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ORIGINAL ARTICLE

Preliminary investigation of changes in pathogen presence in the vaginal microbiome in association with age

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ABSTRACT

Background and Objectives: The vaginal microbiome represents a dynamic ecosystem that undergoes significant transformations throughout a woman's lifespan, influenced by hormonal fluctuations and physiological changes. Interpreting pathogen distribution and developing suitable therapeutic care techniques for women's reproductive health depends on an understanding of these age-related patterns. This study aims to thoroughly describe age-related changes in the makeup of the vaginal microbiome and the distribution of pathogenic species.

Materials and Methods: Vaginal swab samples were collected from 29 subjects, categorized into different age groups (A: 15-30 years, B: 31-40 years, C: 41-50 years, and D: 51-60 years old females). Microbiome DNA was extracted from the collected vaginal swabs and shotgun next generation sequencing was performed. Post-sequencing, data was analysed using in-house pipeline followed by statistical analysis using R programming.

Results: The results showed that microbial diversity varied significantly with age. Group C displayed the most severe pathogenic burden; Group A had the highest overall species diversity with 350 bacterial species. Group D displayed the greatest overall relative abundance levels of microorganisms, primarily due to *Lactobacillus rhamnosus* dominance.

Conclusion: This study shows that the composition of the vaginal microbiome changes fundamentally over the course of a woman's life, with each stage bringing with it its own set of microbial signatures, pathogenic risks, and therapeutic prospects.

Keywords: Vaginal microbiome; *Lactobacillus*; Aging; Perimenopause; Estrogen

INTRODUCTION

The vaginal microbiome is a dynamic, complex community of microbes, including bacteria, fungi, viruses, and other microbial populations that inhabit the female reproductive tract. It forms an individualized ecosystem with a particular composition and set of functional attributes distinct from other microbial communities such as the gut microbiome (1, 2). A healthy vaginal microbiome plays a crucial role in supporting the overall well-being of women. Vaginal microbiome structure can vary significantly between

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and even within individuals throughout the lifespan. This could be influenced by genetics, age, ethnicity, hormonal status, sexual intercourse, hygiene practices and antibiotic usage (3). Despite the variability of the vaginal microbiome, it is typically characterized by the dominance of *Lactobacillus* species (1, 2). *Lactobacillus*, a dominant member of healthy vaginal microbiome undergoes anaerobic glycolysis, resulting in the secretion of lactic acid, hydrogen peroxide (H_2O_2), and bacteriocins which are antimicrobial in nature (4, 5). The release of these antimicrobial compounds into the vaginal environment reduces vaginal pH and prevents the growth of pathogenic species, which promotes vaginal health (2, 6-9). Additionally, these antimicrobial compounds adhere to vaginal epithelial cells and compete with other microbes for the binding sites thus inhibiting pathogen growth (10).

Although many of the bacteria present within vaginal microbiome are beneficial, some opportunistic pathogens also exist within the microbiome population (11). While the proportions of bacteria present within the vaginal environment remain at normal levels, these opportunistic pathogens cannot achieve significant growth by dominating *Lactobacillus* species (5). However, disruptions to the microbiome composition, specifically decreases in abundance of *Lactobacillus* (7, 12) allow opportunistic pathogens to rapidly populate the vaginal environment, resulting in disease conditions such as bacterial vaginosis (BV) (11, 13). For instance, in BV, with reducing abundance of *Lactobacillus* species, bacteria such as *Gardnerella vaginalis* and *Atopobium vaginae* can form a biofilm that further disintegrate the vaginal ecosystem. Significant effects on vaginal and reproductive health results from these changes in the vaginal microbiome thus leading to an increased risk of HIV and HPV infections and complicated pregnancy outcomes such as preterm labor, miscarriage, and infertility (3, 14, 15).

There are many factors, both modifiable and non-modifiable which influence the composition of the vaginal microbiome, such as age, ethnicity, diet, estrogen levels and hygiene (15-17). Among these, age plays a particularly significant role as it reflects hormonal and physiological changes that can significantly impact diversity and stability. With age, changes in estrogen level, immune function and vaginal structure can affect vaginal microbiome. Puberty marks the onset of significant estrogen production; it also helps the development of vaginal epithelium alongside begins to produce glycogen which promotes healthy vaginal microbiome (1). Age-associated fluctuations and reduction of estrogen level results in dysbiosis, leading to potential pathogenic infections in the older population (18). Understanding the effects of age, on vaginal microbiome composition can offer critical insights not only to the interventions needed, but also when said interventions are required to maintain the healthy composition of the vaginal microbiome and promote vaginal health at all stages of life.

There are several studies on vaginal microbiome of reproductive-age women, evaluating the differences between the overweight and healthy weight reproductive-aged (19-20) or comparison of older reproductive-age vs younger reproductive-aged women for preterm birth risk (21). However, this is the first investigation on women of different age group, focussing reproductive age, perimenopause, menopause, and age-related changes in vaginal microbiome due to the presence of vaginal pathogens.

MATERIALS AND METHODS

Study population and design. This study was conducted following ethical guidelines of Health Research Ethics Board of Alberta (HREBA.CHC-25-0013, 15-May-2025). Participants consented for the study, and significant safeguards were implemented to protect privacy and confidentiality of participants. BioAro Inc. has its own database of vaginal microbiome data collected from the samples for next generation sequencing and analysis. This cross-sectional retrospective study comprised of 29 subjects divided into four age groups: A: 15 years-30 years, B: 31-40 years, C: 41-50 years and D: 51-60 years. Table 1 provides a concise summary of participant data, including age group, hygiene, information about children, and sexual activity. The age group, hygiene, child information, and sexual activity of the participants are all clearly defined in the Table 1. The study's exclusion criteria involved participants using antibiotics, hormonal therapy or expectant and nursing moms, while its inclusion criteria comprised the availability of participant data and informed consent. Vaginal microbiome samples were collected by subjects using Biofemme kit as per manufacturer's instructions, transported to laboratory and stored at -20°C for future analysis. We added vaginal microbiome data of

Table 1. Characteristics of 29 Vaginal Microbiome Samples

Sample Name	Age Group	Regular Periods	No of kids	Hygiene product usage	Sexually Active	Shannon Diversity
Sample1	15-30	no	0	yes	Yes	3.97944929
Sample2	15-30	yes	0	no	No	1.88943841
Sample3	15-30	yes	0	no	Yes	1.5519298
Sample4	15-30	no	0	no	No	1.67788763
Sample5	15-30	no	0	no	Yes	2.32026648
Sample6	15-30	yes	0	no	Yes	2.68878504
Sample7	15-30	yes	0	no	Yes	0.80488064
Sample8	15-30	no	1	no	Yes	0.12982472
Sample9	31-40	yes	0	yes	Yes	0.12768751
Sample10	31-40	Yes	1	yes	Yes	1.20789265
Sample11	31-40	no	1	yes	Yes	0.16927622
Sample12	31-40	yes	2	yes	Yes	0.39692182
Sample13	31-40	yes	1	no	Yes	0.17047565
Sample14	31-40	yes	1	no	Yes	0.77460762
Sample15	31-40	no	1	yes	Yes	0.10619593
Sample16	31-40	yes	1	no	Yes	1.41124303
Sample17	41-50	yes	1	yes	Yes	0.02785247
Sample18	41-50	yes	2	yes	Yes	1.15513649
Sample19	41-50	yes	2	no	No	3.48261075
Sample20	41-50	no	1	yes	Yes	1.38801303
Sample21	41-50	yes	1	yes	Yes	0.18217491
Sample22	41-50	yes	1	yes	Yes	0.23851154
Sample23	41-50	yes	1	no	Yes	2.8531604
Sample24	41-50	yes	2	yes	Yes	1.03637078
Sample25	51-60	yes	2	yes	Yes	0.60644017
Sample26	51-60	no	3	no	No	1.00715951
Sample27	51-60	no	2	yes	Yes	0.67807771
Sample28	51-60	no	2	yes	No	2.62928632
Sample29	51-60	no	2	yes	No	0.0356026

14 healthy control subjects collected from HMP site (website URL: https://hmpdacc.org/ last accessed on May 24th 2025).

Extraction of DNA. Genomic microbial DNA was extracted from samples using the Zymo BIOMICS DNA Miniprep Kit (Zymo Research, Cat. No. D4300) according to manufacturer's procedures. In summary, the protocol involved cell lysis with enzymatic digestion and bead beating, and purification of genomic DNA by spin columns and washes. Purified DNA was eluted in DNase-free water.

Next generation sequencing. The isolated DNA from each of the samples was quantified using a Qubit fluorometer strictly according to the instructions from the manufacturer. Each DNA sample was diluted to a pre-decided concentration (i.e., 200 ng) with TE buffer to achieve even representation in the library preparation. Using T4 DNA Ligase in the MGIEasy FS DNA library prep kit, the sequencing adapters containing sequences were ligated complementary to the MGI Rapid sequencing flow cell plus unique barcode sequences to the ends of the fragmented DNA following the manufacturer's protocol.

The circular ssDNA was produced using enzymatic circularization. According to manufacturer's instruction, circularization, was completed using commercially available MGIEasy circularization module reaction kits. DNA Nano Ball (DNB) preparation was

carried out following circularization and sequencing was performed on DNBSeq-G400RS for paired-end shotgun sequencing. A negative control and positive control was included throughout the procedure to test all materials and reagent contaminations.

Analysis of microbiome. The raw reads were processed using an in-house pipeline PanOmiQ at BioAro. The software utilizes databases featuring rapid, curated processes, in addition to an exhaustive taxonomic delineation and classification platform. It includes over 27,000 microbial genomic DNA sequences for speedy identification. The raw reads were analyzed for its quality, and low-quality reads and adaptors were trimmed. Further, host DNA and rRNA were taken out from the sequences by assembling with human reference genome. The taxonomy classification and species identification were performed.

Statistical analysis. R studio (version 2024.04.0-735) was used to perform all analysis. Venn diagrams were created using the ggVennDiagram package. ggplot2 was used to create relative abundance visualizations and comparative bar plots. Fischer exact test was used to compare microbiome of different age groups and the number of pathogens observed in these age groups. The Kruskal-Wallis rank sum test was used as the main statistical technique to evaluate significant variations in bacterial abundance among the four age groups (Groups A, B, C, and D) and control samples because microbiome count data typically have a non-normal distribution.

RESULTS

Vaginal microbiome composition and age. From the sequencing data, a comprehensive list of the bacteria, including beneficial and pathogenic was generated for each age group. The diversity of the microbes reveals a unique pattern that varies with age. Group A (15-30 years) had the highest diversity with 350 species while Group C (41-50 years) had 267 species, making it the second most diverse group. Meanwhile, Group B (31-40 years) showed a significant drop in diversity with just 68 species. Group D (51-60 years) also showed decline in number of species. Group A (15-30 years) showed the most distinctive microbial species with 197 unique species indicating microbial diversity which are unique to younger reproductive age. This diversity indicates hormonal fluctuations, sexual activity patterns, and maturation of immune system which are characteristic of this age group. Group C (41-50 years) contained118 unique species, representing the second highest proportion of unique bacteria. Some of the key unique species included *Bifidobacterium bifidum*, *Corynebacterium kefirresidentii*, and multiple *Prevotella* species, suggesting perimenopause and early menopause create distinct microbial niches. Furthermore, Fig. 1 also showed that the four groups had more shared species which were not present in the healthy control group. This suggests that although there was core vaginal microbiome across groups, physiological or hormonal changes led to vaginal dysbiosis due to the presence of certain species that were not expressed in the control subjects.

Fig. 2 illustrates the complex and varied microbial ecosystem of the youngest demographic (15-30 years). This age group displayed a heterogeneous bacterial composition with multiple species contributing

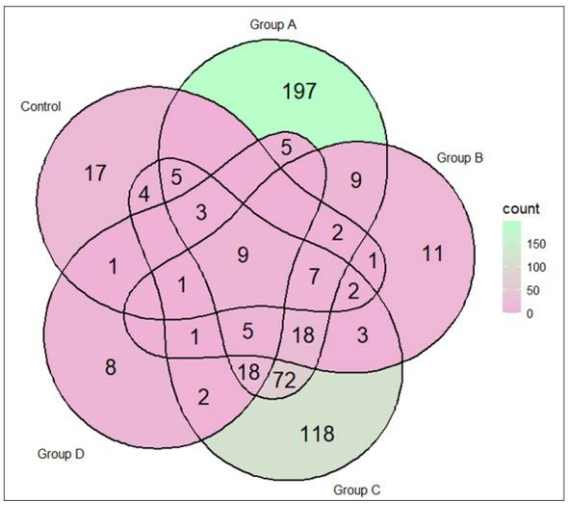

Fig. 1. Comparison of number of microorganisms in healthy and group A, B, C and D subjects: Venn diagram showing the overlapping of microbial species in vaginal microbiome samples in three groups (Group A: 15-30 years old; Group B: 31-40 years old; Group C: 41-50 and Group D: 51-60 years old) and controls. Each set represents one experimental group, with the total area proportional to the group size. Numbers within each region indicate the count of microbes that were shared between the overlapping groups or unique to individual groups. The color intensity corresponds to the count magnitude with darker green indicating higher counts and lighter pink representing lower counts.

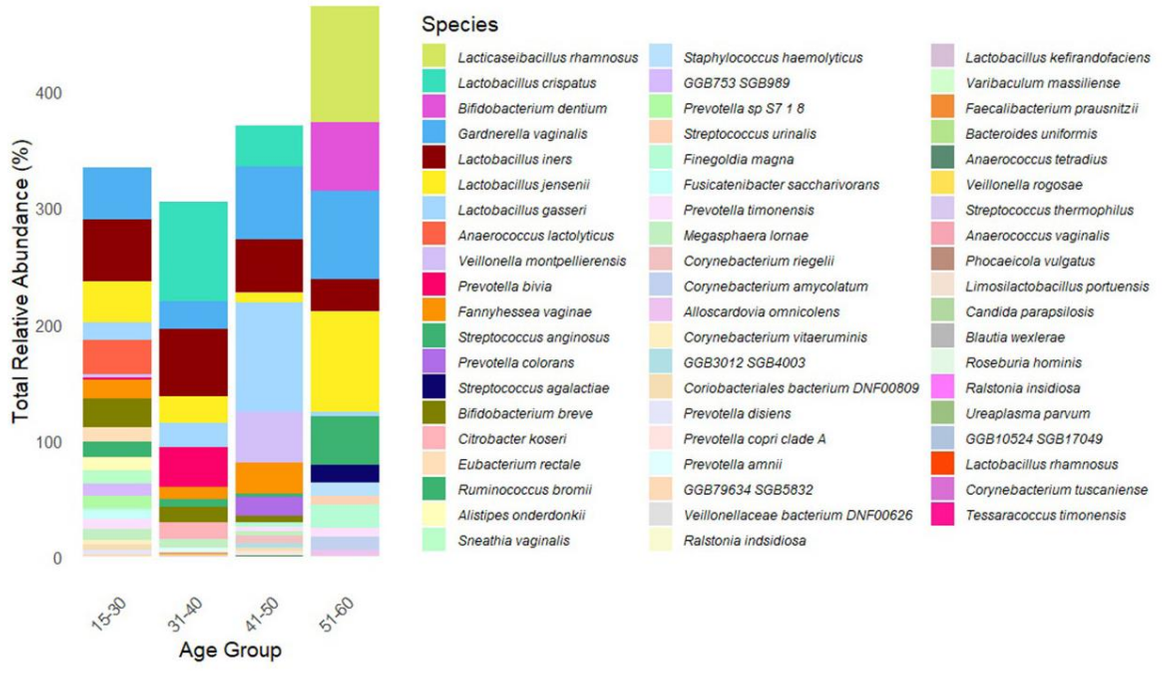

Fig. 2. Relative abundance of prominent species, present in different age group and healthy subjects: Bar plot showing the relative abundance of the top 15 microbial species identified in the vaginal microbiomes of each Group A (15-30 years), Group B (31-40 years), Group C (41-50 years), and Group D (51-60 years). Each segment showcases relative contribution of a specific microbial species to the total vaginal microbiome. This figure compares microbial abundance patterns among different age groups, highlighting both common and distinct species present within the examined populations.

to the overall community structure, including significant representation of *Gardnerella vaginalis*, *Lactobacillus iners*, and *Lactobacillus jensenii*, which were significantly represented along with a number of other bacterial taxa that created a microbial environment typical of women of reproductive age (18). Additionally, in the 31-40 age group (Group B), *Lactobacillus crispatus* dominated while in comparison, the 41-50 years (Group C) showed moderate levels of total relative abundance, indicating a transitional phase in the makeup of the vaginal microbiome with the continuous presence of varied bacterial species but also some changes in community structure. *Gardnerella vaginalis* was increased, with significantly lower levels of *L. crispatus*, *L. gasseri*, and *L. jensenii*, indicating that this age group had a greater number of microorganisms residing in the vaginal environment and that there may be dysbiosis taking place due to menopause. Most notably, the 51-60 years age (Group D) exhibited the highest total relative abundance levels of microrganisms. This was predominantly attributed to an overwhelming dominance of *Lactobacillus rhamnosus*, which constituted the vast majority of the microbial community. These data indicate that there might be a transient change from protective environment to a more diverse and potentially pathogenic microbiota with increasing age and hormonal changes.

Comparing pathogenic species in vaginal microbiome. The analysis of pathogen distribution across different age groups reveals significant patterns in microbial diversity and composition (Fig. 3). Group A (15-30 years) demonstrated a moderate pathogenic burden with distinct pathogenic species including *Fannyhessea vaginae*, *Fusobacterium nucleatum*, *Gardnerella vaginalis*, *Prevotella bivia*, *Staphylococcus epidermidis*, *Streptococcus anginosus*, and *Veillonella montpellierensis*. All pathogenic species present in this age group were also found in other groups, as this age group did not exhibit any distinct pathogens that were specific to this demographic. This depicts a transitional stage during which opportunistic infections start to form, showcasing colonization patterns, however, the vaginal environment remains stable overall. The most striking finding emerged in Group C (41-50 years), which demonstrated the highest pathogen`s burden with 26 distinct pathogenic species. This age group contained 16 unique pathogenic species not found in

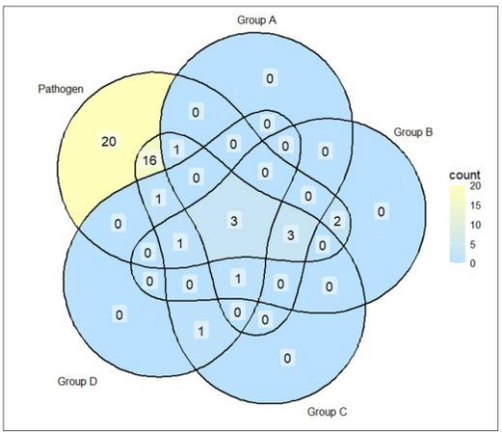

Fig. 3. Venn diagram for the number of pathogens in different groups: Venn diagram illustrating the distribution and overlap of pathogenic species identified in the vaginal microbiomes of subjects from four age groups (Group A: 15-30 years, Group B: 31-40 years, Group C: 41-50 years and Group D: 51-60 years). Each group is represented by each circle, and the number of common or unique items within each intersection region is indicated by a numerical value. The color intensity reflects the element count magnitude, with lighter blue denoting lower counts and deeper yellow denoting greater counts.

other demographics, including *Mageeibacillus indolicus, Neisseria gonorrhoeae, Olegusella massiliensis,* 13 different *Prevotella* species, and *Veillonella atypica*. The predominance of *Prevotella* species in this age group indicates a fundamental shift toward anaerobic, gram-negative bacterial colonization that is characteristic of perimenopausal vaginal microbiome changes (22). The presence of *Neisseria gonorrhoeae* exclusively in this age group may reflect increased sexual activity patterns or decreased immune surveillance during hormonal transitions (23).

Abundance of species across age groups. Fig. 4 presents $\log_{10}$ abundance patterns of five key bacterial species across different age groups divided into Groups A-D. The *E. coli* abundance data. Fig. 4a shows relatively low levels across age groups, with $\log_{10}$ abundance values ranging from approximately -3.5 to -1.8. Group A (15-30 years) and control group shows the lowest abundance levels, while there is a slight increase in Group D. This pattern is consistent with studies which show that *E. coli* colonizes the vaginal tract and might be linked to UTIs, especially in menopausal women (1).

G. vaginalis exhibits the most significant age-related alterations with Group A and B showing the maximum abundance (Fig. 4b). From there, it gradually decreases through Group C and D and reaching the lowest levels in the control group. According to epidemiological data, the incidence of *G. vaginalis* is highest in women under the age of 19 (71.5%) and those between the ages of 20 and 29 (68.4%), with notable declines beyond the age of 40 (11). The pattern seen in our study is consistent with those findings. Across age groups, *Prevotella* abundance levels are somewhat constant in all groups except Group A (Fig. 4c), with $\log_{10}$ values regularly falling between 0.5 and 1.0. *Prevotella* species can be linked to bacterial vaginosis and their presence may explain higher susceptibility to age related vaginal infections and diseases (22). *Streptococcus* remains low across all groups, with log10 values ranging from approximately -0.7 to 0.8 (Fig. 4d). This shows relatively stable levels throughout, with minimal age-related variation. This stability is characteristic of *Streptococcus* species role as core background colonizers rather than dominant members of the microbiome, not susceptible to fluctuations due to hormones or environment (17).

Lactobacillus species exhibit a consistency across all age groups with minimum fluctuation. This constancy is remarkable given the vast study on age-related changes in *Lactobacillus* populations. According to studies, the abundance of *Lactobacillus*, notably *L. crispatus* and *L. iners*, declines dramatically after age 45 due to hormonal changes and estrogen loss (18).

Statistical analysis of the microbial abundance data across age groups showed some significant differences between groups. We used a statistical analysis test called Kruskal-Wallis test which yielded p-values for several key species: *Escherichia coli* ($p = 0.0111$), *Gardnerella vaginalis* ($p = 0.0186$), *Lactobacillus* sp ($p = 0.0221$), *Prevotella* sp ($p = 0.00907$), and *Streptococcus* sp ($p = 0.0144$). These results confirm that samples were statistically significant and the observed differences in bacterial abundance between age groups were not due to random variation, it depicts age-related changes in vaginal microbiome composition.

Diversity of vaginal microbiome across age groups. The composition of vaginal microbiome across different age groups compared to control group is shown in Fig. 5 as a visual representation, demonstrating age-related clustering patterns. The youngest group (15-30 years) showed tight clustering, suggesting a more homogenous microbiome composition in the

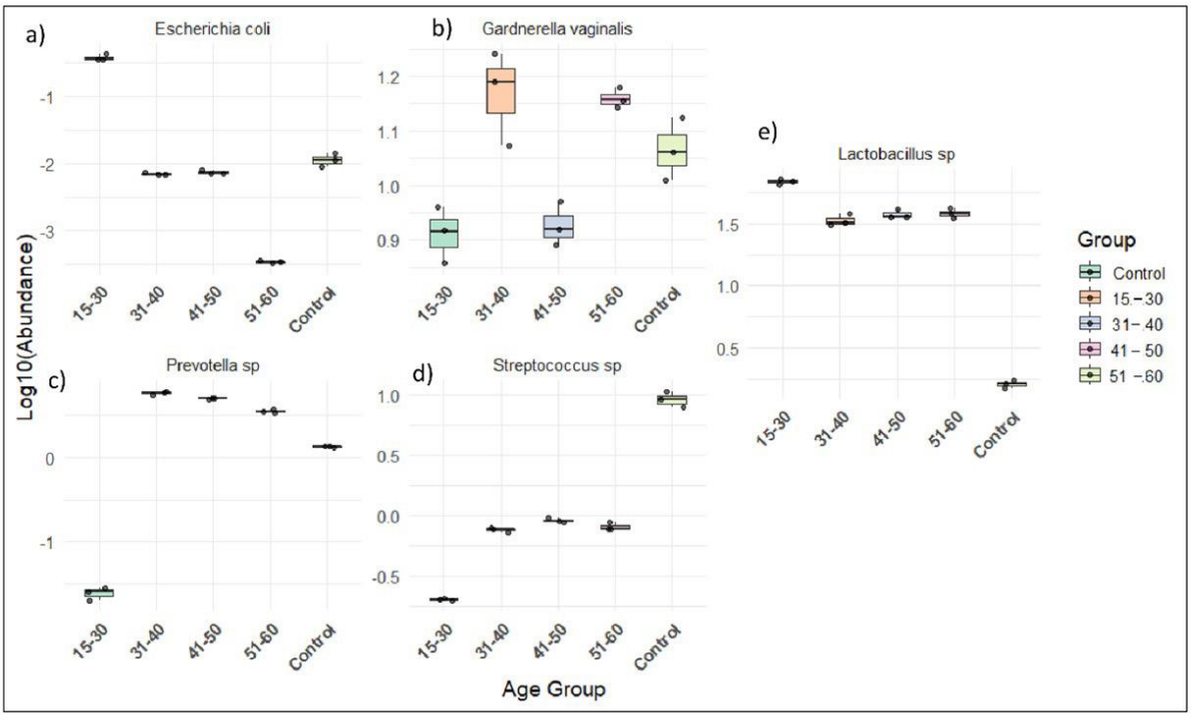

Fig. 4. Comparative bar plot of pathogens in different groups: Comparative bar plot showing the number of pathogenic species identified in the vaginal microbiomes of Group A (15–30 years), Group B (31-40 years), Group C (41-50 years) and Group D (51-60 years).

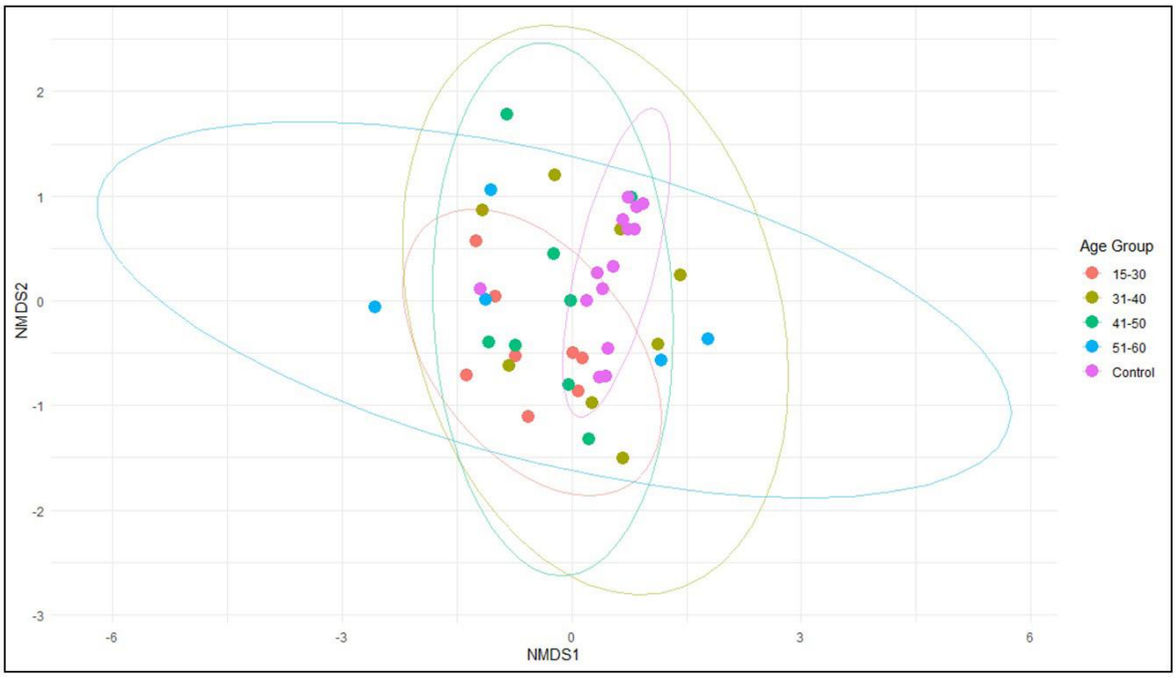

Fig. 5. Non-metric multidimensional scaling (NMDS) ordination of vaginal microbiome composition by age group. NMDS plot showing vaginal microbiome samples from different age groups (15-30, 31-40, 41-50, 51-60 years) compared to control samples. Each point represents an individual vaginal microbiome sample, with colors indicating age groups.

vagina. Group B (31-40 years) showed less scattering of samples, thus indicating some diversity in microbial community structures in this age group. Group C (41-50 years), which mostly comprised of subjects in their perimenopausal phase, showed an increased dispersion suggesting changes in microbiome associated with hormonal fluctuations. Group D (51-60 years), which constituted the menopausal or postmenopausal group, displayed the most diverse pattern, indicating greater variation in vaginal microbiome composition, consistent with the loss of estrogen related microbial regulation.

DISCUSSION

The vaginal microbiome represents a dynamic and complex ecosystem that undergoes significant transformations throughout a woman's lifespan, fundamentally influenced by hormonal fluctuations, age-related physiological changes, and microbial community dynamics. While healthy vaginal environments are typically characterized by *Lactobacillus* spp. dominance and low microbial diversity, substantial variations exist across different populations and life stages. Understanding these age-related patterns is crucial for interpreting pathogen distribution and establishing appropriate clinical management strategies for women's reproductive health (10, 24).

Our study has revealed a striking difference in microbial diversity and pathogen abundance across four distinct age cohorts, together with some striking conclusions in Group C, which showed a high pathogen burden and a particularly diverse microbial community. These findings provide information about the relationship between *Lactobacillus* spp., abundance and colonization patterns. Our results demonstrate subspecies specific differences in protective capacity among *Lactobacillus* spp, challenging the assumption of uniform protective effects across all *Lactobacillus* strains.

The increased diversity in group C, which represents perimenopausal woman, can possibly be attributed to the decrease in estrogen levels (25). It has been reported that estrogen levels are proportional to glycogen levels (12, 2), which aids in the growth of *Lactobacillus* spp. and allows for their dominance in the vaginal environment (9, 2). With lower estrogen levels, there is less glycogen (12, 16, 25) and thus the survivability of *Lactobacillus* spp. in the vaginal environment greatly declines (2). The reduced survivability of the *Lactobacillus* sp. prevents their dominance of the vaginal microbiome and gives opportunities for different species of bacteria to grow within the vaginal environment resulting in a more diverse vaginal microbiome (25). The vaginal microbiome results for this group align with findings in current literature (24, 25). This group exhibited a significantly diverse vaginal microbiome compared to other age groups, and the relative abundance of *Lactobacillus* sp. was the lowest.

The large number of pathogens unique to group C could be attributed to low abundance of *Lactobacillus* spp. observed within this group. It is known that *Lactobacillus* spp., through the production of lactic acid and other antimicrobial compounds such as hydrogen peroxide, inhibit the growth and presence of pathogens within the vaginal environment (2, 5, 9). These results highlight the continuous nature of the relationship between *Lactobacillus* spp. and pathogen presence, where lower abundances of *Lactobacillus* spp. allow for a greater range of pathogens to develop within the vagina (7, 12, 17). The current investigation highlights that the efficacy of *Lactobacillus* spp. is to deter pathogen growth. The unique pathogens in group C are only present in conjunction with the low *Lactobacillus* spp. abundance. This indicates that the antimicrobial properties of *Lactobacillus* spp. are very effective at preventing the growth of the unique pathogens. Pathogens are able to grow only when there is a low abundance of *Lactobacillus* spp.. Pathogens common among all groups include *Gardnerella vaginalis* and *Prevotella bivia*, while *Lactobacillus* spp. abundance varies. This demonstrates that the presence of these pathogens is independent of *Lactobacillus* spp. abundance, suggesting that the antimicrobial properties of *Lactobacillus* spp. are less effective against them. The idea that the positive effects of *Lactobacillus* spp. depend on pathogens somewhat deviates from the general concept that high *Lactobacillus* spp. abundance indicates vaginal health (5, 13, 21), suggesting other potential key factors may fill the gaps left by *Lactobacillus* spp. and prevent pathogen growth.

The vaginal microbiomes of groups B and C differed not only in pathogen variety but also in the abundance of *Lactobacillus* spp., with group B showing a significantly higher abundance. This, paired with the significant difference in pathogens between groups C and B, indicates that the decrease in *Lac-*

tobacillus spp. abundance that occurs as women age and transition into menopause allows pathogens to thrive within the vaginal microbiome (25, 12, 16). Decreased *L. crispatus* with age is linked with an increase in inflammation of the cervicovaginal region (26). This re-enforces the notion found in literature that *Lactobacillus* spp. play a crucial role in deterring the growth of pathogens and maintaining vaginal health (12, 27). This result also demonstrates the necessity of a high abundance of *Lactobacillus* spp. in the vaginal environment and supports the potential application of compounds that encourage *Lactobacillus* spp. growth such as glycogen (2, 5) in ensuring vaginal and reproductive health as woman age and enter menopause.

Another important observation from this study is the elevated number of pathogens found within age group A. Despite having similar abundance of all *Lactobacillus* spp. (*L. crispatus, L. gasseri, L. jensenii* and *L. iners*) when compared to group B, group A contains significantly more pathogens, which contradicts findings indicating the positive relationship between *Lactobacillus* sp. abundance and vaginal health (7, 12, 27) and highlights a deeper nuance in the relationship between *Lactobacillus* sp. abundance and pathogen presence within the vaginal microbiome. One potential explanation for this is the difference in *L. iners* abundance between groups A and B, where group A had a nearly 40 times larger average abundance (23.9%) compared to group B (0.653%). *Lactobacillus iners* can produce only L-lactic acid, which helps to prevent the growth of pathogens and maintain homeostasis. However, other *Lactobacillus* spp., such as *L. crispatus, L. gasseri,* and *L. jensenii,* can produce both D-lactic acid and L-lactic acid (26, 28, 29). Unlike these species, *L. iners* only has the gene that encodes for L-lactic acid, and therefore can only produce that isomer (30). L-Lactic acid has been shown to be a less effective antimicrobial agent, (30) so the increased presence of L-lactic acid, due to elevated *L. iners* abundance, may result in lower proficiency in inhibition of pathogen growth, which corroborates with the elevated pathogen presence in group A.

The highest diversity in Group C might be linked to hormonal shifts that occur during perimenopause. These changes could create a more favourable environment for various microbes to thrive. On the other hand, the sharp decline observed in Group D may suggest that post-menopausal changes are leading to a more limited microbial landscape. The presence of *Gardnerella vaginalis* across all age groups indicates that this organism resides in the vaginal environment irrespective of age. In contrast, the age-specific emergence of certain pathogens, like *Neisseria gonorrhoeae* in Group C, could point to behavioral or immune system factors that are unique to that age group.

The age-related clustering pattern shows the influence of reproductive hormones, particularly estrogen, on vaginal microbiome composition. Estrogen promotes glycogen deposition in vaginal epithelial cells, which serves as a substrate for *Lactobacillus* species to produce lactic acid and maintain an acidic environment (pH < 4.5) (24). The transition from a tight cluster in Group A to a more diversified cluster in Group D might corresponds to the decline in estrogen levels during perimenopause and menopause. Perhaps this hormonal shift might leads to reduced *Lactobacillus* spp. and colonization by potentially pathogenic bacteria, reflected in the microbial composition. Microbial diversity exhibits a varied trajectory across age groups, according to the current study. This pattern, which is marked by high diversity in youth, decreased diversity during peak reproductive years, increased diversity during perimenopause, and subsequent stabilization in postmenopausal years, contradicts the conventional wisdom that age-related changes are linear (12). Our findings highlight the critical role of *Lactobacillus* species composition in determining pathogenic susceptibility, revealing that not all *Lactobacilli* provide equivalent protective benefits. This finding underscores the importance of species-specific rather than genus-level analysis in microbiome research. These findings imply that age-specific approaches to vaginal health are required, which has urgent implications for women's healthcare. The identification of the 41-50 years age group as a period of heightened pathogenic susceptibility indicates the need for targeted preventive interventions during perimenopause.

CONCLUSION

The vaginal microbiome plays a key role in maintaining vaginal and reproductive health and there are many factors that affect its composition and thus its ability to protect and maintain vaginal health. This study focused on age, and its association with the

vaginal microbiome and health, specifically pathogen presence within the vaginal microbiome. The study found that microbial diversity follows a complex trajectory throughout age groups, with Group A (15-30 years) having the most species diversity, followed by Group C (41-50 years), while Groups B and D showed less variations in microbial species. These results challenge the assumption of age-related changes, indicating high diversity in youth, decreased diversity during peak reproductive years, increased diversity during perimenopause, and subsequent stabilization in the postmenopausal years (31). These findings are relevant for women's healthcare, implying that age-appropriate approaches to vaginal health management are required. The identification of the 41-50-year age group as a phase of increased pathogenic susceptibility highlights the importance of introducing preventive measures during perimenopause. Finally, our study shows that the vaginal microbiome undergoes a fundamental change during a woman's life, with each phase providing distinctive microbial signatures, pathogenic risks, and therapeutic prospects. Understanding these age-related trends is important for developing tailored approaches to women's reproductive health.

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