Female reproductive microbiome in fertility care

Samiya Dash, B.S.,[a,#] Dong Zhao, Ph.D.,[a,b,#] Ina Schuppe-Koistinen, Ph.D.,[a] and Juan Du, Ph.D.[a]

[a] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden; [b] Department of Gastroenterology, the First Affiliated Hospital (Southwest Hospital) of Third Military Medical University, Chongqing, People's Republic of China

The microbiome has emerged as a critical determinant of female reproductive health and fertility outcomes. Although conventional infertility evaluations, encompassing medical history, ovulation assessment, uterine and tubal evaluation, genetic screening, hormonal profiling, and reproductive tract imaging, provide essential diagnostic information, a substantial proportion of infertility cases remain unexplained, prompting increased attention to microbial factors. This review provides a comprehensive, critical evaluation of the methods for assessing the female reproductive microbiome, spanning traditional culture-based microbiology to contemporary molecular approaches. We systematically discuss the diagnostic performance, clinical utility, and established techniques, including microscopic examination, Nugent scoring, and Amsel criteria, alongside modern molecular methods such as quantitative PCR panels, 16S rRNA gene sequencing, shotgun metagenomics, and other multiomics. Critically, we evaluate the current microbiome testing platforms in clinical validity and utility. We identify significant gaps between research-grade methodologies and clinically actionable diagnostics, including a lack of standardized protocols, inconsistent reporting of absolute bacterial loads vs. relative abundances, and limited validation against reproductive outcomes. We propose evidence-based criteria for selecting appropriate diagnostic approaches on the basis of clinical context and discuss emerging technologies, including multiomics integration for implementing microbiome assessment in fertility care. (Fertil Steril® 2026;125:558–73. ©2026 by American Society for Reproductive Medicine.)

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Globally, an estimated 110 million women of reproductive age (15–49 years) experienced infertility in 2021, with prevalence continuing to rise (1). In clinical practice, the standard female infertility evaluation typically includes assessment of ovulatory function through menstrual history and hormonal profiling (follicle-stimulating hormone, antimüllerian hormone, thyroid-stimulating hormone, and prolactin), evaluation of tubal patency via hysterosalpingography, and pelvic imaging with transvaginal ultrasound to assess uterine and ovarian anatomy. Additionally, conditions such as endometriosis, characterized by the presence of endometrial-like tissue outside the uterus, can significantly impact fertility and may be evaluated through imaging or laparoscopy. Polycystic ovary syndrome (PCOS), a common endocrine disorder causing irregular ovulation and hyperandrogenism, is also assessed during this evaluation (2). Additional investigations, such as sonohysterography, hysteroscopy, or genetic screening, are pursued on the basis of clinical findings, maternal age, or a history of recurrent loss. Despite these comprehensive assessments, approximately 30% of infertility cases remain unexplained, prompting investigation into additional factors such as the reproductive tract microbiome (3–5).

An essential factor that is often underestimated in routine tests is the female reproductive microbiome. The Human Microbiome Project has provided a foundation for understanding the significant impact of the human microbiome on reproductive physiology and disease (6–8). Although the causal relationship between reproductive microbiome dysbiosis and adverse reproductive outcomes remains incompletely understood, accumulating evidence links microbial imbalance to implantation failure, early pregnancy loss and reduced success rates in assisted reproductive technologies (9–14).

A healthy vaginal microbiome is typically characterized by the dominance of *Lactobacillus* species. The functions of these species include producing lactic acid to maintain low pH, as well as certain species generating hydrogen peroxide (H_2O_2) and bacteriocins, which contribute to inhibiting

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[#] S.D and D.Z. contributed equally.

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Correspondence: Juan Du, Ph.D. (E-mail: juan.du@ki.se).

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pathogen colonization (15–16). Using community state types (CSTs), vaginal microbiomes are categorized on the basis of *Lactobacillus* dominance. Over half of healthy women exhibit *Lactobacillus* dominance, classified as CST I, II, III, or V. CST I is primarily characterized by *Lactobacillus crispatus*, whereas CST II features *Lactobacillus gasseri*, CST III is dominated by *Lactobacillus iners*, and CST V by *Lactobacillus jensenii*. In contrast, CST IV represents a more diverse community with lower *Lactobacillus* concentrations, often linked to dysbiosis and increased risk of infections like bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC). This classification highlights the critical role of *Lactobacillus* in maintaining vaginal health (10, 16–18).

The prevalence of CST IV has been reported to be higher in certain demographic groups. However, these associations also likely reflect complex interactions among environmental, socioeconomical, habits, other diseases conditions and stress-related factors (19–23). Moreover, although the CST framework has been widely adopted, it should be noted that studies also further divided the subcommunities in the vaginal microbiome, including VALENCIA, metagenomic community state types (mgCSTs), and vaginal community dynamics (VCDs) (17, 24, 25). VALENCIA CSTs have been increasingly applied in recent large population studies and pregnant cohorts (17, 18, 26–29).

The vaginal microbiome has been shown to exhibit dynamic changes in response to age, pregnancy, and external perturbations, such as antibiotic use, sexually transmitted infections, and douching (17, 25, 30–34). The vaginal microbiome changes composition, structure, and physiology in response to hormonal fluctuations and menstrual cycle variations under both normal and disease conditions, with these bacterial features potentially exerting reciprocal effects on reproductive health (25, 30). BV, aerobic vaginitis (AV), and VVC are common vaginal conditions with alterations in the vaginal microbiome. BV has a significant decrease in *Lactobacillus* species, whereas AV is characterized by vaginal inflammation, and VVC is caused by fungal overgrowth. These conditions often present with similar, nonspecific symptoms (e.g., abnormal discharge, irritation) that do not clearly distinguish one condition from another, creating diagnostic challenges in clinical practice (35–37). Significantly, BV, AV, and VVC are associated with increased risks of miscarriage, preterm birth, and infertility, underscoring the need for accurate diagnosis and timely, effective treatment (38–41).

Furthermore, the endometrium was previously recognized as a sterile environment, which has delayed research into its microenvironment compared with that of the vagina. Sequencing techniques have demonstrated that it harbors a low-biomass microbiome that extends into the fallopian tubes and is essential for embryo implantation (42–44). Although potential transient contamination from the lower reproductive tract during sampling remains a concern, current data suggest the human endometrial microbiome is highly diverse, predominantly composed of Firmicutes (including *Lactobacillus*), Bacteroidetes, Proteobacteria, and Actinobacteria, despite a lack of consensus across studies (45, 46). In various uterine diseases, this composition shifts toward increased levels of *Proteobacteria* (e.g., *Escherichia coli*), *Bacteroidetes* (e.g., *Prevotella*), and *Actinobacteria* (e.g., *Gardnerella*) (47–51). An abnormal endometrial microbiota is associated with implantation failure, pregnancy loss, and various gynecological and obstetrical conditions (52, 53).

The gap between scientific progress and clinical application underscores the need for evaluating the reproductive microbiome in fertility care. Therefore, we aim to review and compare current methods for assessing vaginal dysbiosis, from traditional microbiology to modern molecular techniques. We advocate for greater integration of microbiome-based diagnostics into fertility practice.

Traditional clinical tests for vaginal microbiome

Traditional vaginal microbiome assessment remains fundamental to fertility practice. Accurate diagnosis requires understanding normal discharge variability across the menstrual cycle, necessitating standardized collection protocols. Many cases of BV remain asymptomatic or present with only mild symptoms, causing them to be overlooked without specific clinical tests (54–58). Given the impact of vaginal dysbiosis on reproductive success, early microbiome evaluation, using conventional and molecular approaches, has become fundamental to comprehensive fertility care.

Microscopic examination

Microscopic examination allows direct observation of both microbial morphotypes and host cells using complementary techniques, commonly wet mount microscopy and Gram staining. Together, these techniques enable healthcare professionals to evaluate the cellular makeup and microbial composition in vaginal samples. In a physiologically normal vaginal specimen, wet mount microscopy reveals predominantly superficial and intermediate squamous epithelial cells from the upper stratified layers, minimal to absent polymorphonuclear leukocytes (PMNs), resulting in a low leukocyte-to-epithelial cell ratio characteristic of a noninflammatory state. Women with BV who experience subsequent spontaneous preterm birth exhibit a significantly higher mean PMN/ epithelial cell ratio (e.g., 3.4 ± 6.0), making this ratio a better predictor of adverse outcomes than merely observing the presence of clue cells (59). Additionally, Gram staining is a differential technique classifying bacteria by cell wall structure and morphology. In a healthy vaginal microbiome, there is a marked predominance of *Lactobacillus* species, observed with Gram-positive bacilli (60, 61). In contrast, BV is characterized by abundant clue cells (epithelial cells with adherent coccobacilli obscuring cell borders), a marked reduction or absence of lactobacilli, and increased mixed microbiota, including *Gardnerella*. Additionally, BV also typically has symptoms such as altered color, odor, volume, or

TABLE 1

Clinical test for vaginal dysbiosis using traditional methods. AU: Please verify and confirm the table used is correct.

Method	Standard	Indicators of dysbiosis	Advantage	Limitation
Microscopy (wet mount, Gram staining)	Morphological visualization of cells and microorganisms	Increased leukocytes; reduced *Lactobacillus*; presence of yeast or clue cells	Rapid, low-cost, BV/VVC indicators	Subjective, limited sensitivity
Functional tests (pH, H_2O_2, enzymes)	Acidity detection; Lactobacillus activity; pathogens-associated enzymes	Vaginal pH>4.5, reduced H_2O_2, or elevated pathogen enzyme activities	Rapid, low-cost, self-test	Low specificity
Culture-based method	Growth and identification on selective media; biochemical tests	Isolation of bacterial or fungal pathogens	Species identification; antimicrobial susceptibility testing	Limited to cultivable species; time-consuming
Nugent score	Gram staining, morphological examination for scoring *Lactobacillus* (0-4), *G. vaginalis* (0-2), and *Mobiluncus* (0-2)	Scores: (1) 0-3, normal Lactobacillus-dominant flora (BV-negative); (2) 4-6, intermediate flora (dysbiosis but not meeting BV criteria); (3) 7-10, BV	Standardized BV diagnosis	Semi-quantitative, requires trained personnel, time-consuming
Amsel criteria	Vaginal pH, odor test, and microscopy	≥3 positive clinical features:(1) thin, homogeneous discharge; (2) vaginal pH >4.5 (3) positive "whiff test" (10% KOH) (4) presence of clue cells on microscopy.	Simple, rapid; widely used in clinics	Subjective, low reproducibility

Dash. Female reproductive microbiome. Fertil Steril 2026.

consistency, representing a dysbiosis yet not an inflammatory condition (62–65).

AV is defined by a severe inflammatory state (vaginal pH > 6.0) distinguishable by the presence of numerous PMNs, parabasal epithelial cells resulting from epithelial thinning. Microbiologically, it involves a shift from *Lactobacillus* dominance to an overgrowth of aerobic commensals (such as *E. coli* or *Group B Streptococcus*) rather than just anaerobic cocci (66, 67). Trichomoniasis is identified by motile, flagellated *Trichomonas vaginalis* protozoa, often accompanied by significant leukocytosis and inflammatory exudate, although they are not required for diagnosis (68, 69). In addition to presence of clinical symptoms, potassium hydroxide (KOH) wet mount microscopy or Gram staining can be used to identify budding yeast cells, with pseudohyphae observed in some cases of VVC (70, 71). These distinct microscopic patterns enable rapid differentiation of vaginal pathologies and guide appropriate therapeutic intervention to restore normal vaginal microbiome homeostasis (61, 72, 73).

In the fertility clinic setting, microscopic examination serves as an important first-line diagnostic tool for rapid assessment of the vaginal and cervical microenvironment, which provides clinically useful context in fertility care (74). Microscopy offers immediate, direct visualization of cellular morphology and microbial morphotypes, providing semiquantitative information on microorganisms and host inflammatory responses that are relevant to vaginal health, including symptoms associated with BV and other conditions linked to adverse reproductive outcomes. The presence of BV has been associated with an increased risk of adverse pregnancy outcomes, including miscarriage, as well as adversely affecting sperm by immune-mediated dysfunction (75). This rapid turnaround, often providing actionable information within minutes, makes microscopy an invaluable point-of-care tool in fertility management, bridging the gap between clinical presentation and comprehensive microbiological diagnosis (Table 1).

Functional tests

Functional testing plays a central role in the primary evaluation of vaginal microenvironment abnormalities. Vaginal pH is intrinsically linked to microbial homeostasis, with physiologic values of 3.8–4.5 reflecting *Lactobacillus* dominance and optimal reproductive health (76). Although hydrogen peroxide (H_2O_2) production by certain *Lactobacillus* species has been widely discussed in mechanistic studies and sometimes used in vaginal functional tests, its concentrations in vivo are highly variable and generally insufficient to provide consistent antimicrobial protection. Therefore, H_2O_2 is not considered a reliable marker for clinical diagnosis and needs to be used together with other tests (15, 77, 78). In clinical practice, combined vaginal pH testing and clinical symptom assessment significantly improve diagnostic sensitivity for vaginitis (77, 79), with pH typically measured using phenaphthazine (Nitrazine) indicator paper (80).

From research development perspective, enzymatic biomarkers enhance diagnostic precision beyond pH-based assessment. Leukocyte esterase indicates active polymorphonuclear leukocyte infiltration, reflecting the intensity of the inflammatory response. Sialidase activity, produced by BV-associated communities that commonly include *Gardnerella vaginalis* and *Prevotella spp.* are strongly associated with BV, making it a good clinical marker for BV diagnosis (81). Elevated β-glucuronidase activity suggests *Escherichia coli* or Group B *Streptococcus* colonization, whereas coagulase positivity indicates *Staphylococcus aureus*, a key pathogen in AV (82–84).

Recent advances in point-of-care testing have facilitated integration of multiple diagnostic parameters. Commercial platforms such as the Hygeia Touch Self-Testing Kit employ dry-chemistry enzymatic methods to assess vaginal pH and enzymatic markers in simplified formats (85). These tests are designed for screening, providing a rapid, user-friendly assessment with reported performance comparable with established screening approaches, with overall accuracy reported to be of approximately 88% and sensitivity and specificity of approximately 87% and 89%, respectively, compared with traditional diagnostic methods relying on clinician judgment (86). Their main aim is to improve accessibility and facilitate early detection in both clinical and home-based settings, rather than replacing microscopy or laboratory diagnostics. Ultimately, these tools should be viewed as complementary screening aids rather than standalone diagnostic tests.

Culture-based methods

Culture-dependent diagnostics remain a classic, clinically valuable strategy for identifying imbalances in the vaginal microbiome, particularly for specific bacterial and fungal pathogens. Direct culture is used for specific vaginal pathogens, with appropriate selective or differential media targeted for specific organisms, followed by identification on the basis of colony morphology, Gram staining, and biochemical testing. (87–89). Nevertheless, although some BV-associated anaerobes, such as *Prevotella* species, *Fannyhessea vaginae*, *Sneathia*, and *Megasphaera,* are technically culturable on specialized anaerobic media, the fastidious nature, strict oxygen sensitivity, extended incubation requirement, and technical complexity make routine clinical culture impractical for most BV-associated bacteria. Species Bacterial Vaginosis-Associated Bacteria (BVAB1-3) pose challenges for detection using standard culture methods (90–93).

For AV, blood agar and MacConkey agar are common agar plates that enable isolation of aerobic pathogens, including *Streptococcus agalactiae*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Escherichia coli*, with subsequent antibiotic susceptibility testing guiding targeted therapy, particularly important in fertility contexts. For the case of VVC, culture on routine fungal media or fungal differential media followed by MALDI-TOF mass spectrometry is the standard approach for species-level identification (94, 95).

Culture-based methods provide simple, practical diagnostics that yield viable organisms for primary diagnosis, antimicrobial susceptibility testing, and resistance profiling. However, they demonstrate low sensitivity in capturing the full vaginal microbiome diversity, as many fastidious, slow-growing, or strictly anaerobic bacteria remain unculturable by standard techniques, potentially missing clinically relevant organisms that contribute to dysbiosis and adverse reproductive outcomes (96, 97). The limitations have driven the development of culture-independent molecular diagnostic approaches to complement traditional microbiology in comprehensive vaginal microbiome assessment (Table 1).

Diagnostic criteria on the basis of the traditional method

The two most widely employed diagnostic frameworks for BV diagnosis are the Nugent scoring system and the Amsel criteria, each offering distinct advantages and limitations in the fertility context. The Nugent score provides a semi-quantitative microscopic assessment on the basis of Gram-stained vaginal smear morphology, using a standardized scoring system to evaluate bacterial morphotypes. The Amsel criteria represent a clinical diagnostic approach on the basis of four parameters: presence of a thin homogenous vaginal discharge, vaginal pH > 4.5, a positive amine "whiff test" after the addition of 10% KOH, and the presence of clue cells (Table 1). The diagnosis of BV is made when at least three of the four criteria are met (98). In addition, some fertility clinics offer specialized reproductive microbiome testing, such as endometrial microbiome analysis using commercial assays (EMMA/ALICE, Igenomix), in cases of recurrent implantation failure (RIF), unexplained infertility, or recurrent pregnancy loss.

In fertility practice, the Nugent score and Amsel criteria serve complementary diagnostic roles: Amsel criteria enable rapid point-of-care assessment for immediate treatment decisions during time-sensitive cycles, whereas Nugent score provides a structured framework for in vitro fertilization (IVF) or intrauterine insemination (IUI) screening (62, 98–100). The intermediate Nugent category may be clinically relevant in fertility settings as it reflects partial shifts in vaginal microbial composition rather than symptoms. Even though sometimes, both intermediate and BV-range scores can be asymptomatic, reduced *Lactobacillus* dominance and associated changes in microbial composition have been linked to adversely affect implantation and early pregnancy outcomes (101, 102).

However, both methods provide a graded evaluation of vaginal cells and microbes, which require specialized expertise in microscopy and laboratory support; therefore, they may exhibit interobserver variability (103). Critically, neither provides species-level taxonomic resolution, cannot detect fastidious or unculturable anaerobes, and fails to distinguish clinically significant *Lactobacillus* species (*L. crispatus* vs. *L. iners* vs. *L. gasseri* vs. *L. jensenii*) with distinct reproductive outcome associations (16, 104). Consequently, comprehensive vaginal microbiome assessment in modern fertility practice requires integrating traditional diagnostic methods with advanced molecular and multiomics technologies to achieve the species-level resolution, functional characterization, and personalized therapeutic targeting necessary to optimize reproductive outcomes.

MOLECULAR METHODS FOR VAGINAL MICROBIOME ANALYSIS

The inherent limitations of traditional culture-based and microscopic diagnostic methods have driven a paradigm shift toward technologies in fertility microbiome assessment. These technologies have fundamentally transformed our understanding of the vaginal microbiome-fertility axis, enabling identification of specific microbial signatures associated with implantation success, pregnancy outcomes, and treatment response, thereby facilitating more accurate diagnostic assessment and personalized, microbiome-targeted therapeutic interventions in clinical fertility practice.

Quantitative PCR (qPCR) and Digital Droplet PCR (ddPCR)

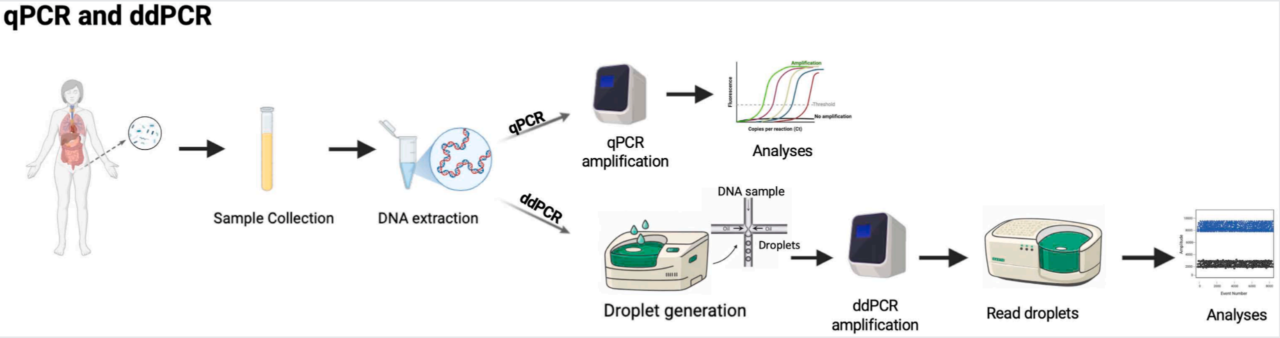
qPCR and ddPCR enable precise enumeration of specific microbial targets, including bacteria, fungi, and viruses, through targeted amplification and fluorescence-based detection. qPCR enables real-time quantification of determined microbial DNA targets, allowing rapid assessment of taxa relevant to the reproductive microbiome, with custom qPCR panel kits available that target the main species found in the reproductive microbiome (105, 106). The subsequent emergence of ddPCR advanced this capability by partitioning samples into thousands of nanoliter droplets, enabling absolute quantification of DNA copy numbers without reliance on standard curves and achieving superior sensitivity for low-abundance targets (107) (Figure 1).

These complementary technologies have proven particularly valuable in fertility microbiome research: qPCR analysis of vaginal and endometrial samples from women with unexplained infertility revealed significantly depleted *Lactobacillus* spp. and elevated *G. vaginalis* and *Mycoplasma hominis* compared with fertile controls (108). Zhou et al. developed a ddPCR assay targeting the 23S rRNA gene of *G. vaginalis* that reported good analytical performance, with no cross-reactivity observed for common vaginal commensals or pathogenic bacteria (109). ddPCR also provides a unique advantage in precise absolute quantification of low abundant nucleic acids, enabling detection of subtle allelic imbalances, such as maternally inherited mutations in noninvasive prenatal testing (110). However, both technologies are inherently limited to detecting taxa with known target sequences, precluding the discovery of novel or uncharacterized microorganisms. Additionally, ddPCR exhibits reduced dynamic range at very high bacterial concentrations and requires specialized instrumentation, rigorous assay optimization, and greater technical expertise, resulting in higher costs and longer turnaround times compared with qPCR, particularly in clinical fertility practice. Moreover, the high genomic similarity among prevalent vaginal microbiome species poses significant challenges for taxonomic discrimination using targeted PCR approaches. Although numerous studies have employed species-specific primers or probes for vaginal bacteria, rigorous validation of their specificity against closely related taxa is frequently lacking, potentially leading to cross-reactivity and misidentification that compromise diagnostic accuracy in clinical fertility applications (111–113).

16S rRNA gene sequencing

16S rRNA gene sequencing identifies bacteria by analyzing a highly conserved molecular marker containing nine hypervariable regions (V1–V9) that enable genus- and species-

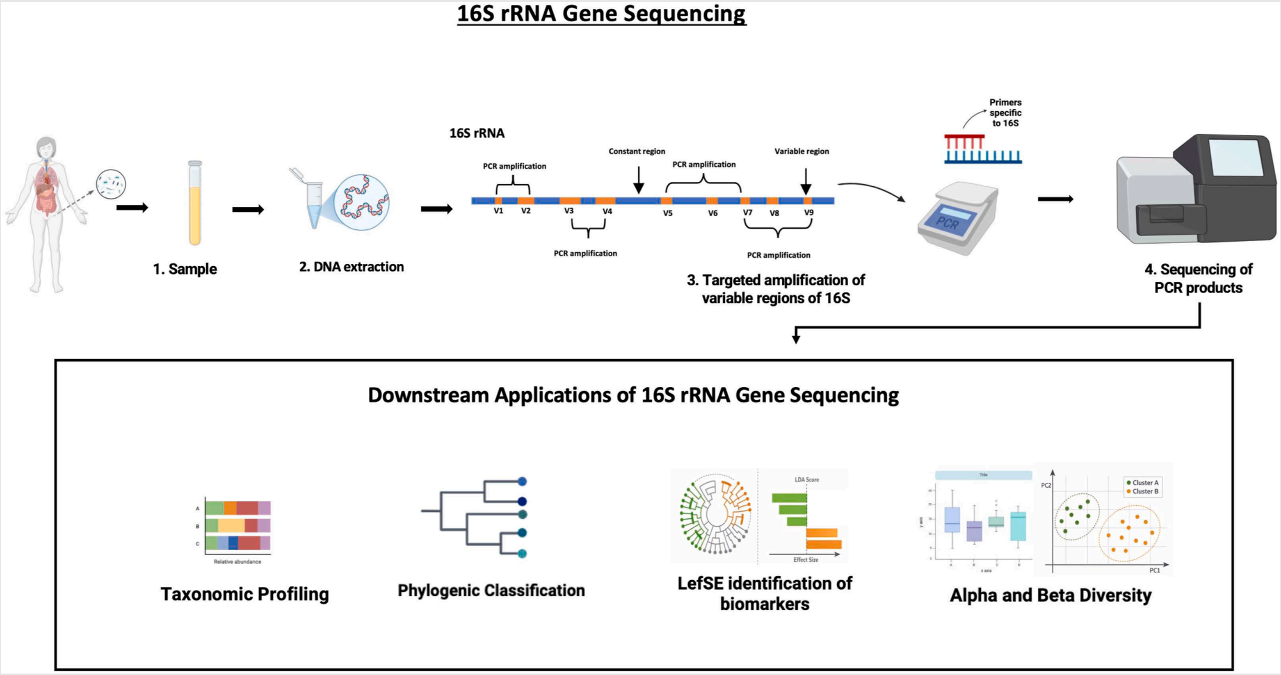
FIGURE 1



Graphical schematic of the workflow of qPCR and ddPCR. DNA is extracted from samples, and specific target sequences are amplified using sequence-specific primers. In qPCR, amplification is measured using fluorescent dyes or probes, allowing quantification based on cycle threshold (Ct) values. In ddPCR, the PCR reaction mix is first partitioned into thousands of nanoliter-sized droplets through droplet generation, with each droplet acting as an independent PCR microreaction. Endpoint PCR amplification occurs within each droplet, followed by droplet reading, during which fluorescence is measured to classify droplets as positive or negative. Created using Biorender.com.

Dash. Female reproductive microbiome. Fertil Steril 2026.

FIGURE 2



16S rRNA gene sequencing workflow. DNA was extracted from samples using standard protocols. The bacterial 16S rRNA gene was amplified with targeted primers and sequenced using high-throughput sequencing. Resulting sequences were used for taxonomic profiling and phylogenetic classification of microbial communities. Microbial diversity was assessed using alpha diversity metrics to evaluate community richness and evenness, and beta diversity metrics to evluate the dissimilarity in community composition between samples. LEfSe analysis was applied to identify differentially abundant taxa and potential microbial biomarkers. This single-gene amplicon approach enables high-throughput profiling of microbial communities. Created using Biorender.com.

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level taxonomic discrimination. Compared with other commonly used 16S regions (e.g., V1–V2, V1–V3, V3–V4), direct comparisons have shown that V3–V4 provide improved representation of key vaginal taxa and more accurate CST assignment compared with V1–V2, likely due to reduced primer mismatches leading to biased detection of clinically relevant organisms like *Gardnerella* and *Chlamydia* (114). Nevertheless, full-length 16S rRNA gene sequencing using long-read technologies (PacBio, Oxford Nanopore) maximizes resolution yet incurs higher costs vs. short-read platforms (115–116) (Figure 2).

16S rRNA gene sequencing has revealed critical vaginal microbiome-fertility associations. Infertile women exhibit significantly reduced vaginal *Lactobacillus* abundance compared with fertile controls. Vaginal *L. gasseri* enrichment enhanced fecundability while elevated *Fannyhessea vaginae* (previously called *Atopbium*) reduced it, and *L. iners*-dominant microbiomes conferred approximately 55% reduced per-cycle conception rates compared with *L. crispatus/L. gasseri*-enriched profiles (117). A longitudinal vaginal microbiome analysis by D. MacIntyre et al. in a British pregnancy cohort revealed dramatic postpartum shifts toward reduced *Lactobacillus* dominance and increased diversity regardless of pregnancy composition or ethnicity (9). Furthermore, vaginal *Lactobacillus* dominance was associated with higher IVF success rates. In contrast, the presence of *Streptococcus* spp., *Staphylococcus* spp., and *Escherichia coli* was associated with reduced live birth rates and lower embryo transfer success (118, 119). Prospective studies collecting vaginal samples immediately before embryo transfer have confirmed these associations in patients undergoing assisted reproductive technology (ART) with single euploid blastocyst transfer (120).

A multicenter study of 342 infertile patients showed that dysbiotic endometrial microbiota comprised genera such as *Fannyhessea, Gardnerella, Klebsiella, Neisseria, Staphylococcus, and Streptococcus*. At the same time, *Lactobacillus* dominance was associated with live birth (121). 16S rRNA gene sequencing of endometrial samples showed that 38% of IVF patients had a *Lactobacillus*-dominant microbiome, compared with 85.7% in healthy volunteers. In the same study, among women who have achieved pregnancy, the median relative abundance of *Lactobacillus* is approximately 96%, suggesting that higher endometrial *Lactobacillus* levels may favor implantation (122). Microbiomes in a receptive endometrium that are dominated by non-*Lactobacillus* species have been shown to significantly decrease rates of implantation, pregnancy, ongoing pregnancy, and live births (8, 43, 123–126).

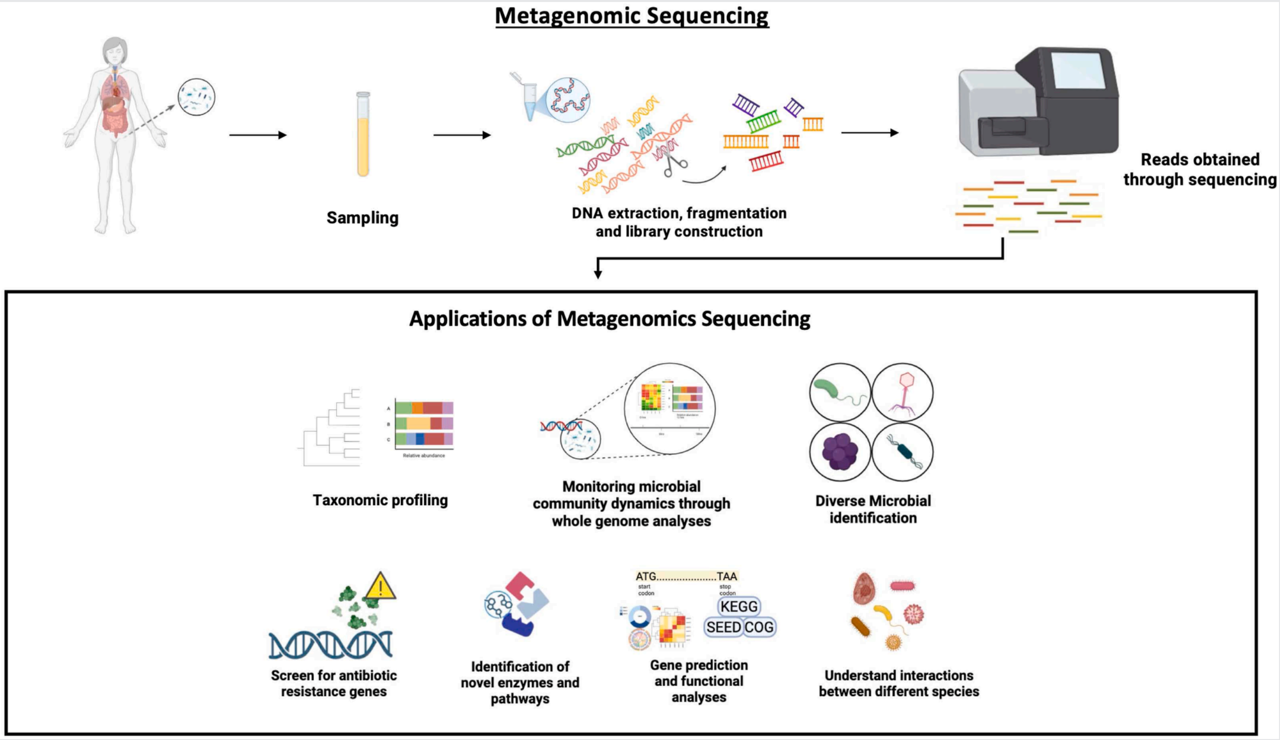
Although 16S rRNA gene sequencing provides cost-effective bacterial community profiling, fundamental biological and technical limitations constrain its taxonomic resolution. The method, particularly in short-read 16S rRNA amplicon sequencing, may not reliably distinguish closely related species or strains, as many taxa previously considered monophyletic comprise multiple genomovars (127). Intragenomic heterogeneity further complicates taxonomic assignment. Intragenomic heterogeneity of the 16S rRNA gene has been documented; certain *Lactobacillus* species with multiple highly similar but nonidentical 16S copies within a single genome complicating species level resolution (128). Database quality represents an additional confounding factor. A United Kingdom sovereign audit of 1,399 reference sequences revealed ~5% contained substantial errors, including chimeric artifacts, sequencing errors, or taxonomic misannotations (127, 129). Full length 16S rRNA sequencing can reliably identify dominant *Lactobacillus* species (130); however, in contexts where finer taxonomic resolution or function insight is of interest, complementary approaches, including whole-genome sequencing or shotgun metagenomics, can provide additional genetic information. These methods enable strain-level characterization and functional profiling and are primarily used to address specific mechanistic questions rather than routine community classification.

Shotgun metagenomics

Shotgun metagenomics, enabled by next-generation sequencing platforms, revolutionized microbiome science by allowing culture-independent, unbiased analysis of entire microbial communities directly from clinical specimens. Unlike amplicon-based methods that target specific marker genes, shotgun metagenomics sequences all DNA present in a sample, providing comprehensive taxonomic profiling across kingdoms (bacteria, archaea, fungi, and viruses) alongside functional gene characterization, including metabolic pathways, virulence determinants, and antibiotic resistance mechanisms (131) (Figure 3).

Sequencing-based methods, initially using 16S rRNA gene profiling, then subsequently expanded through shotgun metagenomics, have provided evidence against the long-standing assumption of sterility in the female upper reproductive tract by demonstrating the presence of microbial DNA across the cervical canal, uterine cavity, and

FIGURE 3



Metagenomic sequencing workflow. Samples were collected, DNA was extracted using standard protocols, and the resulting DNA was fragmented, and a library was constructed for high-throughput sequencing. This whole-genome approach enables multiple applications, including taxonomic profiling, monitoring microbial community dynamics, assessing diversity, identifying antibiotic resistance genes, discovering novel enzymes and pathways, predicting gene functions, and exploring interspecies interactions. Metagenomic sequencing provides comprehensive, high-resolution insights into both the taxonomic composition and functional potential of microbial communities. Created using Biorender.com.

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peritoneal fluid (132). Building on classical CSTs, mgCSTs are novel categories of microbiomes that reflect unique combinations of metagenomically resolved species, demonstrate associations with demographic factors and vaginal health indicators, particularly BV, and offer a framework for investigating functional diversity and its implications for women's genital health (133). While mgCSTs represent a promising approach for capturing microbial diversity, their relevance for guiding fertility care has yet to be established. Furthermore, Feehily et al. used shotgun metagenomic sequencing to show that *L. crispatus*-dominated communities associated with full-term pregnancy differ structurally and functionally from preterm communities, including strain-level differences within *Gardnerella* and distinct metabolic profiles that extend beyond the resolution of 16S rRNA gene profiling. This highlights distinct structural and functional differences in the vaginal microbiota of women at high risk for preterm birth (PTB) compared with low-risk controls, which may help identify microbial traits that influence PTB (134). These findings demonstrate the clinical relevance of metagenomic approaches in advancing personalized medicine for reproductive health and better fertility outcomes (135).

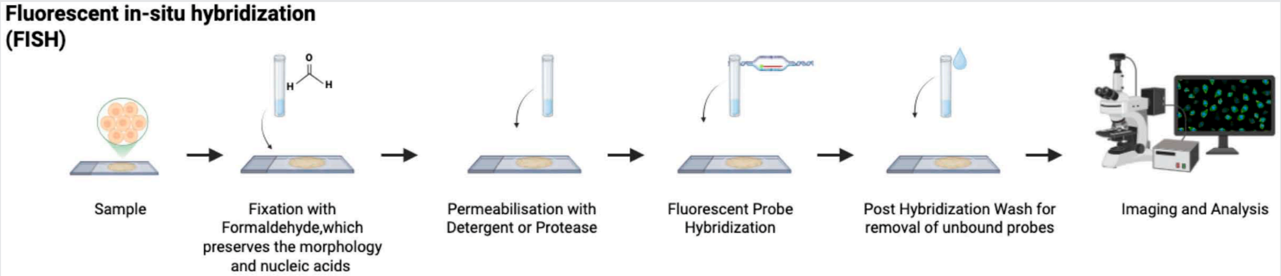
Shotgun metagenomics faces substantial technical challenges in low-biomass reproductive tract environments, such as the endometrium, requiring substantial sequencing depth, specialized bioinformatic expertise, and comprehensive reference databases to address functions that remain incomplete for many reproductive taxa. Host DNA contamination poses a critical confounding factor, with human DNA constituting >99% of sequencing reads in endometrial samples, necessitating microbial enrichment strategies, such as methylation-based host DNA depletion (e.g., NEBNext Microbiome Enrichment) or differential lysis methods to achieve adequate microbial genome coverage. However, standardized protocols for low-biomass reproductive samples remain under development (134, 136). Like other sequencing methods, contamination during sampling or sequence library preparation is an issue, and proper controls are needed for each step. Additional limitations include computational intensity, high per-sample costs, and potential false-positive taxonomic assignments from database contamination or misannotation. Despite these challenges, shotgun metagenomics is promising to be used for comprehensive characterization of the reproductive microbiome, enabling combined analysis of community structure, functional potential, and strain-level variation, which could be important for understanding microbiome-fertility relationships and developing targeted microbiome-based interventions (137).

Fluorescent in situ hybridization (FISH)

FISH employs fluorescently labeled nucleic acid probes to visualize specific microorganisms within their spatial context (138). FISH combined with immunohistochemical detection of bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA) identified Gram-negative and Gram-positive bacteria in endometrial tissues from women with and without endometriosis (139). FISH has also been used to visualize structured polymicrobial *G. vaginalis* biofilms adherent to endometrial and fallopian tube epithelium, demonstrating vaginal microbiota ascension into the upper genital tract (34, 140). These findings have significant implications for understanding bacterial vaginosis-associated adverse pregnancy outcomes (Figure 4).

Although not directly applicable to the vaginal microbiome, next-generation single-cell spatial methods have dramatically enhanced FISH capabilities. SRS-FISH (Stimulated Raman Scattering-FISH) combines phylogenetic identification with metabolic activity profiling via deuterium incorporation (141). GenomeFISH employs metagenome-assembled genome-derived probes to achieve strain-level resolution with up to 27-fold higher signal intensity than conventional rRNA-FISH, successfully distinguishing *Prevotella copri*, *Agathobacter rectalis*, and *Faecalibacterium*

FIGURE 4



Graphical schematic of the workflow of fluorescent in situ hybridization (FISH). Microbial samples are first fixed to preserve cell structure and nucleic acids. Cells are then permeabilized to allow for entry of fluorescently labeled oligonucleotide probes, targeting bacterial 16S rRNA sequences. Probes hybridize to their complementary rRNA sequences within bacterial cells. Unbound sequences are removed by washing, and fluorescence microscopy is used to detect and visualize target bacterial taxa; created with Biorender.com.

Dash. Female reproductive microbiome. Fertil Steril 2026.

prausnitzii species in human fecal samples (142). Despite these advances, FISH is limited by probe availability for only a subset of commensal bacteria, insufficient resolution for closely related taxa, restricted multiplexing because of spectral overlap, complex probe design, and the inability to capture live-cell dynamics because of sample fixation. Future efforts enhance signal sensitivity and taxonomic specificity to advance spatially resolved FISH studies of the reproductive microbiome.

MULTIOMICS APPROACHES

Advanced multiomics technologies are revolutionizing reproductive microbiome research by integrating complementary layers. Although not applied in the fertility clinic yet, metatranscriptomics captures actively expressed microbial genes through RNA sequencing, revealing real-time metabolic activity rather than mere taxonomic presence (143). Proteomics characterizes microbial and host-derived proteins, while metabolomics profiles small-molecule metabolites that mediate host-microbe crosstalk (143–146).

Both metatranscriptomics and metaproteomics remain in their infancy because of limited reference databases, a lack of standardized protocols, and an abundance of uncharacterized microbial genes and proteins. Researchers have begun developing innovative analytical tools (147). Metatranscriptomic analysis by the J. Ravel group revealed that microbial abundance does not predict transcriptional activity, with non-*Lactobacillus*-dominated communities exhibiting distinct mucin-degradation patterns potentially linked to adverse outcomes. It demonstrated that the pathogenic potential of *L. iners* and *G. vaginalis*, particularly their cytolysin expression, varies with community composition (145). Metaproteomic analysis offers valuable functional insights into microbial communities relevant to disease mechanisms and holds promise for future clinical diagnostic applications (144, 146, 148).

The evidence consistently demonstrates that *Lactobacillus* produces distinct metabolic signatures compared with BV-associated polymicrobial states. The relationship between these profiles and fertility outcomes shows a coherent pattern: *Lactobacillus* dominance is associated with beneficial metabolite production (lactate, mannitol, indole-3-lactate) and improved fertility outcomes (149), whereas dysbiosis characterized by increased diversity and anaerobic overgrowth correlates with metabolic shifts toward biogenic amines, short-chain fatty acids, and amino acid catabolites that may impair implantation and pregnancy success (150–153). Using glycan microarrays, V. Tajadura-Ortega et al. (154) demonstrated that vaginal pathogens (*E. coli, F. nucleatum, S. agalactiae*) exhibit distinct glycan-binding profiles compared with *Lactobacillus* species, with chondroitin sulfate-mediated epithelial adhesion being competitively inhibited by *L. crispatus*, highlighting glycan interactions as key determinants of vaginal microbiome composition and reproductive health and pregnancy outcomes. The mechanistic explanations proposed across studies provide a coherent understanding, whereby *Lactobacillus* species maintain low pH through lactate production and exclude pathogens through competitive mechanisms, including mannitol-mediated tonicity optimization and immunomodulation via aryl hydrocarbon receptor binding (149). Conversely, BV-associated bacteria use amino acids as carbon and nitrogen sources, producing catabolites and biogenic amines that may create an inflammatory microenvironment unfavorable for implantation (149, 150, 155). The metabolic changes observed in successful vs. unsuccessful IVF cycles support the concept that a metabolic state may be optimal for embryo implantation (152, 153).

The convergence of spatial transcriptomics and single-cell technologies with microbiome research represents a methodological shift from bulk compositional analysis toward spatially and cellularly resolved profiling. The technologies examined fall into two complementary categories: spatial approaches (10x Visium, GeoMx, MaPS-seq, HiPR-FISH) that preserve tissue architecture and reveal microbial localization patterns (156, 157), and single-cell methods (INVADEseq, SPLiT-Seq, SAG sequencing) that capture cellular heterogeneity and individual cell responses (158). The main challenge lies in the mismatch between capture methods for eukaryotic mRNA (which rely on poly-A) and those for prokaryotic RNA, which lacks polyadenylation. This discrepancy leads to low capture efficiency for microbial sequences, often resulting in the recovery of ribosomal RNA instead of messenger RNA. Additional sample preparation issues include bacterial cell wall permeabilization, aggregation, and contamination. The field is advancing with solutions such as lysozyme treatment to improve microbial capture, random hexamer primers to replace poly-T capture, and specifically designed capture oligos for bacterial sequences. As these technologies develop, their use is expected to extend from high-microbial-burden areas, such as the intestine, to more sterile tissues, enabling exploration of microbial presence in previously inaccessible environments.

Integrating genomics, transcriptomics, proteomics, metabolomics, and microbiome profiling has highlighted interactions driving age-related fertility decline (159). Characterization of extracellular vesicles in uterine fluid revealed dynamic shifts in miRNA, transcripts, and surface proteins across the menstrual cycle, reflecting molecular preparation for implantation (160). In endometriosis, combined analyses of microbial, metabolic, hormonal, immune, and genetic pathways revealed how chronic inflammation, oxidative stress, and endometriosis impair fertility (161). These findings illustrate how functional multiomics can inform fertility care, as successful implantation and pregnancy outcomes are increasingly understood to depend on microbial functional activity and host-microbe interaction, rather than microbial composition alone. However, substantial clinical translation challenges remain. Metatranscriptomics provides only transient snapshots of gene expression, as

RNA degrades rapidly and fluctuates with sampling conditions. Proteomics faces technical hurdles, including low-abundance protein detection, sample complexity in mixed host-microbe specimens, and ambiguous protein identification. Metabolomics cannot definitively attribute metabolite origins when multiple microbial species and host tissues contribute to shared biochemical pathways. All these approaches require expensive instrumentation, such as mass spectrometry and high-throughput sequencing platforms, specialized bioinformatic expertise, and computationally intensive data integration across platforms. For fertility clinics, these limitations translate to high per-sample costs, prolonged turnaround times spanning weeks to months, and interpretation challenges that currently preclude routine clinical implementation (162).

The reproductive tract virome, which includes bacteriophages and eukaryotic viruses, adds a layer of microbial complexity; however, it is comparatively understudied relative to bacterial communities, with its relevance in fertility care remaining unclear (163). Viral infections such as Human Immunodeficiency Virus (HIV), Human Papillomavirus (HPV), and Herpes Simplex Virus can disrupt this balance, leading to dysbiosis and increased susceptibility to BV, VVC, and sexually transmitted infections (164–166). Studies are increasingly focusing on characterizing the phage populations present in the vagina, understanding their dynamics, and exploring their therapeutic potential as a novel approach to restore healthy microbial communities (25, 167–169). Understanding the interactions between viral infections and the female microbiome is crucial for developing targeted therapeutic strategies to restore microbial balance and improve reproductive health.

Perspective

The integration of microbiome assessment into fertility practice offers substantial promise for addressing unexplained infertility. However, a critical gap exists between the wealth of multiomics research data and the paucity of validated clinical tools. Although commercial vaginal microbiome testing using qPCR and 16S rRNA sequencing is now available, widespread adoption requires addressing methodological limitations, establishing clinical utility through rigorous trials, and developing standardized interpretive frameworks.

Importantly, many microbial patterns discussed in relation to fertility are supported primarily by associative evidence, and the extent to which these represent mechanistic drivers rather than downstream biomarkers reflecting broader host, hormonal, immunological, or behavioral changes remains uncertain. This limitation extends beyond the reproductive tract itself. Recent evidence suggests that microbiomes, such as those of the gut and oral cavity, may be associated with fertility outcomes. However, most current data remain largely associative and lack causal validation (170, 171). Clarifying this distinction is critical for clinical translation and will require longitudinal and interventional studies linked to clinically meaningful endpoints such as implantation success and live birth.

Although endometrial microbiome profiling may more directly correlate with reproductive outcomes, vaginal microbiome assessment offers practical advantages: noninvasive sampling, self-collection capability, and established correlations with upper tract colonization. Additionally, although a low-biomass endometrial microbiome has been proposed, it remains unclear if detected bacterial DNA represents a stable autochthonous community or transient allochthonous bacteria repeatedly introduced by the lower reproductive tract. This distinction has important implications for interpretation and clinical translation (172). A hybrid approach integrating traditional microbiology with molecular methods may therefore optimize clinical implementation. As cross-cohort datasets expand, validating key microbial biomarkers enables the development of user-friendly diagnostic platforms requiring minimal specialized training. Traditional culture rapidly identifies pathogens and guides antimicrobial therapy, while molecular approaches provide comprehensive taxonomic resolution and detect fastidious anaerobes. This integrated strategy streamlines workflows and facilitates broader adoption of microbiome diagnostics in reproductive medicine.

In addition to diagnostic applications, emerging evidence suggests potential clinical utility of microbiome-targeted interventions, although current findings are variable and predominantly derived from limited, nonrandomized studies. In women with RIF, one study reported higher pregnancy rates following targeted antibiotics and probiotics (64.5%) compared with controls (33.3%) (173); however, these findings require validation in larger randomized trials with standardized outcome measures. Probiotic trials for BV and VVC demonstrate variable efficacy, which shows the need for ”the right bug in the right place” (174). In a landmark, randomized trial, intravaginal *Lactobacillus crispatus* CTV-05 (LACTIN-V), administered after antibiotic therapy, reduced recurrence compared with placebo (175). More recent early-phase trials of multistrain vaginal biotherapeutics have demonstrated successful colonization of *Lactobacillus*-dominant community states (176, 177). Furthermore, increasing evidence suggests that BV-associated microbiota can be sexually transmissible, with male partner treatment reducing recurrence, though the implications of these findings for fertility outcomes are yet to be established (178).

Whole vaginal microbiome transplantation represents an emerging approach that demonstrates modification of vaginal microbiome community structure, most clearly observed in refractory BV (179). Evidence to date remains limited, consisting primarily of case series without controls, in which microbiome shifts variably and with some individuals requiring repeated administration. Isolated case reports, including microbiome restoration in a woman with recurrent late pregnancy losses who subsequently delivered a healthy infant (180), serve as preliminary observations. Together, these findings suggest potential relevance beyond BV while underscoring the need for cautious interpretation and further validation of clinical efficacy before clinical implementation.

A pragmatic framework for integrating microbiome assessment into fertility practice would treat testing as a complement to already established diagnostic workflows rather than a substitute. In this context, noninvasive vaginal sampling in patients with unexplained infertility or RIF offers a practical starting point, with molecular profiling used to characterize broad dysbiosis patterns rather than basing interpretation on the absence or presence of individual taxa. These findings would primarily inform hypothesis-driven interventions or stratify patients for clinical trials, rather than guide routine treatment decisions. Equally important is a stewardship-oriented approach to interpretation, as overdiagnosis or indiscriminate antimicrobial use risks disrupting microbial homeostasis and contributing to resistance. This reinforces the need to treat microbial assessment as an adjunctive input to clinical interpretation rather than a basis for routine intervention.

Realizing this potential will require standardized protocols for specimen collection and processing, validated reference databases linking microbial functions to reproductive physiology and machine learning frameworks integrating multiomics data with clinical endpoints, including live birth rates and implantation success. While microbiome profiling is extremely data-rich, its clinical uptake has been limited by interpretive complexity, motivating the development of tools aimed at supporting nonspecialist use. AI-driven platforms are being developed to support the interpretation of complex microbiome data in clinical settings, with the aim of improving consistency and interpretability rather than automating clinical decisions. Current efforts largely focus on identifying broad dysbiosis patterns across multiomics layers, alongside the development of digital tools intended to support longitudinal monitoring and more consistent data interpretation. However, these approaches remain contingent on robust clinical validation through randomized controlled trials, as well as practical considerations such as cost, automation, and clinically feasible turnaround times before routine integration into fertility care can be justified.

Despite current limitations, multiomics approaches hold transformative potential for precision reproductive medicine. As understanding of microbial-host-reproductive interactions deepens, microbiome-informed care may transition from investigational to a standard fertility evaluation component, offering new therapeutic avenues for patients with previously intractable infertility.

CONCLUSION

In addition to traditional methods for evaluating the reproductive microbiome, multiple molecular approaches offer complementary insights. Targeted methods such as qPCR and droplet digital PCR (ddPCR) provide sensitive quantification of specific taxa. At the same time, broad profiling techniques, including 16S rRNA sequencing, shotgun metagenomics, and multiomics platforms, reveal community composition, functional potential, and host-microbe interactions. Each method presents distinct advantages and limitations, and no single approach captures the full complexity of microbiome-fertility relationships. Translating these technologies into fertility clinic practice will require integrating complementary techniques, developing clinically validated biomarkers, and demonstrating improved patient outcomes through rigorous clinical trials. Such advances are essential for establishing evidence-based diagnostics and microbiome-targeted therapeutic strategies for infertility and reproductive disorders.

CRediT Authorship Contribution Statement

Samiya Dash: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Dong Zhao:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Ina Schuppe-Koistinen:** Writing – review & editing. **Juan Du:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Interests

S.D. has nothing to disclose. D.Z. has nothing to disclose. I.S-K. has nothing to disclose. J.D. has nothing to disclose.

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