

# A pilot study evaluating microbiome profiles of breast tissue, vaginal samples and stool in patients with benign breast lesions (fibroadenoma)

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## Article

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# Abstract

Although breast microbiota has been studied in healthy and malignant tissues, its composition in benign lesions such as fibroadenoma remains largely unknown. This pilot study compared the microbiota of fibroadenoma tissue, the vagina, and the gastrointestinal tract to identify microbial taxa shared across these anatomically distinct niches. Tumor, vaginal, and fecal samples were collected from three women with fibroadenoma. Samples were analyzed by quantitative culture, 16S rRNA gene sequencing, MALDI-TOF identification, and PCR detection of selected atypical bacterial pathogens. Culture-based analysis revealed abundant bacterial colonization of the gastrointestinal and vaginal niches, whereas no cultivable microorganisms were detected in fibroadenoma tissue. However, 16S rRNA sequencing identified diverse bacterial taxa across all niches. Despite distinct microbial communities, several taxa were shared among niches. Beta diversity analysis showed significant inter-niche differences (PERMANOVA,  $p = 0.01$ ) and greater similarity between fibroadenoma and gut microbiota than between fibroadenoma and reproductive tract microbiota. PCR additionally detected *U. urealyticum* and *M. hominis*. In summary, fibroadenoma tissue harbors a distinct microbiota while sharing selected taxa with the gastrointestinal and reproductive tracts, supporting its integration within the broader host-associated microbial ecosystem.

## Introduction

Breast cancer is the most commonly occurring cancer among women worldwide and the leading cause of cancer-related deaths in this group. According to data from the International Agency for Research on Cancer and the World Health Organization, approximately 2.3 million new cases of breast cancer in women were diagnosed worldwide in 2022, and it is estimated that by 2050 the number of new cases may increase to 3.2 million annually [1–3]. Scientific research focuses primarily on malignant cancers; however, benign breast lesions, such as fibroadenomas, occur in over 50% of the female population and also represent a significant clinical and diagnostic challenge [4].

For a long time, breast tissue was considered a sterile environment. A breakthrough occurred in 2014 with the study by Urbaniak et al., who confirmed the presence of viable bacteria in mammary gland tissue [5]. Shortly thereafter, it was demonstrated that the microbiota present within breast tumors, including benign lesions such as fibroadenomas, is richer and more diverse than that found in healthy tissue, and that these microorganisms reside intracellularly, enabling them to directly modulate host cell biology [6,7]. The most commonly reported bacterial genera present in breast tissue include *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Enterococcus* [5,8]. Distinct dysbiosis patterns have been described in both malignant and benign breast lesions. Among fibroadenomas, *S. aureus* has been detected and has been hypothesized to play a role in the induction of MED12 gene mutations, a key molecular event associated with the development of these tumors [9,10]. Moreover, the presence of genotoxic bacteria such as *E. coli* and *S. epidermidis* has been demonstrated. The genotoxins produced by these microorganisms, including colibactin, are capable of inducing DNA double-strand breaks, thereby contributing to genomic instability [8]. It has been suggested that the abundance of such

bacteria may be lower in benign lesions than in breast cancer, potentially preventing full malignant transformation [4,8].

Although we are increasingly understanding the roles that microorganisms play in oncological processes, their origin and presence within breast tissue remain largely hypothetical. Numerous studies point to the existence of a gut–breast axis, suggesting that under dysbiotic conditions bacteria may translocate from the intestines and reach the mammary glands via the bloodstream or lymphatic system [4,11,12]. This is further supported by observations showing that orally administered probiotics can subsequently be detected in human milk [13]. The skin may also serve as a source of bacteria present in breast tissue. Bacteria commonly associated with the skin microbiota, such as *Staphylococcus* spp., can enter the mammary gland through the openings in the nipple and subsequently spread via the milk ducts [12,14,15]. The contribution of microbial reservoirs other than the gastrointestinal tract and the skin remains largely unexplored. In this context, the female reproductive tract represents a particularly interesting microbial niche. Although direct microbial transmission from the female reproductive tract to breast tissue has not yet been demonstrated, women with breast cancer have been reported to exhibit significant alterations in the composition of the vaginal microbiota. Specifically, these changes involve a decrease in protective bacteria traditionally classified as *Lactobacillus* and an increase in microorganisms such as *Staphylococcus* spp., *Gardnerella vaginalis*, and representatives of *Mycoplasma* and *Ureaplasma* [16,17]. Among these microorganisms, atypical bacteria are of particular interest because of their capacity for persistent colonization, close interactions with host cells, and potential involvement in chronic inflammation and alterations of cellular processes [18,19].

The relationships between the microbiota of benign breast lesions and microbial communities inhabiting other body sites remain largely unexplored. This pilot study aimed to characterize and compare the microbiota of fibroadenoma tissue, the gastrointestinal tract, and the female reproductive tract in the same patients. Using culture-based microbiological methods, 16S rRNA gene sequencing and targeted PCR for selected atypical bacteria, we assessed whether these anatomically distinct ecological niches share common microbial signatures and whether selected microorganisms associated with the reproductive tract are also detectable in fibroadenoma tissue. Due to the exploratory nature of the study and the limited number of participants, the obtained results should be interpreted as preliminary observations.

## Results

### Culture-Based Characterization of the Microbiota

Qualitative and quantitative analyses of culture-based results revealed high bacterial loads and a diverse microbiota composition in fecal and vaginal samples, whereas no viable microorganisms were detected in the fibroadenoma tissue specimens from any of the participants. The isolated microorganisms and their quantitative abundance are summarized in Table 1.

The number of microorganisms in the vaginal samples ranged from  $10^4$  to  $10^8$  cfu/mL. All women harbored bacteria formerly assigned to the genus *Lactobacillus* in the vagina, including *L. crispatus* ( $10^8$  cfu/mL, patient A01), *L. iners* ( $10^7$  cfu/mL, patient A02), or *L. jensenii* ( $10^7$  cfu/mL, patient A03). Besides lactic acid bacteria, opportunistic pathogens were also detected in the reproductive tract, including *S. anginosus* (patient A02) and *S. epidermidis* (patient A03). In addition, two women were found to be colonized by yeasts of the genus *Candida* (patients A02 and A03). Bacterial counts in fecal samples reached up to  $10^8$  cfu/g. The isolated species represented the dominant members of the gut microbiota and were mainly affiliated with the phyla Bacillota (e.g., *Enterococcus faecalis* and *Eubacterium limosum*) and Bacteroidota (e.g., *Phocaeicola vulgatus* and *Bacteroides uniformis*).

All three patients exhibited distinct microbiological profiles across the examined ecological niches. Importantly, no bacterial species were shared between the female reproductive tract and fecal samples of the studied women.

**Table 1.** Culture-based characterization of microbial communities in the studied ecological niches. The presented values are expressed as colony-forming units (cfu) per gram (g) of tissue or stool and per milliliter (mL) of vaginal specimen.

| Patient | Microorganism                        | Fibroadenoma tissue [cfu/g] | Vaginal sample [cfu/mL] | Stool sample [cfu/g] |
|---------|--------------------------------------|-----------------------------|-------------------------|----------------------|
| A01     | <b>Bacillota</b>                     |                             |                         |                      |
|         | <i>Limosilactobacillus jensenii</i>  | -                           | 1 x 10 <sup>4</sup>     | -                    |
|         | <i>Limosilactobacillus fermentum</i> | -                           | -                       | 8 x 10 <sup>5</sup>  |
|         | <i>Lactobacillus crispatus</i>       | -                           | 1 x 10 <sup>8</sup>     | -                    |
|         | <i>Enterococcus faecalis</i>         | -                           | -                       | 5 x 10 <sup>6</sup>  |
|         | <i>Enterococcus faecium</i>          | -                           | -                       | 3 x 10 <sup>5</sup>  |
|         | <b>Bacteroidota</b>                  |                             |                         |                      |
|         | <i>Bacteroides uniformis</i>         | -                           | -                       | 1 x 10 <sup>8</sup>  |
|         | <b>Pseudomonadota</b>                |                             |                         |                      |
|         | <i>Escherichia coli</i>              | -                           | -                       | 2 x 10 <sup>6</sup>  |
|         | <b>Actinomycetota</b>                |                             |                         |                      |
|         | <i>Collinsella aerofaciens</i>       | -                           | -                       | 3 x 10 <sup>8</sup>  |
| A02     | <b>Bacillota</b>                     |                             |                         |                      |
|         | <i>Eubacterium limosum</i>           | -                           | -                       | 1 x 10 <sup>8</sup>  |
|         | <i>Enterococcus faecium</i>          | -                           | -                       | 1 x 10 <sup>6</sup>  |
|         | <i>Lactobacillus iners</i>           | -                           | 1 x 10 <sup>7</sup>     | -                    |
|         | <i>Streptococcus anginosus</i>       | -                           | 1 x 10 <sup>6</sup>     | -                    |
|         | <b>Bacteroidota</b>                  |                             |                         |                      |
|         | <i>Phocaeicola vulgatus</i>          | -                           | -                       | 1 x 10 <sup>8</sup>  |

**Pseudomonadota**

|                         |   |   |                 |
|-------------------------|---|---|-----------------|
| <i>Escherichia coli</i> | - | - | $2 \times 10^6$ |
|-------------------------|---|---|-----------------|

**Actinomycetota**

|                                |   |   |                 |
|--------------------------------|---|---|-----------------|
| <i>Bifidobacterium bifidum</i> | - | - | $5 \times 10^6$ |
|--------------------------------|---|---|-----------------|

|                     |   |                 |   |
|---------------------|---|-----------------|---|
| <i>Winkia neiri</i> | - | $1 \times 10^6$ | - |
|---------------------|---|-----------------|---|

**Ascomycota**

|                         |   |                 |   |
|-------------------------|---|-----------------|---|
| <i>Candida glabrata</i> | - | $1 \times 10^3$ | - |
|-------------------------|---|-----------------|---|

**A03****Bacillota**

|                                     |   |                 |   |
|-------------------------------------|---|-----------------|---|
| <i>Limosilactobacillus jensenii</i> | - | $1 \times 10^7$ | - |
|-------------------------------------|---|-----------------|---|

|                                     |   |   |                 |
|-------------------------------------|---|---|-----------------|
| <i>Ligilactobacillus salivarius</i> | - | - | $2 \times 10^4$ |
|-------------------------------------|---|---|-----------------|

|                                   |   |                 |   |
|-----------------------------------|---|-----------------|---|
| <i>Staphylococcus epidermidis</i> | - | $1 \times 10^4$ | - |
|-----------------------------------|---|-----------------|---|

|                                    |   |   |                 |
|------------------------------------|---|---|-----------------|
| <i>Staphylococcus haemolyticus</i> | - | - | $4 \times 10^3$ |
|------------------------------------|---|---|-----------------|

|                            |   |   |                 |
|----------------------------|---|---|-----------------|
| <i>Enterococcus durans</i> | - | - | $2 \times 10^4$ |
|----------------------------|---|---|-----------------|

**Bacteroidota**

|                            |   |   |                 |
|----------------------------|---|---|-----------------|
| <i>Phocaeicola plebius</i> | - | - | $6 \times 10^8$ |
|----------------------------|---|---|-----------------|

**Ascomycota**

|   |   |                 |   |
|---|---|-----------------|---|
| - | - | $1 \times 10^3$ | - |
|---|---|-----------------|---|

|                         |  |  |  |
|-------------------------|--|--|--|
| <i>Candida albicans</i> |  |  |  |
|-------------------------|--|--|--|

**Detection of Atypical Bacteria by PCR**

Targeted PCR analysis for selected atypical bacteria revealed the presence of DNA from species belonging to the family *Mycoplasmataceae* in the female reproductive tract, the gastrointestinal tract, and, most importantly, in fibroadenoma tissue. The results of PCR screening are summarized in Table 2.

One noteworthy finding was the detection of *U. urealyticum* DNA across all sampled niches in patient A01, including fibroadenoma tissue, the vaginal swab, and the stool sample. Furthermore, patient A01 was found to harbor *M. hominis* in both the vaginal and fecal samples, however, this species was not detected in the breast tissue. In patient A02, *M. hominis* and *U. urealyticum* DNA was detected solely in

the vaginal swab, with no corresponding detection in the other analyzed samples. For patient A03, PCR testing yielded negative results for all targeted atypical bacteria across the three anatomical sites examined. Moreover, *C. trachomatis*, *M. genitalium*, and *U. parvum* were not identified in any of the clinical samples collected.

**Table 2.** PCR detection of atypical bacteria in the studied ecological niches.

| Patient | Microorganism                 | Fibroadenoma tissue | Vaginal sample | Stool sample |
|---------|-------------------------------|---------------------|----------------|--------------|
| A01     | <i>Chlamydia trachomatis</i>  | –                   | –              | –            |
|         | <i>Mycoplasma genitalium</i>  | –                   | –              | –            |
|         | <i>Mycoplasma hominis</i>     | –                   | +              | +            |
|         | <i>Ureaplasma urealyticum</i> | +                   | +              | +            |
|         | <i>Ureaplasma parvum</i>      | –                   | –              | –            |
| A02     | <i>Chlamydia trachomatis</i>  | –                   | –              | –            |
|         | <i>Mycoplasma genitalium</i>  | –                   | –              | –            |
|         | <i>Mycoplasma hominis</i>     | –                   | +              | –            |
|         | <i>Ureaplasma urealyticum</i> | –                   | +              | –            |
|         | <i>Ureaplasma parvum</i>      | –                   | –              | –            |
| A03     | <i>Chlamydia trachomatis</i>  | –                   | –              | –            |
|         | <i>Mycoplasma genitalium</i>  | –                   | –              | –            |
|         | <i>Mycoplasma hominis</i>     | –                   | –              | –            |
|         | <i>Ureaplasma urealyticum</i> | –                   | –              | –            |
|         | <i>Ureaplasma parvum</i>      | –                   | –              | –            |

**16S rRNA Microbiota Profiles**

Overall Microbial Composition:

The taxonomic composition of microbial communities differed between fibroadenoma tissue, vaginal swabs, and stool samples. Relative abundances of the ten most prevalent bacterial phyla and genera identified by 16S rRNA gene sequencing are presented in Figure 1.

At the phylum level, the vaginal microbiota was overwhelmingly dominated by members of Bacillota. Stool samples harbored a more diverse microbial community, primarily represented by Bacillota and Bacteroidota. In contrast, fibroadenoma tissue displayed the most heterogeneous composition,

characterized by variable proportions of Bacillota, Pseudomonadota, and other less abundant bacterial phyla (Figure 1A).

Genus-level analysis revealed a marked predominance of *Lactobacillus* spp. in the vaginal microbiota of all study participants. The stool microbiota was primarily composed of bacterial genera commonly found in the human gut, such as *Faecalibacterium*, *Ruminococcus*, and *Bacteroides*. By contrast, the microbial communities identified in fibroadenoma tissue were mainly represented by the genera *Sporosarcina*, *Bradyrhizobium*, and *Lactobacillus*, with their relative abundances differing between patients (Figure 1B).

To gain a more detailed insight into the microbiome composition of the studied ecological niches, the 20 most abundant species in each niche were compared based on their CLR-transformed abundance values (Figure 2). The heatmap clearly shows differences in the microbial species composition of the vagina, feces, and fibroadenoma tissue. The key species identified in the vaginal samples were *Lactobacillus crispatus* and *Lactobacillus iners*. In contrast, all fecal samples were enriched in bacterial taxa commonly associated with the gut commensal microbiota, including *Faecalibacterium prausnitzii*, *Ruminococcus bromii*, *Phocaeicola vulgatus*, *Blautia luti*, and *Blautia wexlerae*. Notably, fibroadenoma tissue was characterized by a marked enrichment of *Sporosarcina aquimarina*, particularly in patients A01 and A02, whereas this species was virtually undetectable in the vaginal and fecal samples. Fibroadenoma tissue also harbored lactic acid bacteria, including *L. crispatus* and *L. iners*, as well as taxa typically associated with the intestinal microbiota, such as *F. prausnitzii*, *R. bromii*, *B. wexlerae*, *B. luti*, and *Agathobacter rectalis*. However, these microorganisms were detected at substantially lower relative abundances than those observed in their corresponding source niches.

Despite the presence of niche-specific microbial profiles, several species were detected in individual patients as being common to both fibroadenoma tissue and the vaginal microbiota, including *L. crispatus* (patient A03) and *L. iners* (patients A01 and A02). Similarly, species shared between fibroadenoma tissue and fecal samples included *F. prausnitzii* and *Faecalibacterium duncaniae* (patient A01), *R. bromii* (patients A01, A02, and A03), *B. wexlerae* (patients A01 and A02), *B. luti* (patients A01, A02, and A03), and *A. rectalis* (patients A02 and A03). However, in most cases, these species did not constitute a consistent pattern shared across all study participants, indicating substantial inter-individual variability in the species-level composition of the microbiota.

#### Alpha Diversity:

To compare the richness and diversity of microorganisms in fibroadenoma tissues, vaginal swabs, and fecal samples, an alpha diversity analysis was performed. Diversity was assessed using the Chao1, Shannon, Simpson, and Observed Richness indices. The results of alpha diversity analyses are presented at the genus level (Figure 3). Additional analyses at other taxonomic levels are provided in the Supplementary Materials online (Supplementary Figure S1).

Alpha diversity differed between sample types primarily in terms of taxonomic richness. Stool samples exhibited the highest richness, tissue samples intermediate richness, and vaginal samples the lowest richness across all taxonomic levels. Kruskal–Wallis tests identified significant differences between sample types for richness-based metric (Observed) across all taxonomic levels after Benjamini–Hochberg correction (adjusted  $p \sim 0.04$ ). In contrast, diversity indices incorporating both richness and evenness (Shannon and Simpson) did not reach statistical significance after multiple-testing correction (adjusted  $p \sim 0.054$ – $0.066$ ), although they showed similar trends. These results indicate that the primary difference between sample types lies in the number of taxa present, rather than in the distribution of relative abundances among taxa. Additional post hoc analyses using Wilcoxon rank-sum tests did not reveal any statistically significant differences between individual sample types.

In summary, alpha diversity analyses revealed consistent trends in diversity differences among the ecological niches examined; however, these findings should be interpreted with caution due to the small sample size and the limited statistical power of the comparative analyses.

#### Beta Diversity:

Beta diversity analysis was conducted to evaluate variations in the microbial community structure among fibroadenoma tissue, vaginal samples, and fecal specimens. Community dissimilarity was assessed using three complementary distance metrics: Bray–Curtis dissimilarity, which accounts for differences in taxon abundance, Jaccard distance, based on the presence or absence of taxa, and Jensen–Shannon divergence (JSD). For consistency with earlier microbiome analyses, only the genus-level beta diversity is presented in the main manuscript (Figure 4), while the corresponding results for the phylum, family, and species levels are provided in Supplementary Figure S2 online.

PCoA plots based on Bray–Curtis distances revealed clear clustering of samples according to niche type across multiple taxonomic levels. Stool, vaginal, and fibroadenoma tissue samples formed clearly separated clusters within the ordination space. The strongest separation was observed at the phylum level, where the first principal coordinate explained 74.2% of the total variance (Supplementary Figure S2A online). Although minor overlap between groups was observed at lower taxonomic levels, the overall clustering pattern was consistent with the PERMANOVA results, confirming that microbial community structure differs markedly between the three sample types.

Global PERMANOVA analysis confirmed significant differences in microbial community composition among fibroadenoma tissue, vaginal swabs, and stool samples (adjusted  $p < 0.05$ ). Similar results were obtained using Bray–Curtis, Jaccard, and Jensen–Shannon distance metrics, indicating that the observed clustering was robust irrespective of the distance metric applied. Dispersion differences between sample types were not statistically significant ( $p > 0.05$ ), indicating that the observed PERMANOVA results likely reflect true differences in microbial community composition rather than differences in within-group variance.

Pairwise comparisons revealed the greatest difference in microbial community composition between stool and vaginal samples ( $R^2 \approx 0.90$ ), indicating a clear separation of these microbial communities. Moderate differences were observed between vaginal samples and fibroadenoma tissue samples ( $R^2 \approx 0.71$ ), whereas the smallest differences were detected between stool samples and fibroadenoma tissue samples ( $R^2 \approx 0.60$ ). The observed  $R^2$  values indicate marked differences in microbial community structure among the analyzed sample types; however, none of the pairwise comparisons reached statistical significance ( $p = 0.1$ ), likely due to the small sample size in each group ( $n = 3$ ).

Overall, beta diversity analysis demonstrated distinct microbial community compositions across the investigated ecological niches.

#### Differential Abundance Analysis:

Differential abundance analysis was performed to identify bacterial taxa that differed significantly in relative abundance between fibroadenoma tissue, vaginal swabs, and stool samples. The complete results for the 20 most relevant bacterial taxa in each pairwise comparison are presented in Supplementary Tables S1–S3 online.

Differential abundance analysis using ALDEx2 did not identify taxa with statistically significant differences after multiple-testing correction. However, several taxa showed relatively large effect sizes, suggesting potential biological differences between sample types. Owing to the limited sample size and the higher variability characteristic of low-biomass samples, these results should be regarded as exploratory.

The strongest differences were observed between stool and vaginal samples (Supplementary Table S1 online). Genera associated with the vaginal microbiome showed very large effect sizes, particularly *Lactobacillus*, which displayed the largest difference between groups (effect  $\sim 9.7$ ) and markedly higher abundance in vaginal samples. Other genera enriched in the vaginal environment included *Limosilactobacillus*, *Ureaplasma*, and *Peptoniphilus*.

When comparing stool to fibroadenoma tissue, several genera were found to be strongly enriched in the tissue specimens (Supplementary Table S2 online). These included *Sporosarcina*, *Bradyrhizobium*, *Cutibacterium*, and *Staphylococcus*. Conversely, several typical gut-associated genera such as *Bacteroides*, *Faecalibacterium*, and *Phocaeicola* showed slightly higher CLR values in stool samples compared to tissue, although the effect sizes for these taxa were smaller. Overall, these results suggest that the microbial signal detected in tissue samples includes both taxa commonly associated with environmental sources and a subset of bacteria typically observed in the gut microbiome.

The comparison between vaginal and fibroadenoma tissue samples further underscored the intermediate ecological position of the fibroadenoma microbiota (Supplementary Table S3 online). While the vagina remained dominated by *Lactobacillus* (effect  $\sim -6.4$ ), the tissue samples showed noticeably higher abundances of several genera typically associated with the gut microbiome, including

*Faecalibacterium*, *Blautia*, *Ruminococcus*, *Bacteroides*, and *Agathobacter*. These findings are consistent with the beta-diversity analyses and clustering patterns observed in the taxonomic heatmaps, where tissue samples occupy an intermediate position between stool and cervical microbiomes.

#### Comparison of 16S rRNA sequencing with culture- and PCR-based analyses:

The comparative analysis of conventional culture, 16S rRNA gene sequencing, and targeted PCR demonstrated both areas of agreement and significant divergence in the obtained results, highlighting the distinct sensitivity and specificity profiles of each diagnostic method.

The presence of several bacterial genera within the investigated ecological niches was confirmed by both culture-based/PCR-based analyses, as well as by 16S rRNA gene sequencing. The greatest agreement between methods was observed for vaginal and fecal samples (Figure 5). Both approaches confirmed the dominance of *Lactobacillus* and the presence of *Ureaplasma* spp. within the female genital tract microbiota. In fecal samples, 16S rRNA gene sequencing and conventional culture concordantly identified genera including *Phocaeicola*, *Limosilactobacillus*, *Enterococcus*, *Eubacterium*, *Collinsella*, and *Bacteroides*, although these findings were not consistent across all participants.

As expected, 16S rRNA gene sequencing detected a substantially broader spectrum of microorganisms than conventional culture. This was particularly evident for anaerobic commensals, such as *Bacteroides* spp. identified in fecal and tissue samples, as well as for low-abundance microorganisms present in tissue specimens that could not be recovered by culture-based methods, including representatives of the genera *Streptococcus*, *Staphylococcus*, *Phocaeicola*, *Lactobacillus*, *Enterococcus*, and *Bacteroides*. Conversely, conventional culture enabled the detection of certain taxa, such as *Escherichia* spp., which were not identified by 16S rRNA gene sequencing. Targeted PCR further complemented the microbiological analyses by enabling the detection of atypical bacteria, including *M. hominis* and *U. urealyticum*, which were not assessed by conventional culture and may be underrepresented in 16S rRNA gene sequencing datasets, particularly in low-biomass samples.

Together, culture, targeted PCR, and 16S rRNA sequencing provided complementary information regarding the microbial composition of fibroadenoma tissue, vaginal swabs, and stool samples.

## Discussion

To the best of our knowledge, this pilot study is the first to compare the microbiota of the gastrointestinal tract, reproductive tract, and fibroadenoma tissue within the same patients. The complementary application of conventional culture, 16S rRNA gene sequencing, and targeted PCR provided a comprehensive characterization of the microbial communities across the analyzed niches, revealing distinct niche-specific microbial profiles. Despite these differences, several taxa were shared among niches, indicating partial overlap between microbial communities.

The marked differences in microbiota composition observed across the analyzed niches suggest that each represents a distinct environment that supports the growth of specific microbial communities. Vaginal samples were dominated by bacteria of the genus *Lactobacillus*, consistent with the well-documented role of this genus in maintaining vaginal health and homeostasis. While a depletion of *Lactobacillus* has been observed in the reproductive tract microbiota of women with breast cancer, the dominance of this genus is commonly reported in the urogenital niches of women without malignant disease [16,17,22]. Stool samples exhibited a characteristic gut microbial profile, with obligate anaerobic genera, including *Faecalibacterium*, *Bacteroides*, and *Blautia*, among the most abundant taxa. The microbiological composition of fibroadenoma tissue was the most diverse, including both commensal gut-associated taxa (*Faecalibacterium*, *Blautia*), microorganisms associated with the skin surface or vaginal environment (*Staphylococcus*, *Lactobacillus*) and the genera *Sporosarcina* and *Bradyrhizobium*, which were unique to this tissue. A similar heterogeneity of benign breast lesions was reported by Hieken et al. and Ukraincev et al, who emphasized that the breast harbors its own unique microbial signature [23,4].

Despite the clear differences in the composition of microbial communities inhabiting the various niches, several taxa were present in more than one anatomical site. An example is the presence of *Lactobacillus* species in both the reproductive tract and fibroadenoma tissue. Moreover, in some patients, the same species were detected in both niches, including *L. iners* in patients A01 and A02 and *L. crispatus* in patient A03. Members of the family *Lactobacillaceae*, including *Lactobacillus*-related taxa, have been reported as components of the microbiota of healthy breast tissue [24]. It has been suggested that, due to their immunomodulatory and anti-inflammatory properties, certain strains may play a protective role against neoplastic transformation [25]. However, across different studies, the relative abundance of *Lactobacillus* species present in benign and malignant breast lesions remains inconsistent [8,23,25]. Also noteworthy is the presence of *U. urealyticum* DNA in the tumor tissue, vaginal swab, and fecal sample of patient A01, as confirmed by targeted PCR. Although *U. urealyticum* has traditionally been associated with urogenital tract infections, its presence within the tumor microenvironment, including both benign and malignant lesions, is increasingly being reported in the context of promoting chronic inflammation and potentially contributing to host DNA damage [26,27]. Other taxa commonly detected in both fibroadenoma tissue and the vagina and/or stool included the genera *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Phocaeicola*, *Bacteroides*, and *Eubacterium*. These microorganisms are frequently found in various ecological niches associated with the human body.

The presence of the same bacterial taxa in anatomically distant niches raises the question of what accounts for their presence in breast tissue. As mentioned earlier, several potential routes have been proposed through which microorganisms may reach breast tissue. Bacteria originating from anatomically distant mucosal surfaces may potentially access breast tissue through hematogenous or lymphatic dissemination [4,14]. Another proposed mechanism is the entero-mammary pathway. This concept assumes the translocation of selected intestinal bacteria to the mammary gland through mechanisms involving immune cells, particularly dendritic cells and CD18+ lymphocytes. [4,28,29]. There is also the concept of an exogenous cutaneous pathway, which suggests that the mammary gland may

become colonized by skin-associated bacteria entering through the nipple orifices and subsequently migrating upstream through the lactiferous ducts [12,14, 30]. The actual contribution of the mechanisms described above to the establishment and composition of the breast tissue microbiota remains unclear. Even less is known about the potential relationships between the microbiota of the female reproductive tract and the breast microbiome. The detection of shared bacterial taxa, including species-level taxa (*L. crispatus*, *L. iners*, and *U. urealyticum*), in both vaginal samples and fibroadenoma tissue raises the possibility of biological connections between these anatomically distant ecological niches. Nevertheless, the presence of the same taxon in different anatomical locations cannot be considered evidence of microbial translocation. Widely distributed host-associated bacterial taxa may independently colonize multiple ecological niches. Establishing whether microorganisms detected at these sites are genetically related requires higher-resolution methods capable of strain-level characterization.

While the breast cancer microbiome has been extensively investigated, the microbial communities associated with benign breast lesions, including fibroadenoma, remain poorly characterized. In the present study, fibroadenoma tissue harbored a heterogeneous bacterial community that was clearly distinct from both vaginal and intestinal microbiota. The analyzed tissue samples contained, among others, members of the genera *Lactobacillus*, *Staphylococcus*, *Streptococcus*, and *Enterococcus*, which have been reported in the literature as common constituents of the microbiota of healthy breast tissue [5,6,8]. At the same time, previous studies have suggested a potential association between the presence of the genus *Staphylococcus* and the pathogenesis of fibroadenomas [4,9,10]. One of the most noteworthy findings of the present study was the consistent presence of the genera *Sporosarcina* and *Bradyrhizobium* in fibroadenoma tissue samples obtained from all three patients. Although this pattern may indicate a potential association of these bacteria with the fibroadenoma microenvironment, the small study cohort does not allow conclusions to be drawn regarding their specificity for benign breast lesions. Notably, previous studies have detected *Sporosarcina* in tissue adjacent to triple-negative breast cancer (TNBC) tumors, raising the possibility that this taxon may be linked to inflammatory or proliferative microenvironments and is not necessarily specific to malignant lesions [31,32]. The genus *Bradyrhizobium* has been infrequently reported in the literature. Limited evidence suggests its presence in both malignant tumors and adjacent non-malignant breast tissue [23,33,34]. At present, the available evidence is insufficient to clearly define a microbiome characteristic of fibroadenoma. Larger studies with greater statistical power are needed to determine whether these taxa are indeed characteristic of benign breast lesions.

A major strength of the present study was the simultaneous application of three microbiological approaches: conventional culture, 16S rRNA gene sequencing, and targeted PCR. Each method provided distinct yet complementary information on the microbial communities analyzed. Conventional microbiological methods confirmed the viability of selected microorganisms and provided quantitative information on bacterial load, whereas 16S rRNA gene sequencing enabled the identification of a broad range of bacterial taxa. Targeted PCR further complemented these approaches by detecting *M. hominis* and *U. urealyticum*, which are difficult to culture and may be overlooked in analyses based on whole-community sequencing. However, each of the methods used has its own limitations, making it

impossible to fully capture the microbial profile using a single analytical tool alone. Therefore, the integration of these complementary approaches provides the most comprehensive and reliable framework for studying microbial communities, especially in low-biomass environments such as breast tissue, as has also been demonstrated in previous studies [35-37].

Several limitations of the present study should also be acknowledged. First, the small number of participants (N = 3) limits the statistical power of the analyses and precludes generalization of the findings. Second, breast tissue represents a low-biomass sample, making microbiome analyses particularly susceptible to technical bias and potential contamination originating from laboratory reagents or the surrounding environment. Furthermore, it should be noted that 16S rRNA gene sequencing has limited species-level resolution, which in some cases hampers the precise identification of microorganisms. Therefore, the results obtained in this study should be interpreted cautiously and viewed mainly as hypothesis-generating rather than as conclusive evidence of the observed relationships. Nevertheless, the consistency of several observations across individual cases supports the need for larger prospective studies.

In conclusion, this pilot study provides additional evidence that breast fibroadenoma tissue is not a sterile environment but rather harbors a unique and diverse microbiome, whose composition exhibits significant similarities to the microbial communities of the gastrointestinal and female reproductive tracts. While the source of microorganisms detected in benign breast lesions cannot be determined based on the present data, the findings suggest that their microbiota should be viewed in the context of the broader microbial ecosystems of the host. Validation of these observations will require larger patient cohorts together with strain-resolved metagenomic approaches to provide a more detailed characterization of the microbial communities involved.

## Methods

### Study Design and Patient Characteristics

This pilot study was conducted to compare the microbiota of three different ecological niches - breast lesion tissue, vaginal sample and stool - in women with benign breast tumors. Patients were recruited at the Oncology Outpatient Clinic of University Hospital in Krakow, Poland between May and June 2025. Three patients (aged 26 - 40 years) with histologically confirmed fibroadenoma were included in the study. Only patients who declared no antibiotic use within the previous 30 days and no chronic diseases, such as diabetes, autoimmune diseases, or inflammatory bowel diseases, were eligible for inclusion. A detailed characterization of the patients enrolled in the study is presented in Table 3. The study protocol was approved by the Bioethics Committee of the Jagiellonian University Medical College (approval no. 1072.6120.76.2024). Written informed consent was obtained from all participants prior to enrollment. All procedures involving human participants and biological samples were performed in accordance with the relevant guidelines and regulations.

**Table 3.** Characteristics of patients. Abbreviations: **BMI** (Body Mass Index)

| PARAMETER                                                                     | PATIENT A01  | PATIENT A02  | PATIENT A03  |
|-------------------------------------------------------------------------------|--------------|--------------|--------------|
| Age                                                                           | 32           | 40           | 26           |
| BMI (kg/m <sup>2</sup> )                                                      | 23           | 24           | 21           |
| Chronic diseases (diabetes, autoimmune diseases, inflammatory bowel diseases) | no           | no           | no           |
| Menopausal status (pre/postmenopausal)                                        | pre          | pre          | pre          |
| Antibiotic therapy (<30 days)                                                 | no           | no           | no           |
| Histopathological diagnosis                                                   | fibroadenoma | fibroadenoma | fibroadenoma |

## Sample Collection

### Breast Lesion Tissue:

Material for the study was collected during routine biopsy procedures under sterile conditions. A portion of the collected tissue was sent for histopathological examination to the Department of Pathomorphological Diagnostics, University Hospital in Krakow, to establish the final diagnosis of the lesion. The remaining portion, intended for microbiological analyses, including culture-based and molecular methods, was divided into two parts and placed into sterile RNase- and DNase-free tubes. The tubes were then placed on dry ice and transported directly to the laboratory of the Department of Microbiology, Jagiellonian University Medical College, where the samples were stored at -80 °C until further analysis.

### Stool Samples:

Stool samples were self-collected by the patients using specialized, sterile “Kałszyk” stool sample collection kits (Kosowski, Poland). The samples were delivered to the Department of Microbiology, Jagiellonian University Medical College, where they were stored at -80 °C until further analysis.

### Vaginal Swabs:

Vaginal swabs from the posterior fornix were collected by a gynecologist using a sterile speculum. Two swabs were obtained from each patient: one designated for conventional culture and the other for DNA isolation and molecular analyses. All swabs were transported to the Department of Microbiology, Jagiellonian University Medical College. Samples intended for culture, secured in Amies transport medium (Deltalab, Spain), were immediately inoculated onto appropriate culture media. In contrast, swabs for molecular analyses, collected into sterile tubes without transport medium, were stored at -80 °C until DNA extraction.

## Culture-based Microbiological Analysis

A qualitative and quantitative assessment of viable microorganisms present in the examined clinical samples was performed using culture-based methods. To prepare the initial suspensions, fragments of breast lesion tissue (approximately 10–50 mg), after thawing, were homogenized in 1 ml of sterile phosphate-buffered saline (PBS) using a mortar. From the thawed stool samples, 1 g of material was weighed and suspended in 9 ml of sterile Schaedler broth (Oxoid, UK). Vaginal swabs delivered to the laboratory were suspended in 1 ml of Tryptic Soy Broth (TSB, Oxoid, UK). All suspensions prepared in this way were subjected to a serial dilution procedure in sterile physiological saline. From the prepared dilutions, 100 µl aliquots were collected and plated onto the following culture media: Columbia Blood Agar, MacConkey Agar, MRS Agar, Schaedler Agar, Sabouraud Agar (all Oxoid, UK), and Bile Esculin Agar (BD, USA). This enabled the cultivation of: aerobic bacteria, Gram-negative rods, lactic acid bacteria, anaerobic bacteria, yeasts and filamentous fungi, as well as fecal enterococci. Plates with aerobic cultures were incubated for 24 hours at 37 °C, whereas media inoculated with anaerobic microorganisms were incubated under anaerobic conditions at 37 °C for 48–72 hours. After incubation, the grown bacterial and fungal colonies were counted, and the results were expressed as CFU/g for stool and tissue samples (taking into account the weight of individual samples) and as CFU/ml for vaginal swabs. Species-level identification of the cultured microorganisms was performed using mass spectrometry with the MALDI Biotyper (Bruker Scientific LLC, USA). Taxonomic assignments were verified using the MALDI Biotyper for In Vitro Diagnostic (MBT IVD) Library of mass spectra.

### **DNA Extraction and 16S rRNA Sequencing**

Genomic DNA from all sample types was isolated using the DNeasy PowerSoil Kit (Qiagen, Germany), according to the manufacturer's protocol. The purity and quantity of isolated DNA were assessed using two methods: spectrophotometry with a NanoDrop device (Thermo Fisher Scientific, USA) and fluorometry with the Qubit dsDNA High Sensitivity Assay Kit on a Qubit Fluorometer (Thermo Fisher Scientific, USA). Sequencing was performed at the Małopolska Centre of Biotechnology, Jagiellonian University. In order to prepare libraries for sequencing using Oxford Nanopore Technologies, the 16S Barcoding Kit 24 V14 (SQK-16S114.24) (ONT, Oxford, UK) was used. Amplification of the 16S rRNA gene was performed using the primers provided in the kit (27F and 1492R) and LongAmp Hot Start Taq 2X Master Mix (New England Biolabs, Ipswich, MA, USA), using 10 ng of DNA as a template. A C1000 Touch thermocycler (Bio-Rad, Hercules, CA, USA) was used for amplification, and the following reaction conditions were applied: initial denaturation at 95 °C for 1 min; 25 cycles at 95 °C for 20 s, 55 °C for 30 s, 65 °C for 2 min; elongation at 65 °C for 5 min. The concentration of the PCR products was measured fluorimetrically, as before. The amplicons were mixed in equal molar ratios, and the resulting pool was purified using AMPure XP (Beckman Coulter, Indianapolis, IN, USA). 100 fmol of amplicons were measured for sequencing. Sequencing was performed on a P2 Solo sequencer using a PromethION R10.4.1 flow cell (FLO-PRO114M) (ONT, Oxford, UK) in accordance with the manufacturer's instructions. MinKNOW ver. 25.03.7 was used for data collection, using default settings for 16S sequencing and high-accuracy basecalling.

### **Targeted PCR for Selected Atypical Bacteria**

The previously extracted genomic DNA was also utilized as a template for the detection of selected atypical bacterial species. The presence of *C. trachomatis*, *M. hominis*, *M. genitalium*, *U. urealyticum*, and *U. parvum* was assessed using classical PCR with species-specific primers, according to previously published protocols with modifications where applicable [20,21]. Detailed PCR conditions and primer sequences are provided in the Supplementary Materials (Supplementary Table S4 online). Amplification products were separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (Bio-Rad, Hercules, Kalifornia, USA) to confirm the presence and size of the amplified fragments.

## **Bioinformatic and Statistical Analyses**

### Taxonomical Classification:

For the identification of microbiota at the species level, fastq files were analyzed with the EPI2ME Labs wf-16 workflow. Samples were classified using the NCBI 16S/18S targeted-loci database, with reads filtered to a length range of 800-2000 bp. Taxon-level read counts were exported from the Kraken2 report and used as the input abundance table for all downstream analyses.

### Data Processing:

Taxon-level read counts from the Kraken2 output were aggregated to phylum, family, genus, and species level, and converted to relative abundances. All analyses were conducted in R using the packages *vegan*, *phyloseq*, *compositions*, *ALDEx2*, *pairwiseAdonis*, and *philentropy*, with a fixed random seed (42) for reproducibility.

### Alpha Diversity:

Alpha diversity was estimated at each taxonomic level using Shannon diversity, Simpson diversity, observed richness, and the Chao1 richness estimator. Observed richness and Chao1 produced identical values across all samples, so observed richness was used as the representative richness metric. Differences between sample types were assessed using the Kruskal-Wallis test at each taxonomic level, with BH-adjusted p-values; pairwise Wilcoxon rank-sum tests (BH-adjusted) were performed as post hoc comparisons only where the global test reached significance.

### Beta Diversity:

Community-level differences between sample types were assessed using Bray-Curtis dissimilarity, Jaccard distance, and Jensen-Shannon divergence, calculated on relative abundance data at each taxonomic level. Differences in composition were tested by PERMANOVA, with BH-adjusted p-values across taxonomic levels. Homogeneity of multivariate dispersion was evaluated for Bray-Curtis distances to rule out dispersion as a confound. PCoA (Principal Coordinate Analysis) based on Bray-Curtis dissimilarities was used to visualize compositional relationships. Pairwise PERMANOVA comparisons between sample types were performed at the genus level, given the constraint on minimum achievable p-values at n = 3 per group, these were treated as exploratory.

## Differential Abundance Analysis:

Pairwise differential abundance testing between sample types was performed at the genus level using ALDEx2. For each comparison, the BH-adjusted Kruskal-Wallis p-value, median between-group CLR difference, standardized effect size, and within-group geometric mean CLR abundance were reported. Given the limited power of rank- and permutation-based testing at  $n = 3$  per group, effect size rather than p-value was used as the primary metric for ranking differentially abundant taxa.

## Declarations

### Generative AI-assisted writing

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) to improve the language, manuscript organization, and structure of the text. All AI-assisted content was critically reviewed, edited, and verified by the authors. The scientific interpretation of the results, conclusions, and final content of the manuscript remains the sole responsibility of the authors.

### Data availability statement

The datasets generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA 1522804 <https://www.ncbi.nlm.nih.gov/sra/PRJNA1522804>. All other data are included in this article and its Supplementary Information files.

### Author contributions

**A.T.P:** coordinated the project, performed the culture-based microbiological analyses and wrote the original manuscript; **J.S and D.H.Z:** were responsible for patient recruitment, clinical sample collection and interpretation of results. They revised the manuscript for important intellectual content **A.M:** performed the bioinformatic and statistical analyses and prepared the corresponding data visualizations; **M.S:** conceived and designed the study, contributed to data interpretation. All authors have read and approved the final version of the manuscript.

### Competing interests:

The authors declare no competing interests.

### Funding Declaration

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## Figures

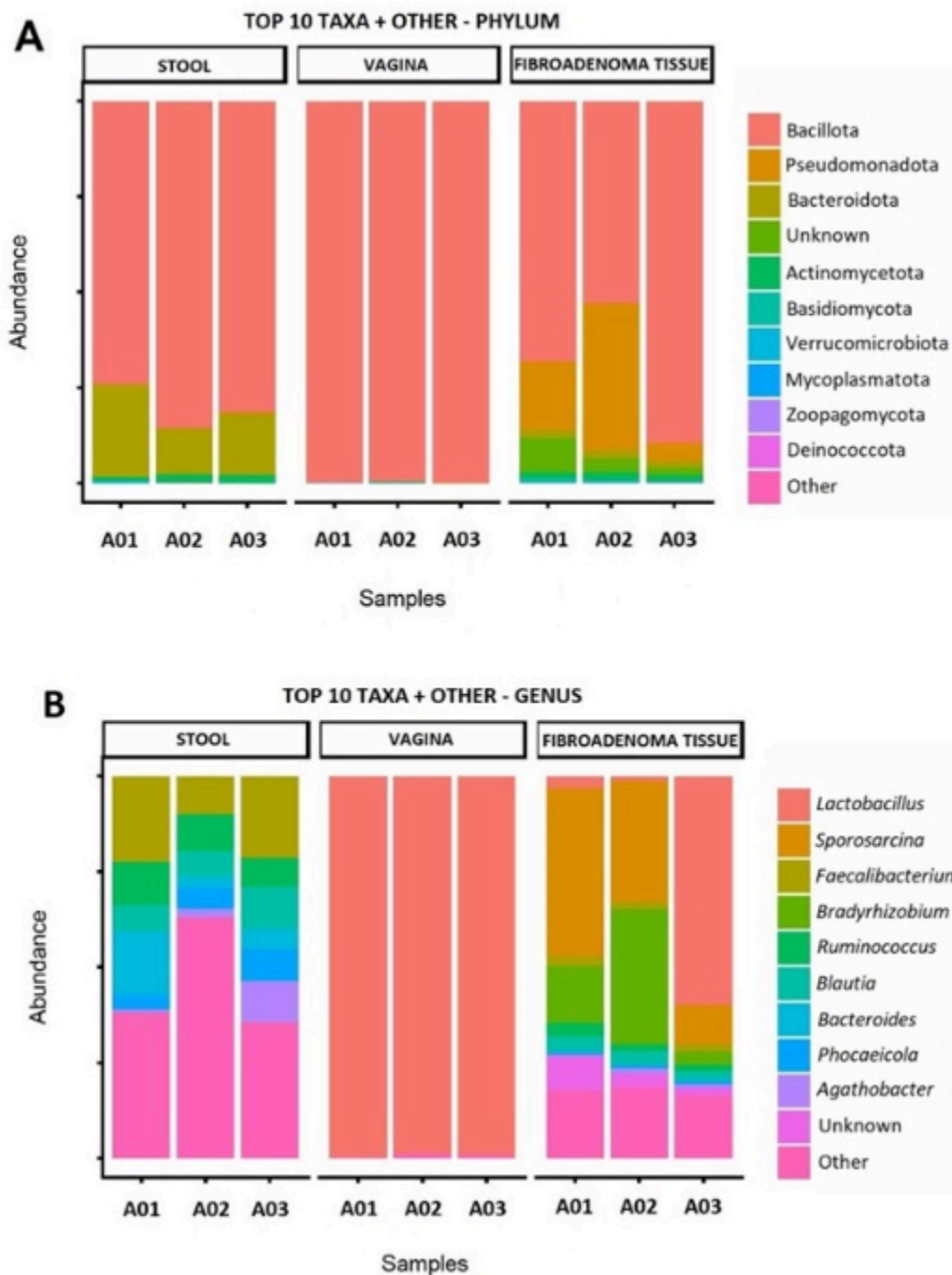


**Figure 1**

Overall microbial composition across fibroadenoma tissue, vaginal swabs, and stool samples determined by 16S rRNA gene sequencing. Relative abundances of the ten most abundant bacterial taxa are shown at the (A) phylum and (B) genus levels. Each bar represents an individual sample, and colors indicate the relative abundance of individual taxa.

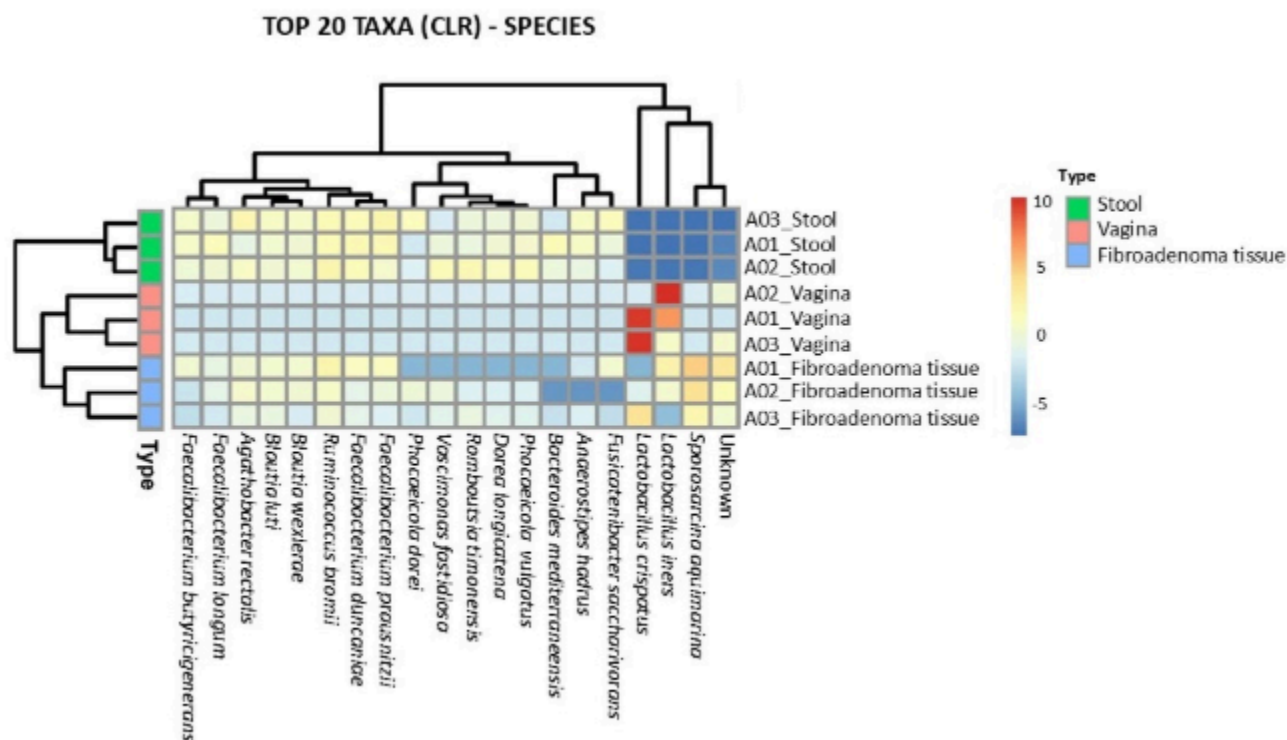


**Figure 2**

Heatmap of the twenty most abundant bacterial species identified by 16S rRNA gene sequencing across fibroadenoma tissue, vaginal swabs, and stool samples. Color intensity represents centered log-ratio (CLR)-transformed relative abundances. Positive values mean that the species is more abundant than the average microbial component in that sample. Negative values mean that the species is less abundant than the average microbial component in that sample. Values close to zero indicate average relative abundance within that sample.

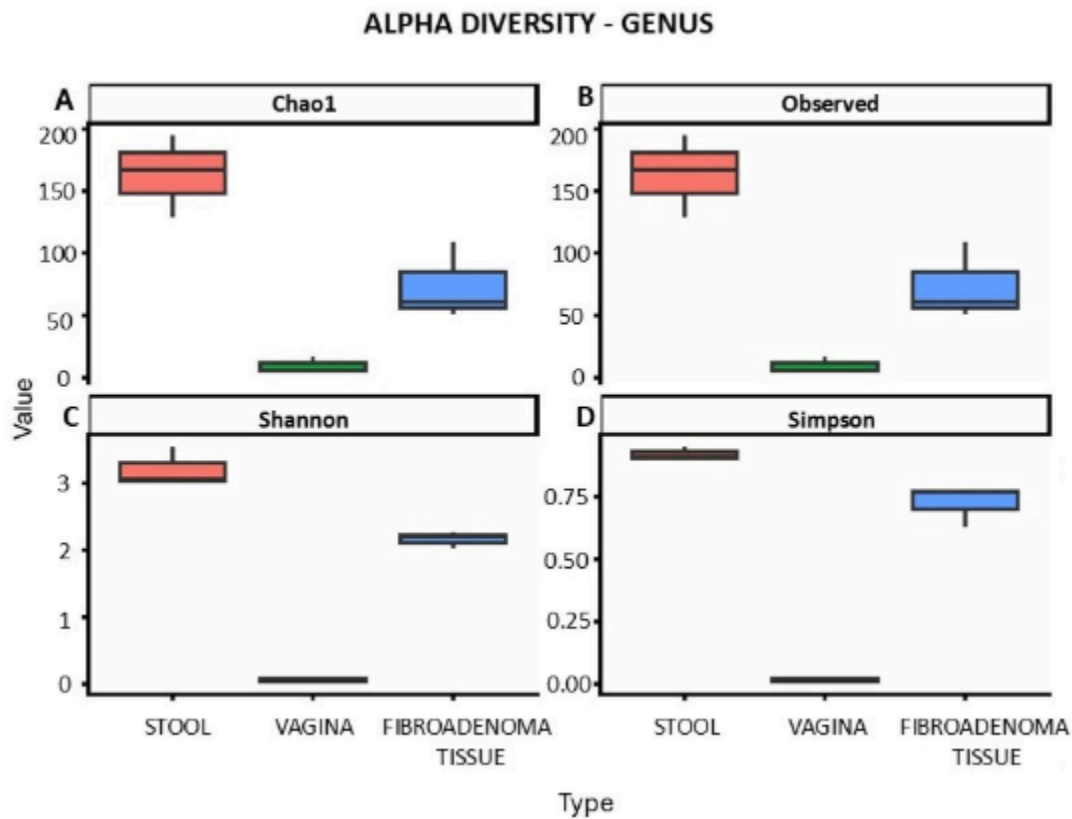


**Figure 3**

Alpha diversity of microbial communities at the genus level across stool samples, vaginal swabs, and fibroadenoma tissue. Boxplots show (A) Chao1 richness, (B) Observed richness, (C) Shannon diversity, and (D) Simpson diversity indices calculated from 16S rRNA gene sequencing data. Boxes represent the interquartile range (IQR), and the horizontal line indicates the median. Statistical comparisons were performed using the Kruskal-Wallis test with Benjamini-Hochberg correction, followed by pairwise Wilcoxon rank-sum tests.

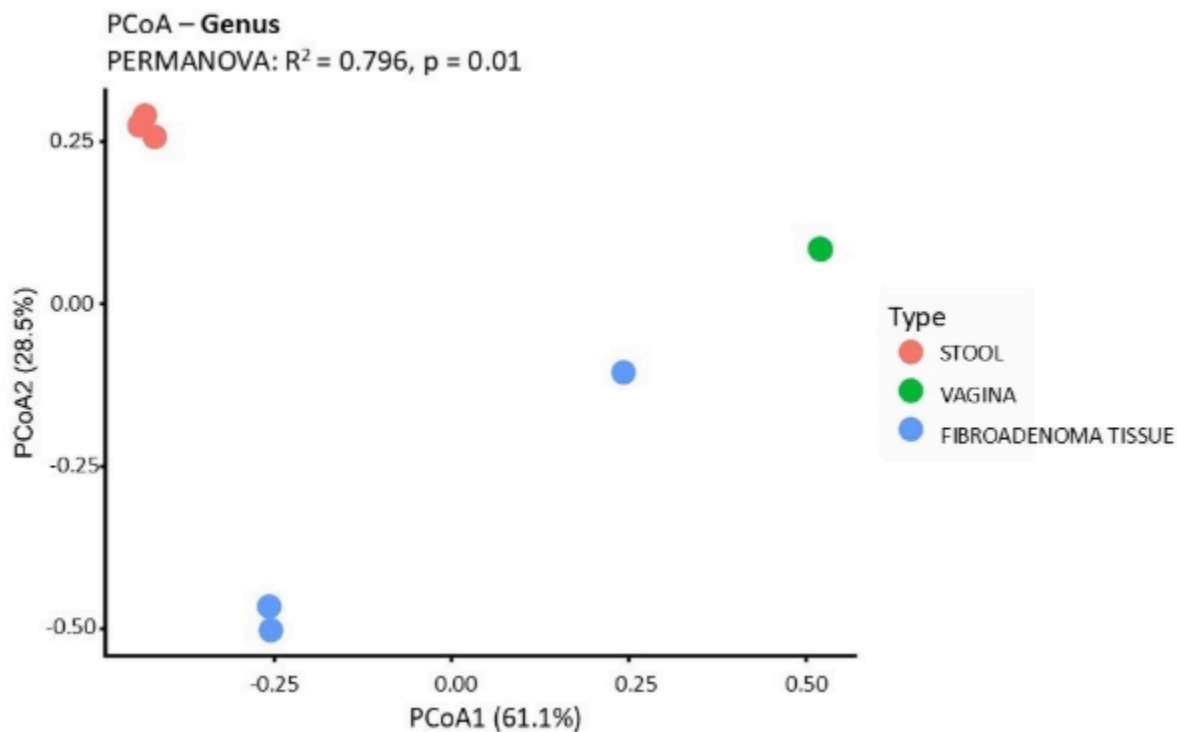
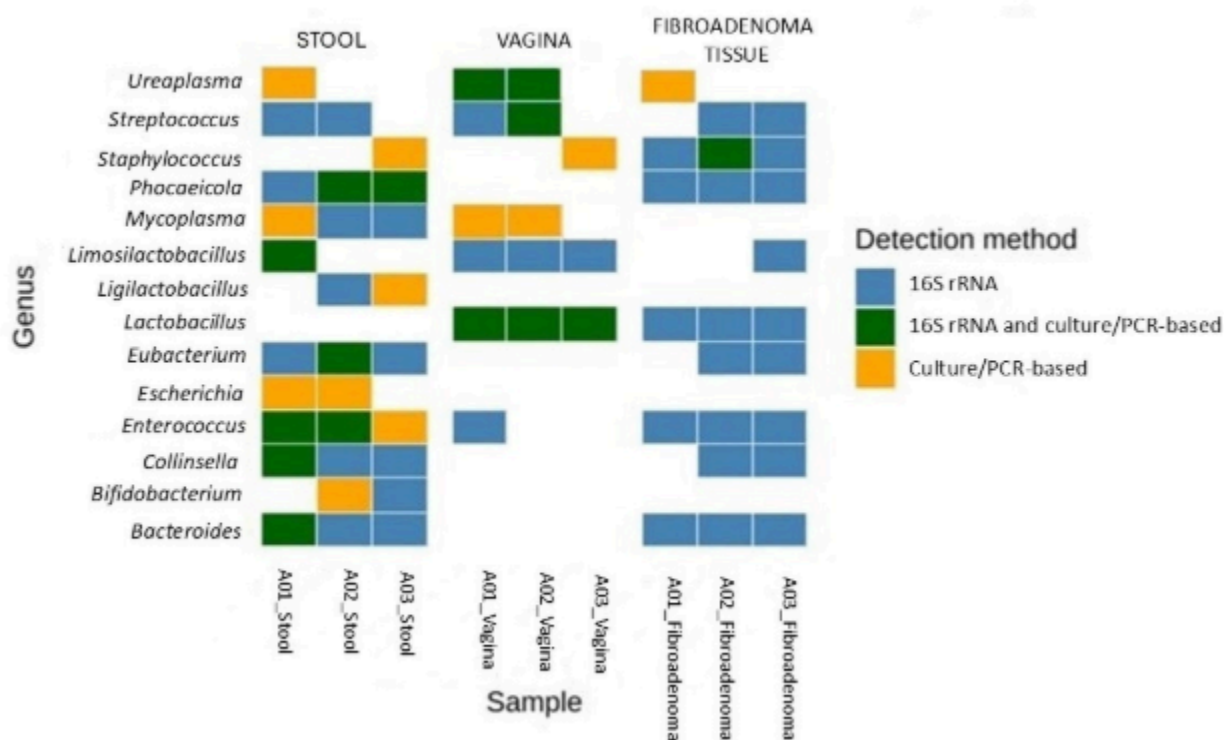


Figure 4

Beta diversity of microbial communities at the genus level across fibroadenoma tissue, vaginal swabs, and stool samples. PCoA based on Bray–Curtis dissimilarity illustrates the differences in microbial community composition among the three ecological niches. Each point represents an individual sample, and colors indicate sample type.



## Figure 5

Comparison of bacterial taxa at the genus level identified by 16S rRNA gene sequencing with culture/PCR-based results across the analyzed ecological niches.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterial.pdf](#)