Title: Temporal Dynamics of the Vaginal Microbiome and Host Immune Markers Before, During, and After Metronidazole Treatment for Bacterial Vaginosis

Running Title: Vaginal Microbiome and Immune Responses to Metronidazole in BV

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Abstract This study analyzed metagenomic and immune marker profiles of seven individuals before, during, and after a 7-day course of metronidazole treatment for bacterial vaginosis (BV). Treatment reduced BV-associated bacteria and immune marker levels, with distinct early (days 1-4) and late (days 5-7) phases. Post-treatment, variability in microbial and immune marker profiles demonstrated a rapid resurgence of certain BV associated bacteria, highlighting the need for additional strategies like probiotics to maintain a healthy vaginal microbiome. The study found significant host and microbial influences on immune response variance, with IP-10 and sEcad highly correlated with the vaginal microbiome. The findings identify optimal timing for administering live biotherapeutics to restore D-lactic acid-producing *Lactobacillus* species dominance and underscore the complexity of BV infection and treatment response among different people.

Importance Bacterial vaginosis (BV), a common condition associated with an increased risk of preterm birth and sexually transmitted infections among others, is characterized by a dysbiotic vaginal microbiome associated with dominance of a diverse assortment of anaerobic bacterial species. Metronidazole is the first-line treatment recommended by the CDC for BV when patients report symptoms. Despite treatment, BV recurrence is common. There is limited data regarding the effects of oral metronidazole on the vaginal microbiome starting at initiation of treatment as most studies have compared measurements taken before and after treatment completion. This study utilized metagenomic sequencing, pan-bacterial qPCR, and immune marker measurements to analyze the longitudinal dynamics of the vaginal microbiome and host immune response before, during and after metronidazole treatment.

Keywords vaginal microbiota, vaginal dysbiosis, antibiotics, metagenome, inflammatory markers

INTRODUCTION

Bacterial vaginosis (BV) is a condition with associated significant adverse outcomes, including an increased risk of sexually transmitted infections (STIs)[1-7], pelvic inflammatory disease[8], and premature birth[9,10], and recurrence is often rapid and frequent following antibiotic treatment[11]. BV is characterized by a non-optimal vaginal microbiome, in which optimal *Lactobacillus* species are depleted, and a diverse array of anaerobic bacteria, particularly *Gardnerella* species, predominates[12]. Rapid shifts in microbial composition can be transient or persistent depending on the individual's microbiome stability[13,14]. Although BV etiology is multifactorial, BV generally stems from disruptions to an optimal vaginal microbiome that lead to dysbiosis[15].

Both symptomatic and asymptomatic BV induce genital proinflammatory cytokines, such as interleukin-1ß (IL-1ß)[16], and cause epithelial disruption with the release of soluble E-cadherin (sEcad)[17]. The epithelial damage may be directly caused by local inflammation[18], and the resulting increase in cervical epithelial permeability enhances susceptibility to pathogenic microbes responsible for some STIs, such as HIV[19]. Furthermore, the loss of *Lactobacillus* leads to a less acidic cervicovaginal pH, creating an environment that favors the growth of these pathogens[20]. Comparatively, *Lactobacillus* spp. capable of producing D-lactic acid, such as *L. crispatus*, *L. jensenii*, or *L. gasseri*, exist in a mutualistic relationship with the host. An optimal vaginal microbiome is associated with increased STI protection and immune quiescence, achieved through low-level immune system stimulation, the production of lactic acid[21], and antimicrobial byproducts[15,22]. *L. iners* presents a more complex relationship with reproductive health, because unlike other *Lactobacillus* spp., *L. iners* can only produce L-lactic acid[23]. Studies suggest that *L. iners* can persist in BV-associated microbial communities, potentially facilitating shifts between

dysbiotic and lactobacilli-dominant states[24-26], and it is frequently associated with an increased risk of BV recurrence, STI acquisition, and negative birth outcomes[27,28].

The recommended clinical treatment for BV is topical or oral metronidazole (MET)[29]. While MET can partially restore a balanced vaginal microbiota, studies have shown that the composition does not fully return to its pre-BV state[30]. Following MET treatment, the vaginal microbiota is typically dominated by *L. iners*[11,31-33]. Some people may experience a rebound in beneficial *Lactobacillus* species, leading to the reestablishment of an optimal vaginal microbiome dominated by *L. crispatus*, *L. jensenii*, or *L. gasseri*. However, Mayer *et al*.[11] observed that although MET treatment led to a notable decrease in BV-associated bacteria and an increase in beneficial *Lactobacillus* species, these changes were often temporary, with many participants experiencing a recurrence of BV, along with a resurgence of the BV-associated bacteria within weeks of completing the treatment. Similarly, Turner *et al*. found that while *Lactobacillus* species increased in most participants within 4–14 days post-treatment, persistently high levels of *Gardnerella* species Gsp07 were associated with refractory responses to treatment[34]. A study examining the effects of a 7-day course of oral MET on the vaginal microbiota of Rwandan women with BV found that treatment resulted in a modest reduction of BV-associated anaerobes and an increase in *L. iners*[35]. Yet, the overall cure rate was only 54.5%, with treatment failure associated with higher pretreatment concentrations non-optimal species, particularly *G. vaginalis*. These findings were confirmed by Gustin *et al*.[36], who observed women with greater vaginal microbiota richness at the time of BV diagnosis were more likely to experience recurrent BV following oral MET treatment. And in the case of *L. iners* predominated microbiota, Lee *et al*.[37] reported that MET efficacy decreases in individuals with higher abundances of *L. iners* relative to *G. vaginalis* before treatment. Ultimately, the diversity of vaginal microbiota prior to treatment and the

incomplete restoration of *Lactobacillus* spp. post-treatment leave the vaginal microbiome vulnerable to BV persistence or recurrence[38,39].

These studies, amongst others, underscore the complexity of the vaginal microbiota and the challenges associated with re-establishing and maintaining a *Lactobacillus*-dominated microbiota following antibiotic treatment, regardless of pre-treatment vaginal microbiome composition. A study of South African women treated for BV with a single dose of MET found that while treatment induced short-term shifts in vaginal microbiota and mucosal cytokines, treatment failures led to persistently elevated concentrations of pro-inflammatory cytokines in the genital tract. These persistent inflammatory profiles were associated with an increased risk of HIV acquisition[40]. Armstrong *et al*.[41] observed that treatment of BV with topical MET reduced vaginal inflammation and these benefits were specifically associated with a loss of BV-associated bacteria. The study, which collected genital immune marker and vaginal metagenomic data before and after MET treatment, found that levels of proinflammatory cytokines, chemokines, and soluble immune markers of epithelial barrier disruption were reduced following the 7-day course of antibiotics compared to pre-treatment vaginal concentrations. This was concordant with a reduction in BV-associated bacteria total loads rather than increases in *Lactobacillus* abundance. However, the study did not characterize host and microbiota responses during MET treatment, leaving a critical knowledge gap regarding the immunological and microbiological effects initiated by MET treatment[16].

To address this gap, we leveraged samples from a prior observational study in which participants self-collected daily vaginal swabs over 10 weeks to examine how host immunology, microbiomes, and host immunology-microbiome interactions change throughout BV treatment. In this study, we characterized vaginal immune markers and metagenomes from daily samples

collected before, during, and after a 7-day course of oral MET (500 BID) in seven participants who experienced clinical BV while in the study. Both the immune marker and microbiome composition exhibited inter and intra-personal variation, reflecting the complex interactions between the vaginal microbiome and human immune system, particularly during BV and MET treatment. Nevertheless, D-lactic acid-producing *Lactobacillus* species increased in abundance during the first four days of MET before declining, suggesting a potential window for secondary treatment or adjunctive therapies to enhance *Lactobacillus* restoration and prevent BV recurrence. Additionally, host immune marker analysis showed that IP-10 and sEcad exhibited distinct and complementary interaction patterns with *Lactobacillus* and BV-associated species prior to and during symptomatic BV, highlighting their potential as biomarkers for molecular BV diagnosis.

RESULTS

Participant characteristics. As previously reported, 25 participants experienced symptomatic BV (SBV) while participating in a 10-week observational study[13]. Two vaginal swabs stored dry collected at time points before, during and after MET treatment by seven participants (representing nine SBV events) were available for immune marker and metagenomic analyses. For each SBV event, we aimed to analyze swabs collected at specific time points: two days before SBV onset, the day of BV diagnosis (BVDX), three days during MET treatment, and two days post-treatment, equating 66 data points utilized for this study (**Figure 1**). Two participants (UAB003 and UAB128) experienced two SBV events within the original 70-day study period. The mean age of the participants was 24.5 years; five participants self-identified as Black and two as White. One participant, UAB128, experienced vaginal bleeding throughout the study, but the information collected in their daily diary did not indicate the cause of bleeding. Of the nine SBV events

analyzed, eight resulted in a vaginal microbiome dominated by *L. iners* (CST III) for the first two days after concluding MET. However, only two participants (UAB130 and UAB135) maintained CST III throughout the end of the study, while the remaining participants fluctuated between CST III and CST IV post-MET. Of note, UAB127 maintained CST IV post-MET.

Bacterial species abundance changes. Although longitudinal patterns of total bacterial abundance (measured by panbacterial qPCR) varied per participant, the overall bacterial abundances decreased over the 7-day MET treatment course ($R^2 = 0.19$; $\log_2$ fold change = -1.29; $p = 0.05$; TableS1; Table S2; **Figure 2**). On the day of BV diagnosis (BVDX), the combined estimated absolute abundance of BV-associated taxa (BVT: *Gardnerella* spp., *Sneathia* spp., *Fannyhessea vaginae*, *Ca.* Lachnocurva vaginae, *Prevotella* spp., and *Megasphaera lornae*) was significantly higher than that of D-lactic acid producing *Lactobacillus* (*L. crispatus, L. jensenii, L. gasseri*, *L. paragasseri*, and *L. mulieris*; DL), with a $\log_2$FC of 8.01 ($p = 0.0004$; Table S3; **Figure 2**). During the first four days of MET treatment (early MET), BVT absolute abundance decreased ($\log_2$FC = -2.16; $p = 0.038$) while the aggregate estimated absolute abundance of DL increased ($\log_2$FC = 3.07; $p = 0.026$). However, between days 5 –7 of MET treatment (late MET), DL absolute abundances plateaued ($\log_2$FC = 1.36; $p = 0.19$) and subsequently declined after treatment completion, returning to levels comparable to pre-treatment but with greater variability between participants. No statistically significant *p*-values were observed after late MET treatment. Notably, DL estimated absolute abundances never surpassed that of the BVT at any point during the study (Table S4). Throughout the study, the estimated absolute abundance of *L. iners* did not exhibit any significant changes relative to BVT or between study phases. However, by day 6 of MET treatment, *L. iners* estimated absolute abundance exceeded that of BVT (Table S4). In a regression

model, where the independent variable was the study day relative to BVDX, *L. iners* exhibited the least variation explained by day of study (R^2 = 0.20), followed by BVT (R^2 = 0. 48), and DL (R^2 = 0.59).

Individual species did not necessarily follow the overall trends observed in the combined estimated absolute abundance of BVT, nor DL (**Figure 3; Figure S1**). Throughout the study, *Lactobacillus* species exhibited dynamic shifts in estimated absolute abundance across the different phases of BV treatment. Between the pre-SBV phase and BVDX, all *Lactobacillus* spp. showed either slight decreases or minimal changes, with *L. gasseri* displaying the most significant decrease (log_2FC = -1.36; p = 0.042). However, from BVDX to the end of early MET treatment, several *Lactobacillus* species showed increasing trends. *L. crispatus* significantly increased in abundance with a log_2-fold change of 2.99 (p = 0.029), as did *L. jensenii* (log_2FC of 1.86, p = 0.03), and *L. gasseri* (log_2FC = 1.56, p = 0.04). During the late MET phase, trends were mixed, with some species like *L. gasseri* exhibiting moderate increases, whereas others, such as *L. mulieris* declined. By the end of the 7-day MET treatment course, most species either stabilized or experienced a slight decrease in estimated absolute abundance, suggesting that the initial increases in *Lactobacillus* spp. during early MET treatment were not sustained, and a plateau or reduction occurred post-treatment. Across the entire study, *L. jensenii* and *L. mulieris* had the most variation explained by study day during MET treatment (R^2 = 0.65 and R^2 = 0.57; Table S1).

Vaginal soluble immune factors. The differential abundance of soluble immune markers was assessed across the study phases (**Figure 4**; Table S5). R^2 values ranged from 0.18 to 0.51, indicating variability in model fit across different immune markers (Table S6). Among all samples, matrix metallopeptidase 9 (MMP-9) and soluble E-cadherin (sEcad) were the most abundant

185 immune markers. From pre-SBV to BVDX, MMP-9 significantly increased (FC = 2.95, $p = 0.03$),
186 while sEcad concentrations decreased (FC = -0.35, $p = 0.01$). Throughout and after MET treatment,
187 MMP-9 levels did not significantly change, whereas sEcad concentrations continued to decrease
188 (FC = -1.20, $p = 0.04$). Pro-inflammatory markers MIP-1β, IL-1α, and IL-1β also showed
189 significant increases from pre-SBV to BVDX, followed by non-significant reductions during MET
190 treatment. After MET treatment completion, MIP-1β and IL-1β exhibited another, though less
191 significant, increase (FC = 0.99, $p = 0.06$). No immune markers changed significantly between the
192 early and late MET treatment phases.
193
194 **Relationships between the vaginal microbiome and immune markers.** PERMANOVA tests
195 identified microbiome composition and participant ID as major contributors to the variance
196 observed in immune marker abundances (**Table 1**). IP-10 and sEcad abundance profiles were
197 primarily driven by the microbiome (46.66 and 43.83%, respectively), while participant ID
198 explained more than 40% of the observed variance of IL-1β, MIG, MIP-3α, IL-6, and IL-1α.
199 Further, sEcad and IL-1α were impacted by the study phase more than any other immune marker
200 (18.46 and 14.53%, respectively).
201 Given the significant influence of the vaginal microbiome on IP-10 and sEcad, correlations
202 between the concentrations of these immune markers and the estimated absolute abundances of
203 bacterial taxa were examined across the different study phases. Each phase revealed distinct
204 patterns in both the direction and magnitude of these associations. DL species exhibited positive
205 correlations with IP-10 until the completion of MET treatment, whereas BVT species were
206 predominantly negatively correlated during this period (**Figure 5A**). Conversely, correlations with
207 sEcad followed an inverse trend: DL species consistently maintained negative associations

208 throughout the study, while BVT species demonstrated positive correlations that persisted until
209 post-MET (**Figure 5B**). The strongest correlations were observed during the BVDX and early
210 MET treatment phases, while the correlations between immune markers and bacterial taxa were
211 attenuated in the late and post-MET treatment phases. Post-MET samples were predominantly
212 characterized by negative correlations between bacterial species and the immune markers
213 analyzed.
214

215 **DISCUSSION**

216 The primary treatment for BV has been topical or oral metronidazole since the late 1970's, yet its
217 efficacy in restoring an optimal vaginal microbiome composition[30] and preventing recurrence
218 remains limited[42]. Improved treatments for BV are crucial not only for alleviating symptoms and
219 preventing recurrences, but also reducing its serious consequences, such as increased susceptibility
220 to STIs, urogenital infections, and reproductive complications[1-7,9,10,15,26]. This study employed a
221 high-resolution temporal analysis of the vaginal microbiome and immune marker profiles in seven
222 individuals before, during, and after treatment with oral metronidazole 500 mg twice daily for 7
223 days, addressing a critical knowledge gap in understanding the effects of BV treatment on the host
224 immune response and the vaginal microbiome composition[43]. A key strength of this study was the
225 use of estimated absolute abundance measurements, which provide a more comprehensive view
226 of microbiome dynamics than traditional 16S rRNA gene amplicon sequence-based or
227 metagenomic abundance data. By accounting for variations in total microbial load, this approach
228 enabled precise comparisons across samples and conditions.

229 The effects of MET on the vaginal microbiome composition and soluble immune markers
230 aligned with previous findings; the estimated absolute abundance of BV-associated bacteria

231 decreased, while that of D-lactic acid producing *Lactobacillus* species showed minimal change.
232 Additionally, a reduction in the host immune response was observed when comparing samples
233 pre- and post-MET treatment[41]. However, leveraging the study's higher temporal resolution, we
234 identified two distinct phases of MET treatment based on the estimated absolute abundances of
235 key bacterial species: early MET phase (day 1 – 4) and a late MET phase (day 5 – 7). In particular,
236 DL and *L. iners* estimated absolute abundances increased with a concomitant decrease in most
237 BVT (i.e. *Gardnerella* spp., *Prevoltella* spp., and *Fannyhessa vaginae*). However, other BVT,
238 such as *Megasphaera lorna* and "*Ca.* Lachnocurva vaginae", did not change in abundance during
239 treatment.

240 The post-treatment period, which has been proposed as a critical period for long-term
241 vaginal health[42], exhibited increased variability in both BVT and *Lactobacillus* species estimated
242 absolute abundances across participants compared to earlier stages of the study, suggesting a
243 reestablishment phase of the microbiome. Notably, several bacterial species exhibited a rapid and
244 pronounced increase shortly after MET treatment ended. These species, *P. amnii*, *Prevotella*
245 spNov1, *Prevotella* spNov2, *Anaerococcus prevotii*, *Gemella asaccharolytica*, *Berryella*
246 sp001552935, *Dialister micraerophilus*, and *Disalister* sp001553355, represent taxa with diverse
247 and adaptive characteristics. For instance, *G. asaccharolytica*, a facultative anaerobe[44], may
248 exhibit variable susceptibility to MET. *Prevotella* spp., while strict anaerobes, efficiently
249 metabolize host-derived glycans and mucins[45,46], which may become more accessible as other
250 bacteria decline during treatment. Furthermore, many of these species exhibit reduced
251 susceptibility to MET[47-49]. Evidence suggests that several of these taxa are capable of integrating
252 into biofilms[50-52], where antibiotic penetration is limited, thereby allowing them to persist during
253 treatment and rapidly proliferate post-treatment[53]. Further research is needed to determine whether

these taxa contribute to biofilms formation in the vaginal microenvironment. Collectively, these observations suggest that these species possess mechanisms for surviving treatment and rapidly proliferating in the transient ecological niche created by MET treatment-induced shifts in the microbiome.

Though significant changes in the microbiome were observed during MET treatment, such associations were not quantifiable among most immune markers, underscoring the individualized nature of the vaginal microbiome-host immune interactions[25]. When evaluating factors influencing immune response variance, host and microbiome composition were the strongest contributors, with the microbiome exerting a greater influence on IP-10 and sEcad than the host. Interestingly, these immune markers were influenced by the vaginal microbiome more than the host. This underscores the microbiome's role in modulating immune response, consistent with previous studies[40,54]. Although study phase contributed to immune marker variance in sEcad, IL-1α, and IP-10, its effect was modest compared to host and microbiome factors. However, the substantial unexplained variance in several immune markers, particularly IL-6, IFNα2a, and IL-17A, suggests that additional, unmeasured factors influence immune marker levels.

Recognizing the substantial impact of the vaginal microbiome on IP-10 and sEcad, correlations between these immune markers and the estimated absolute abundances of DL and BVT across study phases were analyzed. The strongest correlations were observed at BVDX and during the early MET treatment phase, signifying that this as a period of heightened microbiome-immune interactions. By contrast, the attenuation of correlations attenuated in the late and post-MET treatment phases, possibly reflecting the diminished bacterial load or a transient ecological state dominated by *L. iners*.

More specifically, IP-10 and sEcad demonstrated distinct interaction patterns between DL and BVT species during BV, further supporting their potential as biomarkers for molecular BV diagnosis. Prior to MET treatment completion, DL species were positively correlated with IP-10, supporting previous findings[41,55] and the role DL species in host anti-inflammatory regulation[7]. IP-10 (CXCL10) is a chemokine that plays a key role in immune regulation and defense against pathogens. Its primary function is to recruit immune cells, particularly activated T cells, natural killer cells, and monocytes, to sites of inflammation or infection via binding to its receptor, CXCR3[56]. Notably, IP-10 and IL-1β comprise the rapid point-of-care Genital Inflammation Test [GIFT] test for both BV and STIs[57]. In contrast, sEcad exhibited an inverse relationship with DL and BVT. BVT species and sEcad were positively correlated prior to and during MET treatment, while DL species remained negatively correlated with sEcad throughout the study. As an essential component of epithelial cell-cell junctions, sEcad is essential for maintaining epithelial barrier integrity that prevents the passage of harmful substances and pathogens[58]. The study by Liu *et al*.[19] identified sEcad as a potential biomarker for vaginal epithelial disruption, indicating that elevated levels of this protein may reflect damage to the genital epithelium and could be used to assess epithelial integrity in reproductive health. However, our findings support the use of sEcad as a general biomarker for molecular BV diagnosis.

Despite the limited sample size, the study design, which included sampling prior to SBV, at BV diagnosis, during MET treatment, and post-treatment, provided novel insights into the effects of MET on the microbiome composition, immune responses, and their interplay. The significant variance in immune marker profiles was largely influenced by both the microbiome and the individual participant. While participant variation was evident, sEcad and IP-10 emerged as promising biomarkers of vaginal microbiome dynamics during a symptomatic BV event and

MET treatment due to their levels strongly impacted by microbiome factors and their distinct interaction patterns with DL and BVT species.

Understanding the mechanisms driving the rapid resurgence of BV-associated bacteria provides critical insights into post-MET treatment microbial recovery dynamics and informs the development of targeted interventions to prevent recurrence. In this study, no participant transitioned to a protective *Lactobacillus*-dominated vaginal microbiome[12,59]. Instead, individuals either maintained a CST III microbiome, fluctuated between CST III and CST IV microbiomes, or experienced BV recurrence. These findings underscore the limitations of antibiotic therapy alone and emphasize the need for adjunctive strategies that promote *D*-lactic acid-producing *Lactobacillus* spp. growth during the early phase of MET treatment and foster post-therapy resilience to enhance treatment outcomes[60-65].

Therapies aimed at modulating the composition of the vaginal microbiome, including probiotics combined with MET treatment for BV, have had mixed success but remains promising. A prospective, parallel-group, randomized, controlled study found that oral *L. rhamnosus* GR-1 and *L. reuteri* RC-14[66] (aka Lactogyn), when administered alongside MET, had no effect on the 30-day BV cure rate compared to MET alone[67]. Similarly, EcoVag vaginal probiotic capsules containing *L. gasseri* and *L. rhamnosus*, given after a seven-day course of 2% vaginal clindamycin cream, did not improve the 30-day cure rate[68]. The inefficacy of these products may be due to the fact that the species comprised in these probiotic formulations are not naturally present in the vaginal microbiome[69]. In contrast, LACTIN-V, a live biotherapeutic product containing *L. crispatus* CTV-05[70], significantly reduced BV recurrence when administered vaginally after MET therapy. Participants receiving LACTIN-V were more likely to achieve long-term BV remission compared to placebo groups[70,71]. Vaginal microbiome transplantation is another promising

322 approach for introducing beneficial *Lactobacillus* species to promote long-term BV remission in
323 combination with MET therapy[72]. Notably, all these studies have attempted to modulate the
324 vaginal microbiome after MET therapy. Interestingly, our study suggests that the optimal window
325 for intervention with probiotics, live biotherapeutics or VMT may be early in MET treatment,
326 when D-lactic acid-producing species are naturally increasing relative to BV-associated taxa. This
327 finding warrants further investigation.

328 Comprehensive analyses of the vaginal microbiome and immune factors during MET
329 treatment has the potential to provide a wealth of information regarding microbial-host temporal
330 dynamics while attempting to clear BV, and therefore methods to improve diagnosis and treatment
331 outcome. This study paves lays the groundwork for future research aimed at understanding the
332 complexities of BV infection and nuanced treatment responses among different people, optimizing
333 the live biotherapeutic delivery timing to restore DL species dominance, and advancing biomarker-
334 based BV diagnostics. For improved methods of clearing BV and alleviating its adverse sequelae
335 to become a reality, longitudinal studies with larger cohorts will be essential to validate and expand
336 upon these findings.

337

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343

344 **MATERIAL AND METHODS**

Human cohort. This analysis used samples collected between September 2008 and July 2010 from 135 non-pregnant female participants of reproductive age enrolled in a 10-week prospective longitudinal study at the University of Alabama at Birmingham in which two vaginal dry swabs were self-collected daily[13]. Participants received clinical evaluations at enrollment, at weeks 5 and 10, as well as when the participant experienced vaginal symptoms. For this secondary analysis, we analyzed swabs surrounding symptomatic BV (SBV) events clinically diagnosed using Amsel's criteria[73] and treated with MET 500 BID for 7 days. Of the original 25 participants that experienced SBV and received treatment, swabs were available for all study phases from seven participants and nine SBV events. For each of these, swabs collected two days prior to SBV (n = 19), the day of BV diagnosis (BVDX, n = 8), three days during MET (n = 20), and two days after MET (n = 17) were used to generate genomic DNA extractions, metagenomic sequencing, and immune marker profiles in this pilot study. The swab designated for immune marker profiling was shipped to the University of Toronto. These swabs were collected as part of previously published study[13] and the clinical study protocol was approved by the Institutional Review Board of the University of Alabama at Birmingham and the University of Maryland School of Medicine. Written informed consent was appropriately obtained from all participants.

Immune marker measurements. Eluates from vaginal swabs in PBS were thawed and centrifuged at 4500 rpm for 30 minutes prior to soluble immune factor measurement. Supernatant was then removed and analyzed with the MSD platform according to the manufacturer's instructions as previously described[13]. Cytokines and soluble immune factors included sEcad, IFNα2a, IL-17A, IL-1α, IL-1β, IL-6, IL-8, IP-10, MIG, MIP-1β, MIP-3α, and MMP-9. Cytokine

and other soluble immune factor data were log10 transformed to minimize effects of highly abundant immune markers.

DNA extraction, qPCR, and metagenomic sequencing. Genomic DNA was extracted as previously described[13]. The total 16S rRNA gene copy number was determined using a PanBacterial qPCR method previously developed by Liu et al [74]. Barcoded genomic DNA libraries were constructed using the NEBNext® DNA Sample Prep Master Mix Set 1 (New England Biolabs). Metagenomic libraries were sequenced on a NovaSeq 6000 platform on an S2 Flowcell and generate 55 million reads per sample. Each sequencing plate included wells with nuclease-free water as a negative control and positive extraction controls using the ZymoBIOMICS Microbial Community Standard. The negative controls were nuclease-free water. Host reads were removed using BMTagger[75] with the GRCh38 human genome as reference. The remaining metagenomic reads were quality filtered using fastp (v.0.21)[76] and then mapped to VIRGO2[77]. The abundance of genes mapped to VIRGO2[77] were normalized by gene and average library length (gene abundance x 150 / gene length). The gene abundance counts for each species were summed to represent the abundance of that species and normalized by sequencing depth. The species counts were then multiplied by the panbacterial qPCR abundance within each sample to get the estimated absolute abundance of each microbial taxa. The data was log10 transformed to minimize effects of highly abundant species. Metagenomic data are available (PRJNA208535).

16S rRNA gene amplicon sequencing data. For this analysis we also examined existing amplicon sequencing data (PRJNA208535)[13]. Taxonomy was assigned using SpeciateIT and vSpeciateDB[78] and CSTs assigned using VALENCIA[79].

Statistical analysis. Wilcoxon's matched-pairs signed-rank test for each immune marker and microbial taxa were performed using the samples collected on BVDX and the day after the 7-day course of MET was complete with the function wilcox.test (alternative = TRUE, exact = FALSE) as part of the R stats package v4.3.1[80]. The mixed effects polynomial regression models for the immune marker and microbiome data were produced and tested using the R Statistical Software stats, lme4 v1.1-35.1[81], performance v.0.12.2[82], and splines v4.3.1 packages[80]. R^2 values were obtained using the R package MuMIn v1.47.5[83]. Within the formula, time point was the fixed effect, species or immune marker concentrations were the dependent variables, and participant ID was considered a random effect. Model selection criteria, including Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), and Log-Likelihood, were calculated using associated functions as part of the stats R package. Residuals were extracted and the Shapiro-Wilk test was applied to assess the normality of the residuals. Bootstrapping was conducted to assess the robustness of the model's estimates using the bootMer function[81].

Differential abundance analysis was performed for immune markers and taxa between the first day of SBV and BVDX, BVDX and day 4 of MET, day 4 of MET and the final day of MET, and day 7 of MET and the final day of the study. Taxa only present in 10% of samples across both timepoints were removed. For each retained taxa and immune marker, a two-sample t-test was performed to assess significant differences in abundance between the two treatments. Variable importance in projection (VIP) scores were calculated with the mixOmics v.6.26.0 R package[84].

The immune response variance explained by each factor considered in this study was estimated through permutational multivariate ANOVA (PERMANOVA) tests. The vaginal microbiome composition was represented by the first 12 principal coordinates, which explained

413 >70% of the variation in the microbiome, to avoid species-species correlations producing
414 overestimations. Principal coordinate analysis (PCoA) was performed on the log10 normalized
415 microbiome data using the R package vegan v2.6-4[85]. The input data for the PCoA was a Bray-
416 Curtis dissimilarity matrix, obtained through the vegdist function (method = "bray"). PCoA was
417 performed using the wcmdscale function (eig = TRUE, add = "cailliez"). PERMANOVA tests for
418 each immune marker were conducted using the adonis2 function (permutations = 5040, method =
419 "bray). The results were filtered to only include significant contributors ($p < 0.2$) and then the
420 principal coordinates were summed to get the variance caused by the microbial composition, in
421 addition to participant ID and phase of BV treatment (i.e. prior to SBV, BVDX, early MET, late
422 MET, and after MET).
423
424 **Correlation analysis.** The R stats package v4.3.1[80] was used to determine whether significant
425 correlations exist between the metagenomic and immune marker data for each stage of BV
426 treatment. The initial species abundance table was filtered before normalization, removing species
427 with a frequency of less than 200 reads across all samples to eliminate low abundant species that
428 could skew the correlation tests. The normalized estimated absolute abundance data at the species
429 level was used to calculate pairwise correlations with the soluble immune marker profiles using
430 the Spearman method, as well as adjustment of *p*-values using the Benjamini-Hochberg method
431 with the corr.test function. For simplicity, only major *Lactobacillus* spp. and BV-related taxa were
432 included and correlations were retained if the associated *p*-value was less than 0.05. Correlations
433 were visualized with heatmap plots generated using the Heatmap function in the circlize v0.4.15
434 R package[86].
435
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Table 1. Estimated immune abundance variation. The observed variation (%) in the cytokine concentrations explained by the vaginal microbiome, participant ID, and phase of BV treatment (*i.e.,* prior to SBV, BVDX, early MET, late MET, and after MET) were calculated by applying PERMANOVA tests to each cytokine. The vaginal microbiome composition was represented by the first 12 principal coordinates to avoid species-species correlations producing overestimations and significant contributors were summed to get the percent variance explained by the microbiome. The remaining variation not accounted for by the above factors was considered unexplained.

Cytokine	Microbiome	Participant ID	Study Phase	Remaining Unexplained
IP-10	46.66	19.98	10.51	22.85
sEcad	43.83	25.05	18.56	12.56
MIG	42.17	45.7	4.61	27.52
IL-8	37.33	36.54	2.67	23.46
MIP-1β	36.71	35.13	1.5	26.67
IL-1α	36.15	40.03	14.53	9.3
IL-17A	34.73	25.5	4.82	34.96
MMP-9	33.51	38.67	0.99	26.83
IFNα2a	33.4	28.67	2.23	35.7
MIP-3α	28.81	44.33	0.85	26.01
IL-1β	18.88	52.69	3.03	25.41
IL-6	14.25	43.56	2.83	39.36

660

661 **FIGURE LEGENDS**

662 **Figure 1. Pilot study design.** Participants clinically diagnosed with BV and prescribed a 7-day
663 oral MET regimen as part of the UMB-HMP study[13] provided daily self-collected vaginal swabs
664 over a 10-week period, with samples selected to represent days preceding symptomatic BV, the
665 day of diagnosis, the MET treatment period, and post-treatment days. The color of each day
666 signifies the CST assigned based on 16S-rRNA sequencing and shape denotes the study phase.
667 Days for which immunological and metagenomic sequencing data were generated or only 16S-
668 rRNA sequencing was available are indicated by larger and smaller point sizes, respectively. Red
669 lines designate days during which the participant experiences menses.

670 **Figure 2. Bacterial estimated absolute abundance through study phases.** The log10
671 transformed panbacterial total abundances (qPCR; black), in addition to the combined estimated
672 absolute abundance of BVT (*Gardnerella* spp., *Sneathia* spp., *Fannyhessea vaginae*, *Ca.*
673 Lachnocurva vaginae, *Prevotella* spp., and *Megasphaera lornae*; blue), D-lactic acid producing
674 *Lactobacillus* (DL; *Lactobacillus crispatus, L. jensenii, L. gasseri*, *L. paragasseri*, and *L.*
675 *mulieris*; red), and *L. iners* (orange) were plotted for all participants through each phase of the
676 pilot study (prior to SBV, BVDX, early MET, late MET, and after MET). Mixed effects models
677 were generated for each group (solid lines), with 95% confidence intervals applied.

678

679 **Figure 3. Bacterial abundances over time.** The change in estimated absolute abundance (log10
680 transformed) from BVDX of key taxa included in DL (red headers) and BVT (light blue headers)

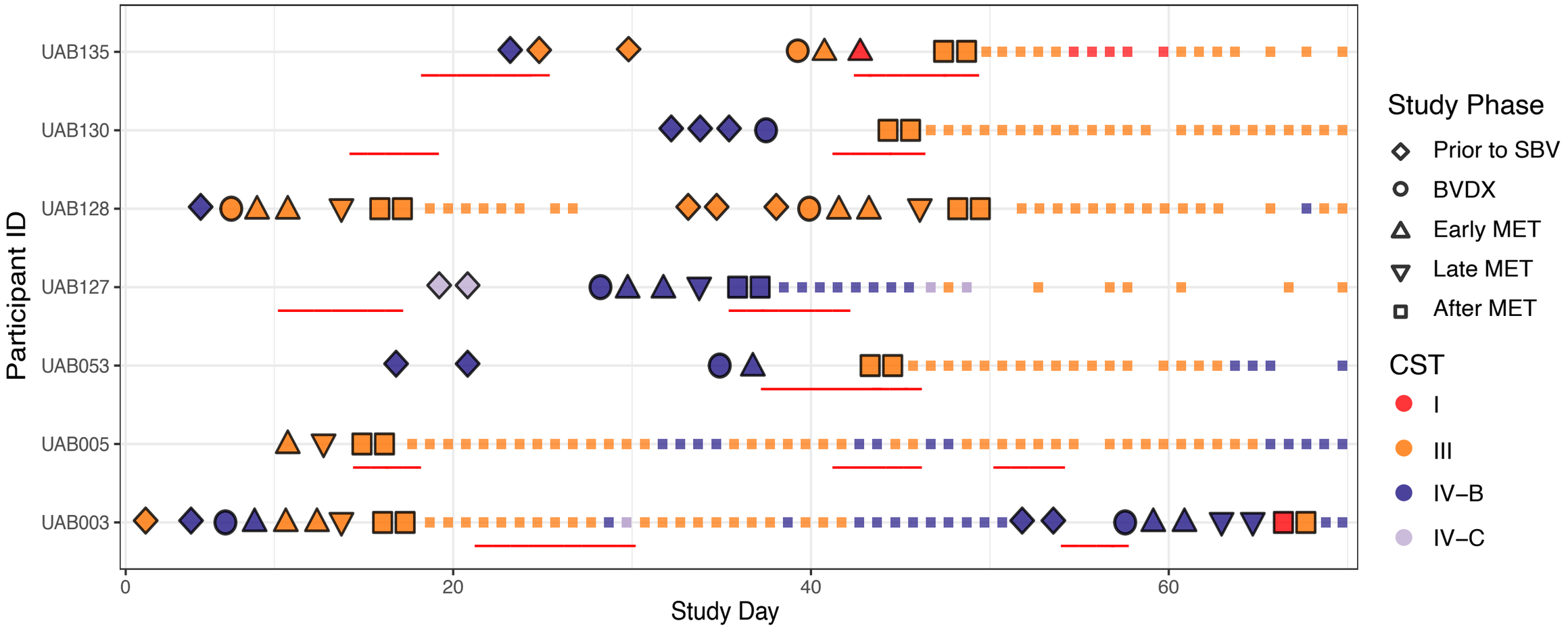
for all participants were plotted across the study, with days relative to BVDX, which is represented by day 0. Negative days are those prior to SBV, and day 1 represents the first day the participant started their 7-day course of oral MET. Mixed effects models (solid red lines) and 95% confidence intervals (dotted red lines) were generated for each species, with the study day as the fixed effect, estimated absolute abundance as the response variable, and participant ID considered a random effect. The conditional R^2 values associated with the respective taxa are displayed.

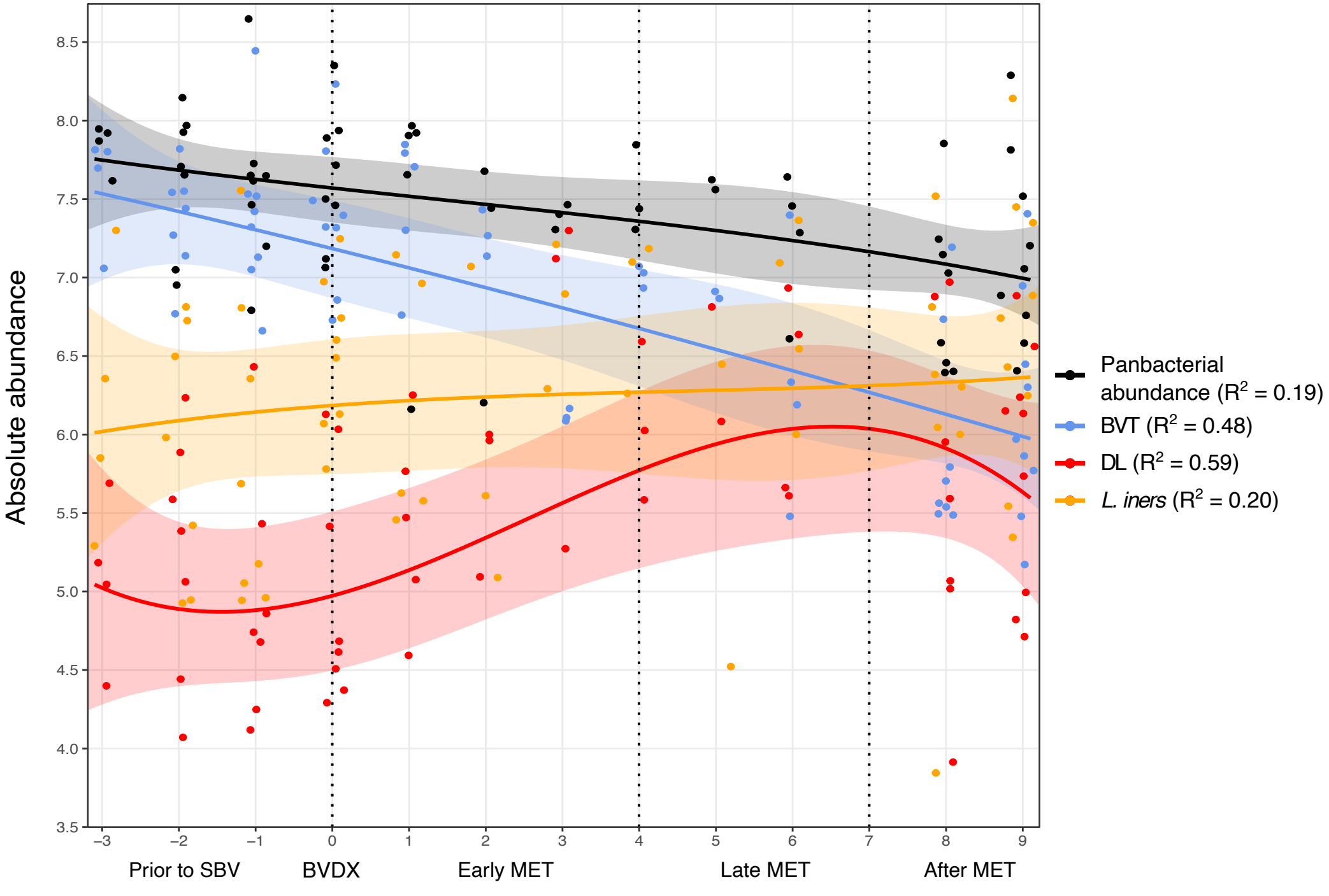
Figure 4. Immune concentrations over time. The concentrations (pg/mL) of each immune marker for all participants was plotted for each day of the study, with day of study relative to BVDX (day 0). Negative days are those prior to SBV, and day 1 represents the first day the participant started their 7-day course of oral MET. Immune marker concentrations were log10 transformed and mixed effects models (solid red lines) and 95% confidence intervals (dotted red lines) were generated for each immune marker, with the study day as the fixed effect, immune marker concentration as the response variable, and participant ID considered a random effect. The conditional R^2 values associated with the respective cytokine are present in the plot titles. * indicates the cytokine had a p-value < 0.05 associated with its differential abundance during any phase of the study.

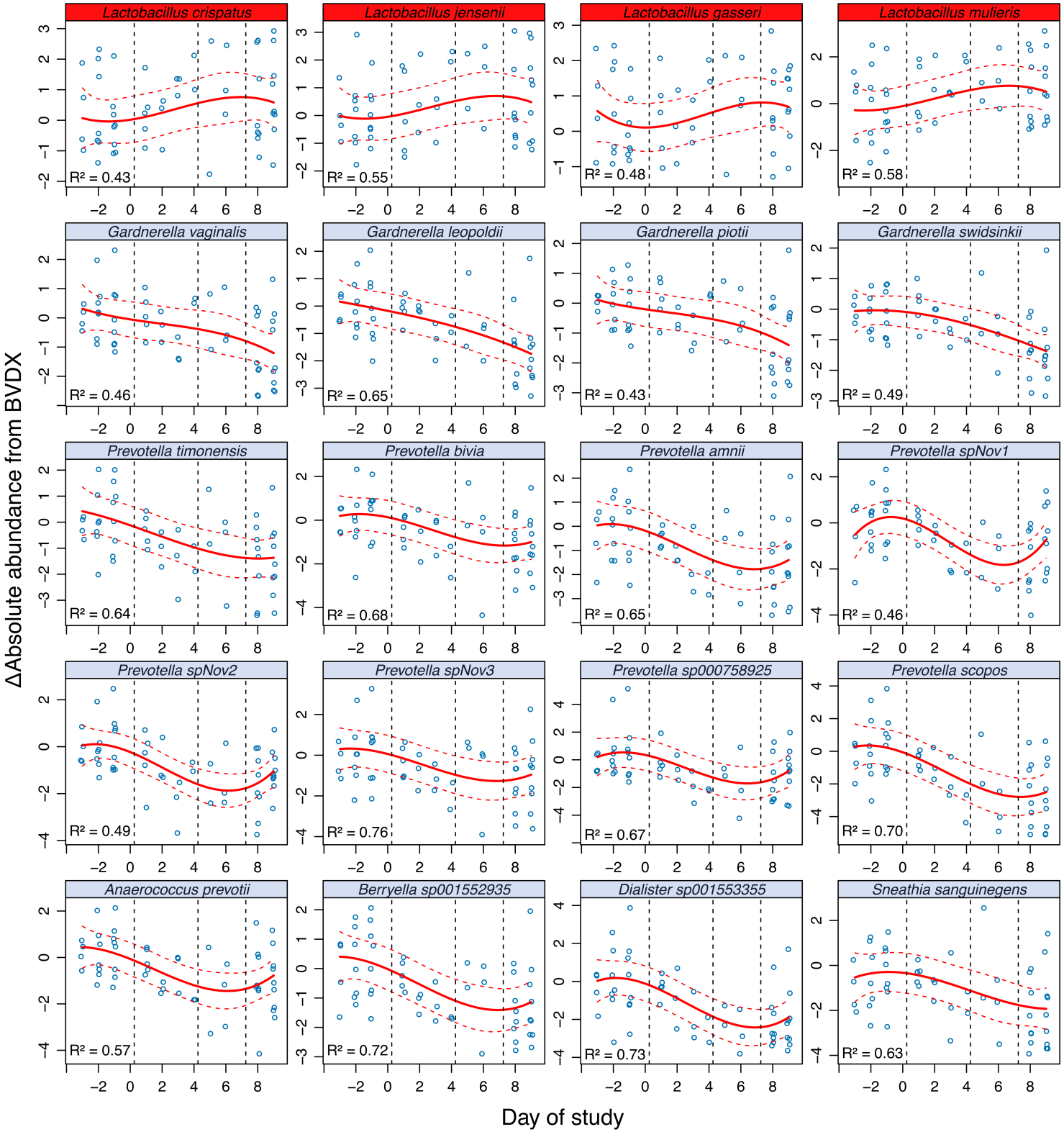
Figure 5. Correlations between immune markers and the vaginal microbiome taxa. Spearman correlations between concentrations of the immune markers most influenced by the vaginal microbiome (IP-10 and sEcad) and the estimated absolute abundance of key taxa were plotted across each phase of the study. A) IP-10 was negatively correlated with many BV-related

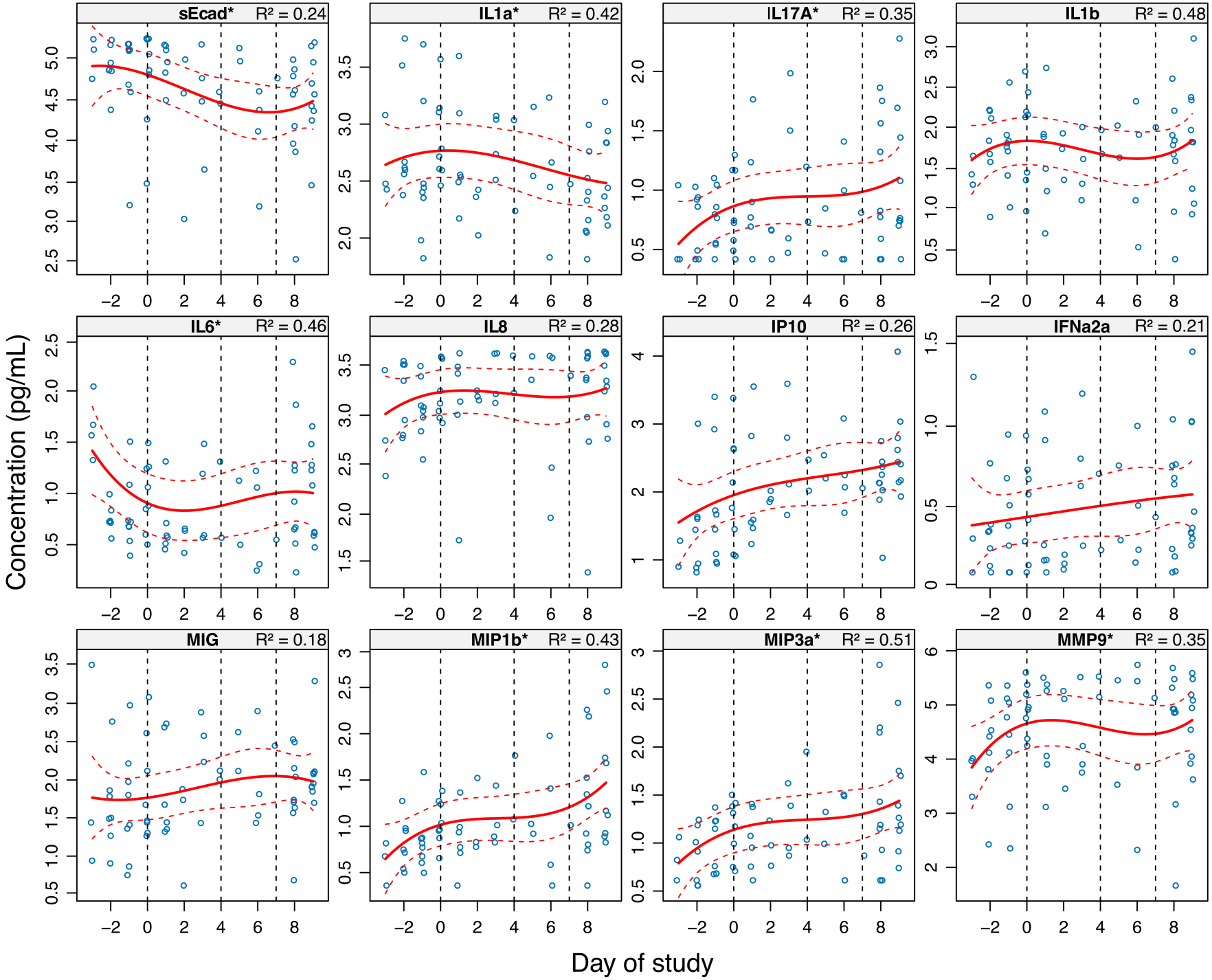
704 and lower abundance vaginal species, and B) sEcad had positive correlations with BV-related

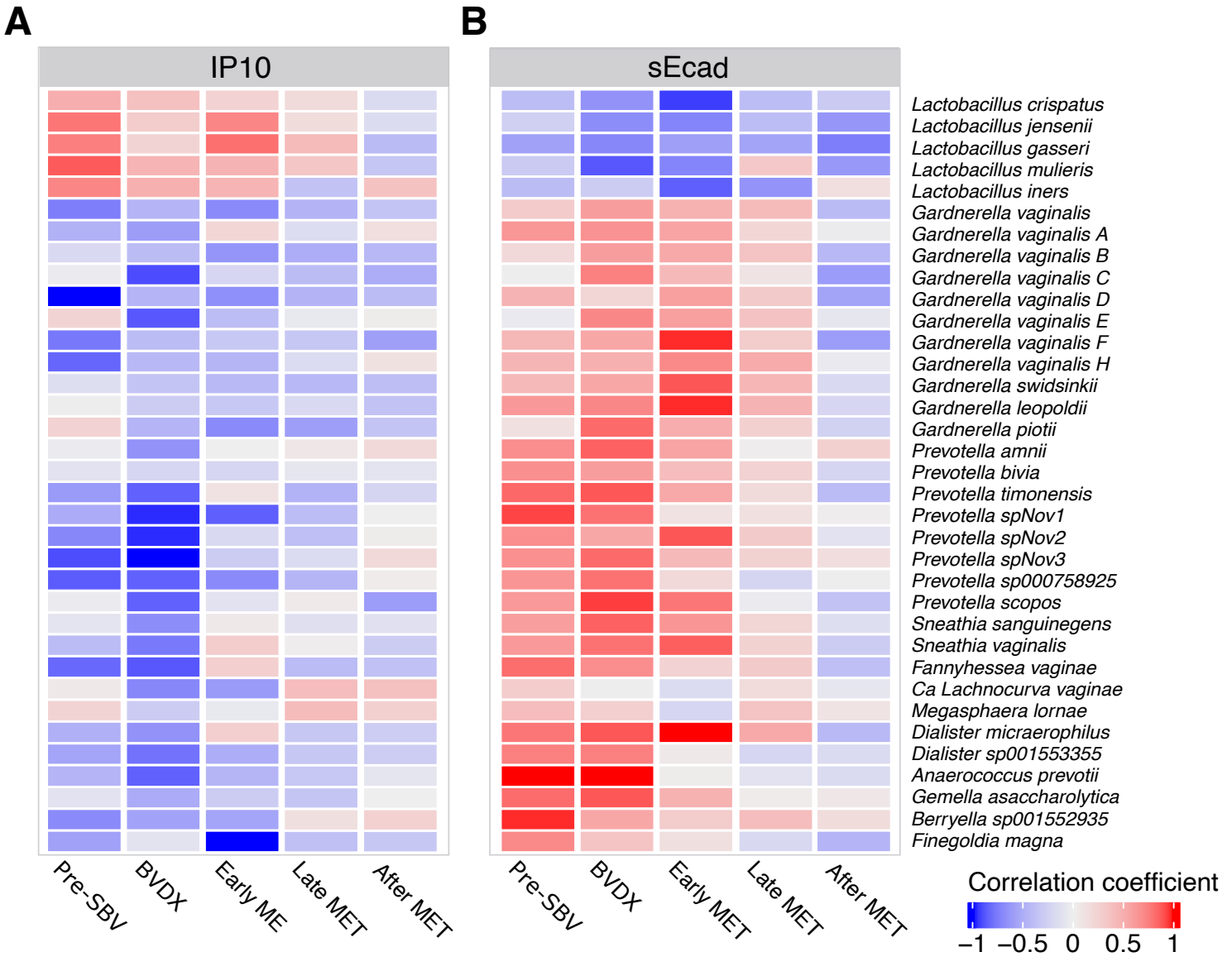
705 species and negative correlations with vaginal *Lactobacillus* species.


Participant ID
UAB135
UAB130
UAB128
UAB127
UAB053
UAB005
UAB003
0
20
40
60
Study Day
Study Phase
Prior to SBV
BVDX
Early MET
Late MET
After MET
CST
I
III
IV–B
IV–C


Absolute abundance
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
−3
−2
−1
0
1
2
3
4
5
6
7
8
9
Prior to SBV
BVDX
Early MET
Late MET
After MET
Panbacterial abundance (R^2 = 0.19)
BVT (R^2 = 0.48)
DL (R^2 = 0.59)
L. iners (R^2 = 0.20)






A
IP10
B
sEcad
Lactobacillus crispatus
Lactobacillus jensenii
Lactobacillus gasseri
Lactobacillus mulieris
Lactobacillus iners
Gardnerella vaginalis
Gardnerella vaginalis A
Gardnerella vaginalis B
Gardnerella vaginalis C
Gardnerella vaginalis D
Gardnerella vaginalis E
Gardnerella vaginalis F
Gardnerella vaginalis H
Gardnerella swidsinkii
Gardnerella leopoldii
Gardnerella piotii
Prevotella amnii
Prevotella bivia
Prevotella timonensis
Prevotella spNov1
Prevotella spNov2
Prevotella spNov3
Prevotella sp000758925
Prevotella scopos
Sneathia sanguinegens
Sneathia vaginalis
Fannyhessea vaginae
Ca Lachnocurva vaginae
Megasphaera lornae
Dialister micraerophilus
Dialister sp001553355
Anaerococcus prevotii
Gemella asaccharolytica
Berryella sp001552935
Finegoldia magna
Pre-SBV
BVDX
Early ME
Late MET
After MET
Pre-SBV
BVDX
Early MET
Late MET
After MET
Correlation coefficient
−1 −0.5 0 0.5 1