

Ecological Indices of Bacterial Vaginosis Associated Facultative Bacteria

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ABSTRACT

Bacterial vaginosis (BV), a common vaginal dysbiosis characterised by a disparity in the commensal of vaginal polymicrobial community. Facultative anaerobic bacteria are more often found in patients due to impaired immune system. However, the effective diagnosis and understanding the prevalence and type of bacteria in BV is essential for the treatment. To find out the frequency, distribution, and diversity of the bacterial isolates in vaginal samples of reproductive-age women who were diagnosed with BV. 150 vaginal swab samples were collected from reproductive-age women. Samples cultured in different agar media to isolate facultative anaerobic bacteria from the BV-positive samples. The isolated bacteria were identified by microscopic examination and biochemical tests, followed by a statistical analysis, viz., Shannon diversity index, Simpson's diversity index, and species evenness, to determine the diversity and distribution of the bacterial species among the vaginal samples. Shannon and Simpson values indicate moderate species diversity, with dominance by bacteria such as *Klebsiella spp.*, *E. coli*, and *Enterococci spp.* The use of culture and statistical analysis contributes for understanding the microbial community of the vagina in BV. They also provide insight into bacterial population stability, dominance patterns, and the extent of variation of microbial communities. This confirms bacterial vaginosis as a polymicrobial condition.

Keywords

Bacterial vaginosis (BV), vaginal dysbiosis, Facultative Bacteria

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Introduction

Among the different types of vaginal infections, bacterial vaginosis (BV) is the most common vaginal infection

affecting women of reproductive age worldwide (Pramanick *et al.*, 2019). The replacement of the normal *Lactobacillus*-dominated vaginal flora with an overgrowth of a diverse community of facultative and

obligate anaerobic microorganisms are well characterized (Bitew *et al.*, 2021). This replacement of dominant lactobacillus from other harmful bacteria is responsible for the symptoms such as abnormal vaginal discharge, odour, and inflammation, and most cases that remains asymptomatic (Holdcroft *et al.*, 2023).

The etiology of BV is not exactly known yet, but various species engage in complex metabolic interactions in the vaginal environment (Turovskiy *et al.*, 2011). Even though *Gardnerella vaginalis*, an anaerobic bacterium, is well studied (Morrill *et al.*, 2020), other facultative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* are also found to be associated with the BV-positive women (Ahmed Aliyi *et al.*, 2022). These facultative bacteria can cause prolonged infections and complicate treatment due to their ability to form biofilms and develop resistance to antimicrobial therapies (Donlan & Costerton, 2002).

In this study, to gain knowledge regarding the distribution and diversity of facultative bacteria, their association with BV which is essential for developing effective treatment strategies and improving clinical results. However, the knowledge regarding the facultative bacterial communities in BV is limited, which needs further attention (Muzny & Schwebke, 2016; Onderdonk *et al.*, 2016). Therefore, the present study focuses on isolating and characterising facultative bacterial isolates obtained from vaginal swabs of reproductive-age women with BV. By determining the major facultative pathogens, a full picture of the BV microbial community can be assessed, which helps in designing improved therapeutic approaches.

BV is the most common vaginal infection among women of reproductive age, characterised by a shift from the dominated lactobacillus vaginal microbiome to a diverse mix of anaerobic and facultative bacteria, so it is a polymicrobial disorder (Djogic *et al.*, 2025). Several reproductive health outcomes, like preterm birth, increased risk of sexually transmitted diseases, and pelvic inflammatory diseases, is also linked with BV (Murphy & Mitchell, 2016).

The pathogenesis of BV is unclear, but it is considered as multifactorial and involves complex interactions between microbes (Muzny *et al.*, 2020). The principal pathogen is often believed as *Gardnerella vaginalis*, as it can initiate biofilm formation on vaginal epithelial cells, which can facilitate the colonization of other anaerobes, such as

Atopobium vaginae and *Prevotella* spp (Castro *et al.*, 2019). The frequently isolated facultative anaerobes from BV cases indicate their significant role in disease development and progression.

Facultative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus* spp are opportunistic pathogens. The infections, including urinary tract infections and pelvic inflammatory disease, are commonly associated with BV. The ability of these bacteria to form biofilms complicates the BV treatment because of increased resistance to antibiotics and the host's immune response. This biofilm-associated resistance contributes to the high recurrence rate of BV, after standard antimicrobial therapy and highlights its prevalence and potential role in influencing diagnostic results (Machado *et al.*, 2016; Shang *et al.*, 2024).

The coexistence of facultative and anaerobic bacteria in BV is studied by molecular techniques, such as 16S rRNA gene sequencing which helps to understand the vaginal microbiota. But still, the culture-based methods are important in isolating and characterizing the clinically relevant bacterial species. Although the importance of facultative bacteria in BV is increasingly recognised, data specific to regions and populations remain limited (Fredricks *et al.*, 2005).

Even though BV is widely studied, research is mainly focused on the anaerobic bacteria like *Gardnerella vaginalis* and *Atopobium vaginae*. The role of facultative anaerobic bacteria is underexplored, even though they are capable of surviving in the variable conditions of the vaginal environment and are also capable of forming biofilms (Chen *et al.*, 2021).

There is limited data on the regional variations in vaginal microbiota, specific frequency, diversity, and the pathogenic potential of facultative bacteria isolated from BV-positive women among different populations. Therefore, the present study focuses on the isolation, identification, and frequency analysis of facultative bacteria in BV-positive vaginal samples.

Materials and Methods

Study Design and Population

A cross-sectional study was conducted involving women of reproductive age (18-45years) attending the

gynaecology outpatient department at Basaveshwar Teaching and General Hospital, Kalaburagi, between June to December 2023. Samples were collected from patients who visited the hospital for routine examination after obtaining informed consent.

Sample Collection.

A total of 150 samples were collected from patients using sterile vaginal cotton swabs. Soon after the collection, samples were immediately transported to the microbiology laboratory for analysis.

Diagnosis of Bacterial Vaginosis

Amsel's criteria were used to diagnose the BV, which requires the presence of at least three of the following criteria

- Vaginal pH > 4.5
- Positive whiff test
- Presence of clue cells on microscopic examination
- Homogeneous, thin, greyish-white vaginal discharge

Apart from that, Gram staining was performed, and Nugent scoring was used to confirm BV status, with a score ≥ 7 indicating a positive diagnosis.

Isolation and Identification of Facultative Bacteria

31 BV-positive vaginal samples were cultured on different media, like Nutrient agar, Brain Heart Infusion agar, and Lauria Bertani, as well as selective and differential media, followed by incubation for 24 to 48 hours at 37°C.

The identification was based on colony morphology, Gram stain, and the standard biochemical tests, like Catalase, Indole, Methyl red, Voges-Proskauer, H₂S, and motility tests.

Data Collection and Analysis

After isolation followed by identification of organism X in total BV-positive samples, the percentage of occurrence of X was calculated using the formula-

Percentage of Organism X = (Number of times the organism X was isolated/Total number of isolates) x 100

Shannon's Diversity index

Shannon's Diversity index is a metric used to quantify the diversity of a community in ecology and microbiology. It explains both richness (number of different species present) and evenness, denoted as H' . The higher the H' values, the greater the diversity. The formula to calculate H' is-

$$H' = -\sum_{i=1}^S P_i \ln(P_i)$$

Where,

S = Total number of species in the community.

P_i = Proportion of the i^{th} species in the community ($P_i = n_i/N$)

n_i = number of individuals of species i

N = total number of individuals of all species.

\ln = natural logarithm.

$$\text{Evenness (E)} = H' / \ln(S)$$

Interpretation for H'

- < 1.0 indicates Low diversity, which means the community is dominated by one or a few species, low evenness and richness.
- 1.0-2.0 indicates moderate diversity, which means several species are present with some dominance; moderate evenness.
- 2.0-3.0 indicates a high diversity, which means a balanced community with good species richness and even distribution.
- > 3.0 indicates very high diversity, which means a very complex and stable ecosystem, rare in microbial or clinical samples.

Simpson's diversity index

Simpson's diversity index is another measure of diversity in a community, but it focuses more on dominance than richness. It gives the probability that two individuals randomly selected from a sample belong to the same species.

$$\text{Formula used: } D = \sum_{i=1}^S P_i^2$$

Where,

S= Total number of species

P_i = Proportion of the i^{th} species in the community ($P_i = n_i/N$)

Simpson's diversity index is represented as 1-D.

Interpretation of 1-D values

- < 0.20 indicates very low diversity, which means strong dominance of one species; poor diversity
- 0.20-0.50 Indicating low diversity, which means few species dominate; uneven distribution.
- 0.5-0.7 indicates moderate diversity, which means some balance; moderate diversity
- 0.7-0.9 indicates high diversity, which means several species with fairly even distribution.
- > 0.90 indicates very high diversity, which means a highly even, complex, and stable community.

Results and Discussion

Sample collection and isolation

The 31 BV-positive vaginal swab samples were analysed based on Nugent score and Amsel criteria, and each sample was cultured on selective and differential media. The predominant isolates were *Enterococcus*, *Klebsiella* and *E. coli*, along with a few co-occurring opportunistic species. Gram staining, morphological characters of colonies and biochemical characterisation methods were used to confirm the pure cultures.

Percentage and Distribution of Isolates

Within 31 Samples, *Klebsiella* and *E. coli* were the most frequently detected species, followed by *Enterococcus* and *S. aureus*. The frequency matrix showed most samples representing the growth of two to four bacterial species regularly when cultured, indicating the mixed bacterial colonisation patterns.

Diversity Index

Shannon (H'), Simpson (1-D), and Evenness (E) indices of isolated bacteria were calculated for vaginal samples.

Shannon Diversity index (H')

Shannon's diversity index (H') 1.9606, representing

moderately high diversity among the isolated bacterial species. The evenness (E) is 0.892, suggesting a fairly even distribution, representing no single species is dominating strongly. Species such as *Klebsiella*, *E. coli*, and *Enterococcus* have slightly higher frequencies compared to other bacterial isolates among the BV-positive samples. This also suggests the mixed colonisation and the interspecies interaction in the vaginal microbiota of the BV samples.

Simpson's diversity index

In the present study, the Simpson's index of dominance "D" is 0.162, indicating that there is only a 16.2% probability that two randomly selected bacterial isolates from the community belong to the same species. The Simpson's diversity index 1-D value is 0.8377, representing a rich and diverse bacterial population with low dominance by a single species.

Bacterial vaginosis is a polymicrobial condition characterised by the shift of a lactobacillus-dominated vaginal microbiome to the increased diversity of anaerobic or facultative bacteria. The findings from these bacterial isolates regarding frequency, distribution, and diversity help establish an understanding of BV pathogenesis and its clinical implications among women of reproductive age.

The Shannon diversity and Simpson indices indicate complexity and the structure of bacterial communities associated with BV. The H' and 1-D values from the study reflect the moderately high bacterial diversity with consistent species distribution among nine predominant isolates. This reflects the polymicrobial state, where several bacteria are found together. The balanced coexistence of facultative and opportunistic pathogens contributes to the dysbiosis of the vaginal environment. Similar reports were found in the study made by (Srinivasan *et al.*, 2012), where *Gardnerella vaginalis*, *Atopobium vaginae*, and anaerobes such as *Prevotella* and *Mobilincus* with Shannon values (1.8-2.2) reflected the complex bacterial interaction. In contrast, a study made by (Muzny *et al.*, 2020) indicated a lower H' value of less than 1, which represents the dominance of *Lactobacillus* species in healthy microbiota.

The higher evenness E and low Simpson's index of dominance D in the study support the balanced microbial group with limited dominance by any single species.

Fig.1 Flow chart representing the selection of samples

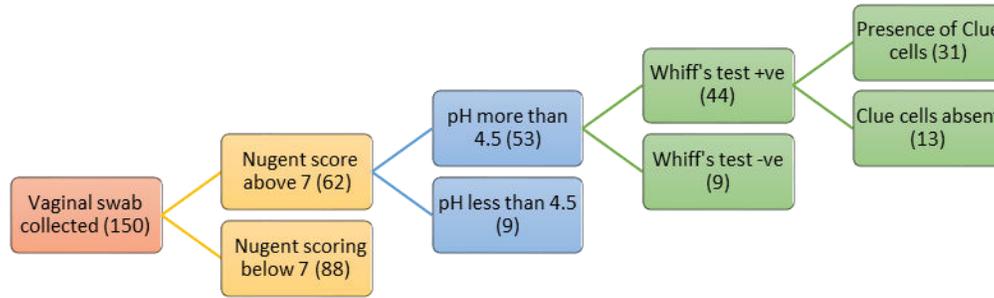
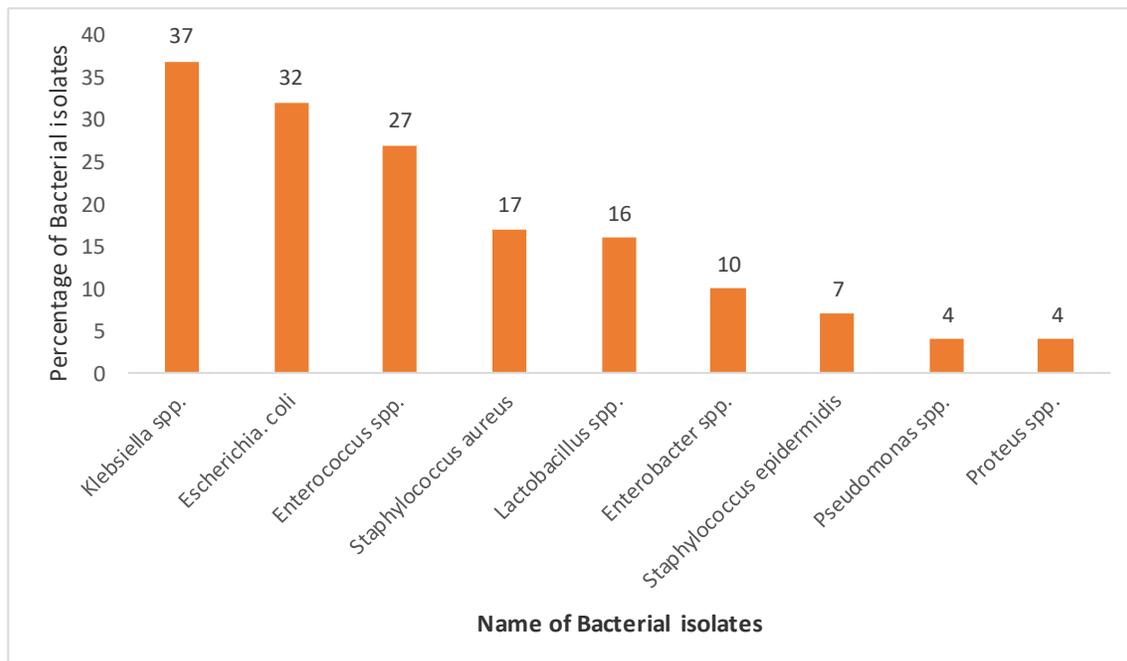


Fig.2 Represents the percentage of bacterial isolates.



This balance indicates microbial cooperation and quorum sensing. This increases the stability of a community and biofilm formation (Hardy *et al.*, 2017; Liu *et al.*, 2020). The observed diversity may play an important role in quorum-sensing-mediated interactions.

In this study, a total of 150 samples were isolated and screened for BV using the standard diagnostic criteria. 62 samples were identified as BV positive based on Nugent scoring. According to the Amsel criteria, the presence of any three signs of the four (pH, whiff test, Presence of clue cells, type of discharge) is regarded as BV positive. While 53 samples were positive for the pH test, 44 samples were positive by the whiff test, and 31 samples were positive for the presence of clue cells.

The culture method and the ecological diversity indicate that the Bacterial Vaginosis is a polymicrobial community, dominated by *Klebsiella*, *E. coli*, and *Enterococcus*.

Shannon's diversity indices, evenness, and Simpson's diversity index among the samples reflect the dynamic and heterogeneous state of BV-associated microbiota, characterised by mixed colonisation and significant interspecies interactions with no single species dominating in BV-positive Vaginal samples.

A higher value of diversity indicates mixed bacterial colonisation, while a lower diversity index values indicate dominance by a few opportunistic bacteria.

These finding highlights the disruption of the dominant lactobacillus, leading to the establishment of the facultative pathogens, which promote quorum sensing and biofilm formation, contributing to the BV. Thus, integration of conventional culture methods and diagnostic criteria may provide a better understanding of microbial ecology and disease progression in bacterial vaginosis.

Author Contributions

Sahanapriya Gubbewad: Investigation, formal analysis, writing—original draft. Jayasimha Rao: Validation, methodology, writing—reviewing. Sujatha Inginshtetty:—Formal analysis, writing—review and editing. Meenakshi S. Devarmani: Investigation, writing—reviewing. R. Chandrakanth Kelmani: Resources, investigation writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical consideration: The ethical permission was obtained by the Institutional Ethics Committee (IEC) at H.K.E. Society's Mahadevappa Rampure Medical College, Kalaburagi.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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