# Vaginal Lactobacillus iners abundance predicts outcome in antibiotic treatment of bacterial vaginosis

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**Abstract**

OBJECTIVES: To examine the distribution of Gardnerella genomospecies in a Chinese cohort, investigate its relationship with BV and elucidate the potential function of L. iners in predicting the clinical outcome of BV. POPULATION: 130 non-pregnant BV patients and 41 healthy women from Peking University First Hospital. MAIN OUTCOME MEASURES: Patients visited clinic again after antibiotic treatment and divided into three groups according to Nugent score. METHODS: Vaginal swabs used for microscopic examination, 16SrRNA sequencing, bacterial culture and isolation and Gardnerella vaginalis, Atopobium vaginae and Lactobacillus iners isolates used for competition tests. RESULTS: Seven Gardnerella genomospecies were presented in all participants and relative abundance of all detected genomospecies were higher in BV patients ($p<0.05$). Cured patients possessed higher GS03 compared to other groups ($p=0.005$, 0.0337). L. iners was significantly higher in cured patients compared to other groups ($p=0.0021$, $p<0.0001$) and it was able to inhibit the growth of Gardnerella vaginalis and Atopobium vaginae. CONCLUSION: Seven Gardnerella genomospecies can be detected in Chinese BV patients, but its distribution is not related to BV. Cured patients possess higher relative abundance of L. iners is higher and L. iners can inhibit growth Gardnerella vaginalis and Atopobium vaginae. L. iners might become a predictive indicator of clinical outcomes of BV patients and its antimicrobial function might be beneficial to BV patients. FUNDING: National Key Research and Development Program of China (2021YFC2301000) and the National Natural Science Foundation of China (81971342). Key words: bacterial vaginosis, Gardnerella genomospecies, Lactobacillus iners, 16SrRNA sequencing, antimicrobial activity.

## INTRODUCTION

Bacterial vaginosis (BV) is the most common lower genital tract infection in reproductive-aged women, affecting about 4-75% reproductive-aged women internationally[1-3]. BV is characterized as a dysbiosis of vaginal microbiome in which the*Lactobacillus spp* dominant flora is lost[1], accompanied by significant increase of anaerobic bacteria, including*Gardnerella* , *Atopobium* , *Prevotella* ,*Mobiluncus* and so on[4-7]. Metronidazole and clindamycin are recommended for BV treatment and the short-term cure rate can reach up to 70%, but up to 60% women will experience at least one episode of BV recurrence within 12 months[8]. The recurrent episodes of BV have been demonstrated to be related to a variety of adverse outcomes in gynecology and obstetrics, such as sexually transmitted diseases, cervical cancer, infertility, premature birth and so on[1,9-11].

However, the exact etiology of BV remains unclear. In the past studies, a variety of anaerobic bacteria are proved to be related to BV, while*Gardnerella* has attracted special attention as 16SrRNA sequencing-based techniques have revealed that it could be detected in almost all BV patients and its ability to form polymicrobial biofilm is related to refractory or recurrent BV[12-14]; yet paradoxically, 40% of healthy women are also positive for such bacteria[13]. Therefore, whether this species is the contributing pathogen for BV remains debatable[3,15,16]. In recent years, 9 genomospecies of *Gardnerella* are recognized through cpn60 gene typing, whole genome sequencing and other methods[17-21]. Many investigations focused on identifying the differences of the ability to adhere to vaginal epithelial cells, virulence and drug resistance among genomospecies and

the relevancy of the distribution of *Gardnerella* genomospecies with the occurrence, symptoms or the clinical outcome of BV, but the results lack consistency[3,22-28].

With respect to the normal vaginal microbiome, researches have also accumulated and concluded that the loss of *Lactobacillus* is an essential part in the progression of BV[1]. Based on 16S rRNA sequencing of women across countries and ethnic groups, it is generally accepted that *L. crispatus* , *L. iners* , *L. gasseri* and *L. jensenii* are the four most commonly detected*Lactobacillus* species in the vaginal microbiome[7]. However, the exact species of*Lactobacillus* that contributes to either development of BV, or the response to treatment, is yet to be examined. Thus, our study profiled the vaginal microbiome in healthy women and BV patients, before and after antibiotic treatments, and explored the potential contributions of *Gardnerella* and *Lactobacillus* in treatment outcome of BV at genomospecies or species level.

## MATERIALS AND METHODS

### Cohort Recruitment and Sample Collection

The samples were collected from 308 reproductive age, HIV-negative, non-pregnant woman patients who came to Peking University First Hospital with major complaints about vulvovaginal discomfort and/or abnormal vaginal discharge during the year of 2020-2021. All par were tested for HIV, HPV, HSV-2, syphilis, *Chlamydia trachomatis* , *Neisseria gonorrhoeae* , vulvovaginal candidiasis, *Trichomonas vaginalis* ,*Ureaplasma urealyticum* , *M. hominis* , and urinary tract infections. Patients infected with either kind of pathogens above or diagnosed with any internal disease were excluded from this study. Meanwhile, 41 healthy women came to Peking University First Hospital for annual physical examination were included as negative control. Three samples of vaginal microbiome samples were collected from the same position of upper 1/3 of anterior vaginal wall with vaginal swabs (Becton, Dickinson and Company) during inspection. The first swab was used for DNA extraction and sequencing and stored in the environment of -80°C immediately. The second swab was used for Gram staining, microscopic examination and evaluation of biological parameters. The last swab was used for bacterial culture, isolation and purification. The studies involved human participants were reviewed and approved by The Ethics Committee of Peking University First Hospital. All participants have signed the informed consent in written form for the publication of any potentially identifiable images or data included in this article and agreed to be involved in our follow-up voluntarily.

### Diagnostic Procedures and Treatment

The presence of BV is diagnosed by Gram stain-based Nugent score (score of 0-3 is considered to be negative for BV, 4-6 intermediate status, 7-10 BV) and Amsel criteria (BV is diagnosed when at least two of the following criteria are fulfilled: vaginal pH>4.5, release of fishy order when addition of 10% potassium hydroxide, and/or 20% clue cells presented in one glass slide). Two experienced technicians were involved in microscopic examination separately and blind to each other to make sure the authenticity of diagnosis. Patients who were diagnosed with BV were prescribed with topical 5% metronidazole gel for 5 days. All patients were asked to visit their gynecologist again within one week after completion of their treatment. Another two vaginal swabs were collected following the procedures above. The same diagnostic procedures mentioned ahead were repeated to confirm patients' clinical outcome and the patients were divided into three groups according to their Nugent score post-treatment: group cured (0-3), group intermediate (4-6) and group failed (7-10).

### Genomic DNA Isolation from Vaginal Samples

The vaginal swab and scrape samples were vortexed and centrifuged for 10 min at 10,000 g to collect the bacterial cells and the supernatant was discarded. All genomic DNA extractions were performed by using the DNeasy(r) Power Soil(r) Pro Kit (Qiagen) following the manufacturer's instructions.

### 16S rDNA Sequencing

A 16S rDNA gene fragment comprising the V3 and V4 hypervariable regions were amplified by using specific V3 forward primer 5'-CCTACGGGNBGCASCAG-3' and V4 reverse primer 5'-GACTACNVGGGTATCTAATCC-3'. The amplified products were checked and analyzed on 2% agarose

gel. Sequencing was performed using a 250-bp paired-end sequencing protocol on the Illumina NovaSeq6000 platform. Sequence analysis was performed following the previous study[29]. The sequences were merged using the FLASH program[30] and subjected to quality filtering using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Exclusion of chimeras was done using the UCHIME command and the 'GOLD' database[31]. After random rarefication of microbiome sizes to 6555 reads, the taxonomic assignment of reads was determined by RDP classifier4 to generate the composition matrices at the level of the phylum to the genus[32]. The rarefied 6,555 reads were also blasted against the 16s rDNA sequences of established*Gardnerella* genomospecies and *Lactobacillus* species (including *L. crispatus* , *L. iners* , *L. jensenii* and*L. gasseri* ) to identify the genomospecies of *Gardnerella*and *Lactobacillus.*

**Bacterial Isolation and Culturing Condition**

Vaginal swabs were immediately inoculated onto both Colombia blood agar, Laked Sheep Blood Agar with Kanamycin and Vancomycin and MRS broth (BD Difcoa) added with IsoVitaleXTM Enrichment (BD BBLTM; 2% v/v) and L-Cys (augmented by L-Gln, with final concentration of 1.1mM, respectively). All broths mentioned above were securely stored until used. The broths were placed into an anerobic environment set at 37degC using AS-580 anaerobic chamber (Anaerobic system), with an atmosphere of 5% carbon dioxide, 5% hydrogen, and 90% nitrogen (AirgasO) for 24-48 hours. All bacteria colonies from both all broths were picked out, purified and identified through 16S sequencing. *G. vaginalis* , *A. vaginae* and *L. iners* were tittered and maintained on Columbia blood agar.

**Antimicrobial Activity Evaluation**

Purified *Gardnerella vaginalis* and *Atopobium vaginae*strains were spread onto Colombia blood agar and coated all boards. Another Colombia blood agar containing isolated *L. iners* or purchased *L. johnsonii* were placed onto the broth coated with either *G. vaginalis* or *A. vaginae* and cultured under the anaerobic condition mentioned above. 2 parallel tests for *L. johnsonii* and 3 parallel tests for *L. iners* were run to make sure the authenticity of our experiments. Diameter of the inhibition zone was measured after culturing for 24-48h.

**Statistical Analysis**

Statistical analysis on bacterial taxonomic identification was performed using R v4.1.1 software. The Wilcoxon test, Kruskal–Wallis' test and Kruskal-Wallis test in ggpubr package were used to the measure the difference in abundance.

## RESULTS

**Cohort description**

A total of 130 out of 308 patients who met the requirement and confirmed to have BV were included in our study. The clinical information of all participants is shown in **(Table S1)** . After standard 5-day metronidazole treatment, patients were divided into three groups according to their clinical outcome: 61 patients were cured (46.9%, group cured), 36 patients turned into intermediate BV (27.7%, group intermediate) while 33 patients remained BV (25.4%, group failed). There's no significant difference in the age between healthy women and BV patients (38.03 vs 37.19, p=0.4764, Kruskal-Wallis test). However, statistically significant differences could be noticed in both Nugent score (0.58 vs 7.88, $p<0.0001$, Kruskal-Wallis test) and pH (4.21 vs 5.02, $p<0.0001$, Kruskal-Wallis test) between two groups. Furthermore, we analyzed the differences of Nugent score and vaginal pH among the three groups before treatment, with no significant differences found **(Figure S1)** .

**BV patients have higher *Gardnerella*, *Prevotella*and *Atopobium***

We analyzed all vaginal microbiota through 16SrRNA sequencing**(Figure 1)** . The results reveal that *Lactobacillus. spp*are the most dominant species in healthy women (78.95%), while 16 (39%) of whom are *L. crispatus* dominant and 14 are *L. iners* dominant (34%). In contrast, BV related bacterial species are the most prevalent taxa in BV patients before taking any medications:*Gardnerella. spp* (35.61%),*Prevotella*(11.66%)

and *Atopobium* (10.69%) were among the top three highest relative abundance in BV patients **(Table S2)** . In terms of relative abundance before treatment, all three bacteria were statistically higher than healthy women ($p<0.0001$, Kruskal-Wallis test), while the relative abundance of each bacterium was similar among groups before the application of metronidazole and not statistically significant ($p>0.99$, Kruskal-Wallis test) **(Figure 2)** .

We then analyzed the vaginal microbiome in BV patients after metronidazole treatment, and found significant differences in microbiome composition can be detected among patients in different clinical outcome groups. In cured patients (group cured), the relative abundance of all three BV-associated bacteria significantly decreased ($p<0.0001$, Wilcoxon test), yet the relative abundance of*Gardnerella. spp* was still higher than healthy cohort ($p<0.0001$, Wilcoxon test). Relative abundance of both*Atopobium* and *Prevotella* decreased posttreatment in intermediate group (group intermediate, $p=0.0103$, $p<0.0001$, Wilcoxon test), but the *Gardnerella. spp* did not change significantly ($p=0.0946$, Wilcoxon test). In contrast to the two groups with improvement, no significant decrease of any bacteria is detected in patients without improvement (group failed). Intergroup comparison shows that patients in group cured has lower relative abundance of*Gardnerella. spp* , *Atopobium* and *Prevotella* than group failed ($p=0.0009$, $p<0.0001$, $p<0.0001$, Kruskal-Wallis test), lower relative abundance of *Atopobium* and*Prevotella* than Group intermediate ($p=0.0002$, $p=0.0038$, Kruskal-Wallis test). Meanwhile, group intermediate contained lower relative abundance of *Atopobium* and *Prevotella* compared to Group failed ($p=0.0022$, $p=0.0254$, Kruskal-Wallis test), but relative abundance of *Gardnerella. Spp* showed no statistical differences between Group cured and Group intermediate, or between Group intermediate and Group failed **(Figure 2)** .

### *Gardnerella* genomospecies were not associated with BV treatment outcome.

Since former studies have recognized nine different *Gardnerella*genomospecies via whole genome sequencing, yet only seven genomospecies are detected in our specimen, namely GS01, GS02, GS03, GS05, GS07, GS08 and GS09, with decreasing abundance. Each detected *Gardnerella*genomospecies in BV patients was increased compared to healthy women pretreatment ($p<0.01$, Kruskal-Wallis test) **(Table S3)** . When comparing between groups of patients with different treatment outcomes, we found that that only the abundance of GS03 in group cured was significantly higher than group intermediate and group failed before treatment ($p=0.005$, 0.0337, Kruskal-Wallis test), while abundances of other genomospecies shows no significant differences among groups**(Figure 3)** .

With respect to treatment outcome, in group cured, relative abundance of every *Gardnerella* genomospecies was decreased posttreatment ($p<0.05$, Wilcoxon test), but only the relative abundance of GS07, GS08 and GS09 was restored to level similar to that of healthy individuals **(Figure 3)** . In Group intermediate or group failed, none significant changes were found in any genomospecies before and after treatment ($p>0.05$, Wilcoxon test). Further analysis showed that relative abundance of GS05, GS07 and GS08 was lower in group cured compared to group intermediate and all genomospecies was significantly lower compared to group failed; and between group intermediate and group failed, only GS03 showed statistical differences ($p=0.0265$, Kruskal-Wallis test) **(Figure 3)** .

### Higher *L. iners* is associated with positive outcome of BV treatment

As the four most commonly seen *Lactobacillus* species in reproductive aged women are *L. crispatus* ,*L. iners* ,*L. gasseri* and *L. jensenii,* we specifically allocated the sequences to the four species with stringent similarity threshold (99%). In the results we found *L. iners* to be the highest in terms of abundance in healthy individuals, with *L. crispatus* ,*L. jensenii* in decreasing order and *L. gasseri* has the lowest proportion. Relative abundance of *Lactobacillus spp* in BV patients was overall significantly lower than healthy people pretreatment, but only *L. crispatus* and *L. iners* were significantly different among BV patients and healthy women ($p<0.0001$, $p=0.0407$, Kruskal-Wallis test) **(Table S3)** . We also discovered that even though the relative abundance of*Lactobacillus spp* in total among three groups of BV patients were similar before treatment, the proportion of *L. iners* is higher in group cured than group intermediate and group failed pretreatment ($p=0.0021$, $p<0.0001$, Kruskal-Wallis test), while not significantly different

between group intermediate and Group failed (p>0.9999, Kruskal-Wallis test) **(Figure 4)** .

In addition, we found that *Lactobacillus spp* abundance in total was restored only in group cured after being treated with metronidazole (p=0.0048, Wilcoxon test), but at species level, only *L. iners*showed statistical difference (p=0.0007, Wilcoxon test) thus it is the most affected species. The same taxa showed no sign of restoration in neither group intermediate nor group failed. The relative abundance of*L. iners* was significantly different among groups with treatment outcomes, as group cured possessed higher *L. iners* relative abundance than group intermediate and group failed (p=0.02, p=0.0274, Kruskal-Wallis test). No difference was found between group intermediate and group failed in with regard to any other *Lactobacillus*species abundance (p>0.05, Kruskal-Wallis test)**(Figure 4)** .

### *Lactobacillus iners* inhibits *Gardnerella vaginalis*and *Atopobium vaginae* in vitro

As our results indicate that higher *L. iners* is associated with positive outcome of BV treatment, we hypothesized that *L. iners*might have inhibitory effect on the growth of BV-associated bacteria and thus facilitate the treatment outcome. We co-cultured clinically isolated *L. iners* with *G. vaginalis* or *A. vaginae,*and used *L. johnsonii* that has been reported to be capable of inhibiting the growth of series of pathogens as positive control. We found that, after culturing for 24-48h, inhibition zones were manifested in all parallel tests, no wonder co-cultured with *G. vaginalis* or*A. vaginae* , indicating the inhibitory effect of *L. iners*against the growth *of G. vaginalis* and *A. vaginae* , in the*in vitro* co-culture system **(Figure S2)** .

## DISCUSSION

### Main findings

7 *Gardnerella* genomospecies were detected in Chinese women. We found that only relative abundance of GS03 was higher in cured patients compared to group intermediate and group failed (p=0.005, 0.0337).*L. iners* was better preserved in cured patients compared to other two groups (p=0.0021, p<0.0001) and it was able to inhibit the growth of *Gardnerella vaginalis* and *Atopobium vaginae* is validated (average radius of inhibition zone: 1.97cm and 2.9cm).

Our study shows *L. iners* (30.33%), *L. crispatus* (29.21%) and *Gardnerella* (7.12%) ranked the top 3 most prevalent bacterium in Chinese healthy women and *Gardnerella* (37.12%),*Prevotella* (10.73%) and *Atopobium* (8.72%) ranked the top 3 in BV patients but no such correlation between relative abundance of*Gardnerella* , *Atopobium* or *Prevotella* and clinical outcomes are found, which is in contrast to the results of Marjin et al[33].

We detected 7 out of 9 genomospecies in Chinese women, with the absence of GS04 and GS06. Though lacking explicit conclusion, studies based on cohorts from other regions and ethics reported that different structures of the *Gardnerella* spp community are related with BV, manifesting as certain genomospecies are more abundant in BV patients, while others not[34,35]. Furthermore, researchers declared that the distribution of Gardnerella genomospecies might differ among different ethical groups[18]. However, based on a Chinese cohort, no differences are found in the distribution of all detectable genomospecies between healthy women and BV patients, with GS01, GS02, GS03 ranked the top three most prevalent and GS08 ranked the least. No genomospecies are thought to be specifically related to BV as the relative abundance of all genomospecies are significantly higher in BV patients compared to healthy women.

Former study has reported an association between high abundance of certain *Gardnerella* genomospecies or combination of several genomospecies with BV[17,25,36-38], and coinfection of GS03 and GS04 was thought to be related to negative clinical outcomes[18,39]. But in our study, we find that relative abundance of GS03 is even higher in cured patients. GS03 is proved to be more susceptible to metronidazole[40], which indicates that higher GS03 percentage might make the *Gardnerella spp*community more fragile to antibiotics treatment, thus predicting a more positive clinical outcome. Nevertheless, we still propose that this statistical difference of GS03 among groups might not be a convincing indicator to foresee patients' prognosis, as it only makes up 3.84% of the whole bacterial taxa and approximate 1/5 of the most abundant genomospecies GS01. Therefore, we think that analyzing the distribution of *Gardnerella* genomospecies based on their relative

abundance might not be useful to predict patients' clinical outcomes. We strongly suggest more attention should be drawn to better understanding the interactions among different BV associated bacteria instead of focusing on one single species, as BV is defined as a polymicrobial dysbiosis.

In the meanwhile, our study noticed that patients with more *L. iners* before treatment might have a better clinical outcome. This is a remarkable finding as it makes *L. iners* an innovative predictive parameter, yet former research pointed out the opposite opinion[41]. Different from other *Lactobacillus*species mentioned in this article, *L. iners* shows unique metabolic and genomic characteristics and its protective function is questionable compared to other*Lactobacilli* [42-45]. Its production of hydrogen peroxide and D-lactic acid is lower and inerolysin it secreted is thought to be a cholesterol-dependent cytotoxins which is homogenous to vaginolysin, expressed by several BV associated bacteria[46-49]. It is widely acknowledged that metronidazole will instantly reduce the load of vaginal microbiota and shift it into an *L. iners* dominant one[33,50,51], but this kind of structure is unstable and has the potential to lead to BV recurrence[52]. Though absent with consistent conclusion, *L. iners* is considered to be a "foe" instead of a "friend"[48,53,54]. However, our study proposed a different point of view as our *in vitro* experiments validated the inhibitory effect of *L. iners* against *G. vaginalis* and*A. vaginae* . We assume that when *L. iners* is preserved, its antimicrobial abilities might facilitate the therapy as it is resistant to metronidazole and able to scavenge pathogens simultaneously. Furthermore, considering *L. iners* is capable of synthesizing L-lactic acid and a small amount of D-lactic acid[55], we hypothesized that the restoration of *L. iners* after treatment might be a crucial part for other *Lactobacillus* to recover by maintaining an acidic environment and countering the growth of BV associated bacteria.

Our study sketches the distribution of *Gardnerella* genomospecies in Chinese BV patients and provides a new perception of the role*L. iners* playing in BV. Our result indicates that the relative abundance of *L. iners* might be a potential predictive marker of patients' clinical outcomes and the latent therapeutic value of *L. iners* is waiting to be unveiled. Furthermore, our study also emphasizes that getting more knowledge of vaginal microbiota pretreatment is essential, as it provides gynecologist with more information, modify their medical decisions subsequently and thus enhance clinical outcomes of BV patients.

**Strengths, limitations and interpretation**

This study is the first attempt to describe the distribution of*Gardnerella* genomospecies in a Chinese cohort, determine its relation with clinical outcomes of BV patients and validate the inhibitory effect of *L. iners* against *G. vaginalis* and*A. vaginae* through co-culture experiments. However, this is still a single-centered study with a small quantity of participants. In future studies, more participants are required to fully understand the*Gardnerella* genomospecies distribution in China and test the authenticity of the predictive value of *L. iners* . Moreover, more experiments are required to clarify the molecular mechanisms underneath the antimicrobial effect of *L. iners* and explore its latent therapeutic value.

**Conclusion**

Our research finds 7 *Gardnerella* genomospecies and reveals their distribution in Chinese cohort, yet no correlation between the distribution of *Gardnerella* genomospecies and patients' clinical outcomes are confirmed. Furthermore, we discovered *L. iners* as a new indicator for BV prognosis and validated its inhibitory effect against the growth against *Gardnerella vaginalis* and*Atopobium vaginae* . Finally, we suggest gynecologist to have better understanding of vaginal microbiota of BV patients to improve their overall health.

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**DISCLOSURE OF INTERESTs**

The authors declare no conflict of interest.

## CONTRIBUTION TO AUTHORSHIP

BX and JW designed the project. BX collected samples. JL ran the experiment procedure. All authors participated in data analysis, writing and discussing the contents of this article and approved the submitted version.

## DETAILS OF ETHICS APPROVAL

The studies involved human participants were reviewed, approved and supervised by The Ethics Committee of Peking University First Hospital. All participants have signed the informed consent in written form for the publication of any potentially identifiable images or data included in this article and agreed to be involved in our follow-up voluntarily.

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**[Figure 1] Vaginal microbiome structure of each participant**

This figure manifests the top 26 most abundant bacteria in participants' vaginal microbiome, organisms ranked 27 and below are all categorized into label "others". Figure-1A shows the vaginal microbiome of healthy participants, with Figure-1B showing BV patients pretreatment and Figure-1C showing posttreatment. The order is arranged according to the relative abundance of dominant *Lactobacillus* species in each patient from low to high.

**[Figure 2] Intergroup comparison of *Gardnerella, Prevotella* and *Atopobium***

Intergroup comparison of *Gardnerella* (Figure-2A),*Prevotella* (Figure-2B) and *Atopobium* (Figure-2C) relative abundance. Only statistically significant $P$ value that has clinical meaning is marked in the graph. Significance is exhibited as: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$; Wilcoxon test for pairwise comparison between pre- and posttreatment and Kruskal-Wallis test for comparisons among different groups.

**[Figure 3] Intergroup comparison of each *Gardnerella*genomospecies**

Intergroup comparison of the relative abundance of each*Gardnerella* genomospecies: GS01 (Figure-3A), GS02 (Figure-3B), GS03 (Figure-3C), GS05 (Figure-3D), GS07 (Figure-3E), GS08 (Figure-3F) and GS09 (Figure-3G). Only statistically significant *P* value that has clinical meaning is marked in the graph. Significance is exhibited as: *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001; Wilcoxon test for pairwise comparison between pre- and posttreatment and Kruskal-Wallis test for comparisons among different groups.

**[Figure 4] Intergroup comparison of 4 *Lactobacillus*species**

Intergroup comparison of the relative abundance of the four most abundant *Lactobacillus* species: *L. crispatus* (Figure-4A),*L. iner* s (Figure-4B), *L. gasseri* (Figure-4C) and *L. jensenii* (Figure-4-D). Only statistically significant *P* value that has clinical meaning is marked in the graph. Significance is exhibited as: *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001; Wilcoxon test for pairwise comparison between pre- and post-treatment and Kruskal-Wallis test for comparisons among different groups.

**[Table S1] Cohort Description**

| *Mean(95CI%)* | Healthy Participants | BV patients | P value[b] |
|---|---|---|---|
| | (N=41) | (N=130) | |
| Age | 38.03(35.09-40.97) | 37.19(35.64-38.74) | |
| Nugent Score | 0.58(0.31-0.83) | 7.88(7.65-8.12) | <.0001 |
| pH | 4.21(4.07-4.36) | 5.02(4.96-5.07) | <.0001 |
| Pregnancy Rate | None | None | |
| Infection of Other STI[a] | | | |

BV, bacterial vaginosis; STI, sexually transmitter infections.

[a]Other STI include HIV, HPV, HSV-2, syphilis,*Chlamydia trachomatis* , *Neisseria gonorrhoeae* , vulvovaginal candidiasis, *Trichomonas vaginalis* , *Ureaplasma urealyticum* , *M. hominis* , and urinary tract infections.[b]Only statistically significant P value is manifested in the table. Kruskal-Wallis test was used for inter-group comparison.

**[Table S2] Relative abundance of top 10 most abundant bacteria at genus level**

| | All Participants$_{(N=171)}$ | All Particip |
|---|---|---|
| Genus | Healthy | BV patients |
| *Relative abundance (%) of each genus, mean(95%CI)* | *Relative abundance (%) of each genus, mean(95%CI)* | *Relative abu* |
| *Lactobacillus* | 78.95(68.00-89.89) | 7.66(5.16-10 |
| *Gardnerella* | 7.12(1.70-12.54) | 35.61(31.73- |
| *Prevotella* | 0.98(0.10-1.85) | 11.66(9.64-1 |
| *Atopobium* | 1.25(-0.23-2.73) | 10.69(8.08-1 |
| *Megasphaera* | 0.12(-0.06-0.30) | 7.56(6.04-9.0 |
| *Aerococcus* | 0.60(-0.40-1.59) | 3.77(2.35-5.1 |
| *Saccharofermentans* | 0.12(-0.096-0.33) | 3.26(2.27-4.2 |
| *Dialister* | 0.30(-1.96-2.55) | 2.95(2.52-3.3 |
| *Streptococcus* | 0.31(0.045-0.57) | 0.95(0.23-1.6 |
| *Enterococcus* | 0.02(0.004-0.05) | 0.82(-0.10-1. |

BV, bacterial vaginosis.

[a] "Group Cured" was defined as patients whose Nugent score were lowered to 0-3 after metronidazole

treatment.[b] "Group Intermediate" was defined as patients whose Nugent score were changed to 4-6 after metronidazole treatment.[c] "Group Failed" was defined as patients whose Nugent score remained at 7-10 after metronidazole treatment.[d] Wilcoxon test was used for comparison between these two groups.

**[Table S3] Relative abundance of each *Gardnerella*genomospecies in each group**

| | All Participants$_{(N=171)}$ | All Participa |
|---|---|---|
| Genomospecies | Healthy | BV patients |
| *Relative abundance (%) of each genus, mean(95%CI)* | *Relative abundance (%) of each genus, mean(95%CI)* | *Relative abu* |
| *GS01* | 5.20(0.37-10.02) | 14.7(11.14-1 |
| *GS02* | 0.65(0.14-1.16) | 9.16(6.98-11 |
| *GS03* | 0.54(-0.16-1.23) | 2.83(1.89-3.7 |
| *GS05* | 0.02(0.00-0.03) | 2.17(1.56-2.7 |
| *GS07* | 0.05(-0.05-0.15) | 0.52(0.16-0.8 |
| *GS08* | 0.00(0.00-0.00) | 0.01(0.00-0.0 |
| *GS09* | 0.01(-0.01-0.02) | 0.28(0.22-0.3 |

BV, bacterial vaginosis; GS, genomospecies

[a] "Group Cured" was defined as patients whose Nugent score were lowered to 0-3 after metronidazole treatment.[b] "Group Intermediate" was defined as patients whose Nugent score were changed to 4-6 after metronidazole treatment.[c] "Group Failed" was defined as patients whose Nugent score remained at 7-10 after metronidazole treatment.[d] Wilcoxon test was used for comparison between these two groups.

**[Table S4] Relative abundance of four *Lactobacillus*species in each group**

| | All Participants$_{(N=171)}$ | All Participa |
|---|---|---|
| Species | Healthy | BV patients |
| Relative abundance (%) of each genus, mean(95%CI) | Relative abundance (%) of each genus, mean(95%CI) | Relative abu |
| *L. crispatus* | 29.21(18.01-40.41) | 0.08(0.06-0.1 |
| *L. iners* | 30.33(18.69-41.41) | 4.83(3.12-6.5 |
| *L. gasseri* | 2.99(-1.20-7.19) | 0.51(-0.14-1. |
| *L. jensenii* | 3.17(-1.09-7.43) | 0.31(-0.20-0. |

BV, bacterial vaginosis.

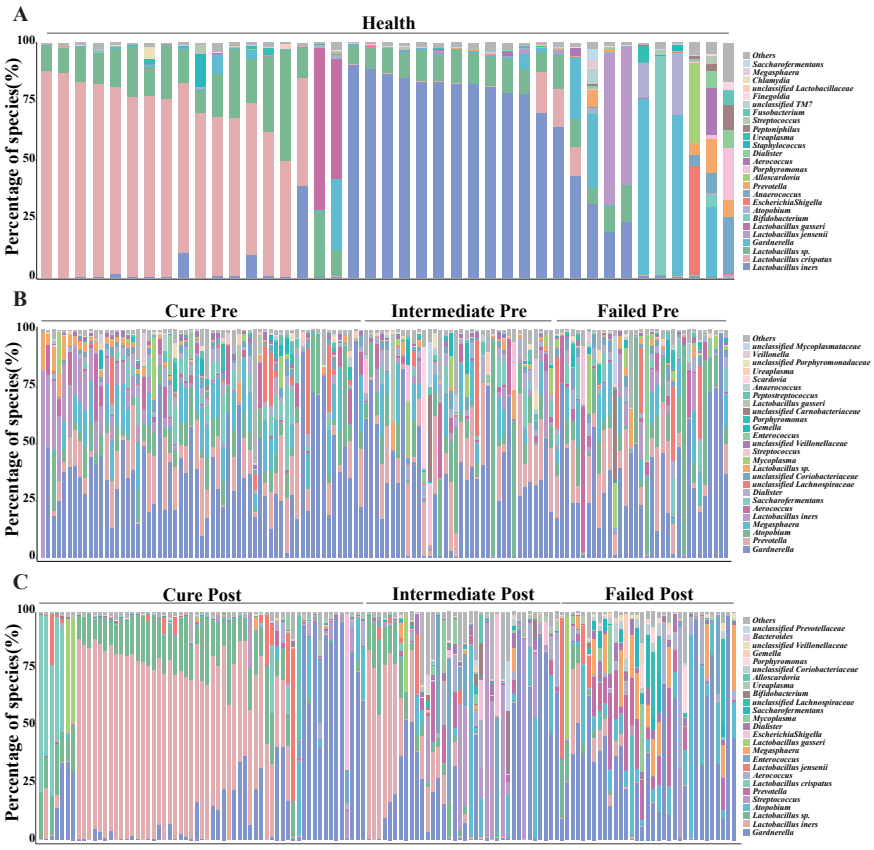
[a] "Group Cured" was defined as patients whose Nugent score were lowered to 0-3 after metronidazole treatment.[b] "Group Intermediate" was defined as patients whose Nugent score were changed to 4-6 after metronidazole treatment.[c] "Group Failed" was defined as patients whose Nugent score remained at 7-10 after metronidazole treatment.[d] Wilcoxon test was used for comparison between these two groups.

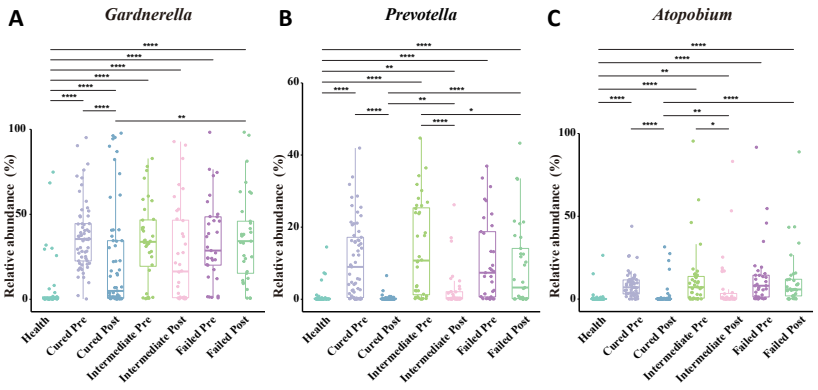
**[Figure S1] Intergroup comparison of Nugent score and vaginal pH**

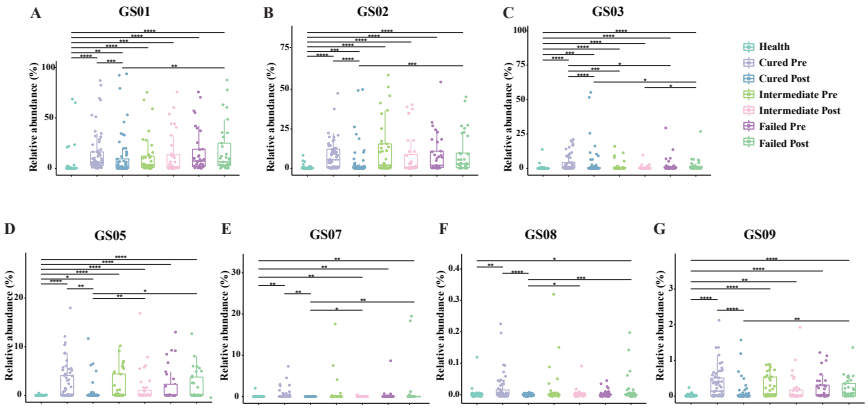
This figure shows the intergroup comparison of Nugent score (Supplementary Figure-1A) and vaginal pH (Supplementary Figure-1B) among Group cured, Group intermediate and Group failed with Kruskal-Wallis test used for statistical analysis. Only statistically significant differences are marked in the figure.

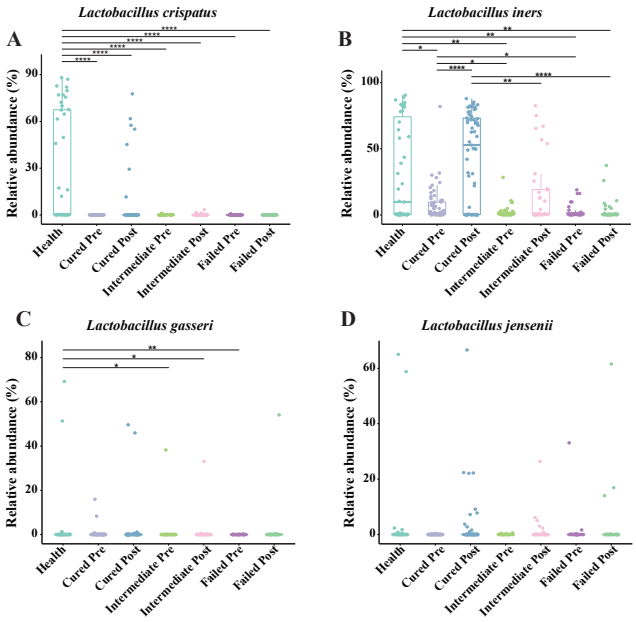
**[Figure S2] Antimicrobial activity test of *L. johnsonii* or *L. iners* against *G. vaginalis* or *A. vaginae***

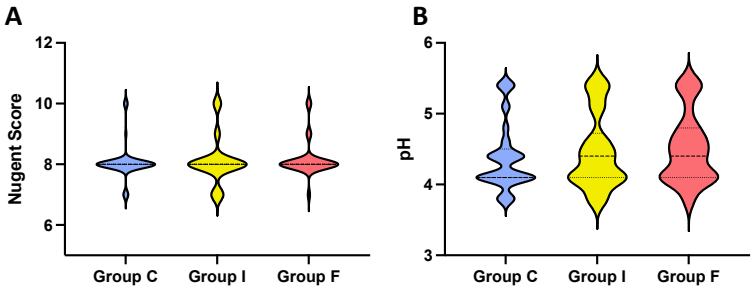
This figure shows the actual pictures of broths we used for antimicrobial activity tests and the diameter of the inhibition zone in each broth.









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