narra j

Short Communication

Impact of semen insemination on the vaginal microbiome profile of candidiasis rat model: A preliminary forensic study on sexual violence evidence

Raja AFW. Iswara[1,2*], Andon Hestiantoro[3], Yuli Budiningsih[4], Retno A. Werdhani[5], Ponco Birowo[6], Puspita E. Wuyung[7], Fadilah Fadilah[8] and Dedi Afandi[9]

[1]Doctoral Program in Medical Science, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; [2]Department of Forensic and Medicolegal, Faculty of Medicine, Universitas Halu Oleo, Kendari, Indonesia; [3]Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; [4]Department of Forensic and Medicolegal, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; [5]Department of Community Medicine, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; [6]Department of Urology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; [7]Department of Pathological Anatomy, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; [8]Department of Chemistry, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; [9]Department of Forensic and Medicolegal, Faculty of Medicine, Universitas Riau, Pekanbaru, Indonesia

*Corresponding author: rajaalfath@uho.ac.id

Abstract

Sexual violence, including sexual intercourse, can occur in women experiencing vaginal discharge, particularly in cases of vaginal candidiasis. In candidiasis, the vaginal microbiome undergoes changes that could serve as a diagnostic indicator or as evidence of sexual activity. The aim of this study was to assess the effects of semen insemination on the vaginal microbiome profile of candidiasis rats and to determine its forensic investigations in cases of sexual violence. An experimental study was carried out using Wistar strain rats (*Rattus norvegicus*), consisting of four male rats (for spermatozoa donors) and twenty-four female rats. The female rats were divided into four groups: normal condition (control), normal condition post-semen insemination, candidiasis rats, and candidiasis rat post-semen insemination. Vaginal microbiome profiles were examined for each group, using alpha diversity (Chao 1, Shannon, Simpson, and Faith PD indices) and beta diversity (Bray Curtis, Jaccard, Unweighted Unifrac and Weighted Unifrac indices). Data were analyzed using the Kruskal-Wallis test for alpha diversity and the PERMANOVA test for beta diversity. There is no significant difference in alpha and beta diversity of the vaginal microbiome between groups of rats in normal conditions and those with candidiasis, both pre- and post-semen insemination ($p>0.05$). In candidiasis rats, the microbiome predominantly consisted of the Eukaryota kingdom, particularly *Candida albicans*. However, the study highlights that the presence of *Eschericia-Shigella, Roseomonas,* and Archaea in the vaginal microbiome post-semen insemination potentially serves as an indicator of sexual intercourse, which may provide evidence of sexual violence in forensic medicine.

Keywords: Alpha diversity, beta diversity, candidiasis, semen insemination, vaginal microbiome profile

Introduction

The human vagina hosts a diverse and dynamic microbiome, which is predominantly dominated by *Lactobacillus* species [1,2]. These beneficial bacteria play a crucial role in maintaining the

https://narraj.org/

Received: August 1, 2024 - Accepted: January 30, 2025 - Published online: February 5, 2025

internal physical and chemical environment, which is essential for protection against infections [2,3]. The vaginal ecosystem operates in a delicate balance, with various components working together to sustain the overall health of the reproductive tract [4,5]. In women of childbearing age, the normal vaginal pH ranges from 3.8 to 5.0, creating a moderately acidic environment that supports this microbial balance [6]. Several factors, including vaginal infections, aging, sexual activity, and vaginal douching, can disrupt the vaginal microbiome and alter the vaginal pH, leading to conditions such as vaginal dysbiosis [7].

One of the most common and debilitating conditions that women of reproductive age face is vulvovaginal candidiasis, or vaginal yeast infection, which often leads to abnormal vaginal discharge [8,9]. It is estimated that 75–80% of women experience vulvovaginal candidiasis at least once in their lifetime, and this infection accounts for more than 25% of all cases of infectious vaginitis [10,11]. During candidiasis, the vaginal microbiome undergoes significant changes, which not only affect the pH but also influence immune responses [12,13]. Specifically, an increase in neutrophils is observed, with various mechanisms such as phagocytosis, extracellular degranulation, neutrophil extracellular trap, and trogocytosis being activated to combat the infection [14,15].

In cases of sexual violence, women may present with vaginal discharge, often with the presence of candidiasis. Data from Bhayangkara Kendari Hospital, a referral center for sexual violence cases in Southeast Sulawesi, Indonesia, shows that candidiasis is the most prevalent vaginal infection observed in victims of sexual violence. However, there is limited study on how sexual intercourse may alter the vaginal microbiome, especially in the context of pre-existing infections like candidiasis. Changes in the vaginal microbiome profile could serve as indicators of either infectious or sexual activity, yet the relationship between these factors remains poorly understood [12,13].

A study involving human subjects presents significant ethical challenges, particularly when studying sensitive topics such as sexual violence and vaginal infections. Consequently, animal models, particularly rats, offer a viable alternative for such studies. Rats' vaginal microbiomes differ from humans in their dominant bacterial communities, with species from *Proteobacteria*, *Firmicutes,* and *Actinobacteria* phyla prevailing in rats [16], as opposed to *Lactobacillus* dominance in humans [4]. Additionally, the vaginal pH in rats is generally neutral to alkaline, whereas in humans more acidic. Furthermore, the number of neutrophils in the rat vagina is influenced by the estrus cycle, which could add variability to microbiome profiles [16]. The aim of this study was to investigate the effect of semen insemination on the vaginal microbiome profile of candidiasis rats. By exploring this relationship, we aim to gain insights into how sexual activity may influence vaginal infections and microbiome dynamics, with potential forensic implications for cases of sexual violence.

Methods

Study design and setting

An experimental study using animal models was conducted at the Animal Research Facilities (ARF) Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia. This study obtained male and female white rats (*Rattus norvegicus*) of the Wistar strain from Bio Farma, Bandung, Indonesia.

The *Candida albicans* cultures used in the study were sourced from patients with candidiasis vaginalis, obtained through the Parasitology Laboratory, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia. These cultures typically formed creamy white or off-white colonies on sabouraud dextrose agar and upon microscopic examination, appeared as oval-to-round yeast cells with pseudohyphae. Microbiome examinations were performed at the Bioinformatics Laboratory IMERI, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.

Selection was carried out using a simple random sampling technique. Four groups were examined for the vaginal microbiome profile: normal condition (control), normal condition post-semen insemination, candidiasis rats, and candidiasis rats post-semen insemination. A minimum sample size of 32 female rats was required based on the Federer formula. The study involved two

experimental groups: the normal female rat group and female rats with candidiasis model, with each group consisting of 16 female rats. For sperm insemination, four male rats were used (sperm samples from each male rat were inseminated into the vaginas of four female rats).

Inclusion and exclusion criteria

The rats included in this study were aged 8–10 weeks, weighing 200–250 grams, healthy, had no wounds or anatomical abnormalities, and had been acclimatized. The criteria for male rats' sperm to be inseminated into female rats' vaginas included a pH range of 7.2–7.8, sperm concentration of over 35 million/mL ejaculate, more than 3.9% normal sperm morphology, total sperm motility of more than 40% with progressive motility exceeding 29%, non-progressive motility at 1%, and immotile sperm at less than 19%. Rats that were sick or died during adaptation or treatment and male rats with asthenozoospermia were excluded from the study. Female rats that died during regular vaginal swab sampling, which prevented the collection of complete serial data until the study's conclusion, were excluded from the analysis.

Animal preparation and acclimatization

The rats were acclimatized for one week and given Ratbio feed (Citra Ina Feedmill, Jakarta, Indonesia), which contains 60% carbohydrates, 20% protein, 4% fat, 4% crude fiber, 12% calcium, and 0.7% phosphorus. Moreover, water was given *ad libitum* and put in a closed system cage to minimize exposure to various factors that can affect the spermatozoa and vaginal group of the rats. The temperature and humidity were maintained within a range of 23±10°C, under 12 hours dark and 12 hours light cycle.

Candidiasis rat models

The procedure for developing candidiasis rat models in this study was based on the guidelines outlined in the animal models for candidiasis protocol [17]. The candidiasis rat group was given a subcutaneous injection of 0.5 mg estradiol valerate in 50 µL of sesame oil in the lower abdomen of female rats every day for five days before vaginal inoculation. For *C. albicans* inoculation, a 2.5×10^6 cell suspension (or desired inoculum in the range between 5×10^4 and 5×10^8) of *C. albicans* blastospores from stationary phase culture in 0.02 mL of sterile phosphate-buffered saline (PBS) was prepared. A total of 0.02 mL of yeast cell suspension in PBS was inoculated into the vagina using a micropipette and sterile tip. The tip was inserted into the end of the vaginal lumen, approximately 1.5 cm close to the cervix. The inoculation was performed under anesthesia to minimize rat distress. After 3–5 days, a vaginal swab was taken using sterile cotton swabs. The vaginal swab was applied to an object glass. After drying, the slide was stained with KOH 10% solution to confirm a *C. albicans* infection, characterized by the appearance of pseudohyphae and spores from *C. albicans*.

Semen collection and examination

The procedure for semen collection in this study was based on the guidelines outlined in the simple method for repeated in vivo sperm collection from laboratory mice protocol [18]. Male rats were euthanized using a lethal dose of ketamine 80 mg/kg body weight (Dexa Medica, Tangerang, Indonesia). Semen samples were taken from the cauda epididymis of male rats. At that location, they were clamped and then cut. The semen was removed from the cut part by squeezing it into a petri dish containing 1.5 mL of Vitrolife Sperm Rinse (GlobalMed Groups, Miami, USA) buffer media containing human serum albumin. Sperm in semen obtained from male rats was confirmed to be present and not experiencing asthenozoospermia by examining motility, viability, and spermatozoa count before insemination.

The procedure for semen examination in this study was based on the guidelines outlined in the WHO laboratory manual for the examination and processing of human semen Sixth Edition protocol [19]. After semen collection from the male rats, pH analysis was conducted using the pH indicator MQuant Merck (Merck, Darmstadt, Germany). The sperm underwent microscopic analysis to evaluate sperm count, motility, viability, and morphology. Sperm count was assessed using a Makler sperm counting chamber (016261 Makler counting chamber, Darmstadt, Germany), motility was directly observed under a microscope with 0.9% NaCl, and eosin staining was used to assess viability and morphology.

Insemination of semen containing sperm into the vagina of female rats

The procedure for semen insemination in this study was based on the guidelines outlined in the sperm harvesting and artificial insemination of mice [20]. Using a micropipette with the tip at a 120° angle, the female rat was held in a supine position for insemination. A total of 200 µL semen from a 1.5 mL sperm rinse in a petri dish was inserted into the vagina of each female rat to a depth of 1.5 cm. After insemination, the rats were kept in a supine position for up to one minute before returning them to their cages.

Vaginal swabs of female rats

The procedure of vaginal swabs in this study refers to vaginal cytology of the laboratory rat and mouse: review and criteria for the staging of the estrous cycle using stained vaginal smears [21]. A sterile cotton swab with a diameter of 3 mm and a length of 12 mm was inserted into the vagina of the female rat to a depth of 1.5 cm and rotated the swab clockwise for 20 seconds at a 45° angle. Swab collection was performed simultaneously across all groups two days after semen insemination.

Vaginal microbiome examination

The vaginal swab was placed in a collection tube containing DNA/RNA shield liquid and stored at -80°C to preserve nucleic acids. DNA extraction was assessed for purity using a NanoDrop2000™ spectrophotometer for purity and a Qubit™4 Fluorometer (Thermo Fisher Scientific, Massachusetts, USA) for DNA concentration. The reference value for DNA quality included a purity index of 1.6–2 and a concentration greater than >5 ng/µL. The V3 and V4 regions of bacterial 16sRNA in the DNA extracts were amplified using PCR. Amplified DNA products, marked with specific tags, were reassessed for concentration using the Qubit™4 Fluorometer.

Sequencing was performed using the Illumina MiSeq™ Next Generation Sequencing platform (Illumina, California, USA), following the manufacturer's protocol, with paired-end reads of 2×300 base pairs. The sequencing reads were combined using the FLASH v1.2.7 (NBACC, Maryland, USA) application to generate operational taxonomic unit (OTU) reads. Quality control of the sequencing process was conducted using the USEARCH algorithm to minimize PCR bias.

Analysis workflow involved importing data and demultiplexing (separating sample data by barcodes), denoising (removing non-biological sequences), OTU clustering and taxonomy assignment, phylogenetic tree construction, alpha and beta diversity measurement using QIIME 2, version 2024.5 (Caporaso Lab, Arizona, USA). OTU reads were referenced against the SILVA 138.1 database for taxonomy assignment.

Statistical analysis

The Kruskal-Wallis test was employed to analyze differences in microbiome alpha diversity (Chao 1, Shannon, Simpson and Faith PD indices) between groups. For microbiome beta diversity (Bray Curtis, Jaccard, Unweighted Unifrac, and Weighted Unifrac indices), differences were analyzed using the permutational multivariate analysis of variance (PERMANOVA) test. The non-parametric nature of PERMANOVA and its reliance on permutation-based testing make it suitable for diverse applications, including ecological studies and genomics.

Results

Twenty-four female rats were successfully used in this study, with none dying during regular vaginal swab sampling, which could have prevented the collection of complete serial data. Vaginal microbiome analysis was performed on four groups to examine the vaginal microbiome profile: normal condition (control), normal condition post-semen insemination, candidiasis rats, and candidiasis rat post-semen insemination, using the SILVA 138.1 database for taxonomy identification which is presented in **Figure 1**. The majority of microbial organisms present in the vaginal microbiome could not be classified down to the specific species level.

In the normal condition (control) group, the microbiome was dominated by the kingdom Bacteria, with the phylum Proteobacteria identified, specifically from the family Pasteurellaceae. In the normal condition post-semen insemination group, the

kingdom Bacteria remained dominant, with the phylum Proteobacteria. Within this phylum, two bacterial families were identified: Pasteurellaceae and Enterobacteriaceae, including the genus *Escherichia-Shigella*.

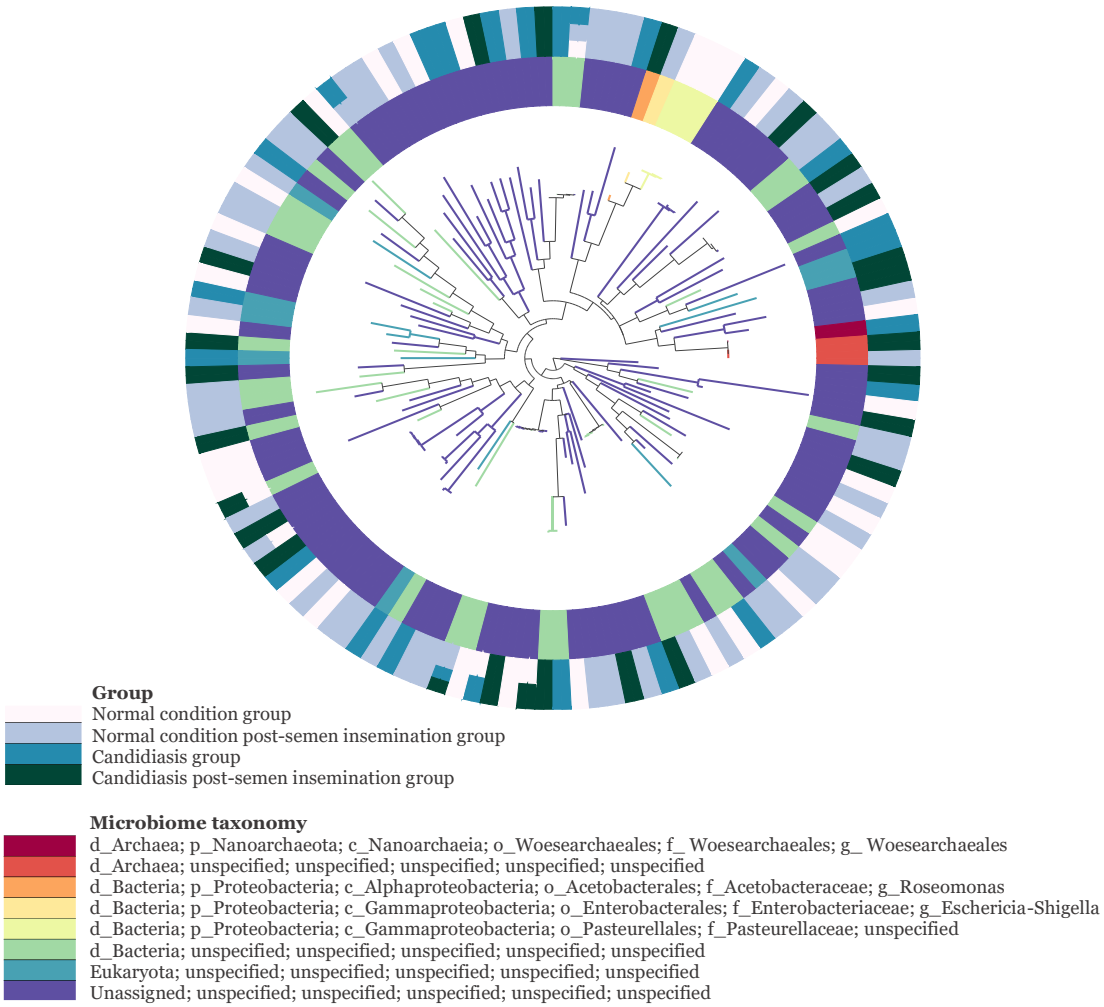

Figure 1. Taxonomy and phylogenetic tree of the rat vaginal microbiome.

In the candidiasis group, the kingdom Bacteria was also present, but fundamental differences in the microbiome were observed. These included the presence of the kingdom Eukaryota, which is believed to represent the domain of the species *C. albicans*, along with the detection of the domain Archaea, including the genus *Woesearchaeales*.

In the candidiasis group after semen insemination, the presence of the microbiome is essentially more diverse, with the kingdom Bacteria and phylum Proteobacteria present. Notably, within the phylum Proteobacteria, the family Roseomonas was identified. Additionally, the presence of the kingdom Eukaryota and the domain Archaea was also observed. There is a similarity in the microbiome of the candidiasis group, characterized by the presence of Eukaryota, and a similarity with the normal group after semen insemination, specifically the Proteobacteria phylum and Archaea.

Alpha diversity describes the abundance and distribution of taxonomic groups within an ecosystem or ecological community. It provides insights into the complexity of a microbial ecosystem, such as the vaginal microbiome in rats. To compare the microbiome profiles of rats under different conditions, such as normal condition (control), normal condition post-semen insemination, candidiasis rats, and candidiasis rats post-semen insemination, alpha diversity can be assessed using several indices, including Chao 1, Shannon, Simpson, and Faith PD indices (**Table 1**).

The Chao 1 index is used to estimate the richness of microbial communities in a population. There was no significant difference in the Chao 1 index between the group of rats with a *p*-value of 0.703. The Shannon diversity index measures microbial diversity by considering both the

number of different species present and their relative abundance. Similarly, there was no significant difference in the Shanon index between the groups of rats with a *p*-value of 0.435.

Table 1. Comparison of alpha diversity of vaginal microbiome between groups of rats in normal conditions and candidiasis, pre- and post-semen insemination

Variables	Groups				*p*-value[a]
	Normal condition, median (min-max)	Normal condition post-semen insemination, median (min-max)	Candidiasis, median (min-max)	Candidiasis post-semen insemination, median (min-max)	
Chao 1 index	6.00 (3.00–27.00)	24.50 (19.00-30.00)	11.50 (11.00–12.00)	12.00 (11.00–13.00)	0.703
Shanon index	2.21 (0.79–3.59)	3.34 (3.11-3.57)	2.65 (2.64–2.66)	2.51 (2.46–2.57)	0.435
Simpson index	0.73 (0.29–0.88)	0.90 (0.88-0.92)	0.83 (0.82–0.84)	0.76 (0.76–0.76)	0.679
Faith PD index	2.43 (1.58–7.86)	6.08 (4.98-7.17)	5.55 (4.92–6.18)	5.19 (4.91–5.48)	0.769

[a] Analyzed using the Kruskal-Wallis test

The Simpson diversity index quantifies overall microbial diversity, emphasizing the evenness of species distribution. No significant difference was observed in the Simpson index between groups, with a *p*-value of 0.679. The Faith PD index evaluates the phylogenetic diversity of the microbial community, incorporating evolutionary relationships. Similarly, there was no significant difference in the Faith PD index between groups with a *p*-value of 0.769.

Beta diversity was analyzed using several indices, including the Bray Curtis, Jaccard, Unweighted Unifrac, and Weighted Unifrac, across four vaginal microbiome groups (**Figure 2**). The Bray-Curtis index measures the dissimilarity in species abundance between different environments. The analysis showed uneven distributions between groups, as depicted in **Figure 2A**.

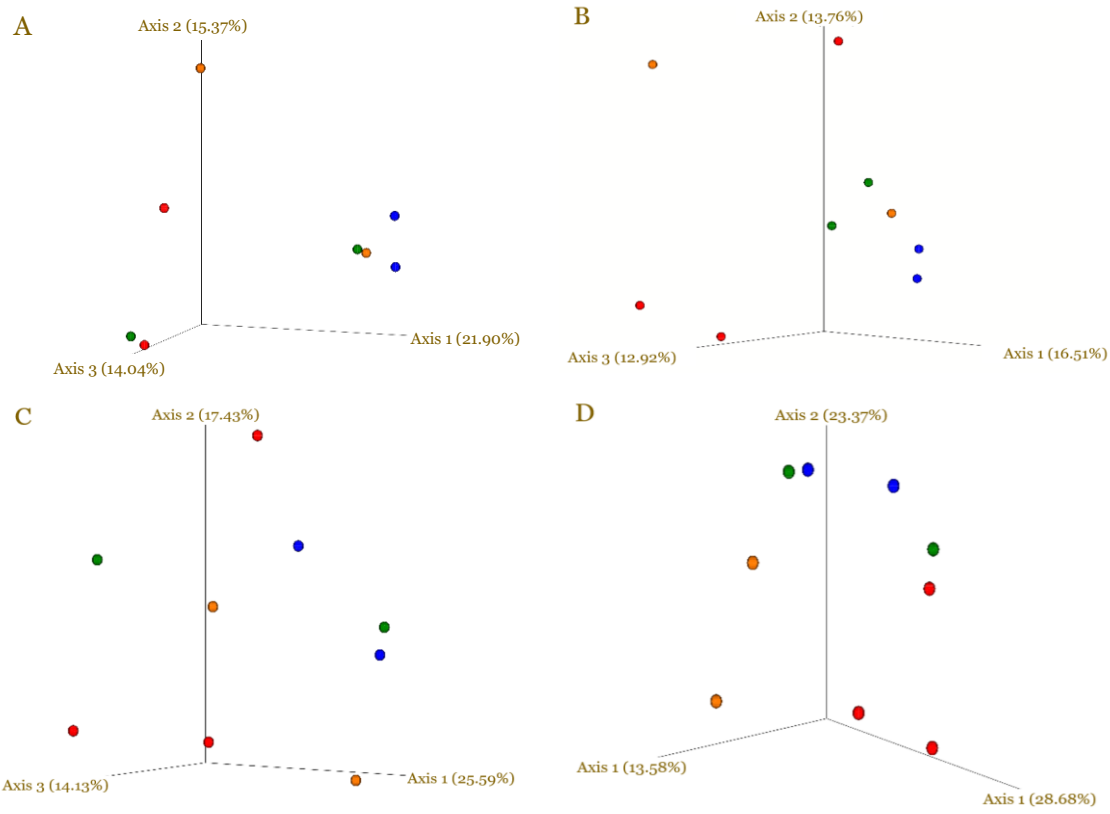

Figure 2. Sporadic distribution of the vaginal microbiome in Bray Curtis index (A), Jaccard index (B), Unweighted Unifrac index (C), and Weighted Unifrac index (D). The circles are colored in red for normal condition group, blue for normal condition post-semen insemination group, yellow for candidiasis group, and green for candidiasis post-semen insemination group.

The Jaccard index measures the similarity of species presence or absence between environments. The analysis revealed a similarity of species between normal condition groups post-semen insemination and the candidiasis group post-semen insemination, as presented in **Figure 2B**. The Unweighted Unifrac index qualitatively examines the phylogenetic relationship between groups. The results are illustrated in **Figure 2C**. The Weighted Unifrac index quantitatively incorporates species abundance in phylogenetic relationships, yielding no significant differences, as presented in **Figure 2D**.

The PERMANOVA test revealed no significant differences in the beta diversity of vaginal microbiome profiles among the four groups for any of the indices. This included the Bray-Curtis index (p=0.25), Jaccard index (p=0.057), Unweighted Unifrac index (p=0.378), and Weighted Unifrac index (p=0.223).

Discussion

The majority of microbial organisms present in the vaginal microbiome could not be classified down to the specific species level. Instead, the identification was more general, often only to a broader taxonomic level like genus, family, or kingdom. This could be due to limitations in the extracted DNA sample being too small, the resolution of the sequencing technology or the reference database used for classification [22,23].

The microbial organisms were only identifiable at the kingdom level, which is a broad taxonomic category that groups organisms into major divisions such as Bacteria, Eukaryota, and Archaea. This suggests that the data available from the sequencing process could only distinguish organisms at a high taxonomic level without providing more specific information like genus or species [22,23].

A significant portion of sequences remain unclassified, indicating that many of the sequences obtained from the microbiome samples could not be matched to any known taxonomic group. These sequences are labeled as "unclassified" because they do not correspond to any identified organism in the reference database used for taxonomy assignment. This can happen if the organism is novel or if the database lacks enough data for a match [22,24].

There were no significant differences in alpha diversity (Chao 1 index, Shannon index, Simpson index, and Faith PD index) of the vaginal microbiome between groups of rats. This indicated that the microbiome composition between these groups is similar statistically, although qualitatively, there appears to be a difference in the microbiome composition in each treatment group. Even though the primers used for microbiome analysis did not specifically target *Candida* and even many bacterial compositions in the rats could not be accurately detected.

OTUs of the rat vaginal microbiome in normal conditions were mostly undetectable. However, the detectable OTUs were dominated by the kingdom Bacteria, with Proteobacteria being the most abundant phylum. In rats with candidiasis, the microbiome was also predominantly composed of the kingdom Bacteria, alongside the kingdoms Eukaryota and Archaea. The most abundant phyla in this group were Proteobacteria and Nanoarchaeota. These findings align with the study of Levy *et al.* [16], which shows that the rat vaginal microbiome under normal conditions is dominated by three main phyla, namely Proteobacteria, Firmicutes and Actinobacteria.

This similarity in the vaginal microbiome composition between candidiasis and normal rats suggests that the native microbiome of the rat vagina supports the establishment of *Candida* infection in the group of candidiasis rats. This observation concurs with the findings of Hameed *et al.* [25], which highlighted that certain components of the normal microbiomes, particularly members of the phyla Firmicutes (*Lactobacillus*, *Streptococcus*) and Proteobacteria (*Escherichia*), influence the growth and pathogenicity of *Candida* infections. There appears to be a synergistic relationship between *C. albicans,* Group B *Streptococcus* (GBS), and *Escherichia coli*, which poses a significant risk to the host. Supporting evidence from several studies validated in vitro models using vaginal epithelial cells, demonstrating that GBS and *C. albicans* can synergistically enhance their ability to coexist and colonize host cells [25,26].

To explore the synergistic relationship between *C. albicans*, Group B *Streptococcus* (GBS), and *Escherichia coli* in a way that poses a significant risk to the host, it is necessary to employ several approaches from microbiology, immunology, and potentially molecular biology. These

approaches may include culturing the pathogens together, biofilm formation analysis, molecular interaction studies, host cell and immune response analysis, resistance and synergy testing, metabolomic and proteomic profiling, or using a host in vivo model [27].

A study by Hoffmann *et al.* [28] demonstrated a strong symbiotic relationship within the human gut microbiome among the kingdoms Archaea, Bacteria, and Fungi. Microbial communities in various environments are known to form syntrophic relationships, where metabolic by-products of one microorganism serve as nutrients for another. This dynamic enables the presence of Archaea and Bacteria to support the growth of fungi like Candida. Consequently, the candidiasis model in this study was successfully established by inoculating rats with *C. albicans*.

Another factor contributing to the absence of differences in the microbiome profile between the four groups is the time required for such changes to occur. The genetic stability of the microbiome varies significantly depending on species and location. Research on the human gut microbiome has shown that certain microbial species remain stable over extended periods, sometimes spanning several years. Chen *et al.* [29] observed that microbiome stability and variation persisted over a four-year observation period, with differences in alpha and beta diversity emerging over time. In contrast, semen insemination in this study was evaluated only one day after insemination, and the candidiasis model in this study was evaluated only five days after inoculation, which may not have allowed sufficient time for significant changes to manifest.

Microbiome analysis in this study employed bacterial primers but did not include yeast-specific primers, such as internal transcribed spacers (ITS). As a result, the *Candida* profile could not be directly assessed. However, an examination of the microbiome revealed the presence of the kingdom Eukaryota in nearly all rats in the candidiasis group. At the bacterial level, no significant differences were observed between normal and candidiasis rats, as both groups displayed similar abundance values. In contrast, within the Eukaryota kingdom, the candidiasis group showed an abundance value of up to 20, whereas it was absent in normal rats. This strongly suggests the microorganism was *C. albicans* (Eukaryota group), which had been inoculated into the vagina but was not detected by the equipment due to the specificity of the primer used.

The Jaccard index, which measures species similarity between environments based on presence or absence, was also assessed. The study found no significant differences in the Jaccard index between groups [30,31]. Descriptive data indicated a 100% similarity between groups, reflected by a distance greater than 0.9. The sporadic distribution of the Jaccard index further supported this similarity between the normal condition group with semen insemination and the candidiasis group with semen insemination. This is likely because both groups received semen containing sperm with microbiomes that were similar between the rat vaginal microbiome and semen microbiome.

The Bray-Curtis index evaluated the dissimilarity in species abundance between two environments, making it particularly valuable for analyzing compositional data by comparing the differences in species abundance [32]. In the present study, no significant differences were observed in the Bray-Curtis index between groups. Descriptive data indicated that the index values were predominantly close to 1, with 75% of the samples showing an index value of exactly 1. This high similarity reflects that the microbiome compositions of the groups were nearly identical. The Bray-Curtis index ranges from 0 to 1, with 0 representing completely different microbiome compositions and 1 indicating identical compositions. The finding suggests that the vaginal microbiome of the two groups between the normal group after semen insemination and the candidiasis group after semen insemination were highly similar.

The similarity between these groups indicates that semen insemination may have a homogenizing effect on the vaginal microbiome, potentially overriding the differences caused by candidiasis in the microbiome composition. Semen may introduce external microbes or alter the vaginal environment (e.g., pH or immune response) in a way that reduces the distinct microbial patterns typically associated with candidiasis. This could explain the convergence in microbiome profiles between the normal and candidiasis groups post-semen insemination.

The result might reflect resilience in the vaginal microbiome, wherein sperm insemination restores balance to some extent, even in the presence of candidiasis. Alternatively, it could indicate vulnerability, where semen insemination creates conditions that mask or neutralize

differences in microbial composition caused by infection. In forensic contexts, the similarity might affect the use of microbiome profiling as a marker for identifying infections or sexual activity.

The Unweighted Unifrac index, a beta diversity metric, assesses phylogenetic relationships between species by focusing on the presence of lineages in the phylogenetic tree. It provides insight into evolutionary differences between two environments [30,31]. This study found no significant differences in the Unweighted Unifrac index between groups. Descriptive data showed that index values exceeded 90%, corresponding to a distance of more than 0.75. A distance nearing 1 indicated minimal evolutionary differences between the two environments, attributable to the similar composition of the rat vaginal microbiomes.

The Weighted Unifrac index, an extension of the Unweighted Unifrac index, incorporates lineage abundance in its calculations. By considering species abundance, this metric offers a more ecologically relevant understanding of differences in microbiome composition [29,30]. Consistent with the Unweighted Unifrac findings, no significant differences were observed in the Weighted Unifrac index between groups. Descriptive data indicated index value exceeding 90%, with distances above 0.75. These results reinforce the conclusion that the vaginal microbiomes of the two groups exhibited negligible evolutionary differences due to their high compositional similarity.

Escherichia-Shigella, frequently linked to gastrointestinal infections, its presence in the vaginal microbiome could indicate contamination, an active infection, or other pathological changes possibly connected to sexual activity or hygiene practices [33]. *Roseomonas*, known for its environmental origins, may signify external introduction or unusual microbial shifts, possibly indicating compromised immunity or external factors related to sexual behavior [34]. Archaea, although less studied in the vaginal microbiome, could signal unique environmental or metabolic conditions, such as altered pH or anaerobic niches, potentially relevant in forensic evaluations [35].

These microbial markers might assist in reconstructing events or timelines in forensic cases, such as identifying recent sexual activity or infections that could serve as evidence [36]. They could also distinguish between natural microbial variations and those influenced by external factors like sexual intercourse, infections, or external contaminants. The short-term presence of these microbes might not capture their full impact or persistence in the vaginal environment [37].

Longitudinal studies are essential to determine whether these organisms contribute to chronic conditions or serve as transient markers. Understanding how these microbes interact with the vaginal microbiome could inform diagnostics, particularly in cases of infections or recurrent conditions. In forensic medicine, identifying consistent microbial patterns could improve the reliability of microbiome-based evidence.

Conclusion

In forensic studies, particularly in the context of sexual violence evidence, the finding that semen insemination did not affect the vaginal microbiome profile of the candidiasis rats could have significant implications. The identification of *Escherichia-Shigella*, *Roseomonas*, and Archaea as potential biomarkers could serve as important indicators in forensic microbiome analysis. These microbial signatures may help distinguish between pre-existing infections and those resulting from sexual activity, offering a novel approach to evidence collection in cases of sexual violence. For instance, the presence of certain microbiome components might indicate recent sexual contact, while the absence or presence of others could help identify specific infections, such as candidiasis. Further studies are needed to confirm the reliability of these biomarkers and their role in distinguishing sexual violence evidence from other sources of microbial contamination. By incorporating microbiome analysis into forensic investigations, these findings could improve the accuracy and sensitivity of sexual violence evidence, potentially providing more reliable results in legal contexts.

Ethics approval

The study has received an ethical review and information about passing the ethical review from the Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia/RSUPN dr. Cipto Mangunkusumo Number KET-1147/UN2.F1/ETIK/PPM.00.02/2023.

Acknowledgments

The author would like to thank the Animal Research Facilities (ARF) and Bioinformatics Laboratory IMERI Faculty of Medicine Universitas Indonesia, Bio Farma Bandung, and the Parasitology Laboratory Faculty of Medicine Universitas Indonesia.

Competing interests

All the authors declare that there are no conflicts of interest.

Funding

This study was funded by the Indonesian Education Scholarship (BPI) in collaboration with the Higher Education Financing Center (BPPT), the Ministry of Education, Culture, Research, and the Technology, and the Education and Fund Management Institution (LPDP) of the Ministry of Finance, under Funding Number 02043/J5.2.3/BPI.06/9/2022.

Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

How to cite

Iswara RAFW, Hestiantoro A, Budiningsih Y, *et al*. Impact of semen insemination on the vaginal microbiome profile of candidiasis rat model: A preliminary forensic study on sexual violence evidence. Narra J 2025; 5 (1): e1256 - http://doi.org/10.52225/narra.v5i1.1256.

References

1. Ravel J, Gajer P, Abdo Z, *et al*. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci USA 2011;108(Suppl 1):4680-4687.
2. Miller EA, Beasley DE, Dunn RR, Archie EA. Lactobacilli dominance and vaginal microbiota stability in non-pregnant women. Front Microbiol 2016;7:371.
3. Petrova MI, Reid G, Vaneechoutte M, Lebeer S. Lactobacillus iners: Friend or foe?. Trends Microbiol 2017;25(3):182-191.
4. Smith SB, Ravel J. The vaginal microbiota, host defense, and reproductive physiology. J Physiol 2017;595:451-463.
5. Ahannach S, Spacova I, Decorte R, *et al*. At the interface of life and death: Post-mortem and other applications of vaginal, skin, and salivary microbiome analysis in forensics. Front Microbiol 2021;12:1-10.
6. Murta EF, Filho AC, Barcelos AC. Relation between vaginal and endocervical pH in pre-and post-menopausal women. Arch Gynecol Obs 2005;272:211–213.
7. Lin YP, Chen WC, Cheng CM, Shen CJ. Vaginal pH value for clinical diagnosis and treatment of common vaginitis. Diagnostics 2021;11(1996):1-12.
8. Moghadam FH, Tansaz M, Aminimoghaddam S, *et al*. Vaginitis: Etiology and role of oxidative stress, inflammation and antioxidants therapy. Reprod Med Int 2021;4:1-8.
9. Marrazzo J. Vulvovaginal candidiasis. Clin Evid 2002;6:1784-1796.
10. Devi M, Ismunandar H, Hadibrata E, Nuraisa A. Kandidiasis vulvovaginal. Medula 2021;12(1):118-123.

11. Rao VL, Mahmood T. Vaginal discharge. Obstet Gynaecol Reprod Med 2020;30:11-18.
12. Mania-Pramanik J, Kerkar SC, Mehta PB, *et al*. Use of vaginal pH in diagnosis of infections and its association with reproductive manifestations. J Clin Lab Anal 2008;22:375-379.
13. Nakano FY, Leão R de BF, Esteves SC. Insights into the role of cervical mucus and vaginal pH in unexplained infertility. Med Express 2015;2(2):1-8.
14. Valgardsdottir R, Cattaneo I, Klein C, *et al*. Human neutrophils mediate trogocytosis rather than phagocytosis of CLL B cells opsonized with anti-CD20 antibodies. Blood 2017;129:2636-2644.
15. Mercer F, Ng SH, Brown TM, *et al*. Neutrophils kill the parasite Trichomonas vaginalis using trogocytosis. PLoS Biol 2018;16:1-25.
16. Levy M, Bassis CM, Kennedy E, *et al*. The rodent vaginal microbiome across the estrous cycle and the effect of genital nerve electrical stimulation. PLoS One 2020;15(3):1-20.
17. Conti HR, Huppler AR, Whibley N, Gaffen SL. Animal models for candidiasis. Curr Protoc Immunol 2014; (Suppl 105).
18. Burgstaller SM, Auer KE, Rulicke T. A simple method for repeated in vivo sperm collection from laboratory mice. J Assist Reprod Genet 2024;41:2537-2546.
19. World Health Organization. WHO laboratory manual for the examination and processing of human semen 6th edition. Geneva: World Health Organization; 2021.
20. Duselis AR, Vrana PB. Harvesting sperm and artificial insemination of mice. J Vis Exp 2007;28(3):184.
21. Cora MC, Kooistra L, Travlos G. Vaginal cytology of the laboratory rat and mouse: Review and criteria for the staging of estrous cycle using stained vaginal smears. Toxicol Pathol 2015;43(6):776-793.
22. Caporaso JGKJ, Stombaugh J, Bittinger K, Bushman FD. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2011;7(5):335-336.
23. Navas-Molina JA, Peralta-Sanchez JM, Gonzalez A, *et al*. Advancing our understanding of the human microbiome using QIIME. Methods Enzymol 2013;531:47-58.
24. Agnihotry S, Sarangi AN, Aggarwal R. Construction and assessment of a unified curated reference database for improving taxonomic classification of bacteria using 16s rRNA sequence data. Indian J Med Res 2020;151(1):93-103.
25. Hameed S, Hans S, Monasky R, *et al*. Understanding human microbiota offers novel and promising therapeutic options against candida infections. Pathogens 2021;10(183):1-15.
26. Cools P, Jespers V, Hardy L, *et al*. A multi-country cross-sectional study of vaginal carriage of group b streptococci (GBS) and escherichia coli in resource-poor settings: Prevalences and risk factors. PLoS One 2016;11(1):1-30.
27. Aghazadeh M, Khademi F. The molecular mechanism of candida albicans and bacteria interactions. Biochim Biophys Acta 2018;1862(2):173-183.
28. Hoffmann C, Dollive S, Grunberg S, *et al*. Archaea and fungi of the human gut microbiome: Correlations with diet and bacterial residents. PLoS One 2013;8(6):1-12.
29. Chen L, Wang D, Garmaeva S, e*t al*. The long-term genetic stability and individual specificity of the human gut microbiome. Cell 2021;184(9):2302-2315.
30. Kers JG, Saccenti E. The power of microbiome studies: Some considerations on which alpha and beta metrics to use and how to report results. Front Microbiol 2022;12:1-18.
31. Willis AD. Rarefaction, alpha diversity, and statistics. Front Microbiol 2019;10:1-5.
32. Somerfield PJ. Identification of the Bray-Curtis similarity index: Comment on yoshioka (2008). Mar Ecol Prog Ser 2008,372:303-306.
33. Bassetti M, Giacobbe DR. Infections caused by Escherichia coli and Shigella species: A focus on the clinical and microbiological characteristics of urinary and gastrointestinal infections. Clin Microbiol Infect 2019;25(4):407-414.
34. Dong Y, Zhang X. Roseomonas species and their role in microbial communities. Microb Ecol 2018;76(3):744-757.
35. Revello C, Lombardi A. The role of Archaea in human microbiomes: A review of their significance in health and disease. Microorganisms 2020;8(9):1337.
36. Van Bavel C, Sorensen J. Microbial forensics and the forensic use of microbiome analysis. Forensic Sci Int 2017;277:135-141.
37. Ravel J, Gajer P, Abdo Z. The vaginal microbiome of health women and its association with sexually transmitted infections. Cell Host Microbe 2011;10(3):2017-2014.