L, Rand L, Snyder M, Jiang C. Vaginal microbiomes show ethnic evolutionary dynamics and positive selection of *Lactobacillus* adhesins driven by a long-term niche-specific process. Cell Rep. 2024 Apr 23;43(4):114078.

18. Roachford OSE, Alleyne AT, Nelson KE. Insights into the vaginal microbiome in a diverse group of women of African, Asian and European ancestries. PeerJ. 2022 Nov 29;10:e14449.

19. Tamarelle J, Thiébaut ACM, de Barbeyrac B, Bébéar C, Bourret A, Fauconnier A, Ravel J, Delarocque-Astagneau E, Sabin B, Guillemot D, Morré S, Karsenti I, Claret-Vermeulen MF, Mathiot-Vicaigne E, Régnier C, Aoussou P, Badie-Perez R, Rebouillat-Savy K, Valin N, Tzourio C, Rahis AC, Judlin P, Brun JL, Bongain A, Aegerter P, Méaude-Roufai L, Chemani C, Grimaldi-Bensouda L, Mediouni C, Kersaudy-Rahib D, Lydié N, The i-Predict study group. Vaginal microbiota stability over 18 months in young student women in France. Eur J Clin Microbiol Infect Dis [Internet]. 2024 Sep 20 [cited 2024 Sep 24]; Available from: https://doi.org/10.1007/s10096-024-04943-3

20. France MT, Ma B, Gajer P, Brown S, Humphrys MS, Holm JB, Waetjen LE, Brotman RM, Ravel J. VALENCIA: a nearest centroid classification method for vaginal microbial communities based on composition. Microbiome. 2020 Nov 23;8(1):166.

21. Busonero F, Lenarduzzi S, Crobu F, Gentile RM, Carta A, Cracco F, Maschio A, Camarda S, Marongiu M, Zanetti D, Conversano C, Di Lorenzo G, Mazzà D, De Seta F, Girotto G, Sanna S. The Women4Health cohort: a unique cohort to study women-specific mechanisms of cardio-metabolic regulation. Eur Heart J Open. 2024 Mar 1;4(2):oeae012.

22. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinforma Oxf Engl. 2016 Oct 1;32(19):3047–8.

23. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinforma Oxf Engl. 2014 Aug 1;30(15):2114–20.

24. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High resolution sample inference from Illumina amplicon data. Nat Methods. 2016 Jul;13(7):581–3.

25. Parks DH, Chuvochina M, Chaumeil PA, Rinke C, Mussig AJ, Hugenholtz P. A complete domain-to-species taxonomy for Bacteria and Archaea. Nat Biotechnol. 2020 Sep;38(9):1079–86.

26. Abarenkov K, Nilsson RH, Larsson KH, Taylor AFS, May TW, Frøslev TG, Pawlowska J, Lindahl B, Põldmaa K, Truong C, Vu D, Hosoya T, Niskanen T, Piirmann T, Ivanov F, Zirk A, Peterson M, Cheeke TE, Ishigami Y, Jansson AT, Jeppesen TS, Kristiansson E, Mikryukov V, Miller JT, Oono R, Ossandon FJ, Paupério J, Saar I, Schigel D, Suija A, Tedersoo L, Kõljalg U. The UNITE database for molecular identification and taxonomic communication of fungi and other eukaryotes: sequences, taxa and classifications reconsidered. Nucleic Acids Res. 2024 Jan 5;52(D1):D791–7.

27. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin P, O'Hara R, Simpson G, Solymos P, Stevenes M, Wagner H. Vegan: Community Ecology Package. R package version 2.0-2. 2012 Jan 1;

28. Maaten L van der, Hinton G. Visualizing Data using t-SNE. J Mach Learn Res. 2008;9(86):2579–605.

29. Krijthe J, Van der Maaten L. Rtsne: T-Distributed Stochastic Neighbor Embedding using a Barnes-Hut Implementation [Internet]. 2023 [cited 2024 Dec 16]. Available from: https://cran.r-project.org/web/packages/Rtsne/index.html

30. Bolar K. STAT: Interactive Document for Working with Basic Statistical Analysis [Internet]. 2019 [cited 2024 Dec 16]. Available from: https://cran.r-project.org/web/packages/STAT/index.html

31. Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, Blondel M, Prettenhofer P, Weiss R, Dubourg V, Vanderplas J, Passos A, Cournapeau D, Brucher M, Perrot M, Duchesnay É. Scikit-learn: Machine Learning in Python. J Mach Learn Res. 2011 Nov 1;12(null):2825–30.

32. Kuznetsova A, Brockhoff PB, Christensen RHB, Jensen SP. lmerTest: Tests in Linear Mixed Effects Models [Internet]. 2020 [cited 2024 Dec 16]. Available from: https://cran.r-project.org/web/packages/lmerTest/index.html

33. Peterson RA. Finding Optimal Normalizing Transformations via bestNormalize. R J. 2021 Jun 7;13(1):294–313.

34. Bates D, Maechler M, Bolker B, Walker S, Singmann H, Dai B, Scheipl F, Grothendieck G, Green P, Fox J, Bauer A, simulate.formula) PNK (shared copyright on, Tanaka E, Jagan M. lme4: Linear Mixed-Effects Models using "Eigen" and S4 [Internet]. 2025 [cited 2025 Jan 14]. Available from: https://cran.r-project.org/web/packages/lme4/index.html

35. Blanco-Míguez A, Beghini F, Cumbo F, McIver LJ, Thompson KN, Zolfo M, Manghi P, Dubois L, Huang KD, Thomas AM, Nickols WA, Piccinno G, Piperni E, Punčochář M, Valles-Colomer M, Tett A, Giordano F, Davies R, Wolf J, Berry SE, Spector TD, Franzosa EA, Pasolli E, Asnicar F, Huttenhower C, Segata N. Extending and improving metagenomic taxonomic profiling with uncharacterized species using MetaPhlAn 4. Nat Biotechnol. 2023 Nov;41(11):1633–44.

36. Ahannach S, Delanghe L, Spacova I, Wittouck S, Van Beeck W, De Boeck I, Lebeer S. Microbial enrichment and storage for metagenomics of vaginal, skin, and saliva samples. iScience. 2021 Nov 19;24(11):103306.

37. Muhleisen AL, Herbst-Kralovetz MM. Menopause and the vaginal microbiome. Maturitas. 2016 Sep 1;91:42–50.

38. Holdcroft AM, Ireland DJ, Payne MS. The Vaginal Microbiome in Health and Disease-What Role Do Common Intimate Hygiene Practices Play? Microorganisms. 2023 Jan 23;11(2):298.

39. Tuddenham S, Gajer P, Burke AE, Murphy C, Klein SL, Stennett CA, Wilgus B, Ravel J, Ghanem KG, Brotman RM. *Lactobacillus*-dominance and rapid stabilization of vaginal microbiota in combined oral contraceptive pill users examined through a longitudinal cohort study with frequent vaginal sampling over two years. eBioMedicine. 2023 Jan 1;87:104407.

40. United Nations. Contraceptive Use by Method 2019: Data Booklet [Internet]. United Nations; 2019 [cited 2025 Feb 13]. Available from: https://www.un-ilibrary.org/content/books/9789210046527

41. Doppenberg H. Free pill in the Netherlands: for how much longer? Plan Parent Eur Plan Fam En Eur. 1994 Mar;23(1):8–9.

42. Kwak W, Han YH, Seol D, Kim H, Ahn H, Jeong M, Kang J, Kim H, Kim TH. Complete Genome of Lactobacillus iners KY Using Flongle Provides Insight Into the Genetic Background of Optimal Adaption to Vaginal Econiche. Front Microbiol [Internet]. 2020 May 26 [cited 2025 Jan 14];11. Available from: https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2020.01048/full

43. Holm JB, Carter KA, Ravel J, Brotman RM. Lactobacillus iners and Genital Health: Molecular Clues to an Enigmatic Vaginal Species. Curr Infect Dis Rep. 2023 Apr 1;25(4):67–75.

44. Witkin SS, Mendes-Soares H, Linhares IM, Jayaram A, Ledger WJ, Forney LJ. Influence of Vaginal Bacteria and d- and l-Lactic Acid Isomers on Vaginal Extracellular Matrix

Metalloproteinase Inducer: Implications for Protection against Upper Genital Tract Infections. mBio. 2013 Aug 6;4(4):10.1128/mbio.00460-13.

45. Macklaim JM, Gloor GB, Anukam KC, Cribby S, Reid G. At the crossroads of vaginal health and disease, the genome sequence of Lactobacillus iners AB-1. Proc Natl Acad Sci. 2011 Mar 15;108(supplement_1):4688–95.

46. Anahtar MN, Byrne EH, Doherty KE, Bowman BA, Yamamoto HS, Soumillon M, Padavattan N, Ismail N, Moodley A, Sabatini ME, Ghebremichael MS, Nusbaum C, Huttenhower C, Virgin HW, Ndung'u T, Dong KL, Walker BD, Fichorova RN, Kwon DS. Cervicovaginal Bacteria Are a Major Modulator of Host Inflammatory Responses in the Female Genital Tract. Immunity. 2015 May 19;42(5):965–76.

FIGURE 1

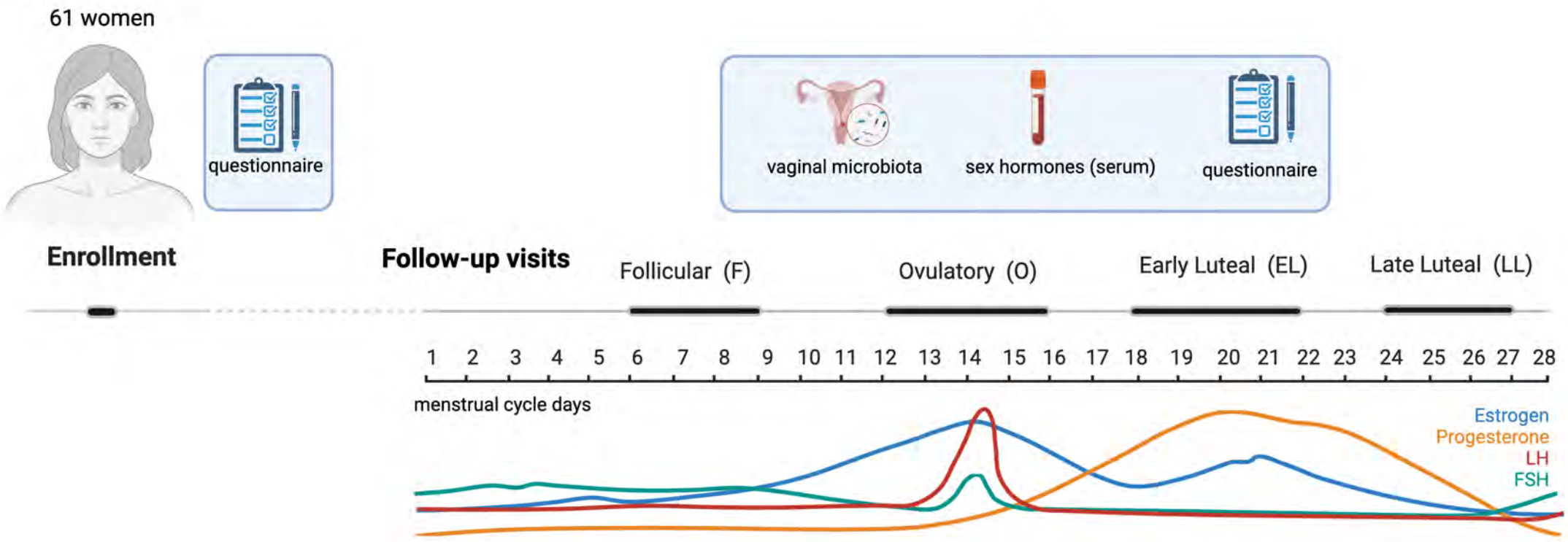

61 women
questionnaire
vaginal microbiota
sex hormones (serum)
questionnaire
Enrollment
Follow-up visits
Follicular (F)
Ovulatory (O)
Early Luteal (EL)
Late Luteal (LL)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
menstrual cycle days
Estrogen
Progesterone
LH
FSH

FIGURE 2

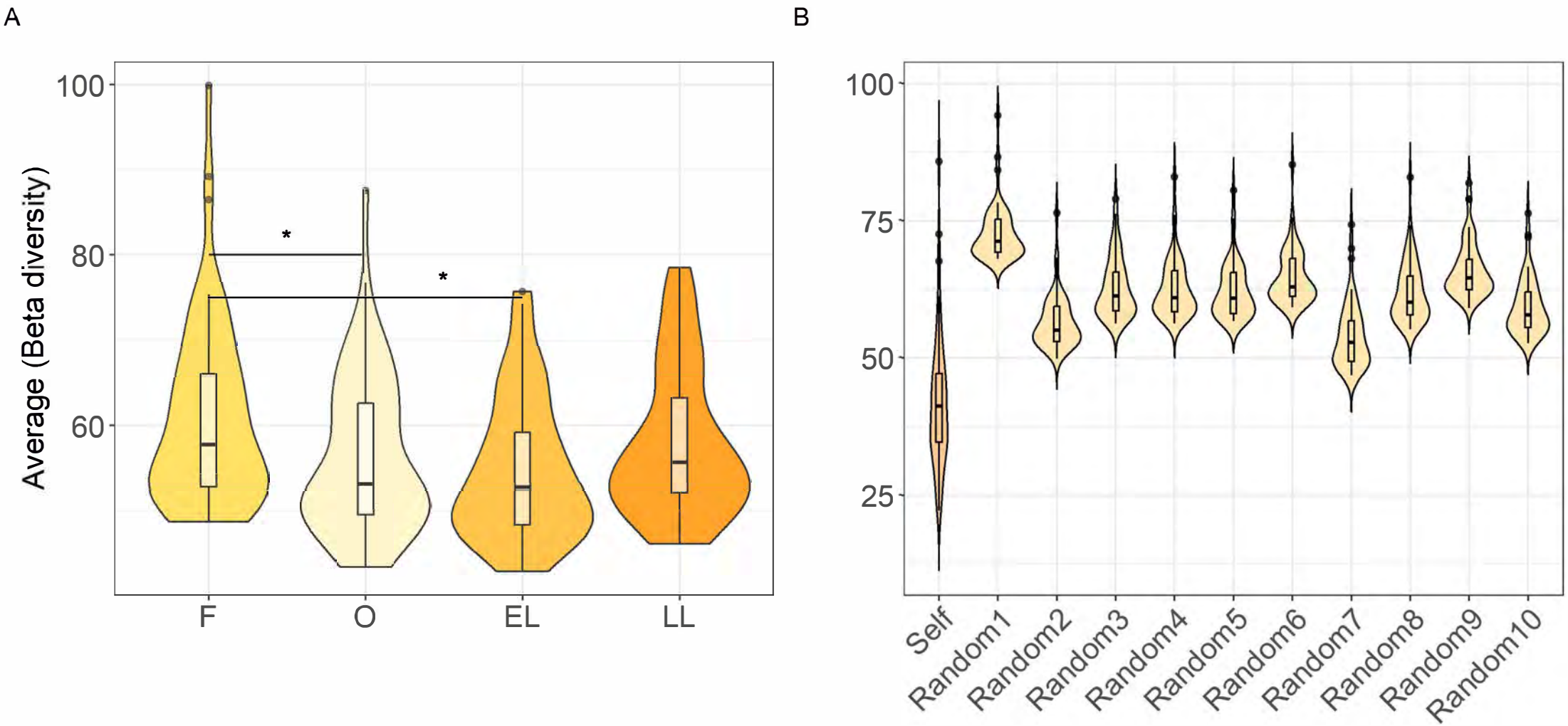

A
B
Average (Beta diversity)
100
80
60
*
*
F
O
EL
LL
100
75
50
25
Self
Random1
Random2
Random3
Random4
Random5
Random6
Random7
Random8
Random9
Random10

FIGURE 3

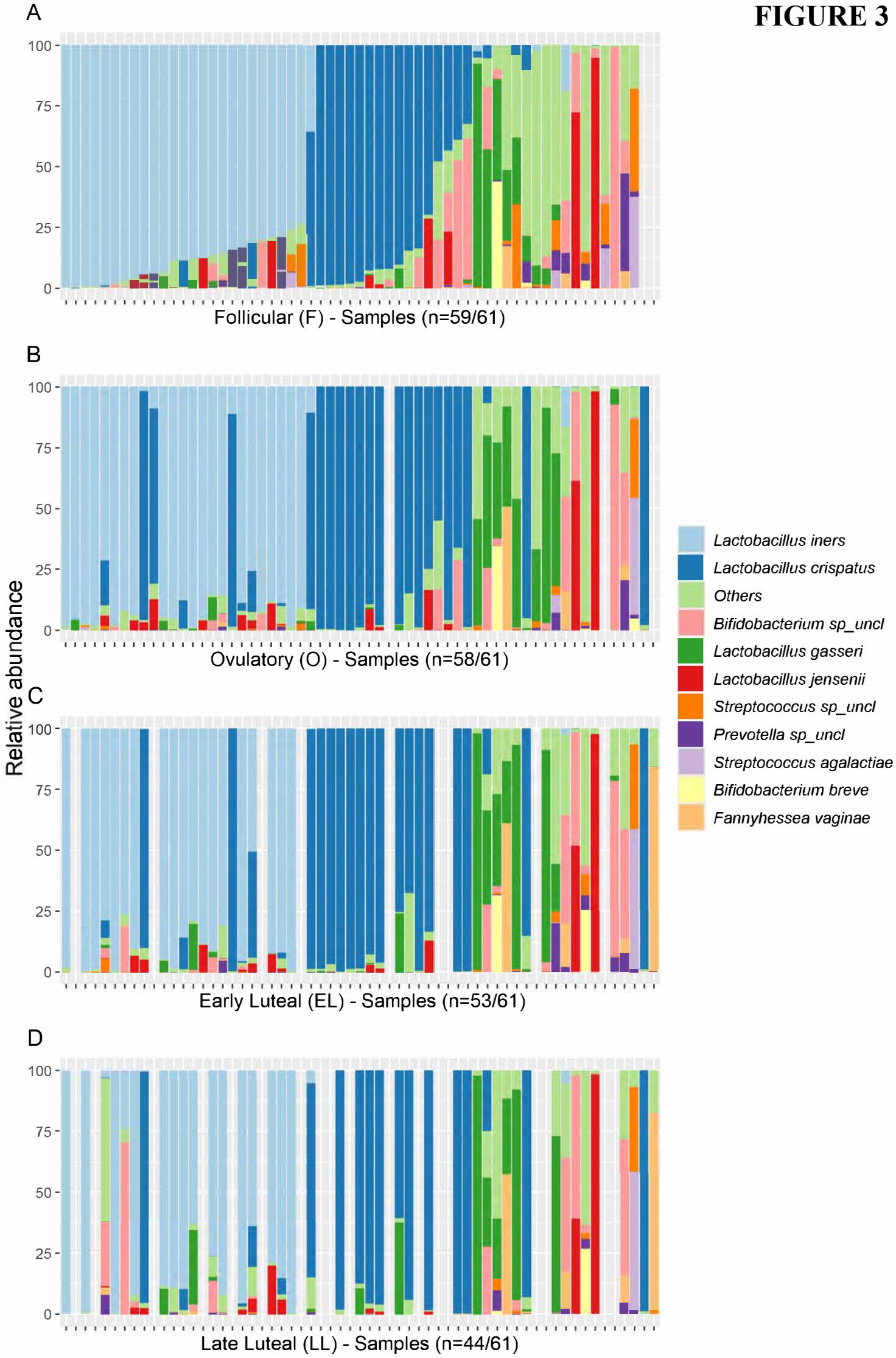
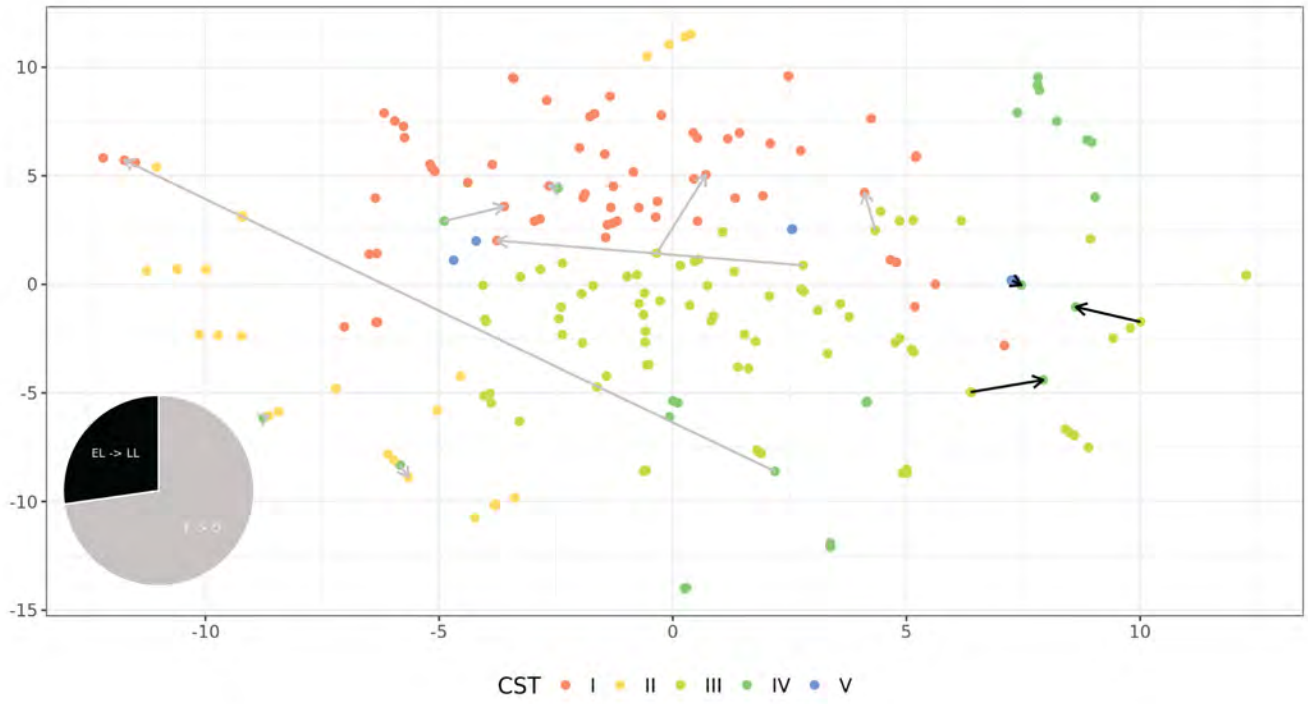

A
B
C
D
Relative abundance
100
75
50
25
0
Follicular (F) - Samples (n=59/61)
Ovulatory (O) - Samples (n=58/61)
Early Luteal (EL) - Samples (n=53/61)
Late Luteal (LL) - Samples (n=44/61)
Lactobacillus iners
Lactobacillus crispatus
Others
Bifidobacterium sp_uncl
Lactobacillus gasseri
Lactobacillus jensenii
Streptococcus sp_uncl
Prevotella sp_uncl
Streptococcus agalactiae
Bifidobacterium breve
Fannyhessea vaginae

FIGURE 4

A


10
5
0
-5
-10
-15
-10
-5
0
5
10
EL -> LL
CST I II III IV V

B

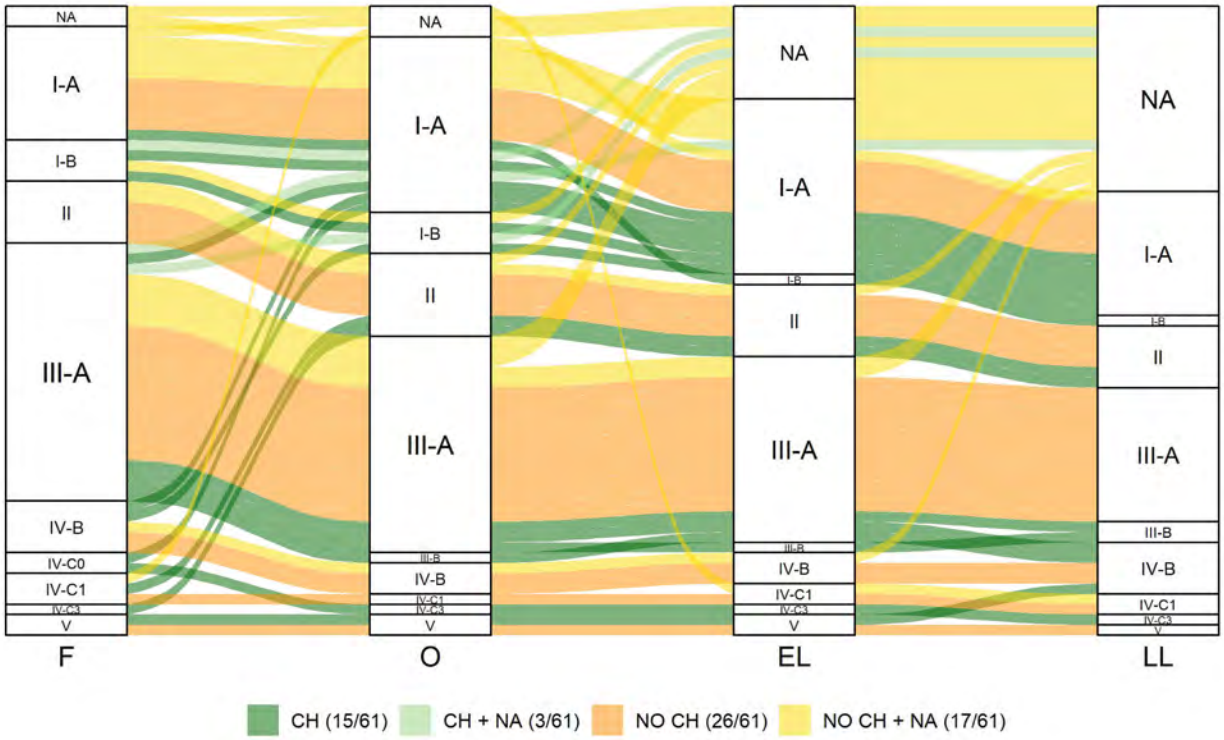

NA
I-A
I-B
II
III-A
IV-B
IV-C0
IV-C1
IV-C3
V
F
O
EL
LL
CH (15/61)
CH + NA (3/61)
NO CH (26/61)
NO CH + NA (17/61)

FIGURE 5

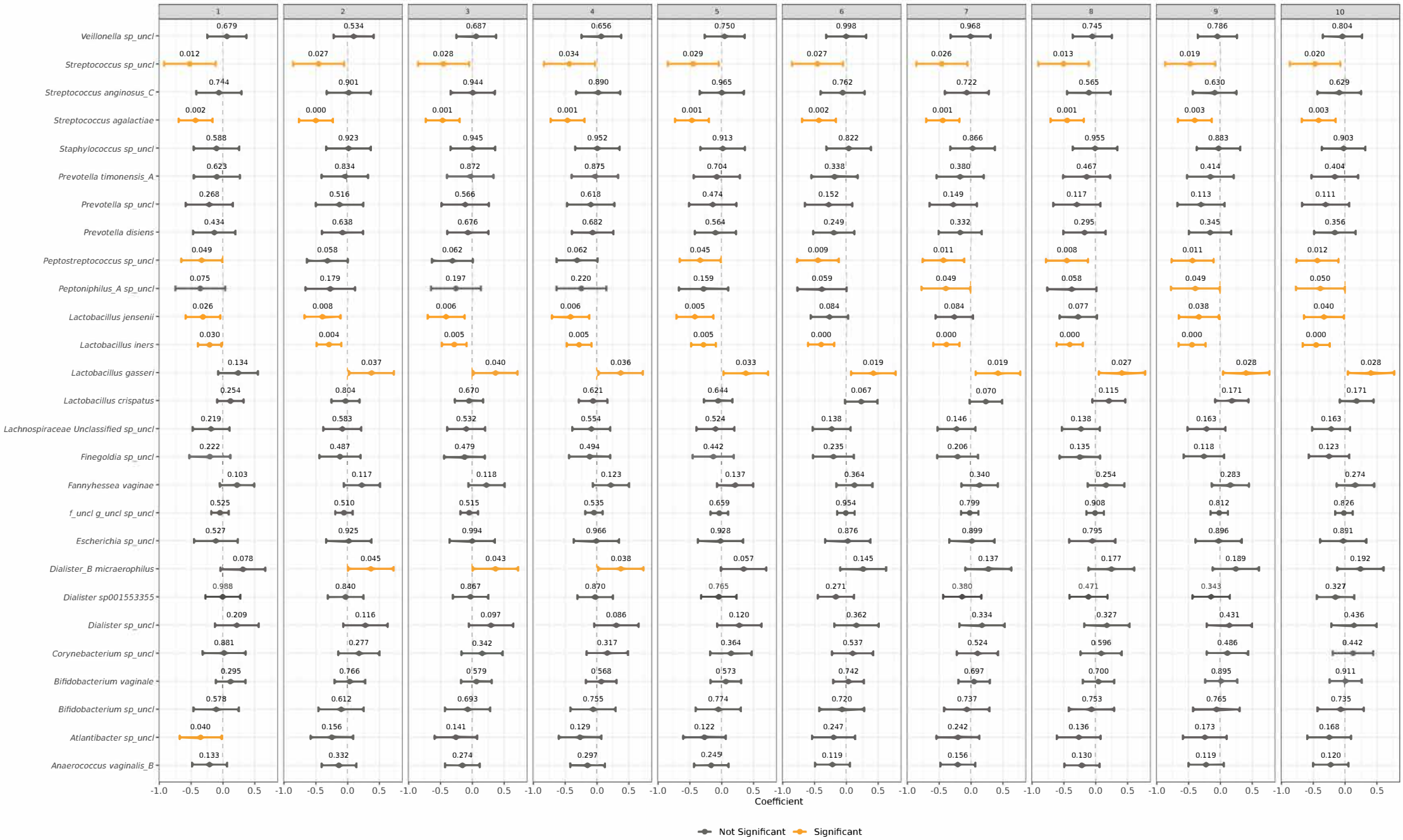
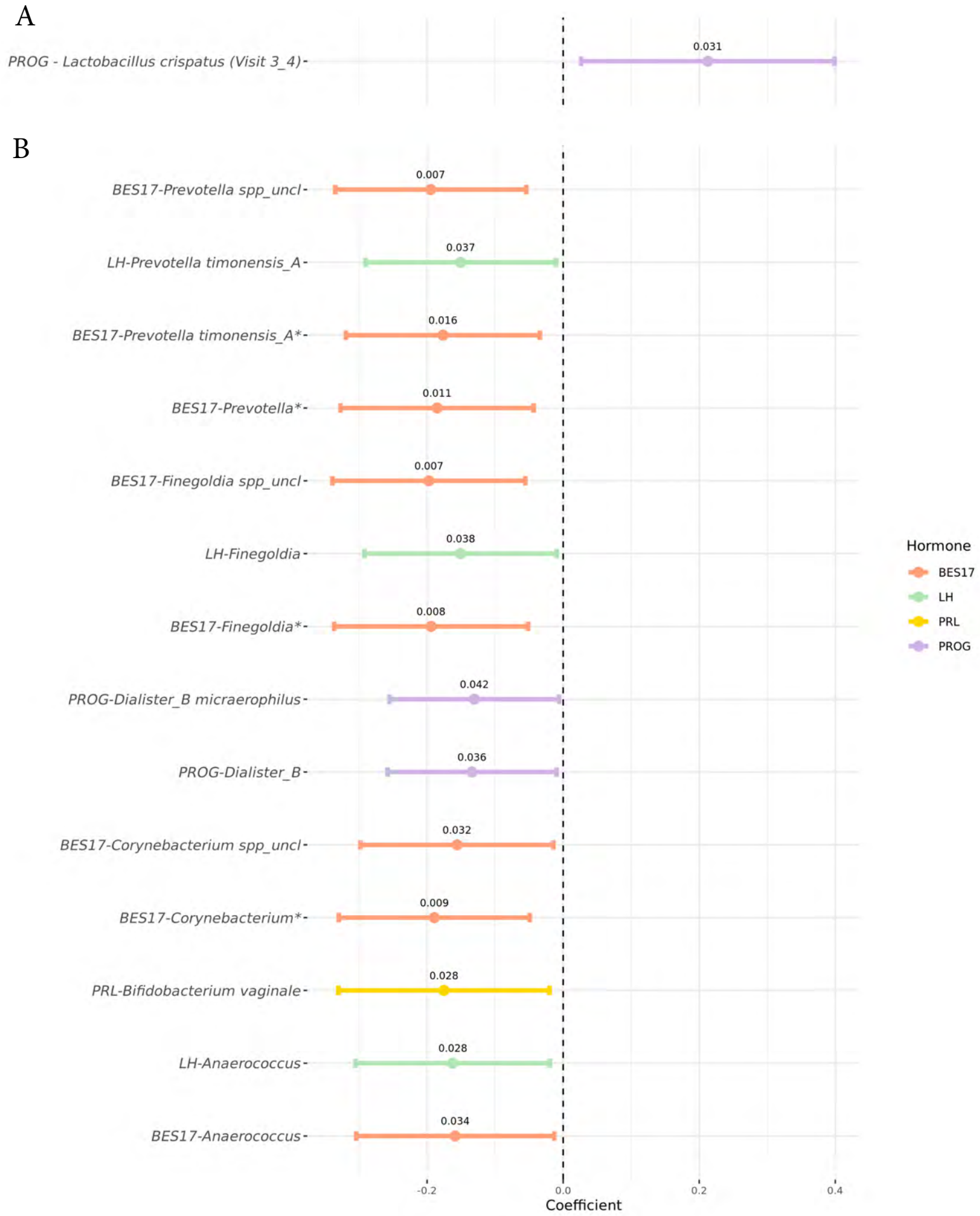

1
2
3
4
5
6
7
8
9
10
Veillonella sp_uncl
Streptococcus sp_uncl
Streptococcus anginosus_C
Streptococcus agalactiae
Staphylococcus sp_uncl
Prevotella timonensis_A
Prevotella sp_uncl
Prevotella disiens
Peptostreptococcus sp_uncl
Peptoniphilus_A sp_uncl
Lactobacillus jensenii
Lactobacillus iners
Lactobacillus gasseri
Lactobacillus crispatus
Lachnospiraceae Unclassified sp_uncl
Finegoldia sp_uncl
Fannyhessea vaginae
f_uncl g_uncl sp_uncl
Escherichia sp_uncl
Dialister_B micraerophilus
Dialister sp001553355
Dialister sp_uncl
Corynebacterium sp_uncl
Bifidobacterium vaginale
Bifidobacterium sp_uncl
Atlantibacter sp_uncl
Anaerococcus vaginalis_B
-1.0 -0.5 0.0 0.5
Coefficient
Not Significant
Significant

FIGURE 6


A
PROG - Lactobacillus crispatus (Visit 3_4)
0.031
B
BES17-Prevotella spp_uncl
0.007
LH-Prevotella timonensis_A
0.037
BES17-Prevotella timonensis_A*
0.016
BES17-Prevotella*
0.011
BES17-Finegoldia spp_uncl
0.007
LH-Finegoldia
0.038
BES17-Finegoldia*
0.008
PROG-Dialister_B micraerophilus
0.042
PROG-Dialister_B
0.036
BES17-Corynebacterium spp_uncl
0.032
BES17-Corynebacterium*
0.009
PRL-Bifidobacterium vaginale
0.028
LH-Anaerococcus
0.028
BES17-Anaerococcus
0.034
-0.2
0.0
0.2
0.4
Coefficient
Hormone
BES17
LH
PRL
PROG