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Integrating epidemiologic and molecular methods to improve vaginal health

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Abstract

Integrating epidemiologic and molecular methods to improve vaginal health

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Challenges measuring the microbiome and limited incorporation of epidemiologic methods in microbiome science have hindered the progress of vaginal microbiome research, and major questions in the field remain unanswered despite decades of work. This dissertation seeks to address these gaps by describing measurement error and resulting bias in vaginal microbiota research; evaluating the state of epidemiologic evidence regarding the role of *Lactobacillus iners*, a controversial vaginal bacterial species, in various sexual health outcomes; and investigating bacterial drivers of BV symptomatology.

The most popular method for characterizing the vaginal microbiota is 16S rRNA gene amplicon sequencing, which provides information on the taxonomic composition of a bacterial community. Shotgun metagenome sequencing characterizes the bacterial genes in a sample and provides information on the community's functional potential, which is more relevant to understanding mechanisms and causal relationships between the microbiome and health

outcomes than taxonomic composition. Metagenome inference methods attempt to bridge the gap between 16S rRNA gene amplicon sequencing and shotgun metagenomics by predicting a bacterial community's metagenome based on its taxonomic composition and annotated whole genome sequences of its members. Several studies have compared metagenome inference performance in different human body sites; however, none specifically reported on the vaginal microbiome.

In **Chapter 2**, we compared the performance of two popular metagenome inference methods, PICRUSt2 and Tax4Fun2, using paired 16S rRNA gene amplicon sequencing and shotgun metagenome sequencing data from vaginal samples from 72 pregnant individuals enrolled in the Pregnancy, Infection, and Nutrition cohort. PICRUSt2 and Tax4Fun2 performed modestly overall (median Spearman correlations between observed and predicted KEGG ortholog [KO] relative abundances 0.20 and 0.22, respectively). Both methods performed best among *Lactobacillus crispatus*-dominated vaginal microbiotas (median Spearman correlations 0.24 and 0.25, respectively) and worst among *L. iners*-dominated microbiotas (median Spearman correlations 0.06 and 0.11, respectively). Differential metagenome inference performance across vaginal microbiota community types can be considered differential measurement error, which often results in differential misclassification. As such, metagenome inference will introduce hard-to-predict bias in vaginal microbiome research. These findings demonstrate the importance of considering common epidemiologic biases in designing and evaluating novel microbiome analytic methods.

The vaginal microbiota of reproductive-age individuals is often dominated by a single *Lactobacillus* species, most commonly *L. iners* or *L. crispatus*. While *L. crispatus*-dominated vaginal microbiotas are widely thought to protect against adverse sexual health outcomes, the role of *L. iners*-dominated microbiotas is less clear and has been debated. To better understand the role of *L. iners*, in **Chapter 3** we conducted systematic reviews of the associations between *L. iners* compared to *L. crispatus* and the following outcomes: bacterial vaginosis (BV); *Chlamydia*

trachomatis, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, human papillomavirus (HPV), HIV, and genital herpes simplex virus type-2 (HSV-2) infections; and cervical dysplasia.

We searched four databases for epidemiologic studies of reproductive-age, nonpregnant, cisgender women that used marker gene sequencing to characterize vaginal microbiota composition and presented an effect estimate for the association between *L. iners* compared to *L. crispatus* and outcomes of interest. For outcomes with ≥3 eligible studies presenting the same form of effect estimate, we conducted random-effects meta-analysis. Three BV studies were included in meta-analysis, which indicated *L. iners*-dominated microbiotas were associated with 2.1-fold higher prevalence of BV compared to *L. crispatus*-dominated microbiotas (95% CI 0.9-4.9). Six *C. trachomatis* studies were included in meta-analysis, which showed *L. iners*-dominated microbiotas were associated with 3.4-fold higher odds of chlamydia compared to *L. crispatus*-dominated microbiotas (95% CI 2.1-5.4). *L. iners*-dominated vaginal microbiotas may be suboptimal compared to *L. crispatus*-dominated microbiotas for BV and chlamydia, which is consistent with prior in vitro, in silico, and genomic work. Evidence was sparse for other outcomes. Nearly all included studies assessed microbiota composition and outcome status cross-sectionally and were at serious risk of bias, critically limiting the quality of evidence reviewed.

In contrast to *Lactobacillus*-dominated microbiotas, BV is a polymicrobial condition characterized by a diverse community of anaerobic and facultative bacteria. It is the most common cause of vaginal discharge worldwide, affecting approximately one quarter of reproductive-age individuals. In high-resource settings, clinical BV diagnosis is typically based on the presence of at least three of four signs and symptoms termed Amsel criteria: amine odor on addition of potassium hydroxide to vaginal fluid; clue cells on vaginal wet prep; thin, gray, homogeneous vaginal discharge; and elevated vaginal pH. Because bacterial colonization and associations with these signs and symptoms may vary between populations, in **Chapter 4** we assessed relationships between vaginal bacteria and Amsel criteria among two distinct populations.

We included Kenyan participants from the placebo arm of the Preventing Vaginal Infections (PVI) trial and participants from a Seattle-based cross-sectional BV study in this analysis. Amsel criteria were recorded at study visits, and the vaginal microbiota was characterized using 16S rRNA gene amplicon sequencing. We fit logistic regression models to evaluate associations between bacterial relative abundance and each Amsel criterion. BV-associated bacterium 1 (BVAB1) was positively associated with all Amsel criteria in both populations. *Eggerthella* type 1, *Fannyhessea* (*Atopobium) vaginae*, *Gardnerella* spp., *Sneathia amnii*, and *Sneathia sanguinegens* were positively associated with all Amsel criteria in the Seattle study, and all but discharge in the PVI trial. This core group of vaginal bacteria may play a key role in the manifestation of BV signs and symptoms across diverse populations, and these findings are consistent with growing evidence regarding the role of biogenic amines and extracellular enzymes in BV etiology and symptom manifestation.

Finally, in **Chapter 5** I discuss the implications of this dissertation work for microbiome science, medicine, and public health. I recommend avenues by which investigators can better incorporate epidemiologic methods and principles into their work, I provide a novel characterization of *L. iners*, and I weigh various strategies to improve BV diagnostics and treatment in high- and low-resource settings.

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Chapter 1. INTRODUCTION

The vaginal microbiota is the community of microorganisms that reside in the vagina. For decades, bacterial cultivation (i.e. culture) and Gram-stain microscopy were the primary methods for characterizing the vaginal microbiota and studying its relationships with sexual and reproductive health outcomes. In the 2000's, the advent of high-throughput sequencing in enabled researchers to investigate the vaginal microbiota with unprecedented granularity and sensitivity. Much of this work has focused on describing vaginal microbiota composition and natural history among reproductive-age individuals, elucidating the etiology of bacterial vaginosis (BV), and examining the roles of vaginal bacteria and BV in sexual and reproductive health outcomes, namely human immunodeficiency virus (HIV) and sexually transmitted infection (STI) acquisition and preterm birth (PTB). These questions remain unanswered to varying degrees, in part due to challenges measuring the microbiota and limited incorporation of epidemiologic methods in microbiota science.[1–5] This dissertation seeks to address these gaps by describing measurement error and resulting bias in vaginal microbiota research; evaluating the state of epidemiologic evidence regarding the role of a common but controversial vaginal bacterial species in various sexual health outcomes; and investigating bacterial drivers of BV symptomatology. Key terms used throughout this dissertation are defined in Table 1.1.

Table 1.1. Key terms used throughout this dissertation.

Term	Description
16S rRNA gene amplicon sequencing	An approach to characterize the taxonomic composition of bacterial communities by amplifying and sequencing hypervariable region(s) of the bacterial 16S rRNA gene
Amsel criteria[6]	System for clinically diagnosing bacterial vaginosis based on the presence of at least three of four signs and symptoms: amine odor on addition of potassium hydroxide to vaginal fluid; >20% clue cells (epithelial cells coated in bacteria) on vaginal wet prep; thin, gray, homogeneous vaginal discharge; and elevated vaginal pH >4.5

Bacterial vaginosis	Polymicrobial condition characterized by a diverse vaginal microbiota of anaerobic and facultative bacteria, including *Gardnerella*, *Prevotella*, *Fannyhessea* (formerly *Atopobium*), and *Sneathia* species; associated with numerous adverse sexual and reproductive health outcomes
Metabolome	All metabolites present in a microbiome, including those produced by microorganisms and the host
Metagenome	All DNA (genes, genomes) present in a bacterial community/microbiome
Metagenome inference	A family of computational approaches that seek to predict metagenome content using taxonomic composition data and reference annotated whole genome sequences; PICRUSt2[7] and Tax4Fun2[8] are two metagenome inference methods
Microbiome	Community of microorganisms and the environment they occupy, includes the microbiota, metagenome, and metabolome
Microbiota	Microbial members of a microbiome, will be used to refer to the bacterial members of a microbiome throughout this dissertation
Nugent score	Gram stain-based scoring system to classify bacterial vaginosis based on bacterial staining and morphotypes on a vaginal smear
Taxon	Generic term for a taxonomic group of any rank (e.g. family, genus, species); plural: taxa

The most popular method for characterizing the vaginal microbiota is 16S rRNA gene amplicon sequencing,[9,10] which provides information on the taxonomic composition of a bacterial community (members and their proportions). Additional sequencing-based approaches include shotgun metagenome sequencing, which characterizes the bacterial genes in a sample and provides information on the community's functional potential, and metatranscriptomics, which characterizes the mRNA transcripts in a sample and provides information on gene/function expression. Analytical chemistry methods can provide information on the activity or functioning of a microbial community by characterizing its metaproteome (all proteins in a sample) or metabolome (all small molecule metabolites in a sample). Compared to taxonomic composition, the functional potential, functional expression, and/or functioning of a bacterial community are

more relevant to establishing mechanistic understandings and causal relationships between the microbiome and health outcomes. However, 16S rRNA gene amplicon sequencing has been and remains the most popular method for characterizing the vaginal microbiome due to the prohibitive costs of shotgun metagenomics, metatranscriptomics, metaproteomics, and metabolomics.[9,10]

Several computational methods referred to as metagenome inference attempt to bridge the gap between 16S rRNA gene amplicon sequencing and shotgun metagenomics by predicting a bacterial community's metagenome based on its taxonomic composition (16S rRNA gene amplicon sequencing data) and annotated whole genome sequences of the community's members.[7,8,11–13] Metagenome inference methods have been evaluated primarily among gut samples, where they appear to perform fairly well.[7,8,11–15] However, no prior studies specifically reported on metagenome inference for the vaginal microbiome. Findings from other body sites cannot easily be generalized to the vagina due to unique features of vaginal microbial ecology and underrepresentation of vaginal samples in reference sequence databases.[16] Without specific information on metagenome inference performance in the vagina, investigators seeking to use these methods in vaginal microbiome research are uninformed about any potential bias these methods may introduce into analyses.

Chapter 2 will address this gap by evaluating the performance of two popular metagenome inference methods, PICRUSt2 and Tax4Fun2, for the vaginal microbiome.[7,8] Moreover, this work examines whether metagenome inference performance varies across sample characteristics and across gene characteristics with the aim of describing measurement error and associated biases incurred when inferred metagenomes are used in vaginal microbiome research. By providing necessary context for investigators to make informed decisions regarding whether to use metagenome inference in their work and how to interpret results from analyses that incorporate inferred metagenomes, this study will ultimately contribute to improving the rigor of vaginal microbiome science.

Despite its limitations, 16S rRNA gene amplicon sequencing has been instrumental in characterizing vaginal microbial ecology and evaluating associations between vaginal bacteria and sexual and reproductive health outcomes. Across populations, the vaginal microbiota of reproductive-age individuals is often dominated by a single *Lactobacillus* species, most commonly *Lactobacillus crispatus* or *Lactobacillus iners*.[17–23] While *L. crispatus*-dominated vaginal microbiotas are widely thought to protect against adverse sexual health outcomes, the role of *L. iners*-dominated microbiotas is less clear and has been debated.[24–26] Moreover, several genomic and metabolic differences between *L. iners* and other common vaginal lactobacilli, including *L. crispatus,* would be consistent with *L. iners* functioning differently in the vagina.[24,25,27–32] As *L. iners* is considered to be the most prevalent and abundant vaginal bacterial species,[25,33] understanding its influence on sexual health is urgently needed to better understand the etiology of adverse sexual health outcomes and develop effective microbiome-based prevention and treatment strategies.

Chapter 3 will evaluate the state of epidemiologic evidence regarding *L. iners* and sexual health outcomes through a series of systematic reviews and meta-analyses targeting the following outcomes: BV; *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, human papillomavirus (HPV), HIV, and genital herpes simplex virus type-2 (HSV-2) infections; and cervical dysplasia. These reviews will elucidate the epidemiology of *L. iners* as compared to other vaginal lactobacilli and inform efforts to develop microbiome-based prevention strategies for BV, HIV, STI, and cervical carcinogenesis. This work will also contribute to improving the epidemiologic rigor of vaginal microbiome science by highlighting common epidemiologic limitations and areas for improvement across the reviewed literature.

In contrast to *Lactobacillus*-dominated microbiotas, BV is a polymicrobial condition characterized by a diverse community of anaerobic and facultative bacteria, including *Gardnerella*, *Prevotella*, *Fannyhessea* (formerly *Atopobium*), and *Sneathia* species. It is the most common cause of vaginal discharge worldwide, affecting approximately one quarter of

reproductive-age individuals.[34] BV etiology remains unclear, in part because vaginal microbiota composition varies between individuals diagnosed with BV and over time within individuals.[35–42] In high-resource settings, clinical BV diagnosis is typically based on the presence of at least three of four signs and symptoms termed Amsel criteria: amine odor on addition of potassium hydroxide to vaginal fluid; >20% clue cells (epithelial cells coated in bacteria) on vaginal wet prep; thin, gray, homogeneous vaginal discharge; and elevated vaginal pH >4.5.[6] Recently, several molecular and point-of-care BV tests have been developed, which seek to make a clinical diagnosis based on the presence/abundance of bacteria or bacterial products that are commonly observed during BV.[43] In research settings, BV is often classified using a Gram stain-based system referred to as the Nugent score.[44] Regardless of classification method, BV is consistently associated with the prevalence and incidence of adverse sexual and reproductive health outcomes including HIV/STI acquisition, cervical disease progression, and preterm birth.[45–52]

The Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) do not recommend BV testing or treatment for asymptomatic individuals,[53,54] so care seeking and subsequent BV treatment are primarily driven by individuals' experiences of their BV symptoms. Among individuals with BV according to Nugent score, as many as 90% of those living in sub-Saharan Africa and 67% of those living in North America are asymptomatic and would receive neither a clinical BV diagnosis nor treatment.[34,55,56] It is unclear what drives heterogeneity in BV presentation between individuals. Moreover, given variations in BV prevalence and vaginal microbiota composition between populations,[34,57–61] it is also unclear whether drivers of BV signs and symptoms are consistent between populations.

Chapter 4 will build on prior work demonstrating associations between specific vaginal bacteria and individual Amsel criteria in an American population[37] by assessing these associations among a Kenyan population as well as the degree to which these associations are consistent between the two populations. Identifying microbial contributions to BV symptomatology can inform design and improve performance of molecular and point-of-care BV diagnostics. These

tests are not yet widely used because they are costly and many health insurance plans do not cover them; however, they will likely become more popular in coming years as costs fall and because they are demonstrated to be cost-effective over a 12-month time horizon.[43,62] Improving test performance before they are more widely implemented will increase the impact of these tests in terms of treating BV, alleviating BV symptoms which can cause substantial psychosocial stress, and potentially reducing the incidence of adverse sexual and reproductive health outcomes associated with BV.[63–65] This work will also contribute to ongoing efforts to elucidate BV etiology, which can in turn aid in improving BV treatment efficacy and developing effective BV prevention strategies.

Chapter 2. VAGINAL MICROBIOME METAGENOME INFERENCE ACCURACY: DIFFERENTIAL MEASUREMENT ERROR ACCORDING TO COMMUNITY COMPOSITION

2.1 ABSTRACT

2.1.1 *Background*

Several studies have compared metagenome inference performance in different human body sites. However, none specifically reported on the vaginal microbiome, and findings from other body sites cannot easily be generalized to the vaginal microbiome due to unique features of vaginal microbial ecology. There is no published evidence to guide investigators in selecting if, when, or which metagenome inference method to use for vaginal microbiome research, or to guide interpretation of vaginal microbiome metagenome inference results.

2.1.2 *Methods*

We compared the performance of PICRUSt2 and Tax4Fun2 using paired 16S rRNA gene amplicon sequencing and whole metagenome sequencing data from vaginal samples from 72 pregnant individuals enrolled in the Pregnancy, Infection, and Nutrition cohort. Participants were selected from those with known birth outcomes and adequate 16S rRNA gene amplicon sequencing data in a case-control design. Cases experienced early preterm birth (PTB, <32 weeks' gestation), and controls experienced term birth (37-41 weeks' gestation).

2.1.3 *Results*

PICRUSt2 and Tax4Fun2 performed modestly overall (median Spearman correlation coefficients between observed and predicted KEGG ortholog [KO] relative abundances 0.20 and 0.22,

respectively). Both methods performed best among *Lactobacillus crispatus*-dominated vaginal microbiotas (median Spearman correlation coefficients 0.24 and 0.25, respectively) and worst among *Lactobacillus iners*-dominated microbiotas (median Spearman correlation coefficients 0.06 and 0.11, respectively). The same pattern was observed when evaluating correlations between univariable hypothesis test p values generated with observed and predicted metagenome data.

2.1.4 *Conclusions*

Differential metagenome inference performance across vaginal microbiota community types can be considered differential measurement error, which often causes differential misclassification. As such, metagenome inference will introduce hard-to-predict bias (towards or away from the null) in vaginal microbiome research.

2.1.5 *Importance*

Compared to taxonomic composition, the functional potential of a bacterial community is more relevant to establishing mechanistic understandings and causal relationships between the microbiome and health outcomes. Metagenome inference attempts to bridge the gap between 16S rRNA gene amplicon sequencing and whole metagenome sequencing by predicting a microbiome's gene content based on its taxonomic composition and annotated genome sequences of its members. Metagenome inference methods have been evaluated primarily among gut samples, where they appear to perform fairly well. Here, we show that metagenome inference performance is markedly worse for the vaginal microbiome, and that performance varies across common vaginal microbiome community types. Because these community types are associated with sexual and reproductive outcomes, differential metagenome inference performance will bias vaginal microbiome studies, obscuring relationships of interest. Results

from such studies should be interpreted with substantial caution and the understanding that they may over- or under-estimate associations with metagenome content.

2.2 INTRODUCTION

Several studies have compared the performance of various metagenome inference methods in different contexts, including human body sites and environmental ecosystems.[7,8,11–15] A common finding of these studies is that performance improves with increasing representation of taxa in reference sequence databases (16S rRNA gene and whole genome), which results in higher-quality metagenome inference for more well studied ecosystems.[7,8,11–15] Namely, metagenome inference methods tend to perform best for the human gut and oral microbiomes and less well for other human body sites, other mammalian gut, and environmental microbiomes.[7,8,11–15] However, no prior studies specifically reported on metagenome inference for the vaginal microbiome (one reported on urogenital samples but gave no additional detail on these samples).[12]

Findings from other body sites cannot easily be generalized to the vaginal microbiome due to unique features of vaginal microbial ecology. For many reproductive-age individuals, the vaginal microbiome is dominated by a single *Lactobacillus* species, and these low-diversity communities are generally associated with positive vaginal, sexual, and reproductive health outcomes.[17,19,20,23,24,66] Conversely, diversity is very common in the gut and is considered a hallmark of gut health.[67,68] Several prevalent vaginal bacteria have only recently been characterized or remain uncharacterized (e.g. bacterial vaginosis associated bacterium 1 [BVAB1, proposed name *Candidatus* Lachnocurva vaginae], BVAB2, *Gardnerella* species, *Mageeibacillus indolicus*, *Megasphaera lornae*).[69–72] Finally, reference sequence databases contain data from >10-fold more gut samples than vaginal samples.[16] As such, it is likely that metagenome inference methods perform poorly for the vaginal microbiome. However, without

specific information on metagenome inference performance in the vagina, investigators seeking to use these methods in vaginal microbiome research are "flying blind" with respect to the potential bias these methods may introduce into analyses. To fill these gaps, we compared the performance of PICRUSt2 and Tax4Fun2 using paired 16S rRNA gene amplicon sequencing and whole metagenome sequencing (WMGS) data from vaginal samples from 72 pregnant individuals. We examined whether performance varied across sample characteristics (hierarchical cluster) and gene characteristics (functional category) to describe the bias PICRUSt2- and Tax4Fun2-inferred metagenomes may introduce into analyses.

2.3 MATERIALS AND METHODS

We used paired 16S rRNA gene amplicon sequencing and WMGS data from the Pregnancy, Infection, and Nutrition (PIN) cohort to evaluate and compare the performance of PICRUSt2 and Tax4Fun2 in the vaginal microbiome.[7,8,73] The PIN cohort is a prospective cohort study approved by the University of North Carolina Institutional Review Board (protocol 16-2166), and all participants provided written informed consent prior to enrollment. For this secondary analysis, we selected participants from among those who consented to additional testing of stored specimens. We report our results according to the Strengthening The Organization and Reporting of Microbiome Studies (STORMS) guidelines.[74]

2.3.1 *PIN cohort participants and study procedures*

PIN cohort participants were recruited from prenatal clinics at the Wake County Human Services Department, WakeMed Medical Center/Wake Area Health Education Center, and University of North Carolina hospitals in North Carolina between 1995 and 2008. Cisgender women were eligible if they were $\geq$16 years old and at $\leq$29 weeks' gestation with a singleton pregnancy. Women

were ineligible if they were <16 years old, at >29 weeks' gestation, did not plan to continue care or delivery at the study site, did not have telephone access, or were non-English speaking. Study staff identified potential participants through medical record review, after which study staff approached potential participants about enrolling in the cohort.

Demographic characteristics were recorded at enrollment, and clinicians collected two cotton-tipped swabs from the posterior vaginal apex between 24-29 weeks' gestation. One swab was used for evaluation of bacterial vaginosis (BV) according to Nugent score and then placed in a Digene Virapap tube with transport media (Digene Diagnostics, Inc, Silver Spring, MD).[44] The second swab was placed in a Roche AMPLICOR Specimen Collection tube containing extraction buffer (Roche Diagnostic Systems, Inc., Branchburg, NJ). Specimens were refrigerated prior to transport and frozen at -70°C within six hours of collection. Following enrollment, a telephone questionnaire was used to collect information on reproductive and medical history; sexual behaviors during pregnancy; and tobacco, alcohol, and other drug use. Birth outcomes were abstracted from medical records following delivery. Gestational age at delivery was defined by early ultrasound (completed <20 weeks' gestation) for 90% of participants, and by last menstrual period date for the remaining participants.

The data used in the current metagenome inference comparison were generated for a nested case-control study of the associations between the vaginal bacterial metagenome and preterm birth (PTB) and whether those associations differ between Black and white individuals (not yet published; Figure 2.1). This nested case-control study was motivated by disparities in PTB rates between Black and white individuals in the US. In 2020, the US overall PTB rate was 10%; however, it was 14% among Black individuals compared to 9% among white individuals.[75] Prior reports of associations between the vaginal microbiota and PTB among US populations have conflicting results regarding taxa that may confer risk of PTB, which is hypothesized to be related to different racial composition of study populations.[60,76–81] Race is socially constructed and not a biological category, and racial disparities in PTB as well as racial differences in vaginal

microbiota and bacterial metagenome composition are caused by systemic and institutional racism in the US.[82–85] As a result of centuries of medical mistreatment and exploitation as well as institutionalized residential segregation (i.e. redlining), Black individuals are less likely to seek medical care, which may contribute to PTB disparities as well as related factors including douching and tobacco use.[86–95] Given documented and interrelated associations between vaginal microbiota composition, douching, tobacco use, and PTB; racial differences in vaginal microbiota composition, douching, and smoking may contribute to racial disparities in PTB in a complex way.[17,60,75–81,96–108] The aim of the nested case-control study was to examine and attempt to tease apart these relationships using both 16S rRNA gene amplicon sequencing and WMGS data.

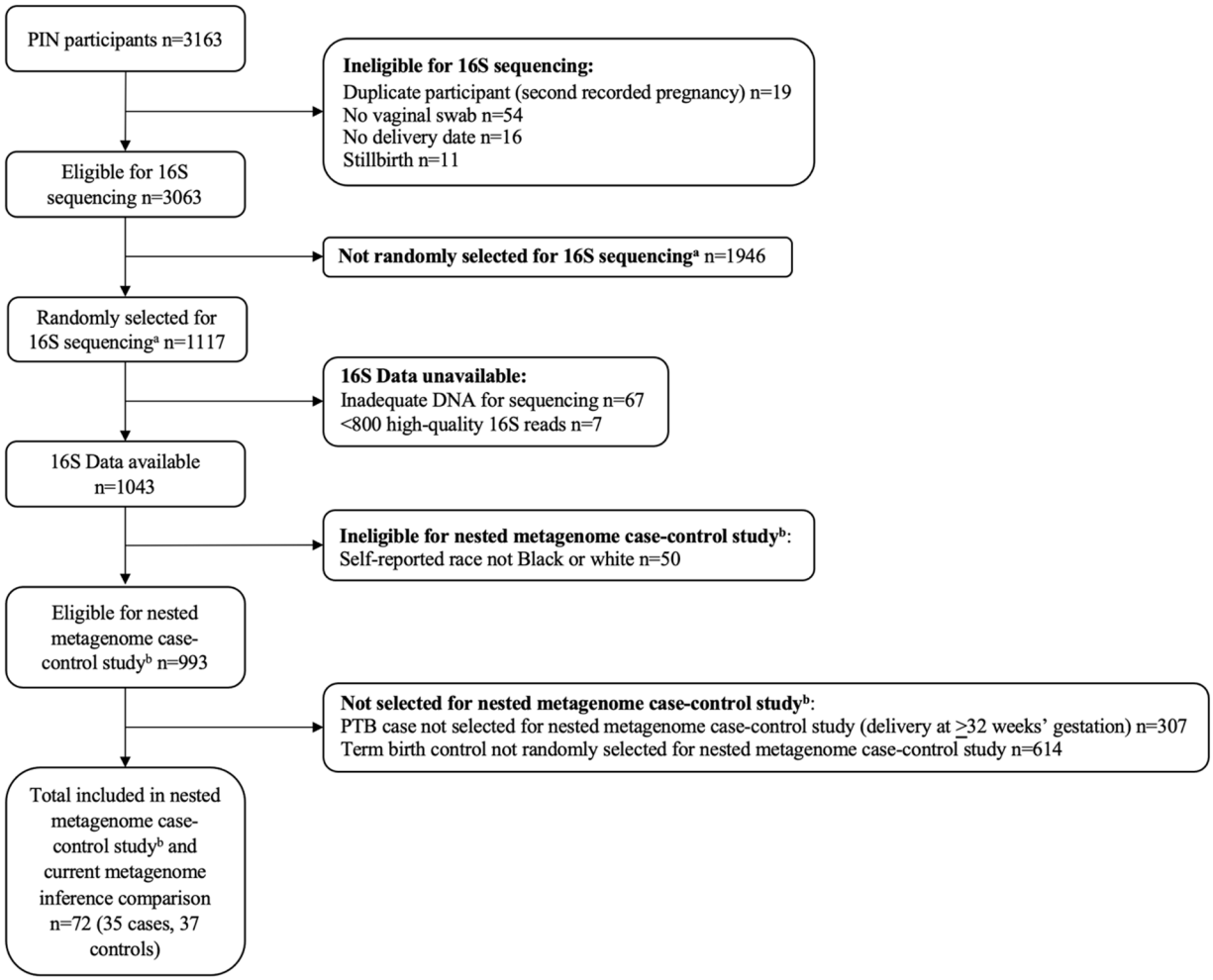

Figure 2.1. PIN participant flowchart for nested case-control study selection.

[a]A random subset of approximately half of the PIN participants were randomly selected to have stored vaginal swabs used for 16S rRNA gene amplicon sequencing.

[b]The nested metagenome case-control study is the nested case-control study of the associations between the vaginal bacterial metagenome and PTB and whether those associations differ between Black and white individuals (not yet published). We used data from the nested metagenome case-control study for the current metagenome inference comparison.

PIN, Pregnancy, Infection, and Nutrition; 16S sequencing, 16S rRNA gene amplicon sequencing; PTB, preterm birth.

For the nested case-control study, cases were participants who experienced early PTB <32 weeks' gestation, and controls were randomly selected from among participants who experienced term birth between 37-41 weeks' gestation. Cases and controls were selected from among participants with adequate extracted DNA for WMGS and ≥800 high-quality reads from 16S rRNA gene amplicon sequencing. Cases and controls were frequency matched on self-reported race and were restricted to participants reporting Black or white race. We used this subset of cases and controls for whom 16S rRNA gene amplicon sequencing and WMGS data are available for the current metagenome inference comparison.

2.3.2 *Microbiome data generation*

Stored cotton-tipped vaginal swabs were used for 16S rRNA gene amplicon sequencing and WMGS as previously reported.[76,81,105] Briefly, frozen swabs were thawed on ice, DNA was extracted using the PowerSoil DNA Isolation Kit (Qiagen, Hilden Germany) according to manufacturer recommendations, and extracted DNA was quantified using PicoGreen. Extracted DNA was amplified with barcoded primers targeting the 16S rRNA gene V1-V3 hypervariable regions using protocols established by the Virginia Commonwealth University (VCU) Vaginal Human Microbiome Project.[97] Amplicon samples were multiplexed (384 samples/run) using a

sample-specific dual-index strategy and sequenced on Illumina MiSeq platform (2 x 300 base paired-end protocol; San Diego, CA). Resulting amplicon sequence data were processed using QIIME2 (version 2019.1.0) and the DADA2 denoise-single method, truncating reads at 120 bases.[109,110] Taxonomy was assigned using the Ribosomal Database Project Naïve Bayesian Classifier and SILVA database (release 138.1).[111–113]

Extracted DNA was also used for whole metagenome library preparation. Paired-end metagenomic DNA libraries were prepared from 250ng of genomic DNA using the Accel-NGS 2S Plus DNA library kit (Integrated DNA Technologies, Coralville, IA) with insert size of approximately 350bp. Samples were pre-processed as previously described.[76,105] Briefly, the barcoded libraries were multiplexed with 10-11 samples per lane and sequenced using the Illumina HiSeq 4000 platform (2 x 150-base paired-end reads; San Diego, CA). Raw sequences were binned based on their barcode sequences using Illumina's bcl2fastq software and quality filtered using MEEPTOOLS,[114,115] where reads shorter than 70 bases and with MEEP quality score > 1 were excluded. WMGS data were screened for duplicate reads, and human sequences were removed by aligning reads to the hg19 build of the human genome using the BWA aligner.[116] Remaining reads were processed according to the approach recommended by Martin and colleagues including read quality trimming, read length filtering, and removal of low complexity reads.[117] Gene families and functional pathways were characterized using HUMAnN2 and the KEGG Orthology database.[118–121]

2.3.3 *Statistical analysis*

We used descriptive statistics to summarize participant characteristics including participant age, self-reported race, gestational age at vaginal swab collection, Nugent score at vaginal swab collection, and gestational age at delivery. We estimated vaginal microbiota alpha diversity measures based on 16S rRNA gene amplicon sequencing data using the estimate_richness function of the phyloseq package (version 1.34.0 throughout the chapter) in R (version 4.0.4

throughout the chapter). We categorized vaginal microbiota composition by hierarchical clustering of 16S rRNA gene amplicon sequencing data based on Jensen-Shannon Divergence distances and Ward linkage using the distance function of the phyloseq package, hclust and cutree functions of the stats package (version 4.0.4), and dendsort function of the dendsort package (version 0.3.4) in R.

We inferred metagenome content from 16S rRNA gene amplicon sequencing data using PICRUSt2 and Tax4Fun2 according to developers' recommendations.[7,8] We implemented PICRUSt2 (version 2.4.1) in Miniconda (version 4.9.2). We implemented Tax4Fun2 with the default reference database and 99% clustering threshold using the runRefBlast and makeFunctionalPrediction functions of the Tax4Fun2 package (version 1.1.5) in R.

For the metagenome inference performance evaluation and comparison, we restricted the observed, PICRUSt2-inferred, and Tax4Fun2-inferred metagenome datasets to KOs present at relative abundance >0% in all three datasets. We evaluated metagenome inference performance using two approaches. First, we estimated Spearman correlation coefficients between observed and predicted KO relative abundances. Second, we estimated Spearman correlation coefficients between univariable hypothesis test p values estimated using observed and predicted KO relative abundances. This approach was proposed by Sun and colleagues following their observation that Spearman correlation coefficients between observed and predicted KO relative abundances are insensitive to random permutations of the data, indicating they may be an unreliable measure for evaluating metagenome inference performance.[14] We used Wilcoxon tests to test the following two null hypotheses: KO relative abundances do not differ between self-identified Black and white participants, and KO relative abundances do not differ between PTB cases and term birth controls. We performed Wilcoxon tests using observed and predicted KO relative abundances separately, and we transformed p values according to equation 2.1 in order to capture the significance and direction of KO relative abundance differences:

Equation 2.1: $P_t = log_{10}(P) * sign((\overline{KO}|group\ 1) - (\overline{KO}|group\ 2))$

Where P_t is the transformed p value, P is the Wilcoxon test p value, $\overline{KO}|group\ 1$ is the mean KO relative abundance in the contrast group for the hypothesis test (self-identified Black participants, cases experiencing PTB), and $\overline{KO}|group\ 2$ is the mean KO relative abundance in the reference group for the hypothesis test (self-identified white participants, controls experiencing term birth). While we performed hypothesis tests comparing KO relative abundance by birth outcome and self-reported race, we only used the results of these hypothesis tests to evaluate metagenome inference performance; we were not seeking to draw inference regarding the relationships between the vaginal microbiota, vaginal bacterial metagenome (observed or inferred), self-reported race, and birth outcome. We selected birth outcome and self-reported race as the explanatory variables to use in the hypothesis tests because the case-control subsample of PIN participants used in the current metagenome inference comparison was well balanced with respect to birth outcome and self-reported race.

For both evaluation approaches, we performed two stratified analyses to evaluate whether metagenome inference performance differs according to vaginal microbiota hierarchical cluster or KO functional category (highest-level categories). We performed each analysis with the original observed and predicted metagenome data, as well as with 100 random permutations of the observed and predicted metagenome data in which KO relative abundances were independently permuted across samples. As proposed by Sun and colleagues, evaluating performance in these permuted datasets serves as a robustness check.[14] Finally, we used R^2 values from linear models to examine relationships between KO functional category relative abundances (response variables) and *L. crispatus* relative abundances, *L. iners* relative abundances, and the ratio of *L. crispatus* : *L. iners* relative abundances (explanatory variables).

2.4 RESULTS

2.4.1 *Description of participants, microbiotas, and metagenomes*

A total of 3063 PIN participants had recorded delivery dates, live births, and stored vaginal swabs and were eligible for selection for into the case-control sub-study. A total of 72 PIN participants were selected and contributed data to this analysis: 35 early PTB cases (<32 weeks' gestation at delivery) and 37 term birth controls (37-41 weeks' gestation at delivery). Among the 72 cases and controls, 45 self-reported Black race (22 cases, 23 controls, ~63% overall and within cases and controls; additional participant characteristics in Table 2.1).

Table 2.1. Participant characteristics overall and by birth outcome.

Characteristic	**Overall N=72**		**Preterm birth cases[a] n=35**		**Term birth controls[a] n=37**	
	N	**%**	**N**	**%**	**N**	**%**
Self-reported race						
Black	45	63	22	63	23	62
white	27	38	13	37	14	38
Age (years)[b]	26	21-30	26	21-29	26	22-33
Gestational age at sample collection (weeks)[b]	27	26-28	27	25-28	28	26-28
Gestational age at delivery (weeks)[b]	37	30-40	30	28-31	40	38-41
Nugent score at sample collection						
Non-BV (0-3)	50	69	25	71	25	68
Intermediate (4-6)	14	19	7	20	7	19
BV (7-10)	8	11	3	9	5	14
Hierarchical cluster[c]						
L. crispatus-dominated	17	24	11	31	6	16
L. iners-dominated	31	43	13	37	18	49
Mixed	24	33	11	31	13	35

[a]Preterm birth before 32 weeks' gestation. Term birth between 37-41 weeks' gestation. Cases and controls were matched on self-reported race.

[b]Continuous characteristics are presented as median and interquartile range.

[c]Based on hierarchical clustering of 16S rRNA gene amplicon sequencing data.

BV, bacterial vaginosis.

Average 16S rRNA gene amplicon sequencing depth was 31049 reads/sample (range 804-54628), and 115 amplicon sequence variants (ASVs) were assigned. We identified three clusters of vaginal microbiota composition based on a priori knowledge of common vaginal bacterial community types and visual inspection of the hierarchical clustering dendrogram and microbiota composition stacked bar plot (Figure 2.2). According to the Connectivity, Dunn, and Silhouette statistics, the optimal number of clusters was two. However, with two clusters, *L. iners*-dominated samples and mixed samples belonged to the same cluster. Because *L. iners* dominance is a commonly-observed vaginal microbiota community type,[17,19,20,23,66] we decided to differentiate between *L. iners*-dominated samples and mixed samples and use three clusters instead of two. Seventeen samples (24% of total) belonged to a cluster dominated by *Lactobacillus crispatus*, including 11 PTB cases (31% of cases) and 16 Black participants (36% of Black participants). Thirty-one (43% of total) belonged to a cluster dominated by *Lactobacillus iners*, including 13 PTB cases (37% of cases) and 23 Black participants (51% of Black participants). Twenty-four (33% of total) belonged to a mixed cluster, including 11 PTB cases (31% of cases) and 16 Black participants (36% of Black participants). According to all alpha diversity metrics estimated (observed richness, Shannon, Simpson, Inverse Simpson), samples from the *Lactobacillus*-dominated clusters appeared to have similar distributions of alpha diversity (Figure 2.3). Alpha diversity values for samples in the mixed cluster overlapped with those from both *Lactobacillus*-dominated clusters, though the mixed cluster showed the widest ranges for all metrics.

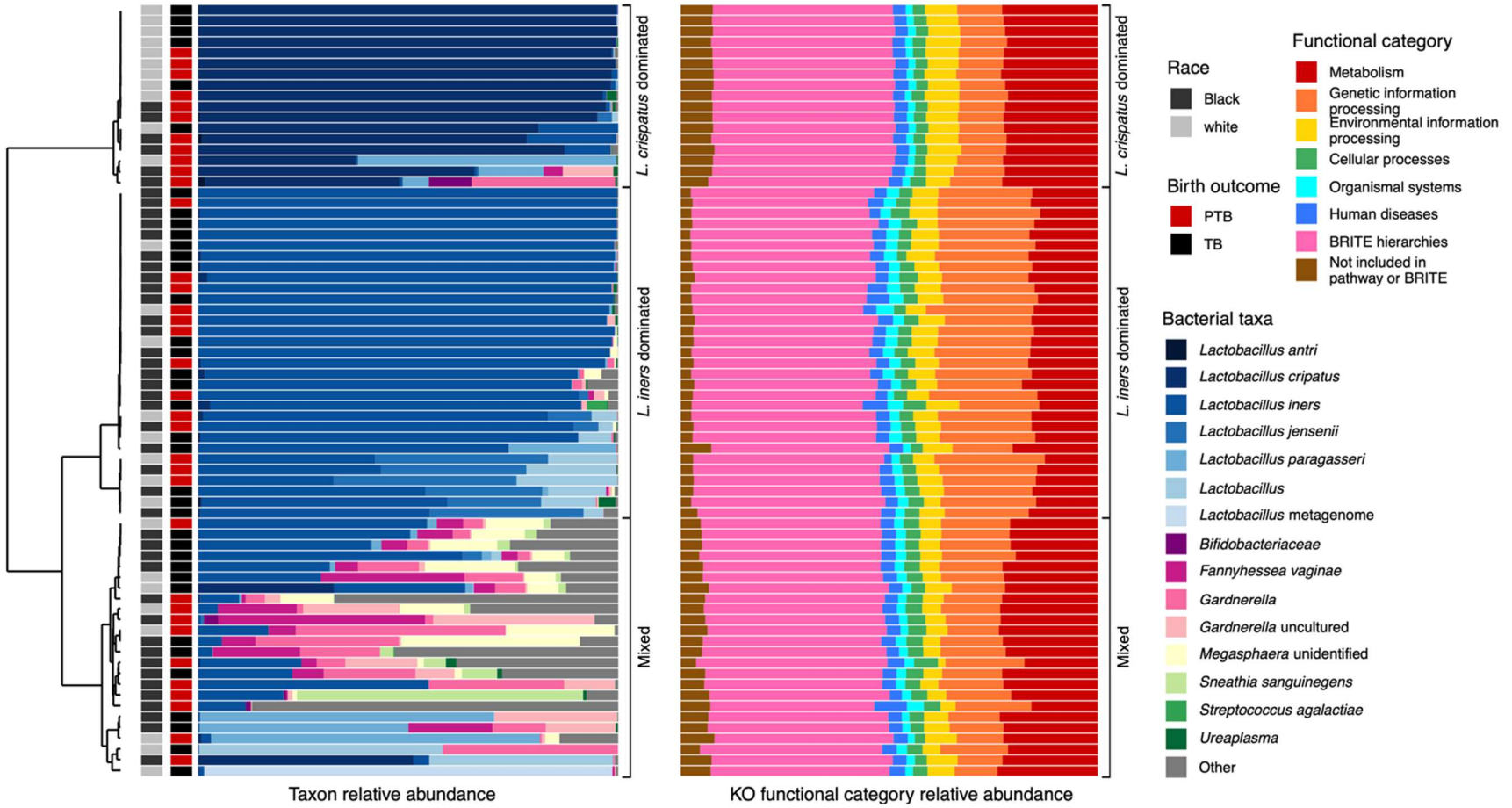

Figure 2.2. Microbiota and metagenome composition, birth outcome, and race of 72 PIN participants included in analysis. Dendrogram from hierarchical clustering of 16S rRNA gene amplicon sequencing data, resulting clusters are indicated by the brackets to the right of each stacked bar plot. Taxon relative abundance from 16S rRNA gene amplicon sequencing data. KO functional category relative abundance from observed whole metagenome sequencing data.

PTB, preterm birth; TB, term birth; KO, KEGG ortholog; KEGG, Kyoto Encyclopedia of Genes and Genomes.

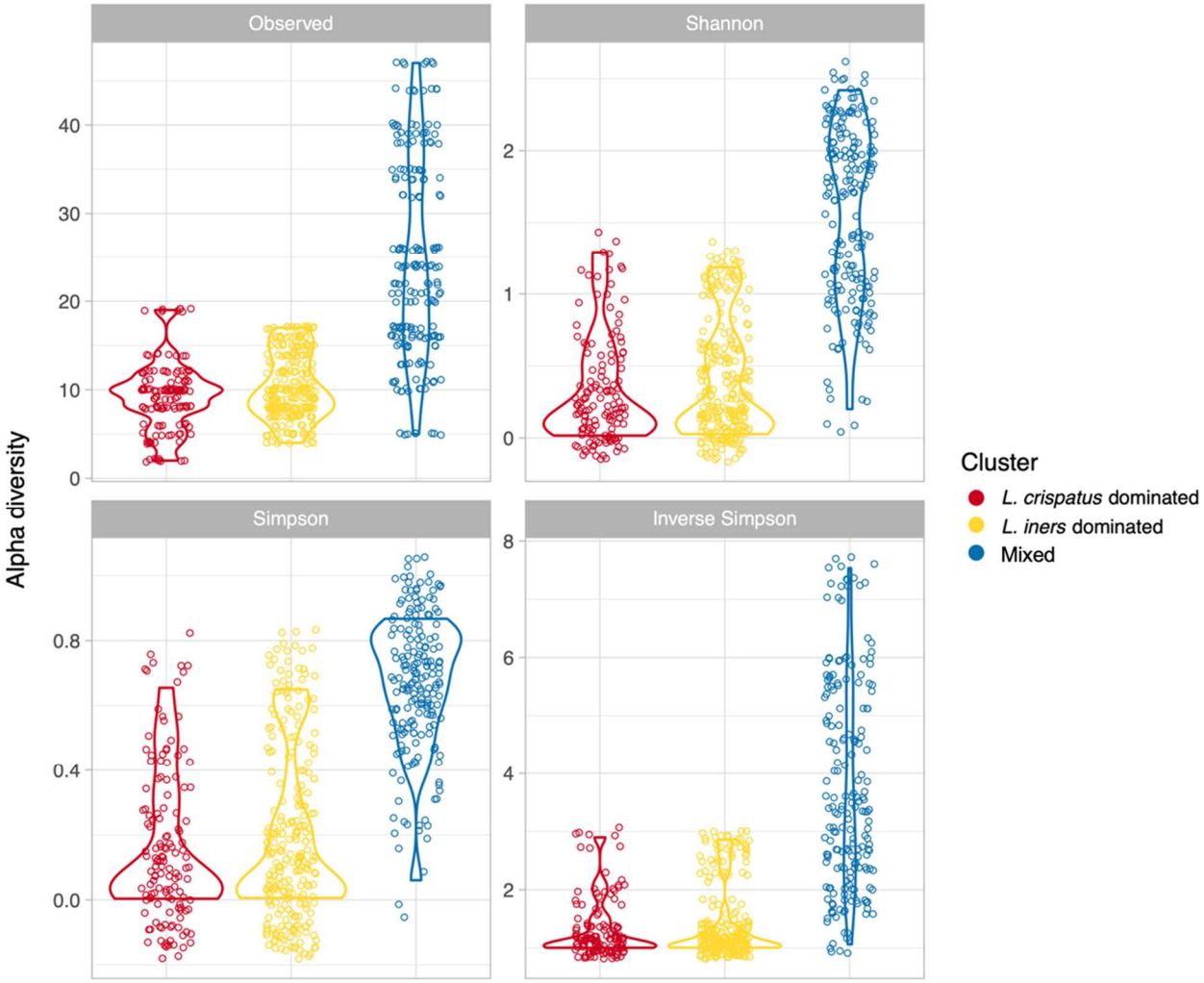

Figure 2.3. Vaginal microbiota alpha diversity by cluster for 72 PIN participants included in analysis.

Alpha diversity estimated using 16S rRNA gene amplicon sequencing data. Alpha diversity metric is indicated by the label at the top of each panel. Observed refers to the number of taxa observed in a sample, or the observed richness. Stratified and colored by hierarchical cluster from hierarchical clustering of 16S rRNA gene amplicon sequencing data.

Average WMGS depth was 1029551 total reads/sample (range 311519-6226056) and 71628 mapped reads/sample (range 424-823811) (150 base pair reads), and 2031 KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs (KO) were assigned. At the highest level of KO

functional category, observed metagenomes of samples belonging to the *L. crispatus*-dominated cluster appeared to be the most homogeneous, and they appeared to be more similar to metagenomes of samples belonging to the mixed cluster than to metagenomes of samples belonging to the *L. iners*-dominated cluster (Figure 2.2, see Appendix A Figure 1 for stacked bar plot colored by the second-highest level of KO functional category). Compared to the other clusters, *L. iners*-dominated metagenomes appeared to be enriched with genes involved in genetic information processing and had lower relative abundances of genes involved in metabolism and uncharacterized genes.

PICRUSt2 predicted the presence of 7049 KOs, of which 1506 (21%) were observed in WMGS data (Figure 2.4). Tax4Fun2 predicted the presence of 5882 KOs, of which 1503 (26%) were observed in WMGS data. A total of 1490 KOs were observed in WMGS data and predicted by PICRUSt2 and Tax4Fun2, and all evaluations and comparisons of metagenome inference performance were restricted to these 1490 KOs.

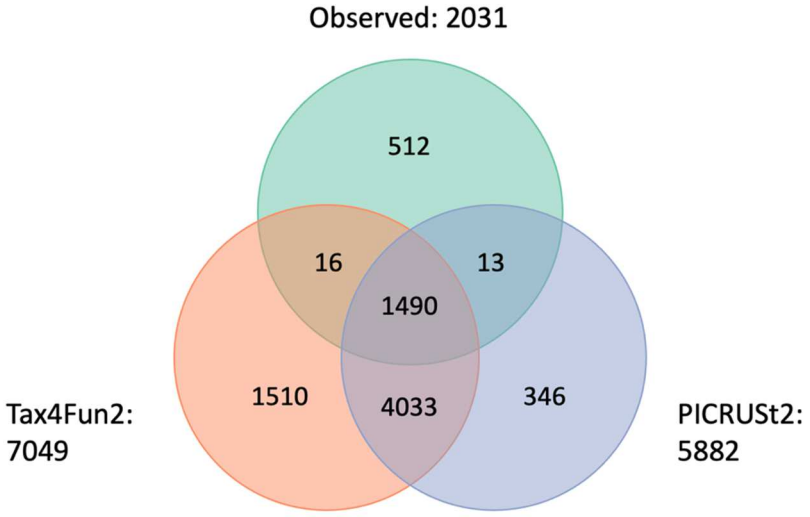

Figure 2.4. Number and overlap of KOs observed and predicted.

KO, KEGG ortholog; KEGG, Kyoto Encyclopedia of Genes and Genomes.

2.4.2 *Metagenome inference performance varied according to dominant Lactobacillus species*

The median Spearman correlation between observed and PICRUSt2-predicted KO relative abundances was 0.20 (range -0.65-0.75) (Figure 2.5A). The median correlation was similar among the *L. crispatus*-dominated (0.24) and mixed (0.21) clusters and lower among the *L. iners*-dominated cluster (0.06). We observed a similar pattern for the Tax4Fun2 predictions, though median correlations were higher for all clusters (overall 0.22, range -0.59-0.79; *L. crispatus*-dominated 0.25; mixed 0.24; *L. iners*-dominated 0.11). Correlations were not robust to permutation (median correlations ~0 for permuted data), indicating this may be a reliable method for evaluating metagenome inference performance for the vaginal microbiome.

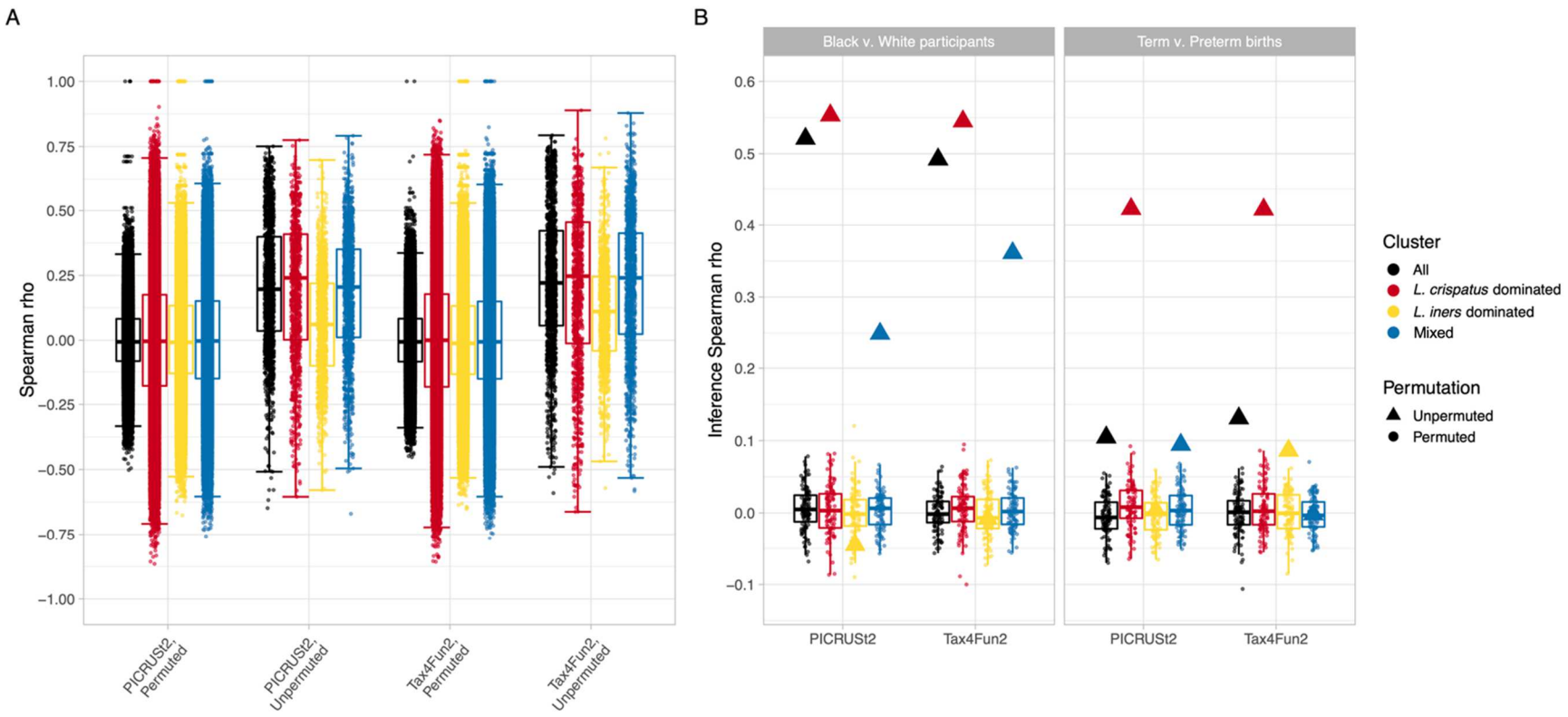

Figure 2.5. Vaginal microbiome metagenome inference performance varies across hierarchical cluster.

Clusters from hierarchical clustering of 16S rRNA gene amplicon sequencing data. Spearman correlation coefficients between observed and predicted KO relative abundances, stratified by hierarchical cluster, metagenome inference method, and permutation (A). Spearman correlation coefficients between univariable hypothesis test transformed p values estimated with observed and predicted KO relative abundances, stratified by hierarchical cluster, metagenome inference method, and permutation (B). We used Wilcoxon tests to test the following two null hypotheses: KO relative abundances do not differ between self-identified Black and white participants (left panel of B, labeled Black v. White participants), and KO relative abundances do not differ between PTB cases and term birth

controls (right panel of B, labeled Term v. Preterm births). We performed Wilcoxon tests using observed and predicted KO relative abundances separately, and we transformed p values according to the following equation in order to capture the significance and direction of KO relative abundance differences:

$$P_t = log_{10}(P) * sign((\overline{KO}|group\ 1) - (\overline{KO}|group\ 2))$$

Where P_t is the transformed p value, P is the Wilcoxon test p value, $\overline{KO}|group\ 1$ is the mean KO relative abundance in the contrast group for the hypothesis test (self-identified Black participants, cases experiencing PTB), and $\overline{KO}|group\ 2$ is the mean KO relative abundance in the reference group for the hypothesis test (self-identified white participants, controls experiencing term birth). We selected birth outcome and self-reported race as the explanatory variables to use in the hypothesis tests because the case-control subsample of PIN participants used in the current metagenome inference comparison was balanced with respect to birth outcome and self-reported race. We were not seeking to draw inference regarding the relationships between the vaginal microbiota, vaginal bacterial metagenome (observed or inferred), self-reported race, and birth outcome.

KO, KEGG ortholog; PTB, preterm birth; KEGG, Kyoto Encyclopedia of Genes and Genomes; PIN, Pregnancy, Infection, and Nutrition.

For the hypothesis test comparing KO relative abundances between Black and white participants, the overall correlation between transformed p values estimated with observed and predicted metagenome data was 0.52 for PICRUSt2 and 0.49 for Tax4Fun2 (Figure 2.5B). For both metagenome inference methods, correlations were higher among *L. crispatus*-dominated samples (PICRUSt2 0.55, Tax4Fun2 0.54), moderate among mixed samples (PICRUSt2 0.25, Tax4Fun2 0.36), and slightly negative among *L. iners*-dominated samples (PICRUSt2 -0.05, Tax4Fun2 -0.01). For the hypothesis test comparing KO relative abundances between cases and controls, transformed p value correlations were highest among *L. crispatus*-dominated samples (PICRUSt2 0.42, Tax4Fun2 0.42). Correlations were low overall (PICRUSt2 0.10, Tax4Fun2 0.13), among *L. iners*-dominated samples (PICRUSt2 0.00, Tax4Fun2 0.09), and among mixed samples (PICRUSt2 0.09, Tax4Fun2 0.00). Correlations were not robust to permutation, and variation across clusters did not appear to be influenced by transformed p value outliers (Appendix A Figure 2). Taken together, these data indicate that PICRUSt2 and Tax4Fun2 perform best among *L. crispatus*-dominated and poorly among *L. iners*-dominated microbiotas.

2.4.3 *Metagenome inference performance varied across KO function*

Median Spearman correlation between observed and PICRUSt2-predicted KO relative abundances were similar across KO functional categories (range: 0.13 for genetic information processing – 0.20 for BRITE hierarchies and uncharacterized) (Figure 2.6A). We also observed little variation in correlations for Tax4Fun2 predictions, though correlations were slightly higher (range: 0.16 for organismal systems – 0.25 for genetic information processing). Correlations were not robust to permutation.

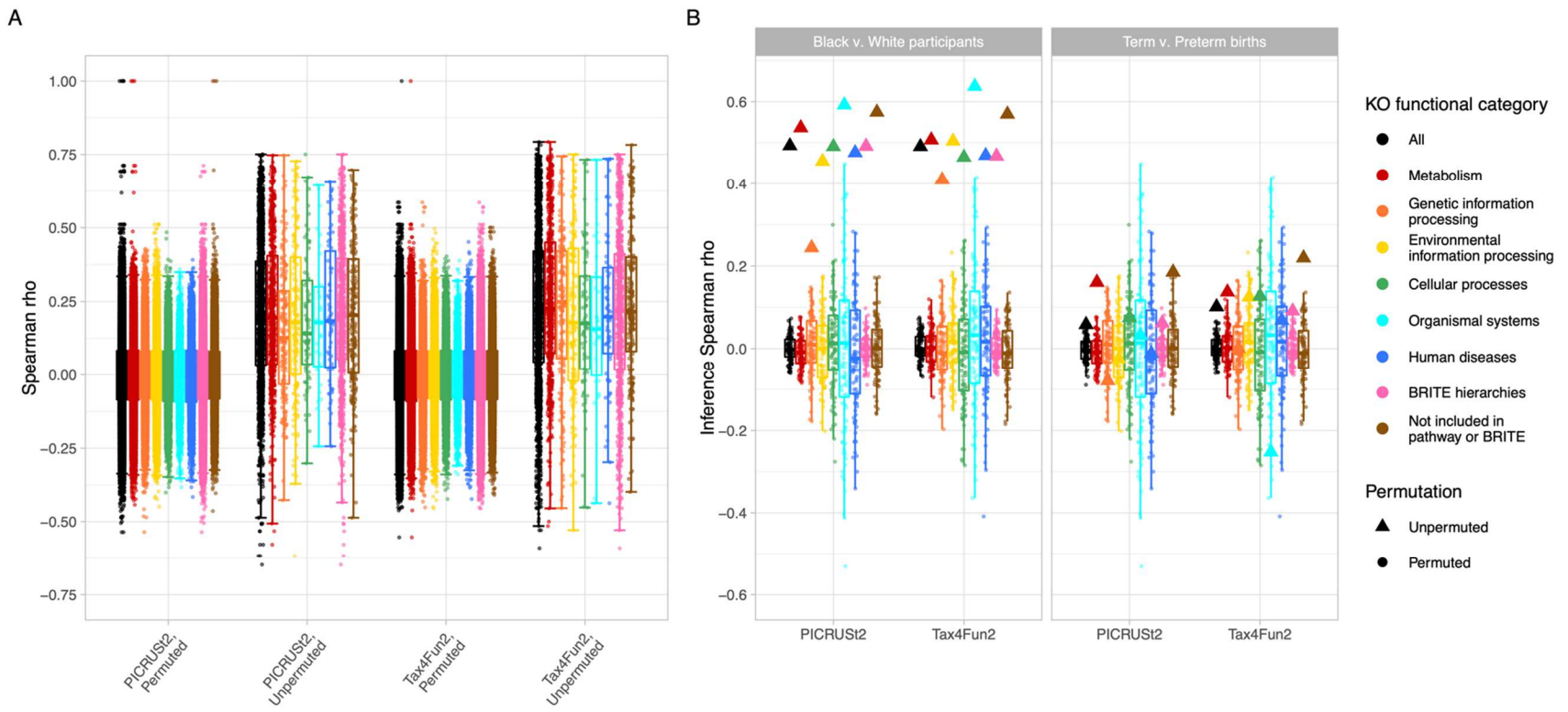

Figure 2.6. Vaginal microbiome metagenome inference performance varies across KO functional category. Spearman correlation coefficients between observed and predicted KO relative abundances, stratified by KO functional category, metagenome inference method, and permutation (A). Spearman correlation coefficients between univariable hypothesis test transformed p values estimated with observed and predicted KO relative abundances, stratified by KO functional category, metagenome inference method, and permutation (B). We used Wilcoxon tests to test the following two null hypotheses: KO relative abundances do not differ between self-identified Black and white participants (left panel of B, labeled Black v. White participants), and

KO relative abundances do not differ between PTB cases and term birth controls (right panel of B, labeled Term v. Preterm births). We performed Wilcoxon tests using observed and predicted KO relative abundances separately, and we transformed p values according to the following equation in order to capture the significance and direction of KO relative abundance differences:

$$P_t = log_{10}(P) * sign((\overline{KO}|group\ 1) - (\overline{KO}|group\ 2))$$

Where P_t is the transformed p value, P is the Wilcoxon test p value, $\overline{KO}|group\ 1$ is the mean KO relative abundance in the contrast group for the hypothesis test (self-identified Black participants, cases experiencing PTB), and $\overline{KO}|group\ 2$ is the mean KO relative abundance in the reference group for the hypothesis test (self-identified white participants, controls experiencing term birth). We selected birth outcome and self-reported race as the explanatory variables to use in the hypothesis tests because the case-control subsample of PIN participants used in the current metagenome inference comparison was balanced with respect to birth outcome and self-reported race. We were not seeking to draw inference regarding the relationships between the vaginal microbiota, vaginal bacterial metagenome (observed or inferred), self-reported race, and birth outcome.

KO, KEGG ortholog; PTB, preterm birth; KEGG, Kyoto Encyclopedia of Genes and Genomes; PIN, Pregnancy, Infection, and Nutrition.

We observed more variation across KO functional category in correlations between transformed p values estimated with observed and predicted KO relative abundances (Figure 2.6B). For the hypothesis test comparing KO relative abundances between Black and white participants, correlations were highest for organismal systems and uncharacterized KOs (PICRUSt2 0.59 and 0.57, respectively; Tax4Fun2 0.64 and 0.57, respectively) and lowest for genetic information processing (PICRUSt2 0.24, Tax4Fun2 0.41). For the hypothesis test comparing KO relative abundances between cases and controls, correlations were highest for uncharacterized KOs and metabolism (PICRUSt2 0.18 and 0.16, respectively; Tax4Fun2 0.22 and 0.14, respectively). PICRUSt2 performed worst for genetic information processing (-0.08), and Tax4Fun2 performed worst for organismal systems (-0.25). Again, correlations were not robust to permutation, and variation across KO functional categories did not appear to be influenced by transformed p value outliers (Appendix A Figure 3). These data indicate that PICRUSt2 and Tax4Fun2 may perform best for uncharacterized genes and poorly for genes involved in genetic information processing.

2.4.4 *L. crispatus-dominated metagenomes were enriched with good-performance KOs, L. iners-dominated metagenomes were enriched with poor-performance KOs*

We examined differences in observed metagenome content between *L. crispatus*- and *L. iners*-dominated samples as a potential cause of differential metagenome inference performance between these clusters. As stated above, *L. iners*-dominated metagenomes appeared to be enriched with genes involved in genetic information processing (poor metagenome inference performance) and had lower relative abundances of uncharacterized genes (good performance); the reverse was true for *L. crispatus*-dominated metagenomes (Figure 2.2). *L. crispatus* relative abundance was strongly positively correlated with uncharacterized KO relative abundance (linear model R^2=50%), whereas *L. iners* relative abundance was strongly negatively correlated with uncharacterized KO relative abundance (linear model R^2=75%) (Figure 2.7A, 2.7B). *L. crispatus*

relative abundance was strongly negatively correlated with genetic information processing KO relative abundance (linear model R^2=45%), while *L. iners* relative abundance was strongly positively correlated with genetic information processing KO relative abundance (linear model R^2=75%) (Figure 2.7A, 2.7B). The ratio of *L. crispatus* : *L. iners* relative abundance was positively correlated with uncharacterized KO relative abundance (linear model R^2=16%) and negatively correlated with genetic information processing KO relative abundance (linear model R^2=16%) (Figure 2.7C).

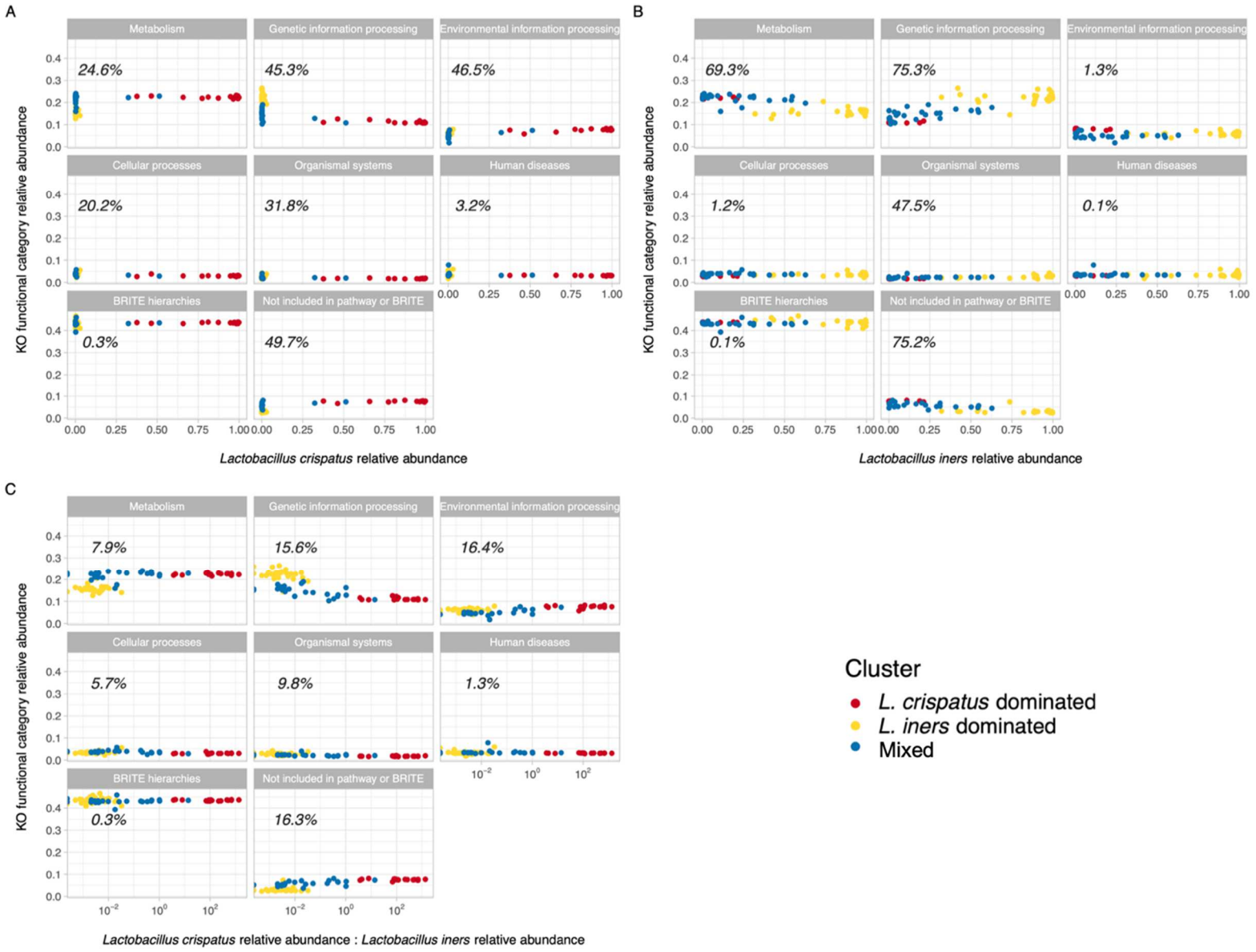

Figure 2.7. Correlations between KO functional category relative abundances and *L. iners* and *L. crispatus* relative abundances.

Scatter plots of *L. crispatus* relative abundance versus KO functional category relative abundance with one panel for each KO functional category (A); *L. iners* relative abundance versus KO functional category relative abundance with one panel for each KO functional category (B); and the ratio of *L. crispatus* relative abundance to *L. iners* relative abundance versus KO functional category relative abundance with one panel for each KO functional category (C). The italicized text in the top left of each panel presents the R^2 from a linear model regressing KO functional category relative abundance (response variable) on *L. crispatus* relative abundance (A), *L. iners* relative abundance (B), or the ratio of *L. crispatus* relative abundance to *L. iners* relative abundance (C) (explanatory variables). The R^2 values represent the proportion of variation in KO relative abundance explained by the variation in *L. crispatus* relative abundance (A), *L. iners* relative abundance (B), or the ratio of *L. crispatus* relative abundance to *L. iners* relative abundance (C). Points are colored by hierarchical cluster from hierarchical clustering of 16S rRNA gene amplicon sequencing data.

KO, KEGG ortholog; KEGG, Kyoto Encyclopedia of Genes and Genomes.

2.5 DISCUSSION

While the performance of various metagenome inference methods has been compared in a number of human body sites and environmental ecosystems, to our knowledge this is the first study specific to the vaginal microbiome.[7,8,11–15] Our cluster-stratified analysis consistently demonstrated that PICRUSt2 and Tax4Fun2 perform best among *L. crispatus*-dominated communities and poorly among *L. iners*-dominated communities. We hypothesize that this differential performance is attributable to biological differences between the communities, as well as reference differences between the communities. In terms of biological difference, our KO functional category-stratified analysis highlighted that *L. iners*-dominated microbiotas tend to be enriched with KO functional categories that are inferred poorly, namely genetic information processing. This is consistent with a prior genomic comparison of 15 *L. iners* and 15 *L. crispatus* strains. The *L. iners* core genome (993 genes) contained a larger proportion of genes involved replication and repair, transcription, and translation than the *L. crispatus* core genome (1442 genes).[30] Likewise, the *L. iners* accessory genome (1223 genes) contained a greater proportion of genes involved in replication and repair and translation than the *L. crispatus* accessory genome (2884 genes).[30] Given that *L. iners* and *L. crispatus* dominated communities within their respective clusters with an average of 86% and 87% relative abundance in the current analysis, differences in genome composition between the species will give rise to differences in metagenome composition between *L. iners*- and *L. crispatus*-dominated communities (in general and with regard to genetic information processing genes), which in turn appear to drive poor metagenome inference performance among *L. iners*-dominated communities. It is unclear why PICRUSt2 and Tax4Fun2 performed poorly for genetic information processing genes in this analysis. One prior function-stratified analysis of gut microbiome samples found that the original implementation of PICRUSt, PICRUSt2, and the original implementation of Tax4Fun tended to perform well for

genetic information processing genes, with the exception of genes involved in transcription, for which all methods performed poorly.[14]

In terms of reference differences between *L. iners*- and *L. crispatus*-dominated communities, *L. iners* genomes are less well represented in public reference databases than *L. crispatus* genomes, which may hinder metagenome inference for *L. iners* and *L. iners*-dominated communities. As of April 22, 2022, the Sequence Read Archive (SRA) contained 40 *L. iners* genomes and 213 *L. crispatus* genomes; 16 *L. iners* strains and 25 *L. crispatus* strains were represented in the DNA Data Bank of Japan (DDBJ); and the European Nucleotide Archive (ENA) contained 508 *L. iners* genomes and 3037 *L. crispatus* genomes. It is a reasonable assumption that reference differences will be ameliorated over time; however, biological differences between *L. iners*-dominated and *L. crispatus*-dominated vaginal microbiotas will persist, as will differential metagenome inference performance.

Taking an epidemiologic lens to these results, we can consider differential metagenome inference performance to be analogous to differential measurement error, which can cause differential misclassification. It is reasonable to think of inferred metagenomes as measurements because they are used as measurements in analyses, so it is in turn reasonable to think of less-than-perfect metagenome inference as measurement error. Differential measurement error occurs when the degree or direction of outcome (response variable) measurement error differs across exposure (explanatory variable) statuses, or when the degree or direction of exposure measurement error differs across outcome statuses. Differential measurement error and subsequent differential misclassification bias effect estimates, and this bias is hard to predict in that it can be in either direction (towards or away from the null value).[122,123] This is in contrast to nondifferential measurement error and nondifferential misclassification (independent of exposure or outcome status), which can often be expected to bias effect estimates towards the null.[122,123] To qualify as differential measurement error, metagenome inference performance must differ across exposure or outcome status, or across a factor that is associated with exposure or outcome

status. While cluster or community type will not be the exposure or outcome of interest in every vaginal microbiome study that uses metagenome inference, we posit that cluster will be associated with either the exposure or the outcome in virtually all of these studies based on the large body of evidence supporting such associations (for example [19,20,66,96,124]). It follows that metagenome inference will introduce differential measurement error, subsequent differential misclassification, and hard-to-predict bias in vaginal microbiome research. Results from these analyses should be interpreted with substantial caution and the understanding that they may over- or under-estimate associations between metagenome content and other variables of interest. This is particularly concerning given that *L. iners*-dominated vaginal microbiotas are consistently observed at high prevalence across populations,[17,19,20,23,66] and poor metagenome inference among these communities will impact similarly large proportions of study populations.

Overall, the PICRUSt2 and Tax4Fun2 performance we observed indicates that metagenome inference methods perform worse for the vaginal microbiome than for the gut microbiome. We will consider results based on observed and predicted KO relative abundance correlations because few studies have examined p value correlations,[13,14] and assessing performance based on p value correlations depends on the hypothesis tested and the true effect size. Among stool samples from various populations, four analyses of six metagenome inference methods report median correlations of approximately 0.60-0.90.[7,12–14] In our analysis, median relative abundance correlations for PICRUSt2 and Tax4Fun2 were 0.19 and 0.22, respectively (Figures 2.5A, 2.6A). Poorer metagenome inference for the vaginal microbiome as compared to the gut microbiome can be attributed, likely in large part, to the vaginal microbiome being relatively understudied compared to the gut microbiome. A number of commonly detected vaginal bacteria (e.g. BVAB1 [proposed name *Ca.* L. vaginae], BVAB2, *Mageeibacillus indolicus*, *Megasphaera lornae*) have only recently been characterized or remain uncharacterized.[69–71] Recent and ongoing vaginal bacterial characterization efforts are indicators that vaginal bacteria are less well represented in 16S rRNA gene and whole genome reference databases than gut bacteria, which

has been shown to decrease metagenome inference performance.[7,8,11–15] Indeed, a recent investigation of all human microbiome datasets in the SRA, DDBJ, and ENA (16S rRNA gene amplicon sequences and shotgun metagenome sequences) revealed that 220017 (49%) of the 444829 samples represented were from the gut, compared to 17784 (4%) from the vagina.[16] Additionally, a taxon formerly believed to be one species, *Gardnerella vaginalis*, was recently speciated into four named species (*Gardnerella leopoldii*, *Gardnerella piotii*, *Gardnerella swidsinskii*, and *G. vaginalis*) and 9 additional genomic species.[72] *Gardnerella* species cannot be differentiated by 16S rRNA gene sequences, so the genomic diversity of *Gardnerella* species cannot be captured by metagenome inference. Considering *Gardnerella* relative abundance was as high as 50% in this analysis (average 18% within diverse cluster), inability to accurately capture *Gardnerella* species genome content can reasonably be expected to decrease metagenome inference performance.

Our results also indicate poorer metagenome inference performance for the vaginal microbiome that has previously been reported for the urogenital microbiome. One study examined the first implementations of PICRUSt and Tax4Fun using urogenital samples and reported median relative abundance correlations of approximately 0.65.[12] Reasons for this discrepancy are unclear, especially considering more specific body site information on the urogenital samples used was not reported.

Our analysis has several strengths. As stated above, this is the first published metagenome inference comparison specific to the vaginal microbiome. Due to ecological differences between the vaginal microbiome and other body site microbiomes, findings from prior reports cannot be easily generalized to the vagina. Focusing on the vaginal microbiome enabled us to identify patterns of metagenome inference performance that are directly related to vaginal microbial ecology, as well as important implications for the risk of bias in vaginal microbiome research that employs metagenome inference. We were able to confidently identify this pattern because we consistently observed differential metagenome inference performance across

clusters in each of the three evaluations we performed (relative abundance correlations, p value correlations from hypothesis tests comparing PTB cases and term birth controls, p value correlations from hypothesis tests comparing Black and white participants). Using three evaluation methods is a second strength of this analysis. Finally, the 16S rRNA gene amplicon sequencing depth (average 31049 reads/sample) and WMGS depth (average 1029551 total reads/sample, 71628 mapped reads/sample) achieved in the parent study indicate the high quality of the sequencing data used.

Our findings should also be interpreted in the context of the study's limitations. With data from 72 participants, we were only able to identify three clusters of vaginal microbiota composition. With a larger dataset, we may have been able to characterize metagenome inference performance for more of the vaginal microbiota community types observed in US populations (e.g. *Lactobacillus gasseri*-dominated*, Lactobacillus jensenii*-dominated, subgroups of *Lactobacillus*-dominated community types; diverse, BV-like community types).[96] Second, all participants were American and were pregnant at the time of sample collection. This limits generalizability of our results to non-US and nonpregnant populations, especially considering documented differences in vaginal microbiota composition between global populations (for example [58,59,98,125,126]) and according to pregnancy status (for example [127–130]). Finally, this analysis was restricted to metagenome features that were observed and predicted. A total of 541 (27%) observed KOs, 4392 (75%) PICRUSt2-predicted KOs, and 5559 (79%) Tax4Fun2-predicted KOs did not contribute to the analysis (Figure 2.3).

In conclusion, our analysis demonstrates that metagenome inference methods perform more poorly for the vaginal microbiome than for the gut microbiome. Moreover, performance was differential according to vaginal microbiota cluster with the best performance among *L. crispatus*-dominated communities and the worst among *L. iners*-dominated communities. Genome content differences between *L. iners* and *L. crispatus* appear to drive this differential performance, which will result in differential measurement error, differential misclassification, and hard-to-predict bias

in vaginal microbiome research that employs metagenome inference. As the cost of whole metagenome sequencing continues to fall, investigators will ideally be able to characterize the actual vaginal bacterial metagenome, obviating the need for metagenome inference and eliminating metagenome measurement error introduced by metagenome inference.

Chapter 3. EPIDEMIOLOGIC EVIDENCE ON THE ROLE OF *LACTOBACILLUS INERS* IN SEXUALLY TRANSMITTED INFECTIONS AND BACTERIAL VAGINOSIS: A SERIES OF SYSTEMATIC REVIEWS AND META-ANALYSES

3.1 ABSTRACT

3.1.1 *Background*

While *Lactobacillus crispatus*-dominated vaginal microbiotas are thought to protect against bacterial vaginosis (BV) and STIs, the role of *Lactobacillus iners*-dominated microbiotas is less clear. To better understand impact of *L. iners* on common cervicovaginal infections, we conducted systematic reviews of the associations between *L. iners ,* compared to *L. crispatus*, and eight outcomes: *Chlamydia trachomatis* (Ct), BV, human papillomavirus (HPV), cervical dysplasia, human immunodeficiency virus (HIV), genital herpes, *Trichomonas vaginalis*, and *Neisseria gonorrhoeae*.

3.1.2 *Methods*

On April 30, 2021, we searched PubMed, Embase, Cochrane Library, and Web of Science for epidemiologic studies of reproductive-age, nonpregnant, cisgender women that used marker gene sequencing to characterize vaginal microbiota composition and presented an effect estimate for the association between *L. iners*, compared to *L. crispatus*, and outcomes of interest. For outcomes with $\geq$3 eligible results presenting the same form of effect estimate, we conducted random-effects meta-analysis. The review protocol was registered prospectively (PROSPERO CRD42020214775).

3.1.3 *Results*

Six Ct studies were included in meta-analysis, which showed *L. iners*-dominated microbiotas were associated with 3.4-fold higher odds of Ct compared to *L. crispatus*-dominated microbiotas (95% CI 2.1-5.4). Three BV studies were included in meta-analysis, which indicated *L. iners*-dominated microbiotas were associated with 2.1-fold higher prevalence of BV compared to *L. crispatus*-dominated microbiotas (95% CI 0.9-4.9). Evidence was too sparse to perform meta-analysis for the remaining outcomes.

3.1.4 *Conclusions*

The epidemiologic evidence reviewed for Ct and BV suggests that *L. iners*-dominated vaginal microbiotas may be suboptimal compared to *L. crispatus*-dominated microbiotas for these outcomes, suggesting *L. iners* dominance may confer risk of acquiring Ct or developing BV. These reviews highlight evidence gaps regarding the remaining outcomes and opportunities to improve epidemiologic rigor in vaginal microbiome science.

3.2 INTRODUCTION

The World Health Organization recognizes sexual health as essential to overall health and wellbeing, and achieving sexual health depends on access to comprehensive sexual health care.[131] As a modifiable risk factor, understanding the vaginal microbiota's role in sexual health outcomes is a promising avenue for developing novel interventions to promote sexual health, which may become an essential component of sexual health care in the future. Across populations, the vaginal microbiota of reproductive-age individuals is often dominated by *Lactobacillus iners* or *Lactobacillus crispatus*.[17–23] While *L. crispatus*-dominated vaginal microbiotas are widely thought to protect against adverse sexual health outcomes, the role of *L.*

iners-dominated microbiotas is less clear and has been debated.[24–26,33] Several recent efforts to define optimal vaginal microbiota composition concluded that *L. crispatus* dominance is optimal, while diverse, bacterial vaginosis (BV)-like microbiotas are suboptimal.[132–135] However, *L. iners* is often omitted from this discourse;[132–135] when included, it is considered suboptimal.[136,137]

As *L. iners* is considered the most prevalent and abundant vaginal bacterial species,[25,33] understanding its influence on sexual health outcomes relative to *L. crispatus* is urgently needed to better understand the etiology of adverse outcomes and develop effective prevention and treatment strategies. To date, BV and BV-associated taxa have been a primary focus of research investigating relationships between the vaginal microbiota and sexually transmitted infections (STI), including systematic reviews,[51,138–143] again omitting *L. iners* from this discourse and meta-discourse. To evaluate the state of epidemiologic evidence regarding *L. iners* and sexual health, we conducted a series of systematic reviews and meta-analyses of the associations between *L. iners*-dominated vaginal microbiotas and common cervicovaginal infections.

3.3 Materials and Methods

We conducted eight separate systematic reviews of studies evaluating associations between *L. iners*, compared to *L. crispatus*, and genital *Chlamydia trachomatis* (Ct) infection, BV, cervical human papillomavirus (HPV) detection, cervical dysplasia, human immunodeficiency virus (HIV) infection, genital herpes simplex virus type-2 (HSV-2) infection, *Trichomonas vaginalis* (Tv) infection, and genital *Neisseria gonorrhoeae* (Ng) infection (outcome definitions in Appendix B Table 1). The reviews were prospectively registered in PROSPERO (CRD42020214775 https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=214775). We conducted the reviews according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses

(PRISMA) 2020 and Conducting Systematic Reviews and Meta-Analyses of Observational Studies of Etiology (COSMOS-E) guidelines.[144,145]

3.3.1 *Systematic review search strategy*

On April 30, 2021, we searched PubMed, Embase, Cochrane Library, and Web of Science (search terms in Appendix B Table 2). Studies were eligible for inclusion in systematic reviews if they were conducted among reproductive-age, nonpregnant, cisgender women; used marker gene (e.g. 16S rRNA gene, *cpn60*) amplification and sequencing to characterize vaginal microbiota composition; and presented an effect estimate for the association between *L. iners*, compared to *L. crispatus*, and the outcome of interest, or presented data which could be used by the reviewers to calculate an effect estimate (exposure and reference definitions in Appendix B Table 3). We defined reproductive-age as post-menarchal and pre-menopausal with the bounds of 13-50 years, inclusive. If search results did not report an age-based eligibility criterion nor participant age range, we assumed the study was conducted among reproductive-age women. If authors did not clearly articulate participants' gender identity, we assumed participants were cisgender women. Studies of cross-sectional, case-control, cohort, or clinical trial designs were eligible. For clinical trials, only data from baseline prior to intervention were included. Case reports and case series were not eligible. Only English, full-text, peer-reviewed, original research manuscripts were eligible, and eligibility was not restricted based on publication year.

3.3.2 *Data collection*

We exported all search results for a given outcome to a Zotero library (one library for each outcome) and manually deduplicated results. Two reviewers independently reviewed full texts of all deduplicated results to determine eligibility. Reviewers settled discrepancies by consensus, or by consulting a third reviewer when consensus was not reached.

Two reviewers independently tracked eligibility decisions and recorded key characteristics of study design, exposure and outcome assessment, and effect estimates with a REDCap survey specifically designed for each outcome. If any of the relevant data were not found in the main text, reviewers examined the corresponding supplemental materials. If any of these data were missing or unclear, we considered them "not reported."

For studies that did not present an effect estimate of interest, but presented sufficient exposure and outcome data to calculate an effect estimate, we calculated effect estimates as follows: prevalence ratios (PR) for cross-sectional studies and cohort studies (when exposure and outcome data were collected at the same time point) that cross-tabulated categorical exposure and outcome data; odds ratios (OR) for case-control studies that cross-tabulated categorical exposure and outcome data; relative abundance ratio of ratios for studies that presented mean *L. iners* and *L. crispatus* relative abundances according to outcome status (Equation 3.1); and Spearman correlation coefficient differences for studies that presented Spearman correlation coefficients between *L. iners* relative abundance and the outcome and between *L. crispatus* relative abundance and the outcome (Equation 3.2). We were not able to estimate measures of uncertainty for relative abundance ratio of ratios or Spearman correlation differences. For one study included for any HPV and hrHPV (Reimers et al. 2016[146]), we estimated odds ratios according to Equation 3.3. We were unable to estimate confidence intervals for these odds ratios.

Equation 3.1:

$$\frac{\frac{Mean(Lactobacillus\ iners\ relative\ abundance)|Outcome\ present}{Mean(Lactobacillus\ crispatus\ relative\ abundance)|Outcome\ present}}{\frac{Mean(Lactobacillus\ iners\ relative\ abundance)|Outcome\ absent}{Mean(Lactobacillus\ crispatus\ relative\ abundance)|Outcome\ absent}}$$

Equation 3.2:

$$Spearman\ correlation\ coefficient(Lactobacillus\ iners\ relative\ abundance, Outcome) - Spearman\ correlation\ coefficient(Lactobacillus\ crispatus\ relative\ abundance, Outcome)$$

Equation 3.3:

$$\frac{OR(Lactobacillus\ iners - dominated\ v. Diverse, (hr)HPV\ v. no\ (hr)HPV)}{OR(Lactobacillus\ crispatus - dominated\ v. Diverse, (hr)HPV\ v. no\ (hr)HPV)}$$

3.3.3 *Systematic review evidence synthesis and meta-analysis*

For BV, we synthesized evidence according to whether BV was assessed using Nugent score or Amsel criteria.[6,44] All studies using Nugent score defined BV as Nugent score ≥7. All studies using Amsel criteria defined BV as ≥3 Amsel criteria present. For HPV, we synthesized evidence according to whether the outcome included detection of any HPV type or was restricted to detection of high-risk HPV (hrHPV) types. We included multiple effect estimates from eligible studies reporting both HPV outcomes. For the remaining outcomes, we synthesized evidence from all included studies.

Studies included in systematic reviews were eligible for meta-analysis if they presented a PR, OR, relative risk (RR), or hazard ratio (HR) for the association between *L. iners*, compared to *L. crispatus*, and the outcome, or if reviewers were able to estimate one of these measures based on data provided in the publication. For each outcome, we conducted a random-effects meta-analysis (RE-MA) if there were ≥3 eligible studies that presented the same form of effect estimate (PR, OR, RR, or HR). We selected random-effects meta-analysis (as opposed to fixed-effects meta-analysis) because random-effects meta-analysis assumes that observed effect estimates vary between studies due to real differences in the effect across studies.[147] This is appropriate for meta-analyses of associations of the vaginal microbiota with sexual and

reproductive health outcomes because there is evidence that these associations vary between populations.[18,59,148,149]

We evaluated heterogeneity in study findings using Cochrane's Q and the I^2 statistic.[150] We used the rma.mv function (study as the random effect) of the metafor package (version 3.0-2 throughout the chapter) in R (version 4.0.4 throughout the chapter) for conducting meta-analyses and estimating Cochrane's Q, and we used code provided on the metafor package website for estimating I^2.[151,152] For each meta-analysis, we constructed a forest plot using the forest and addpoly functions of the metafor package in R.[151] Due to few studies being included in each meta-analysis, we did not explore potential causes of heterogeneity, conduct sensitivity analyses to assess robustness of RE-MA estimates, or assess potential publication bias.

3.3.4 *Risk of bias and quality of evidence assessments*

Two reviewers independently used the instrument developed by Morgan and colleagues for observational studies of etiology to assess risk of bias in included studies.[153] This instrument uses signaling question to assess risk of bias in six domains: confounding, selection bias, exposure measurement, outcome measurement, missing data, and selection of reported results. Signaling question responses are used to rate risk of bias in each domain as low, moderate, serious, critical (highest level), or not enough information to assess. Risks of bias in each domain are then used to evaluate a study's overall risk of bias.

The confounders we considered were vaginal sex (e.g. frequency, number of partners, condom use), hormonal contraception, vaginal washing, race/ethnicity, and variables race/ethnicity may be a proxy for (e.g. socioeconomic status, site/region). We selected these confounders based on expected substantial prevalence/variation of each factor and expected strong confounding. As a baseline, we rated risk of bias due to exposure assessment as moderate for all studies due to the compositional nature of marker gene sequencing data which are, by definition, not well-defined exposures. For the BV outcome, we rated risk of bias due to outcome

measurement as not enough information for studies that did not provide details on training, quality control, or quality assurance for evaluating BV by Nugent score or Amsel criteria. We used the conservative approach of rating overall risk of bias in each study to be equivalent to its highest-rated domain-specific risk of bias. For example, if all domains other than confounding were rated as moderate, but confounding was rated as critical, we rated overall risk of bias as critical. Reviewers settled discrepancies in domain- and study-level risk of bias by consensus.

One reviewer used the Grades of Recommendation, Assessment, Development, and Evaluation (GRADE) system to assess the quality of evidence included for each outcome.[154] The GRADE system applies an initial low quality rating to observational evidence. Quality can be down-rated due to risk of bias, effect estimate inconsistency and imprecision, indirectness, and publication bias. Quality can be up-rated due to large effect, dose response, and if residual confounding increases confidence in effect estimates. We down-rated quality of evidence for indirectness when the majority of studies for an outcome evaluated prevalent outcomes because prevalent outcomes are of less interest to individuals at risk for BV, STI, and cervical dysplasia than incident outcomes. We down-rated quality of evidence for each review for likely publication bias due to insignificant vaginal microbiota*, L. iners*, and/or *L. crispatus* findings being less likely to be published or reported.

3.4 RESULTS

3.4.1 *Overview of search results*

The number of search results reviewed, excluded, and included for each literature search are presented in Table 1 (full PRISMA diagrams in Appendix B Figures 1-8). Deduplicated search results reviewed ranged from 16 for HSV-2 to 320 for BV. For each outcome, ≤8 studies were eligible for inclusion in systematic reviews. For each outcome, between 38-59% of search results

were excluded because they were not peer-reviewed original research manuscripts, 13-42% were excluded because they did not use marker gene sequencing to characterize the vaginal microbiota of reproductive-age, nonpregnant cisgender women, and 14-31% were excluded because they did not present a relevant effect estimate and reviewers were unable to calculate one. Table 2 summarizes key features and effect estimates from each included study. Tables 3 and 4 present risk of bias and quality of evidence assessments, respectively. We begin by presenting results for Ct and BV as these were the only outcomes for which meta-analyses were performed. Next, we present HPV, hrHPV, and cervical dysplasia together, as these outcomes are etiologically related. We then present HIV, and we finish with one subsection for HSV-2 and Tv because these reviews included a limited number of studies. We identified no eligible studies reporting on the relationship between *L. iners* and Ng, so we do not include this outcome in the results.

Table 3.1. PRISMA diagram summary for each systematic review.

Review step	Ct		BV		HPV		Cervical dysplasia		HIV		Genital HSV-2		Tv		Ng	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Total deduplicated results reviewed[a]	42	100	320	100	52	100	39	100	144	100	16	100	22	100	36	100
Excluded because not peer-reviewed original research manuscript[b]	17	40	125	39	23	44	15	38	84	58	8	50	13	59	18	50
Excluded because wrong methods[c]	12	29	133	42	16	31	15	38	32	22	2	13	4	18	10	28
Excluded because no (data to calculate) effect estimate	6	14	54	17	8	15	7	18	25	17	5	31	4	18	8	22
Studies included in review	7	17	8	3	5	10	2	5	3	2	1	6	1	5	0	0
Reports of included studies[d]	7	--	8	--	8	--	2	--	3	--	1	--	1	--	0	--
Reports included in meta-analysis[d]	6	--	3	--	0	--	0	--	0	--	0	--	0	--	0	--

[a]Results from searches of Cochrane Library, Embase, PubMed, and Web of Science.

[b]Reasons include: full text not retrieved, full text not found in English, not peer-reviewed manuscript (e.g. conference abstract), and peer-reviewed review/commentary.

[c]Reasons include: in vitro/in silico/animal studies only, wrong study population, and did not use marker gene sequencing to characterize the vaginal microbiota.

[d]Percentages not provided because reports of studies and studies may have different denominators.

PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; Ct, *Chlamydia trachomatis*; BV, bacterial vaginosis; HPV, human papillomavirus; HIV, human immunodeficiency virus; HSV-2, herpes simplex virus type-2; Tv, *Trichomonas vaginalis*; Ng, *Neisseria gonorrhoeae*.

Table 3.2. Summary of findings for each outcome.

Author Year Country	Study design[a]	N total (N contributed to estimate)[b]	Duration between exposure and outcome assessment[c]	16S rRNA gene hypervariable region(s)	*L. iners*-dominated communities N (%)[d]	*L. crispatus*-dominated communities N (%)[e]	Outcome assessment	Outcome among *L. iners*-dominated N (%)[f]	Outcome among *L. crispatus*-dominated N (%)[g]	Estimate definition[h]	Estimate [CI][i]
Chlamydia trachomatis											
van der Veer 2017[155] Netherlands	Cross-sectional	93 (93)	--	V3-V4	32 (34)	22 (24)	NAAT, cervicovaginal swab	21 (66)	6 (27)	aOR[j]	4.20 [1.20, 15.40]
Balle 2018[156] South Africa	Cross-sectional	72 (72) participants, 144 (144) observations	--	V4	40 (28)	27 (19)	NAAT, vulvovaginal swab	NR	NR	OR	2.50 [0.56, 13.60]
Tamarelle 2018[20] France	Cross-sectional	132 (132)	--	V3-V4	51 (39)	49 (37)	NAAT, cervicovaginal swab	11 (22)	4 (8)	OR	3.09 [0.91, 10.49]
van Houdt 2018[157] Netherlands	Case-control	115 (115)	1 year	V3-V4	38 (33)	43 (37)	NAAT, cervicovaginal swab	42 (prevalence of *L. iners*-dominated microbiotas among those with Ct)	30 (prevalence of *L. crispatus*-dominated microbiotas among those with Ct)	maOR[k]	2.58 [1.01, 6.61]
Filardo 2019[158] Italy	Case-control	138 (100)	--	V4	34 (25)	66 (48)	NR	36 (prevalence of *L. iners*-dominated microbiotas among those with Ct)	26 (prevalence of *L. crispatus*-dominated microbiotas among those with Ct)	mOR[l]	3.92 [1.50, 10.23]
Tamarelle 2020[136] USA	Case-control	240 (240)	--	V3-V4	NR	NR	NAAT, vaginal swab	NR	NR	OR	4.51 [1.41, 16.42]
Ceccarani and Foschi[m] 2019[159] Italy	Cross-sectional	79 (41)	--	V3-V4	Mean RA 20%	Mean RA 26%	NAAT, vaginal swab	Mean RA 28%	Mean RA 28%	RA ratio of ratios	3.01
BV											
Ravel 2012[160] USA	Cross-sectional	148 (58)	--	V1-V2	26 (18)	32 (22)	Nugent score[n]	3 (12)	1 (3)	PR	3.69 [0.41, 33.43]

Balle 2018[156] South Africa	Cross-sectional	72 (NR) participants, 144 (67) observations	--	V4	40 (28)	27 (19)	Nugent score[n]	11 (27)	3 (11)	PR	2.45 [0.76, 8.05]
Haahr 2019[21] Denmark	Cross-sectional	120 (100)	--	V4	51 (43)	49 (41)	Nugent score[n]	4 (8)	3 (6)	PR	1.28 [0.30, 5.43]
Mehta 2015[161] USA	Cross-sectional	64 (64) participants, 581 (581) observations	--	V1-V2	Mean RA 38%	Mean RA 4%	Amsel criteria[o]	Mean RA 58%	Mean RA 2%	RA ratio of ratios	5.61
Campisciano 2017[162] Italy	Cross-sectional	96 (69)	--	V1-V3	Mean RA 29%	Mean RA 22%	Nugent score[n]	Mean RA 15%	Mean RA 6%	RA ratio of ratios	1.76
Ceccarani and Foschi[m] 2019[159] Italy	Cross-sectional	79 (41)	--	V3-V4	Mean RA 20%	Mean RA 26%	Amsel criteria[o]	Mean RA 11%	Mean RA 5%	RA ratio of ratios	5.92
Ravel 2011[17] USA	Cross-sectional	394 (394)	--	V1-V2	135 (34)	101 (27)	Nugent score[n]	13 (10)	0 (0)	Spearman correlation difference	0.21
Smidt 2015[163] Estonia	Cross-sectional	21 (21)	--	V6	Detected 52%	Detected 76%	Nugent score[n]	NR	NR	Spearman correlation difference	-0.04
Any HPV											
Brotman 2014[18] USA	Cohort	32 (32) participants, 930 (930) observations	3-4 days	V1-V2	13 participants (41), observations NR	5 participants (16), observations NR	NAAT, vaginal swab	NR (72)	NR (45)	aTRR[p]	1.79 [0.71, 4.51]
Reimers 2016[146] USA	Cross-sectional	64 (64) participants, 398 (398) observations	--	V1-V2	NR	NR	NAAT, cervicovaginal lavage	NR	NR	aOR[q]	1.90
Onywera 2019[22] South Africa	Cross-sectional	62 (27)	--	V4	24 (39)	3 (5) *Lactobacillus* sp. dominated	NAAT, cervical cytobrush	11 (46)	2 (67)	PR	0.69 [0.28, 1.71]
Borgogna 2020[164] USA	Cross-sectional	39 (20)	--	V1-V3	8 (24)	12 (36)	NAAT, vaginal swab	4 (50)	6 (50)	PR	1.00 [0.41, 2.45]
hrHPV											

Reimers 2016[146] USA	Cross-sectional	64 (64) participants, 398 (398) observations	--	V1-V2	NR	NR	NAAT, cervicovaginal lavage[r]	NR	NR	aOR[q]	4.18
Onywera 2019[22] South Africa	Cross-sectional	62 (27)	--	V4	24 (39)	3 (5) *Lactobacillus* sp.-dominated	NAAT, cervical cytobrush[s]	8 (33)	2 (67)	PR	0.50 [0.19, 1.33]
Berggrund 2020[165] Sweden	Case-control	96 (60)	--	V2-V4, V6-V9	30 (31)	30 (31) *Lactobacillus* sp.-dominated	NAAT, cervical cytobrush[t]	33 (prevalence of *L. iners*-dominated microbiotas among those with hrHPV)	25 (prevalence of *L. crispatus*-dominated microbiotas among those with hrHPV)	mOR[u]	2.04 [0.71, 5.89]
Borgogna 2020[164] USA	Cross-sectional	39 (20)	--	V1-V3	8 (24)	12 (36)	NAAT, vaginal swab[v]	3 (38)	1 (8)	PR	4.50 [0.56, 35.98]
Cervical dysplasia											
Onywera 2019[22] South Africa	Cross-sectional	62 (27)	--	V4	24 (39)	3 (5) *Lactobacillus* sp.-dominated	Cervical cytology, cervical cytobrush	HSIL: 1 (4)	HSIL: 1 (33)	PR	0.13 [0.01, 1.52]
Berggrund 2020[165] Sweden	Case-control	96 (60)	5 months	V2-V4, V6-V9	30 (31)	30 (31) *Lactobacillus* sp.-dominated	Cervical histology, cervical biopsy	29 (prevalence of *L. iners*-dominated microbiotas among those with CIN2+)	34 (prevalence of *L. crispatus*-dominated microbiotas among those with CIN2+)	mOR[u]	0.76 [0.27, 2.13]
HIV											
Spear 2011[166] USA	Case-control	46 (46)	--	V1-V2	Mean RA 24%	Mean RA 11%	Serology	Mean RA 21%	Mean RA 11%	mRA ratio of ratios[w]	0.53
Mehta 2015[161] USA	Case-control	64 (64) participants, 581 (581) observations	--	V1-V2	Mean RA 38%	Mean RA 4%	Serology	Mean RA 51%	Mean RA 4%	mRA ratio of ratios[w]	2.78
Gosmann 2017[124] South Africa	Cohort	236 (236)	11 months	V4	75 (32)	23 (10)	NAAT, blood	9 (12)	0 (0)	HR	3.84 [0.86, 17.18]
Genital HSV-2											

Mehta 2020[23] Kenya	Cross-sectional	231 (117)	--	V3-V4	97 (42)	20 (9)	Serology	51 (53)	6 (30)	PR	1.75 [0.87, 3.51]
Trichomonas vaginalis											
Brotman 2012[167] USA	Cross-sectional	394 (240)	--	V1-V2	135 (34)	105 (27)	NAAT, cervicovaginal swab	2 (1)	1 (1)	PR	1.56 [0.08, 93.14]

[a]Design of the analysis used in the systematic review and meta-analysis (as applicable). Not necessarily the same as the original study's design.

[b]N total refers to the number of participants or observations with vaginal microbiota and outcome data. N contributed to estimate refers to the number of participants or observations whose data were used in generating the effect estimate.

[c]Not applicable for cross-sectional studies, case-control studies that collected exposure and outcome data at a single time point (e.g. Filardo et al. 2019), or cohort studies from which we used exposure and outcome data collected at baseline (e.g. Ravel et al. 2012).

[d]N and prevalence of *Lactobacillus iners*-dominated vaginal microbiotas, unless otherwise noted.

[e]N and prevalence of *Lactobacillus crispatus*-dominated vaginal microbiotas, unless otherwise noted.

[f]N outcome events and outcome prevalence among *Lactobacillus iners*-dominated vaginal microbiotas, unless otherwise noted.

[g]N outcome events and outcome prevalence among *Lactobacillus crispatus*-dominated vaginal microbiotas, unless otherwise noted.

[h]RA ratio of ratios were calculated as: $\frac{Mean(Lactobacillus\ iners\ relative\ abundance)|Outcome\ present}{Mean(Lactobacillus\ crispatus\ relative\ abundance)|Outcome\ present}$ / $\frac{Mean(Lactobacillus\ iners\ relative\ abundance)|Outcome\ absent}{Mean(Lactobacillus\ crispatus\ relative\ abundance)|Outcome\ absent}$.

Spearman correlation coefficient differences were calculated as:

$$Spearman\ correlation\ coefficient(Lactobacillus\ iners\ relative\ abundance, Outcome) -$$

$$Spearman\ correlation\ coefficient(Lactobacillus\ crispatus\ relative\ abundance, Outcome)$$

[i]We were not able to estimate confidence intervals for RA ratio of ratios or Spearman correlation coefficient differences. We were not able to estimate confidence intervals for Reimers et al. 2016 effect estimates for any HPV and hrHPV because these odds ratios were estimated as: $\frac{OR(Lactobacillus\ iners{-}dominated\ v.\ Diverse,\ \ (hr)HPV\ v.\ no\ (hr)HPV)}{OR(Lactobacillus\ crispatus{-}dominated\ v.\ Diverse,\ \ (hr)HPV\ v.\ no\ (hr)HPV)}$.

[j]Adjusted for age (≤ 21 years, > 21 years), type of last sex partner (steady, non-steady).

[k]Matched on age, ethnicity. Adjusted for relationship status (living together, living apart, single, unknown).

[l]Matched on age, race.

[m]Co-first authors.

[n]BV defined as Nugent score ≥7. Non-BV defined as Nugent score ≤6.

[o]BV defined as ≥3 Amsel criteria present. Non-BV defined as ≤2 Amsel criteria present.

[p]Adjusted for normalized menstrual cycle, age (< 30 years, 30-39 years, ≥ 40 years), hormonal contraception (none, non-IUD hormonal contraception, IUD hormonal contraception), study phase (pre- v. post- douching cessation intervention), vaginal sex in the day prior to vaginal swab collection (time-varying; no vaginal sex, vaginal sex with condom, condomless vaginal sex).

[q]Adjusted for vaginal pH (continuous).

[r]hrHPV types evaluated included 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66.

[s]hrHPV types evaluated included 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82.

[t]hrHPV types evaluated included 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59

[u]Matched on age.

[v]hrHPV types evaluated included 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 or 68.

[w]Matched at the index visit for age, smoking status, sexual activity (frequency and number of partners), male condom and contraceptive use.

N, number; CI, confidence interval; NAAT, nucleic acid amplification test; aOR, adjusted odds ratio; NR, not reported; OR, odds ratio; Ct, *Chlamydia trachomatis*; maOR, matched adjusted odds ratio; mOR, matched odds ratio; RA, relative abundance; BV, bacterial vaginosis; PR, prevalence ratio; HPV, human papillomavirus; aTRR, adjusted transition rate ratio; hrHPV, high-risk human papillomavirus; HSIL, high-grade squamous intraepithelial neoplasia; CIN2+, cervical intraepithelial neoplasia grade 2+; HIV, human immunodeficiency virus; mRA ratio of ratios, matched relative abundance ratio of ratios; HSV-2, herpes simplex virus type-2; IUD, intrauterine device.

Table 3.3. Summary of risk of bias assessment for each outcome.

Study	Confounding	Selection bias	Exposure measurement	Outcome measurement	Missing data	Selection of reported results	Overall
Chlamydia trachomatis							
van der Veer 2017[155]	Serious	Low	Moderate	Low	Low	Low	Serious
Balle 2018[156]	Moderate	Low	Moderate	Low	Moderate	Low	Moderate
Tamarelle 2018[20]	Serious	Low	Moderate	Low	Moderate	Low	Serious
van Houdt 2018[157]	Serious	Low	Moderate	Low	Moderate	Low	Serious
Filardo 2019[158]	Serious	Low	Moderate	Moderate	Low	Low	Serious
Tamarelle 2020[136]	Serious	Moderate	Moderate	Moderate	Moderate	Low	Serious
Ceccarani and Foschi[a] 2019[159]	Serious	Low	Serious	Low	Low	Low	Serious
BV							
Ravel 2012[160]	Serious	Moderate	Moderate	Low	Serious	Moderate	Serious
Balle 2018[156]	Serious	Low	Moderate	Not enough information	Low	Low	Serious-to-critical[b]
Haahr 2019[21]	Moderate	Moderate	Moderate	Not enough information	Moderate	Low	Moderate-to-critical[c]
Mehta 2015[161]	Critical	Serious	Serious	Moderate	Moderate	Low	Critical
Campisciano 2017[162]	Serious	Low	Serious	Not enough information	Low	Low	Serious-to-critical[b]
Ceccarani and Foschi[a] 2019[159]	Serious	Low	Serious	Not enough information	Low	Low	Serious-to-critical[b]
Ravel 2011[17]	Serious	Low	Moderate	Not enough information	Low	Moderate	Serious-to-critical[b]

Smidt 2015[163]	Serious	Low	Moderate	Not enough information	Serious	Low	Serious-to-critical[b]
Any HPV							
Brotman 2014[18]	Moderate	Low	Moderate	Moderate	Serious	Low	Serious
Reimers 2016[146]	Serious	Serious	Moderate	Moderate	Low	Moderate	Serious
Onywera 2019[22]	Serious	Low	Moderate	Low	Moderate	Low	Serious
Borgogna 2020[164]	Serious	Not enough information	Moderate	Moderate	Moderate	Low	Serious-to-critical[d]
hrHPV							
Reimers 2016[146]	Serious	Serious	Moderate	Moderate	Low	Moderate	Serious
Onywera 2019[22]	Serious	Low	Moderate	Low	Moderate	Low	Serious
Berggrund 2020[165]	Serious	Serious	Moderate	Serious	Low	Low	Serious
Borgogna 2020[164]	Serious	Not enough information	Moderate	Moderate	Moderate	Low	Serious-to-critical[d]
Cervical dysplasia							
Onywera 2019[22]	Serious	Low	Moderate	Low	Moderate	Low	Serious
Berggrund 2020[165]	Serious	Serious	Moderate	Serious	Low	Low	Serious
HIV							
Spear 2011[166]	Serious	Moderate	Moderate	Low	Low	Low	Serious
Mehta 2015[161]	Serious	Serious	Serious	Low	Moderate	Low	Serious
Gosmann 2017[124]	Serious	Low	Moderate	Low	Low	Low	Serious
Genital HSV-2							
Mehta	Serious	Low	Moderate	Low	Moderate	Moderate	Serious

2020[23]							
Trichomonas vaginalis							
Brotman 2012[167]	Serious	Low	Moderate	Low	Low	Low	Serious
Total							
Total Serious+ N (%)[e]	27 (90)	8 (27)	5 (17)	8 (27)	3 (10)	0 (0)	29 (97)

[a]Co-first authors.

[b]Rated as serious-to-critical because the highest-rated domain-specific risk of bias was serious, but there was not enough information to evaluate risk bias due to outcome measurement. It is possible we would have rated risk bias due to outcome measurement and overall risk of bias as critical with more information to evaluate risk bias due to outcome measurement.

[c]Rated as moderate-to-critical because the highest-rated domain-specific risk of bias was moderate, but there was not enough information to evaluate risk bias due to outcome measurement. It is possible we would have rated risk bias due to outcome measurement and overall risk of bias as critical with more information to evaluate risk bias due to outcome measurement.

[d]Rated as serious-to-critical because the highest-rated domain-specific risk of bias was serious, but there was not enough information to evaluate risk selection bias. It is possible we would have rated risk of selection bias and overall risk of bias as critical with more information to evaluate risk of selection bias.

[e]Sum and proportion of studies rated as moderate-to-critical, serious, serious-to-critical, critical, or not enough information in each bias domain and overall. Studies that contributed to multiple reviews were counted for each review in which they were rated as serious+ because risk of bias was assessed for each review separately.

BV, bacterial vaginosis; HPV, human papillomavirus; hrHPV, high-risk human papillomavirus; HIV, human immunodeficiency virus; HSV-2, herpes simplex virus type-2.

Table 3.4. Summary of quality of evidence assessment for each review.

Review	Risk of bias	Inconsistency	Indirectness	Imprecision	Publication bias	Dose response	Overall
Chlamydia trachomatis	-1 serious	-0	-1 serious	-0	-1 serious	+0	-1 very low
BV	-2 very serious	-0	-2 very serious	-1 serious	-1 serious	+1	-3 very low
Any HPV	-2 very serious	-1 serious	-1 serious	-1 serious	-1 serious	+0	-4 very low
hrHPV	-2 very serious	-2 very serious	-1 serious	-2 very serious	-1 serious	+0	-6 very low
Cervical dysplasia	-1 serious	-0	-0	-1 serious	-1 serious	+0	-1 very low
HIV	-1 serious	-1 serious	-1 serious	-2 very serious	-1 serious	+0	-3 very low
Genital HSV-2	-1 serious	NA	-1 serious	-1 serious	-1 serious	+0	-2 very low
Trichomonas vaginalis	-1 serious	NA	-1 serious	-2 very serious	-1 serious	+0	-3 very low

Large effect, plausible residual confounding in favor of observed effect not included in table because all reviews rated as +0.

BV, bacterial vaginosis; HPV, human papillomavirus; hrHPV, high-risk human papillomavirus; HIV, human immunodeficiency virus; HSV-2, herpes simplex virus type-2.

3.4.2 *Chlamydia trachomatis*

Seven studies were included in the Ct review. Six of the studies evaluated vaginal microbiota composition and Ct at a single time point; the seventh evaluated vaginal microbiota composition one year prior to Ct.[157] Six of seven used nucleic acid amplification tests (NAAT) to evaluate Ct; the seventh did not report Ct testing method.[158] Five were conducted in Europe, one in South Africa, and one in the USA. Six studies were included in meta-analysis,[20,136,155–158] which represented data from >402 participants and >109 Ct infections (one study did not report the number of participants with *L. iners*- or *L. crispatus*-dominated microbiotas,[136] two studies did not report the number of Ct infections among participants with *L. iners*- or *L. crispatus*-dominated microbiotas[136,156]). The summary OR of 3.38 (95% CI 2.12-5.40; Figure 3.1) indicates that individuals with *L. iners*-dominated microbiotas had 3.4-fold significantly higher odds of Ct than individuals with *L. crispatus*-dominated microbiotas. For the remaining study, we estimated a relative abundance ratio of ratios of 3.01, indicating that *L. iners* enrichment during prevalent Ct is 3-fold greater than *L. crispatus* enrichment.[159] We found six of the seven studies to be at serious risk of bias due to confounding, and we rated the overall quality of evidence to be very low due to risk of bias, indirectness (six studies evaluated prevalent Ct), and likely publication bias.

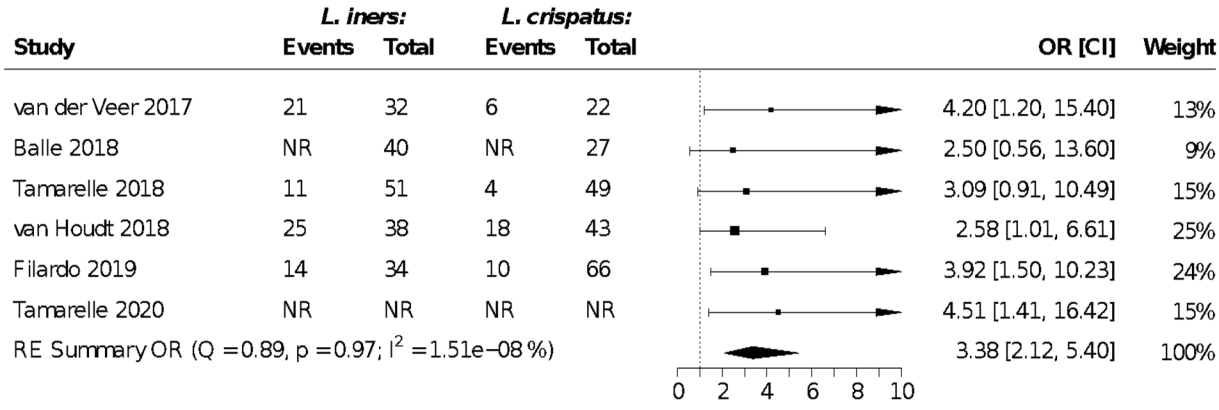

Figure 3.1. *Chlamydia trachomatis* random-effects meta-analysis forest plot.

L. iners heading refers to genital chlamydial infection events among and total number of participants with a *L. iners*-dominated vaginal microbiota. *L. crispatus* heading refers to genital chlamydial infection events among and total number of participants with a *L. crispatus*-dominated vaginal microbiota.

OR, odds ratio; CI, 95% confidence interval; NR, not reported; RE, random effects.

3.4.3 *BV*

Eight studies were included in the BV systematic review, all of which evaluated vaginal microbiota composition and BV status at a single time point (cross-sectionally). Four were conducted in Europe, three in the USA, and one in South Africa. Three studies were included in meta-analysis.[21,156,160] These studies evaluated BV by Nugent score, and they collectively represented data from 225 participants and 25 BV events. The summary PR of 2.10 (95% confidence interval (CI) 0.90-4.88; Figure 3.2) indicates that individuals with *L. iners*-dominated microbiotas had twice the prevalence of BV compared to individuals with *L. crispatus*-dominated microbiotas, though the summary PR was nonsignificant.

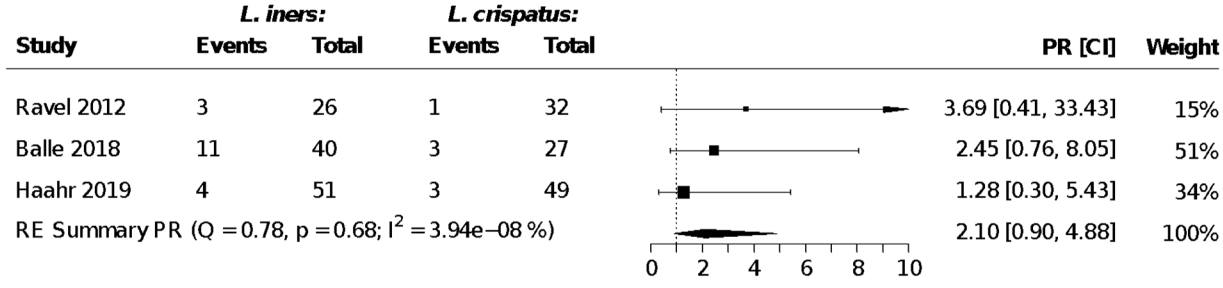

Figure 3.2. BV random-effects meta-analysis forest plot.

L. iners heading refers to BV events among and total number of participants with a *L. iners*-dominated vaginal microbiota. *L. crispatus* heading refers to BV events among and total number of participants with a *L. crispatus*-dominated vaginal microbiota.

PR, prevalence ratio; CI, 95% confidence interval; RE, random effects; BV, bacterial vaginosis.

For three additional studies, we estimated relative abundance ratio of ratios indicating *L. iners* enrichment during prevalent Amsel BV is approximately 5.75-fold greater than *L. crispatus* enrichment,[159,161] whereas *L. iners* enrichment during prevalent Nugent BV is 1.76-fold greater than *L. crispatus* enrichment.[162] The remaining two studies evaluated BV using Nugent score. For these studies, we estimated Spearman correlation differences of 0.21,[17] suggesting *L. iners*-dominated microbiotas may have somewhat more positive correlations with Nugent score than *L. crispatus*-dominated microbiotas, and -0.04,[163] suggesting similar correlations for *L. iners*- and *L. crispatus*-dominated microbiotas. We found seven of the eight studies to be at serious or critical risk of bias due to confounding, and six of eight studies did not provide enough information to evaluated risk of bias due to outcome measurement. We rated the overall quality of evidence to be very low. We down-rated quality of evidence due to risk of bias, indirectness (studies evaluated prevalent BV), imprecision, and likely publication bias. We up-rated quality of evidence based on evidence of a dose-response relationship with all relative abundance ratio of ratios >1.[159,161,162]

3.4.4 *HPV, hrHPV, and cervical dysplasia*

Eight reports from five studies were eligible for inclusion in the HPV review, four of which included any HPV as the outcome, and the remaining four focused on hrHPV. We will summarize the HPV results in this paragraph and the hrHPV results in the next paragraph. All four HPV studies tested for HPV using NAAT, three evaluated vaginal microbiota composition and HPV at a single time point, and one characterized microbiota composition 3-4 days prior to HPV testing. Three were conducted in the USA and one in South Africa. The single longitudinal study presented a transition rate ratio of 1.79 (95% CI 0.71-4.51),[18] and we estimated an OR of 1.90[146] for a cross-sectional study. Both of these estimates suggest individuals with *L. iners*-dominated microbiotas are at 80-90% higher risk of HPV detection than individuals with *L. crispatus*-dominated microbiotas. The two remaining cross-sectional studies presented PRs of 1.00 (95% CI 0.41-2.45)[164] and 0.69 (95% CI 0.28-1.71),[22] indicating null-to-inverse associations. No meta-analysis was performed because

the studies did not present the same form of effect estimate. We rated three studies to be at serious risk of bias due to confounding, and we rated overall quality of evidence as very low due to risk of bias, inconsistency, indirectness (cross-sectional studies evaluated prevalent HPV), imprecision, and likely publication bias.

All four hrHPV studies used NAAT to test for hrHPV, and all characterized the vaginal microbiota and hrHPV at a single time point (Table 2 footnotes list hrHPV types evaluated in each study). Two were conducted in the USA, one in South Africa, and one in Sweden. One study presented an OR of 2.04 (95% CI 0.71-5.89),[165] and for a second study we estimated an OR of 4.18,[146] suggesting *L. iners*-dominated microbiotas are associated with 2-4-fold higher odds of prevalent hrHPV than *L. crispatus*-dominated microbiotas. The remaining two studies presented PRs of 4.50 (95% CI 0.56-35.98)[164] and 0.50 (95% CI 0.19-1.33),[22] providing conflicting evidence regarding hrHPV prevalence. No meta-analysis was performed because the studies did not present the same form of effect estimate. We found all four studies to be at serious risk of bias due to confounding, and two to be at serious risk of selection bias with a third not providing enough information to evaluate risk of selection bias. We rated overall quality of evidence to be very low due to risk of bias, inconsistency, indirectness (studies assessed prevalent hrHPV), imprecision, and likely publication bias.

Two studies were included in the cervical dysplasia review; one used cervical cytology[22] and one used cervical histology[165] to evaluate cervical disease. A cross-sectional study conducted in South Africa classified cervical dysplasia as high-grade squamous intraepithelial lesions (HSIL) and presented a PR of 0.13 (95% CI 0.01-1.52).[22] A case-control study conducted in Sweden characterized the microbiota five months prior to evaluating cervical intraepithelial neoplasia grade 2+ (CIN2+) and reported an OR of 0.76 (95% CI 0.27-2.13).[165] Both studies suggest that *L. iners*-dominated microbiotas are associated with the absence of cervical dysplasia. We found both studies to be at serious risk of bias due to confounding, and we rated the overall quality of evidence to be very low due to risk of bias, imprecision, and likely publication bias.

3.4.5 *HIV*

Three studies were included in the HIV systematic review. Two of the studies were case-control in design, conducted in the USA, and characterized vaginal microbiota composition and tested for HIV by serology cross-sectionally. We estimated relative abundance ratio of ratios of 2.78[161] and 0.53[166] for these studies, which provide conflicting evidence on the relative enrichment of *L. iners* and *L. crispatus* among individuals living with HIV. The third study was a prospective cohort study conducted in South Africa. Vaginal microbiota composition was evaluated at baseline and HIV testing by NAAT occurred twice-weekly during 11 months of follow-up. The authors presented a HR of 3.84 (95% CI 0.86-17.18), indicating individuals with *L. iners*-dominated microbiotas had nearly four-fold higher risk of acquiring HIV than individuals with *L. crispatus*-dominated microbiotas.[124] No meta-analysis was performed because the studies did not present the same form of effect estimate. We found all three studies to be at serious risk of bias due to confounding, and we rated overall quality of evidence as very low due to risk of bias, inconsistency, indirectness (cross-sectional studies assessed prevalent HIV), imprecision, and likely publication bias.

3.4.6 *Genital HSV-2 and Trichomonas vaginalis*

Here we present results for HSV-2 and Tv because these reviews each included one study. The HSV-2 study was cross-sectional and conducted in Kenya. We estimated a PR of 1.75 (95% CI 0.87-3.51), suggesting individuals with *L. iners*-dominated microbiotas have 75% higher prevalence of genital HSV-2 than individuals with *L. crispatus*-dominated microbiotas.[23] The Tv study was cross-sectional, conducted in the USA, and evaluated Tv by NAAT. We estimated a PR of 1.56 (95% CI 0.08-93.14), which, given the wide confidence interval, does not provide evidence of an association in either direction.[167] We found both studies to be at high risk of bias due to confounding.

3.5 DISCUSSION

The Ct and BV evidence reviewer here and their meta-analyses indicate *L. iners*-dominated microbiotas may be suboptimal compared to *L. crispatus*-dominated microbiotas, suggesting *L. iners* dominance may confer risk of acquiring Ct or developing BV. These results should be interpreted with caution as the meta-analyzed studies are at serious risk of bias and represent very-low-quality evidence. These systematic reviews also highlight the dearth and general low quality of epidemiologic evidence on the role of *Lactobacillus iners* in sexual health outcomes. Based on the evidence reviewed, it is challenging, if not impossible, to draw conclusions regarding relationships between *L. iners* and HPV, HIV, genital HSV-2, Tv, or Ng infections, or cervical dysplasia. Claims regarding the relative benefits or risks of *L. iners* and *L. crispatus* as they relate to these outcomes should be interpreted with caution as epidemiologic evidence is limited, conflicting, largely cross-sectional, and at serious risk of bias.

Despite limitations of the epidemiologic evidence, Ct findings are consistent with genomic and *in vitro* evidence. All seven Ct studies and the RE-MA estimate indicate *L. iners* is associated with the presence or acquisition of Ct while *L. crispatus* is associated with its absence. Six of these seven studies assessed vaginal microbiota composition and Ct cross-sectionally.[20,136,155,156,158,159] The *L. iners* genome contains genes for three RNA polymerase σ factors that are absent in other vaginal lactobacilli.[29] This expanded σ factor repertoire enables specific, directed stress responses to a wider array of environmental conditions than in other lactobacilli, which likely contributes to *L. iners*' unique ability to persist during infections including Ct.[29] The seventh study assessed microbiota composition one year prior to Ct.[157] It is unlikely that the vaginal microbiota at a given time point has any direct impact on STI acquisition one year later; however, in vitro evidence suggests *L. iners* may have less capacity to prevent Ct acquisition than *L. crispatus*. *L. iners* lacks genes for D-lactic acid (LA) production,[29,30] and it produces very little D-LA *in vitro*.[168–170] In a three-dimensional cervical epithelial cell model, pretreatment with *L.*

crispatus cell-free supernatant (CFS) reduced Ct infectivity approximately 10-fold more than pretreatment with *L. iners* CFS.[170] This difference appears to be driven by differences in D-LA concentrations, which were 9-fold higher in *L. crispatus* than *L. iners* CFS: *L. iners* CFS supplemented with 1% D-LA achieved similar reductions in infectivity as *L. crispatus* CFS.[170] As the D-LA isomer is more potent than L-LA,[171] differences in D-LA concentration and anti-chlamydial activity are likely maintained between *L. crispatus*- and *L. iners*-dominated vaginal microbiotas *in vivo*.

Genomic, *in vitro*, and *in silico* studies also support the BV findings. All eight BV studies assessed vaginal microbiota composition and BV cross-sectionally, and seven of the studies[17,21,156,159–162] and the RE-MA estimate indicate *L. iners* is associated with the presence of BV whereas *L. crispatus* is associated with its absence. It should be noted that some degree of the association between *L. iners* and Nugent BV can be attributed to measurement error in Nugent scoring. Unlike other lactobacilli, *L. iners* has a thin peptidoglycan layer in its cell wall and stains Gram-negative to Gram-variable.[27] As such, *L. iners* may be mis-scored as *Gardnerella*, artificially inflating Nugent scores.

Measurement error aside, complementary lines of evidence indicate that *L. iners* may contribute to BV development via reduced antagonism of BV-associated taxa, and that *L. iners* may persist during BV to a greater degree than other lactobacilli. As *L. iners* lack genes for D-LA production,[29,30] it likely exhibits little antibacterial activity against BV-associated taxa *in vivo*. Additionally, *L. iners* produces inerolysin, a cholesterol-dependent cytolysin that is unique among lactobacilli and related to vaginolysin, a known *Gardnerella* virulence factor.[31,32] Inerolysin is active at pH characteristic of BV (4.5-6) and is upregulated 6-fold during BV.[28,31,32] Considering its relative lack of metabolism genes, *L. iners* may use inerolysin to obtain nutrients from the host in a commensal manner, particularly during BV.[28–32] Finally, in coculture with cervical epithelial cells, adherent *L. iners* is not displaced by a BV-associated *Gardnerella* strain, and *L. iners* enhances adhesion of this *Gardnerella* strain.[172] These features suggest *L. iners* is uniquely well

poised to persist during BV and transitions in and out of BV. Indeed, a recent in silico mathematical model that successfully recapitulated population prevalences of vaginal microbiota community types corroborates this hypothesis.[173] Measuring microbiota composition at three-month intervals, *L. iners*-dominated microbiotas shifted to diverse, BV-like communities 32% of the time.[173] These BV-like communities likewise transitioned to *L. iners*-dominated communities 20% of the time; they rarely transitioned to *L. crispatus*-dominated communities (1-2%).[173]

The third key finding of these reviews relates to the rigor of epidemiologic studies of the vaginal microbiota and sexual health outcomes. We down-rated quality of evidence for all but one outcome due to indirectness given the substantial proportion of studies that collected exposure and outcome data cross-sectionally (Table 4). Prevalent outcomes are not of particular interest to individuals at risk for the outcome, nor are they of particular epidemiologic interest. Future studies should employ truly longitudinal study designs in which exposure data are collected prior to outcome data, and the interval between exposure and outcome assessment is informed by the natural history of the outcome and the timeframe on which the vaginal microbiota is expected to influence the outcome. Longitudinal designs are the only designs that provide epidemiologic evidence regarding BV development, STI acquisition, or cervical carcinogenesis. Such etiologic evidence is relevant to preventing adverse outcomes, which is of interest to individuals at risk for those outcomes.

In addition to temporality concerns, nearly all 30 studies included across the outcomes were at serious to critical risk of bias overall (29, 97%) and due to confounding (27, 90%; Table 3.3). This is alarming, especially considering 18 of 27 studies at serious to critical risk of bias due to confounding were unadjusted and unmatched.[17,20,22,23,124,136,156,159–164,167] The confounders we considered included vaginal sex, hormonal contraception, vaginal washing, and race/ethnicity (and variables race/ethnicity may be a proxy for). We generally expect these factors to have concordant relationships with our exposure (*L. iners*-dominated v. *L. crispatus*-dominated microbiotas) and outcomes of interest (BV, STI, cervical dysplasia), so we generally expect

uncontrolled confounding due to these factors to bias effect estimates upward (away from 0).[174] The unadjusted and unmatched effect estimates included in these reviews likely overestimate true associations, distorting our understanding of how *L. iners* may influence sexual health outcomes. Ideally, future epidemiologic studies in these areas will collect data on, enroll sufficient participants to adjust for, and adjust for key confounders.

We conducted a series of systematic reviews and meta-analyses to evaluate the state of epidemiologic evidence regarding the role of *L. iners* in eight sexual health outcomes. Our findings indicate *L. iners*-dominated vaginal microbiotas may be suboptimal compared to *L. crispatus*-dominated vaginal microbiotas for Ct and BV, which is consistent with prior in vitro, in silico, and genomic work. Though the focus of this work was to evaluate epidemiologic evidence regarding the relative benefits and risks of *L. iners* and *L. crispatus*, it should also be noted that *L. iners*-dominated microbiotas are associated with lower risk of Ct and BV than microbiotas that are not dominated by a *Lactobacillus* species.[21,136,155,156,158,160] Evidence was sparse for HPV, HIV, genital HSV-2, Tv, and Ng infections, and for cervical dysplasia. Additional epidemiologic and mechanistic studies are needed to further elucidate the role of *L. iners* and identify targets for novel interventions to prevent and treat adverse sexual health outcomes. This research holds great promise as demonstrated by recent work that identified cystine uptake inhibitors as a candidate to promote transition from *L. iners*-dominated to *L. crispatus*-dominated microbiotas.[137]

Chapter 4. ASSOCIATIONS BETWEEN VAGINAL BACTERIA AND BACTERIAL VAGINOSIS SIGNS AND SYMPTOMS: A COMPARATIVE STUDY OF KENYAN AND AMERICAN WOMEN

Citation: Carter KA, Balkus JE, Anzala O, et al. Associations between vaginal bacteria and bacterial vaginosis signs and symptoms: A comparative study of Kenyan and American women. Front Cell Infect Mi 2022; 12: 801770.

4.1 ABSTRACT

4.1.1 *Background*

Bacterial colonization and associations with bacterial vaginosis (BV) signs and symptoms (Amsel criteria) may vary between populations. We assessed relationships between vaginal bacteria and Amsel criteria among two populations.

4.1.2 *Methods*

Kenyan participants from the placebo arm of the Preventing Vaginal Infections (PVI) trial and participants from a Seattle-based cross-sectional BV study were included. Amsel criteria were recorded at study visits, and the vaginal microbiota was characterized using 16S rRNA gene sequencing. Logistic regression models, accounting for repeat visits as appropriate, were fit to evaluate associations between bacterial relative abundance and each Amsel criterion.

4.1.3 *Results*

Among 84 PVI participants (496 observations) and 220 Seattle participants, the prevalence of amine odor was 25% and 40%, clue cells 16% and 37%, vaginal discharge 10% and 52%,

elevated vaginal pH 69% and 67%, and BV 13% and 44%, respectively. BV-associated bacterium 1 (BVAB1) was positively associated with all Amsel criteria in both populations. *Eggerthella* type 1, *Fannyhessea* (*Atopobium) vaginae*, *Gardnerella* spp., *Sneathia amnii*, and *Sneathia sanguinegens* were positively associated with all Amsel criteria in the Seattle study, and all but discharge in the PVI trial.

4.1.4 *Conclusions*

Core vaginal bacteria are consistently associated with BV signs and symptoms across two distinct populations of women.

4.2 INTRODUCTION

Bacterial vaginosis (BV) is the most common cause of vaginal discharge worldwide, affecting approximately 26% of reproductive-age women.[34] BV is a polymicrobial condition characterized by a diverse microbiota of anaerobic and facultative bacteria, including *Gardnerella*, *Prevotella*, *Fannyhessea* (*Atopobium*), and *Sneathia* species. BV etiology remains unclear, in part because vaginal microbiota composition varies between individuals diagnosed with BV and over time within individuals.[35–42] Clinical BV diagnosis is based on the presence of at least three of four signs/symptoms termed Amsel criteria: amine odor on addition of potassium hydroxide to vaginal fluid; >20% clue cells on vaginal wet prep; thin, gray, homogeneous vaginal discharge; and elevated vaginal pH >4.5.[6] In research settings, BV is often identified using a Gram stain-based classification system developed by Nugent and Hillier.[44] BV symptomatology may vary between women and between global regions, with some studies in sub-Saharan Africa reporting as few as 10% of women with Nugent-BV are symptomatic compared to one third of women with Nugent-BV in North America.[34,55,56]

It is unclear what drives heterogeneity in BV presentation. A cross-sectional study of women in Seattle, USA identified independent associations between vaginal bacteria and individual Amsel criteria.[37] These findings suggest BV symptomatology may be driven by the presence or abundance of specific bacteria; however, this has not been assessed in non-US populations. Growing evidence demonstrates that vaginal microbiota composition and associations with clinical outcomes may vary between global populations.[18,57–60,64,148,149,175,176] To investigate the generalizability of findings from the Seattle study, we conducted a comparative analysis of the associations between vaginal bacteria and individual Amsel criteria in a population of Kenyan women enrolled in the Preventing Vaginal Infections (PVI) trial and the Seattle population described above. We performed parallel de novo statistical analyses in each study population to facilitate direct comparison of results between the populations.

4.3 MATERIALS AND METHODS

The PVI trial was a randomized, double-blinded, placebo-controlled trial of periodic presumptive treatment (PPT) for reducing BV and vulvovaginal candidiasis (VVC).[177] The trial and the current study were approved by the Institutional Review Boards of Kenyatta National Hospital, the University of Washington, and the University of Alabama at Birmingham (ClinicalTrails.gov NCT01230814, October 6, 2014). All participants provided written informed consent prior to screening and a second written informed consent if they were eligible and agreed to enroll. The Seattle BV study was cross-sectional and was approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.[37] All participants provided written informed consent prior to enrolling.

4.3.1 *PVI trial participants and study procedures*

PVI trial participants were recruited from four research clinics: one in Mombasa, Kenya, two in Nairobi, Kenya, and one in Birmingham, Alabama.[177] The Mombasa clinic and one Nairobi clinic recruited cisgender women who reported transactional sex. The other two clinics recruited cisgender women from the general population. Eligible participants were 18-45 years old, HIV-1 seronegative, and sexually active. Women had to have a vaginal infection at screening: BV (Nugent score $\geq$7),[44] VVC, and/or *Trichomonas vaginalis* (Tv) infection. Women with $\geq$4 episodes of treatment for symptomatic vaginal infections in the prior year were excluded due to expected need for frequent open-label treatment. At screening, women with symptomatic BV or VVC received open-label treatment, and all women with Tv infection received open-label treatment. Women who met enrollment criteria at screening were invited to enroll within 7-28 days. Returning women with no vaginal symptoms were eligible to enroll. During follow-up, participants with symptomatic vulvovaginitis, vaginal discharge, or vaginal itching received open-label treatment. Pelvic speculum examinations were performed and cervicovaginal swabs collected by clinicians at enrollment and follow-up months 2, 4, 6, 8, 10, and 12. At these visits, vaginal saline wet mounts were examined for clue cells, vaginal secretions tested for amine odor, vaginal pH measured using a test strip (EMD Millipore), and vaginal discharge characteristics recorded by clinicians.

Vaginal fluid was collected using a push-off polyester/polyethylene terephthalate swab (FitzCo) for PCR targeting the 16S rRNA gene (V3-V4 hypervariable regions) and Illumina MiSeq sequencing. Approximately 33,000 sequence reads were generated per sample, giving robust detection of minority species. Results were analyzed using a bioinformatics pipeline that overcomes the tendency of sequencing to overestimate species richness.[37] Sequence data were submitted to the NCBI Short Read Archive (PRJNA638104).

4.3.2 *Seattle study participants and study procedures*

Seattle study participants were recruited from the Public Health Seattle & King County STD Clinic.[37] Nonpregnant women age 18-50 were eligible, regardless of BV status. Pelvic speculum examinations were performed and cervicovaginal swabs collected by clinicians. Vaginal swabs were used for Gram staining, measuring vaginal pH, saline microscopy, and testing for amine odor. Clinicians recorded vaginal discharge characteristics.

Vaginal fluid was collected using polyurethane foam swabs (Epicentre Biotechnologies) for PCR targeting the 16S rRNA gene (V3-V4 hypervariable regions) and 454 FLX pyrosequencing. Approximately 1,600 sequence reads were generated per sample. Results were analyzed using the same bioinformatics pipeline as above.[37] Sequence data were submitted to the NCBI Short Read Archive (SRA051298).

4.3.3 *Statistical analysis*

The primary aim of this work was to characterize relationships between vaginal bacteria and Amsel criteria that are qualitatively consistent between the PVI and Seattle populations. Parallel de novo statistical analyses were conducted for both populations to enable direct comparison of results between the populations. The current PVI analysis was limited to participants enrolled in Kenya and randomized to placebo because the PPT intervention impacted vaginal bacterial colonization.[61] PVI trial observations immediately following open-label treatment were excluded from this analysis. Descriptive statistics were used to summarize participant demographic and behavioral characteristics; Amsel criteria prevalence; and bacterial detection and relative abundance. For each study population, 16S rRNA gene sequencing data were restricted to bacterial taxa whose mean relative abundance was in the top 25% of mean relative abundances for that study and whose prevalence of detection was $\geq$5% for that study. These filters limited the current analyses to taxa that were commonly detected and that are present at high enough abundances that they may reasonably have a biological impact on the vaginal environment.

Amsel criteria (amine odor, clue cells, vaginal discharge, and elevated vaginal pH) were analyzed as individual outcomes and modeled as binary variables.[6] Hypothesis tests were considered significant at $p<0.05$, and a 5% Benjamini-Hochberg false discovery rate was applied to all hypothesis tests. All analyses were conducted separately in each study population, were complete case analyses, and were performed in R (version 3.6.1).

For each study population, taxa of interest for modeling were identified using Analysis of Composition of Microbiomes (ANCOM).[178] ANCOM detects taxa that are differentially abundant between sample types (samples with and without a given Amsel criterion present). ANCOM was specifically designed to be used with compositional 16S rRNA gene sequencing data, as compared to elasticnet regression, which was used in the original Seattle analysis and does not account for compositionality.[178,179] ANCOM was performed separately for each outcome in both study populations.

Bacterial taxa identified as differentially abundant between samples with and without an Amsel criterion present in ANCOM analysis were included in modeling analysis. For each study, four sets of logistic regression models were fit. Each set of models had a single Amsel criterion as the outcome. Within each set, individual models were fit to evaluate associations between the Amsel criterion and each taxon identified as differentially abundant between samples with and without that Amsel criterion. Bacterial relative abundances were modeled using four-level ordinal variables with levels: no detection (reference), tertile 1, tertile 2, and tertile 3. Tertiles of bacterial relative abundance were calculated for each taxon separately in each study population using all samples in which the taxon was detected. Using ordinal exposure variables generates a single odds ratio (OR) for the association between a given taxon and a given Amsel criterion. This OR represents the relative change in odds of the Amsel criterion comparing: samples in relative abundance tertile 1 to samples with no detection; samples in tertile 2 to tertile 1; and samples in tertile 3 to tertile 2. Generalized estimating equations (independent correlation structure) were used for PVI models to account for correlated data due to repeated measures within participants

and different numbers of measurements between participants. No exposure variables were lagged in PVI models to allow for cross-sectional interpretation, enabling direct comparison of PVI and Seattle results. All models for both study populations were unadjusted due to the a priori hypothesis that no available Seattle study covariates would act as confounders, and to enable direct comparison of PVI and Seattle results.

4.4 RESULTS

Of 234 PVI participants, 221 (94%) returned for at least one follow-up visit and consented to additional testing of stored specimens. One hundred ten (50%) of these participants were randomized to placebo, of whom 84 (76%) were enrolled in Kenya and included in this analysis. Median age was 29 years (interquartile range (IQR): 23.8–33 (Table 4.1). Of 242 Seattle participants, 220 (91%) participants' 16S rRNA gene sequencing data met previously-described quality measures and were included in this analysis.[37] Median age was 27 years (IQR: 22-33). Prevalence of BV assessed by Amsel criteria was 13% during PVI follow-up and 44% at the Seattle study visit (Amsel criteria prevalence in Table 4.1). Additional demographic and clinical data are presented in Table 4.1.

Table 4.1. Baseline characteristics and Amsel criteria prevalence in the PVI trial and Seattle study populations.

Characteristic	**PVI trial enrollment (N=84)**		**Seattle study (N=220)**	
	N	**%**	**N**	**%**
Race				
American Indian or Alaskan Native	0	0	7	3
Asian	0	0	15	7
Black	84	100	75	34
Mixed race	0	0	12	6
Native Hawaiian or Pacific Islander	0	0	5	2
white	0	0	97	44

Age[a]	29	24-33	27	22-33
Nugent score				
Non-BV (0-3)	36	43	90	41
Intermediate (4-6)	18	21	13	6
BV (7-10)	30	36	117	53
Clinical factors[b]				
Chlamydia trachomatis infection	6	7	--	--
Trichomonas vaginalis infection	0	0	15	7
Herpes simplex virus type-2 seropositive	55	66	--	--
Vulvovaginal candidiasis	17	20	17	8
Cervicitis	2	2	--	--
	PVI trial follow-up (N=496)		**Seattle study (N=220)[c]**	
	N	**%**	**N**	**%**
Amsel criteria & BV				
Amine odor	124	25	87	40
Clue cells	79	16	81	37
Vaginal discharge	51	10	103	52
Elevated vaginal pH	344	69	145	67
BV assessed by Amsel criteria[d]	66	13	97	44

[a]Age is reported as median, interquartile range.

[b]Testing for *Chlamydia trachomatis*, Herpes simplex virus type-2, and cervicitis was not performed in the Seattle study. No PVI trial participants tested positive for *Neisseria gonorrhoeae* at enrollment, and testing for *N. gonorrhoeae* was not performed in the Seattle study.

[c]Outcomes were not assessed for all participants of the Seattle study. Amine odor, clue cells, and BV were only assessed for 219 participants, vaginal discharge for 200 participants, and vaginal pH for 218 participants. Percentages for these variables used the number of participants for which the outcome was assessed as the denominator.

[d]BV was defined based on the presence of at least three of four Amsel criteria.

PVI, Preventing Vaginal Infections; BV, bacterial vaginosis.

Prevalence of detection and relative abundance of bacterial taxa identified by ANCOM (for any outcome in either population) are summarized in Figure 4.1. *Lactobacillus vaginalis*, *Sneathia* spp. (closely related to *Sneathia amnii* and *Sneathia sanguinegens* but not sufficiently different in the V3-V4 region of the 16S rRNA gene sequences for species-level placement), *Streptococcus*

mitis, and *Veillonella montpellierensis* were detected more frequently among PVI samples. *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, BV associated bacterium 1 (BVAB1), *Megasphaera lornae*, and *Prevotella bivia* were detected more frequently among Seattle samples. Among samples in which a given taxon was detected, median relative abundances of *L. crispatus*, *Lactobacillus iners*, BVAB1, Candidate Division TM7, and *Gardnerella* spp. were higher for PVI samples. Median relative abundance of *S. amnii* was higher for Seattle samples.

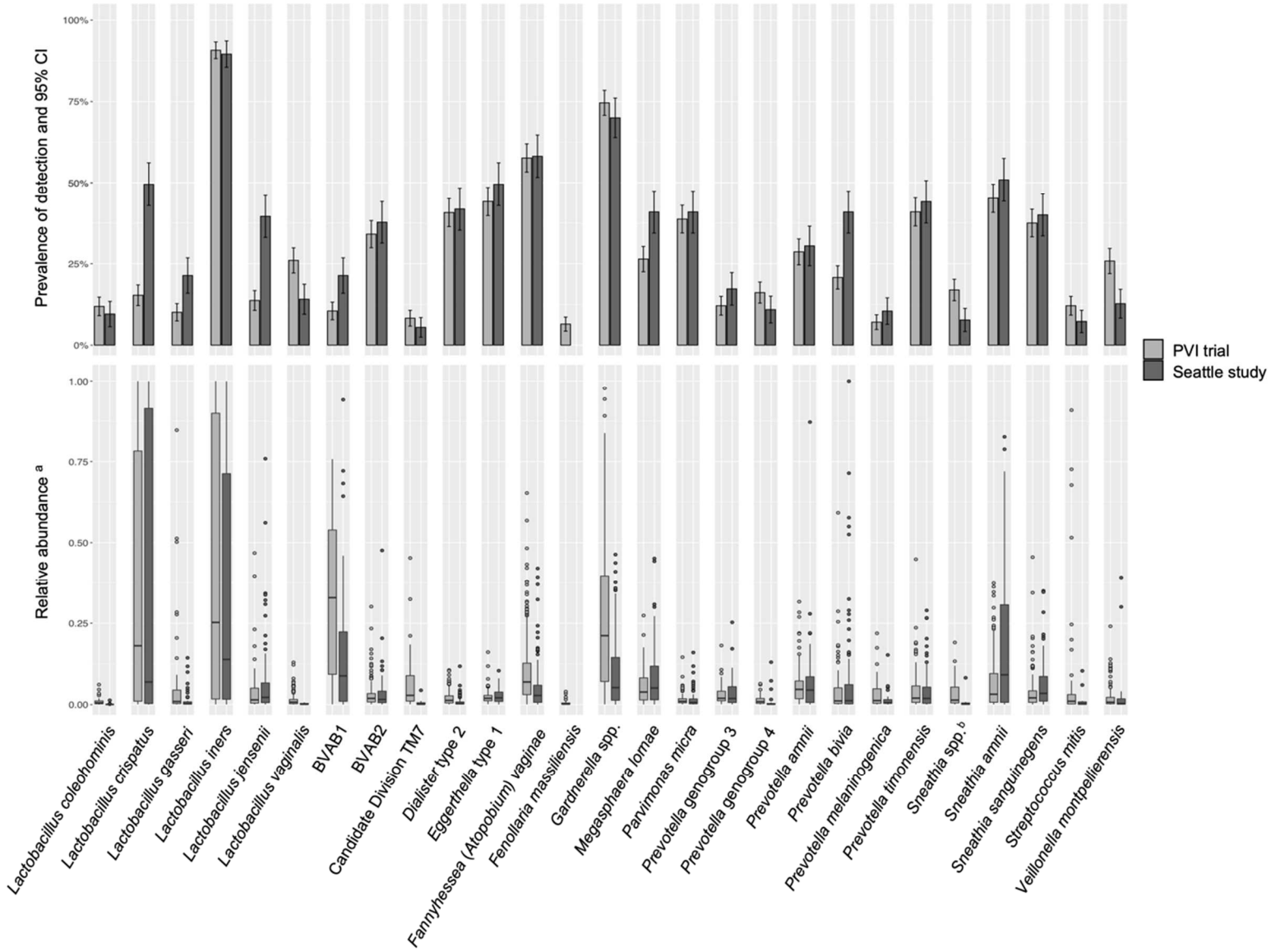

Figure 4.1. Detection and relative abundance of bacteria included in modeling analysis.

All bacteria identified by ANCOM as significantly differentially abundant between samples with and without an Amsel criterion in either study population were included as exposures in modeling analysis and are presented in this figure.

[a]Relative abundances are presented for samples in which a given taxon was detected.

[b]*Sneathia* spp. includes those not classified at the species level as *S. amnii* or *S. sanguinegens,* but closely related to these two species. Sequences are not sufficiently different at the V3-V4 region of the 16S rRNA gene to classify at the species level, hence classified at the genus level.

PVI, Preventing Vaginal Infections; CI, confidence interval; BVAB, bacterial vaginosis associated bacterium; ANCOM, analysis of composition of microbiomes.

Associations between *Lactobacillus* species and Amsel criteria were largely population-dependent (Figures 4.2-4.5). Higher relative abundance of *L. crispatus* was associated with the absence of all Amsel criteria among Seattle participants, whereas it was only associated with the absence of elevated vaginal pH among PVI participants. Higher relative abundances of *L. gasseri* and *L. jensenii* were associated with the absence of all Amsel criteria among Seattle participants and were not associated with any criteria among PVI participants. Higher *L. iners* relative abundance was associated with the absence of amine odor and clue cells in both populations, the absence of vaginal discharge among Seattle participants, and the absence of elevated vaginal pH among PVI participants.

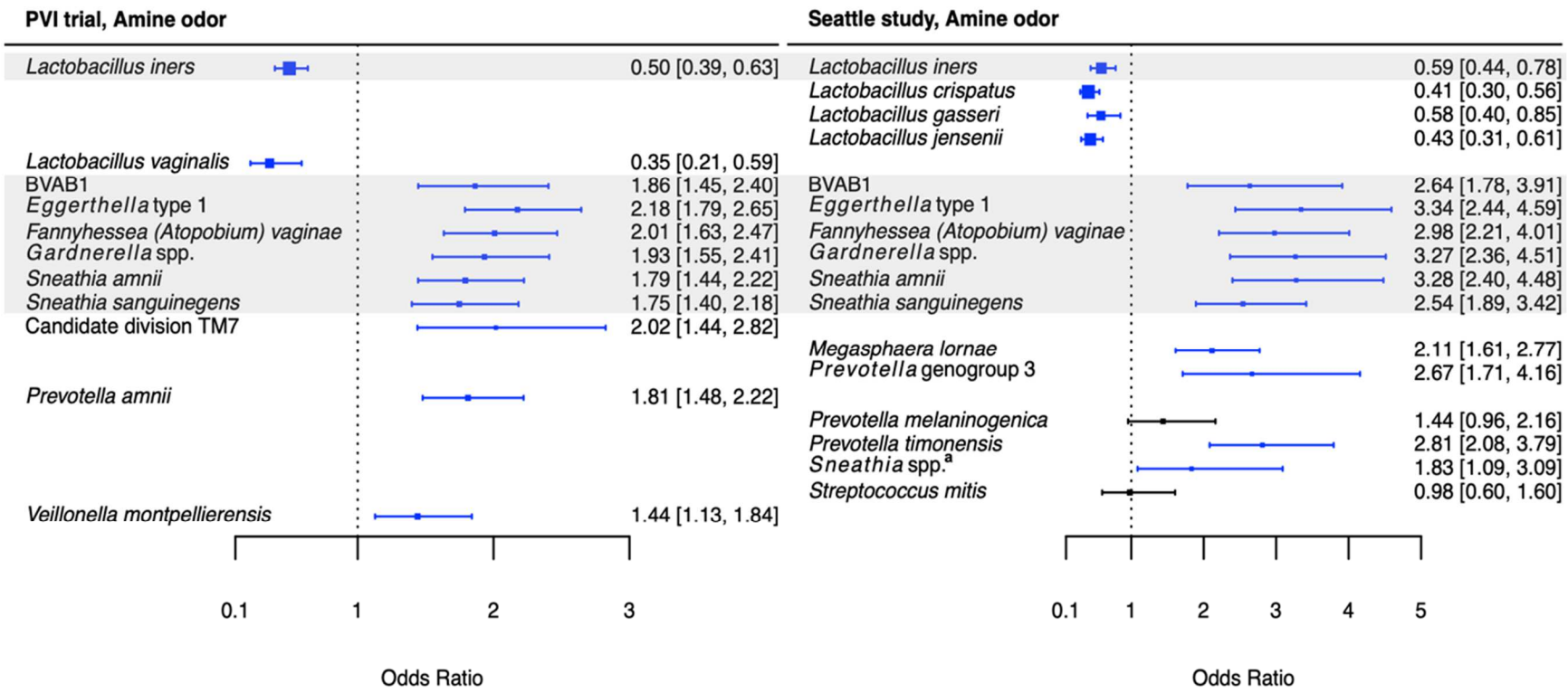

Figure 4.2. Logistic regression modeling results for the association between vaginal bacteria and amine odor. Gray shading indicates taxa that were modeled in both study populations. Blue box and whiskers indicate associations that were significant following Benjamini-Hochberg multiple comparisons adjustment (5% false discovery rate), and black box and whiskers indicate associations that were not significant following multiple comparisons adjustment. All models were univariable logistic regression models with the Amsel criterion as the outcome and bacterial relative abundance as the exposure. Bacterial relative abundances were modeled as tertiles with the reference level being no detection (four-level ordinal variables with levels: no detection,

tertile 1, tertile 2, tertile 3). Generalized estimating equations with an independent correlation structure were used for PVI trial models, and no variables were lagged in PVI models.

[a]*Sneathia* spp. includes those not classified at the species level as *S*. *amnii* or *S*. *sanguinegens,* but closely related to these two species. Sequences are not sufficiently different at the V3-V4 region of the 16S rRNA gene to classify at the species level, hence classified at the genus level.

PVI, Preventing Vaginal Infections; OR, odds ratio; CI, confidence interval; BVAB, bacterial vaginosis associated bacterium.

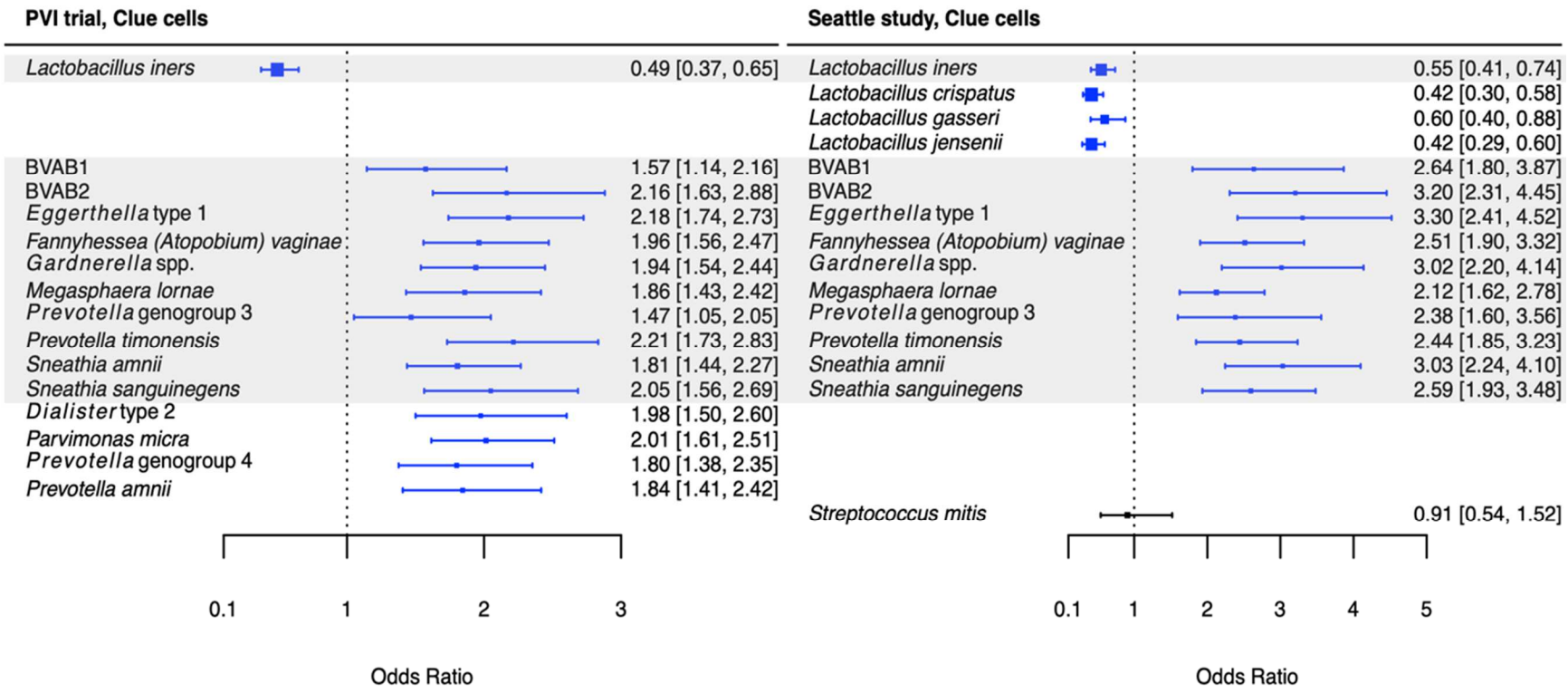

Figure 4.3. Logistic regression modeling results for the association between vaginal bacteria and clue cells. Gray shading indicates taxa that were modeled in both study populations. Blue box and whiskers indicate associations that were significant following Benjamini-Hochberg multiple comparisons adjustment (5% false discovery rate), and black box and whiskers indicate associations that were not significant following multiple comparisons adjustment. All models were univariable logistic regression models with the Amsel criterion as the outcome and bacterial relative abundance as the exposure. Bacterial relative abundances were modeled as tertiles with the reference level being no detection (four-level ordinal variables with levels: no detection,

tertile 1, tertile 2, tertile 3). Generalized estimating equations with an independent correlation structure were used for PVI trial models, and no variables were lagged in PVI models.

PVI, Preventing Vaginal Infections; OR, odds ratio; CI, confidence interval; BVAB, bacterial vaginosis associated bacterium.

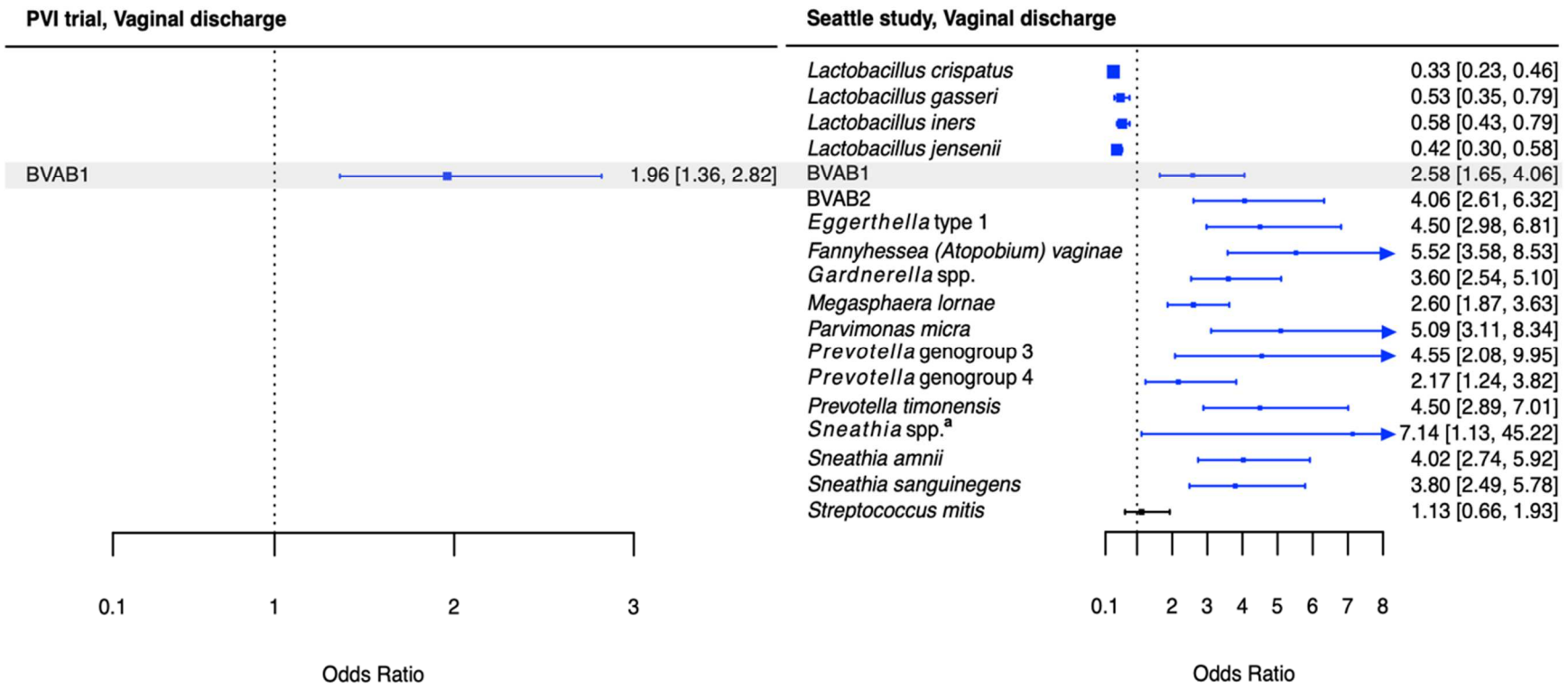

Figure 4.4. Logistic regression modeling results for the association between vaginal bacteria and vaginal discharge. Gray shading indicates taxa that were modeled in both study populations. Blue box and whiskers indicate associations that were significant following Benjamini-Hochberg multiple comparisons adjustment (5% false discovery rate), and black box and whiskers indicate associations that were not significant following multiple comparisons adjustment. All models were univariable logistic regression models with the Amsel criterion as the outcome and bacterial relative abundance as the exposure. Bacterial relative abundances were modeled as tertiles with the reference level being no detection (four-level ordinal variables with levels: no detection,

tertile 1, tertile 2, tertile 3). Generalized estimating equations with an independent correlation structure were used for PVI trial models, and no variables were lagged in PVI models.

[a]*Sneathia* spp. includes those not classified at the species level as *S*. *amnii* or *S*. *sanguinegens,* but closely related to these two species. Sequences are not sufficiently different at the V3-V4 region of the 16S rRNA gene to classify at the species level, hence classified at the genus level.

PVI, Preventing Vaginal Infections; OR, odds ratio; CI, confidence interval; BVAB, bacterial vaginosis associated bacterium.

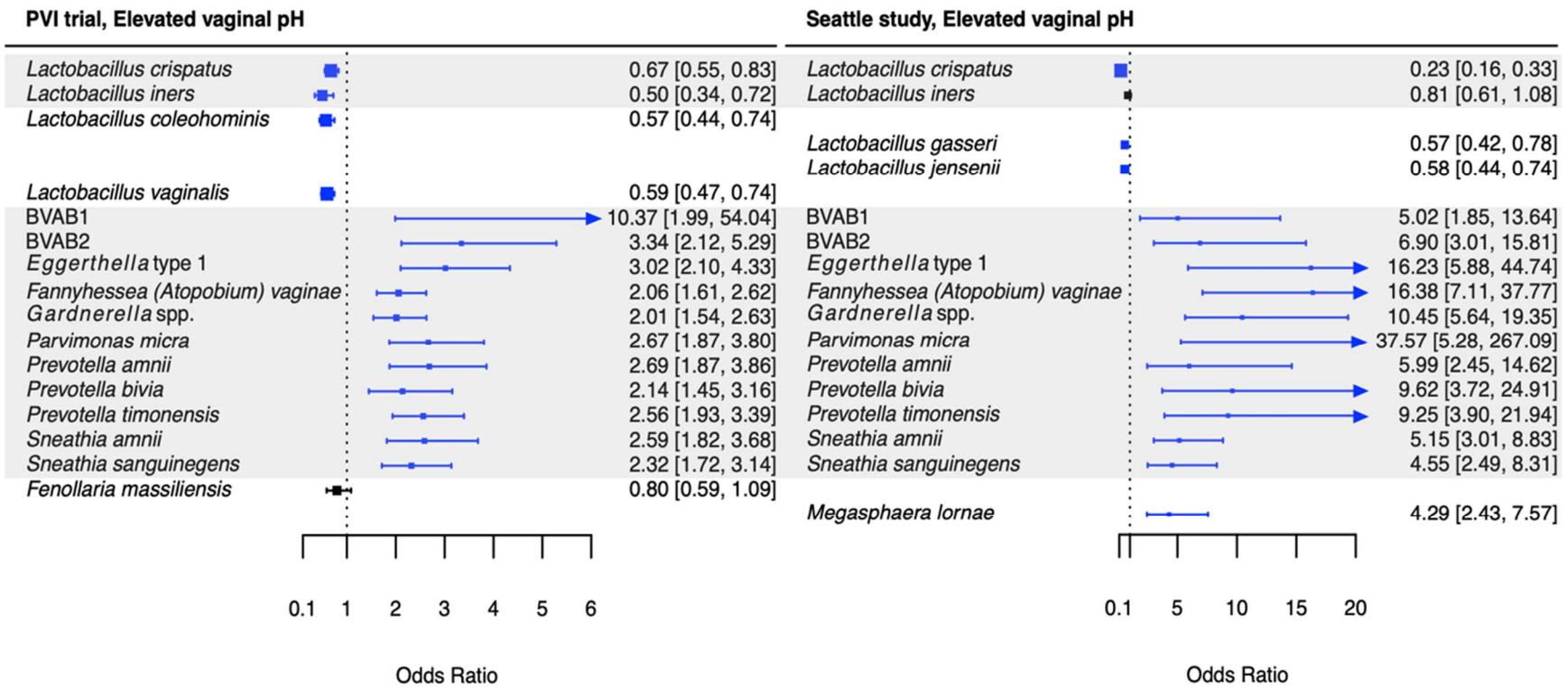

Figure 4.5. Logistic regression modeling results for the association between vaginal bacteria and elevated vaginal pH. Gray shading indicates taxa that were modeled in both study populations. Blue box and whiskers indicate associations that were significant following Benjamini-Hochberg multiple comparisons adjustment (5% false discovery rate), and black box and whiskers indicate associations that were not significant following multiple comparisons adjustment. All models were univariable logistic regression models with the Amsel criterion as the outcome and bacterial relative abundance as the exposure. Bacterial relative abundances were modeled as tertiles with the reference level being no detection (four-level ordinal variables with levels: no detection,

tertile 1, tertile 2, tertile 3). Generalized estimating equations with an independent correlation structure were used for PVI trial models, and no variables were lagged in PVI models.

PVI, Preventing Vaginal Infections; OR, odds ratio; CI, confidence interval; BVAB, bacterial vaginosis associated bacterium.

Several associations between BV-associated taxa and Amsel criteria were consistent between the populations (Figures 4.2-4.6). Higher BVAB1 relative abundance was associated with the presence of all Amsel criteria in both populations. Higher *Eggerthella* type 1, *Fannyhessea* (*Atopobium*) *vaginae*, *Gardnerella* spp., *S. amnii*, and *S. sanguinegens* relative abundances were associated with the presence of all criteria among Seattle participants, and all criteria except discharge among PVI participants. Higher *P. bivia* relative abundance was associated with elevated vaginal pH in both populations. The magnitude of these shared associations was consistently larger among Seattle participants than PVI participants (Figures 4.2-4.5).

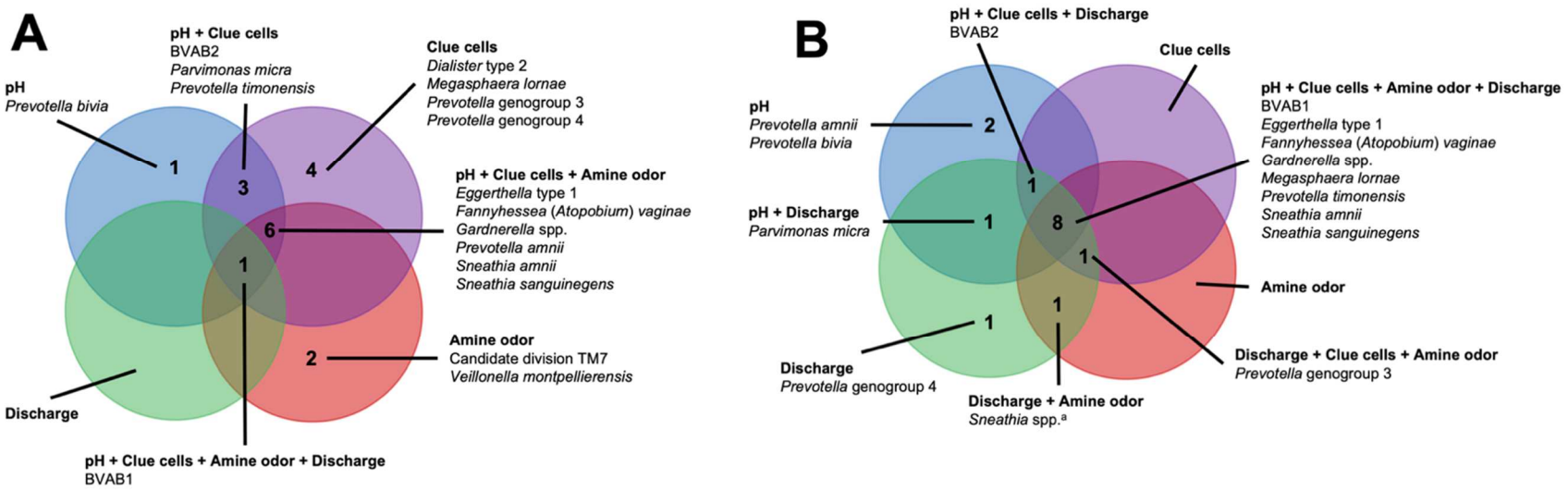

Figure 4.6. Vaginal bacteria significantly positively associated with Amsel criteria in the PVI trial population and Seattle study population.

Vaginal bacteria significantly positively associated with at least one Amsel criterion in (A) the PVI trial population and (B) the Seattle study population. Results are from logistic regression models fit separately for each study population. In each model, a single Amsel criterion was the outcome, and a single bacterial taxon was the exposure. Bacterial relative abundances were modeled as tertiles with the reference level being no detection (four-level ordinal variables with levels: no detection, tertile 1, tertile 2, tertile 3). Generalized estimating equations with an independent correlation structure were used for PVI trial models, and no variables were lagged in PVI trial models. All models were univariable. All associations represented in the Venn diagrams were significant following Benjamini-Hochberg multiple comparisons adjustment (5% false discovery rate).

[a]*Sneathia* spp. includes those not classified at the species level as *S*. *amnii* or *S*. *sanguinegens,* but closely related to these two species. Sequences are not sufficiently different at the V3-V4 region of the 16S rRNA gene to classify at the species level, hence classified at the genus level.

PVI, Preventing Vaginal Infections; BVAB, bacterial vaginosis associated bacterium.

For several additional BV-associated taxa, only some associations were shared between the PVI and Seattle populations (Figures 4.2-4.6). Higher *Prevotella timonensis* and BVAB2 relative abundances were associated with the presence of clue cells and elevated vaginal pH in both populations. Both taxa were associated with the presence of discharge among Seattle participants, and *P. timonensis* was associated with the presence of amine odor among Seattle participants. Higher *Parvimonas micra* relative abundance was associated with elevated vaginal pH in both populations. It was also associated with the presence of clue cells among PVI participants and with the presence of discharge among Seattle participants. Higher *Prevotella amnii* relative abundance was associated with elevated vaginal pH in both populations. It was also associated with the presence of amine odor and clue cells among PVI participants. Higher *Prevotella* genogroup 3 and *M. lornae* relative abundances were associated with the presence of clue cells in both populations. Both taxa were associated with the presence of amine odor and discharge among Seattle participants. *M. lornae* was also associated with elevated vaginal pH among Seattle participants.

4.5 DISCUSSION

In this comparative study, a core group of six vaginal bacteria, BVAB1, *Eggerthella* type 1, *F. vaginae*, *Gardnerella* spp., *S. amnii*, and *S. sanguinegens*, were associated with the presence of three to four Amsel criteria in research populations enrolled in Mombasa and Nairobi, Kenya and Seattle, USA. This core group of vaginal bacteria may play a key role in the manifestation of BV signs and symptoms across diverse populations. Although the etiology of BV remains unclear, these results support recent hypotheses that emphasize the formation of a polymicrobial biofilm, possibly initiated by *Gardnerella* and with early inclusion of *F. vaginae.*[40–42,180–182] The shared associations between these six bacteria and Amsel criteria may reflect bacterial cooccurrence in

the BV biofilm that persists across populations. They may also indicate bacterial interactions within the BV biofilm, which have been documented for *Gardnerella* and *F. vaginae.*[183]

Consistent with the current Seattle analysis, *Eggerthella* type 1 and *S. amnii* were associated with the presence of all Amsel criteria in the original Seattle analysis published by Srinivasan and colleagues, and *F. vaginae* and *Gardnerella* spp. were associated with three criteria.[37] However, BVAB1 was only associated with amine odor in the original analysis, and *S. sanguinegens* was not associated with any criteria.[37] These and other differences in Seattle analysis results are likely due to differences between elasticnet regression and univariable logistic regression, used in the original and current analyses, respectively.[37] Elasticnet regression is a predictive modeling approach in which features retained in the final model are those that are most strongly predictive of the outcome, conditional on other features retained in the model.[184] The lack of association between Amsel criteria and BVAB1 and *S. sanguinegens* in the original analysis reflects that these taxa's relative abundances did not substantially improve the elasticnet model's accuracy in predicting Amsel criteria. In the current univariable logistic regression analysis, associations between higher BVAB1 and *S. sanguinegens* relative abundances and the presence of Amsel criteria indicate that these taxa's relative abundances were significantly higher among samples with Amsel criteria present. The original and current Seattle results are not necessarily inconsistent, and both analytic approaches are hypothesis-generating and require mechanistic studies to understand the role of vaginal bacteria in generating BV signs and symptoms.

Studies investigating vaginal bacterial functions during BV suggest two biologically plausible mechanisms by which this core group of six bacteria may contribute to BV symptomatology. The first potential mechanism is through biogenic amine production. Genomic analyses demonstrated that *Eggerthella* isolates contain genes encoding biogenic amine-producing proteins, and a metabolomics study found that *Eggerthella* type 1, *F. vaginae*, *Gardnerella* spp., *S. amnii*, and *S. sanguinegens* were highly correlated with several biogenic amines, which were in turn associated with BV and individual Amsel criteria.[185,186] Biogenic

amines may contribute to transudation of fluid into the vagina and squamous cell exfoliation, resulting in vaginal discharge and clue cell formation.[186–188] They are also involved in amine odor, and production of biogenic amines may consume protons, increasing vaginal pH.[185] The second potential mechanism involves production of enzymes that degrade cervicovaginal mucus and epithelium, including sialidases and phospholipases. Studies evaluating the role of extracellular enzymes in vaginal epithelial cell sloughing showed that *Gardnerella* spp. produced sialidases and phospholipases, and high abundances of *F. vaginae* and *Sneathia* were associated with increased sialidase activity during BV.[40,189,190] These enzymes may contribute to squamous cell exfoliation and subsequent clue cell formation, and they likely enhance vaginal discharge.[189]

While these potential mechanisms do not appear to involve BVAB1, it is notable that BVAB1 was the only taxon associated with the presence of vaginal discharge in both the PVI and Seattle populations. In addition to BVAB1, 12 other bacteria were associated with vaginal discharge among Seattle participants. The presence of vaginal discharge characteristic of BV was recorded by clinicians in both studies, so these differences are not due to differences in vaginal discharge self-report between the populations. Instead, they can be attributed, at least partially, to the fact that ANCOM only identified BVAB1 as differentially abundant between PVI samples with and without vaginal discharge, making BVAB1 the only bacterial exposure modeled for discharge in the PVI population. Vaginal discharge was only present at 10% of PVI visits, compared to 52% of Seattle visits, and ANCOM may be underpowered to detect taxa that are differentially abundant between PVI samples with and without discharge present. These differences may also indicate that vaginal discharge arises through differing microbial pathways in different populations. Metabolomics evidence again indicates that biogenic amines may contribute to vaginal discharge in the Seattle population. In addition to the core group bacteria, BVAB2, *M. lornae*, *P. micra*, *P. timonensis*, and *Sneathia* spp. were associated with the presence of discharge among Seattle participants. A metabolomics study reported that these taxa

correlated strongly with the polyamine cadaverine, which was associated with vaginal discharge.[186]

Unlike for vaginal discharge, several taxa in addition to the core group bacteria were associated with the presence of clue cells in both the PVI and Seattle populations: BVAB2, *M. lornae*, *P. timonensis*, and *Prevotella* genogroup 3. Prior analysis of the Seattle data also found that BVAB2 and *M. lornae* were associated with clue cells.[37] Results from the current analyses and prior Seattle analysis are supported by observations from studies evaluating vaginal bacterial functions in BV. Fluorescence in situ hybridization targeting BVAB2 showed that BVAB2 can attach to vaginal epithelial cells resulting in similar appearance to clue cells.[191] Other studies investigating vaginal epithelial cell sloughing showed *Prevotella* can produce sialidases and glycosidases, suggesting *Prevotella* may contribute to squamous cell exfoliation and the formation of clue cells.[189,190,192,193] Likewise, high abundance of *Megasphaera* spp. during BV was associated with high sialidase activity.[190] Metabolomics analyses demonstrated that BVAB2, *M. lornae*, and *P. timonensis* were highly correlated with the lipid deoxycarnitine, which was in turn associated with the presence of clue cells.[186] *Prevotella* are also associated with the polyamine putrescine and contain genes encoding biogenic amine-producing proteins, and polyamines may be involved clue cell formation as discussed above.[185,187,188] These reports suggest biologically plausible mechanisms that support the epidemiologic associations of BVAB2, *M. lornae*, *P. timonensis*, and *Prevotella* genogroup 3 with the presence of clue cells.

Our results also suggest *Prevotella* may contribute to elevated vaginal pH. In both populations, *P. amnii*, *P. bivia*, *P. timonensis,* BVAB2, and *P. micra* were associated with elevated vaginal pH, in addition to the core group bacteria. Srinivasan and colleagues' prior analysis of the Seattle data also reported that *P. bivia* was associated with elevated vaginal pH.[37] Metabolomic and genomic evidence again suggests biogenic amines may underlie these associations. One metabolomics study observed that cadaverine and tyramine, both amines, and N-acetylputrescine, a degradation product of the amine putrescine, were associated with elevated

vaginal pH.[186] These amines were highly correlated with BVAB2 and *P. timonensis*, and cadaverine and N-acetylputrescine with *P. micra.*[186] A second metabolomics study reported that *Prevotella* are associated with putrescine, and genomic data indicate that various *Prevotella* species contain genes encoding biogenic amine-producing proteins.[185,188] These prior reports and the current analyses' results are consistent with Nelson and colleagues' proposed model that biogenic amine production by vaginal bacteria consumes protons, increasing vaginal pH.[185]

In addition to diagnosing BV by Amsel criteria, several point-of-care and molecular BV diagnostic assays have been developed in recent years. Current point-of-care tests evaluate the presence of sialidases and amines in a sample.[43] These approaches are supported by our findings and prior evidence suggesting sialidases and biogenic amines may be involved in BV etiology. Current molecular assays test for the presence and/or abundance of BVAB2, *F. vaginae*, *Gardnerella* spp., and/or *Megasphaera* spp.[43] Our findings indicate that additionally targeting BVAB1, *Eggerthella* type 1, *S. amnii*, and/or *S. sanguinegens* may improve assay performance in various populations, especially considering individuals receiving these tests would likely be those presenting with BV signs and symptoms. While existing molecular tests perform well (reported sensitivities of 91-99% and specificities of 86-94%),[43] even modest improvements in performance could translate into thousands of individuals receiving more accurate diagnoses and treatment in the US alone given the high prevalence of BV.

Several features of the Seattle study, PVI trial, and current analyses are important to consider in interpreting these findings. Although statistical analyses were conducted to enable direct comparison of PVI and Seattle results, technical variation between the studies may influence differences in results. Mean sequencing depth of Seattle samples was 1,620 reads per sample, compared to 33,467 reads per sample for PVI samples as different sequencing platforms (pyrosequencing versus Illumina Mi-Seq, respectively) were used based on availability.[37] Minority taxa were less likely to be detected in Seattle samples due to lower sequencing depth, and relative abundances may be overestimated in Seattle data.[194] Additional study design differences and

sources of technical variation between the parent studies may limit our ability to detect associations that are consistent between the two populations, which is why we focus on associations that are consistent between the populations. Moreover, that we were able to detect several shared associations between the populations despite this variation strengthens the evidence for these relationships. Finally, 16S rRNA gene sequencing facilitates taxonomic identification at the genus or species levels for most taxa. For example, 16S rRNA gene sequencing does not differentiate between the recently identified *Gardnerella* species,[72] which may have different relationships with BV signs and symptoms. We were unable to examine heterogeneity between *Gardnerella* species because both original studies used 16S rRNA gene sequencing and therefore classified *Gardnerella* at the genus level.

This comparison study indicates that a core group of vaginal bacteria may contribute to BV symptomatology across populations, and that additional vaginal bacteria may play a role in the manifestation of specific BV signs and symptoms. While these relationships should be investigated in additional populations, these findings help contextualize heterogeneity in BV symptomatology between women and across global regions. These findings are also consistent with growing evidence regarding the role of biogenic amines and extracellular enzymes in BV etiology and symptom manifestation. Taken together, this work and prior reports suggest that bacteria highlighted here may consistently contribute to BV symptomatology in various populations, despite differences in overall vaginal microbiota composition.

Chapter 5. DISCUSSION

5.1 EPIDEMIOLOGIC RIGOR IN VAGINAL MICROBIOME SCIENCE

Microbiome science is a fairly young field that has experienced rapid methodologic advancements in recent decades. Thanks to these advances and associated falling costs of data generation, the field is entering a new stage that will likely be defined by a pivot from reliance on 16S rRNA gene amplicon sequencing to multi-omic studies that integrate data on strain-level taxonomic composition, gene content and expression, metabolome content, and interactions with the host. While this multi-omic era has immense potential to advance our understanding of the role of the vaginal microbiome in sexual and reproductive health outcomes, these advances will be constrained by the epidemiologic rigor of multi-omic studies. To date, integration of epidemiologic methods and concepts in microbiome science has been limited by several factors, including the fact that high-dimensional, compositional data can be challenging to incorporate in epidemiologic analyses. This challenge will only be made greater when analyzing multiple such datasets generated in multi-omic studies; however, the costs of conducting epidemiologically naïve research will also be greater. In order to fully take advantage of this multi-omic era, it is imperative that vaginal microbiome researchers make epidemiologic rigor a priority in their work.

Each chapter of this dissertation highlights an avenue by which investigators can better incorporate epidemiology into their work, with **Chapter 3**'s being perhaps the most apparent. The quality of evidence was very low for all outcomes considered in the *Lactobacillus iners* systematic reviews, due in large part to uncontrolled confounding and cross-sectional study designs. Adjusting for key confounders is critical for estimating minimally biased associations and generating high-quality observational evidence. Epidemiologic studies often inform future efforts to establish/identify causal relationships (mechanistic studies) and develop and evaluate interventions to prevent or treat adverse outcomes (intervention studies/trials). Minimally biased,

adjusted effect estimates can guide future scientific inquiry more precisely than unadjusted estimates, which can in turn foster more rapid and impactful advances in knowledge, clinical practice, and public health. The same is true of longitudinal study designs. The vaginal microbiota can be highly dynamic, and cervicovaginal infections and cervical disease are demonstrated to alter the vaginal microbiota.[35,36,41,59,140,161,195–202] Given the possibility and plausibility of reverse causation, it is unreasonable to assume that evidence from cross-sectional studies represents the vaginal microbiome's effect on the outcome of interest. Instead, evidence from longitudinal studies in which the exposure is known to precede the outcome is necessary for understanding causal relationships and identifying promising bacterial, genomic, and metabolic targets for future mechanistic and interventional research.

Chapter 4 illustrates the importance of generalizing with caution. In epidemiology, generalizability, or external validity, concerns the extent to which associations or effects measured in a given population/setting hold in other populations/settings. This was a central focus of **Chapter 4**'s comparative study of the associations between vaginal bacteria and Amsel criteria in Kenyan and American populations. Though there was substantial qualitative overlap (direction and significance of associations) between the populations, many associations were unique to either population. Some degree of qualitative generalizing across populations is likely appropriate for many vaginal microbiome research questions; however, the extent to which it is reasonable to generalize will be unclear without directly comparing between populations. Alternatively, comparative studies like **Chapter 4** directly address questions of generalizability and obviate the need to make potentially dubious generalizations between populations of interest. Comparative studies are also superior to comparing independent studies of the same question conducted in different populations as they allow the investigator to employ the same study design, laboratory, bioinformatic, and analytic methods in all populations of interest, enabling similar interpretation and direct comparison of results between populations.

Finally, **Chapter 2** provides something of a cautionary tale for researchers developing novel computational methods for analyzing microbiome data. The coming years will likely see many such methods published to address both novel challenges and novel opportunities of integrating data across multiple omics measures. It will be necessary to validate or test these methods in various body site and environmental microbiomes. Building on the idea of generalizing with caution, **Chapter 2** clearly demonstrated that metagenome inference performance for the gut and oral microbiomes cannot be generalized to the vaginal microbiome. Given major differences in taxonomic composition and ecology between body site microbiomes as well as systematic differences in representation of body site microbiomes in public databases, this inability to generalize performance across body sites will likely be true for many other analytic and computational methods. It will also be important to consider if and how these methods might introduce or exacerbate common epidemiologic biases. Despite several prior published metagenome inference comparisons, **Chapter 2** is, to our knowledge, the first to conceptualize variation in metagenome inference performance as differential measurement error and differential misclassification, common forms of information bias. These two approaches, evaluating method performance in various body site and environmental microbiomes and considering common epidemiologic biases in evaluating these methods, should be taken in tandem. Microbiome science subfields focusing on different niches have adopted different ways of operationalizing microbiome composition that reflect the ecological differences between niches. These niche-specific operationalizations are often exposures of interest and as such must be considered when evaluating whether a method may introduce epidemiologic bias. Without having examined whether metagenome inference performance varied across common vaginal microbiota community types or clusters (often the exposures of interest in vaginal microbiota research), we would not have been able to identity the information bias metagenome inference methods introduce in vaginal microbiome analyses.

5.2 *LACTOBACILLUS INERS* – AN OPPORTUNISTIC BYSTANDER

Despite being considered the most prevalent and abundant vaginal bacterial species,[25,33] *L. iners* has received less attention than other common vaginal lactobacilli because it is difficult to culture and was not recognized as a species until 1999.[203] By then, the paradigm that low-diversity vaginal microbiotas with an abundance of *Lactobacillus* promote vaginal health had been established.[44] In the following years, culture-independent methods for characterizing vaginal microbiota composition revealed that *L. iners* was often present in high abundances, it dominated the vaginal microbiota of more than a third of individuals, and it was often present in substantial quantities during bacterial vaginosis (BV).[17,204–206] These associations with BV differentiated *L. iners*' epidemiology from that of other vaginal lactobacilli, and reports of *L. iners*' small genome, inability to synthesize D-lactic acid (D-LA), production of a cholesterol-dependent cytolysin, and Gram-negative to Gram-variable staining differentiated its biology from that of other vaginal lactobacilli.[27,29–32,207] These unique features of *L. iners* challenged the field's decades-old understanding of the *Lactobacillus* genus and began a debate as to the role of *L. iners* in sexual and reproductive health outcomes.[24–26]

Chapter 3's systematic reviews and meta-analyses contribute to this debate in two ways. First, meta-analyses indicate *L. iners* may be suboptimal for BV and *Chlamydia trachomatis* infection compared to *Lactobacillus crispatus*. These findings certainly do not settle the debate, as they are limited by the low quality of evidence they represent and the fact that the reviews only included studies that evaluated the vaginal microbiota using marker gene sequencing (excluded studies that targeted *L. iners* and *L. crispatus* by quantitative PCR). Second, the reviews demonstrate the dearth of peer-reviewed epidemiologic evidence that addresses the relative benefits/risks of *L. iners* and *L. crispatus* for common cervicovaginal infections and cervical carcinogenesis (it should be noted that since the literature searches were conducted, a study was published that would be eligible for inclusion in the *Neisseria gonorrhoeae* review; the

associations between *L. iners*, compared to *L. crispatus*, and *Neisseria gonorrhoeae* infection was null[208]). While there is likely a wealth of publicly available data that could be used to answer these questions, many of these potential analyses have not been conducted or published, limiting the evidence base that informs the *L. iners* debate. To make meaningful progress in understanding how *L. iners* functions in the vaginal microbiome, investigators will have to pay more specific attention to the species in conducting and reporting their work.

Limitations of the evidence notwithstanding, I will offer my thoughts on what *L. iners* may or may not be doing in the vagina. At the risk of over-anthropomorphizing, I will situate these thoughts in the context provided by the title of Petrova and colleagues' 2017 review: "*Lactobacillus iners*: Friend or foe?"[25] Simply stated, it is neither friend nor foe. Genomic comparisons suggest *L. iners* is much more highly specialized to the vaginal environment than other lactobacilli.[29,209] Of note, *L. iners* has lost many metabolism genes and acquired genes encoding a set of unique RNA polymerase σ factors as well as inerolysin, a cholesterol-dependent cytolysin that is closely related to the known *Gardnerella* virulence factor vaginolysin.[29–32] Its expanded σ factor repertoire enables stress responses to a wider range of environmental conditions than in other lactobacilli, which contributes to *L. iners*' unique ability to persist during infections, particularly BV.[29] Inerolysin likely also contributes to *L. iners*' persistence during BV. When present in individuals without BV, *L. iners* often dominates the microbiota and experiences little competition for nutrients. During BV on the other hand, *L. iners* is part of a much more diverse bacterial community and must compete with various BV-associated taxa for nutrients. In this setting, its loss of metabolism genes would likely disadvantage *L. iners*; however, inerolysin is most highly expressed and most active at higher pH characteristic of BV.[28] Because inerolysin preferentially forms arciform slits in lipid bilayers (as opposed to full ring pores formed by vaginolysin), it is hypothesized that *L. iners* uses inerolysin to obtain nutrients from host cells in a commensal manner, particularly during BV.[32] Based on this evidence, I would argue that *L. iners* is neither friend nor foe but instead an "opportunistic bystander" – it takes advantage of the dynamic vaginal environment in ways other

lactobacilli cannot to sustain itself as a member of the microbiota. This characterization is consistent with in vitro evidence that adherent *L. iners* is not displaced by *Gardnerella*, and *L. iners* enhances *Gardnerella* adherence.[172] It is also consistent with recent mathematical modeling work demonstrating *L. iners*-dominated microbiotas serve as a transitional state between *L. crispatus*-dominated microbiotas and diverse, BV-like microbiotas.[173] Finally, I would argue that it is even consistent with *L. iners*' inability to produce D-LA as antagonizing BV-associated taxa through D-LA production would inhibit *L. iners*' ability to persist during BV.[29,30]

I carefully and intentionally choose the words "opportunistic bystander" to describe *L. iners* with the hope of avoiding moral judgements on the goodness or badness of the species, which is important for three reasons. First, bacteria are not sentient and do not have personal objectives in their interactions with a human host. Anthropomorphizing can be helpful in conceptualizing and describing differences between taxa. However, without staying grounded in the truth that bacteria do not have intention, anthropomorphizing can contribute to investigators' personal biases, which may result in inappropriate interpretation of findings and/or selective pursuit of research questions that align with anthropomorphized views at the cost of more well-founded research questions. Second, *L. iners* appears so sit somewhere between *L. crispatus* and diverse, BV-like microbiotas on the spectrum of the benefits/risks the vaginal microbiota may confer to the host. With no true-neutral vaginal microbiota to benchmark benefit and risk, characterizing *L. iners*-dominated microbiotas as either good or bad depends on the comparison being made. Given my characterization of *L. iners* as an "opportunistic bystander" and the high prevalence of *L. iners*-dominated vaginal microbiotas,[25,33] perhaps *L. iners*-dominated microbiotas are a reasonable choice for a neutral state to benchmark benefit and risk. The third reason relates to scientific racism and the fact that race is socially constructed as opposed to being a biological category. Assigning moral value to vaginal microbial communities runs the risk of projecting those moral judgements onto individuals. Because the prevalence of *L. iners*-dominated microbiotas is higher among Asian, Black, African, and Latine populations than it is among white and European

populations,[17,25,96–99] projecting moral judgements about *L. iners* onto individuals will directly contribute to and perpetuate scientific racism. Microbiome science and epidemiology have harmful histories of mishandling race as a scientific variable and failing to meaningfully engage with Critical Race Theory or the Public Health Critical Race Praxis.[82–85,210,211] Anti-racist microbiome science can actively work towards alleviating health disparities and dismantling the systems of oppression that create those disparities; however, this will require investigators to hold themselves and each other accountable for critically considering race as a social construct in the design, conduct, analysis, and reporting of their work. Assigning moral value to vaginal bacteria or common vaginal microbiota community types is antithetical to anti-racist microbiome science and must be avoided if the field is to produce the greatest benefit for the greatest number of people.

5.3 IMPLICATIONS FOR BV CARE

Current (2021) Centers for Disease Control and Prevention sexually transmitted infection (STI) treatment guidelines recommend evaluating BV status for individuals with vaginal symptoms using Amsel criteria, point-of-care tests, or nucleic acid amplification tests.[53] Treatment is recommended for those with a BV diagnosis for the primary benefit of relieving symptoms; other benefits may include reducing the risk of HIV/STI acquisition and adverse pregnancy outcomes.[53] Current (2021) World Health Organization (WHO) guidelines recommend syndromic management of vaginal discharge, including BV treatment for all individuals presenting with vaginal discharge or based on microscopy results.[54] Unfortunately, as many as 69% of treated individuals will experience recurrent BV within 12 months of treatment, which can severely negatively impact self-esteem and sexual relationships.[63,212] Given its high global prevalence, high post-treatment recurrence rate, severe negative psychosocial and sexual impacts, and possible

etiologic role in various adverse sexual and reproductive sequelae, even incremental improvements in BV diagnosis and treatment can improve sexual, reproductive, and social-emotional health for many individuals worldwide.

This dissertation may potentially contribute to improving BV diagnosis and treatment in two ways. As discussed in **Chapter 4**, the associations observed between Amsel criteria and BV-associated bacterium 1 (BVAB1), *Eggerthella* type 1, *Sneathia amnii*, and *Sneathia sanguinegens* indicate that targeting these taxa may improve the performance of molecular BV diagnostic assays. Importantly, that these associations were observed among Kenyan and American populations suggests that performance improvements would likewise be observed in various populations. Though current molecular BV diagnostics have sensitivities of 90-99% and specificities of 81-97%,[43] even small improvements in test performance could yield substantial improvements in predictive values, particularly among individuals with symptoms of cervicovaginal infections. However, molecular BV assays are not yet widely used, which constrains any potential impact of improving test performance.

Currently, greater strides in increasing the accuracy of BV diagnosis can be made by improving diagnostic stewardship in the US and building capacity for BV diagnostics in low-resource settings. A recent multi-site study conducted in the US reported that clinicians evaluated the presence of vaginal discharge for all individuals presenting with vulvovaginal symptoms.[213] However, they only evaluated amine odor for 21% of these individuals, clue cells for 17%, and vaginal pH for 15%.[213] These practices resulted in inappropriate treatment for many individuals: 30% of those with laboratory-confirmed BV received either no treatment or antifungals alone, 12% of those with laboratory-confirmed vulvovaginal candidiasis received antibiotics for BV, and 23% of individuals with no vaginal infection received antibiotics for BV.[213] Further, among those with no laboratory-confirmed vaginal infection, individuals who received unnecessary treatment were >3-fold more likely to return with repeat vulvovaginal symptoms in the following 90 days than those who did not receive treatment.[213] These data clearly demonstrate the urgent need to

improve BV diagnostic stewardship in the US, and they suggest the potential for substantial gains in BV treatment accuracy and patient outcomes through improving diagnostic stewardship.

In low-resource settings, syndromic management approaches are often used to presumptively diagnose and treat various causes of vaginal discharge. A recent meta-analysis evaluated the performance of several syndromic management approaches for diagnosing BV and *Trichomonas vaginalis* infection (Tv).[214] Risk assessment alone and risk assessment with speculum examination performed moderately with 52-58% of individuals treated appropriately, 1-39% of those with BV or Tv not receiving treatment (missed treatment), and 38-58% of those without BV or Tv receiving treatment (over treatment).[214] Additionally including microscopy for wet mount and Gram stain boosted appropriate treatment (95%), yielded a low level of missed treatment (8%), and eliminated over treatment.[214] A cross-sectional study conducted in Rwanda compared WHO vaginal discharge syndromic management (treat all presenting with vaginal discharge for BV) with a vaginal pH-based approach (treat all individuals with pH $\geq$5 for BV).[215] The pH-based approach demonstrated substantial gains in appropriate treatment (51% v. 18%) and reductions in missed treatment (5% v. 77%) and over treatment (59% v. 86%).[215] This evidence suggests that building capacity for vaginal pH measurement and/or microscopy and Gram staining in facilities using syndromic management may considerably improve BV diagnostic accuracy, treatment, and patient outcomes. While microscopy and Gram staining require costly instruments, a source of electricity, and substantial training, measuring vaginal pH can be as simple as using pH paper. As such, incorporating vaginal pH measurement is a low-barrier option for improving vaginal discharge syndromic management.

The second way this dissertation may contribute to improving BV care concerns identifying novel *L. iners*-related targets/candidates for BV treatment. As stated above, as many as 69% of those treated for BV will experience a recurrence within one year of treatment.[212] Post-treatment BV recurrence is likely driven, at least in part, by failure to establish an optimal *L. crispatus*-dominated microbiota following treatment. Indeed, metronidazole treatment appears to favor the

expansion of *L. iners* over other lactobacilli, and it is often associated with *L. iners*-dominated vaginal microbiotas post-treatment.[36,216–218] *L. iners*-dominated communities are more likely to transition to diverse, BV-like communities than they are to transition to *L. crispatus*-dominated communities,[35,173] creating a cycle between BV and *L. iners*-dominated microbiotas. Further, *L. crispatus*-dominated communities have been observed to persist for anywhere between 6 weeks and 9 months on average, after which they are likely to transition to *L. iners*-dominated communities.[35,173] This creates an entry point for *L. crispatus*-dominated microbiotas into the *L. iners* dominance – BV cycle.

Conceptualizing *L. iners* as an opportunistic bystander may be useful for framing the apparently cyclic relationship between BV and *L. iners*-dominated microbiotas, a relationship that likely hampers the establishment of *L. crispatus*-dominated microbiotas. Understanding *L. iners* as an opportunistic bystander implies that interventions seeking to promote *L. crispatus* dominance should both make the vaginal environment more hospitable to *L. crispatus* and actively antagonize *L. iners*. This conclusion is supported by recent mathematical modeling work that indicates interventions promoting transitions from *L. iners* dominance to *L. crispatus* dominance and blocking transitions from *L. crispatus* dominance to *L. iners* dominance may increase prevalence of *L. crispatus*-dominated communities to a substantially greater degree than interventions impacting transitions between diverse, BV-like communities and either *L. iners*-dominated or *L. crispatus*-dominated communities.[173]

To build on this work, the same group explored the different nutritional requirements for *L. crispatus* and *L. iners* in monoculture. They demonstrated that vaginal lactobacilli lack canonical cysteine (Cys, an amino acid) biosynthesis pathways; however, *L. iners* is unique in that it also lacks transport systems for uptake of exogenous Cys and Cys-containing molecules.[137] *L. iners* instead required exogenous L-cystine to synthesize Cys, and cystine uptake inhibitors caused species-specific growth inhibition of diverse *L. iners* strains.[137] Further, in mock BV communities of *Gardnerella*, *Fannyhessea vaginae*, *Prevotella bivia*, *L. iners*, and *L. crispatus*, treatment with

cystine uptake inhibitors alone reduced *L. iners* abundance, metronidazole alone reduced BV-associated taxa abundances, and treatment with cystine uptake inhibitors and metronidazole reduced BV-associate taxa and favored expansion of *L. crispatus* over *L. iners*.[137] Cystine uptake inhibitors may be a promising complement to existing BV treatment regimens as this combination may also promote post-treatment *L. crispatus* dominance and block the *L. iners* dominance – BV cycle in vivo.[137]

I will not attempt to claim that these investigators specifically considered *L. iners* an opportunistic bystander as I do. However, I would argue that this conceptualization is congruent with their research and that it could spur similar lines of inquiry seeking to identify therapeutic targets to promote *L. crispatus* dominance over *L. iners* dominance. Additional alternative strategies for treating and preventing BV, including partner treatment, periodic presumptive treatment, biofilm disruptors, probiotics, vaginal microbiome transplantation, and phage therapy, are in various stages of investigation and have seen limited success in vivo.[65,177,219–227] Given substantial intra- and inter-individual variation in vaginal microbiota composition during BV[35–42] and inter-individual differences in treatment acceptability, it is unlikely any single BV treatment or prevention strategy will work for all individuals. Borrowing a lesson from contraceptives research, a variety of BV treatment options with different modes of action and delivery may allow individuals to select a method that works best for them (both in terms of treatment effectiveness and personal preferences), which may in turn increase adherence to BV treatment regimens and improve patient outcomes.[228–230] For these reasons, treatments that promote *L. crispatus* dominance over *L. iners* dominance may contribute to expanding the BV treatment repertoire and should be a focus of future research.

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APPENDIX A: CHAPTER 2 SUPPLEMENTAL FIGURES

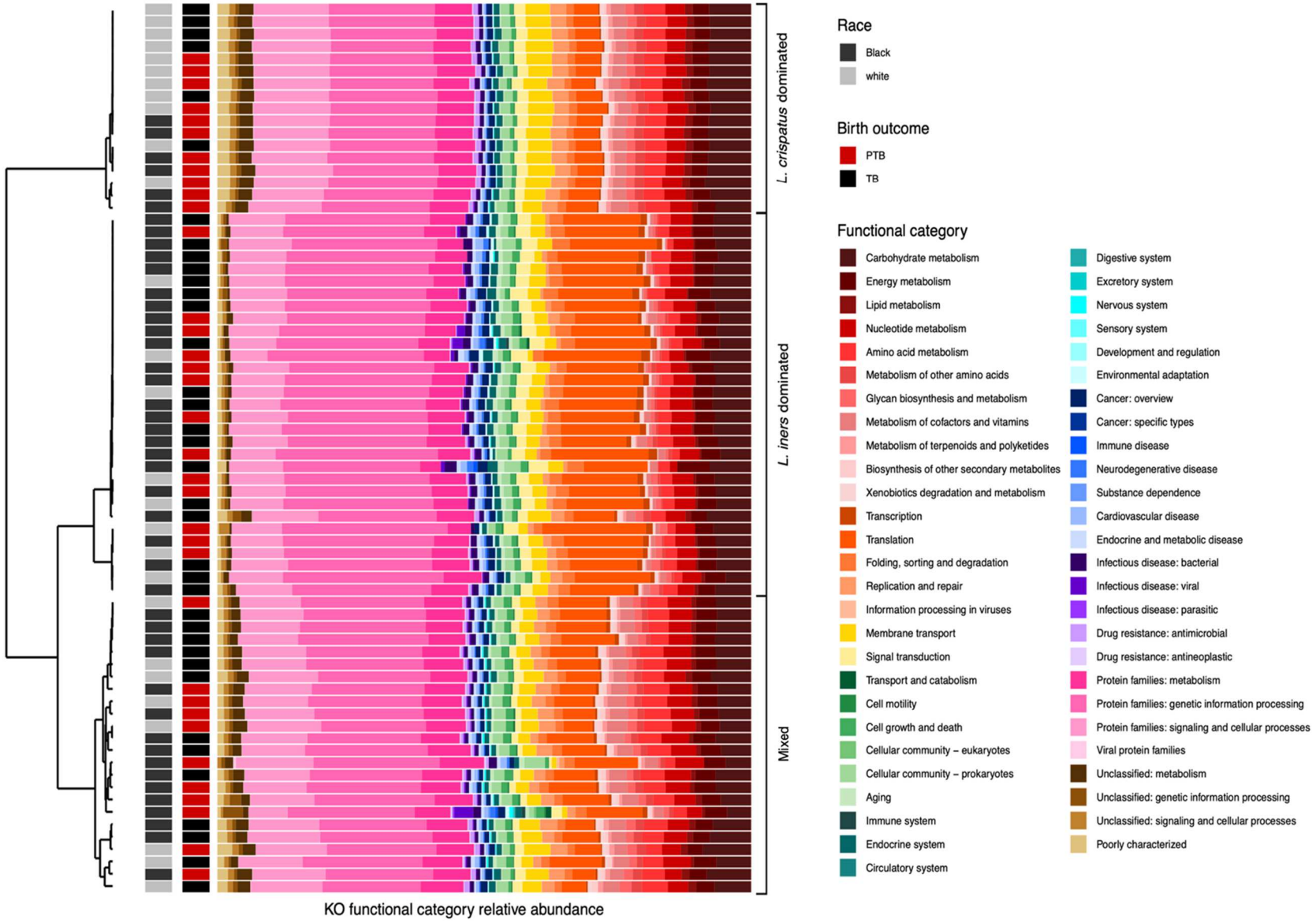

Figure 1. Metagenome composition colored by second-highest level of KO functional category, birth outcome, and race of 72 PIN participants included in analysis.

Dendrogram from hierarchical clustering of 16S rRNA gene amplicon sequencing data, resulting clusters are indicated by the brackets to the right of the stacked bar plot. KO functional category relative abundance from observed whole metagenome sequencing data. PTB, preterm birth; TB, term birth; KO, KEGG ortholog; KEGG, Kyoto Encyclopedia of Genes and Genomes.

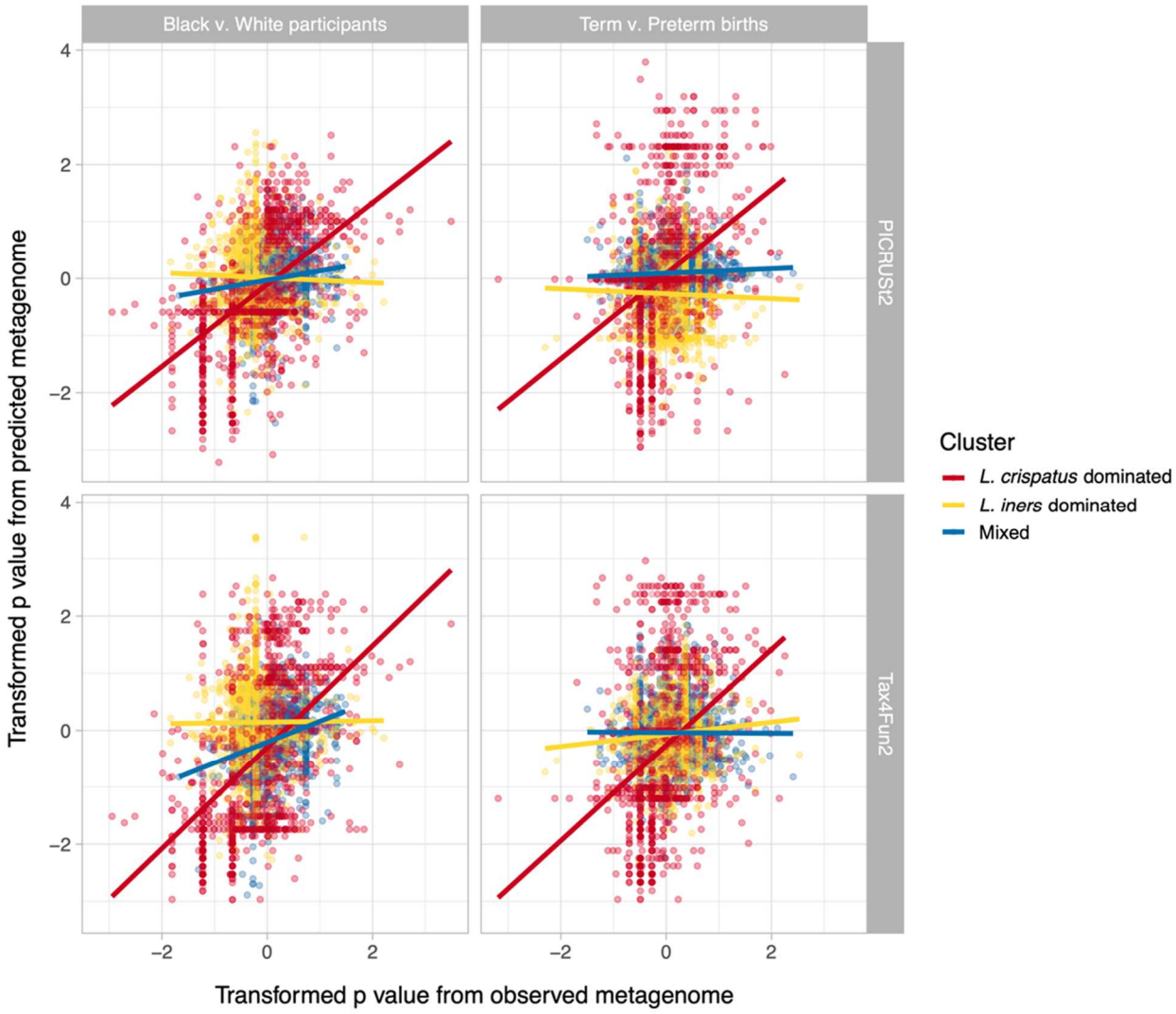

Figure 2. Scatter plots of transformed p values estimated from observed and predicted KO relative abundances for the cluster-stratified analysis.

Colored by cluster from hierarchical clustering of 16S rRNA gene amplicon sequencing data. We used Wilcoxon tests to test the following two null hypotheses: KO relative abundances do not differ between self-identified Black and white participants (left column of plots, labeled Black v. White participants), and KO relative abundances do not differ between PTB cases and term birth controls (right column of plots, labeled Term v. Preterm births). We performed Wilcoxon tests using observed and predicted KO relative abundances separately, and we transformed p values

according to the following equation in order to capture the significance and direction of KO relative abundance differences:

$$P_t = log_{10}(P) * sign((\overline{KO}|group\ 1) - (\overline{KO}|group\ 2))$$

Where P_t is the transformed p value, P is the Wilcoxon test p value, $\overline{KO}|group\ 1$ is the mean KO relative abundance in the contrast group for the hypothesis test (self-identified Black participants, cases experiencing PTB), and $\overline{KO}|group\ 2$ is the mean KO relative abundance in the reference group for the hypothesis test (self-identified white participants, controls experiencing term birth). Results from PICRUSt2-predicted genomes in the top row of plots (labeled PICRUSt2). Results from Tax4Fun2-predicted genomes in the top row of plots (labeled Tax4Fun2).

KO, KEGG ortholog; KEGG, Kyoto Encyclopedia of Genes and Genomes; PTB, preterm birth.

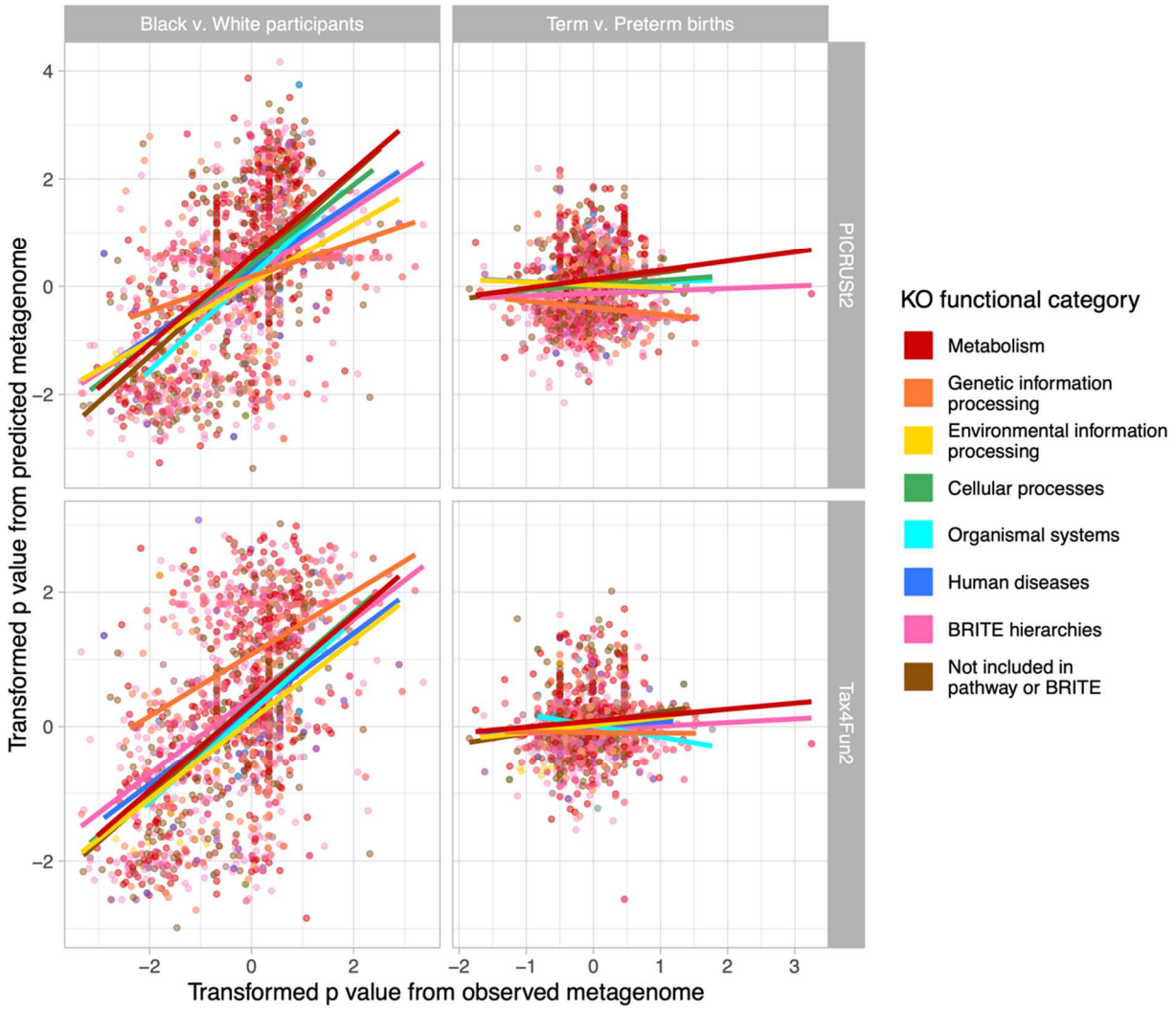

Figure 3. Scatter plots of transformed p values estimated from observed and predicted KO relative abundances for the KO functional category-stratified analysis.

We used Wilcoxon tests to test the following two null hypotheses: KO relative abundances do not differ between self-identified Black and white participants (left column of plots, labeled Black v. White participants), and KO relative abundances do not differ between PTB cases and term birth controls (right column of plots, labeled Term v. Preterm births). We performed Wilcoxon tests using observed and predicted KO relative abundances separately, and we transformed p values according to the following equation in order to capture the significance and direction of KO relative abundance differences:

$$P_t = log_{10}(P) * sign((\overline{KO}|group\ 1) - (\overline{KO}|group\ 2))$$

Where P_t is the transformed p value, P is the Wilcoxon test p value, $\overline{KO}|group\ 1$ is the mean KO relative abundance in the contrast group for the hypothesis test (self-identified Black participants, cases experiencing PTB), and $\overline{KO}|group\ 2$ is the mean KO relative abundance in the reference group for the hypothesis test (self-identified white participants, controls experiencing term birth). Results from PICRUSt2-predicted genomes in the top row of plots (labeled PICRUSt2). Results from Tax4Fun2-predicted genomes in the top row of plots (labeled Tax4Fun2).

KO, KEGG ortholog; KEGG, Kyoto Encyclopedia of Genes and Genomes; PTB, preterm birth.

APPENDIX B: CHAPTER 3 SUPPLEMENTAL FIGURES AND TABLES

Table 1. Outcome definitions.

Outcome	Primary analysis definition	Sub-analysis notes
BV	Detection methods: ≥3 Amsel criteria[a] present or Nugent score[b] ≥7	Sub-analyses according to whether Amsel criteria or Nugent score was used for BV classification. If sufficient results (≥3 results in each category), sub-analyses restricted to incident outcomes and prevalent outcomes.
Chlamydia trachomatis	Sample types: Cervical, cervicovaginal, vaginal, or vulvovaginal swab; or urine sample Detection methods: NAAT or culture	If sufficient results (≥3 results in each category), sub-analyses restricted to incident outcomes and prevalent outcomes.
HPV	Sample types: Cervical cytobrush; cervical, cervicovaginal, vaginal, or vulvovaginal swab; or cervicovaginal lavage Detection methods: NAAT	Sub-analysis considering any HPV as outcome, sub-analysis considering hrHPV[c] as outcome. If sufficient results (≥3 results in each category), sub-analyses restricted to incident outcomes and prevalent outcomes.
Cervical neoplasia	Sample types: Cervical cytobrush or cervical biopsy Detection methods: Cervical cytology or histology Classifications: ASUCS, CIN, ICC, SIL	If sufficient results (≥3 results in each category), sub-analyses restricted to incident outcomes and prevalent outcomes.

HIV	Sample types: Blood or saliva Detection methods: NAAT or immunoassay; laboratory-based testing or home-based/self testing	If sufficient results (≥3 results in each category), sub-analyses restricted to incident outcomes and prevalent outcomes.
Genital HSV-2	Sample types: Lesion sample or blood Detection method: NAAT, serology, or culture	If sufficient results (≥3 results in each category), sub-analyses restricted to incident outcomes and prevalent outcomes.
Trichomonas vaginalis	Sample types: Cervical, cervicovaginal, vaginal, or vulvovaginal swab; or urine sample Detection methods: Wet-mount microscopy, antigen detection test, DNA hybridization, NAAT, or culture	If sufficient results (≥3 results in each category), sub-analyses restricted to incident outcomes and prevalent outcomes.
Neisseria gonorrhoeae	Sample types: Cervical, cervicovaginal, vaginal, or vulvovaginal swab; or urine sample Detection methods: NAAT or culture	If sufficient results (≥3 results in each category), sub-analyses restricted to incident outcomes and prevalent outcomes.

[a]Amsel et al. Am J Med. 1983;74(1):14-22.

[b]Nugent et al. J Clin Microbiol. 1991;29(2):297-301.

[c]hrHPV types as defined in eligible search result publications.

BV, bacterial vaginosis; NAAT, nucleic acid amplification test; HPV, human papillomavirus; hrHPV, high-risk human papillomavirus; ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; ICC, invasive cervical cancer; SIL, squamous intraepithelial lesion; HIV, human immunodeficiency virus; HSV-2, herpes simplex virus type-2.

Table 2. Search terms for each systematic review.

Outcome	Search terms[a]
Bacterial vaginosis	(*Lactobacillus iners* OR *L. iners*) AND (bacterial vaginosis OR BV) AND (female OR woman OR women OR vagina)
Chlamydia trachomatis infection	(*Lactobacillus iners* OR *L. iners*) AND (*Chlamydia trachomatis* OR chlamydia OR *C. trachomatis*) AND (female OR woman OR women OR vagina)
HPV infection	(*L. iners* OR *Lactobacillus iners*) AND (human papillomavirus OR HPV) AND (female OR woman OR women OR vagina)
Cervical neoplasia	(*Lactobacillus iners* OR *L. iners*) AND (cervical cancer OR cervical neoplasia OR cervical precancer OR cervical intraepithelial neoplasia OR squamous intraepithelial lesion OR atypical squamous cell OR ASCUS OR atypical glandular cell) AND (female OR woman OR women OR vagina)
HIV infection	(*Lactobacillus iners* OR *L. iners*) AND (human immunodeficiency virus OR HIV) AND (female OR woman OR women OR vagina)
Genital HSV-2 infection	(*Lactobacillus iners* OR *L. iners*) AND

	(herpes simplex virus type 2 OR herpes simplex virus 2 OR HSV-2 OR HSV 2 OR HSV type 2 OR genital herpes) AND (female OR woman OR women OR vagina)
Trichomonas vaginalis infection	(*Lactobacillus iners* OR *L. iners*) AND (*Trichomonas vaginalis* OR *T. vaginalis*) AND (female OR woman OR women OR vagina)
Neisseria gonorrhoeae infection	(*Lactobacillus iners* OR *L. iners*) AND (*Neisseria gonorrhoeae* OR *N. gonorrhoeae* OR gonorrhoea or gonorrhea) AND (female OR woman OR women OR vagina)

[a]All searches were full-text searches (not restricted to title/abstract or any other portion of the manuscript).

BV, bacterial vaginosis; HPV, human papillomavirus; ASCUS, atypical squamous cells of undetermined significance; HIV, human immunodeficiency virus; HSV-2, herpes simplex virus type-2.

Table 3. Exposure and reference definitions.

Interest level[a]	Exposure definition	Reference definition
Primary	*L. iners*-dominated vaginal microbiota[b]	*L. crispatus*-dominated vaginal microbiota[b]
Secondary	*L. iners* relative abundance	*L. crispatus* relative abundance

[a]For eligible search results reporting (data to calculate) relevant effect estimates based on primary and secondary exposure and reference definitions, we included the effect estimate based on the primary definition. For eligible search results reporting (data to calculate) relevant effect estimates based on secondary exposure and reference definitions only, we included the effect estimate based on the secondary definition.

[b]*L. iners*-dominated and *L. crispatus*-dominated as defined in eligible manuscripts from hierarchical clustering or assignment based on species relative abundances.

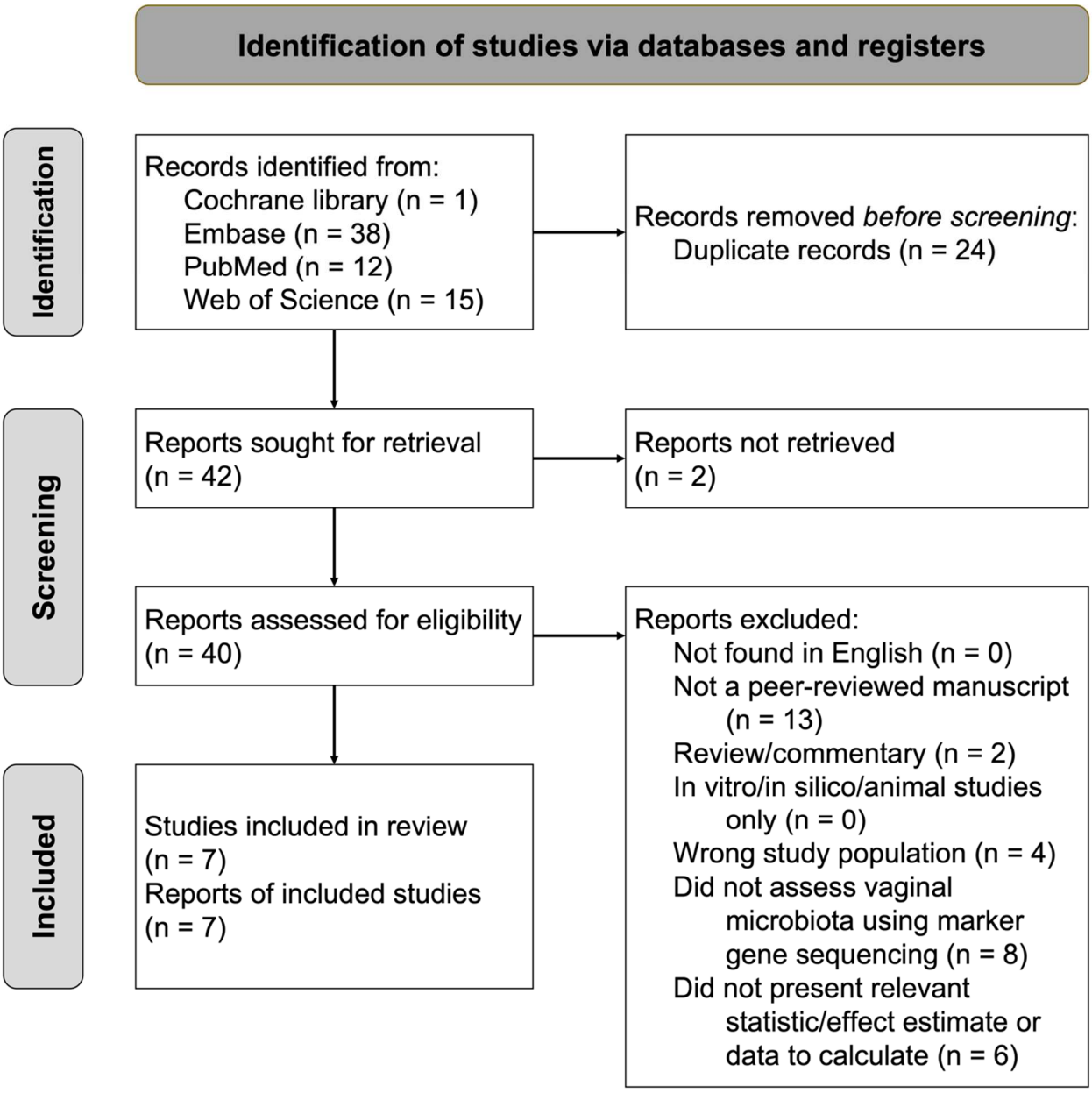

Figure 1. *Chlamydia trachomatis* systematic review PRISMA diagram.

Diagram adapted from Page et al. BMJ Brit Med J. 2021;372:n71.

PRISMA; Preferred reporting items for systematic reviews and meta-analyses.

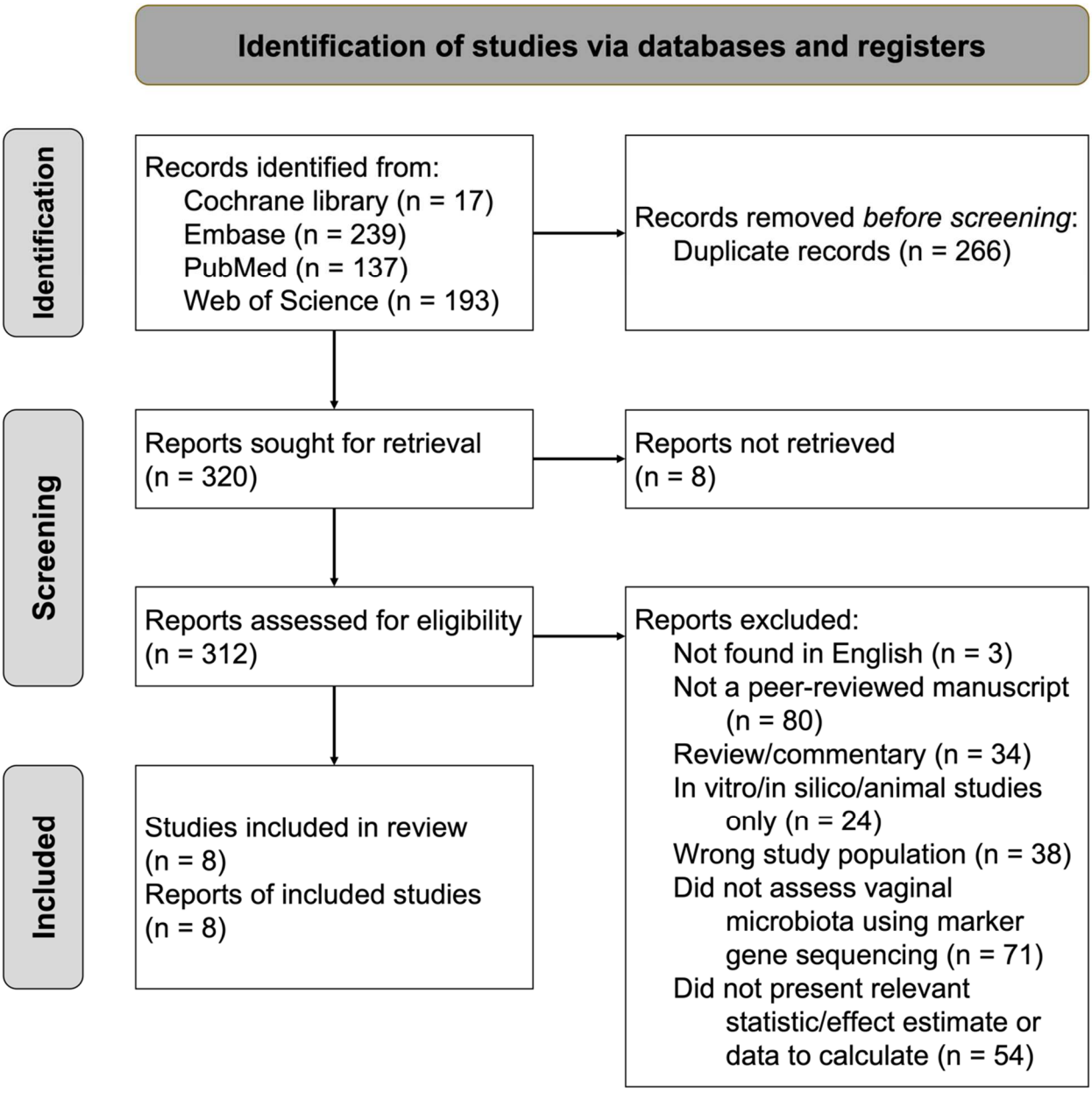

Figure 2. BV systematic review PRISMA diagram.

Diagram adapted from Page et al. BMJ Brit Med J. 2021;372:n71.

BV, bacterial vaginosis; PRISMA; Preferred reporting items for systematic reviews and meta-analyses.

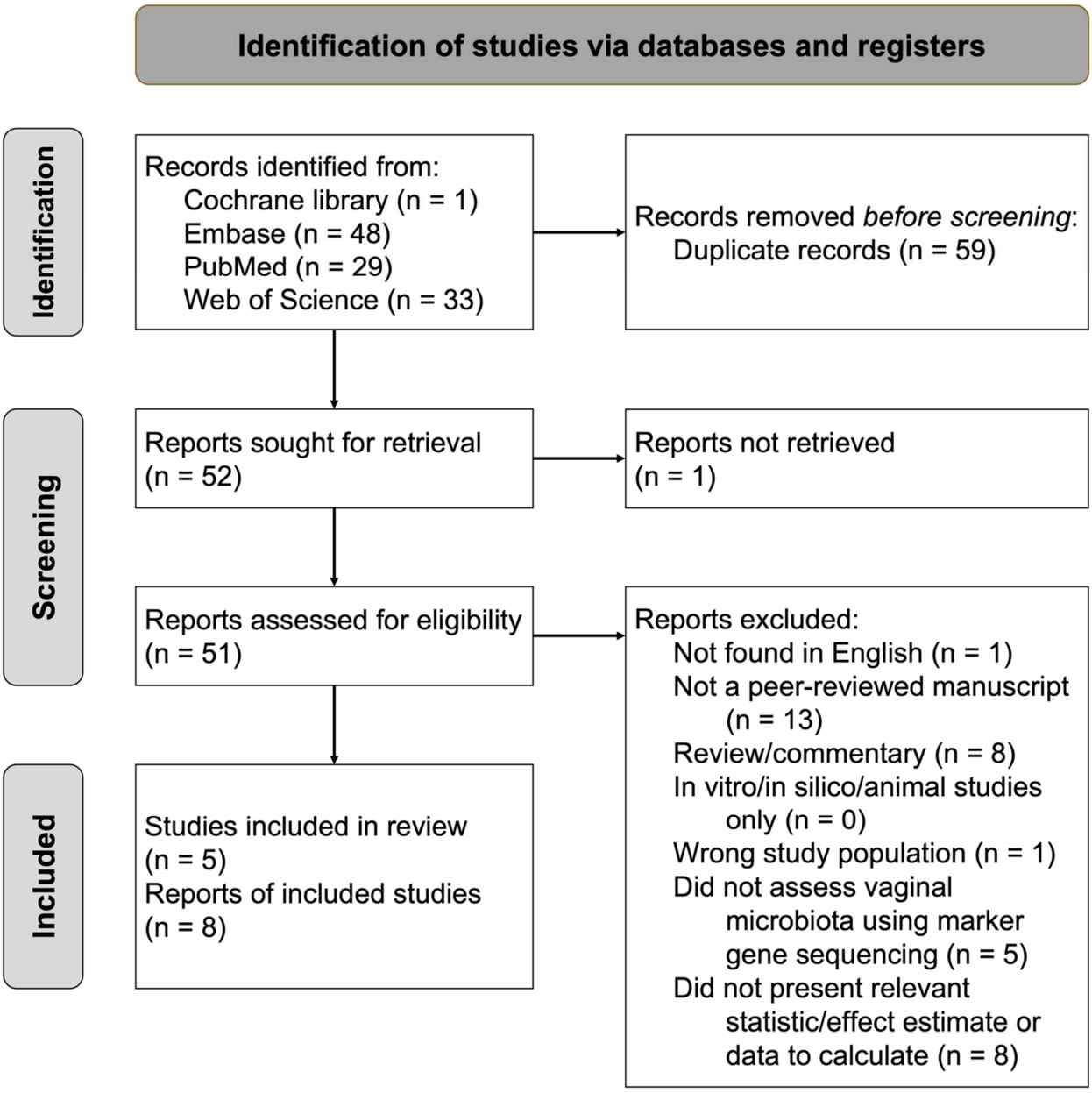

Figure 3. HPV systematic review PRISMA diagram.

Diagram adapted from Page et al. BMJ Brit Med J. 2021;372:n71.

HPV, human papillomavirus; PRISMA; Preferred reporting items for systematic reviews and meta-analyses.

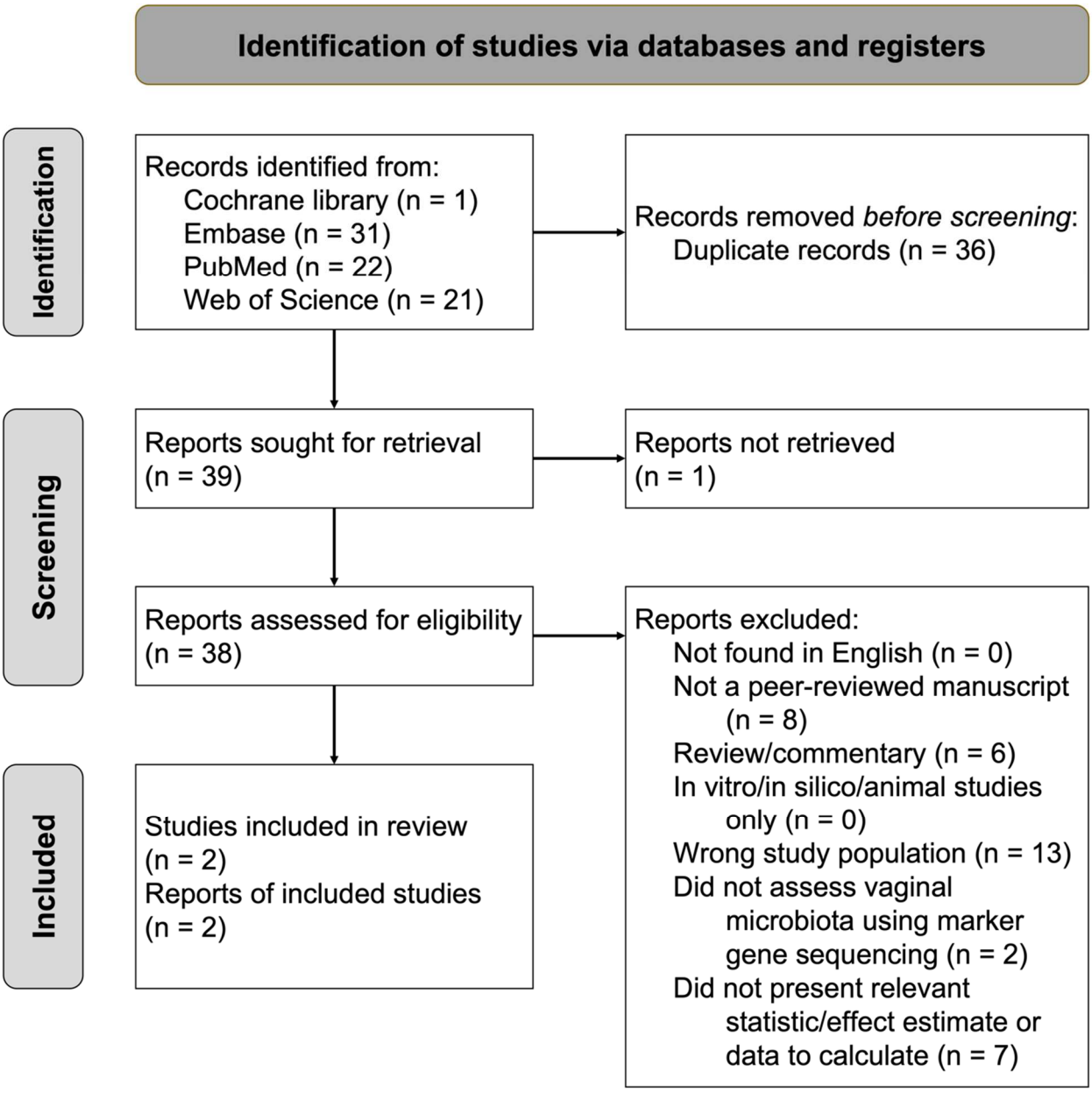

Figure 4. Cervical dysplasia systematic review PRISMA diagram.

Diagram adapted from Page et al. BMJ Brit Med J. 2021;372:n71.

PRISMA; Preferred reporting items for systematic reviews and meta-analyses.

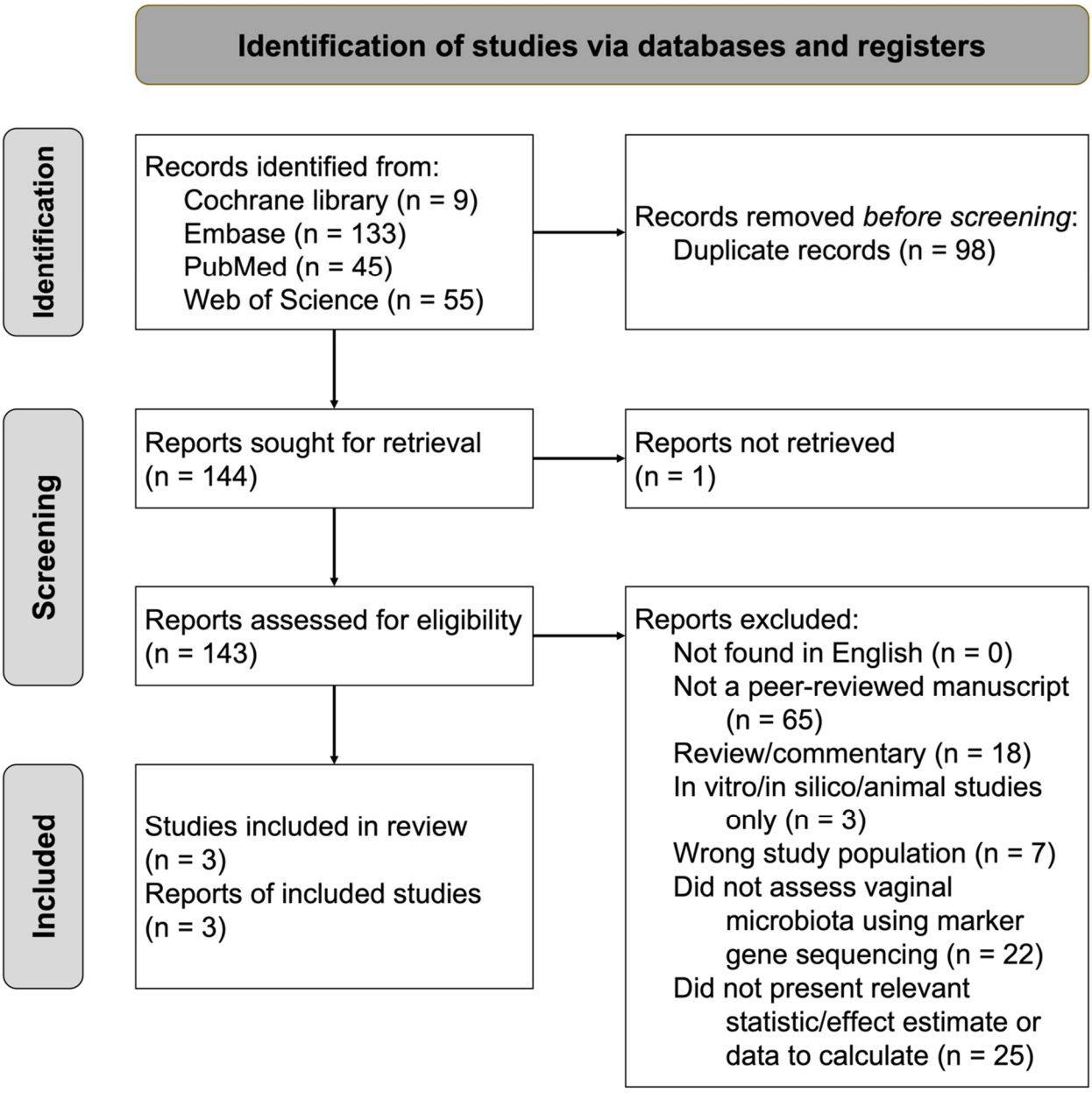

Figure 5. HIV systematic review PRISMA diagram.

Diagram adapted from Page et al. BMJ Brit Med J. 2021;372:n71.

HIV, human immunodeficiency virus; PRISMA; Preferred reporting items for systematic reviews and meta-analyses.

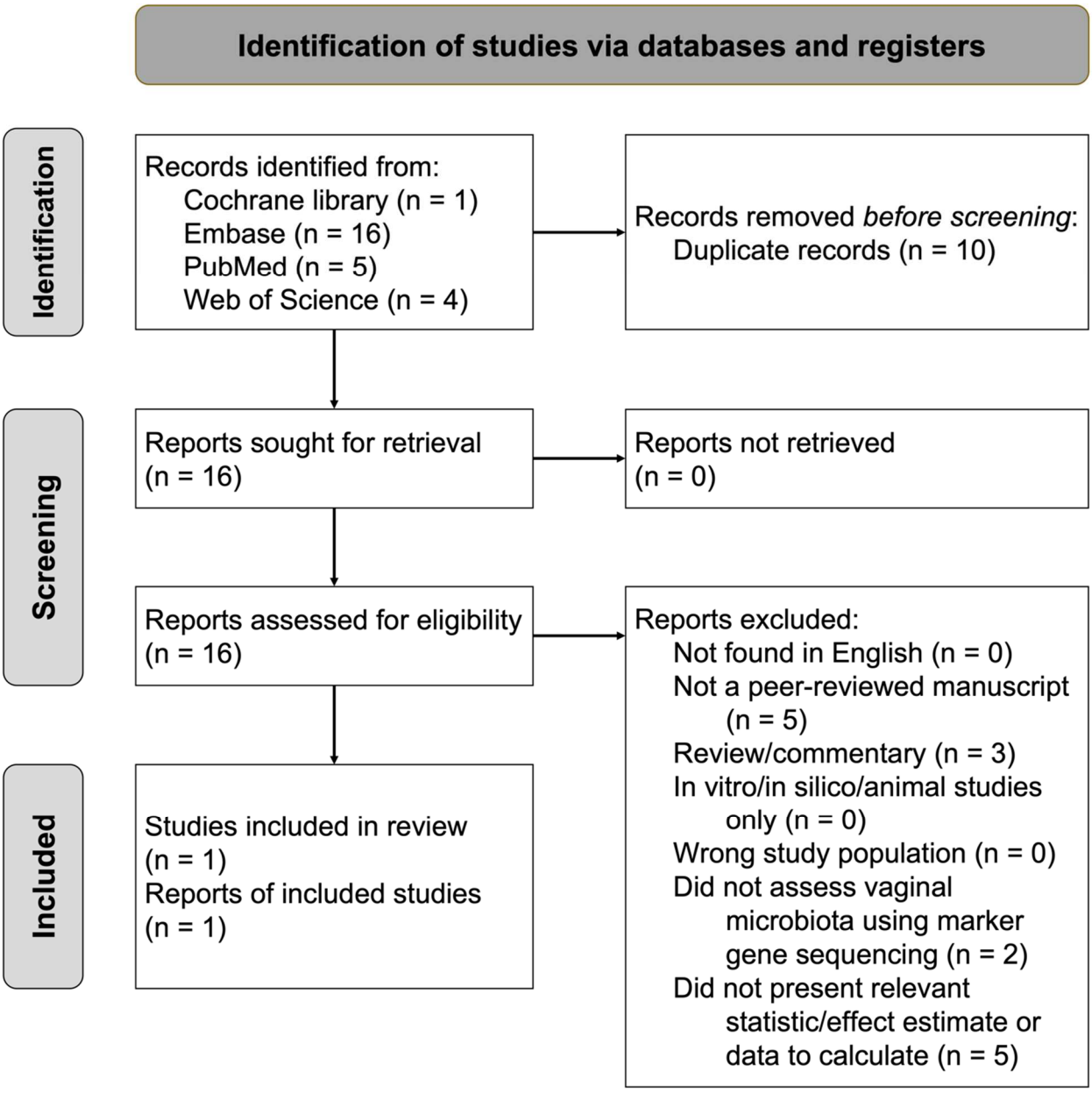

Figure 6. Genital HSV-2 systematic review PRISMA diagram.

Diagram adapted from Page et al. BMJ Brit Med J. 2021;372:n71.

HSV-2, herpes simplex virus type-2; PRISMA; Preferred reporting items for systematic reviews and meta-analyses.

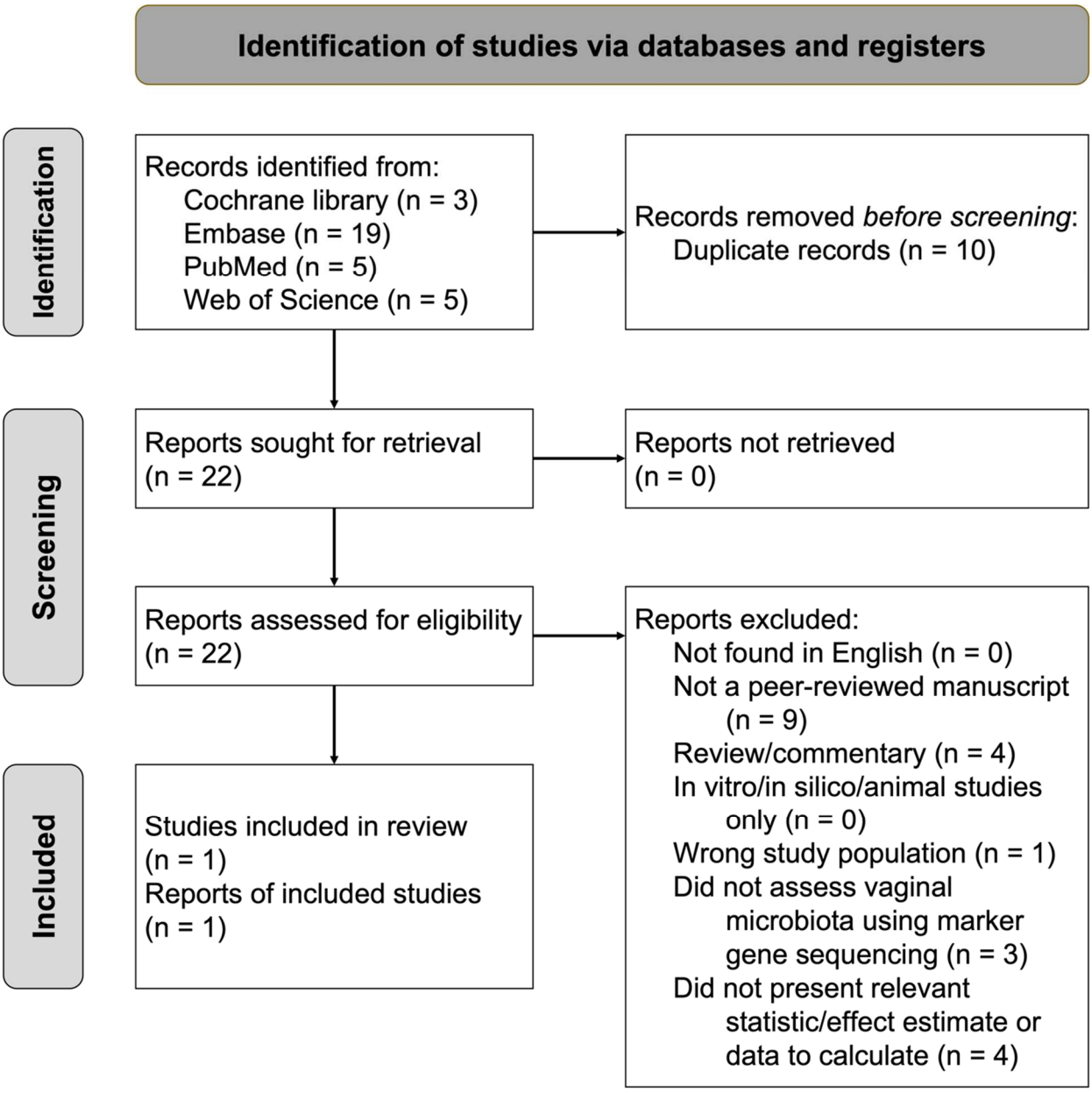

Figure 7. *Trichomonas vaginalis* systematic review PRISMA diagram.

Diagram adapted from Page et al. BMJ Brit Med J. 2021;372:n71.

PRISMA; Preferred reporting items for systematic reviews and meta-analyses.

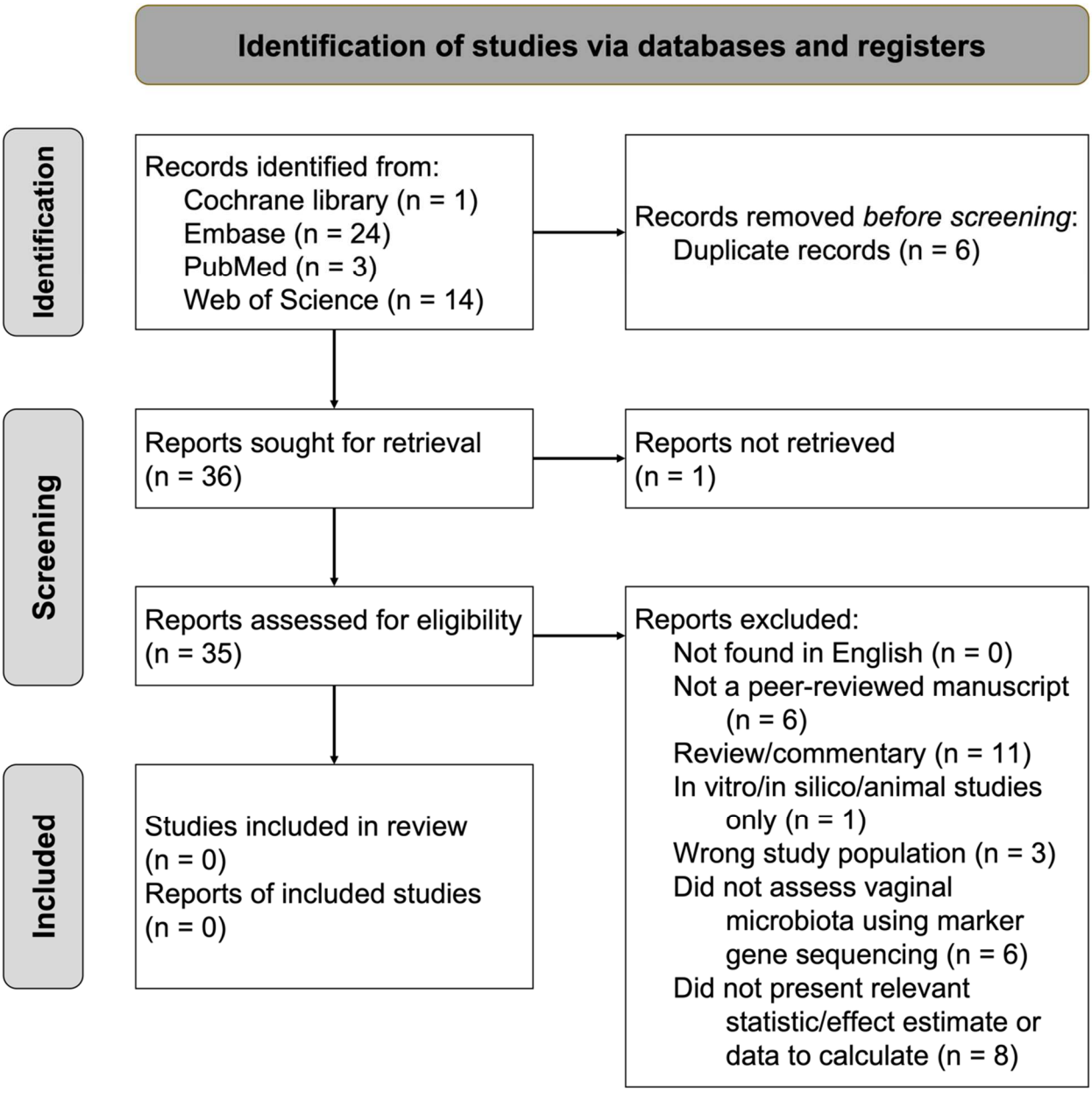

Figure 8. *Neisseria gonorrhoeae* systematic review PRISMA diagram.

Diagram adapted from Page et al. BMJ Brit Med J. 2021;372:n71.

PRISMA; Preferred reporting items for systematic reviews and meta-analyses.