Metatranscriptome analysis of the vaginal microbiota reveals potential mechanisms for protection against metronidazole in bacterial vaginosis

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Running title: Functional activity profiling of the vaginal microbiota during metronidazole treatment of bacterial vaginosis

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Abstract

Bacterial vaginosis (BV) is a prevalent multifactorial disease of women in their reproductive years characterized by a shift from the healthy *Lactobacillus sp.* dominated microbial community towards a highly diverse anaerobic community. BV can initially be cured by antibiotic therapy in most women, but the high recurrence frequency represents a serious challenge. Moreover, for unknown reasons, some women do not respond to therapy. In a clinical study, out of 37 women diagnosed with BV, 31 were successfully treated with a single peroral dose of metronidazole, while 6 still had BV after treatment. Here, we performed a metatranscriptome analysis of the vaginal microbiota of the subgroup of those non-responding patients (n = 6), comparing them to patients who responded (n = 8) over three months (5 time-points). In health, *Lactobacillus iners* and *L. crispatus* contributed more than 90% of all bacterial transcripts, and the communities were dominated by either one or the other. Their functional activity profiles *in vivo* showed that the inerolysin was highly expressed in *L. iners* and hydrogen peroxide producing genes were among the most highly expressed genes in *L. crispatus*. In BV, *Gardnerella vaginalis* contributed on average 40% of all transcripts. In non-responding patients, 7 out of 8 Cas genes and one DNA methyltransferase gene of *G. vaginalis* were highly up-regulated, suggesting that Cas genes of *G. vaginalis*, in addition to protecting against phages, might be involved in DNA repair thus mitigating the bactericidal effect of DNA damaging agents like metronidazole.

Importance:

The vaginal microbiota is relatively uniform in health but shifts towards diverse during bacterial vaginosis (BV). BV is a prevalent multifactorial disease of women in their reproductive years and usually treated with antibiotics like metronidazole. The functional

alterations of the vaginal microbiota during the treatment have never been discovered, despite many studies have identified the key vaginal microbial members in health and BV. Our previous study using 16S rRNA gene sequencing approach failed to detect the difference between patients responded to the treatment and non-responders. By using metatranscriptome, we found out of 8 Cas genes in *G. vaginalis*, 7 were highly up-regulated in non-responding patients. This finding suggests Cas genes of *G. vaginalis*, might play a role in defense against DNA damaging agents like metronidazole. The *in vivo* functional activity profiles of *Lactobacillus iners* and *L. crispatus* confirmed the pathogenic role of *L. iners* and protective role of the latter.

Introduction

The healthy vaginal microbiome (VMB) is characterized by low pH and low diversity and can be categorized into community state types (CSTs) that are dominated by different *Lactobacillus sp.* such as *L. crispatus*, *L. iners*, *L. gasseri* and less frequently *L. jensenii* or a more diverse community (47). The shift of VMB towards dysbiosis has been shown associated with bacterial vaginosis (BV) (54), preterm birth (7,24) and neonatal sepsis (7). BV is a frequent multifactorial disease of women in their reproductive years that is characterized by a shift of this *Lactobacillus sp.* dominated bacterial community to a community of various mostly anaerobic bacteria (54). BV is related to a higher risk of acquiring sexually transmitted infections such as HIV (42). The most common bacteria found in BV, identified by 16S rRNA gene sequencing, are *Gardnerella*, *Atopobium*, *Prevotella*, *Bacteroides*, *Peptostreptococcus*, *Mobiluncus*, *Sneathia*, *Leptotrichia*, *Mycoplasma* and BV associated bacterium 1 (BVAB1) to BVAB3 of the order Clostridiales. Recently, three CSTs dominated by *Gardnerella vaginalis*, Lachnospiraceae and *Sneathia sanguinegens*, respectively, have been described (11) and our recent study identified *S. amnii* as the best biomarker for BV (16).

Studies have shown treatment with antibiotic metronidazole, tinidazole or/plus probiotics increased the relative abundance of *Lactobacillus* species in VMB (27,31,36). Metronidazole is a widely applied chemotherapeutic agent used to treat infectious diseases caused by anaerobic bacteria, and it is the first-line antibiotic for treating BV (6,28). It is a prodrug which requires enzymatic reduction within the cell to transform it into an active form (37). Metronidazole acts by damaging DNA, and resistance mechanisms have been studied in various pathogens (37). Previous studies have shown that failure of BV treatment by metronidazole is relatively rare (16,20), and it is unclear if it is caused by resistance of the BV pathogens to metronidazole, and which mechanisms are acting *in vivo*. A recent study has

demonstrated that failure of treatment of BV with metronidazole is not associated with higher loads of *G. vaginalis* and *A. vaginae* (11). Therefore, the lack of response may result from the activity of the community instead of the abundance of the pathogens and their taxonomic composition. The high rate of recurrence is another crucial problem for BV treatment. It has been reported that the one-year recurrence rate of BV ranges from 40% to 80% after treatment with metronidazole (5). The recurrent rate is similar for other therapies such as clindamycin cream (50). It has been hypothesized that patches of surviving bacterial biofilm may be one of the reasons for recurrence (5). The predominant species of this multi-species biofilm is *G. vaginalis* (30). *G. vaginalis* is frequently found in biofilms during BV but it is also a member of the healthy VMB (53). *L. iners* is characteristic for a community state type in health but may be disadvantageous when the community shifts to a dysbiotic state (32,43,53). Until now, the majority of studies regarding the VMB have focused on 16S rRNA gene sequencing, answering only questions on the taxonomic composition of bacterial communities but not on their functions (54). A metatranscriptome analysis comparing vaginal swabs from two women with BV with two healthy subjects showed that *L. iners* upregulates transcription of the cholesterol-dependent cytolysin (CDC) and of genes belonging to the clustered regularly interspaced short palindromic repeats (CRISPR) system in BV (32). No study has investigated the functional shifts of the VMB during antibiotic treatment of BV.

We had previously analyzed the VMB in the context of a clinical trial using 16S rRNA gene sequencing (16). Of 37 patients diagnosed with BV and included in this study, 31 were initially cured by a single oral dose of metronidazole. Six patients did not respond, i.e. they were diagnosed with BV according to Nugent score at visit 2. Moreover, some patients developed recurrent BV during the three months of the study. Since we did not find differences in the taxonomic composition of the microbiota of non-responding and recurrent

patients, we hypothesized that shifts in microbiota activity might be responsible for those different responses. We therefore chose the non-responding patients (n = 6) from the clinical study and compared their metatranscriptomes to those of 8 patients that were initially cured. For the analysis of recurrence, we chose two recurrent patients from the lactic acid arm of the study (see below) and compared their metatranscriptomes to those of two cured patients that were also provided with lactic acid pessaries, and followed their transcriptomes over the three months of the study (5 time-points). Our analysis is thus comprised of 14 patients, of which six were cured, six did not respond to treatment, and two developed recurrence. Including the time series samples, we analyzed 40 metatranscriptome samples in total.

We could identify genes potentially leading to lack of response to metronidazole treatment and suggest a novel mechanism of *G. vaginalis* to mitigate the DNA damaging effect of metronidazole. Moreover, we demonstrate the importance of *G. vaginalis* for BV, which can be massively underestimated using 16S rRNA gene sequencing, confirm the beneficial role of *L. crispatus* in comparison to *L. iners*.

Results

Study population and overview of sequencing results

We studied the vaginal microbiome of 14 patients during and after metronidazole treatment of BV using metatranscriptome sequencing (Fig. 1). Patients were part of a clinical trial described elsewhere (16). For ten patients, samples were derived from two time points (before (visit 1) and after metronidazole treatment (visit 2). Six of those patients did not respond to treatment with the antibiotic, so were still BV positive according to Nugent score and four of them responded to the treatment and were cured. For the other four patients, 5 time points were analyzed, covering a period of 3 months after treatment. Two of those patients

experienced recurrence, while the other two were cured without recurrence (details in Table S1 sheet 1). In total, we analyzed 40 vaginal fluid samples, 22 with BV status and 18 without. Metatranscriptome sequencing resulted in a total of 1,879,945,342 reads. Of these, 1,377,516,082 reads (73%) were left after quality filtering and removal of ribosomal RNA (Table 1). On average, 34 million reads were analyzed per sample.

Construction of the reference genome and gene databases for taxonomic and functional activity profiling

Based on the standard Kraken (58) database, ~11% of the total putative mRNA reads could be assigned to human for BV and ~56% for health (Table S1 sheet 2). This suggests that the bacterial load is much lower in health than in BV since the human contamination read is much higher in health. Using this standard database, only 41% of total putative microbial (non-human) mRNA reads could be assigned taxonomically (Table 2). Therefore, a refined Kraken database constructed based on the genomes from HMP (38) urogenital tract database plus 16 additional genomes (see methods for details) was finally used for taxonomy classification of putative microbial mRNA reads. Using this database, the rate of classified putative microbial mRNA reads could be improved to 86% on average (Table 2). Interestingly, transcripts from the most abundant 11 species contributed more than 90% of the mapped reads in BV while the two most abundant species accounted for >90% of the classified sequences (Fig. 2), which is much less diverse than the periodontal metatranscriptome in which more than 100 species were required to cover 90% of mappable reads (9).

For functional assignment, we used a customized reference gene database (Table S1 sheet 4) containing the genes from the HMP (38) urogenital tract database, and added the genes of additional 9 genomes (see method section for details). To investigate the functional activity shifts of the communities, we mapped the cleaned metatranscriptomic reads to this

customized reference gene database using BWA (26). In total, around 78% of total putative microbial mRNA reads could be mapped to this microbial reference gene database with a MAPQ >10 on average. The sequencing provided on average 8.9 million mappable microbial mRNA reads per sample, which allowed the functional activity profiling for highly abundant species in the community. The mapping results per sample can be seen in Table S1 sheet 2.

Shifts in the taxonomic composition of the active community during 3 months following metronidazole treatment

The taxonomic composition of transcripts was determined using Kraken. Fig. 1 shows that all communities with BV status (before metronidazole treatment, at recurrence, and in patients with no response to treatment) were dominated by *G. vaginalis*, *A. vaginae*, *S. amnii* and *Prevotella timonensis*. In patients that responded to treatment and were cured, the metatranscriptomes were dominated by *L. crispatus*, *L. iners* and *L. jensenii*. Two patients who developed recurrence were all dominated by *L. iners* after the treatment while only 3 out of 6 patients without recurrence were dominated by *L. iners* after treatment.

The bladder was thought to be sterile in healthy women, but it has now been shown that specific microorganisms are residing in the healthy bladder (35). Therefore, the bladder has to be considered as a possible reservoir for pathogens. The high pH of urine may be a favorable environment for several pathogens, including *Gardnerella sp.*. Here we observed that *Gardnerella sp.* isolated from the bladder (*Gardnerells sp*. 26-12 and *Gardnerella sp.* 30-4) (35) contributed 6% of all classified reads in BV. In several samples of BV patients, transcripts from bladder isolates were extremely abundant (between 10-19% of all transcripts) (samples 07_001_a/b, 13_022_a, 15_006_a/b, 17_004_a) . Some samples ((04_001_a, 06_004_c (recurrence) and 06_001_a/b, 06_006_a/b (without response to treatment)) were dominated by *S. amnii* which has been discovered in the urine microbiome previously (17,57).

Comparison of the taxonomic composition of vaginal fluid samples between metatranscriptome and 16S rRNA sequencing

To discover transcriptionally active bacteria which may contribute to the functional activity shifts in BV, we compared the taxonomic composition determined using 16S rRNA sequencing (16) and the taxonomic composition of the metatranscriptome determined by Kraken with the refined reference database. In health, we did not observe any considerable difference (Table S1 sheet 6), while in BV large differences between the two datasets were found. Fig. 3A-B shows that most of the abundant species identified in the mRNA sequencing data set were also identified using 16S rRNA gene amplicon sequencing, although usually at different abundances. For example, *A. vaginae* comprised 15% of all reads based on 16S rRNA gene sequencing, but only 10% in the metatranscriptome dataset. The most pronounced difference was observed for *G. vaginalis* which comprised on average 40% of relative abundance in the metatranscriptome and on average only 6% in the 16S rRNA sequencing data. By contrast, the two bladder isolates of *Gardnerella sp.* were only identified in the metatranscriptome, probably due to the low resolution of the 16S rRNA gene and the lack of their 16S rRNA genes in the reference database. This finding suggests that *G. vaginalis* and other *Gardnerella* species are extremely transcriptionally active in the BV community. In addition, the commonly used 27F primer has been shown to underrepresent *G. vaginalis* (14).

L. iners and *L. crispatus* were dominant in health and *G. vaginalis* was the most active species in BV

Based on the taxonomic composition of transcripts, the samples were clearly clustered into three groups, namely health communities dominated by *L. crispatus*, health communities dominated by *L. iners* and BV communities (Fig. 3C). The most abundant active species after metronidazole treatment were *Lactobacillus* species of which *L. iners* and *L. crispatus* were

the most abundant ones contributing >90% of the metatranscriptome on average. They showed a negative correlation in occurrence (Spearman correlation coefficient =-0.851, p =7.422e-06). High abundant *L. iners* was never accompanied by high level *L. crispatus* and high abundant *L. crispatus* was only twice (08_003_b, 08_006_d) accompanied by low abundant (lower than one fifth of the abundance of *L. crispatus*) *L. iners*. *L. gasseri* and *L. jensenii* had much lower abundances and were found together with both *L. crispatus* and *L. iners.* In one patient, the active healthy community was dominated by *L. jensenii*.

In BV, *G. vaginalis* was the most abundant active species. Only two communities of patients with BV were dominated by other species, namely *A. vaginae* and *S. amnii*. Additional BV associated species such as *S. sanguinegens* or different *Prevotella sp.* were frequently found in BV samples, and M*obiluncus mulieris* was occasionally found. The only species of *Lactobacillus* found to be active in BV was *L. iners*.

Functional activity profiles in health and disease

In order to profile the function of the communities, all cleaned putative mRNA reads (Table S1 sheet 2, column C) were mapped using BWA onto a customized reference database annotated with KEGG ortholog (KO) genes. We compared the functional activity profiles of samples from patients in BV (22 samples) with samples from health (after successful metronidazole treatment, 18 samples). The PCA based on the expression of KO genes clearly showed that there was a large functional difference between BV and health; the functional activity profiles formed clearly separated clusters (Fig. 4A). Moreover, the functional composition in health was more variable than in BV. This is a striking finding, because 16S rRNA gene sequencing showed a massive increase in diversity in BV (16). The reason might be that the different species of *Lactobacillus* which dominate in different individuals in health

have different functional activity profiles, while the different species that dominate in BV all have similar functional activity profiles.

Time course of functional activity profiles during recurrence

To identify functional difference between women with and without recurrence, the functional activity profiles of two women with recurrence (patients 04_001 and 06_004) were compared to those of two women without (patients 08_006 and 13_019) over a period of three months. Five time points were analyzed of which the first represented acute BV and the others were after metronidazole treatment. The principal components analysis of the functional activity profiles is shown in Fig. 4B. In acute BV, samples from all four patients clustered together (red circle). After the treatment, samples from patient 08_006 who was cured without recurrence moved into a very dense and distinct cluster (illustrated by the arrow 1 and enclosed by a green circle). Samples from patient 13_019 who was also cured, moved to a different cluster after treatment, shown by arrow 2 and encircled blue.

The functional activity shifts in patient 06_004, who experienced recurrence at visit 3, were especially noteworthy: After treatment, the community moved towards a functional activity profile distinct from all others (arrow 3). The recurrence of BV caused the community to shift back to the BV cluster (red circle, arrow 4). At visit 4, the community moved to the heathy cluster (blue circle) and the patient became healthy according to the Nugent score. We speculate that there was an unknown intervention after visit 3 which changed the microbiome but this was not recorded. Interestingly, the other case of recurrence (patient 04_001) had a different progression. From visit 2 to 4, patient 04_001 seemed to be cured, because these samples clustered together in the healthy cluster indicated by the blue circle. At visit 5, however, patient 04_001 had recurrent BV and the community shifted back again to the BV cluster.

Interestingly, the microbial community of patient 08_006 was the only one dominated by *L. crispatus* after metronidazole treatment, while all others, including those in the second healthy cluster, were dominated by *L. iners*. This suggests that communities dominated by *L. crispatus* are more protected from recurrence.

The inerolysin gene was highly expressed in *L. iners* and genes involved in the production of H_2O_2 were highly active in *L. crispatus*

As shown above, *L. iners* and *L. crispatus* dominated different patients after treatment, and *L. crispatus* seemed to have a protective role against recurrence. Therefore, we compared their genomes and functional activity profiles with each other. *L. iners* has the smallest genome encoding around 1500 proteins compared to other *Lactobacillus* species like *L. crispatus* which encodes much more proteins (12). It is known that *L. crispatus* encodes proteins producing hydrogen peroxide (H_2O_2) (1), while *L. iners* encodes a pore-forming toxin protein termed inerolysin (INY) (43). Hydrogen peroxide provides protection against pathogens for the symbiotic vaginal community (8). INY, a cholesterol-dependent cytolysin (CDC), is activated by reducing agents and inhibited by excess cholesterol (43). CDCs were formerly annotated as “thiol-activated cytolysins” (33). However, it is unknown that whether those genes are transcriptionally active in the VMB *in vivo*. To gain more understanding of the different roles of these two bacteria in the healthy vaginal microbiota, we performed a differential expression (DE) analysis based on their KO genes comparing the expression of KO genes of *L. crispatus* from *L. crispatus* dominated samples (n = 4) with the expression of KO genes of *L. iners* from *L. iners* dominated samples (n = 11). The Venn diagram in Fig. 4C shows that the two species share 569 KO genes, while 58 are unique to *L. iners* and 244 are unique for *L. crispatus*. The DE analysis identified 584 significantly differentially expressed KO genes, of which 355 were up-regulated in *L. crispatus* (Table S1 sheet 8). Among the top

100 most differentially expressed KO genes in terms of FDR value, 89 were up-regulated in *L. crispatus*, indicating *L. crispatus* possesses far more diverse functions than *L. iners*. Remarkably, genes assumed to be involved in the production of H_2O_2 (pyruvate oxidase, NADH oxidase, glycolate oxidase) (19,41) were highly expressed in *L. crispatus* (Fig. 4C) suggesting a high level of hydrogen peroxide in vaginal milieu when the VMB is dominated by *L. crispatus.* An iron transport system was found to be highly expressed in *L. crispatus* and it is absent in the genome of *L. iners*. On the other hand, we found that inerolysin (INY) was highly expressed (log2 counts per million mapped reads =10.18) in *L. iners*, but absent in the genome of *L. crispatus* (Fig. 4C, Table S1 sheet 8). Interestingly, we also found the orthologous gene (K11031) of inerolysin known as vaginolysin (15) highly expressed in *G. vaginalis* (details in Table S1 sheet 7). These findings show that *L. iners* exerts a similar functional activity as the BV-associated pathogen *G. vaginalis*.

In vivo* expression of putative metronidazole resistance associated genes in *G. vaginalis

Metronidazole is a prodrug which requires anaerobic conditions for enzymatic reduction within the cell to transform it into an active form (37). Several enzymes are involved in the reduction of metronidazole such as ferredoxin, ferredoxin/ferredoxin-NADP reductase (FNR), ferric uptake regulator (Fur) and nitroreductase (21,24,37,52,55). The reduced metronidazole can introduce DNA strand breaks thus leading to the death of the pathogens. Accordingly, genes responsible for DNA repair like recA and recA-mediated autopeptidase (Rma) might contribute to resistance against metronidazole (51). Moreover, a gene named nitroimidazole resistance gene (NIM) was shown to be related to the resistance against metronidazole in *Bacteroides fragilis* (29). To clarify the possible contribution of these genes to metronidazole resistance of the vaginal microbiota, we examined the expression (Table S1 sheet 10) of these genes in *G. vaginalis* by comparing their expression in the communities with later response to

metronidazole and without at visit 1 (acute BV). For this analysis, BV communities from 10 patients were analyzed in which the level of *G. vaginalis* transcripts was >20%. Six of these patients did not respond to treatment and four were initially cured. Although *A. vaginae* and *S. amnii* are also key players in BV we did not analyze them here since there were two few samples dominated by them. As shown in Fig. S1, ferredoxin, nitroreductase, Fur, FNR were shown to be less active in *G. vaginalis* in the vaginal microbiota of no-responding patients (Fig. S1). FNR was 4.2 times less active (Wilcoxon test P-value = 0.038, based on relative abundance = read count of given genes of *G. vaginalis* / read count of *G. vaginalis* %) in *G. vaginalis* in those communities. FNR is involved in activating metronidazole (21) thus its weak expression indicates the lack of the active antibiotic, in accordance with the lack of response in those patients. Unexpectedly, the NIM gene which was annotated with nitroimidazole resistance gene was also less active in no-responders. RecA and RecA-mediated autopeptidase (Rma) of *G. vaginalis* were more active in some but not all communities from patients that did not respond to metronidazole (Fig. S1). RecA and RecA-mediated autopeptidase are essential genes in SOS response (39) responsible for DNA repair in bacteria. They may play a crucial role in survival of bacteria after treatment with a DNA damaging agent like metronidazole.

CRISPR associated protein and adenine methyltransferase genes of *G. vaginalis* were strongly up-regulated in vaginal fluids of patients not responding to treatment

We then performed a global analysis of differential expression of KO genes of *G. vaginalis* in these same communities (visit 1, 10 BV samples with >20% transcripts from *G. vaginalis* (6 patients that did not respond to treatment and 4 that were initially cured). We observed that there were 24 KO genes highly up-regulated with FDR <= 0.05 (log2 fold change up to 12.4) in communities without response. Strikingly, among the top ten most strongly up-regulated

KO genes, seven were *cas* genes (44) (*cas*1-3, *cas*A-D) (Fig. 5). In total there were 8 different *G. vaginalis* CRISPR-associated (Cas) genes found in its genome, namely *cas*1-3, *cas*A-E. Cas proteins have a dual function; they can perform homology-directed repair as well as degrade foreign DNA (3).

yhdJ and *licD* were the most strongly up-regulated genes with log2 fold change above 10, of which *yhdJ* encodes an adenine-specific DNA-methyltransferase and *licD* encodes a lipopolysaccharide cholinephosphotransferase. The DNA adenine methyltransferase is an essential part of the restriction modification system that protects bacteria from digesting their own genome via their restriction enzymes (45).

There were 32 KO genes down-regulated but the fold change values were not as high as for the up-regulated genes. *galK* (galactokinase) was identified as the most strongly down-regulated gene with a log2 fold change of -5.6.

These findings suggest that CRISPR-Cas, a phage defense mechanism with a dual function, might mitigate the DNA damaging effect of metronidazole and thus may be one of the reasons for lack of response of the vaginal microbiota to treatment. In addition, lack of expression of enzymes (FNR, Fur, ferredoxin, nitroreductase) that help transforming the metronidazole prodrug into the active form might also contribute to lack of response to treatment.

Discussion

The aim of this study was to identify functional activity patterns in the vaginal fluid microbiota in health and BV. In particular, we compared metatranscriptomes of recurrent and non-recurrent patients and of patients who did and did not respond to metronidazole treatment. This is the first study to investigate the compositional as well as functional activity alterations of the vaginal microbiota from patients with BV during treatment with the

antibiotic metronidazole *in vivo* using the metatranscriptomic approach. We found clear patterns which allowed us to formulate novel hypotheses. Since our analysis is based on a relatively small number of 14 patients, our findings should be followed up in larger patient groups with more cases of recurrence and more cases of lack of response to antibiotic treatment.

We observed that *G. vaginalis* was much more dominant based on the abundance of its transcripts than based on the 16S rRNA amplicon sequencing analysis. This suggests that *G. vaginalis* is transcriptionally more active than other vaginal bacteria; moreover, the commonly used 27F primer was previously shown to underrepresent *G. vaginalis* (14). We found that the functional activity profiles in health and BV were massively different. Interestingly, *L. crispatus* and *L. iners* were the dominant species in health and communities were dominated by either one or the other. The patients with recurrence were dominated by *L. iners* after metronidazole treatment. There were many factors observed by laboratory or genomic studies (13,41,46) which show that more protection is provided by *L. crispatus* rather than by *L. iners*. *L. crispatus* has the ability to produce hydrogen peroxide to prevent pathogen colonization, whereas *L. iners* does not (41). A recent longitudinal study also shows that the dominance of *L. iners* in the vaginal microbiota is a risk factor for preterm birth, whereas *L. crispatus* dominance is protective against preterm birth (22). Furthermore, an iron transport system was found in the genome of *L. crispatus* and not in *L. iners* suggesting that *L. crispatus* can capture iron released by the host during menstruation away from potential pathogens (13). On the other hand, *L. iners* can produce inerolysin, a pore-forming cytolysin that enables nutrient acquisition from host cells providing it with an advantage when conditions are scarce (46), and this gene was highly expressed in *L. iners*. Strikingly, an ortholog of inerolysin named vaginolysin was found highly expressed in *G. vaginalis* in BV.

These findings suggest that *L. iners* might initiate the dysbiotic shift of the vaginal flora and contribute to the pathogenesis of BV as well as to recurrence.

Moreover, *Gardnerella* species isolated from the bladder were highly active in several patients with BV, including two patients with recurrent BV. Many potential reasons are discussed for recurrence and the occasional lack of response to metronidazole treatment. For example, it has been reported that sexual activity can introduce pathogens thus may also contribute to the recurrence of BV (5), dosage of metronidazole may have been too low for the patient, or highly virulent strains of *G. vaginalis* may be residing in this particular non-responding patient.

In our metatranscriptome analysis we found evidence for mechanisms that hinder the activation of the metronidazole prodrug, or mitigate the damage that metronidazole inflicts on DNA, and thus could be important reasons for the lack of response in some women.

We show for the first time *in vivo* that the FNR, Fur, ferredoxin, nitroreductase genes of *G. vaginalis* were less active in those patients that did not respond to metronidazole. These genes are required for the activation of metronidazole in anaerobes (21).

Adenine methyltransferase was the most strongly up-regulated gene of *G. vaginalis* in communities from patients without response to metronidazole suggesting that it may play a role in protecting DNA from damage. Studies have provided compelling evidence for DNA methyltransferases 1 (DNMT1) acting as a regulator of genome integrity and as an early responder to DNA double strand breaks in human cells (18), but the explicit role of DNMTs in DNA strand break repair for bacteria remains to be elucidated.

Remarkably, *cas* genes of *G. vaginalis* were highly up-regulated in *G. vaginalis* from vaginal microbiota of patients that did not respond to metronidazole treatment. The CRISPR-Cas genes are present in about half of all Bacteria and most Archaea (34); they represent a

mechanism of adaptive immunity which protects the prokaryotic cell against foreign DNA and has been developed into a universal tool for genome editing (10). The *cas* genes of *G. vaginalis* belong to the *E. coli* subtype and were found in about half of the clinical isolates (44). Their up-regulation might reflect increased phage attacks in BV. Phages have been hypothesized to be crucial for the etiology of BV by causing the collapse of *Lactobacillus* populations (4); accordingly, *L. iners* upregulates its CRISPR-Cas system in BV (32). More than 400 annotated prophage sequences were found in 39 *Gardenerella* isolates from the bladder (35). They might be induced to enter the lytic cycle by the change in pH accompanying the shift to BV.

The upregulation of CRISPR-Cas system genes in *G. vaginalis* from those patients that did not respond to treatment by metronidazole suggests that the CRISPR-Cas system might have a role in not only defending the host against phages, but also in mitigating the DNA damaging effect of metronidazole. In addition to providing adaptive immunity, CRISPR-Cas systems can have various additional functions (56) and it was shown that they can protect the cell against DNA damaging agents (3). The Cas1 enzyme of *E. coli* (YgbT) physically and genetically interacts with the DNA repair system (RecBC, RuvB) and is recruited to DNA double strand breaks; moreover, YgbT is necessary for resistance of *E. coli* to DNA damage caused by the genotoxic antibiotic mitomycin C or UV light (3). Our findings suggest that the CRISPR-Cas system may protect the vaginal microbiota against the DNA damaging effect of metronidazole. If experimentally confirmed, this finding might open a new path to fight bacterial resistance against DNA damaging agents.

Conclusions

We hypothesize that colonization by *L. iners* contribute to recurrence of BV. CRISPR-Cas, a phage defense mechanism with a dual function, might mitigate the DNA damaging effect of metronidazole and thus may be one of the reasons for lack of response of the vaginal microbiota to antibiotic treatment. This first *in vivo* study of the metatranscriptome landscape of vaginal fluid microbiota during metronidazole treatment provides a wealth of new understanding towards the possible mechanisms of lack of response to metronidazole and recurrence in BV. These findings may help to develop more efficient novel therapies to improve the treatment of BV and prevent recurrence. A completely new role for Cas proteins in microbial communities is hypothesized which warrants closer inspection.

Material and Methods

Study design

Vaginal fluid samples of women analyzed here were a subset of the samples obtained during a randomized controlled clinical trial described previously (16). The trial protocol was approved by the local ethics committee (Ärztekammer Nordrhein - Medical Association North Rhine) and written consent was obtained from all participants. The clinical trial was conducted in accordance with the Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects. Principles and guidelines for good clinical practice were followed. The study was registered on ClinicalTrials.gov with the identifier NCT02687789. Briefly, women were included into the clinical trial if they were BV positive according to Amsel criteria and Nugent score and were biofilm positive on vaginal epithelial cells and positive for extracellular polysaccharides (EPS) in urine. For treatment of acute BV, they received 2 g of metronidazole orally and were afterwards treated with an intravaginal pessary

for three weeks, twice a week. Samples were taken during acute BV (visit 1), after receiving metronidazole 7 to 28 days after visit 1 (visit 2), after pessary application one week after visit 2 (visit 3), after continued pessary application two weeks after visit 3 (visit 4) and during follow up three months after visit 4 (visit 5).

The aim of the clinical trial had been to compare the effectiveness of two different types of pessary. The results and the taxonomic composition of the vaginal microbial communities have been reported (16). For the metatranscriptome analysis reported here, we chose a subset of 14 patients from the clinical trial. These 14 patients consisted of two groups named “with response to treatment” (n = 8) and “no response to treatment” (n = 6) (Fig. 1). Among the eight patients who responded to treatment, six were cured without recurrence and two experienced recurrence during the three months follow-up. For the analysis of recurrence, samples from all 5 visits were analyzed for 4 patients (two cured and two with recurrence) (20 samples in total). These four patients all received the commercially available lactic acid pessary after metronidazole therapy at visit 3 and 4. For the analysis of lack of response to metronidazole, samples from all 14 patients were analyzed at two time points, acute BV (visit 1) and 7 to 28 days after antibiotic treatment (visit 2) (28 samples in total). Nugent score > 6 was used to determine BV status, since it is considered the gold standard for BV diagnosis (40) (Table S1 sheet 1). The BV status at visit 5 was determined by Amsel criteria since there was no Nugent score available at that time point. The sample ID was obtained by concatenating the patient ID and letters “a” to “e” indicating visit 1 to 5.

Sample collection and transport

Vaginal fluid was obtained by infusing 2 ml of saline solution into the vagina followed by rotation against the vaginal wall with a speculum and then collecting the vaginal fluid with a syringe. Approximately 700 µl were immediately transferred to a tube containing 2 ml

RNAprotect (Qiagen, Germany). Tubes were immediately frozen at -20°C, transported at -20°C within a week and stored at -70°C.

RNA extraction and mRNA enrichment

RNA was extracted from 1 ml vaginal fluid suspension using the MO BIO PowerMicrobiome™ RNA Isolation Kit (Qiagen, Germany) with pretreatment: Vaginal fluid was centrifuged at 13,000 rpm for 1 minute. The pellet was resuspended in MoBio lysis buffer and this suspension was added to the supplied bead tubes filled with 500 µl ice-cold phenol-choloroformisoamylalcohol solution (Carl Roth, Germany). The bead-suspension mix was shaken at 5 m/s for 1 minute in 3 intervals which were 2 minutes apart using the MO BIO PowerLyzer™ (Qiagen, Germany). After centrifugation for 1 minute at 13,000 rpm and 4°C the upper phase containing the RNA was further processed according to the manufacturer's instructions including DNAse I treatment. RNA was eluted in 100µl nuclease free water and vaccum concentrated to 50 µl. The Ribo-Zero Gold rRNA Removal Kit (Epidemiology) by Illumina (USA) was then used for mRNA enrichment with ethanol precipitation according to the manufacturer's instructions. Integrity of RNA was evaluated using a Bioanalyzer 2100 (Agilent, Germany).

Library preparation, sequencing and preprocessing of sequencing data

Paired-end mRNA Illumina sequencing libraries were constructed with the Script Seq Illumina Kit. Strand specific paired end sequencing was performed on the HiSeq 2500 Sequencer to yield 2 × 110 bp paired-end reads. Primers and sequencing adaptors were removed from raw sequencing data, followed by clipping the bases with quality score < 20 from the reads using Fastq-Mcf (2). After clipping, the remaining reads shorter than 50 were

removed. Thereafter, the ribosomal RNA reads were eliminated using SortMeRNA v2.0 (23) with the default parameters.

Taxonomy assignment using Kraken

Kraken (58), an accurate and ultra-fast taxonomy assignment tool for metagenomes was used to determine the taxonomic composition of the metatranscriptome data. Kraken uses the K-mer strategy and the lowest common ancestor (LCA) algorithm to affiliate a given read to a taxon. The standard Kraken database from the Kraken program with addition of the human genome was used to identify human reads. This standard database consists of prokaryote genomes (2786), virus genomes (4418) and with addition of human genome (ver. GRCh38) was downloaded from NCBI.

The refined Kraken database contained 163 bacterial genomes from 105 species of bacteria (Table S1 sheet 3), including 147 genomes from urogenital subset of HMP reference genome sequence data (HMRGD) (38). To be able to identify transcripts from bladder isolates, we added the genomes of *Gardnerella sp.* 26-12 and *Gardnerella sp.* 30-4 which were isolated from the bladder recently (35). To make sure that the transcripts mapped to the *Gardnerella sp.* bladder isolates did not actually originate from *G. vaginalis*, we included 5 additional genomes (original HMP urogenital tract database contains 4 *G. vaginalis*) from distinct *G. vaginalis* strains in the refined Kraken database. The complete list of reference genomes in the refined Kraken database was compiled as Table S1 sheet 3. Subsequently, the cleaned putative microbial mRNAs reads were mapped against this refined database. All results on the taxonomic composition in this study were achieved based on this database.

Short reads alignment by BWA

Since Kraken was only used for taxonomy classification, BWA was applied to determine the expression of functional genes. A customized reference gene database combining the genes from the urogenital tract subset of the HMRGD (38) and 9 additional genomes (Table S1 sheet 4) was used as mapping reference for BWA to map all cleaned putative mRNA reads for functional activity profiling. The genes of *Gardnerella sp.* 26-12, *Gardnerella sp.* 30-4 were not included due to the poor quality of the genomes. The short reads alignment was performed using BWA with the BWA-MEM (26) algorithm. A mapping seed length of 31 which is much longer than the default seed length 19 was applied to achieve more reliable alignments. To avoid a biased mapping, the reads mapped with mapping quality score (MAPQ) lower than 10 were excluded. MAPQ contains the Phred-scaled posterior probability that the mapping position is wrong (25). A MAPQ of 10 indicates a probability of 10% that the alignment position is wrong.

KEGG Orthologous (KO) gene annotation of customized reference database

The customized short reads alignment reference gene database which was used for BWA was annotated using KEGG prokaryote protein sequences to calculate the expression level of KO genes for differential KO gene expression analysis and KEGG pathway enrichment analysis. The KEGG prokaryote protein sequence database represents a non-redundant protein dataset of prokaryote (Bacteria and Archaea) on the species level and contains about 7 million non-redundant peptide sequences grouped into 14,390 distinct KO genes. A KO gene contains several genes from different species with similar function. DIAMOND (7), a much faster alternative to BLASTX was applied to map the customized short reads alignment reference sequences against the KEGG prokaryote protein sequence database with its “more sensitive

mode". To obtain reliable annotation, only alignments with sequence identity >=50 and E-value <= 1e-5 and query coverage >= 70% were taken into account. By annotating the reference genes to KO genes, we were able to determine the expression profile of KO genes, and investigate the functional activity shifts in BV based on differential expression analysis of KO genes and perform KEGG pathway enrichment analysis.

Differential expression (DE) analysis

All differential expression (DE) analyses were performed using the R package edgeR (48). The Benjamini Hochberg (BH) method was used to correct the p value of DE analysis with the false discovery rate (FDR) for multiple comparisons. Genes with FDR smaller than 0.05 were considered as significantly differentially regulated, which were then used for KEGG pathway enrichment analysis. The sample groups defined for each comparison are listed in Table S1 sheet 1. The groups defined in column "group for G vaginalis" is for the DE analysis of KO genes between *G. vaginalis* in communities with no response to metronidazole and with response. To avoid the analysis bias introduced by zero-inflated data, only the samples with relative abundance of *G. vaginalis* higher than 20% according to the taxonomy assignment were taken into account. In these two columns, "NR1" represents samples at visit 1 from patients with no response to metronidazole after treatment, "NR2" indicates the samples from the same patients at visit 2. And "WR1" denotes samples at visit 1 from patients with response to metronidazole after treatment, while "WR2" means samples from the recurrent time point. Column "group for LC LI" is for the DE analysis for KO genes between *L. crispatus* in the communities dominated by *L. crispstus* ("LC") and *L. iners* in the communities dominated by *L. iners* ("LI").

Detection of putative metronidazole resistance related genes

To detect the expression of previously reported putative metronidazole resistant genes such as recA, recA-mediated autopeptidase (Rma), peroxiredoxin, nitroimidazole resistance protein (NIM), ferredoxin/ferredoxin-NADP reductase (FNR), nitroreductase, sialidase and ferredoxin, we examined the expression level of these genes for *G. vaginalis* in the vaginal community from patients without response to metronidazole treatment (n = 6) as well as with response (n = 4). As most of these genes do not have corresponding KO genes, we annotated the BWA reference database based on the sequences of these genes using BLASTN. These sequences were retrieved from ENA by key words of each "ferredoxin, ferric uptake regulator, NADPH flavin oxidoreductase, nitroreductase, peroxiredoxin, pyruvate ferredoxin oxidoreductase, recA, nitroimidazole resistance, sialidase" plus *G. vaginalis*. In total, 155 unique sequences of *G. vaginalis* were obtained for the annotation of BWA reference database. The identification of duplicate sequences was done by SeqKit (49).

Availability of data and material

The metatranscriptome sequencing data of this subgroup analysis have been deposited in European Nucleotide Archive and are accessible through study accession number PRJEB21446.

Authors' contributions

This study was designed by IWD and CG. CM and CA provided the clinical samples. RNA extraction and mRNA enrichment was performed by CG. SB prepared cDNA libraries, and performed Illumina sequencing. Z-LD performed all the data analyses. Data interpretation and visualization were done by IWD, Z-LD and CG. Z-LD and CG wrote the manuscript draft, and all authors reviewed the manuscript.

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Figures, tables and additional files

Fig. 1: Taxonomic composition of vaginal fluid metatranscriptomes in BV and after treatment with metronidazole. Species with average relative abundance smaller than 0.5% were grouped into “Others”. The red dot on top of the samples indicates BV. The digits indicate the patient ID, while the letters a-e denote visit 1-5. After the first sampling at visit 1 the patients were treated with metronidazole. Total putative bacterial mRNA reads were mapped to the refined reference database using Kraken (see Methods for details). BV status was determined by Nugent score.

Fig. 2: : The cumulative dominance of the vaginal microbiota in BV and health based on metatranscriptome. Red denotes the dominance curve in BV while blue represents the dominance in health. Y-axis indicates the cumulative relative abundance.

Fig. 3: Average taxonomic composition of vaginal fluid samples in BV determined by 16S rRNA amplicon sequencing (A) and metatranscriptome sequencing (B) and taxonomic composition of communities on the species level determined by metatranscriptome sequencing in health and BV (C). In A, amplicon sequencing was performed as described in our previous study (16) using primers V1-V2. For B and C, taxonomy was assigned based on all cleaned reads after removal of human reads using Kraken and the refined reference database. Species with average relative abundance over 2% are shown in A and B. Relative average abundance was calculated based on all mapped reads. Mean and standard error are shown. In C, only species with relative abundance over 2% in at least 1 sample are shown. Red and pink dots on top of the heatmap indicate paired samples of patients which developed recurrence.

Fig. 4: Distinct functional activity profiles in health and BV (A), temporal dynamics of the metatranscriptome during treatment (B), and differential KO gene expression of *Lactobacillus crispatus* and *L. iners* (C). A) Principal components analysis of KO gene expression shows highly similar community activity in BV which is clearly distinct but much more variable in health. **B)** Time course based on PCA of functional activity profiles from four women during treatment and recurrence over a period of three months (five sampling time points). Two women with recurrence (pink and blue color range) and two women without recurrence (green and orange color range) are shown. In the figure legend, BV indicates time point with BV, R indicates the recurrence and H represents health. The green and blue circles highlight healthy clusters, respectively, while the red circle highlights samples from BV. The arrows denote the temporal shifts of the communities during the treatment. **C)** *L. crispatus* and *L. iners* exhibit distinct function activities. The Venn diagram indicates the unique KO genes of *L. crispatus* and *L. iners* as well as their shared KO genes. The innermost ring denotes the expression of KO genes by log2CPM, the outer ring illustrates the fold change of the expression of KO genes between *L. crispatus* dominated communities and *L. iners* dominated communities by log2FC. The small red triangle marks the inerolysin gene, while blue triangles mark the genes involved in the production of hydrogen peroxide.

Fig. 5: Seven *cas* genes and a methyltransferase gene of *G. vaginalis* were highly up-regulated in communities which did not respond to the treatment. KO genes with FDR <= 0.05 are colored in red or turquoise (significantly differentially regulated) while FDR > 0.05 are in grey.

Table 1. Overview of sequencing results

Overview of sequencing results	BV (22 samples)	Health (18 samples)
raw reads	44,503,332 ± 8,934,125	50,048,447 ± 10,989,072
cleaned reads after quality control and rRNA removal	32,506,992 ± 5,955,546	36,797,904 ± 6,934,913
putative microbial mRNA reads	27,524,573 ± 6,035,462	8,686,750 ± 5,867,867

Table 2. Mapping statistics using Kraken and BWA

Mapping statistics	% mapped putative microbial mRNA reads
standard Kraken database	40.55 ± 23.93
refined Kraken database	85.88± 13.99
customized reference gene database using BWA	~ 78.02 ± 16.31

Fig. S1: Differential expression of putative metronidazole resistance associated genes of *G. vaginalis* in vaginal fluid microbiota that did not respond to treatment. The expression value was calculated based on relative abundance of reads mapped onto *G. vaginalis* using BWA. "NR1" (No Response 1) indicates the samples from BV that did not respond to metronidazole treatment later; "NR2" (No Response 2) indicates the samples after treatment with no response to the treatment. "WR1" (With Response 1) represents the samples with acute BV which afterwards responded to metronidazole.

Supplementary Table S1: All supplementary data

Sheet 1. Sample description

Sheet 2. Read summary

Sheet 3. Genomes in the refined Kraken reference genome database

Sheet 4. Customized reference gene database for BWA

Sheet 5. Species composition determined by Kraken based on refined database

Sheet 6. The comparison of the community composition identified by 16S and metatranscriptome (average relative abundance >= 2%)

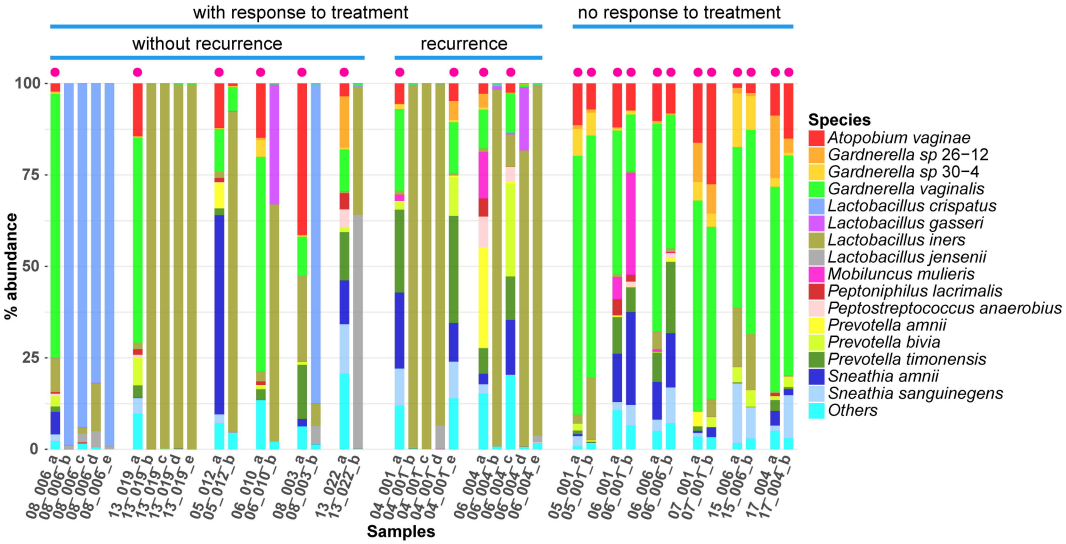
Sheet 7. The gene expression based on customized reference gene database with KO annotation

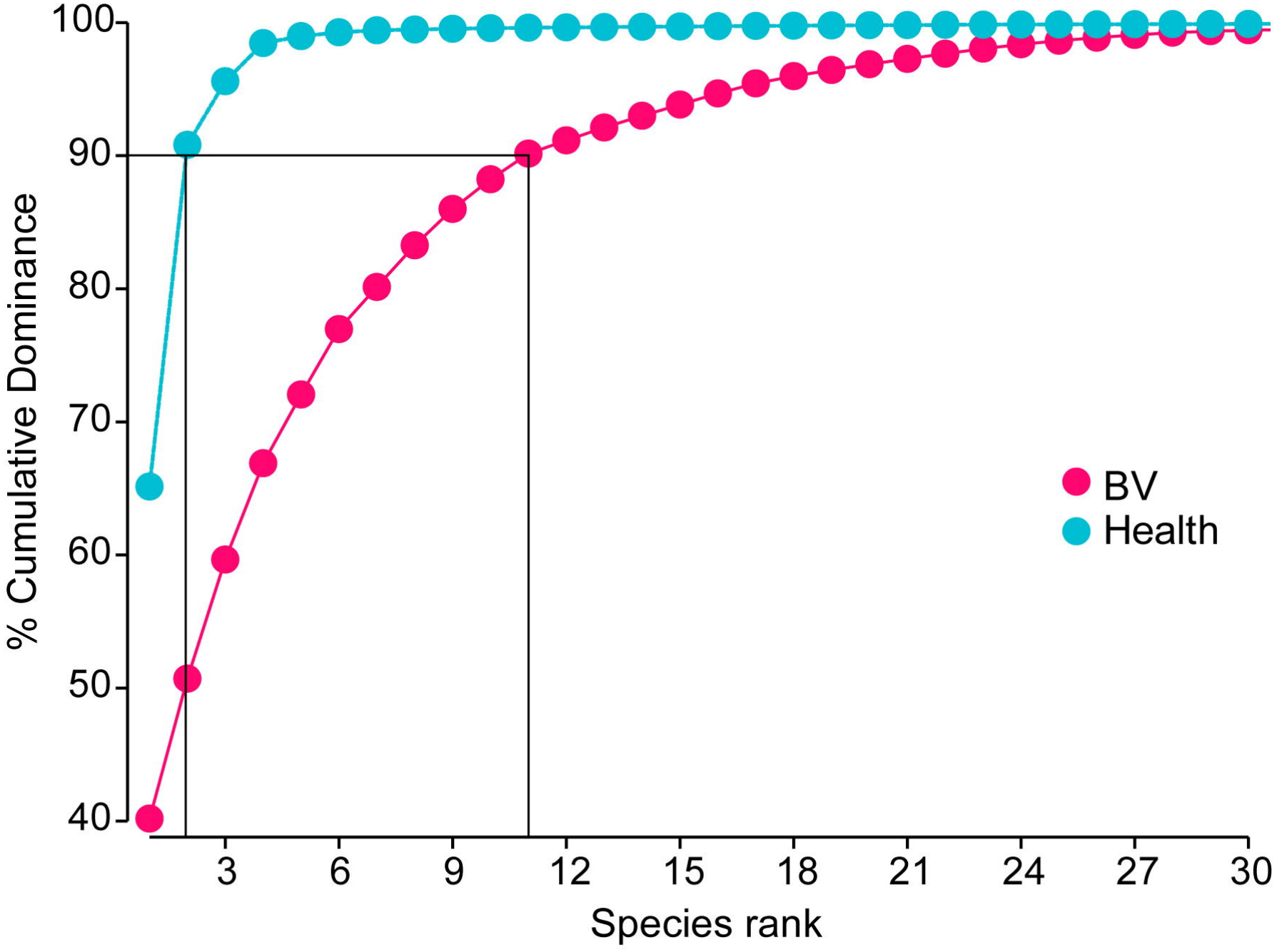
Sheet 8. The differential expression of KO genes between *L. crispatus* and *L. iners*

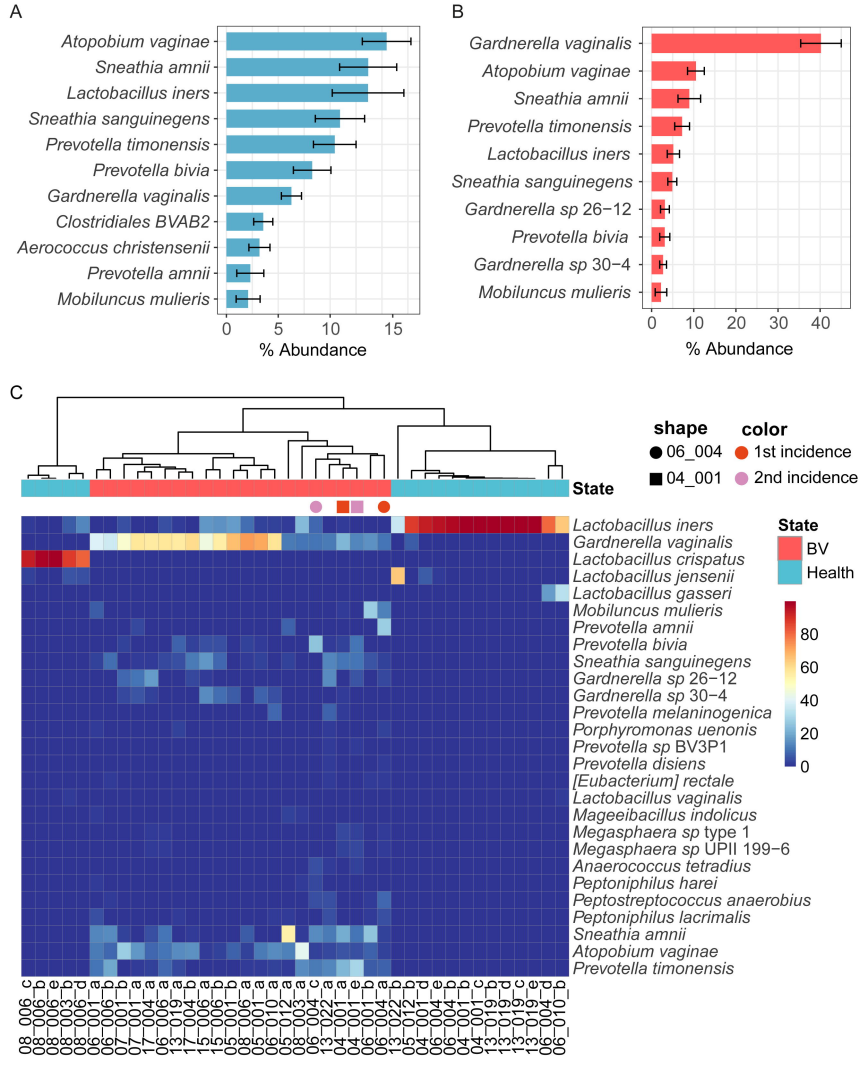
Sheet 9. KEGG pathway enriched in up-regulated KO genes in *L. crispatus*

Sheet 10. Expression of metronidazole associated genes in *G. vaginalis*

Sheet 11. The differential expression of KO genes of *G. vaginalis* from community without response to metronidazole treatment and with response


with response to treatment
no response to treatment
without recurrence
recurrence
% abundance
100
75
50
25
0
Samples
08_006_a
08_006_b
08_006_c
08_006_d
08_006_e
13_019_a
13_019_b
13_019_c
13_019_d
13_019_e
05_012_a
05_012_b
06_010_a
06_010_b
08_003_a
08_003_b
13_022_a
13_022_b
04_001_a
04_001_b
04_001_c
04_001_d
04_001_e
06_004_a
06_004_b
06_004_c
06_004_d
06_004_e
05_001_a
05_001_b
06_001_a
06_001_b
06_006_a
06_006_b
07_001_a
07_001_b
15_006_a
15_006_b
17_004_a
17_004_b
Species
Atopobium vaginae
Gardnerella sp 26−12
Gardnerella sp 30−4
Gardnerella vaginalis
Lactobacillus crispatus
Lactobacillus gasseri
Lactobacillus iners
Lactobacillus jensenii
Mobiluncus mulieris
Peptoniphilus lacrimalis
Peptostreptococcus anaerobius
Prevotella amnii
Prevotella bivia
Prevotella timonensis
Sneathia amnii
Sneathia sanguinegens
Others


100
90
80
70
60
50
40
% Cumulative Dominance
3
6
9
12
15
18
21
24
27
30
Species rank
BV
Health


A
Atopobium vaginae
Sneathia amnii
Lactobacillus iners
Sneathia sanguinegens
Prevotella timonensis
Prevotella bivia
Gardnerella vaginalis
Clostridiales BVAB2
Aerococcus christensenii
Prevotella amnii
Mobiluncus mulieris
0
5
10
15
% Abundance
B
Gardnerella vaginalis
Atopobium vaginae
Sneathia amnii
Prevotella timonensis
Lactobacillus iners
Sneathia sanguinegens
Gardnerella sp 26−12
Prevotella bivia
Gardnerella sp 30−4
Mobiluncus mulieris
0
10
20
30
40
% Abundance
C
shape
06_004
04_001
color
1st incidence
2nd incidence
State
State
BV
Health
80
60
40
20
0
Lactobacillus iners
Gardnerella vaginalis
Lactobacillus crispatus
Lactobacillus jensenii
Lactobacillus gasseri
Mobiluncus mulieris
Prevotella amnii
Prevotella bivia
Sneathia sanguinegens
Gardnerella sp 26−12
Gardnerella sp 30−4
Prevotella melaninogenica
Porphyromonas uenonis
Prevotella sp BV3P1
Prevotella disiens
[Eubacterium] rectale
Lactobacillus vaginalis
Mageeibacillus indolicus
Megasphaera sp type 1
Megasphaera sp UPII 199−6
Anaerococcus tetradius
Peptoniphilus harei
Peptostreptococcus anaerobius
Peptoniphilus lacrimalis
Sneathia amnii
Atopobium vaginae
Prevotella timonensis
08_006_c
08_006_b
08_006_e
08_003_b
08_006_d
06_001_a
06_006_b
07_001_b
07_001_a
17_004_a
06_006_a
13_019_a
17_004_b
15_006_a
15_006_b
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13_022_a
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13_022_b
05_012_b
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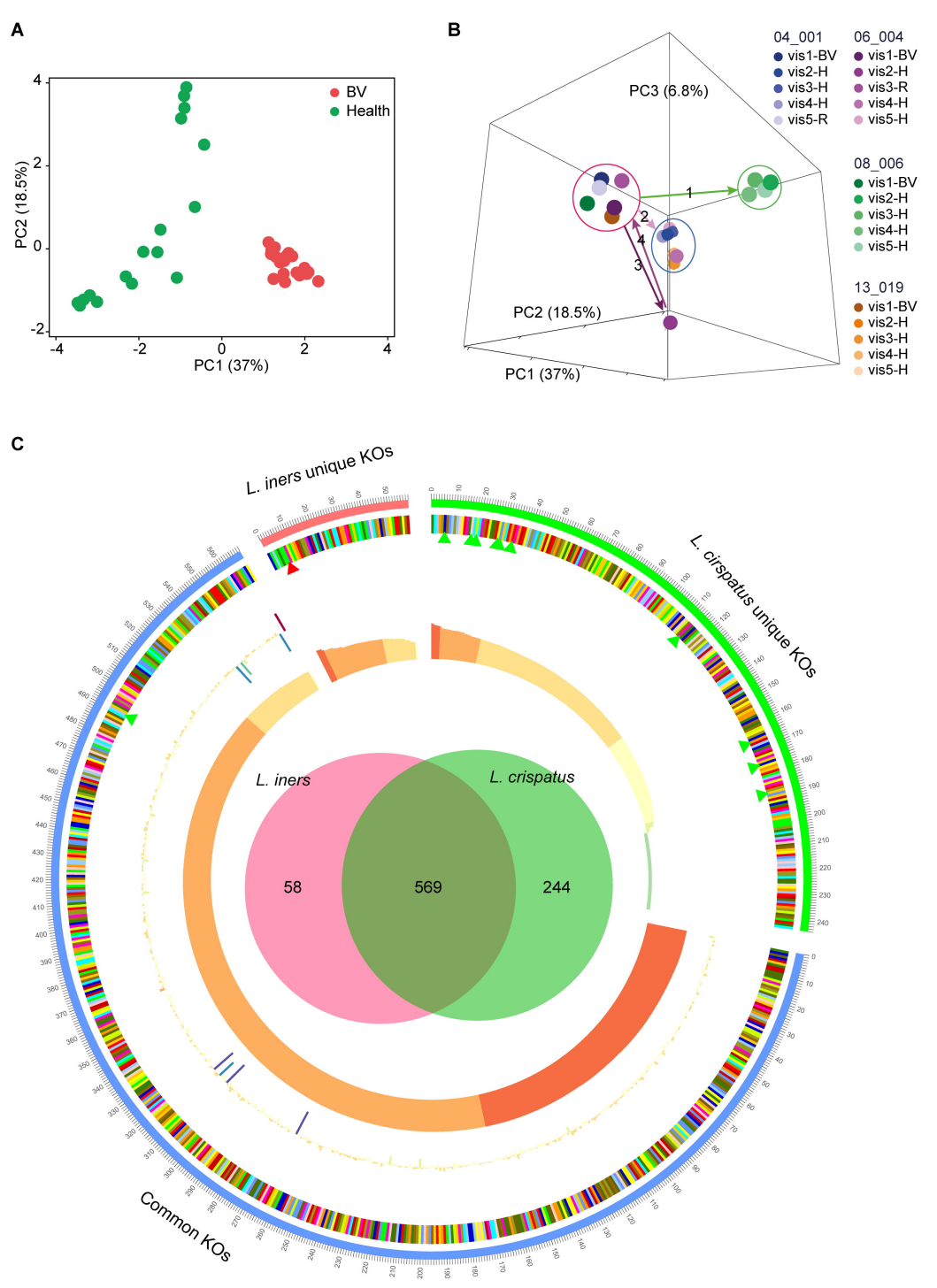
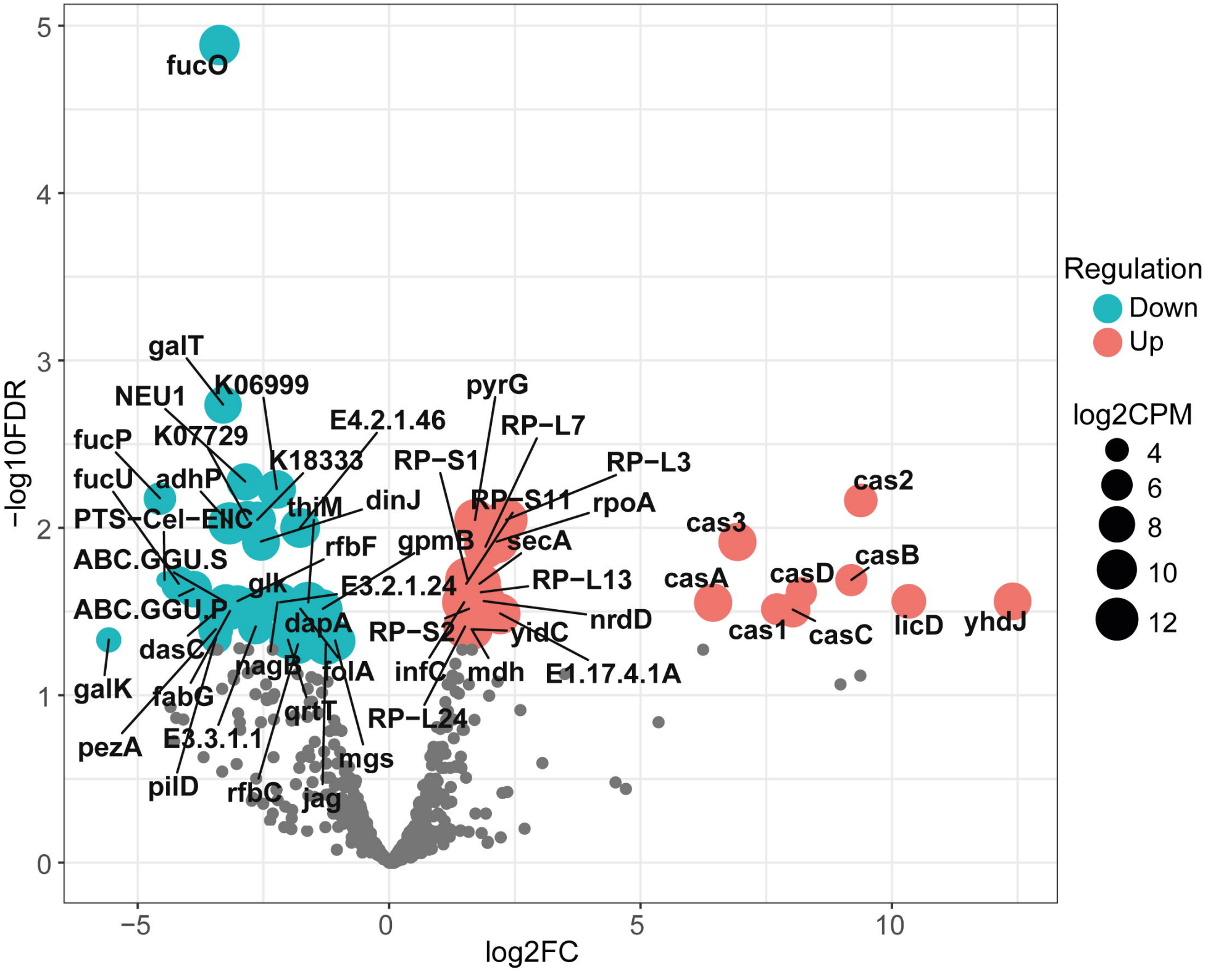
06_010_b


A
BV
Health
PC2 (18.5%)
PC1 (37%)
B
04_001
vis1-BV
vis2-H
vis3-H
vis4-H
vis5-R
06_004
vis1-BV
vis2-H
vis3-R
vis4-H
vis5-H
08_006
vis1-BV
vis2-H
vis3-H
vis4-H
vis5-H
13_019
vis1-BV
vis2-H
vis3-H
vis4-H
vis5-H
PC3 (6.8%)
PC2 (18.5%)
PC1 (37%)
C
L. iners unique KOs
L. cirspatus unique KOs
L. iners
L. crispatus
58
569
244
Common KOs



fucO
galT
K06999
NEU1
pyrG
E4.2.1.46
RP−L7
fucP
K07729
K18333
RP−S1
RP−L3
fucU
adhP
thiM
dinJ
RP−S11
rpoA
cas2
PTS−Cel−EIIC
cas3
rfbF
gpmB
secA
ABC.GGU.S
casB
glk
E3.2.1.24
RP−L13
casA
casD
ABC.GGU.P
dapA
nrdD
RP−S2
yidC
cas1
casC
licD
yhdJ
dasC
nagB
folA
infC
mdh
E1.17.4.1A
galK
fabG
qrtT
RP−L24
pezA
E3.3.1.1
mgs
pilD
rfbC
jag
−log10FDR
log2FC
Regulation
Down
Up
log2CPM
4
6
8
10
12