

# Metabolic Signatures of Bacterial Vaginosis

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**ABSTRACT** Bacterial vaginosis (BV) is characterized by shifts in the vaginal microbiota from *Lactobacillus* dominant to a microbiota with diverse anaerobic bacteria. Few studies have linked specific metabolites with bacteria found in the human vagina. Here, we report dramatic differences in metabolite compositions and concentrations associated with BV using a global metabolomics approach. We further validated important metabolites using samples from a second cohort of women and a different platform to measure metabolites. In the primary study, we compared metabolite profiles in cervicovaginal lavage fluid from 40 women with BV and 20 women without BV. Vaginal bacterial representation was determined using broad-range PCR with pyrosequencing and concentrations of bacteria by quantitative PCR. We detected 279 named biochemicals; levels of 62% of metabolites were significantly different in women with BV. Unsupervised clustering of metabolites separated women with and without BV. Women with BV have metabolite profiles marked by lower concentrations of amino acids and dipeptides, concomitant with higher levels of amino acid catabolites and polyamines. Higher levels of the signaling eicosanoid 12-hydroxyeicosatetraenoic acid (12-HETE), a biomarker for inflammation, were noted in BV. *Lactobacillus crispatus* and *Lactobacillus jensenii* exhibited similar metabolite correlation patterns, which were distinct from correlation patterns exhibited by BV-associated bacteria. Several metabolites were significantly associated with clinical signs and symptoms (Amsel criteria) used to diagnose BV, and no metabolite was associated with all four clinical criteria. BV has strong metabolic signatures across multiple metabolic pathways, and these signatures are associated with the presence and concentrations of particular bacteria.

**IMPORTANCE** Bacterial vaginosis (BV) is a common but highly enigmatic condition that is associated with adverse outcomes for women and their neonates. Small molecule metabolites in the vagina may influence host physiology, affect microbial community composition, and impact risk of adverse health outcomes, but few studies have comprehensively studied the metabolomics profile of BV. Here, we used mass spectrometry to link specific metabolites with particular bacteria detected in the human vagina by PCR. BV was associated with strong metabolic signatures across multiple pathways affecting amino acid, carbohydrate, and lipid metabolism, highlighting the profound metabolic changes in BV. These signatures were associated with the presence and concentrations of particular vaginal bacteria, including some bacteria yet to be cultivated, thereby providing clues as to the microbial origin of many metabolites. Insights from this study provide opportunities for developing new diagnostic markers of BV and novel approaches for treatment or prevention of BV.

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The composition of small molecule metabolites in the human vagina is largely shaped by bacterial metabolism of human-derived nutrients. These bacterial metabolites may impact human cell function, inflammation, and disease susceptibility. However, few studies have comprehensively examined the associations between vaginal bacteria and metabolites, and no study has used quantitative PCR (qPCR) to link concentrations of fastidious vaginal bacteria to metabolite concentrations. Individual metabolites associated with bacterial vaginosis (BV) status have been identified and shown to be useful markers of BV, such as lactate (no BV) and succinate (BV) (1–7). However, there are hundreds of other compounds that have not

been identified in vaginal fluid and linked to particular bacterial communities or clinical outcomes.

The composition of the human vaginal microbiota ranges from communities dominated by a limited number of *Lactobacillus* species to complex communities of diverse anaerobes, most evident with the common condition BV (8–11). BV affects up to 29% of women in the United States (12) and has been associated with several adverse reproductive and health outcomes, including elevated risks for acquisition of sexually transmitted infections (13–15), transmission of HIV to male sex partners (16), preterm birth (17), pelvic inflammatory disease (18), and cervicitis (19). The microbiota in BV is heterogeneous; different groups of

women have vaginal microbiotas dominated by different bacteria, such as BV-associated bacterium 1 (BVAB1), *Leptotrichial Sneathia* species, *Prevotella* spp., *Gardnerella vaginalis*, or *Lactobacillus iners*, while others are colonized with diverse bacteria and no single dominant bacterium (11, 20). BV is diagnosed in clinical settings using Amsel criteria, in which a set of four signs or symptoms are evaluated, with at least three required for a diagnosis of BV (21). We have previously shown that different bacteria are correlated with each of these four clinical criteria (11). Notably, small molecule metabolites may affect at least three of these clinical criteria, including vaginal discharge, pH, and amine odor. The diagnosis of BV can also be made using Gram stain interpretation of vaginal fluid smears with enumeration of bacterial morphotypes (22).

Polyamines, such as putrescine and cadaverine, are found in women with BV and contribute to the fishy amine odor under this condition (1, 23–25). These polyamines are likely produced from decarboxylation of amino acids mediated by bacteria (23). Chen et al. demonstrated that *in vitro* production of polyamines by mixed anaerobic vaginal bacteria was inhibited in the presence of metronidazole, suggesting a role for bacterial production of polyamines (26). Likewise, trimethylamine has a fishy odor, is found in women with BV, and is also thought to be a product of bacterial metabolism (27). These amines become volatile when pH is elevated, a property utilized in the clinical diagnosis of BV when employing the “whiff” test (4, 28, 29). The amine odor can also be noted *in vivo* with elevated pH during menses and after vaginal intercourse with men, as the pHs of blood and semen are close to 7, leading to enhanced volatilization. It has been suggested that the amines are associated with increased vaginal transudation of fluid and squamous cell exfoliation, resulting in the thin homogeneous grayish-white discharge that is typically associated with BV (4).

Mass spectrometry (MS) (when coupled with chromatography) offers the opportunity to measure small molecule metabolites in the vagina and to assign identity to hundreds of compounds simultaneously. One recent study described the metabolite profiles in eight women with BV using this approach and classified these women into two groups based on their vaginal metabolite compositions (30). Additional insights into the functions of these bacterial consortia can be gained by linking bacterial community composition and concentrations of bacteria to metabolic signatures. This information may be useful in the diagnosis of BV, establishing metabolic profiles associated with increased disease risk and developing new approaches to treat BV based on interrupting metabolic networks. In this study, we compared global metabolomic profiles in cervicovaginal lavage (CVL) fluid obtained from 40 women with BV to profiles in 20 women without BV. For a targeted set of select metabolites, we validated relative quantities using a different metabolomics platform in a second, independent cohort of women to ensure reproducibility. We further investigated correlations between these metabolites and concentrations of key individual bacterial species (using quantitative PCR [qPCR]) in order to understand the role of particular bacteria in shaping metabolic profiles.

## RESULTS

**Primary study (cohort A).** Characteristics of the 60 women from cohort A are presented in Table 1. There was a low prevalence of other infections, including *Candida* (8%), *Trichomonas vaginalis*

(3%), *Chlamydia trachomatis* (3%), and *Neisseria gonorrhoeae* (0%). Cases included women with BV by both Amsel criteria and Nugent score; controls did not have BV by both criteria. An elevated pH of >4.5 was the only Amsel criterion noted in all cases ( $n = 40$ ); vaginal fluid from 20% of control women had a pH of >4.5 ( $n = 4$ ). The presence of >20% clue cells and a positive whiff test (amine odor) were both observed in 83% of cases ( $n = 33$ ); clue cells and a positive whiff test were not detected in the control group. A thin homogeneous discharge that is present in some women with symptomatic BV was noted in 85% of cases ( $n = 34$ ) and 5% ( $n = 1$ ) of women in the control group.

**Metabolite summary.** We detected 279 known metabolites in vaginal fluid from women with and without BV. There were significant differences in levels of 173 metabolites (62% of the 279 detected) on comparison of women with and without BV ( $q$  value = 0.02). Women with BV had higher levels of 55 biochemicals (20%) and lower levels of 118 biochemicals (42%). A summary of metabolites altered in BV based on pathway is provided in Table 2.

Hierarchical clustering depicting this set of the most variable metabolites measured in CVL samples from all women resulted in the creation of four groups, of which 2 clusters were populated by women with BV. Cluster I had 12 women without BV, and cluster II comprised 15 women with BV. Cluster III included 7 women without BV. Cluster IV included 25 women with BV and 1 woman without BV (subject 58) (Fig. 1). Vaginal fluid from subject 58, the outlier in cluster IV, had an elevated pH of 5.6 and a Nugent score of 6, defined as intermediate on this scale.

Below, we summarize alterations in metabolism in BV for each of the areas outlined.

**Amino acids.** We detected 19 of 20 important amino acids (95%) that are incorporated into proteins using the Metabolon platform (see Table S1 in the supplemental material). Levels of 18 of 19 amino acids detected (94.7%) were lower among cases; this was statistically significant for 16 of 18 amino acids (84.9%). We also noted higher concentrations of amino acid catabolites in women with BV (see Table S1). Examples of such catabolites include cadaverine ( $P < 0.001$ ) and pipercolate ( $P < 0.001$ ) in the lysine degradation pathway, tyramine ( $P < 0.001$ ), 4-hydroxyphenylacetate ( $P < 0.001$ ), and 3-(4-hydroxyphenyl)propionate ( $P < 0.001$ ) in the tyrosine pathway, tryptamine ( $P < 0.001$ ) in the tryptophan pathway, and citrulline ( $P < 0.001$ ) in the arginine pathway. There was marked elevation of the polyamine putrescine ( $P < 0.001$ ), along with lower levels of putrescine precursors arginine ( $P < 0.001$ ) and ornithine ( $P < 0.001$ ), in women with BV. The polyamine spermine ( $P < 0.001$ ), a typical product of putrescine degradation, was lower in BV. Enhanced protein/amino acid catabolism in BV was further supported by detection of lower levels of dipeptides ( $n = 28$ ); this was statistically significant for 24 dipeptides (85.7%). Levels of oxidized ( $P < 0.001$ ) and reduced ( $P = 0.049$ ) glutathione were lower in BV.

**Carbohydrates.** Of the four amino sugars detected, *N*-acetylneuraminic acid, commonly known as sialic acid, was higher in BV ( $P < 0.001$ ), while glucosamine levels were lower ( $P = 0.0059$ ). Glucose oligosaccharides of various lengths, including maltotriose ( $P = 0.0095$ ), maltotetraose ( $P = 0.0016$ ), maltopentaose ( $P < 0.001$ ), and maltohexaose ( $P < 0.001$ ) were all lower in BV. Likewise, lower levels of simple sugars and sugar alcohols, such as lactate ( $P < 0.001$ ), fructose ( $P = 0.0012$ ), and mannitol ( $P = 0.001$ ), were noted; galactose ( $P < 0.001$ ) and threitol ( $P < 0.001$ ) were significantly higher in BV. Succinate levels were also higher in BV ( $P < 0.001$ ).

TABLE 1 Characteristics of study participants according to case or control status<sup>a</sup>

Characteristic	Cohort A			Cohort B		
	Total (n = 60)	Cases (n = 40)	Controls (n = 20)	Total (n = 60)	Cases (n = 40)	Controls (n = 20)
Age, yr, median (range)	26 (17–56)	26 (19–42)	26 (17–56)	29 (19–49)	29 (19–45)	30 (21–49)
Race (self-defined), no. (%) <sup>b</sup>						
Black or African American	13 (22)	10 (25)	3 (15)	24 (40)	22 (55)	2 (10)
White	41 (68)	27 (68)	14 (70)	28 (47)	14 (35)	14 (70)
Other	4 (7)	2 (5)	2 (10)	7 (12)	4 (10)	3 (15)
Do not know/declined to provide data	2 (3)	1 (3)	1 (5)	1 (1)	0 (0)	1 (5)
Menses at visit, no. (%)	4 (7)	3 (8)	1 (5)	5 (8)	4 (10)	1 (5)
Vaginal douching, past week, no. (%) <sup>c</sup>	4 (7)	3 (8)	1 (5)	3 (5)	2 (5)	1 (5)
Antibiotic use in past mo, no., (%) <sup>d</sup>	5 (8)	5 (13)	0 (0)	5 (8)	3 (7.5)	2 (10)
Hormonal contraceptive in past mo, no. (%) <sup>e</sup>	7 (60)	3 (8)	4 (20)	18 (30)	9 (23)	9 (45)
Sexual activity, no. (%)						
Sex in past 3 mo <sup>f</sup>	55 (92)	37 (93)	18 (90)	49 (82)	32 (80)	17 (85)
Sex with female partners <sup>g</sup>	13 (22)	9 (23)	4 (20)	9 (15)	7 (18)	2 (10)
Sex with male partner <sup>h</sup>	50 (83)	33 (83)	17 (85)	49 (82)	34 (85)	15 (75)
Amsel criteria, no. (%)						
Vaginal discharge <sup>i</sup>						
Normal	18 (30)	5 (13)	13 (65)	18 (30)	0 (0)	18 (90)
Abnormal	35 (58)	34 (85)	1 (5)	35 (58)	34 (85)	1 (5)
Other	7 (11)	1 (3)	6 (30)	7 (12)	6 (15)	1 (5)
pH						
4.5 and less	16 (27)	0 (0)	16 (80)	16 (27)	1 (2)	15 (75)
>4.5	44 (73)	40 (100)	4 (20)	44 (73)	39 (98)	5 (25)
Clue cells						
Absent	21 (35)	2 (5)	19 (95)	18 (30)	0 (0)	18 (90)
<20% (few)	5 (8)	4 (10)	1 (5)	4 (7)	2 (5)	2 (10)
>20% (many)	33 (55)	33 (83)	0 (0)	38 (63)	38 (95)	0 (0)
Whiff test						
Negative	27 (45)	7 (18)	20 (100)	28 (47)	8 (20)	20 (100)
Positive	33 (55)	33 (83)	0 (0)	32 (53)	32 (80)	0 (0)
Other infections, no. (%)						
<i>Trichomonas vaginalis</i>	2 (3)	2 (5)	0 (0)	1 (2)	1 (2)	0 (0)
<i>Chlamydia trachomatis</i> <sup>j</sup>	2 (3)	1 (3)	1 (5)	0 (0)	0 (0)	0 (0)
<i>Neisseria gonorrhoeae</i> <sup>k</sup>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>Candida</i>	5 (8)	1 (3)	4 (20)	4 (7)	3 (8)	1 (5)

<sup>a</sup> Except where noted, the data represent the number (percentage) of participants. Cases included women with BV by both Amsel criteria (21) and by interpretation of Gram stain using the Nugent score (22). Controls included women who did not have BV by both criteria.

<sup>b</sup> Study participants were able to select more than one category for race. “Black/African American” and “White” indicate women who selected only these categories.

<sup>c</sup> Douching data were not available for 1 study participant who did not provide data.

<sup>d</sup> Antibiotic use data were not available for 4 study participants.

<sup>e</sup> Hormonal contraceptive data were not available for 4 study participants who did not provide data.

<sup>f</sup> Sex data were not available for 2 study participants who did not provide data.

<sup>g</sup> Female sex partner data were not available for 9 study participants who did not provide data.

<sup>h</sup> Male sex partner data were not available for 2 study participants who did not provide data.

<sup>i</sup> Abnormal vaginal discharge indicates women with a thin homogeneous discharge that is consistent with BV. Women with vaginal discharge that was not normal or who did not have a thin homogeneous discharge were placed in the “Other” category.

<sup>j</sup> *Chlamydia trachomatis* and *Neisseria gonorrhoeae* data were not available for 5 study participants.

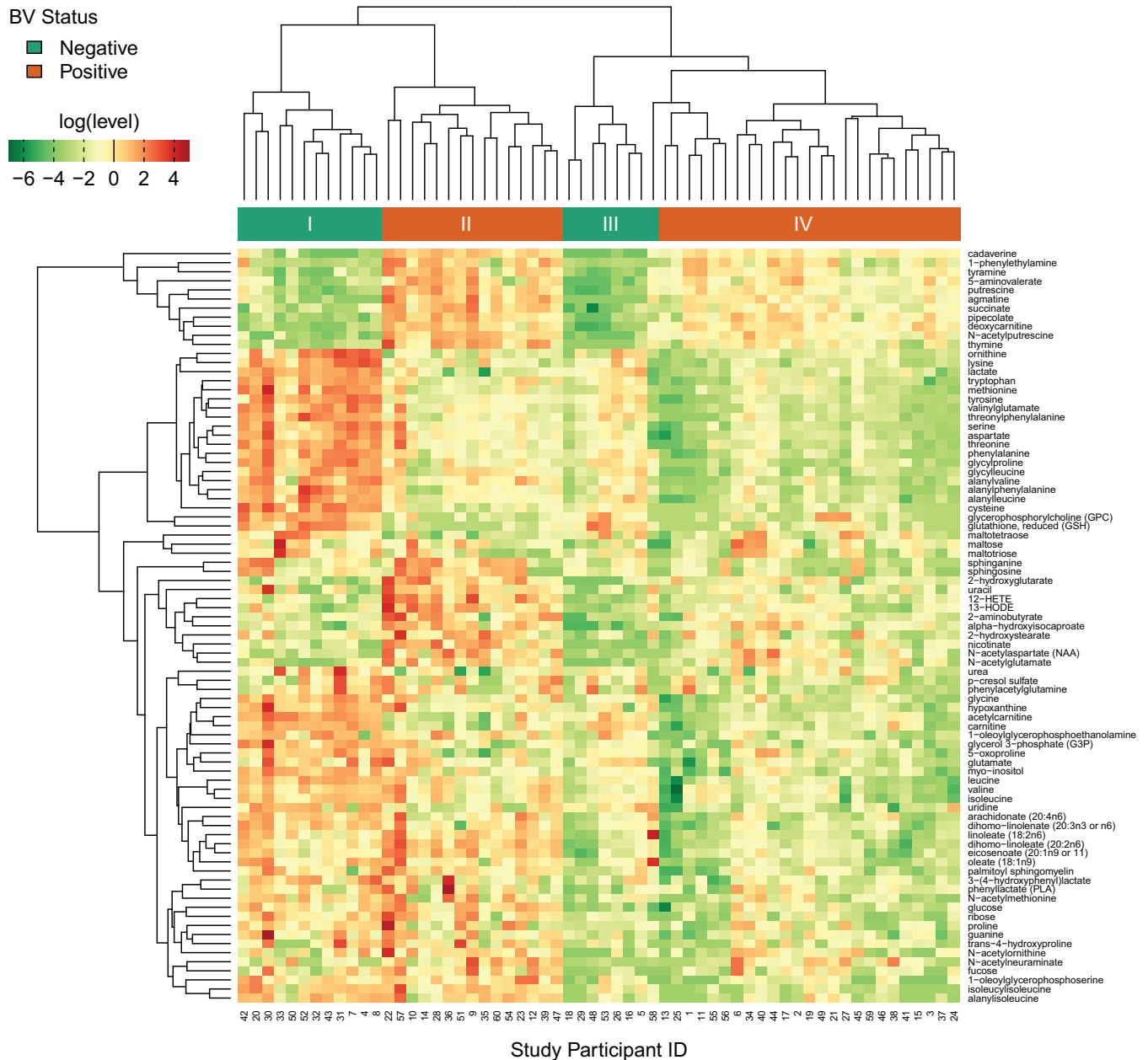
TABLE 2 Summary of biochemicals altered based on super pathways

Super pathway <sup>a</sup>	Total no. altered	No. (%) of biochemicals:	
		Higher in BV	Lower in BV
Amino acid	90	32 (35.6)	35 (38.9)
Peptide	28	0 (0.0)	24 (85.7)
Carbohydrate	27	3 (11.1)	15 (55.6)
Energy	6	2 (33.3)	0 (0.0)
Lipid	74	12 (16.2)	27 (26.5)
Nucleotide	17	3 (17.6)	8 (47.1)
Cofactors and vitamins	13	3 (23.1)	6 (46.2)
Xenobiotics	24	0 (0.0)	3 (12.5)

<sup>a</sup> Biochemicals categorized according to KEGG pathways.

**NAD.** NAD, an essential cofactor for energy metabolism, was below the limit of detection in 82.5% of BV cases and in only 10% of controls ( $P < 0.001$ ). Levels of nicotinamide, a precursor to NAD biosynthesis, were also lower in BV ( $P = 0.06$ ), while nicotinate ( $P = 0.002$ ) levels were higher.

**Lipids.** Multiple components of lipid metabolism were affected in BV. The arachidonic acid catabolite 12-hydroxyicosatetraenoic acid (12-HETE) was higher in BV ( $P < 0.001$ ), while arachidonate was lower ( $P = 0.0075$ ). Deoxycarnitine, a precursor to carnitine, was higher in BV ( $P < 0.001$ ), while carnitine was lower ( $P < 0.001$ ). Ascorbic acid, which is essential to the synthesis of carnitine, was lower in BV ( $P < 0.001$ ). Acyl-carnitines such as acetylcarnitine ( $P < 0.001$ ), propionylcarnitine ( $P < 0.001$ ), and butyrylcarnitine ( $P <$



**FIG 1** Hierarchical clustering of metabolites. A dendrogram shows associations of 30% of most variable metabolites with BV status, determined using the Amsel and Nugent criteria. Clustering of the metabolites resulted in four groups (clusters I to IV). The clustering algorithm was not informed by BV status. Study participant identification numbers (IDs) are provided on the x axis, and metabolites are listed on the y axis. The heat map depicts log-transformed concentrations of the most variable metabolites between the clusters. Values ranged from  $-6.956$  (dark green) to  $4.818$  (dark red).

0.001), were also lower in BV. Of the four monohydroxy fatty acids detected, levels of 4-hydroxybutyrate ( $P < 0.001$ ) and 13-hydroxyoctadecadienoic acid (13-HODE) ( $P = 0.0042$ ) were higher in BV. Levels of biochemicals involved in glycerol metabolism, including glycerol ( $P = 0.046$ ) and glycerol-3-phosphate ( $P < 0.001$ ), were lower in BV.

#### Association between metabolites and bacterial abundance.

Pearson correlation coefficients were used to investigate associations between bacterial abundance and metabolite abundance in vaginal samples. Specifically, we analyzed the abundance of 30% of the most variable metabolites and the relative abundance of

bacteria detected by broad-range PCR with pyrosequencing in a subset of 20 women with BV and 10 women without BV. A group of 10 BV-associated bacteria were highly correlated with metabolites typically associated with BV, including succinate, cadaverine, putrescine, tyramine, and deoxycarnitine (Fig. 2). *Megasphaera* sp. type 1 had the highest correlation coefficient with the fatty acid 12-HETE (0.48). A second cluster of BV-associated bacteria, including BVAB1, BVAB3, *Megasphaera* sp. type 2, and several *Prevotella* species, such as *P. amnii*, *P. disiens*, *P. buccalis*, and *P. bivia*, showed similar positive associations with the above-mentioned metabolites, but the correlations were less pronounced. The three

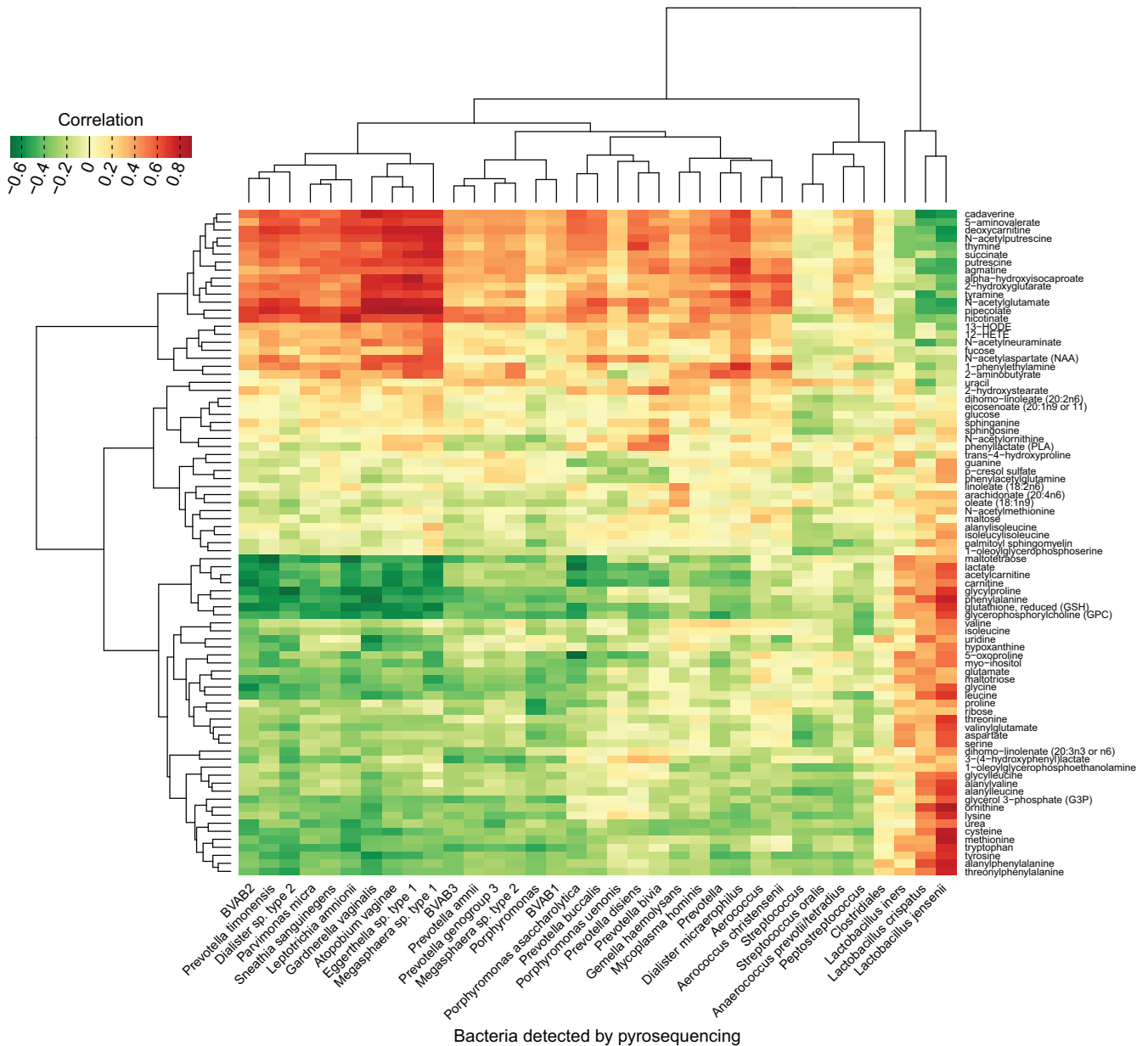
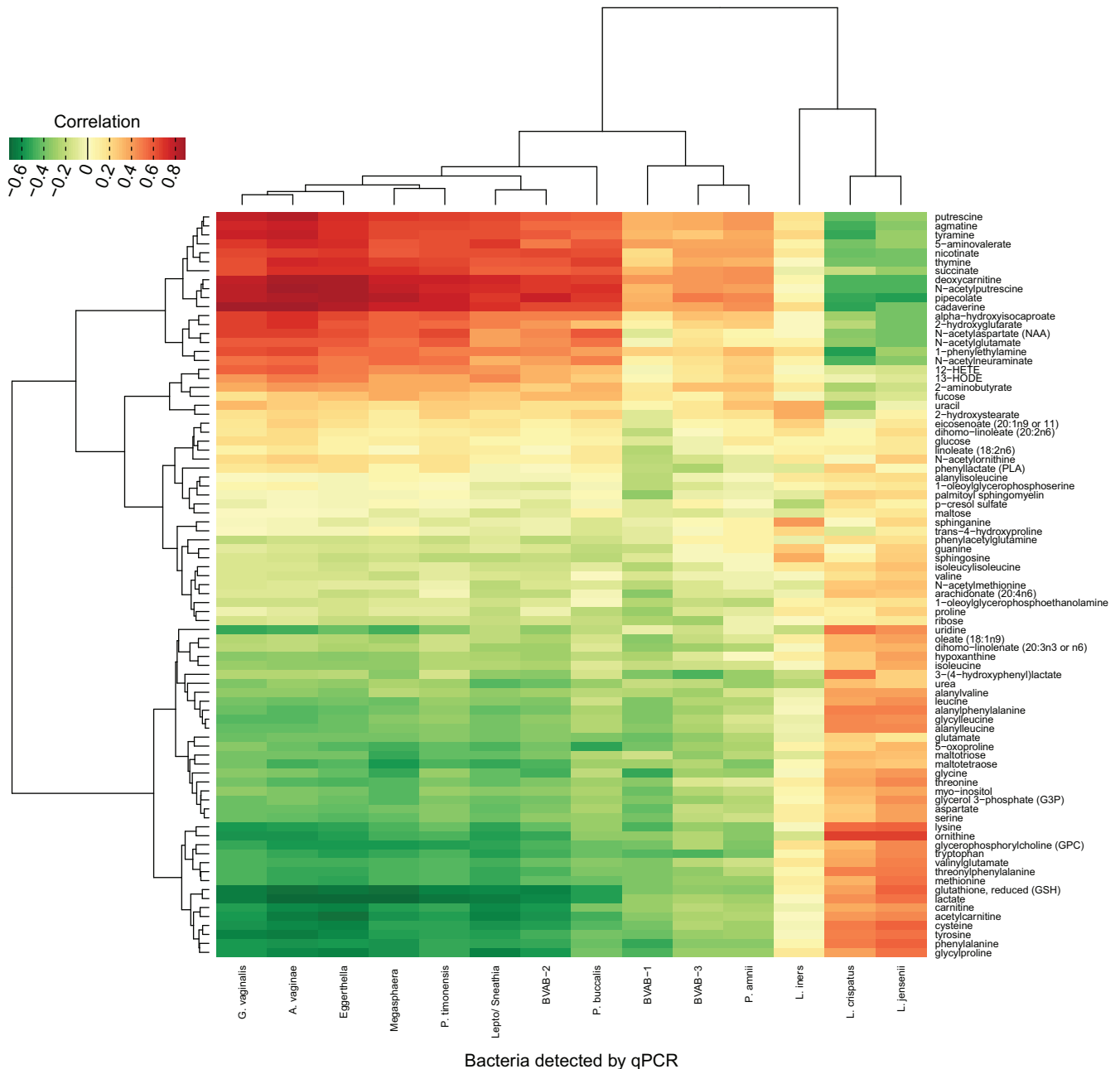


FIG 2 Association between metabolites and bacterial abundance. Hierarchically clustered Pearson correlation coefficients are displayed in a heat map to demonstrate associations of vaginal bacteria (relative abundance) detected using broad-range PCR and pyrosequencing (x axis) with the abundance of 30% most variable metabolites (y axis). Correlation values ranged from  $-0.7$  (dark green) to  $0.84$  (dark red).

lactobacilli *L. crispatus*, *L. jensenii*, and *L. iners* clustered together and exhibited metabolite correlation patterns that overall were in contrast to the patterns found with BV-associated bacteria.

**Association between metabolites and bacterial concentrations.** Bacteria were selected for qPCR based on relative abundance measured by broad-range PCR and pyrosequencing in a subset of samples (Fig. 2) and on associations made in previous studies that have demonstrated the sensitivity, specificity (31), and significance of these bacteria in BV (11, 20, 32). Associations of concentrations of key vaginal bacteria with the 30% most variable metabolites were investigated using Pearson correlation coefficients (Fig. 3). *L. crispatus*, *L. jensenii*, and *L. iners* clustered together, while the BV-associated bacteria clustered as two groups.

Group 1 comprised *Leptotrichia/Sneathia* spp., BVAB2, *Megasphaera* spp., *Prevotella timonensis*, *Atopobium vaginae*, *Gardnerella vaginalis*, *Eggerthella*-like bacterium, and *Prevotella buccalis*. Group 2 comprised BVAB1, BVAB3, and *Prevotella amnii*. The clustering patterns noted here also reflected patterns observed in our association analysis of metabolites with bacteria detected by broad-range PCR and pyrosequencing. *L. crispatus* and *L. jensenii*, lactobacilli typically associated with vaginal health, exhibited metabolite correlation patterns that were similar; these were in striking contrast to those exhibited by BV-associated bacteria (Fig. 3). *L. iners* exhibited correlation patterns that were intermediate between those of BV-associated bacteria and those of *L. crispatus* and *L. jensenii*. Concentrations of *L. crispatus* and *L. jensenii* had strong



**FIG 3** Association of specific vaginal bacteria with metabolites. Hierarchically clustered Pearson correlation coefficients are displayed in a heat map to demonstrate associations of key vaginal bacterial concentrations (*x* axis) measured using taxon-directed qPCR with the 30% most variable metabolites (*y* axis). Correlation values range from  $-0.71$  (dark green) to  $0.85$  (dark red). Two subgroups of BV-associated bacteria were observed, and clustering patterns were similar to those noted in Fig. 2 using an untargeted approach for bacterial community analysis. *L. crispatus* and *L. jensenii* exhibited correlation patterns that were similar and opposite to those by BV-associated bacteria.

positive correlations with several amino acids and dipeptides but were negatively correlated with amino acid catabolites (tyramine, piperolate, and cadaverine) and polyamines (putrescine and agmatine). *L. iners* was negatively correlated with some amino acids and dipeptides (glutamate and glycylleucine) but positively correlated with others (proline, threonine, aspartate, serine, and valinylglutamate). *L. crispatus* and *L. jensenii* were positively correlated with sugars, such as maltose, maltotriose, and maltohexose, lipid metabolism biochemicals, such as arachidonate and carnitine,

as well as lactate, urea, and reduced glutathione. These two lactobacilli were negatively correlated with *N*-acetylneuraminic acid, succinate, the carnitine precursor deoxycarnitine, the eicosanoid 12-HETE, the fatty acid 13-HODE, and the nucleobase uracil. BV-associated bacteria typically exhibited correlation patterns with metabolites that were opposite to those exhibited by *L. crispatus* and *L. jensenii*. Among BV-associated bacteria, BVAB1 displayed specific differences in metabolite correlation patterns compared with BV-associated bacteria from group 1. BVAB1 was

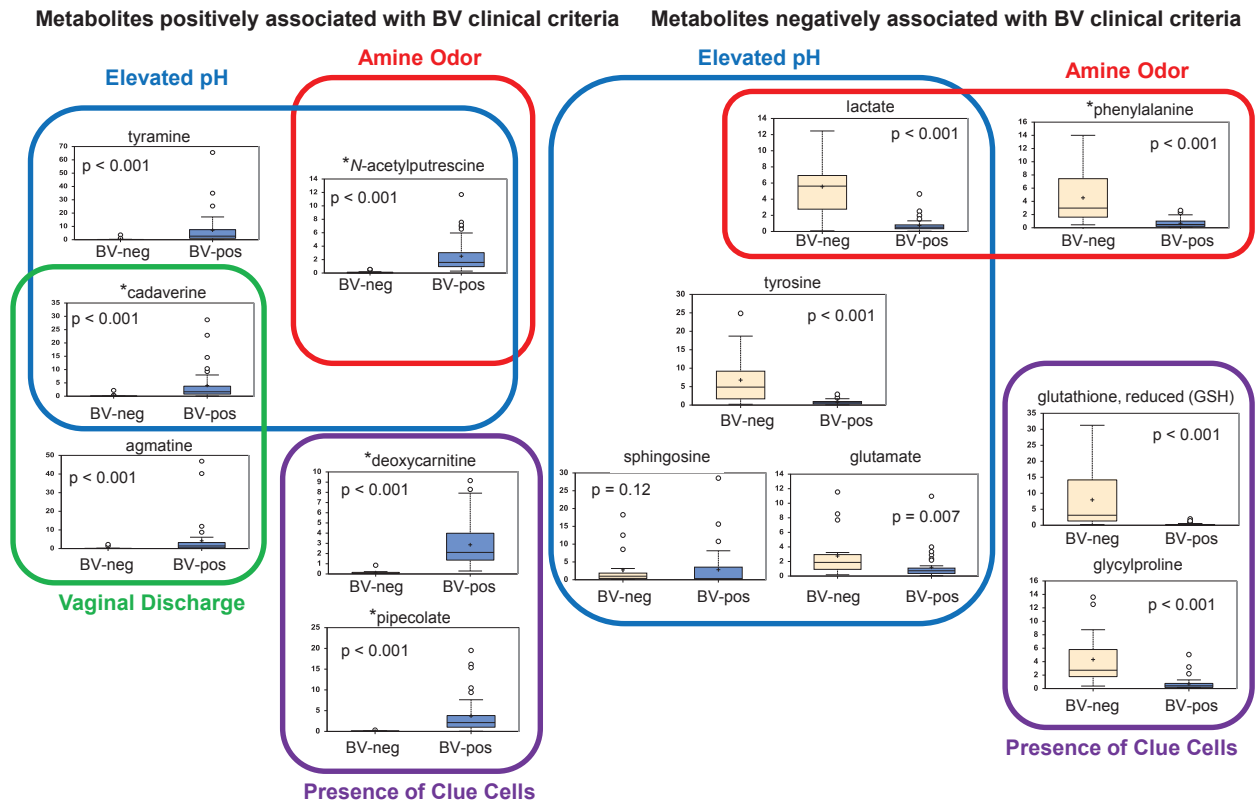


FIG 4 Association of metabolites with Amsel clinical criteria. Shown is a model depicting metabolites associated with individual clinical criteria used in BV diagnosis (21). Stars denote metabolites that were positively or negatively associated with BV status. BV status is indicated on the x axis of box plots. The y axis of box plots represents scaled concentrations of metabolites. The lines in box plots represent the mean, and whiskers denote 95% confidence intervals.

negatively correlated with *N*-acetylaspargate, 12-HETE, and fatty acids, such as eicosenoate and dihomolinoleate; these metabolites had positive associations with group 1 BV-associated bacteria. BVAB3 and *P. amnii*, members of the group 2 BV-associated bacteria, exhibited metabolite correlation patterns that were similar to each other.

**Associations between metabolites and individual clinical characteristics.** We examined associations of metabolites with each Amsel criterion for BV using penalized linear regression models (Fig. 4 and Table 3). No metabolite was associated with all four individual criteria. Lactate was negatively associated with elevated pH and an amine odor. Cadaverine was associated with elevated pH and thin homogeneous discharge, while *N*-acetylputrescine was associated with elevated pH and an amine odor. Deoxycarnitine and pipecolate were associated with the presence of clue cells, while the reduced form of glutathione (GSH) and glycylproline were negatively associated with the presence of clue cells. Deoxycarnitine, *N*-acetylputrescine, pipecolate, and cadaverine were positively associated with BV overall, while phenylalanine was negatively associated with BV.

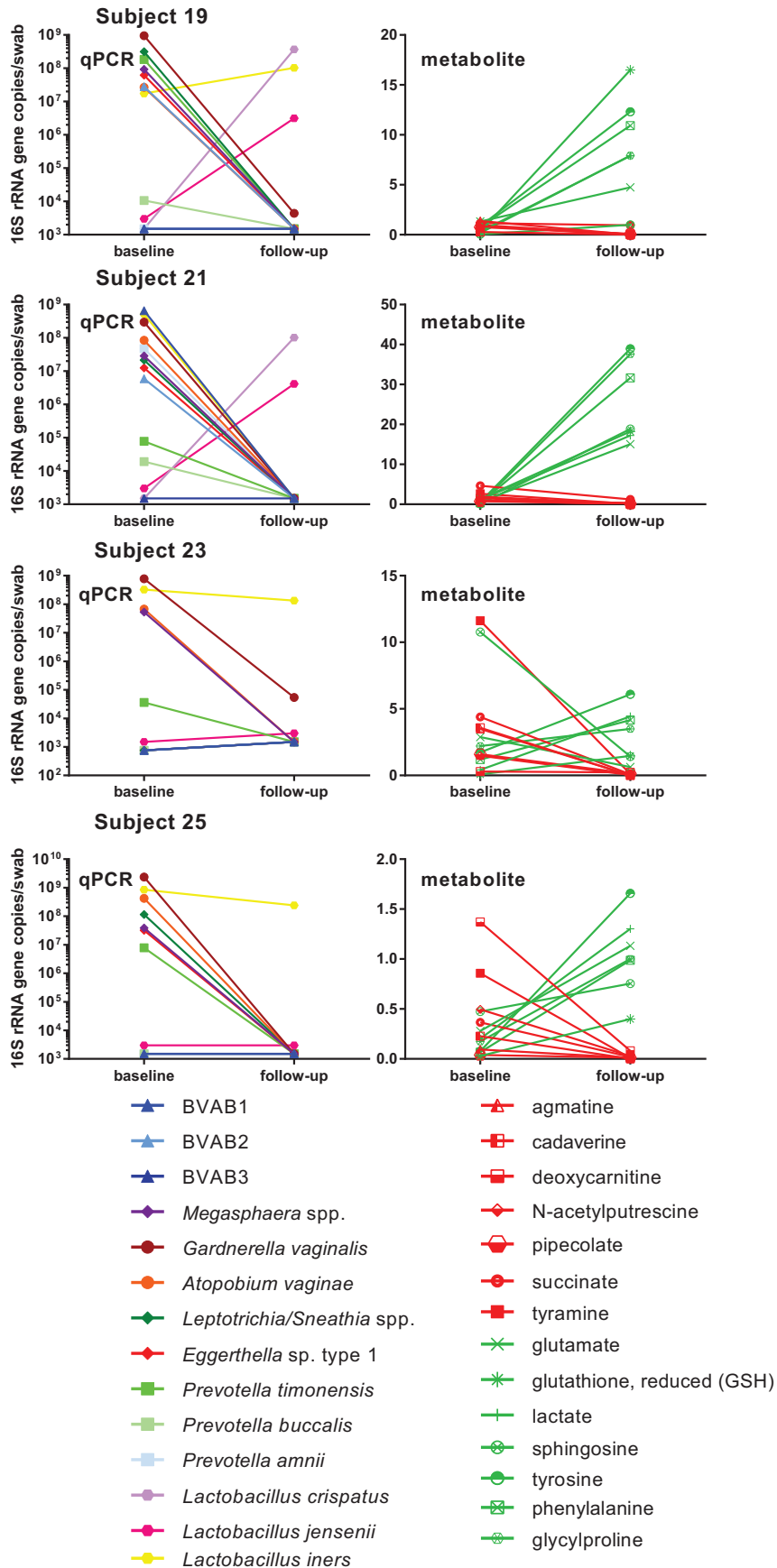
**Alterations in key vaginal bacteria and metabolite concentration with changes in BV status.** Limited longitudinal data are presented for 8 women who returned for follow-up visits 4 weeks after baseline sample collection per the study protocol (Fig. 5; see Fig. S1 in the supplemental material). Participants diagnosed with BV were treated with metronidazole. Study participants 19, 21, 23, and 25 were cured of BV at the follow-up visit (Fig. 5). All four

TABLE 3 Association of metabolites with individual clinical criteria

Clinical criterion	Regression coefficient <sup>a</sup>
<b>BV status</b>	
Deoxycarnitine	0.5
Phenylalanine	-0.299
<i>N</i> -Acetylputrescine	0.254
Pipecolate	0.148
Cadaverine	0.025
<b>Elevated pH</b>	
Cadaverine	0.0657
<i>N</i> -Acetylputrescine	0.0577
Lactate	-0.0505
Glutamate	-0.0249
Sphingosine	-0.0137
Tyrosine	-0.0083
Tyramine	0.0033
<b>Presence of clue cells</b>	
Deoxycarnitine	0.2519
Glycylproline	-0.203
GSH <sup>b</sup>	-0.0311
Pipecolate	0.0024
<b>Presence of amine odor</b>	
<i>N</i> -Acetylputrescine	0.302
Lactate	-0.072
Phenylalanine	-0.049
<b>Vaginal discharge</b>	
Agmatine	0.14
Cadaverine	0.11

<sup>a</sup> A negative value indicates a negative association with the clinical criterion, and a positive value indicates a positive association with the clinical criterion.

<sup>b</sup> Reduced glutathione.





participants had high concentrations of lactobacilli at their follow-up visits and increased concentrations of metabolites negatively associated with BV. Women with high concentrations of *L. crispatus* at follow-up had high concentrations of metabolites negatively associated with BV. Women with high concentrations of *L. iners* also showed increased concentrations of metabolites negatively associated with BV, but these shifts were not as dramatic as increases seen in women with *L. crispatus* dominant communities (Fig. 5). Study participants 4 and 7 did not have BV at either time point and had high concentrations of lactobacilli at both time points (see Fig. S1). High concentrations of metabolites negatively associated with BV were also noted at baseline and follow-up. Study participants 12 and 28 had recurrent BV after antibiotic treatment. In both participants, concentrations of metabolites associated with BV declined at the follow-up visit, but there was no associated increase in metabolites negatively associated with BV (see Fig. S1). In this longitudinal analysis, changes in the microbiota were associated with shifts in metabolites in the same subject, whereas stability of the microbiota was associated with fewer changes, even after patients received antibiotic therapy.

**Validation study (cohort B).** An important question is whether metabolites identified by the global metabolic screen in the primary study (cohort A) are reproducible in other samples using a different platform to measure the metabolites. Hence, in the validation study (cohort B) we evaluated a targeted set of metabolites that were identified as significant in the primary study, including amino acids, amino acid catabolites, sialic acid, and eicosanoids 12-HETE and 13-HODE (see Table S2 in the supplemental material). The validation cohort included 40 women with BV and 20 women without BV; BV was diagnosed by both Amsel criteria and Nugent score (Table 1). Of the 101 metabolites that were detected by the Northwest Metabolomics Research Center (NW-MRC) platform, 57 metabolites (56.4%) were significantly different between women with and without BV ( $q < 0.05$ ). Of the 57 metabolites, 37 were lower in BV (64.9%) and 20 were higher (35.1%). Most observations made in the primary study were confirmed in the validation study. Examples include lower levels of amino acids in BV, and this was significant for 16/20. Similarly, amino acid catabolites cadaverine ( $P < 0.001$ ), tyramine ( $P < 0.001$ ), and tryptamine ( $P = 0.003$ ) were higher in women with BV. The polyamine putrescine was higher in BV ( $P < 0.001$ ), while the precursors arginine ( $P = 0.007$ ) and ornithine ( $P = 0.009$ ) were lower. *N*-Acetylneuraminic acid (sialic acid) ( $P < 0.001$ ) and succinate ( $P < 0.001$ ) were higher, and lactate was lower ( $P < 0.001$ ). We also corroborated components of lipid metabolism, including higher levels of 12-HETE ( $P = 0.006$ ) and 13-HODE ( $P = 0.0004$ ) and lower levels of the 12-HETE precursor, arachidonic acid ( $P = 0.016$ ). In some cases, such as ascorbate ( $P = 0.775$ ) and oxidized glutathione ( $P = 0.162$ ), similar trends were observed in the primary and validation studies (both lower in BV),

but fold change was not statistically significant in the validation study. Likewise, glucose was higher in women without BV ( $P < 0.001$ ) but not statistically significant in the primary study. One example of a notable difference in the two studies is piperolate, which was lower in BV ( $P = 0.018$ ) in the validation study but was found to be higher in BV in the primary study ( $P < 0.001$ ) and was associated with the presence of clue cells.

As in the primary study, we used Pearson correlation coefficients to link bacteria detected by broad-range PCR and pyrosequencing with the metabolites detected in the validation study (see Fig. S2 in the supplemental material). BV-associated bacteria were highly correlated with metabolites associated with BV, such as the amino acid catabolites tyramine and histamine, the polyamines putrescine and cadaverine, and the fatty acids 13-HODE and 12-HETE. In contrast, the lactobacilli were correlated with intact amino acids and glucose, among other metabolites. The clustering patterns were similar to that observed in the primary study, with the BV-associated bacteria clustering as two groups and the lactobacilli clustering together. *L. iners* exhibited correlation patterns that were intermediate between those of the BV-associated bacteria and the lactobacilli (see Fig. S2).

## DISCUSSION

Research on the human microbiome has been largely focused on describing the composition of microbial communities under conditions of health and disease. Our knowledge of the functional properties of vaginal microbes is just emerging (30, 33). Small molecule metabolites (the metabolome) reflect the enzymatic pathways and complex metabolic networks that drive microbial transformation of host-derived products. We examined the physiological state of the vagina using approaches that integrate microbial community composition with metabolite profiles present in human vaginal fluid. First, we determined metabolites present in vaginal fluid from women with and without BV. Second, we associated vaginal bacterial species with metabolite concentrations using broad-range 16S rRNA gene PCR (to assess representation) as well as taxon-directed qPCR (to measure bacterial concentrations). Third, we correlated metabolites with individual clinical criteria used to diagnose BV. Fourth, we validated a set of select metabolites of interest that were identified in the primary study by measuring them in a second set of samples using a different metabolomics platform.

There is a notable delineation of metabolite profiles in women with and without BV across multiple pathways (Fig. 1 and Table 2; see Tables S1 and S2 in the supplemental material). The range and magnitude of these differences are striking. Within women with BV, there are at least two subtypes based on the metabolic profiles, reflecting different concentrations rather than the presence/absence of particular metabolites (Fig. 1). In a recent metabolomics study of eight women with symptomatic BV, clustering analysis

**FIG 5** Shifts in concentrations of key vaginal bacteria and metabolites with treatment for BV. Longitudinal data are presented for four study participants (19, 21, 23, 25) who were cured of BV posttreatment with metronidazole at the 1-month follow-up visit. Bacterial concentrations are displayed as 16S rRNA copies per swab on the *y* axis of the *x-y* plots. Scaled metabolite concentrations (*y* axis) of 14 metabolites associated with individual clinical criteria from Fig. 4 (Table 3) have been classified as positively associated with BV and are higher in women with BV (red) and negatively associated with BV and are lower in women with BV (green). *y* axis scales are different for each subject reflecting differences in metabolite concentrations. All four participants had high concentrations of lactobacilli at their follow-up visits and increased concentrations of metabolites negatively associated with BV (green). Women with high concentrations of *L. crispatus* at follow-up had high concentrations of metabolites negatively associated with BV (green). Women with high concentrations of *L. iners* also showed increased concentrations of metabolites negatively associated with BV (green), but these shifts were not as dramatic as increases seen in women with *L. crispatus* dominant communities.

resulted in two groups, differentiated by 48 metabolites (30). In our study, we detected 12/48 metabolites that were reported by Yeoman et al. Of the 12 metabolites, tryptophan, urea, and valine contributed to the differences in the four clusters; the other 9 were not among the top 30% of most variable metabolites presented in Fig. 1. Our data support previous studies that have demonstrated that BV is associated with high concentrations of putrescine and cadaverine and low concentrations of lactate (2, 3, 5, 6, 23–27, 30, 34, 35). However, our data provide additional insight into the global changes in metabolite profiles in BV reflecting numerous pathways, particularly amino acid metabolism.

Lower levels of amino acids and higher levels of amino acid catabolites suggest increased utilization of amino acids in BV. Alternatively, lower amino acid levels in BV could be explained by decreased production of amino acids, but this would not explain the increase in amino acid decarboxylation products. Concomitant with lower levels of amino acids, there were lower levels of dipeptides as well, supporting increased catabolism (Table 2; see Table S1 in the supplemental material). Specific BV-associated bacteria were correlated with the presence of amino acid catabolites, whereas *L. crispatus* and *L. jensenii* were associated with the presence of intact amino acids and dipeptides. These data support the notion that BV-associated bacteria may use amino acids as a carbon and nitrogen source, in contrast to lactobacilli, which are known to metabolize sugars, such as glycogen. In line with our findings, another study that performed comparative analysis of *Lactobacillus* genomes from the human vagina suggested that *Lactobacillus* genomes were underrepresented in amino acid transport and metabolism, while being overrepresented for carbohydrate transport and metabolism (36). Furthermore, genome analysis of *L. iners* AB-1 showed that it lacks genes necessary for the *de novo* synthesis of amino acids, with the possible exception of serine, and that ~15% of its genome is dedicated to various transport mechanisms, suggesting that it acquires metabolites, such as amino acids, crucial for survival from the environment (37). This is consistent with our observations that the metabolite profile of *L. iners* is intermediate to those of *L. crispatus*/*L. jensenii* and the BV-associated bacteria.

The well-known amines putrescine, cadaverine, and tyramine were detected in women with BV (Fig. 1; see Tables S1 and S2 in the supplemental material). *N*-Acetylputrescine, a degradation product of putrescine, was associated with an amine odor and elevated pH (Fig. 4 and Table 3). Cadaverine was associated with vaginal discharge and elevated pH, and tyramine was associated with elevated pH (Fig. 4 and Table 3). Cadaverine has been shown to be highly correlated with an amine odor, but it was not associated with an amine odor in our model. The lasso and other regularization approaches often include only one of several tightly correlated variables in the fitted model, and this may explain the exclusion of cadaverine in the model of association with an amine odor. BV-associated bacteria were strongly associated with these metabolites, and *L. iners* was also positively associated. Trimethylamine (TMA) was not detected in our study due to its volatility (gas at room temperature) and the nature of the detection platforms, but it has been linked to BV in other studies (24, 27, 35). However, we did measure trimethylamine oxide (TMAO) in the validation study and found that the levels were lower in BV.

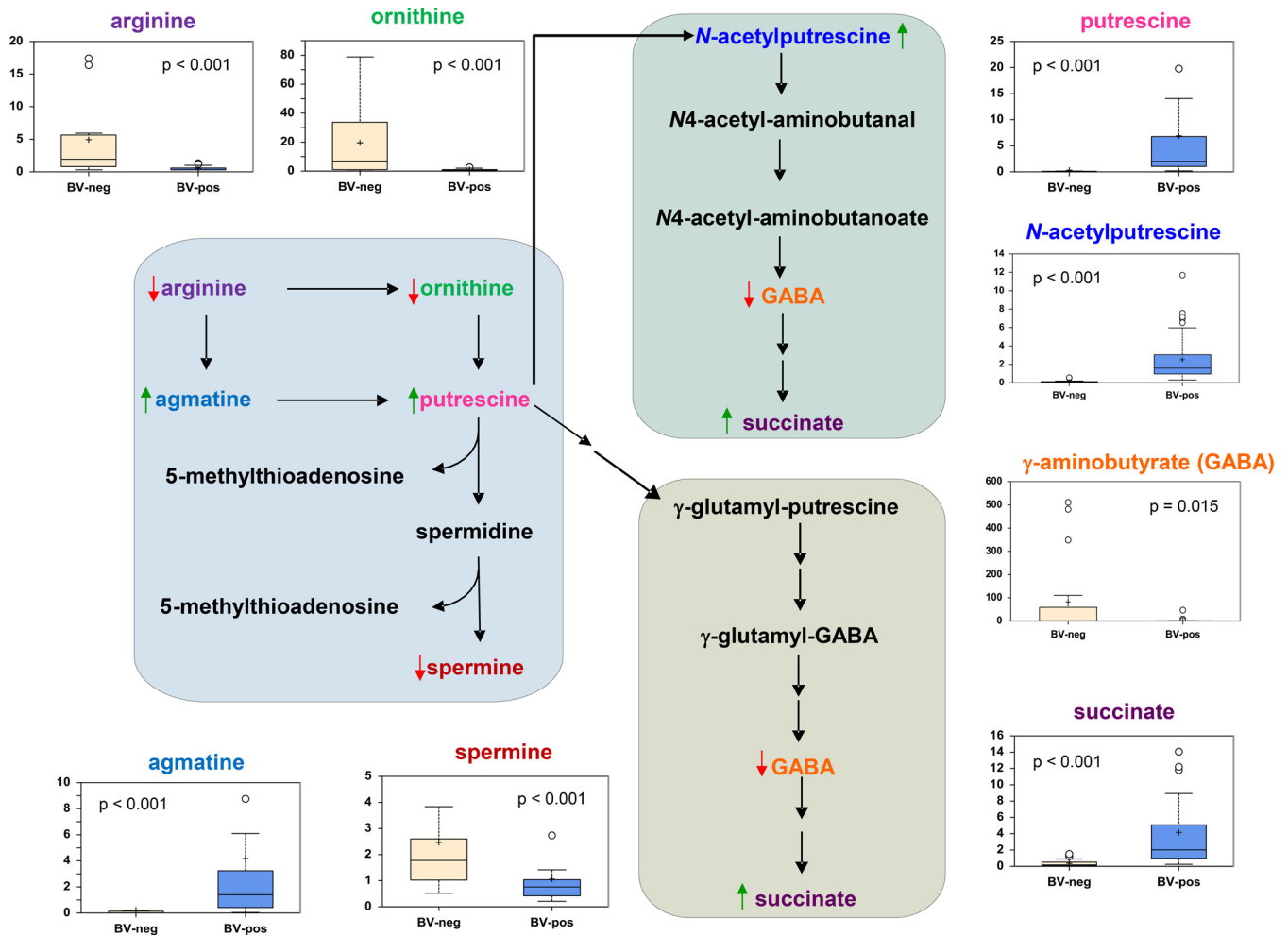
The amino acid arginine typically serves as a precursor for the generation of the polyamines putrescine, spermidine, and spermine. Lower arginine levels and higher putrescine levels in BV sup-

port conversion of arginine to putrescine. However, lower spermine and higher succinate levels suggest the presence of alternate pathways in BV (Fig. 6; see Tables S1 and S2 in the supplemental material). One possible explanation is that putrescine is converted to succinate via  $\gamma$ -aminobutyrate (GABA), a novel alternate putrescine utilization pathway that was recently discovered in *Escherichia coli* (38, 39), wherein putrescine is not converted to spermine. This is also supported by observations of higher levels of *N*-acetylputrescine in our study, which also leads to the formation of GABA (Fig. 6). Additional pathways may account for the higher levels of succinate in BV.

Carnitine, a product of lysine or methionine degradation, was lower in BV, while levels of the precursor, deoxycarnitine, were high. One possible explanation for lower levels of carnitine is conversion to TMA by BV-associated bacteria, a process shown to be mediated by bacteria in the gut (40). TMA is produced from L-carnitine found in meat and is absorbed and converted to trimethylamine oxide (TMAO) in the liver. Production of TMAO can be inhibited by suppressing the intestinal microbiota with antibiotics, demonstrating another role for bacteria in TMA-TMAO metabolism. An alternate explanation for low carnitine in BV is that production is inhibited by lack of ascorbate, which is a necessary cofactor for synthesis.

There were significant alterations in lipid metabolism in BV. The higher levels of 12-HETE and lower levels of its precursor arachidonate in BV observed in both the primary and validation studies (see Tables S1 and S2 in the supplemental material) suggest conversion of arachidonate to 12-HETE by BV-associated bacteria. 12-HETE is a signaling eicosanoid that can mediate inflammatory response pathways, is a biomarker for inflammation (41–43), and is a fatty acid that serves membrane structural roles as a component of phospholipids (44). BVAB1 was negatively associated with 12-HETE ( $-0.028$ ), while other BV-associated bacteria (measured by qPCR) were positively associated with 12-HETE (0.143 to 0.55). Interestingly, 12-HETE has been shown to be correlated with human parturition, wherein 12-HETE levels are significantly elevated in laboring women (45). Furthermore, BV is associated with preterm birth (46, 47). Clearly, not all BV-associated bacteria are positively associated with 12-HETE, which suggests that the different subtypes of BV may be correlated with distinct risk factors.

The vaginal epithelium is in a reduced state in BV (48). Nonetheless, there are suggestions of perturbed redox homeostasis in BV, implying oxidative stress in this reduced environment. Ratios of reduced glutathione (GSH) to oxidized glutathione (GSSG) are a good estimate of the redox environment; a decrease in the GSH/GSSG ratio is suggestive of oxidative stress (49). The GSH/GSSG ratio in women without BV was found to be +3.29, and that in women with BV was 0.23 in the primary study, and similar ratios were noted in the validation study (see Table S2 in the supplemental material). GSH/GSSG ratios are critical in maintaining a reduced environment in the gut (50). All BV-associated bacteria (detected by qPCR) and *L. iners* were negatively correlated with the presence of GSH. *L. crispatus* and *L. jensenii* were positively correlated with the presence of GSH. In addition, GSH was negatively correlated with the presence of clue cells. Another metabolite indicative of oxidative stress is ascorbate (vitamin C), a major antioxidant whose levels were significantly lower in BV. A third metabolite that may affect the redox status is spermine, whose concentrations are lower in BV. Spermine is present in semen and



**FIG 6** Model representing putative putrescine metabolism pathways in bacterial vaginosis. Box plots show selected metabolites that were detected in our study. BV status is indicated on the x axis of box plots. The y axis of box plots represent scaled concentrations of metabolites. The lines in box plots depict the mean, and whiskers denote 95% confidence intervals. Arginine typically serves as a precursor for the generation of polyamines putrescine, spermidine, and spermine. Levels of putrescine were higher in BV, while levels of spermine were lower. Higher levels of succinate are a hallmark in BV. Recently, a novel putrescine utilization pathway has been discovered in *Escherichia coli* via  $\gamma$ -aminobutyrate (GABA), resulting in succinate production. This pathway may play a role in BV. Higher levels of *N*-acetylputrescine were observed in BV, which can also lead to GABA formation. Red or green arrows next to metabolites indicate metabolites detected in our study and show whether concentrations were higher or lower in women with BV.

has antioxidant properties. Spermine is thought to protect spermatozoa, which contain high levels of polyunsaturated fatty acids that can be susceptible to reactive oxygen species (51). It is unclear why BV is associated with multiple metabolic indicators of oxidative stress when the environment is otherwise considered anaerobic and reduced. There is typically a lack of leukocytes in vaginal fluid from women with BV, so leukocytes are unlikely to contribute to the production of reactive oxygen species.

There were substantial changes in carbohydrate and energy metabolism in BV. One example includes the higher levels of *N*-acetylneuraminic acid in vaginal fluid from women with BV (see Tables S1 and S2 in the supplemental material). *N*-Acetylneuraminic acid is the most ubiquitous sialic acid and is a component of glycoproteins, glycolipids, and oligosaccharides (52). A recent study also observed increased quantities of *N*-acetylneuraminic acid in vaginal fluid from women with BV (53). There have been many reports of increased activity of sialidases in BV (54–57); sialidases hydrolyze *N*-acetylneuraminic acid residues from oligosaccharides, glycoproteins, and

glycolipids. A point-of-care diagnostic test for BV that is currently available relies on the measurement of sialidase activity in vaginal fluids (58, 59). Importantly, high sialidase activity in BV has been associated with increased risks for preterm birth (55, 56, 60, 61). Glycoproteins such as mucins are present in mucus and are reported to create a physical barrier between the host epithelium and bacteria (62, 63); this may be the source of the free *N*-acetylneuraminic acid present in vaginal fluid in BV. While little is known about the utilization of this metabolite by vaginal bacteria, a recent study showed that *G. vaginalis* has the metabolic machinery required for the transport and subsequent breakdown of *N*-acetylneuraminic acid as a carbon and energy source (53). Interestingly, *N*-acetylneuraminic acid has been shown to play a role in bacterial biofilms (64). Indeed, biofilms may play a role in BV, and *G. vaginalis* has been shown to be a key member of these biofilms (65–67). Interfering with production or liberation of *N*-acetylneuraminic acid in the vagina may be one approach to prevent BV if this metabolite plays a critical role in biofilm formation.

We confirm in our study that women with BV had high levels

of succinate and low levels of lactate. Consistent with this, BV-associated bacterial concentrations were positively correlated with succinate, while the lactobacilli were negatively correlated. As expected, lactate was also negatively correlated with the presence of an amine odor and elevated pH, two criteria used in the clinical diagnosis of BV (Fig. 4 and Table 3).

There are some limitations in this study. First, the set of 279 identified metabolites detected certainly does not represent all the small metabolites present in vaginal fluid. For example, some volatile compounds, such as TMA, acetate, and butyrate, are missed by our approach, and many low concentration or unknown/unusual metabolites are also not accounted for. Second, this is a cross-sectional study. Limited longitudinal data are presented to show that changes in bacterial concentrations in individual women are associated with shifts in metabolite levels as BV status changes (Fig. 5; see Fig. S1 in the supplemental material). More frequent sampling in future studies will help to advance our understanding of the dynamic nature of metabolites and how alterations in bacterial concentrations may drive these fluxes. Third, we focused our analysis on women with BV or without BV based on accepted criteria, but there are some women with intermediate vaginal microbiota by Gram stain and Nugent score that were not studied in the primary cohort. We examined the metabolites in a small set of 10 women with intermediate Nugent scores as part of the validation study. Indeed, some women with intermediate scores had metabolite profiles that were intermediate between the metabolic profiles of women with BV and those without, as shown in a multidimensional scaling plot (see Fig. S3 in the supplemental material). However, some women with intermediate Nugent scores had metabolite profiles that were similar to profiles in women without BV (Nugent scores of 0 to 3), while others had profiles similar to those found in women with BV (Nugent scores of 7 to 10). A larger sample size is needed to assess the metabolic patterns in women with intermediate microbiota, and longitudinal studies are needed to determine if a transitional microbiota is associated with a shifting metabolic profile. Fourth, we measured metabolites in cervicovaginal lavage (CVL) in both the primary and validation cohorts. We performed additional experiments as part of the validation studies to address whether dilution in CVL impacts detection of particular metabolites and found that no metabolite was detected only in swab samples. As expected due to dilution, lower concentrations of metabolites were detected in CVL than with swabs (see Fig. S4 in the supplemental material).

In conclusion, metabolic signatures of BV are distinct and broad, consistent with dramatic changes in vaginal bacterial compositions. There are at least two metabolic subtypes in BV. It is not known if these different profiles are associated with increased risks for specific adverse health outcomes associated with BV, but these associations should be investigated. Insights from this metabolomics analysis could be used to develop new diagnostic markers of BV and new approaches for prevention or treatment of BV.

## MATERIALS AND METHODS

**Cohort A. (i) Study population and sample collection.** The initial study population was derived from two clinic sites in Seattle with enrollment between November 2003 and June 2010: the Public Health, Seattle and King County Sexually Transmitted Diseases Clinic (STD clinic) ( $n = 30$ ) and the University of Washington's Women's Research Clinic (WRC) ( $n = 30$ ). We conducted a case-control study comprising 40 women with BV and 20 women without BV. BV was defined by both Amsel criteria (21) and Gram stain interpretation using the Nugent method (22); women

were classified BV negative if negative by both diagnostic measures. Cases and controls were randomly selected from both clinics in a 2:1 ratio, with the goal of having a better representation of women with BV given the increased bacterial heterogeneity associated with this condition. All women provided written informed consent, and the studies were approved by Institutional Review Boards at the Fred Hutchinson Cancer Research Center and the University of Washington.

A pelvic examination with speculum was performed for collection of samples. Polyurethane foam swabs (Epicenter Biotechnologies, Madison, WI) were brushed against the lateral vaginal wall and stored at  $-80^{\circ}\text{C}$  until DNA extraction for bacterial PCRs. Vaginal fluid was also collected for Gram staining, pH, saline microscopy, and potassium hydroxide preparation. Cervicovaginal lavage (CVL) fluid was collected by instilling 10 ml sterile saline into the vagina, aspirating it, and storing it at  $-80^{\circ}\text{C}$  prior to processing for metabolomics studies.

**(ii) DNA extraction and qPCR.** DNA was extracted using the Ultra Clean soil DNA kit or the Bacteremia kit (Mobio, Carlsbad, CA), which gave similar results. DNA was eluted in 150  $\mu\text{l}$  buffer and diluted 1:1 in 1 mM Tris–0.1 mM EDTA (TE) buffer. Sham swabs without human contact were processed as controls to assess contamination from reaction buffers or sample collection swabs. Samples were evaluated for presence of PCR inhibitors using a qPCR assay targeting a segment of exogenously added jellyfish DNA; inhibition was defined as a delay in the threshold cycle of  $>2$  cycles compared with no-template controls (68). Control assays targeting the human 18S rRNA gene were performed to ensure that swabs contained human tissue (68). DNA from each sample was subjected to a panel of 14 qPCR assays to measure concentrations of bacteria. The bacteria targeted included three members of the order *Clostridiales* which are highly specific for BV, designated BV-associated bacterium 1 (BVAB1), BVAB2, and BVAB3 (8). Other vaginal bacteria targeted included *Gardnerella vaginalis*, *Leptotrichia/Sneathia* spp., *Megasphaera*-like bacterium (type 1 and type 2), *Atopobium vaginae*, *Lactobacillus crispatus*, *Lactobacillus jensenii*, and *Lactobacillus iners* (69, 70). We developed four additional qPCR assays targeting an *Eggerthella*-like bacterium, *Prevotella amnii*, *Prevotella timonensis*, and *Prevotella buccalis* using 16S rRNA gene-specific primers and taxon-directed hydrolysis probes. The assay conditions and primer and probe sequences for qPCR assays developed in this study are presented in Table 4. Core PCR reagents were obtained from Applied Biosystems (Carlsbad, CA), and master mixes contained buffer A (1 mM), deoxynucleotide triphosphates (1 mM), magnesium (3 mM), AmpErase uracil-*N*-glycosylase (0.05 U), and AmpliTaq gold polymerase (1 to 1.5 U) per reaction. Primers were added at 0.8  $\mu\text{M}$  per reaction, and the final probe concentration was 150  $\mu\text{M}$ . Assays underwent 45 cycles of amplification on the StepOnePlus real-time PCR system (Life Technologies, Grand Island, NY). Plasmid standards were run in duplicate from  $10^6$  to 2.5 copies. Specificity and sensitivity testing was conducted as previously described (69). Two microliters of DNA (diluted 1:1 with TE buffer) was added to each qPCR mixture, and values are reported as 16S rRNA gene copies per swab.

**(iii) Broad-range PCR and pyrosequencing of 16S rRNA gene amplicons.** We performed broad-range 16S rRNA gene PCR with pyrosequencing using 454 Life Sciences FLX technology (Roche, Branford, CT) targeting the V3–V4 region of the 16S rRNA gene for a subset of samples from 20 women with BV and 10 women without BV, a subset of the cohort described previously (11). Sequence reads were classified using the phylogenetic placement tool pplacer (71) and a curated reference set of vaginal bacteria (11).

**(iv) Measurement of levels of biochemicals.** Chromatographic separation and full-scan mass spectroscopy (MS) were performed using the Metabolon (Durham, NC) platform (72–74). Briefly, CVL samples were extracted to remove the protein fraction while allowing maximal recovery of small molecules using the MicroLab Star system (Hamilton, Reno, NV). The resulting extract was split into equal parts for analysis by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). Samples were dried by placing them

TABLE 4 Primer and probe sequences developed in this study

PCR assay	PCR conditions	Amplicon size (bp)	Primer/probe	Primer/probe sequence <sup>a</sup>
Eggerthella-like	56°C annealing, 39 s 72°C extension, 30 s	84	Egger-like_126F	5' GACCAACCTGCCTTACATT 3'
			Egger-like_210R	5' GCATACATCATGTGATATGTGC 3'
Prevotella buccalis	57°C annealing, 39 s 72°C extension, 30 s	171	Egger-like_155-178_pb	5'-FAM-AAAAGAAATCTGGCTAATACCAA-MGBNFQ-3'
			Pbuccalis_455F	5' GCGCGACGTGTCTGCGCA 3'
			Pbuccalis_626R	5' CCGGTTGAGCCGGTACA 3'
			Pbuccalis_587-604_pb	5'-FAM-CGCCAGRTAAGCGTGTG-MGBNFQ-3'
Prevotella timonensis	64°C annealing/ extension, 60 s	90	Ptimonensis_578F	5' GAGCGTAGGCTGTCTATTAAGC 3'
			Ptimonensis_668R	5'-CTTCCTGCATACTCAAGTCGCAC 3'
Prevotella amnii	59°C annealing, 39 s 72°C extension, 30 s	69	Ptimonensis_609-629_pb	5'-FAM-ATTTACCGCTCAACCGGTGG-MGBNFQ-3'
			Pamnii_989F	5' GGCTTGAATTGCAGATGTTTATAT 3'
			Pamnii_1058R	5' CCATGCAGCACCTCACAAAT 3'
			Pamnii_1014-1033_pb	5'-FAM-AGATGATATATCCCTTCGG-MGBNFQ-3'

<sup>a</sup> FAM, 6-carboxyfluorescein; MGBNFQ, minor groove binder-nonfluorescent quencher.

on a Zymark TurboVap (American Laboratory Trading, East Lyme, CT) and stored at  $-80^{\circ}\text{C}$ . Aliquots of water and solvents used in the extraction were included as controls to assess the contribution of the separation process and extraction methods to the compound signals. Additional samples with known compositions were also included with every run to ensure that there were no process deviations in each run. A reference library of 1,000 purified standards with known chemical structures was used to identify the small molecules from our samples by matching chromatographic properties and mass spectral signatures.

(v) **Data analysis.** Metabolite data were median centered, and missing values were imputed as the minimum observed for that metabolite; median values were then log transformed. Pyrosequencing taxonomic counts and qPCR measures were log transformed. Welch's two sample *t* tests were used to compare metabolites between BV-positive and BV-negative groups. An estimate of false discovery rate (*q* value) was calculated to account for multiple comparisons. A low *q* value ( $<0.1$ ) indicates high confidence in a result. Eighty-three metabolites (30%) with the largest variability (interquartile range) were used to hierarchically cluster samples using Euclidean distance (Fig. 1); identification of four sample groups maximizes silhouette width. To explore the relationship between relative abundances of metabolite and pyrosequencing reads (Fig. 2) or qPCR measures (Fig. 3), Pearson correlations were calculated between metabolite and pyrosequencing reads or qPCR levels; these were used in hierarchical clustering using Euclidean distance. Data transformation was performed in R (<http://cran.r-project.org>) (75).

Lasso regression with binomial response was used to identify metabolites strongly associated with Amsel clinical criteria (Table 3). The number of metabolites retained was set to the largest value within 1 standard deviation of the cross-validation error. Analysis was performed with the R package glmnet (76).

**Cohort B. (i) Study population and sample collection.** Cohort B in the independent validation study also comprised 40 women with BV and 20 women without BV enrolled in the STD clinic between April 2011 and August 2013. The BV definitions and sample collection procedures were the same as those in cohort A. We also included an additional 10 women with intermediate Nugent scores of 4 to 6 to determine if their metabolite profiles differed from those who had BV (Nugent scores of 7 to 10) or those who had Nugent scores of 0 to 3. Of these 10 women, 6 were BV negative and 4 were BV positive by Amsel clinical criteria. All women provided written informed consent, and the study was approved by Institutional Review Board at the Fred Hutchinson Cancer Research Center.

(ii) **DNA extraction and molecular methods.** DNA was extracted using the Bacteremia kit (Mobio, Carlsbad, CA), eluted in 150  $\mu\text{l}$  buffer, and diluted 1:1 in 1 mM Tris–0.1 mM EDTA (TE) buffer. We targeted the V3-V4 region of the 16S rRNA gene for broad-range 16S rRNA gene PCR with pyrosequencing using 454 Life Sciences titanium technology (Roche, Branford, CT). Reads were processed using the same methods as cohort A.

(iii) **Measurement of levels of biochemicals. (a) Sample preparation.** Commercially available pooled serum (Innovative Research, Novi, MI) was used as the quality control (QC) sample, and aqueous metabolites were extracted using methanol (250  $\mu\text{l}$ ). The supernatant (200  $\mu\text{l}$ ) obtained postcentrifugation ( $20,800 \times g$  for 10 min) was dried for 90 min using a Vacufuge Plus evaporator (Eppendorf, Hauppauge, NY) and reconstituted in 600  $\mu\text{l}$  40% solvent A (40 mM ammonium acetate in  $\text{H}_2\text{O}$  plus 0.3% acetic acid) and 60% solvent B (acetonitrile plus 0.3% acetic acid) containing 5.13  $\mu\text{M}$  L-tyrosine- $^{13}\text{C}_2$  and 22.54  $\mu\text{M}$  sodium-L-lactate- $^{13}\text{C}_3$  (Cambridge Isotope Laboratory). The isotope-labeled internal standards helped monitor LC-MS assay performance. Samples were filtered through 0.45- $\mu\text{m}$ -pore polyvinylidene difluoride (PVDF) filters (Phenomenex, Torrance, CA) prior to LC-MS analysis. Acetonitrile, ammonium acetate, methanol, and acetic acid (LC-MS grade) were obtained from Fisher Scientific (Pittsburgh, PA), and stable isotope-labeled tyrosine and lactate internal standards ( $>99\%$  pure) were obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). The QC sample was injected every 10 vaginal samples. Vaginal swab samples and CVL fluid (100  $\mu\text{l}$ ) were processed using the same procedure as the QC sample but extracted using 850  $\mu\text{l}$  of methanol. Sham samples (saline solution and swabs without human contact) were included to assess background signals (chemical impurities present in materials used to collect vaginal fluid). Blank saline solution and swab samples were injected once each for every 10 vaginal fluid samples.

(b) **Chromatography conditions.** The LC system was composed of two Agilent 1260 binary pumps, an Agilent 1260 auto-sampler and Agilent 1290 column compartment containing a column-switching valve (Agilent Technologies, Santa Clara, CA). Each sample was injected twice, at 10  $\mu\text{l}$  for analysis using the negative-ionization mode and 2  $\mu\text{l}$  for analysis using the positive-ionization mode. Both chromatographic separations were performed in the HILIC mode on two parallel SeQuant ZIC-cHILIC columns (150 by 2.1 mm, 3.0- $\mu\text{m}$  particle size) (Merck KGaA, Darmstadt, Germany). While one column was performing the separation, the other column was reconditioning in preparation for the next injection. The flow rate was 0.300 ml/min, the autosampler temperature was kept at  $4^{\circ}\text{C}$ , the column compartment was set at  $40^{\circ}\text{C}$ , and the total separation time for each ionization mode was 18 min. The mobile phase was composed of solvents A and B. The gradient conditions for both separations were identical and are as follows: 0 to 2 min, 25% solvent A and 75% solvent B; 2 to 5 min, 25 to 70% solvent A, linear gradient; 5 to 9 min, 70% solvent A; 9 to 11 min, 70 to 25% solvent A, linear gradient; 11 to 18 min, 25% solvent A.

(c) **MS and data processing.** After the chromatographic separation, MS ionization and data acquisition were performed using an AB Sciex QTrap 5500 mass spectrometer (AB Sciex, Toronto, Ontario, Canada) equipped with an electrospray ionization (ESI) source. The instrument was controlled by analyst 1.5 software (AB Sciex, Toronto, Ontario, Can-

ada). Targeted data acquisition was performed in multiple-reaction-monitoring (MRM) mode. We specifically developed additional assays for 19 metabolites of interest based on our results from cohort A, wherein we measured biochemicals using the Metabolon platform (see Table S2 in the supplemental material). We monitored 104 and 76 MRM transitions in the negative and positive modes, respectively (180 transitions total) (see Table S2). The source and collision gas was N<sub>2</sub> (99.999% purity). The ion source conditions in the negative mode were curtain gas (CUR) = 25 lb/in<sup>2</sup>, collision gas (CAD) = high, ion spray voltage (IS) = -3.8 kV, temperature (TEM) = 500°C, ion source gas 1 (GS1) = 50 lb/in<sup>2</sup>, and ion source gas 2 (GS2) = 40 lb/in<sup>2</sup>. The ion source conditions in the positive mode were CUR = 25 lb/in<sup>2</sup>, CAD = high, IS = 3.8 kV, TEM = 500°C, GS1 = 50 lb/in<sup>2</sup>, and GS2 = 40 lb/in<sup>2</sup>. The extracted MRM peaks were integrated using MultiQuant 2.1 software (AB Sciex, Toronto, Ontario, Canada). Standard compounds corresponding to measured metabolites were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (95 to 99% pure).

(iv) **Data analysis.** Metabolite data and pyrosequencing reads were processed and analyzed as described for cohort A.

**Accession numbers.** Sequence reads for cohort A have been previously deposited in the NCBI Short Read Archive SRA051298 (11). Sequence reads for cohort B have been deposited in the NCBI Short Read Archive under the accession no. SRP056030.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00204-15/-/DCSupplemental>.

- Figure S1, PDF file, 0.1 MB.
- Figure S2, PDF file, 0.02 MB.
- Figure S3, PDF file, 0.01 MB.
- Figure S4, PDF file, 0.01 MB.
- Table S1, XLSX file, 0.1 MB.
- Table S2, XLSX file, 0.03 MB.

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S.S., J.M.M., D.R., and D.N.F. conceived and designed the experiments. T.L.F. performed the biological experiments, including DNA extractions and PCRs. D.D. performed the experiments evaluating the metabolites from samples in cohort B. S.S., M.T.M., and N.G.H. analyzed the data. S.S. and D.N.F. wrote the paper. All authors edited the manuscript and approved the final draft.

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