

Gardnerella Vaginalis in Recurrent Urinary Tract Infection is Associated with Dysbiosis of the Bladder Microbiome

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Abstract

Background: Recent studies on the urine microbiome have highlighted the importance of the gut-vagina-bladder axis in recurrent cystitis (RC). In particular, the role of *Gardnerella* as a covert pathogen that activates *E. coli* in animal experiments has been reported. Herein, we conducted a human bladder microbiome study to investigate the effect of *Gardnerella* on RC.

Methods: Urine 16S ribosomal RNA gene sequencing via transurethral catheterization was conducted in the normal control group (NC) (n=18) and RC group (n=78).

Results: The positive detection rate of *Gardnerella* species did not differ between the NC and RC groups (22.2% vs. 18.0%, $p = 0.677$). In addition, the *Gardnerella*-positive NC and *Gardnerella*-positive RC groups showed similar levels of microbiome diversity. The *Gardnerella*-positive group was categorized into three subgroups: *Escherichia*-dominant group, *Gardnerella*-dominant group, and *Lactobacillus*-dominant group, respectively. All of the *Escherichia*-dominant groups were associated with RC. *Gardnerella*-dominant or *Lactobacillus*-dominant groups expressed RC with symptoms when risk factors such as degree of *Gardnerella* proliferation or causative agents of bacterial vaginosis were present.

Conclusion: *Gardnerella* can act as a covert pathogen of RC depending on other risk factors, and *Gardnerella* infection should be considered in patients with RC. New guideline recommendations regarding antibiotics selection based on a novel method to detect the cause of RC may be required to reduce antibiotics resistance.

Introduction

Urinary tract infections (UTI) are about eight times more common in women than in men, and 50-60% of adult women will experience at least one UTI in their lifetime.^{1,2} Of these, 30% of UTI patients develop recurrent cystitis (RC) within 6 months.^{1,2} Clinically, UTI can develop into systemic diseases such as severe kidney disease and sepsis. Also, UTI is problematic as it can affect quality of life or cause depression.³⁻⁵ UTI treatment is based on the assumption that urine is sterile, and antibiotics are treatment of choice based on standard urine culture. However, in reality, the causative bacteria of many patients are not detected in standard urine culture, and RC develops in about 25-30% of patients despite the use of antibiotics.⁶

Traditionally, colonization of the vaginal introitus or periurethra by pathogenic gut bacteria is known to cause RC by retrograde infection.^{7,8} Due to this gut-bladder axis etiology, the vaginal microbiome, particularly *Gardnerella vaginalis*, is frequently excluded from the causative agent of UTIs, although they can also cause the infection. The clinical significance of *G. vaginalis* has been underestimated to date, due to its low detection rate of traditional urine culture.⁹ In general, uropathogenic *Escherichia coli* (UPEC) is known as the most common causative agent of RC, and thus clinical studies on polyinfection by Gram-positive bacteria or co-infective UTI by vaginal microbiota are few in number.^{10,11}

Recently, 16S ribosomal RNA gene amplification and sequencing and the EQUIC (enhanced quantitative urine culture) technique have revealed that a complex bladder microbiome exists in addition to classical bacteria.¹² Through a previous pilot study, we demonstrated the difference in bacterial diversity and patterns between RC and acute uncomplicated cystitis.¹³ Due to these new techniques, a new disease pathway through gut-vagina-bladder crosstalk has gained focus apart from the traditional mechanism of UTI, which was mainly caused by gut-bladder crosstalk.^{7,8,14,15} Such changes in study perspectives can provide good therapeutic clues, particularly in RC, which is difficult to treat with frequent recurrence.

Recent animal studies have shown that *Gardnerella*, a major pathogen of bacterial vaginosis, triggers UPEC and causes RC.^{14,16} Based on these results, we conducted a 16S ribosomal RNA gene sequencing study to investigate the differences in the urinary microbiome of RC and the effect of *Gardnerella*, a major strain of bacterial vaginalis in patients with RC.

Materials And Methods

Patients and study protocol

Between April 2020 and June 2021, we collected information on patients who underwent the urine NGS test (16S ribosomal RNA gene amplification). Patients who fulfilled the following inclusion criteria were eligible for this study: (a) patients 20 years of age or older (b) who underwent urinalysis, urine culture, and urine NGS. Patients with single acute uncomplicated cystitis, anatomical or structural abnormalities such as a prolonged indwelling catheter, pregnancy, or urinary stone were excluded from the study. In addition, patients with intrauterine contraceptive devices, vaginitis, and vaginal discharge were excluded. As a result, 96 patients who met the criteria were included in the study.

The patients were divided into two groups according to the following definitions. The normal group consisted of patients displaying no cystitis symptoms for at least the past year, and those that underwent a urine NSG test for the purpose of examination at a health promotion center, with no abnormal findings in abdominal image or urinalysis. The RC group was defined as consecutive patients who visited the outpatient clinic with symptomatic RC between April 2020 and June 2021. RC was defined as positive repetitive urine cultures (twice in six months, or three times in a year) with typical cystitis symptoms.

The study protocol was approved by the Institutional Review Board of Soonchunhyang University Bucheon Hospital (IRB number SCHBC 2021-10-011-01). The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki. The need of informed consent was waived by Institutional Review Board of Soonchunhyang University Bucheon Hospital due to the retrospective design of this study.

DNA extraction and 16S rDNA sequencing

The overall process is similar to the previous research protocol.¹³ All urine samples were collected by transurethral catheters. Upon collection, samples were immediately sent to the lab to be stored and

refrigerated with boric acid, and transported to the genetic lab within one day. The urine specimens (25ml) were centrifuged at 3,300 rpm for 30 min, and the urinary pellets were harvested and processed for DNA extraction using MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. To check the contamination of the DNA extraction process, we performed a negative DNA extraction consisting of only reagents without urine specimens during the test setup.

Prepared DNA was used for 16S library construction using the NEXTflex 16S V4 Amplicon-Seq (Bioo Scientific, Austin, TX, USA), and the resulting library was sequenced using the Illumina MiSeq Reagent Kit v2 (500 cycles) following the manufacturer's protocol.

Bioinformatics analysis and data processing

We used QIIME 2 to analyze the 16S sequence data. Demultiplexed and primer-trimmed data were quality-filtered and denoised using DADA2.^{17,18} Amplicon sequence variants (ASVs) with fewer than 10 reads or present in only a single sample were removed, and taxonomy was assigned to each ASV using the naive Bayes machine-learning taxonomy classifiers in the q2-feature-classifier against the NCBI RefSeq database with taxonomic weight assembly using q2-clawback.^{19,20}

Contamination was removed separately for each pair of urine sample and negative control with the R package microDecon²¹ which uses the proportions of contaminant operational taxonomic units (OTUs) or amplicon sequence variants (ASVs) in blank samples to systematically identify and remove contaminant reads from metabarcoding data sets. After decontamination, samples with more than 1% of *Gardnerella* were classified as *Gardnerella* positive.

Statistical analysis

The proportions of *Gardnerella* (+) urinary microbiota samples of the NC group and RC group was compared using the Chi-squared test. Next, we compared the characteristics of the microbial community between the *Gardnerella* (+) NC group and *Gardnerella* (+) RC group. Differences in alpha diversity between the *Gardnerella* (+) NC group and *Gardnerella* (+) RC group were analyzed based on the Shannon's diversity index using the Wilcoxon test. Principal coordinates analysis based on weighted Unifrac distances was used to construct a visualization of the data. Permutational multivariate analysis of variance (PERMANOVA)²², implemented in the *adonis* function of the R/*vegan* package (v2.5-2), was performed to identify microbial community dissimilarity of the *Gardnerella* (+) NC group and *Gardnerella* (+) RC group. To discriminate *Gardnerella* (+) urine samples into subgroups according to microbial community similarity regardless of the disease group, we used the k-medoids clustering algorithm, which clusters samples with the smallest total pairwise distance.²³ The number of clusters was assessed with the silhouette method.²⁴ In addition, hierarchical clustering using complete linkage was performed to visualize relationships among the samples based on the similarity of microbial composition. The procedure was also operated on a phylogenetically informed distance matrix which was computed using

the weighted UniFrac metric. Through hierarchical clustering, a heatmap of relative abundance of the compositional genera based on Spearman's correlation coefficient was represented and each subgroup was named on the basis of the dominant member of the respective subgroup.

Results

Baseline characteristics

The clinical characteristics and the urinalysis results of the patients are presented in Table 1. The patients were all female and the mean age was 54.5 ± 14.9 years. The NC (NC) group consisted of 18 patients, and the RC (RC) group was of 78 patients. The mean age of the RC group was slightly higher than that of the control group, but it was not statistically significant (56.2 vs 47.1 years, $P = 0.291$). There were no differences between the groups in terms of menopause or diabetes.

Gardnerella positive detection rate in the NC and RC groups

Next, we compared the positive detection rate of Gardnerella in the NC and RC groups (Table 2). Gardnerella was detected in 18 of 96 patients (18.8%). Regarding the positive detection rate, there was no significant difference between the two groups with 22.2% in the NC group and 18.0% in the RC group ($P = 0.677$).

Microbiome diversity of the NC and RC groups

Bacteria frequently detected in Gardnerella-positive patients were classified according to the NC group and RC group, respectively (Table 3). In the Gardnerella-positive NC group, Lactobacillus (55.67%), Gardnerella (30.94%), Haemophilus (7.58%), and Kocuria (3.6%) groups were frequently detected. On the other hand, in the Gardnerella-positive RC group, Gardnerella (42.18%), Lactobacillus (24.87%), Escherichia (22.57%), and Haemophilus (6.52%) groups were frequently detected. In particular, Atopobium, Megasphaera, and Ureaplasma, known to be associated with bacterial vaginosis, were detected only in the RC group (Figure 1).

To determine the distribution of various microorganisms present in one sample (alpha diversity), we calculated the Shannon index (Figure 2A). There was no significant difference regarding alpha diversity between the RC group and NC group ($P = 0.96$). To determine whether the microbial community was different between the two cystitis groups (beta diversity), we evaluated the weighted UniFrac distances (Figure 2B). The composition of the microbiome did not differ between the two groups ($P = 0.127$).

Three urotypes of bladder microbiome associated with Gardnerella

We investigated the presence of any patterns in the Gardnerella-positive group. The distribution of Gardnerella (+) urinary microbiota was analyzed with K-medoids clustering (Figure 3A), hierarchically clustering (Figure 3B), and bar plot (Figure 3C), and was classified into three groups as follows (Figure 3D): Group 1 Escherichia dominant group (Gardnerella is very few and Escherichia is dominant), Group 2

Gardnerella dominant group (Gardnerella accounted for more than 50%), and Group 3 Lactobacillus dominant group (Gardnerella is present in some cases, but Lactobacillus is predominant in more than 50%). All of Group 1 (Escherichia dominant) was associated with RC (5 RC, 0 NC). In Group 2 (Gardnerella dominant: 5 RC, 2 NC) or Group 3 (Lactobacillus dominant: 4 RC, 2 NC), both RC and asymptomatic NC were mixed (Figure 3A). In particular, bacterial vaginosis-associated strains such as Atopobium, Megasphaera, and Ureaplasma were detected only in the RC group. However, in Group 2 and Group 3, the proportion of Gardnerella was higher in the RC group than the NC group, although not significant (Group 2, $P = 0.095$; Group 3, $P = 0.13$) (Figure 4).

Discussion

In this study, we found that there was no significant difference in urine microbiota results between the Gardnerella-positive NC group and Gardnerella-positive RC group. The Gardnerella-positive group could be divided into three urotypes: 1) Escherichia-dominant group, 2) Gardnerella-dominant group, and 3) Lactobacillus-dominant group, respectively. All Escherichia-dominant groups were associated with RC. In the Gardnerella-dominant and Lactobacillus-dominant groups, the NC and RC groups were mixed. In particular, bacterial vaginosis-associated strains such as Atopobium, Megasphaera, and Ureaplasma were detected only in the RC group.

Our research group has completed two papers related to the urine microbiome. The core of the first paper was that *E. coli* was the most causative strain in acute and recurrent cystitis, but the base from which *E. coli* grew, that is, the bladder condition (commensal or pathogenic organism) was completely different.¹³ That is, the first paper is a study on the *E. coli* dominant urotype. In this paper, the second study, we found out that Gardnerella affects the dominant urotype of recurrent cystitis in the process of newly elucidating the pathophysiology of recurrent cystitis.

A recent UTI guideline is based on antibiotic treatment based on urine culture for RC.²⁵ In particular, continuous low-dose antimicrobial prophylaxis is recommended if RC persists after behavioral interventions have failed.²⁵ In reality, 75% of the patients of clinical practice with RC are taking empirical antibiotics without undergoing tests.²⁶ Consequently, antibiotic resistance has increased while RC prevalence has not decreased.²⁷

Recently, new bacterial technology of 16s RNA sequencing and EQUIC revealed that various bladder microbiomes play an important role in addition to the past classical uropathogens in RC.^{6,13} According to a previous classical urine culture-based study, the most common causative bacteria of uncomplicated UTI were *E. coli* (58%), mixed flora (13.4%), and *K. pneumoniae* (6.5%).²⁸ However, the pattern was different in our group's previous pilot study.¹³ This study based on NGS additionally discovered *Sphingomonas*, *Staphylococcus*, *Streptococcus*, and *Rothia* spp., which were not found in the existing culture, especially for the RC group.

With the accumulation of the knowledge regarding this microbiome, the importance of the gut-vagina-bladder axis for RC has been increasingly emphasized. Clinical evidence of an association between the vagina and bladder is as follows. First, bacterial vaginosis is a risk factor for UTI;²⁹ second, UTI decreases when hormone treatment such as vaginal estrogen is administered;^{30,31} and lastly, there are many patients who complain of frequent UTI after sex.³² On the microbiological basis, it has been reported that about two-thirds of bladder microbiota overlap with gut microbiota, and about one-third of bladder microbiota exists only in the vagina.³³

The first finding of our study was that there was no difference in the detection rate of Gardnerella between the NC and RC groups. Similar with our result, a previous study reported that Gardnerella was detected in 27% of the normal population, and this ratio was not significantly different from patients with urinary symptoms.⁹

Second, our study suggested three patterns of Gardnerella -positive patients. First, in the *E. coli* dominant group, this is the first study of humans showing that Gardnerella can act as a covert pathogen that activates *E. coli*. It has already been shown that the vagina acts as a reservoir for uropathogens such as *E. coli*. Moreover, even short exposure of the bladder to Gardnerella caused bladder cell damage such as urothelial exfoliation and urothelial apoptosis in an animal study.¹⁶ The second group (Gardnerella dominant group) provides clues that increased amounts of Gardnerella itself may be associated with RC. Although rare, it has been reported that Gardnerella acts as a causative agent of UTIs.²⁹ Considering the low culture positive rate of Gardnerella, the clinical significance is likely to be higher in practice. In the case of the third Lactobacillus-dominant group, since the protectivity effect of Lactobacillus is different depending on the type of Lactobacillus strain, cystitis can occur even with a small percentage of Gardnerella in Lactobacillus strains with poor protectivity. However, in this study, the Lactobacillus strain was not analyzed, and thus further follow-up studies are required to investigate the difference between normal and RC strains.

Considering that both NC and RC exist in group 2 (Gardnerella dominant) and group 3 (Lactobacillus-dominant), it can be inferred as follows. Asymptomatic Gardnerella infection is present in the vagina, and some are self-treated.³⁴ Likewise, the presence of Gardnerella does not necessarily cause RC, just as the presence of Gardnerella does not necessarily cause bacterial vaginosis. Although Gardnerella itself does not have high virulence, it is affected by other risk factors for causing symptoms. Risk factors such as host immunity, Gardnerella proliferation, microbiome environment, and residence environment seem to influence the development of the phenotype (asymptomatic or RC). For example, it seems that cystitis always occurs when Gardnerella and other bacterial vaginosis strains are accompanied which can be interpreted as the influence of the microbiome environment. In addition, the fact that the proportion of Gardnerella was higher in cystitis patients could be the evidence that the amount of Gardnerella proliferation had an effect on phenotype expression. Overall, our results provided clues for a new pathophysiology of RC. The usage of antibiotics based on traditional uropathogens in group 2 or group 3 has weak clinical effects and may cause side effects of antibiotics-resistance to occur.

Our study has several advantages. First, we demonstrated the clinical importance of gut-vagina-bladder axis in humans focusing on *Gardnerella* for the first time. The gut-vagina-bladder axis has been explained to some extent through microbiological and animal experiments, but studies on humans are still lacking.^{7,16,33} Second, in our study, the specimen was collected by transurethral catheterization, so the bladder microbiome is well reflected without contamination. Finally, our study suggested several types and novel mechanisms of RC that had not been previously elucidated.

However, our study also has several limitations. First, since it is not a prospective study, it cannot be free from selection bias, especially regarding the NC group. Also, although not statistically significant, the NC group was younger than the RC group and had a lower rate of menopause, which may have affected the results. Second, our study revealed the importance of *Gardnerella* infection as a covert pathogen, but did not provide a cut-off for the required amount of infection for clinical symptoms to appear. Third, the difference between the *Lactobacillus* strains in the NC group and the RC group could not be suggested. This requires follow-up studies and is believed to provide clues about *Lactobacillus* prophylaxis in patients with RC in the future. Fourth, this study did not suggest whether treating *Gardnerella* could actually improve the clinical symptoms of RC. In general, antibiotics used for bacterial vaginosis and those used for RC are different.³⁵ The choice of antibiotics for UTIs can also affect the vaginal microbiome. For example, the use of beta-lactam antibiotics is less effective against vaginal colonized *E. coli*, and recurrent UTIs caused by vaginal *E. coli* easily occur with such beta-lactam antibiotics.^{36,37} Furthermore, *Gardnerella* is difficult to treat due to biofilm formation.³⁸ Metronidazole or tobramycin, which were previously recommended as therapeutic agents for *Gardnerella*, can prevent the formation of a new biofilm, but are known to have less effect on previously formed biofilm.³⁸ Therefore, further studies are needed to determine which antibiotic is most suitable for RC caused by *Gardnerella*.

In summary, if conventional uropathogens are not detected in patients with RC, *Gardnerella* infection should be considered. Asymptomatic urine *Gardnerella* (asymptomatic bacteriuria) does not require treatment, but urine *Gardnerella* in symptomatic patients is considered to be the causative agent of cystitis, so treatment is necessary. Also, even for asymptomatic cases, treatment should be considered if *E. coli* or the causative agent of bacterial vaginosis is detected in addition to *Gardnerella*.

Declarations

Ethics approval and consent to participate: The study protocol was approved by the Institutional Review Board of Soonchunhyang University Bucheon Hospital (IRB number SCHBC 2021-10-011-01). The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki. The need of informed consent was waived by Institutional Review Board of Soonchunhyang University Bucheon Hospital due to the retrospective design of this study.

Consent for publication: N/A

Availability of data and material: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest statement: All authors have no conflicts of interest relevant to this study.

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- 4) Data analysis and interpretation: All authors
- 5) Manuscript writing: Jeong-Ju Yoo, Ju Sun Song
- 6) Final approval of manuscript: All authors.

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Tables

Table 1. Baseline characteristics of patients

| | Total (N = 96) | Normal control (N = 18) | Recurrent cystitis (N = 78) | p |
|--------------------------|-------------------|----------------------------|--------------------------------|-------|
| Age (years) | 54.5 ± 14.9 | 47.1 ± 11.8 | 56.2 ± 15.1 | 0.291 |
| Female | 96 (100) | 18 (100) | 78 (100) | 0.999 |
| Menopause | 61 (63.5) | 8 (44.4) | 53 (67.9) | 0.062 |
| Diabetes | 15 (15.6) | 2 (11.1) | 13 (16.7) | 0.558 |
| Urinalysis | | | | |
| Urine RBC (per HPF) | 0 [0 - 400] | 0 [0 - 400] | 0 [0 - 30] | 0.051 |
| Urine WBC (per HPF) | 0 [0 - 204] | 0 [0 - 29] | 0 [0 - 204] | 0.252 |
| Urine bacteria (per HPF) | 0 [0 - 3+] | 0 [0 - 3+] | 0 [0 - 3+] | 0.720 |
| Urine protein | 0 [0 - 2+] | 0 [0 - 1+] | 0 [0 - 2+] | 0.679 |
| Urine glucose | 0 [0 - 3+] | 0 [0 - 3+] | 0 [0 - 3+] | 0.710 |

NOTE: The data are presented as the mean ± standard deviation or median [min - max] for continuous variables and n (%) for categorical variables.

Abbreviations: RBC, red blood cell; WBC, white blood cell; HPF, high-power field

Table 2. Positive detection rate of *Gardnerella* species

| | Number of patients | <i>Gardnerella</i> positive samples | P-value |
|--------------------|--------------------|-------------------------------------|---------|
| Normal control | 18 | 4 (22.2%) | 0.677 |
| Recurrent cystitis | 78 | 14 (18.0%) | |
| Total | 96 | 18 (18.8%) | |

Table 3. Contribution of most abundant urinary bacterial genera to *Gardnerella* positive group

| Genera | Percent contribution in G (+) Normal Control group | | | Percent contribution in G (+) Recurrent Cystitis group | | |
|-------------------|---|------|-------|---|------|-------|
| | Mean | Min | Max | Mean | Min | Max |
| Lactobacillus | 55.67 | 8.19 | 96.00 | 24.87 | 0.00 | 86.38 |
| Gardnerella | 30.94 | 3.70 | 61.49 | 42.18 | 1.13 | 99.09 |
| Haemophilus | 7.58 | 0.00 | 30.33 | 6.52 | 0.00 | 91.33 |
| Kocuria | 3.60 | 0.00 | 14.38 | 0.01 | 0.00 | 0.13 |
| Tumebacillus | 1.91 | 0.00 | 7.65 | 0.00 | 0.00 | 0.00 |
| Escherichia | 0.23 | 0.00 | 0.60 | 22.57 | 0.00 | 96.93 |
| Ureaplasma | 0.06 | 0.00 | 0.12 | 0.32 | 0.00 | 2.56 |
| Atopobium | 0.00 | 0.00 | 0.00 | 1.74 | 0.00 | 12.73 |
| Pseudoxanthomonas | 0.00 | 0.00 | 0.00 | 0.40 | 0.00 | 5.46 |
| Megasphaera | 0.00 | 0.00 | 0.00 | 0.35 | 0.00 | 4.96 |
| Ruminococcus | 0.00 | 0.00 | 0.00 | 0.29 | 0 | 4.08 |
| Clostridium IV | 0.00 | 0.00 | 0.00 | 0.25 | 0.00 | 3.48 |
| Blautia | 0.00 | 0.00 | 0.00 | 0.33 | 0.00 | 3.20 |
| Alloscardovia | 0.00 | 0.00 | 0.00 | 0.15 | 0.00 | 2.03 |

Abbreviations: G (+), Gardnerella positive

Figures

Figure 1

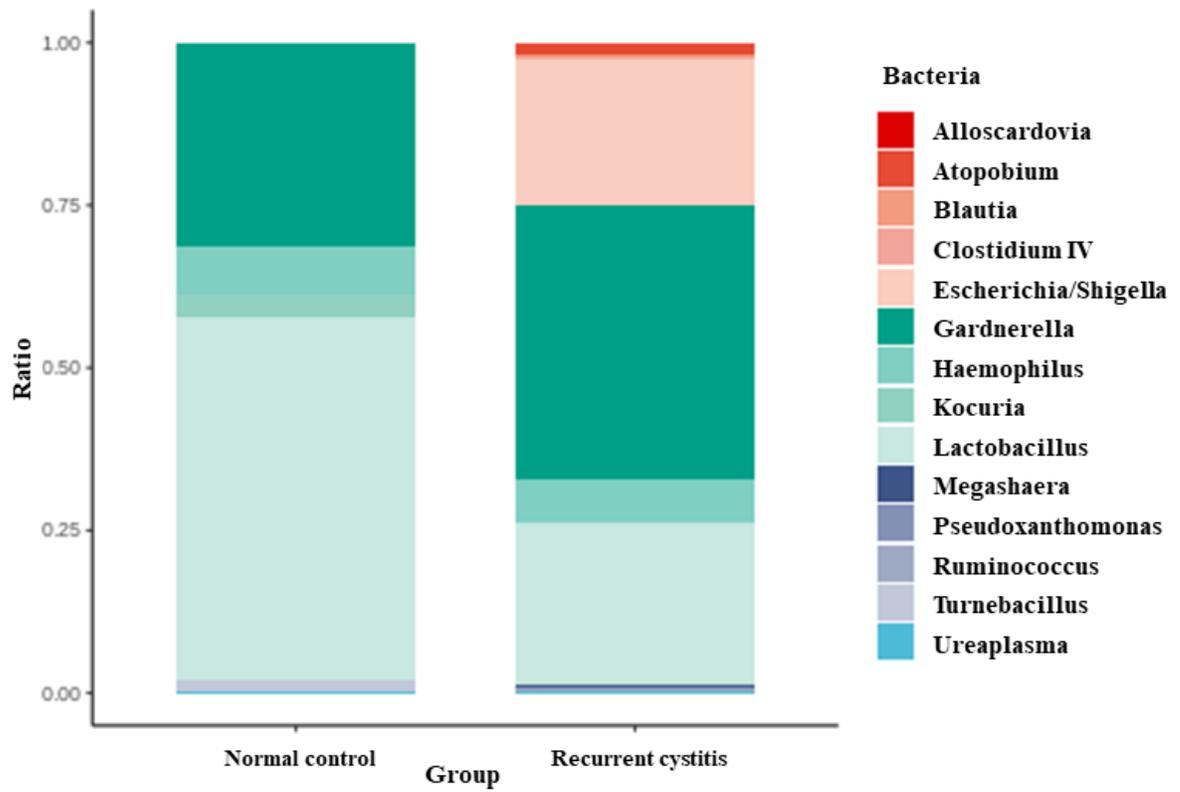


Figure 1

Relative abundance of urinary microbiota in Gardnerella (+) normal control group and Gardnerella (+) recurrent cystitis group

Gardnerella (+) urinary microbiota revealed three distinct subgroups by (A) K-medoids clustering and (B) hierarchical clustering, (C) bar plot, (D) pie chart

Figure 4

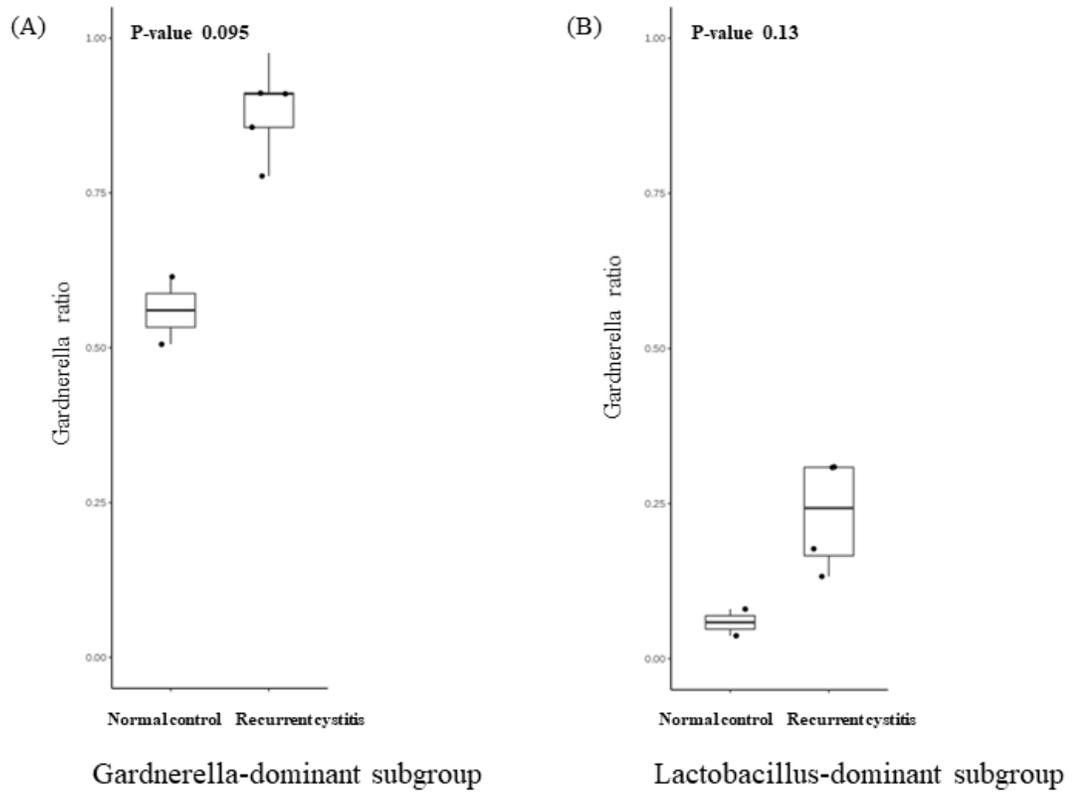


Figure 4

Relative abundance of Gardnerella in (A) Gardnerella-dominant subgroup, and (B) Lactobacillus-dominant subgroup