Cervicovaginal microbial features predict *Chlamydia trachomatis* spread to the upper genital tract of infected women

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ABSTRACT

Introduction: *Chlamydia trachomatis (CT)* infection can lead to pelvic inflammatory disease, infertility and other reproductive sequelae when it ascends to the upper genital tract. Factors including chlamydial burden, co-infection with other sexually-transmitted bacterial pathogens and oral contraceptive use influence risk for upper genital tract spread. Cervicovaginal microbiome composition influences CT susceptibility and we investigated if it contributes to spread by analyzing amplicon sequence variants (ASVs) derived from the V4 region of 16S rRNA genes in vaginal samples collected from women at high risk for CT infection and for whom endometrial infection had been determined.

Results: Participants were classified as CT negative (CT-, n=77), CT positive at the cervix (Endo-, n=77), or CT positive at both cervix and endometrium (Endo+, n=66). Although we were unable to identify many significant differences between CT infected and uninfected women, differences in abundance of ASVs representing *Lactobacillus iners* and *L. crispatus* subspecies but not dominant lactobacilli were detected. Twelve informative ASVs predicted endometrial chlamydial infection (AUC=0.74), with CT ASV abundance emerging as a key predictor. We also observed a positive correlation between levels of cervically secreted cytokines previously associated with CT ascension and abundance of the informative ASVs.

Conclusion: Our findings suggest that vaginal microbial community members may influence chlamydial spread directly by nutrient limitation and/or disrupting endocervical epithelial integrity and indirectly by modulating pro-inflammatory signaling and/or homeostasis of adaptive immunity. Further investigation of these predictive microbial

factors may lead to cervicovaginal microbiome biomarkers useful for identifying women at increased risk for disease.

INTRODUCTION

Chlamydia trachomatis (CT) is the most common, sexually transmitted, bacterial infection globally (1), with young adults at greatest risk for infection. During childbirth, CT can also be transmitted to a newborn through contact with infected cervical tissue and secretions, resulting in infection of mucous membranes of the eye, oropharynx, urogenital tract, and rectum (2). In addition, CT has been extensively studied as a potential co-factor for human papillomavirus (HPV) enhancing cervical cancer risk through cellular transformation, viral load enhancement, and oncogene overexpression (3). When CT ascends from the cervix to the endometrium or uterus, it can lead to upper genital tract infection, increasing risk for pelvic inflammatory disease and reproductive sequelae such as ectopic pregnancy, or infertility (2). Despite the availability of diagnostics and effective treatments, the asymptomatic nature of CT infection often leads to undiagnosed cases, contributing to its importance as a global health burden (4).

The cervicovaginal microbiome (CVM) is frequently classified into community state types (CSTs), based on the dominant bacterial species present. Using 16s rRNA gene sequencing, Ravel et al. defined the most common CSTs (I-V) (5). CSTs I, II, III, and V are dominated by specific *Lactobacillus* species with overall lower diversity of other microbiota and are generally associated with healthy reproductive health outcomes (6, 7). However, CST IV is comprised of heterogeneous, predominantly anaerobic bacterial genera and *Lactobacillus* species are sparse (5). CST IV is associated with adverse health outcomes such as bacterial vaginosis (BV) (8), pregnancy failure (9), and pre-term birth (10). Vaginal dysbiosis, strongly associated

with CST IV, has also been linked to increased STI vulnerability (7, 8, 11-13). In particular, *in vitro* (14, 15) and *in vivo* (16, 17) studies have associated indole-producers within the CVM with CT evasion of IFN-mediated depletion of tryptophan, an essential chlamydial nutrient (18, 19). Anaerobes also produce biogenic amines and proinflammatory short-chain fatty acids, promoting symptoms and disease (20, 21). In contrast, *Lactobacillus crispatus*-dominated CST I is considered protective against CT infection (8, 13, 22). Mechanisms contributing to protection include production of lactic acid that can directly kill CT (23) while D(-) lactic acid produced by some *Lactobacillus sp.* inhibits CT infection by down-regulating host epithelial cell proliferation (6). CVM-derived metabolites may also act on their host to stimulate DC activation and accelerate immunity (24). STI co-pathogens may also act directly, or in conjunction with microflora, to influence chlamydial infection. We previously observed that women with PID caused by *Neisseria gonorrhoeae* (NG) and CT carried systemic blood transcriptional signatures with greatest activation of cell death pathways and suppression of responses essential for adaptive immunity and CT infection clearance (25). Although these studies have established that CVM composition influences risk for chlamydial cervical infection (8, 13, 22, 26), the extent to which it influences ascending infection has yet to be investigated.

In this study, we sought to investigate the contribution of the CVM to ascending chlamydial infection using high-throughput amplicon sequencing based on 16S ribosomal RNA (16S rRNA) genes, by determining if bacterial members, especially those previously associated with susceptibility to cervical infection, can be implicated in chlamydial spread to the upper genital tract. We analyzed the amplicon sequence

variants (ASVs) derived from the V4 region of 16S rRNA genes to investigate the CVM of study participants enrolled in T cell Response Against Chlamydia (TRAC), a highly characterized cohort of women at high risk for CT infection and for whom endometrial infection had been determined. When participants were categorized into CSTs based on their CVM composition, CST III (dominated by *Lactobacillus iners*; 30.0%) and CST IV (characterized as diverse; 54.5%) predominated. At the cervical level, ASV analysis revealed no significant overall differences in CVM composition between the CT-, Endo-, and Endo+ groups. Nevertheless, our analysis revealed that certain cervicovaginal microbial features were predictive of ascended chlamydial infection (Endo+). Including CT ASV abundance in the analysis significantly improved prediction accuracy, indicating that CT burden at the cervix is a critical factor in the progression of infection. Furthermore, we detected a positive correlation between microbial features predicting CT ascension and levels of selected cytokines in cervical secretions, indicating that these CVM members may influence ascending infection through their interaction with host immune responses, acting on CT indirectly and directly. This study underscores the potential of developing mucosal biomarkers to identify women at higher risk of CT upper genital tract infection, which could be valuable for clinical practice and future vaccine trials.

RESULTS

Classification of participants based on STI clinical diagnostic tests

Cervicovaginal swab samples and endometrial tissue biopsy specimens were collected from 246 women aged 18-35 years in the TRAC cohort (27, 28).

Demographics and sexual history of the cohort were previously described (27). Cervical and endometrial infection status of study participants was determined using nucleic acid amplification tests (NAATs) on relevant samples (27). Twenty-six vaginal swab-derived samples were excluded from this study for the following reasons: three had quality concerns identified during DNA extraction, while the infection status of the remaining twenty-three samples was undetermined because they yielded equivocal results regarding chlamydial detection at the cervix or endometrium during clinical diagnostic testing.

The remaining 220 participants were classified into the following three groups: Endo+, Endo-, and CT- (**Supplementary Table S1**). A total of 143 women who tested positively for CT at their cervix (CT+) were subsequently divided into two groups based on confirmed upper genital tract infection. Women testing positively for CT infection at their cervix and endometrium (n=66) were defined as Endo-positive (Endo+) and the remaining CT infected women who tested positive for CT at their cervix but tested negative for endometrial CT infection (n=77) were grouped as Endo-negative (Endo-). The control group (n=77) was comprised of women testing negatively for cervical chlamydial infection (CT-). However, among the 77 uninfected women, seven samples from participants that were initially diagnosed as CT- by NAAT clinical testing but subsequently identified as CT+ by the detection of CT reads in 16S analysis, were later confirmed to be CT+ by separate quantitation of CT burden in cervical samples by qPCR (29). Consequently, these participants were excluded from 16S microbiome analyses comparing CT+ to uninfected, comparing Endo+ to Endo-, and were reserved for a proof-of-concept analysis. Clinical diagnostic testing at enrollment additionally

determined cervical infection with the bacterial STI pathogens *Neisseria gonorrhoeae* (NG) and/or *Mycoplasma genitalium* (MG) in all participants (**Supplementary Table S1**) (27).

Characteristics of cervicovaginal microbiota in the TRAC cohort

The CVM of TRAC participants were profiled using 16S rRNA gene V4 region sequencing and ASV-based analysis. ASVs identifying common STI bacterial pathogens were detected in cervicovaginal 16S rRNA including CT, NG, and MG (**Figure 1A**). CT ASV vaginal abundance amongst participants testing positively for CT infection positively correlated with CT cervical burden (Spearman correlation coefficient 0.575) (29) previously assayed by qPCR (27). CT ASV sequences were detected in 83 of the 143 CT-infected women (58%) and in 7 of 77 women who were diagnosed as CT- (9.1%) (**Figure 1A, Supplementary Table S1**). Of 20 NG infected women identified by clinical diagnostic testing, NG ASV sequences were detected in 17 of 20 (85%), while NG ASV sequences were detected in 5 out of 200 women testing negatively (2.5%). Assaying via qPCR indicated these discrepancies most likely were a consequence of low burden and/or differential site sampling (29). In contrast, MG-identifying ASV sequences were detected in only 6 of the 38 participants clinically diagnosed with MG (15.8%), an observation explained by the overall low burden of MG detected in infected TRAC participants (30). MG abundance in cervical specimens, previously determined by qPCR (30), was significantly associated with detection of MG ASV in sample libraries (Mann-Whitney, P=0.0206). Overall, these results support the high quality of this 16S rRNA based sequencing dataset.

Based on ASV relative abundance from 213 women, the most prevalent genera in these women were *Lactobacillus, Gardnerella, Prevotella*, and *Megasphaera* (**Figure 1A, Supplementary Figure 1A**). The three most abundant ASVs observed overall were *Lactobacillus iners, Lactobacillus crispatus,* and *Gardnerella vaginalis* (**Supplementary Figure 1B**). Within each group (CT-, Endo-, and Endo+), individuals exhibited a spectrum of *Lactobacillus* abundance, from *Lactobacillus*-predominant compositions to *Lactobacillus*-sparse (**Figure 1A, Supplementary figure 1A-B**). Alpha diversity indices were assessed at the ASV level to investigate whether diversities of cervicovaginal microbial communities differed among the Endo+, Endo-, and CT- groups. Neither Shannon nor Inverse Simpson indices were significantly different between among three groups (**Supplementary Figure 1C;** Wilcoxon rank-sum test p-values > 0.05).

Community State Types (CSTs) represented within TRAC cohort.

The cervicovaginal microbial composition of TRAC participants was further evaluated by categorizing each sample according to CST, taxonomic profiles that represent discrete vaginal microbial communities (**Figure 1B**) (5, 31). The TRAC cohort exhibits a high prevalence of CT infection (65%, 143 of 220 participants) with CST IV (characterized as diverse) as the predominant CST type (54.5%, 120 of 220, **Figure 1**). Almost all the remaining participants were assigned as CST I (dominated by *L. crispatus;* 14.1%, 31 of 220) or CST III (dominated by *L. iners*; 30.0%, 66 of 220) (**Figure 1**). Two participants were assigned to CST II (dominated by *L. gasseri*) and one participant was assigned to CST V (dominated by *L. jensenii*) (**Figure 1**). The seven participants that tested false CT negative were assigned to CST I, III, and IV (**Supplementary Table S1**).

To assess a potential relationship between CST and epidemiological information in the TRAC cohort, we first evaluated the association between CST and cervical CT infection status for 213 women excluding seven false negatives (**Supplementary Table S1, Supplementary Table S2**). However, the association between CT infection status and CST was not significant (**Supplementary Table S2;** Fisher's exact test P-value > 0.05). Second, the association between CST and demographics and physiologic information was assessed for 220 women (**Supplementary Table S1, Supplementary Table S2**). The CST type was significantly associated with Nugent score or race (**Supplementary Table S2;** Fisher's exact test p-value < 0.05). Of the 119 women in CST IV, 107 (89.9%) were classified as having BV based on the Nugent score, compared to lower proportions of BV in the other CST types. CST IV was more common among Black and Multiracial participants, whereas CST I was more common among Caucasian participants, consistent with previous studies (5, 12, 32-35).

Significant differences in abundance were observed between the CT+ and CT- groups for *Lactobacillus* and *Prevotella* species.

Since it has been reported that cervicovaginal microbial profiles differ between CT- and CT+ individuals (36-40), we investigated CVM compositions of CT+ and CT- TRAC participants. We used sparse partial least squares discriminant analysis (sPLS-DA) (41) because this method is effective for supervised classification and feature selection, aiming to identify the best discriminating features between groups while handling high-dimensional data. The statistical discrimination performance for classifying CT- and CT+ women using the first two sPLS-DA components was

estimated by a mean area under the curve (AUC) of 0.70 (**Figure 2A**), indicating that overall CVM profiles in the presence of CT ASV tended to differ between CT+ and CT- women. However, when CT ASV was excluded from the discrimination analysis, performance dropped to a mean AUC of 0.51 (**Figure 2B**).

We also performed sPLS-DA analysis within CSTs, to examine if differences in CVM composition (excluding CT ASV) could be detected between CT+ and CT- individuals. In CST I (dominated by *L. crispatus*), CT- women appeared to cluster in a manner visually distinct from CT+ women but discrimination performance was statistically inconsistent, with a mean AUC of 0.49, likely due to the small number of samples assigned to CST I (n=30) (**Supplementary Figure 2A**). Within CST III (dominated by *L. iners*), cervical CT infection status was moderately discriminated (AUC mean = 0.71, AUC s.d. = 0.04, n = 63) (**Supplementary Figure 2B**) selecting six low-abundant *L. crispatus* ASVs for the first sPLS-DA component (**Supplementary Table S3**). Within CST IV-assigned participants, the discrimination between CT+ and CT- groups was weak (AUC mean = 0.50, AUC s.d. = 0.04, n = 117) (**Supplementary Figure 2C**).

To identify cervicovaginal microbiome features that are differentially abundant between CT- and CT+ groups, we used Microbiome Multivariate Association with Linear Models (MaAsLin2) (42), which produces a list of significant microbial features with false discovery rate correction. A total of 28 ASVs differed in abundance between CT+ and CT- groups, including ASVs assigned to *Fannyhessea vaginae*, *Gardnerella vaginalis* and multiple *Prevotella* spp, microorganisms previously associated with enhanced sensitivity to STI infection and BV (7, 8, 11-13) (**Supplementary Table S4**, unadjusted

P-value < 0.05). CT (ASV sequence # ZOtu76) and NG (ASV sequence # ZOtu95) were more abundant in CT+ women, with Benjamini Hochbergh (BH)-adjusted P-values < 0.0001 and unadjusted P-values < 0.05, respectively. Two *Dialister* ASVs (ASV sequence #'s ZOtu105 and ZOtu220) were (**Supplementary Table S4**, unadjusted P-value < 0.05). Four of the 28 ASVs, including NG and 3 ASVs assigned to *Dialister invisus,* a member of the gut microbiome that has been associated with infections at other mucosal sites*, Prevotella sp.*, and *L. crispatus* (ASV sequence #'s Zotu96, Zotu220, Zotu304, and Zotu2052), were also identified as informative ASVs discriminating cervical CT infection status by sPLS-DA analysis (**Supplementary Table S3 and S4**).

Although no significant differences in abundance of dominant *Lactobacillus spp.* ASVs (ASV sequence #s ZOtu1, ZOtu2, ZOtu11, ZOtu32, and ZOtu374) were detected between CT+ and CT- participants in the overall cohort based on their predominant ASVs, differences were observed in 7 uncommon ASVs assigned to *L. iners*, *L. crispatus*, *L. jensenii*, *L. gasseri* and *L. colini* (**Supplementary Table S4, Supplementary Figure 3**). To investigate if these low-abundance ASVs were sequencing artifact, we conducted multiple sequence alignments to identify the position of mismatches between these low-abundant *Lactobacillus* ASV sequences and their corresponding reference sequence, reasoning that errors generated by merging of paired-end reads would be disproportionately concentrated in the middle of sequences. The 7 ASV sequences were aligned with the corresponding ASV sequences of their most abundant *Lactobacillus sp.* ASVs listed in **Supplementary Table S4**, and V4 region sequences of *Lactobacillus sp*. curated in the RDP 16S No18 reference

database (43) (**Supplementary Figure 4**). The most abundant *Lactobacillus* ASVs (ASV sequence #s ZOtu1, ZOtu2, ZOtu11, ZOtu32, and ZOtu374) exactly matched their corresponding V4 region reference sequences (**Supplementary Figure 4**). In contrast, the 7 low-abundant *Lactobacillus* ASV sequences contained mismatches at 20 of 253 nucleotides in the V4 region, except for one ASV (ASV sequence # ZOtu459). Intriguingly, these nucleotide variants frequently occurred at the same 20 positions along the *Lactobacillus* V4 region, rather than being concentrated in the middle of ASV sequences (**Supplementary Figure 4**). These results suggested that the observed sequence variants in these low-abundant *Lactobacillus* ASVs reflected biological variation in *Lactobacillus* subspecies or strains that composed the CVM of TRAC participants.

We investigated whether CVM features could predict CT infection status within this high STI risk cohort, the majority of whom had BV at enrollment (27) (**Supplementary Table S1**). We performed a random forest-based analysis using ASV abundance, while excluding the CT ASV. In brief, we randomly partitioned the samples from CT+ and CT- groups into a training dataset and a separate test dataset. We built a predictive model using the training dataset and evaluated its performance by predicting the CT infection status using the independent test dataset (see Methods for details). The prediction performance was assessed using the average receiver operating characteristic (ROC) curves across 100 replicates. The corresponding average AUC was found to be 0.61 for the prediction using all ASVs excluding the CT ASV (**Supplementary Figure 5**). Thirteen ASVs contributed to prediction of absent CT infection (**Supplementary Table S5**). NG ASV (ASV sequence# ZOtu95), more

abundant in CT+ women (**Supplementary Table S4**), was the strongest contributor to the prediction. NG is not considered a member of the CVM, thus its contribution to this prediction likely derives from the frequency of co-infection with CT in women reporting high risk behaviors for STI acquisition. Four *Syntrophococcus sucromutans*-assigned ASVs and four *Prevotella spp*. ASVs, which showed differential abundance between CT+ and CT- women (**Supplementary Table S4**), were found to be predictors of absent CT infection (**Supplementary Table S5**). However, the ASVs of *S. sucromutans* (ASV sequence #s ZOtu709, ZOtu1481, ZOtu1814, and ZOtu2372) have low confidence genus-level assignments, indicating that these strains, currently classified as members of the class *Clostridia*, require further investigation to resolve their taxonomic classification.

Cervicovaginal microbial features are predictive of susceptibility to ascended chlamydial infection

Given the severe sequelae that can arise after CT infection ascends to involve the endometrium and fallopian tubes and the absence of diagnosing this outcome non-invasively, we investigated if CVM profiles of CT+ women were reflective of CT endometrial infection. We first examined the overall differences in CVM compositions between Endo- and Endo+ patients using sPLS-DA analysis, as before (see also Methods). Endo+ and Endo- women showed distinguishable separations by the first two sPLS-DA components including CT ASV (**Figure 2C**; AUC mean = 0.76, AUC s.d. = 0.01). The CT ASV (ASV sequence # ZOtu76) and two *Lactobacillus* ASVs (ASV sequence #s ZOtu915 and ZOtu914) contributed the most to the first component, while

more than 50 ASVs were identified as important features for the second component discriminating Endo+ and Endo- women (**Supplementary Table S6**). Despite the high number of important features identified, in the absence of CT ASV, discrimination between Endo+ and Endo- weakened with an AUC of 0.55 (**Figure 2D**).

We also examined the CVM profiles within CST I, CST III, and CST IV types separately for CT endometrial infection status using sPLS-DA analysis. Endo- women were visually separable from Endo+ women within CST I with the first sPLS-DA component including 8 *Lactobacillus* ASVs (**Supplementary Table S6**), but the statistical performance was very low, with a mean AUC of 0.47 (**Supplementary Figure 2D**), again likely due to the small number of women within CST I (n=23). The discrimination of endometrial infection status within CST III was also weak (AUC mean = 0.58, AUC s.d. = 0.09, n=43) (**Supplementary Figure 2E**). However, endometrial infection status within CST IV (characterized as diverse) can be discriminated (AUC mean = 0.71, AUC s.d. = 0.03, n=76) (**Supplementary Figure 2F**) with the CT ASV solely contributing to the first sPLS-DA component and the interaction of 19 ASVs including 4 *Prevotella* ASVs and a *Mycoplasma microti*-assigned ASV (# ZOtu45) contributing the second sPLS-DA component (**Supplementary Table S6**).

To identify CVM features that would be most informative in predicting CT ascension, we performed random forest-based analysis using ASV abundances as features to predict individual patients in Endo+ or Endo- groups. Since CT is not only a key factor of ascended infection but potentially modulates the cervical and endometrial environment for other microbes, we assessed the predictive performance of the model in three scenarios: (A) prediction with all ASVs including CT ASV (# ZOtu76) (**Figure**

3A), (B) prediction with all ASVs but excluding CT ASV (**Figure 3B**), and (C) prediction with only CT ASV (**Figure 3C**). Average AUCs were 0.74 for the model with all ASVs, and 0.57 for the model that excludes the CT ASV (**Figure 3A-B**), indicating the importance of CT ASV abundance for prediction. When the model was trained by the CT ASV alone it resulted in an average AUC of 0.74 (**Figure 3C**).

In the prediction with all ASVs including CT ASV, CT and 11 additional ASVs were identified as informative features using selection frequency based on mean decrease in accuracy (**Table 1**; feature selection frequency 80 out of 100 replicates). These 11 ASVs were also selected as informative features in the prediction using all ASVs but excluding CT ASV (**Table 1**; feature selection frequency $\geq$ 80 out of 100 replicates). Notably, there was no overlap between these features and those selected as predictors of absent CT cervical infection (**Supplementary Table S5**). Among the identified 12 ASVs, four were identified with higher abundance in Endo+ women (ASV sequence #s ZOtu76, ZOtu233, ZOtu1808, ZOtu567) (**Table 1**). Of the four? highly abundant ASVs in Endo+ women, three were also identified as important features discriminating Endo+ and Endo- patients in the above sPLS-DA analysis (ASV sequence #s ZOtu76, ZOtu233, and ZOtu1808) (**Table 1** and **Supplementary Table S6**). All three ASVs assigned to *Sutterella stercoricanis* (ASV sequence #'s ZOtu110, ZOtu184, and ZOtu180) exhibited lower abundance in Endo+ women (**Table 1**). *Actinotignum schaalii* ASV (ASV sequence # Zotu281) and *Haemophilus haemolyticus* ASV (ASV sequence # Zotu103) were also identified as predictors, which were less abundant in Endo+. *Mycoplasma microti* (ASV sequence # Zotu45), a species previously isolated from the respiratory tracts of prairie voles (43), seemed an unlikely

component of the CVM. Subsequent examination determined that the assigned ASV shared 100% identity with uncultured *Mycoplasma sp.* clone Mnola (44), now *Candidatus* Malacoplasma girerdii (45), a strict endosymbiont of *Trichomonas vaginalis* (TV). Within TRAC, we observed that detection of *Ca.* M. girerdii ASV reads coincided with a positive TV clinical diagnostic finding in 14 of 37 participants (37.8%) but was only detected in 7 of 183 (3.8%) of participants who tested negatively for TV suggesting that this was the more correct assignment. Overall, these results suggest that multiple CVM features together can be predictive of susceptibility to CT ascension but the amount of CT at the cervix is a key factor.

Cervicovaginal microbial features associated with ascending CT infection are positively correlated with cytokines in cervical secretions associated with endometrial CT infection

To better understand possible functional consequences of these 12 CT ascension-related microbial features on host immune responses, we explored their associations with levels of 7 cytokines in TRAC cervical secretions that had been previously associated with CT ascension (44). The following cytokines, CXCL10, TNF- α, IL17A, CXCL9, CXCL11, CCL4, and CXCL13, were positively associated with endometrial infection (unadjusted $P<0.05$) in a univariable regression model. Using canonical correlation analysis (CCA) (45), we investigated whether there was an overall correlation between these cytokine levels and the predictive microbial features. With 12 ASVs, including CT, we observed a positive correlation between microbial abundances and cytokine levels (**Figure 4A**; canonical correlation = 0.54). There was also a positive

but weaker association between cytokine levels and CVM-derived ASVs when CT ASV was excluded (**Figure 4B**; canonical correlation = 0.43). CT ASV abundance also correlated weakly with the seven cytokines (**Figure 4C**; canonical correlation = 0.49). Together, these results indicate that host immune responses are not exclusively modulated by CT abundance. The CCA-based analyses suggest that the predictive CVM microbial features influence CT ascension directly and indirectly, through interaction with host immune responses.

A proof-of-concept analysis for predicting ascending CT infection using the twelve informative ASVs

We identified twelve ASVs that are predictive of susceptibility to ascending CT infection using random forest-based prediction and ASV abundance of Endo+ and Endo- women (**Table 1 and Figure 3A**). Four false negative CT+ participants (**Supplementary Table S1**) had residual endometrium-derived samples for additional testing and were used for proof-of-concept prediction of Endo+ vs Endo- here. An optimal threshold of 0.55 was derived from the averaged ROC curve of the model informed by all ASVs (**Figure 3A**) and was used to determine between Endo+ and Endo- (**Supplementary Figure 6**). The model correctly predicted CT ascension status for two out of four participants based on confirmed detection of CT DNA (29). CT ASV abundance was considered as a key factor for the prediction of CT ascension but was unable to predict Endo+ vs Endo- status alone (data not shown). This proof-of-concept analysis of four false negative CT+ participants could not validate the 12 ASVs including

CT ASV due to very small sample size. Validation of the 12 CVM features identified requires testing in a larger, independent cohort.

DISCUSSION

Recent literature has revealed that cervicovaginal microbiota may modulate susceptibility to infections such as human papillomavirus (HPV), CT, NG and MG infections (46). CVM composition has been associated with increased risk of incident CT infection (16, 22, 46, 47). In this study, we sought to determine whether CVM composition facilitates CT ascension from the cervix to the upper genital tract by profiling 16S rRNA genes obtained from vaginal swabs collected at enrollment from the TRAC cohort. Although we were unable to identify many significant differences between CT infected and uninfected women, we identified 12 cervicovaginal microbial features that predicted active upper tract infection. Chlamydial cervical burden was a key driver of ascent because vaginal CT ASV abundance alone, effectively predicted this outcome with AUC of 0.74 for sensitivity and specificity. We also observed positive correlation between levels of 7 cytokines previously associated with CT ascension and abundance of CVM-derived ASVs, indicating that local host immune responses during infection were not exclusively modulated by pathogen load.

TRAC is comprised of women at elevated risk for STI infection and a striking feature of this cohort is the very high rate of CT infection, 67%, amongst enrollees, reflective of highly targeted recruitment strategies (27). This may have blunted our ability to detect significant differences in the composition of the CVM, at the level of CST, for individual genera or species that associated with protection against CT infection.

Unsurprisingly, most participants were assigned to CST III or CST IV, previously recognized as neutral or favorable for CT infection (7, 8, 11-13). Within CST III, CT- women were separable from CT+ using sPLS-DA components containing *L. crispatus*-assigned ASVs. Amongst CST I-assigned participants, the CST most highly associated with protection from CT infection (8, 13, 22), CVM composition did not differ sufficiently to cluster CT infected and uninfected participants effectively. TRAC was designed as a longitudinal study to investigate T cell responses important for protection from incident chlamydial infection, but the high rate of CT positivity at enrollment combined with a stringent requirement that participants have completed a minimum of three follow up assessments, provided insufficient power to confirm previously identified associations of CST with infection susceptibility. Nevertheless, a random forest-based analysis using all ASVs (excluding CT) was moderately predictive of CT- women **(Supplementary Table S5)**, while MaAsLin2 analysis identified differentially abundant community members previously associated with susceptibility **(Supplementary Table S4)**. Conserved between both analyses were ASVs assigned to *Prevotella sp.* and *S. sucromutans* that associated with CT infection. Studies, *in vitro* (14, 15) and *in vivo* (16, 17) have associated indole-producers, e.g. *Prevotella sp*., with CT evasion of IFNγ-mediated tryptophan depletion (18, 19). Of the *Prevotella sp*. discriminating CT infection in our analysis (48), only *P. brunnea* is an indole producer (48, 49), but it is also possible that they modulate host factors, indirectly influencing chlamydial susceptibility. *S. sucromutans*, in association with *Prevotella sp*., serves as a biomarker of gut barrier dysfunction (leaky gut) (50) where increased luminal abundance of Zonulin-1, an endogenous tight-junction protein, was associated with decreased abundance of these

species. Directly relevant to our study, recent work by Hinderfield and colleagues (51), demonstrated that CST IV-associated microorganisms promote paracellular permeability of human ectocervical monolayers with coincident down-regulation of Zonulin and Occludin expression, leading them to hypothesize that undermining cervical epithelium integrity enhances susceptibility to TV and other STI pathogens (51). Thus, a CVM that contributes essential substrates and improves pathogen access to the cervical epithelium can increase susceptibility to chlamydial infection.

In contrast, lactic acid produced by *Lactobacillus sp.* directly kills CT (23) while D(-) lactic acid produced by some *Lactobacillus sp.* inhibits CT infection by down-regulating host epithelial cell proliferation (6). In this study, we were unable to detect associations of the most abundant *Lactobacillus* species with CT positivity but identified differences in low abundance strains **(Supplementary Table S4)**. We questioned whether the low-abundant *Lactobacillus* ASVs that were differentially abundant by MaAsLin2 analysis were genuine *Lactobacillus* species or accidental assignments of artificial sequences to *Lactobacillus* species, given the taxonomic complexity among *Lactobacillus* species (52) and the limitations of characterizing these species through 16S rRNA gene sequencing (53). Although ASVs have intrinsic biological meaning independent of reference database or the specific context of a study (54), the taxonomic resolution is much lower compared to the full length of 16S rRNA sequences or metagenome sequences (55). Recent genomic studies identifying genetic elements that control functional differences between and within *Lactobacillus species*, such as the role of bile salt hydrolases that promote colonization of vertebrate-adapted niches (56), and the ability to metabolize extracellular glycogen important for microbial growth in the

female genital tract (57, 58) as well as identification of novel urogenital tract species (59) suggest that it is premature to conclude that the differences we detected between CT infected and uninfected women are artifactual, and future investigations should consider implementing typing approaches with superior resolution that will enable us to determine if further investigation into the biological roles of individual *Lactobacillus* subspecies in cervical CT infection is needed.

The TRAC cohort provided a unique opportunity to investigate the CVM contribution to chlamydial ascension from the cervix to the upper genital tract. Using sPLS-DA, we were able to observe overall differences in CVM compositions between Endo- and Endo+ participants, although discriminatory resolution dropped considerably when the CT ASV was excluded, emphasizing the importance of CT cervical abundance in driving ascension. At the level of CST, discriminatory performance was highest in CST IV, but much reduced for CST I and CST III participants, suggesting that CVM-derived factors that promote susceptibility to CT infection also contribute to its ascent. However, we did not detect much overlap in the specific CVM features differentially abundant between CT- and CT+ participants by MaAsLin2 analysis and those selected by random forest analysis as predictive of spread with the obvious exception of the CT ASV.

Many of the CVM features selected by our analysis have not previously been associated with human chlamydial infection but have been investigated in the context of the respiratory or gut microbiome. Through “nutritional immunity”, the host may limit iron availability as a means of protection against invading pathogens (60). CT requires iron (61, 62) and may experience additional competition for this essential nutrient from CVM

462 members. Iron restriction also impacts host cellular metabolism, leading to differential
463 host gene expression with downstream consequences for availability of other key
464 substrates required for chlamydial developmental success (63). Antithetically,
465 transcription of the *trpBA* operon essential for chlamydial scavenging of exogenous
466 indole to tryptophan is iron-regulated (64) leading to the proposal that a low iron
467 environment could increase risk for sequelae (64). *H. haemolyticus* is a respiratory tract
468 commensal that limits infection with the pathogenic *H. influenzae* through competition
469 (65), because it uses its superior ability to sequester iron to facilitate occupation of their
470 shared niche (66, 67). Its relatively lower abundance in Endo+ participants may indicate
471 that chlamydial need for iron predominates during natural infection and that *H.*
472 *haemolyticus* abundance modulates ascended infection through its negative influence
473 on chlamydial growth.

474 Also less abundant in Endo+ participants, *S. stercoricanis* has not been
475 previously described as a significant member of CVM but within the human gut
476 microbiome, its reduced abundance has been identified as a predictor of enhanced
477 susceptibility to respiratory infection (68). *Sutterella sp.* are epithelium-adherent (69),
478 but do not contribute to gut dysbiosis or loss of gut epithelial integrity. In the context of
479 STI infection, their abundance is significantly increased in the rectum of HIV negative,
480 STI uninfected men, and CD4/CD8 ratio positively correlated with *Sutterella* in HIV-
481 infected patients, consistent with an association with T cell-mediated adaptive
482 responses independent of active bacterial infection (70). They are mildly pro-
483 inflammatory, and it has been proposed that their association with their human host is
484 mutualistic through their contribution to steady state induction of Th17 cell differentiation

485 (71, 72). We previously reported that IL-17A transcript was significantly increased in
486 blood of participants with high cervical *C. trachomatis* burden compared to those with
487 low cervical burden and uninfected participants (25) and that cervical secretion of IL-
488 17A was associated with increased odds of endometrial infection (44). Nevertheless, we
489 observed increased frequencies of CD4 Th17 cells in peripheral blood of CT-infected
490 TRAC participants compared to uninfected participants, and a further increase in
491 participants without recurring infection compared to those who were reinfected with CT
492 within a year after enrollment (73). Together, these results suggest that CT infection
493 elicits local and systemic Th17 responses, and that the systemic Th17 responses
494 detected were associated with reduced reinfection. It is possible that occupation of a
495 niche by benign *S. stercoricanis* within the CVM of Endo- participants protects against
496 expansion of members that positively contribute to chlamydial burden by provision of
497 necessary intermediates or by compromise of epithelial integrity. It is also possible that
498 its reduced abundance in Endo+ women contributed to reduced CD4 T cell activity,
499 ultimately delaying resolution of chlamydial infection. Future studies that are more
500 informative of function, e.g. transcriptomics, metabolomics, may identify common
501 metabolites or pathways that can be associated with CT spread to the upper
502 reproductive tract, while profiling of adaptive immune cell populations recruited to the
503 infection site will lend further insight into mechanisms of chlamydial clearance.

504 We previously observed the importance of CT/NG coinfection on risk for CT
505 reinfection (27). We also reported altered bloodborne transcriptional signatures pointing
506 to suppressed host adaptive responses with CT/NG coinfection in women with pelvic
507 inflammatory disease (25). However, we did not observe TV nor MG co-infection

associating with ascended CT in TRAC participants (27, 30). In this study, an ASV that we ultimately re-assigned to *Ca*. M. girerdii (74, 75) was identified as a CVM feature predictive of ascended infection. Members of this large and diverse class of *Mollicutes,* distinguished by a small genome and lacking a cell wall, such as *M. hominis* and *Ureaplasma sp.*, have been identified as commensals and/or opportunistic genital tract pathogens, although meta-analyses have not effectively resolved the extent of their impact on female infertility or other reproductive outcomes including preterm birth (76, 77). Intriguingly, *M. hominis* and *Ca. M. girerdii*, alone or together, can live intracellularly or on the surface of TV, accelerating the parasite's growth (78, 79) and modulating its pro-inflammatory potential. Relevant to CT infection because of the importance of cell-mediated responses for chlamydial clearance, TV harboring *M. hominis*, but not alone, synergistically upregulates IL-8, IL-1β, and TNF-α production as well as inducing production of the Th-17 polarizing cytokine IL-23 by human monocytic THP-1 cells with coincubation (80). If, as we speculate, recruitment of chlamydia-specific Th1 and Th17 cells to the genital tract is essential for control, TV coinfection, in some but not all instances, might promote this outcome, explaining why *Ca.* M. girerdii ASV was associated with Endo- women. Confusingly, ASVs assigned to the other TV endosymbiont *M. hominis* were also detected in many TRAC participants but were not selected as predictive features. We were not able to immediately resolve this contradictory observation because rRNA from TV was not captured in this analysis since it is a protozoan and the clinical diagnostic was not quantitative, but it highlights the potential for factors that influence pathogen success to be mediated through

interactions between microbiome members, including those that cannot be detected using a 16S rRNA profiling approach.

ASVs contributing to prediction of ascended CT infection that were more abundant in Endo+ women included *Actinobaculum massiliense,* a urinary tract commensal or opportunistic pathogen (81), and reportedly a rare cause of PID (82). A recent proteomic analysis identified two highly expressed subtilisin-like proteases which may cleave cytokines (83), providing potential to modulate the host inflammatory response to itself and CT. ASVs assigned to *Butyrivibrio hungatei* and the genus *Acetothermia* were also more abundant in Endo+ participants. Neither bacterium is well documented as a member of CVM. In the context of gut microflora, *B. hungatei* is thought to act positively because reduced abundance of this butyrate producer has been associated with dysbiosis and disorders of the gut-brain axis such as Alzheimer's Disease (84). Butyrate is a short chain fatty acid (SCFA) that modulates colon health by multiple mechanisms. In low amounts it serves as a carbon source for other commensals, while at higher doses it can be toxic, targeting other members of normal flora or pathogens such as *Salmonella sp*. (85). Colonic enterocytes transport butyrate as a preferred substrate (86) promoting epithelial health and integrity. However, in the female genital tract, SCFAs are associated with the production of pro-inflammatory cytokines and perturbation of epithelial integrity (87).

With potential translational impact, random-forest analysis revealed the importance of cervical CT abundance in prediction of concomitant upper genital tract spread. Using the CT ASV alone is a highly informative and simple feature, capable of predicting chlamydial ascension with equivalent AUC performance to the prediction

using 12 ASV CVM biomarkers. A major barrier to preventing CT disease is delayed diagnosis, because infection is often asymptomatic (88), but both clinical and subclinical upper-tract inflammation can lead to chronic oviduct damage (89). Diagnostic biomarkers of ascended infection would be particularly useful for evaluation of therapeutics and vaccines (90-92) and improve case management, since women with clinical PID receive a longer antibiotic course compared to asymptomatic women with CT (93). Abundance of the CT ASV appears to be a better predictor of ascended CT infection than blood-borne transcriptional biosignatures (94) or profiling circulating immune cells (73) for this cohort of highly exposed women but was unsuccessful when applied to our small proof-of-concept set. Nevertheless, CT ASV with the CVM-derived selective features provided the best correlation with local cytokines, indicating the potential for further improved prediction with inclusion of host immune features. We previously performed a small pilot study that indicated that whole transcriptome sequencing analysis of ribosomal RNA-depleted total RNA isolated from cervical swab samples contained pathogen-specific sequences from women with confirmed sexually transmitted bacterial pathogens (95). Simultaneously, we identified and quantified their active microbial communities and after integration with their associated host-derived reads, we detected clustered host transcriptional profiles reflecting microbiome differences and STI infection. Together, these findings indicate that cervical sampling can yield sensitive and robust biomarkers for evaluation of candidate chlamydial vaccines, such as those currently in development or advancing towards Phase I (96) and Phase II trials.

High throughput sequencing of 16S rRNA enabled us to efficiently identify CVM communities in the TRAC cohort, but taxonomic resolution for microbial species or strains was frequently too low to confidently determine their taxonomy as cervicovaginal microbiome. Nonetheless, we were able to detect STI pathogenic microbes, as well as highly variable microbes such as *Lactobacillus sp.* and *Mycoplasma sp.* using ASV sequences. Copy number of the 16S rRNA gene can vary widely, ranging from 1 to 37 in bacteria and 1 to 5 in archaea (97). This variation in 16S rRNA gene copy number (GCN) limits the accuracy of quantifying 16S rRNA genes and CT 16S reads, potentially introducing biases in subsequent analyses (98). Indeed, this effect may be reflected in our variable success detecting ASVs associated with NG (four copies) versus CT (two copies) and MG (single copy) (99) in samples from women testing positively with highly sensitive clinical diagnostic tests. Although novel methods have been developed to correct for 16S rRNA GCN (100-103), we did not apply 16S rRNA GCN prediction tools for precise quantification of 16S rRNA genes in this study. In future work, we can adopt full-length 16S rRNA sequencing as an alternative to short reads of 16S rRNA to improve taxonomic resolution and quantification accuracy.

This study had several additional limitations. For example, the participants of TRAC cohort were highly exposed to CT infection, which may have introduced bias in the representation of the CVM communities in general. Vaginal swab samples for microbiome sequencing were collected only at enrollment when participants were actively infected, so there were no baseline samples to investigate temporal changes in CVM features related to the development of CT ascending infection. Even if we had chosen to sample participants longitudinally, all received antibiotic treatment at

enrollment, which could have affected CVM composition. Also, 16S rRNA-based sequencing provided information only on the composition of CVM profiles. This limits the ability to understand the functional roles that these microbial communities may play in modulating host immune resistance or susceptibility to CT ascension. We did not investigate other factors such as host genetic heterogeneity and the effect of innate immunity on ascended CT infection.

In future studies, the integration of other techniques such as transcriptomics and metabolomics with 16S rRNA based sequencing, studies that have been initiated with establishment of an additional cohort of CT-infected women (TRAC2) (104) are expected to facilitate the development of deeper functional insights and identification of relevant biological pathways. Additionally, expanding the analysis with other relevant members of the CVM including parasites such as TV and viruses could also enhance our understanding of microbial interactions with the host and improve the prediction of upper genital tract infection spread. These multi-omics analyses may also contribute to the identification of biomarkers of protection or disease risk that could contribute to assessment of vaccine efficacy in future clinical trials.

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Availability of data and material

Raw 16S rRNA gene sequencing data supporting the conclusions of this article is publicly available at NCBI's Short Read Archive (SRA) under BioProject accession number PRJNA1136868 (https://www.ncbi.nlm.nih.gov/bioproject/). **Supplementary Figures S1–S8** and **Supplementary Tables S1–S3, S5**, and **S7** are included in **Additional File 1: Supplementary Materials**. **Supplementary Table S4** is provided in **Additional File 2**, and **Supplementary Table S6** is provided in **Additional File 3.** The legends of **Supplementary Tables S4** and **S6** are provided in **Additional File 1: Supplementary Materials.**

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METHODS

T Cell Response Against Chlamydia (TRAC) Cohort

This cohort was composed of mainly young (median age, 21 years; range, 18-35 years), single (89%), African American (66%) women at high risk for the acquisition of CT (**Supplementary Table S1**). Inclusion criteria indicating high-risk status included > 3 sexual partners in the previous 6 months, ≤ 14 years of age at sexual debut, history of pelvic inflammatory disease (PID), or presentation to the recruitment site with any of the following: presence of mucopurulent cervicitis on exam, or sexual contact with a partner known to be infected with CT or NG or non-gonococcal non-chlamydial urethritis.

Women with a current diagnosis of PID according to the Centers for Disease Control and Prevention guidelines were excluded. Additional exclusion criteria were pregnancy, uterine procedure or miscarriage in the preceding 60 days, menopause, hysterectomy, antibiotic therapy in the preceding 14 days, and allergy to study medications. A total of 246 TRAC participants were enrolled into a longitudinal study designed to investigate T cell responses important for protection from incident chlamydial infection over 12 months of follow-up between February 2011 and August 2014 and were recruited from the Allegheny County Health Department's Sexually Transmitted Diseases Clinic, Magee-Womens Hospital (MWH) Ambulatory Care Clinic, and the Reproductive Infectious Disease Research Unit at MWH in Pittsburgh, PA. This study complied with the Declaration of Helsinki guidelines and all study participants provided informed consent prior to initiation of study procedures. The Institutional Review Boards at the University of Pittsburgh and the University of North Carolina approved the study. Participants completed questionnaires regarding obstetric/gynecologic history, behavioral practices, sexual histories, contraceptive methods, and symptoms according to study protocols. Clinical, histological, and microbiological testing was performed, and blood and endometrial samples were obtained at enrollment, after which all participants received single-dose agents to treat *gonorrhoea* (ceftriaxone, 250 mg intramuscularly) and chlamydia (azithromycin, 1 g orally). Participants in this cohort were assessed for cervical and endometrial infection using Aptima Combo 2 (AC2) (Hologic, Marlborough, MA) NAAT with overall infection rates of 67% and 8.5% for CT and NG, respectively. MG and TV infection was determined using Aptima MG and Aptima TV diagnostics (Hologic) respectively.

16S library preparation and sequencing

DNA was extracted from cervicovaginal swabs stored at -80 ℃ using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA). Before extraction, the manufacturer's protocol was optimized with minor modifications to achieve the best performance in terms of DNA/RNA yield and equal recovery of six different species comprising the ATCC Vaginal Microbiome Whole Cell Mix (MSA-2007). Average yields were 52.8 ng/µl yield of DNA in a final volume of 100µl per sample. The V4 regions of 16S rRNA gene were amplified using the Illumina 16S V4 primer set of 515F [GTGYCAGCMGCCGCGGTAA] and 806R [GGACTACNVGGGTWTCTAAT] (105, 106) designed for dual indexing as described by the Earth Microbiome Project protocol (https://earthmicrobiome.org/protocols-and-standards/16s/). Sequencing libraries were prepared according to the Illumina's 16S Metagenomic Sequencing Library Preparation Guide (https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html; Part # 15044223 Rev. B), and sequenced on an Illumina NextSeq 500 Sequencer (Illumina, San Diego, CA).

16S rRNA-seq data processing

The 16S rRNA data analysis pipeline is outlined in **Supplementary Figure 7.** Raw fastq files containing paired-end read sequences per each sample were demultiplexed and preprocessed into amplicon sequence variants (ASVs) using bioinformatics software, USEARCH v11.0 (107) and PEAR (108). The fastq_eestats2

function in USEARCH was used to determine if trimming was needed to optimize read merging, based on expected errors for 150bp forward and reverse reads across different length cutoffs (100, 110, 120, 130, 140, and 150bp), which was 150bp for our data. Merged reads were expected to be >252bp with short overlap nucleotides (<10bp). PEAR, known for its robustness with short overlapping regions, was employed for read merging, utilizing a minimum overlap size of 8 bases (108). Following read merging, primer sequences were trimmed from both ends, and low-quality reads were filtered at a maximum expected error rate of 1.0.

Sequence denoising (error-correcting) and the generation of zero-radius OTUs (zOTUs), referred to as amplicon sequence variants (ASVs), were achieved using unoise3 function in USEARCH (107). The unoise3 algorithm prioritizes read abundance over sequence differences by processing sequences based on descending order of read abundance (109). As a result, a rare sequence can be incorporated into a highly abundant centroid sequence even if its difference is relatively high and may be discarded by chance depending on the order of pooled unique sequences (107, 109). To reproduce the same set of ASV sequences in TRAC cohort despite this limitation of randomly discarding low-abundant sequences, we added additional steps to the standard denoising and ASV generation process. We reordered unique sequences based on read frequency, read length, entropy, and confidence level of taxonomy assignment, all in decreasing order except for read length. Considering the optimal length of V4 region of 16S rRNA DNA to be 253bp, we calculated the distances between 253bp and the length of read, and then arranged the reads from the smallest distance to the longest distance away from 253bp. Using the rearranged ~995,000

unique read sequences, we consistently reproduced the same total set of 2,777 ASVs from an average of ~151,000 reads per sample. Of 2,777 ASV sequences, the Burrows-Wheeler Alignment tool (110) aligned 569 to the human reference genome (GRCh38) and these were excluded. We also removed ASV sequences shorter than 252bp, with 1,990 ASVs retained for further analysis. The inferred ASVs were assigned taxonomy using the RDP Classifier 16S trainset No. 18 raw training database (https://sourceforge.net/projects/rdp-classifier/files/RDP_Classifier_TrainingData/).

For analyses based on ASV abundances of CT+ and CT- women, 783 out of 1,990 ASVs were excluded because they had fewer than 50 read counts across CT+ and CT- groups because these low-abundance ASVs represented experimental artifacts generated during sequencing. Similarly, of 1,990 ASVs, 1,043 with fewer than 50 reads across Endo+ and Endo- women were excluded from analyses based on ASV abundances of Endo+ and Endo- women.

Multiple sequence alignment of 7 low-abundant *Lactobacillus sp*. ASV sequences (**Supplementary Table S4**) with their corresponding sequences of the most dominant *Lactobacillus sp*. ASVs and the V4 region sequences of *Lactobacillus sp*. obtained from the RDP 16S No18 reference database was performed using MUSCLE 3.8.31v (111). The alignment was visualized using UGENE (112) to with the goal of identifying mismatches in the central region of the 252bp ASV sequences (**Figure S4**).

Modification of reference sequences for V4 regions in RDP Classifier

RDP Classifier 16S trainset No18 raw training database provides both bacterial and archaeal 16S rRNA reference sequences. Among a total of 21,195 reference

sequences, nearly 40% had identical V4 region sequences for multiple species ranging from 2 to 65 different species in the RDP Classifier database (**Supplementary Table S7**). In such cases, sintax function for taxonomic assignment in USEARCH randomly chooses a species among multiple species given by the reference annotation (113). In addition, since the RDP Classifier database is not limited to human-related microbial taxonomy annotation, assigned species may include those not typically observed in the human cervicovaginal microbiota. For example, although *L. crispatus* and *L. jensenii* are commonly found in the cervicovaginal environment, taxonomic assignment based on the V4 region of the RDP Classifier database might incorrectly identify *L. acidophilus* as the predominant *Lactobacillus* species instead (114). To enhance the reliability of species assignments, we implemented a two-step approach. From the ASV abundance table, we selected a single species among those with identical V4 reference sequences based on the highest abundant species driven from the RDP Classifier database. Selected species are indicated with stars (**) in **Tables 1** and **2**. Remaining species and their corresponding V4 reference sequences were removed from the reference database. Using this reduced set of V4 region sequences as our reference database, we repeated taxonomy assignment for ASVs. Despite random, sintax algorithm-driven selection of a species from among multiple alternatives, we were able to track the species chosen from among those with identical V4 sequences.

Statistical analyses

Alpha diversity. To investigate significant differences in alpha diversity among the three groups (Endo+, Endo-, and CT-), we standardized the total read counts per

sample, which ranged from 34,571 to 417,336, by rarefying the reads across samples to the minimum read counts (34,571 reads) using rarefy_even_depth function in phyloseq R package. We assessed Shannon (richness) and Inverse Simpson (evenness) using phyloseq and microbiome R packages. Phylogenetic diversity was calculated using picante R package. The phylogenetic tree for phylogenetic diversity was generated from the 16S rRNA V4 region sequences of swab samples by using MUSCLE (111). Statistical significance in alpha diversity between each pair of groups was determined using the Wilcoxon rank-sum test.

The centered log-ratio transformation. Microbiome-derived read count data obtained through amplicon sequencing is analyzed as compositional data (115) where a proportional change for any microorganism will affect relevant abundances of others, rendering ASV read abundances mutually dependent. Centered log-ratio (CLR) transformation is one approach that generalizes relative abundances with respect to the geometric mean of all sequences in the sample (116). CLR-transformation of read counts enables meaningful comparisons of microbial abundances by mitigating compositional data biases (117). Since CLR-transformation depends on logarithms, handling zero counts is challenging. We treated absolute zero counts by replacing them with the smallest CLR-transformed values calculated by from our read counts.

Community state type (CST) classification of TRAC cohort and discrimination analysis for CT infection status associated with CST. TRAC participants (n=220) were categorized into CST types according to their vaginal microbial compositions using

VALENCIA (VAginaL community state typE Nearest CentroId clAssifier) (118). VALENCIA classifies samples for CST types based on their similarity to a set of reference centroids, and the VALENCIA tool and reference centroids were sourced from GitHub (https://github.com/ravel-lab/VALENCIA). Where necessary, the taxon names from RDP Classifier database were manually adapted to correspond with the VALENCIA taxonomy of the reference centroids.

A sparse partial least squares discriminant analysis (sPLS-DA) (41) was employed to discriminate between CT infection status across all women, and to differentiate between CT infection status within CST I, CST III, and CST IV types. The sPLS-DA was executed using splsda function in mixOmics R package (119). For training across 213 women, 10-fold cross-validation (CV) with 1,000 iterations was used, while within assigned CSTs, sPLS-DA utilized 5-fold CV repeated 1,000 times. To assess the sPLS-DA model, AUC scores were computed from the training CV sets and averaged over 1,000 iterations, yielding the mean and standard deviation (s.d.). The ASVs listed in **Supplementary Table S3** and **Supplementary Table S4** were extracted using the perf function in the mixOmics R package with a threshold of frequencies greater than 0.80 for each component.

Microbiome Multivariate Association with Linear Models (MaAsLin2) for differential abundance analysis. To investigate if CVM of TRAC cohort differ in abundance between CT+ and CT- women, MaAsLin2 (42) was performed using Maaslin2 R package with the embedded CLR transformation method (normalization =

"CLR", transform = "NONE", analysis_method = "LM", correction = "BH", min_prevalence = 0).

Random forest classification. Random forest classification was used to determine if CVM excluding CT is predictive of the lack of CT infection. This method was also utilized to predict chlamydial ascending infection and to identify microbial biomarkers. The random forest was performed using randomForest R package (120), and the processes of parameter selection, and biomarker identification, are outlined in **Supplementary Figure 8**. In the parameter selection, we selected the optimal hyperparameters to improve prediction performance. For model evaluation and biomarker identification, a random forest classification model was generated and evaluated with K-fold CV, simultaneously choosing the microbial biomarkers of chlamydial ascension.

The following parameters were examined: (1) number of folds for CV (K=5 or K=10), (2) level of phylogeny used to aggregate ASVs into species or genus, (3) significance level of T-tests and Wilcoxon rank-sum tests used for aggregated microbial features (0.05 or 0.10), (4) whether to use the default setting of randomForest function or a hyperparameter grid search. Tuning parameters in the randomForest function included three options: mtry (number of trees randomly sampled as candidates at each split), nodesize (minimum size of terminal nodes), and sampsize (sizes of sample to grow). To accomplish this, we split our dataset into training and test datasets using K-fold CV, ensuring that each fold included samples from both the Endo+ and Endo- groups. With training data, we performed two-sample T-tests and Wilcoxon rank-sum

tests on ASVs to aggregate ASVs having p-value < 0.05 and the same direction in abundances within the same genus or species between Endo+ and Endo- groups. Specifically, when ASVs have a p-value less < 0.05, ASVs demonstrating the same directional trend in abundances (i.e., either positive or negative t-statistics) within a species or genus were aggregated into a single microbiome feature. ASVs with p-values >0.05 but with consistent directional trends, were also aggregated into a single microbiome feature. These aggregated microbiome features were tested again using T-tests and Wilcoxon-tests to choose microbial features having p-values less than the significance level (0.05 or 0.10). The aggregated microbiome features were subsequently employed as features of training data and test data for random forest classification. For every combination of the four parameters, AUC values were calculated from 20 replicates of random forest, and parameters were selected based on the highest sum of the mean and median AUC values across the 20 replicates. As a result, the optimal parameters chosen included a significance level of 0.10, a 10-fold cross-validation (CV), aggregation of read counts at the genus level for ASVs, and the default settings of the randomForest R function. These settings were applied twice, once including CT ASV as a microbiome feature, then excluding CT ASV.

With optimal parameters, the process re-implemented for model evaluation and biomarker identification with 100 iterations. Prediction accuracy was assessed by averaging the ROCs and AUCs obtained from the test sets over the 100 iterations. Optimal thresholds for specificity and sensitivity were obtained by pROC R package (121). For biomarker identification, ASVs were ranked based on their mean decrease in accuracy, which indicates the importance of each feature in classifying samples. The

higher the mean decrease in accuracy, the more important the variable is in the model. Using this information, ASVs were identified as biomarkers predicting chlamydial ascension.

For validation of the 12 informative ASVs indicating risk for chlamydial ascension, we performed random forest prediction using the same approach by which the 12 informative ASVs were identified (**Table 1**). A total of 143 samples (both Endo+ and Endo-) were randomly split into training and test datasets using 10-fold cross-validation (CV). The test dataset, including the four samples, was predicted using the CLR-transformed abundance of the 12 ASVs. This entire process was repeated 100 times to ensure consistency in the predictions across different subsets of the 143 samples used as the training dataset. We then evaluated the predictions by calculating the mean, standard deviation, and standard error of Endo+ probabilities for the seven samples, as well as for the Endo+ and Endo- samples.

Canonical Correlation Analysis. Canonical correlation analysis (CCA) focuses on finding linear combinations maximizing the correlation between two sets of multiple variables. To assess the association between cervical cytokine levels and microbial features, CCA was implemented by CCA R package (45).

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TABLES

Table 1. ASVs predictive of ascended CT infection. The ASVs selected for predicting susceptibility to ascending CT infection were determined based on their selection frequency threshold derived from random forest predictions. The selection frequency indicates the number of times an ASV was detected with non-NA values of mean decrease in accuracy, occurring in 9 or 10 out of 10 folds, out of 100 replications. From the RF classification results, 12 ASVs were identified as microbial biomarkers because their selection frequencies exceeded 80. Notably, despite considering two different scenarios (A: prediction with all ASVs including CT ASV and B: prediction with all ASVs but excluding CT ASV), 11 out of 12 ASVs were consistently identified across both scenarios and indicated by "Shared" in Prediction scenario column of **Table 1**. The relative abundance of these ASVs was determined using t statistics, indicating whether an ASV was higher in Endo+ or lower in Endo+. Total read count was calculated for all 220 women, and details of the %ID and Confidence level columns were provided in **Table 1**.

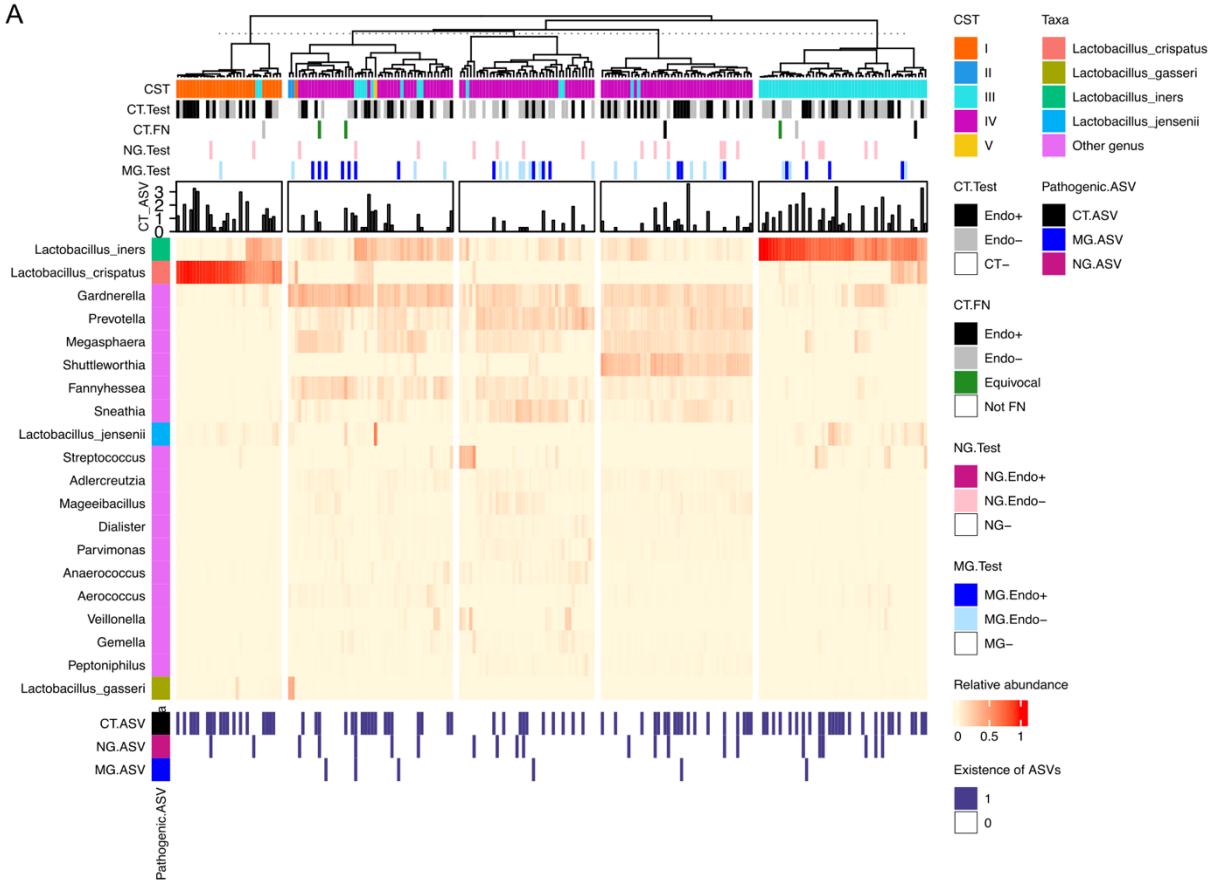
Relative abundance	Pred. Scenario	Species	ASV	Selection frequency	Total read count	%ID	Genus	Confidence level	
								Genus	Species
Higher in Endo+	w/ CT	*Chlamydia_trachomatis*	ZOtu76	100	15,683	100	*Chlamydia*	1	0.96
	Shared	*Acetothermia_genera_incertae_sedis_clone*	ZOtu233	100	312	79	*Acetothermia_genera_incertae_sedis*	0.0064	0.0005
		Butyrivibrio_hungatei	ZOtu1808	98	325	89.4	*Butyrivibrio*	0.2048	0.0266
		Actinobaculum_massiliense	ZOtu567	100	63	100	*Actinobaculum*	0.89	0.6141
Lower in Endo+	Shared	*Actinotignum_schaalii***	ZOtu281	100	200	100	*Actinotignum*	0.96	0.768
		Haemophilus_haemolyticus	ZOtu103	100	6,697	100	*Haemophilus*	0.96	0.7584
		Candidatus Mycoplasma girerdii	ZOtu45†	100	68,190	100	*Mycoplasma*		
		Porphyromonas_bennonis	ZOtu373	100	132	99.2	*Porphyromonas*	1	1
		Propionibacterium _lymphophilum	ZOtu333	100	142	100	*Propionibacterium*	1	1
		*Sutterella_stercoricanis***	ZOtu110	100	4,343	96.4	*Sutterella*	0.894	0.5453
		*Sutterella_stercoricanis***	ZOtu184	99	690	99.6	*Sutterella*	0.9801	0.9605
		*Sutterella_stercoricanis***	ZOtu180	87	756	97.6	*Sutterella*	0.8748	0.6998

** ASVs were assigned to a specific species from among different species that share the same sequence in the V4 region of the 16S rRNA.

† ZOtu45 ASV was initially assigned as *Malacoplasma microti* based on RDP Classifier DB with 89% identity (Confidence level: 0.8209 at the genus level and 0.4351 at the species level), but it was later identified as *Candidatus Mycoplasma girerdii* with 100% identity.

FIGURES

A



B

CST Types	subCST Types	Definition	No. of samples	Sum (%)
I	I-A	Almost completely L. crispatus	18	31 (14.1)
	I-B	Less L.crispatus but still majority	13	
II	II	Dominated by L. gasseri	2	2 (0.9)
III	III-A	Almost completely L. iners	27	66 (30.0)
	III-B	Less L. iners but still majority	39	
IV	IV-A	BVAB1 and G. vaginalis	0	120 (54.5)
	IV-B	G.vaginalis and A. vaginae	114	
	IV_C0	Prevotella spp.	2	
	IV-C1	Streptococcus spp.	4	
V	V	Dominated by L. jensenii	1	1 (0.5)

Figure 1. Profiles of community state types from TRAC cohort associated with Chlamydial infection. A. The taxonomic composition of 220 women, incorporating CST types and pathogen infection status (CT = *Chlamydia trachomatis*, NG = *Neisseria gonorrhoeae* (NG), and MG = *Mycoplasma genitalium*) were presented on the heat map. The testing results for pathogenic infection status at enrollment (CT.Test, NG.Test, MG.Test) were provided as bar graphs on the top of the heatmap. The reassigned

results of seven false negatives for CT infection are labeled as CT.FN. The term 'Equivocal' indicates that the reassignment of these cases as either Endo+ or Endo- was unclear, despite them being confirmed as CT+. CT ASV read counts were converted into logarithmic scale (CT_ASV; log10(read count + 1)). The observations of three pathogenic ASV sequences were illustrated with ‘Existence of ASVs’ at the bottom of the heatmap (CT.ASV, NG.ASV, MG.ASV). **B.** The table shows the definitions of CST types and the number of women belonging to each CST type.

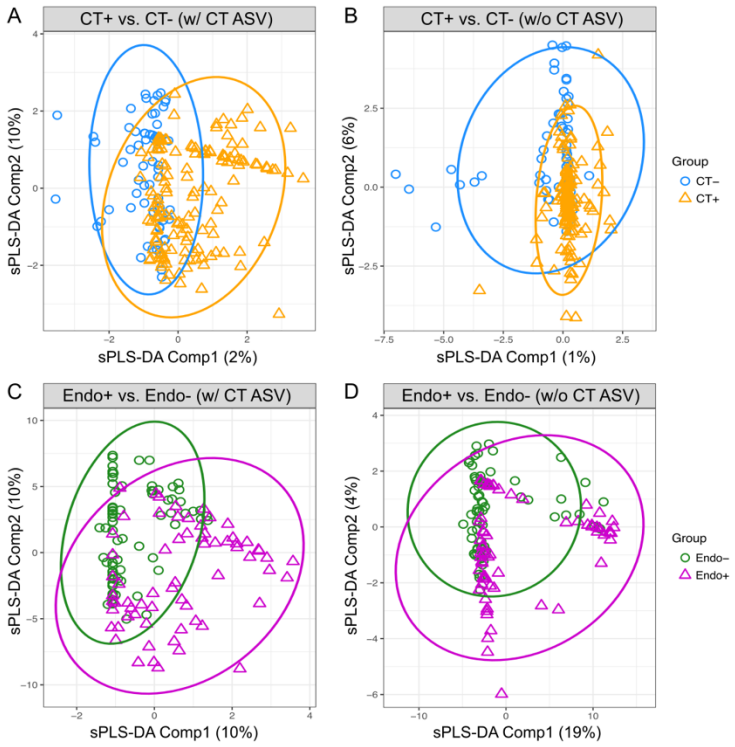


Figure 2. Discrimination of chlamydial infection status at the cervix or endometrium in TRAC cohort using sPLS-DA. Discrimination of chlamydial infection status at cervix (A and B) and endometrium (C and D) was visualized using the first two components of sPLS-DA. The discriminations were performed using ASVs including CT ASV (A and C) or excluding CT ASV (B and D). Each component's contribution to the discrimination was indicated at their axis. The performance was evaluated using the mean Area Under the Curve (AUC) values. **A.** The first two sPLS-DA components discriminate CT+ from CT- women based on ASVs with CT ASV (AUC mean = 0.70, AUC s.d. = 0.02) **B.** The discrimination of CT+ from CT- women based on without CT ASV (AUC mean = 0.51, AUC s.d. = 0.03) **C.** The first two sPLS-DA components discriminate Endo+ from Endo- women (AUC mean = 0.76, AUC s.d. = 0.01) **D.** The discrimination of Endo+ from Endo- women based without CT ASV (AUC mean = 0.55, AUC s.d. = 0.03).

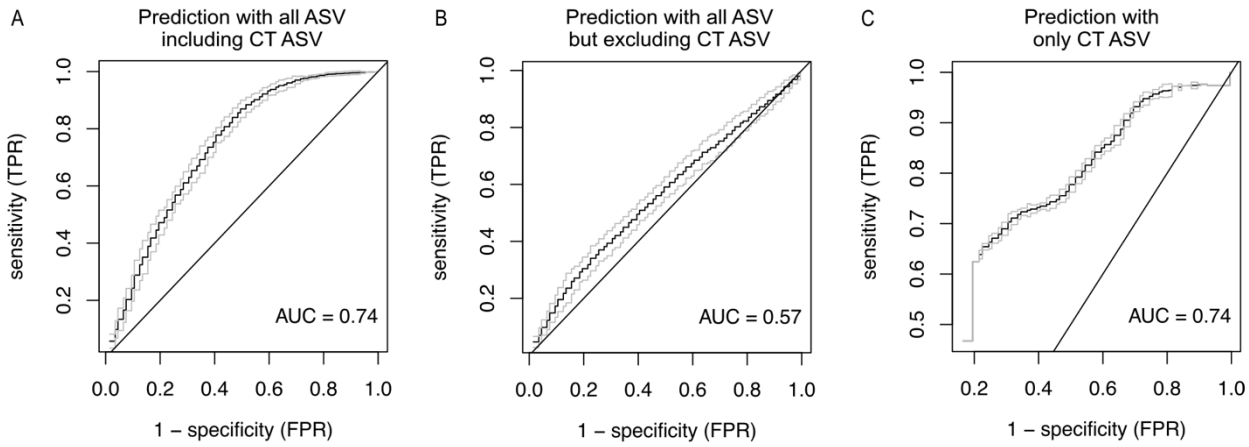


Figure 3. ROC curves and corresponding AUCs from random forest prediction for susceptibility to ascending chlamydial infection. Prediction accuracies were evaluated by averaging AUCs over 100 replicates for each scenario. **A.** The prediction performance for the susceptibility to CT ascension using all ASVs, including CT ASV resulted in an AUC of 0.74. **B.** The prediction performance for excluding CT ASV resulted in an AUC of 0.57. **C.** The susceptibility of ascending CT infection, predicted using only CT ASV, led to an AUC of 0.74.

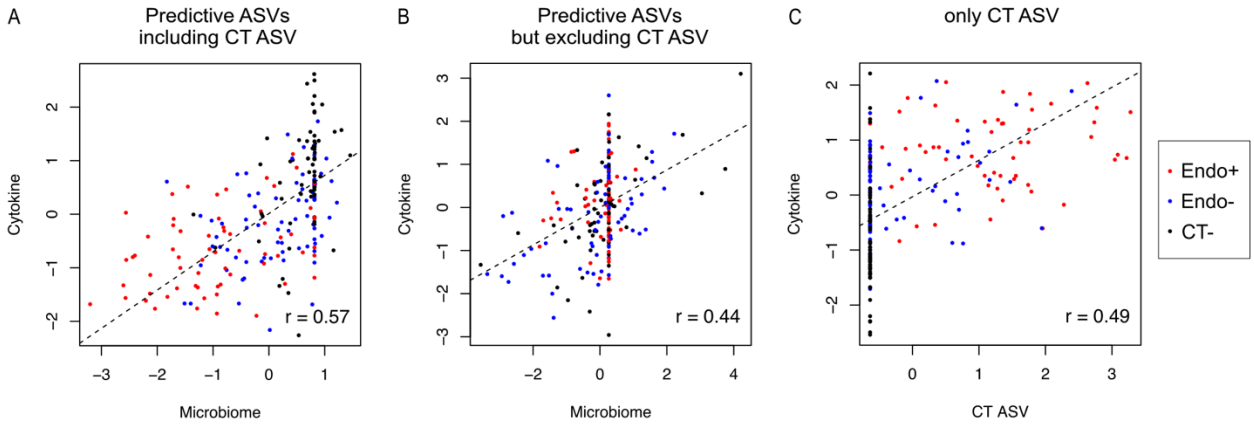


Figure 4. The Correlations between canonical variates of microbiome biomarkers and cervical cytokines. The canonical correlations between cervical cytokines related to chlamydial ascension and microbial biomarkers identified for chlamydial ascension risk were performed under two scenarios: (A) correlating the 7 cervical cytokine levels with 12 informative ASVs, including CT ASV, listed in **Table 1**, and (B) with 11 informative ASVs, excluding CT ASV. **A.** The canonical correlation between the seven cervical cytokine levels and the 12 ASVs, including CT ASV, was visualized using two CCA variates, resulting in a correlation coefficient of 0.57. **B.** The canonical correlation between the seven cervical cytokine levels and the 11 ASVs, excluding CT ASV, yielded a correlation coefficient of 0.44. **C.** Only CT ASV abundance was correlated with the seven cytokine levels, resulting in a correlation coefficient of 0.49.