Screening the vaginal microbiome as a predictive tool in reproductive health

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LAYMAN SUMMARY

Across different environments of the human body exists communities of interacting microbes. These communities are known as ‘microbiomes’. There are many different microbiome sites across the body, including the oral, skin, respiratory, gut and even the underarm microbiome! In healthy individuals, a healthy microbiome is one that is microbially diverse (although this is not always the case, as we will discuss later). Microbiomes typically contain a mixture of bacteria that are ‘good’ and ‘harmful’ to human health. The ‘good’ microbes are symbiotic or commensal organisms which contribute to human health in many different ways, such as vitamin production, enzyme production and preventing the colonisation of pathogens (Rowland *et al.*, 2018). The ‘bad’ microbes consist of pathogens or pathobionts whose harmful effects are typically kept at bay by protective microbes. However, disruptions to the microbiome environment due to illness, dietary changes, hormonal influences, antibiotic usage, etc may create a favourable environment for the harmful microbes, permitting their growth (Zheng *et al.*, 2022).

Though the gut microbiome is the largest and most well documented community, increasing attention is being paid to the microbiome that exists within human vaginas. The vagina is one of the most important organs when it comes to human reproductive and sexual health. Therefore, it is understandable that maintaining a healthy vaginal microbiome is of utmost importance. Although scientists do not yet fully understand the exact make up of a healthy vaginal microbiome, they do have some insights. Unlike other microbiomes in the body which are polymicrobial, a healthy vaginal microbiome is considered as one that has low levels of microbial diversity. Vaginal microbiomes in which *Lactobacillus* species such as *L. crispatus*, *L. iners*, *L. gasseri* or *L. jensenii* are predominant are typically considered to be indicative of a healthy vaginal environment (Jenkins *et al.*, 2023). Together, these bacteria lay the foundation for the five key Community State Types, a collection of well-defined vaginal states describing commonly observed vaginal environments (Ravel *et al.*, 2011). Vaginal microbiomes where these bacteria are not the dominant species are typically termed ‘dysbiotic’ vaginal microbiomes. Dysbiosis of the vaginal microbiome can lead to bacterial vaginosis, a polymicrobial condition associated with vaginal discomfort and adverse health risks such as preterm birth, sexually transmitted infections, fertility issues and even cancer. In reproduction, people with dysbiotic vaginal microbiomes often have more trouble trying to conceive and as a result, many people presenting to assisted reproduction clinics have vaginal dysbiosis (Babu *et al.*, 2017).

Taken together, these factors have provoked researchers to look for a method to examine or screen the vaginal microbiome in order to identify risk factors associated with the aforementioned conditions or issues. Identification of bacterial species or strains at are antagonists of human reproductive and sexual health would give researchers and clinicians the upper hand in preventing and managing disease and fertility issues. For example, the identification of *Prevotella* species as facilitators of HIV-1 infections in dysbiotic vaginas could allow doctors to screen and identify individuals who are at high risk of acquiring this infection (Van Teijlingen *et al.*, 2022). Additionally, researchers have begun to unveil a key bacterial genetic signature that has a high association with preterm births (Nori *et al.*, 2023). The full characterisation of this signature would permit obstetricians and gynaecologists to put suitable interventions in place to monitor the health of expectant mothers whose vaginal microbiomes contain this signature in order to best prevent avoidable infant mortality. Overall, although research in this area is relatively novel, the vaginal microbiome is a very promising site for screening. If fully actualised, using the vaginal microbiome as a predictive tool in healthcare may save or improve countless lives and improve our understanding of the female reproductive tract.

ACRONYMS

AI: Artificial Intelligence
BV: Bacterial Vaginosis
BMI: Body Mass Index
CST: Community State Type
EMMPRIN: Extracellular matrix metalloproteinase inducer membrane protein
FMT: Faecal microbiota transplant
GSM: Genitourinary syndrome of menopause
HIV-1: Human Immunodeficiency Virus 1.
IVF: In vitro fertilisation.
MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionisation-Time of Flight mass spectrometry
mgCST: Metagenomic Community State Type
PICRUSt2: Phylogenetic Investigation of Communties by Reconstruction of Unobserved States
RIF: Recurrent Implantation Failure
STI: sexually transmitted infection
Treg: T-regulatory cell

ABSTRACT

The vagina is an organ of utmost importance in reproductive and sexual health. Its health is largely determined by the status of the niche microbial community that lives within it: the vaginal microbiome. A vaginal microbiome dominated by vaginal *Lactobacillus* species is typically considered an indicator of good health, while deviations from this dogma are considered unstable and a marker for poor health, typically leading to a diagnosis of bacterial vaginosis. A dysbiotic vaginal microbiome has also been associated with other conditions such as infertility, preterm birth, genitourinary syndrome of menopause, etc. Until recently, data on the constitution of the vaginal microbiome has been limited and the true impact of this microbial community on human health was underappreciated. In recent years, as the impact of the gut microbiome on human health has been unravelled and shown promising clinical applications, the vaginal microbiome has now piqued the interest of researchers and clinicians. Compared to other sites of the reproductive tract, the vaginal microbiome can be easily sampled and thus screened, making it a desirable testing target. This raises questions as to the predictive capacity of vaginal microbiome data. Identifying screenable biomarkers within the human vaginal microbiome would be pertinent to human reproductive and sexual health, creating new avenues of intervention for assisted

reproduction, disease prevention and management, reduction of infant mortality, etc. This calls for an in-depth analysis of the feasibility of using the vaginal microbiome as a predictive tool in healthcare by analysing past and current trends from bench to bedside and examining where and how it could be applied, which is the focus of this review.

Key terms: vaginal microbiome, bacterial vaginosis, preterm birth, infertility, *Lactobacillus crispatus*, CST, reproductive health, sexual health.

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1. INTRODUCTION

What makes us human and what exactly are we made of? This is the age-old question that the global multidisciplinary team involved in The Human Genome project thought they had answered upon publishing the human genetic code. However, in recent years, studies on the microbiome have made it clear that the key to gaining a full understanding about human health and disease does not merely lie within our own genetic code. Now, it is virtually impossible to ignore the impact that the microbial communities that occupy various niches across our bodies have on human lives. When considering human disease, it is vital not just to look at our own genetics, but also to the genetics of our microbial compatriots who have co-evolved alongside humans. Indeed, some researchers would go as far to suggest the human microbiome as being “the second human genome” (Relman and Falkow, 2001). Thus far, major milestones in human microbiota research have largely focused on the gut microbiome. Nevertheless, increasing attention has been recently paid to the vaginal microbiome. The human vagina is a hugely important organ in the field of sexual and reproductive care. The health of this organ has far reaching influences on fertility, acquisition of sexually transmitted infections (STIs), miscarriage, preterm delivery, etc (Jašarević *et al.*, 2018; Feehily *et al.*, 2020; Schoenmakers and Laven, 2020). However, making comparisons between the intestinal and vaginal niche is rather complex. Unlike the gut microbiome, where diversity is associated with a plethora of health benefits, microbial diversity is seen as a marker for poor vaginal health. The health of a vagina is typically considered as falling into one of five major community state types (CSTs), based on the presence and predominance of key vaginal bacterial species. Despite this, there is a lack of agreement to the true definition of a healthy vaginal microbiome, thus limiting the strength of using vaginal microbiome data as a tool in healthcare. Indeed, the accuracy of defining vaginal health in line with these CSTs has been a source of debate in recent years.

It is understandable that defining a ‘healthy’ vaginal microbiome would seem to prove invaluable to clinicians. The presence of microbes in the vagina was first documented at the end of the 19th century by German scientist Albert Döderlein, who identified what is now known to be *Lactobacillus* (Döderlein, 1892). Despite this, it wouldn’t be until over a century later that attempts would be made to characterise the vaginal microbiome. The Human Microbiome project defines a healthy microbiome as one that is free of disease. In this respect, a healthy vaginal microbiome is generally viewed as one that is not in a state of dysbiosis and is *Lactobacillus* dominated (The Integrative HMP (iHMP) Research Network Consortium, 2019). However, there is much debate surrounding the language used by microbiome researchers, particularly with the word ‘dysbiosis’. Dysbiosis is a rather nonspecific description which may invoke bias. Its prefix ‘dys’, originating from the Greek word for ‘bad’, may falsely imply preexisting, in-depth knowledge of the state of a healthy microbiome (Shanahan and Hill, 2019). Accordingly, defining the health of an individual's vaginal microbiome solely on the basis of conventional CSTs and the presence or absence of specific species may be a harmful overgeneralization that fails to fully recognise the contribution of vaginal microbes in human health and disease. By assuming that non-dysbiotic states are inherently indicative of good health, it is easy to falsely assume that microbial species present in low abundance are unimportant. It may also cause the vaginal microbiome of people with a CST associated with good health to be overlooked in the clinic. This begs the question of whether the field of sexual and reproductive health has been significantly limited by so stringently defining the health of the vaginal microbiome. Furthermore, the long-standing notion that the vaginal microbiome is lacking diversity and is predominantly monomicrobial may also be holding progression in this field back. It is becoming increasingly evident that the vaginal microbiome is richer than may have been originally appreciated. Thanks to advances in sequencing techniques and culturomics, the vaginal microbial repertoire has expanded significantly in recent years, demonstrating that even a ‘healthy’ vaginal microbiome boasts varying levels of diversity (Chacra *et al.*, 2024). This review will consider if it is time to move away from defining vaginal health in terms of species composition and shift towards defining it in terms of genetic composition and diversity. It will also investigate the predictive potential of vaginal microbiome data and the clinical relevance this may have.

2. THE VAGINAL MICROBIOME

2.1. The human vaginal microbiome

The human vaginal microbiome is associated with the predominance of *Lactobacilli*. *Lactobacillus* species are crucial in establishing a first line of defence against infection and disease in the human vagina. As oestrogen levels increase during the human reproductive cycle, so do the levels of glycogen in vaginal epithelial cells. Upon epithelial cell lysis, it is thought that this glycogen is broken down into smaller sugar units by α-amylase enzymes found in the vaginal tract, though recent studies have suggested that enzymes produced by the vaginal microbiota may also contribute to this process (Jenkins *et al.*, 2023). These in turn are metabolised by *Lactobacilli* to produce lactic acid in the form of one of two isomers; D-lactic acid and/or L-lactic acid. While L-lactic acid can also be produced by vaginal epithelial cells, D-lactic acid is only produced by bacteria, though not all species of *Lactobacillus* can produce both isomers (Witkin *et al.*, 2013). Lactic acid production helps to maintain the acidic pH of the vaginal environment that restricts the growth of invasive organisms. In addition, the anti-inflammatory properties of lactic acid helps to maintain vaginal health by stimulating the production of IL-1RA, which is an antagonist of the IL-1R receptor that mediates the signalling response of proinflammatory cytokines such as interleukin-1(Hearps *et al.*, 2017). Pathogen growth is also limited by competitive binding by *Lactobacilli* to epithelial cells as well as their ability to produce various anti-microbial compounds such as bacteriocins. Should the protective qualities of *Lactobacilli* be absent or inadequate, the vaginal environment may shift away from a healthy, lowly diverse state towards a dysbiotic, polymicrobial state. This may ultimately lead to fluctuations in species concentrations in favour of non-*Lactobacillus* species, a state typically termed as bacterial vaginosis (BV) and subsequently vaginal discomfort. Typically, BV is treated using antibiotics, namely metronidazole, but with only limited success. Over 50% of BV patients treated with antibiotics experience a reinfection within one year (Verwijs *et al.*, 2020). Though the exact aetiology of recurrence is not fully understood, it has been suggested that antibiotic treatment promotes *Lactobacillus iners* predominance of the post-treatment vagina rather than the more protective *Lactobacillus crispatus* (Joag *et al.*, 2019). Because of this, there remains controversy as to whether or not asymptomatic BV requires treatment. Additional research is required in this area in order to effectively promote *L. crispatus* predominance after antibiotic treatments.

One of the most pivotal studies in vaginal microbiota research is that of Ravel *et al.*, 2011 in which different vaginal communities of North American women from four major

ethnic groups (White, Black, Hispanic and Asian) were sampled and characterised. From sequencing of the 16S rRNA gene, vaginal communities were grouped into one of five different community state types (CSTs); CST1 through CST5. CST1, CST2, CST3 and CST5 are dominated by *Lactobacillus* species; *L. crispatus, L. gasseri, L. iners* and *L. jensenii* respectively (Figure 1.). CST4 is the only CST not associated with *Lactobacilli* dominance and contains the highest level of microbial diversity, higher pH levels and higher concentrations of strictly anaerobic bacterial species. People with a CST4 vaginal microbiome typically possess bacterial species associated with BV and thus this CST is largely regarded as resembling a state of dysbiosis. Despite this, it is apparent that CST4 is present in a large proportion of the population with asymptomatic BV, particularly Black and Hispanic populations.

How is it that people with a clinically dysbiotic vaginal microbiome are seemingly healthy, while others are not and experience symptomatic BV? Contemporary studies by the same group may provide some clarity. Analysis of vaginal microbiome metagenomic and metatranscriptomic datasets have revealed and defined the existence of bacterial subspecies (Ma *et al.*, 2020). These are defined as clusters of bacterial strains with shared gene functions between different vaginal samples. Metagenomic subspecies are composed of a variation of species-specific genes with unique functionality. These are used to define metagenomic CSTs (mgCSTs). This approach of characterising the vaginal microbiome helps to explain the difference in disease states in people with a CST4 state vaginal microbiome. For example, *L. iners* dominance has historically been considered an indicator for increased BV risk. However, in a subsequent study, one *L. iners* metagenomic subspecies was demonstrated as being associated with increased BV risk, while another *L. iners* metagenomic subspecies in the same study was negatively associated with a BV diagnosis. (Holm *et al.*, 2023). The differences in CST4 health statuses can further be explained by the range of *G. vaginalis* diversity revealed in this study. *G. vaginalis* is one of the hallmark species associated with BV (Borgdorff *et al.*, 2017). However, of the six *Gardnerella*-mgCSTs described in this study, only three had a significant association to a BV diagnosis. Consequently, it has been recently proposed to further develop CSTs into 27 different mgCSTs, which are unique associations of these metagenomic subspecies. This allows for a deeper qualitative description of vaginal microbiomes and has the potential to improve the predictive capacity of vaginal microbiome data in human health and disease.

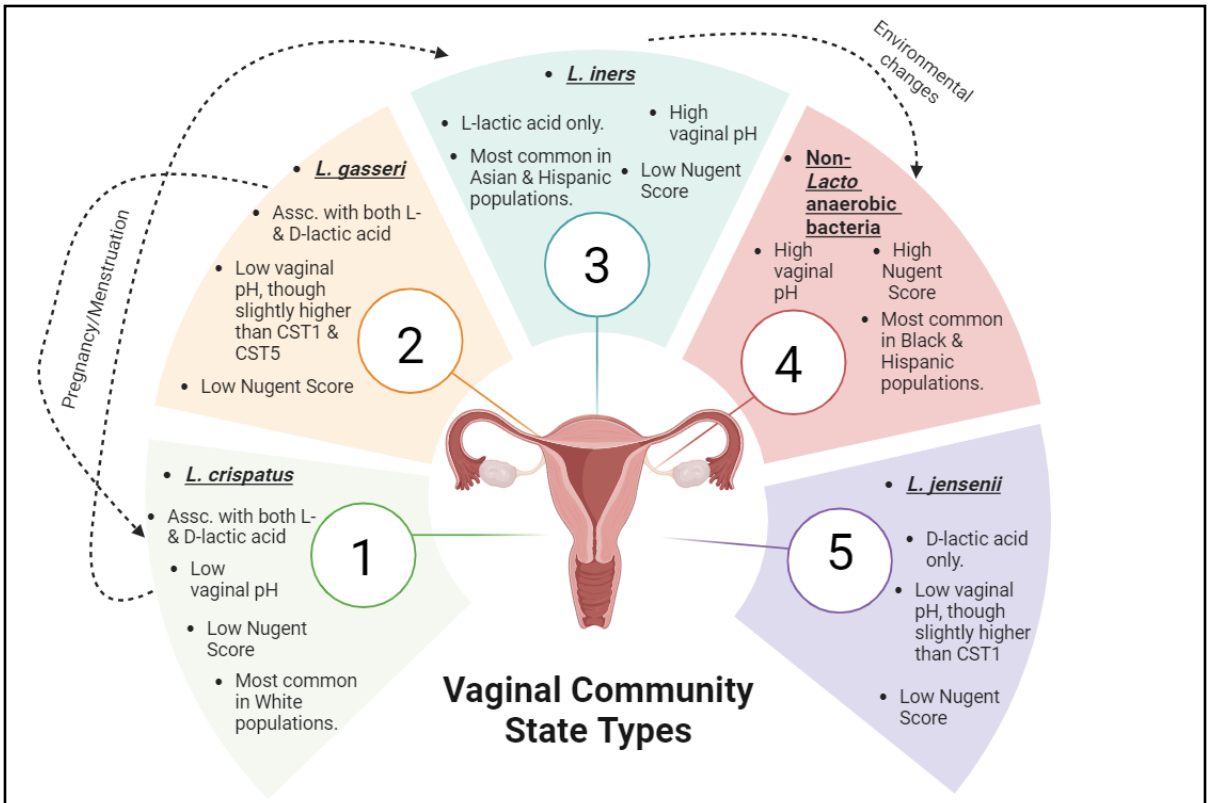

Figure 1: Features of the five primary vaginal community state types (CSTs) of reproductive aged people as outlined by Ravel et al., 2011. Most common and defining bacterial species of each state type are in bold text and underlined. Dotted lines represent some of the most common transitional changes of CSTs. While other microbial species may be present in each state type, they are not used as a criteria basis for defining CSTs. Figure created by the author using *biorender.com.*

2.2. The evolution of the vaginal microbiome

Amongst primates, the human vaginal environment is unique. Humans have the most acidic vaginal environment of all the primates. Humans also exhibit far less vaginal microbial diversity than non-human primate species (Yildirim *et al.*, 2014). Of all primate species studied, humans were surprisingly the only species to exhibit *Lactobacillus* dominance. Even the vaginal microbiomes of our closest relatives, chimpanzees, only have a relative concentration of <3.5% *Lactobacilli* species compared to a range of 60-93% in human populations. As a result, the use of animal models for comparative studies of the healthy human vaginal microbiome is not straightforward and is thus limited. In spite of this, it is evident that the vaginal microbiome of humans has robustly evolved and adapted to complement human life, and several theories have been put forward in an attempt to explain the unique microbial landscape of the human vagina. These include: adaptations to accommodate the fluctuating oestrogen levels of the human ovarian cycle, an increased risk of STDs, the higher risk associated with human pregnancy and delivery, as well as, the starch-rich diet of humans contributing to our higher vaginal glycogen levels (Miller *et al.*, 2016). To date, there is still much debate within the scientific community as to what the leading hypothesis is, though the ‘diet’ theory and the ‘common-function’ theory are exciting

leads for future research. The latter theory proposes that *Lactobacilli* dominance is not universal across primates because vaginal protection may also be provided by other bacteria via varying mechanisms, thus suggesting that the presence of *Lactobacillus* is not necessarily essential for a healthy vagina. An argument supporting this theory is that during clustering analysis, human samples taken from patients with both symptomatic and asymptomatic bacterial vaginosis (BV) showed greater similarity to samples from non-human primates than other infection-free human samples (Yildirim *et al.*, 2014). Regardless, both theories require further research but offer an exciting line of investigation into improving our understanding of the vaginal microbiota.

3. SCREENING THE VAGINAL MICROBIOME.

3.1. Traditional Analytic Techniques.

In order to harness the predictive potential of the vaginal microbiome on human health, its components must be thoroughly analysed and screened. Traditionally, the vaginal microbiome was analysed using techniques such as wet mount microscopy and Gram-staining, both used in the clinical diagnosis of BV. Clinically, patients with vaginal dysbiosis experiencing three out of four 'Amsel criteria' can be diagnosed with symptomatic BV (Amsel *et al.*, 1983), which are as follows: abnormal vaginal discharge, a vaginal pH higher than 4.5, vaginal odour and the presence of clue cells on a microscopic wet mount test. Asymptomatic BV is generally considered to be the absence of symptoms that would contribute to discomfort such as odour and/or abnormal discharge. BV can also be defined via Gram staining of vaginal swabs viewed on a microscopic slide on the basis of Nugent scoring (Nugent *et al.*, 1991). Nugent scoring uses a zero to ten scale system which assigns a score based on bacterial cell morphology. A total score >7 is indicative of a positive BV diagnosis. The latter method is not routinely performed at a clinical level due to the time and resource constraints, though it is still conducted in research studies. Although microscopy can be directly applied to samples to visualise the microbial communities occupying the vagina, it is a vastly limited analytical technique. Such characterisation of the vaginal microbiome is restricted to identifying differences in bacterial cell morphology and thus providing a rather limited description of a microbial community.

3.2 Advancements in Molecular Methods.

Phylogenetic analyses of vaginal microbiota, mainly via sequencing of the highly conserved 16S rRNA gene, has predominated in the field in the last two decades. Despite overcoming the limitations of traditional techniques, the molecular methods initially used in

microbiome research are not without fault. Polymerase chain reaction (PCR) is a powerful tool that can be used to detect causative agents of STIs (Morris *et al.*, 2021) and diagnose BV (Cartwright *et al.*, 2012), although it is largely restricted to only detecting the microbes for which primers were designed and included and thus may allow only a narrow insight into vaginal microbial communities. A vast majority of developments in 16S rRNA sequencing techniques have been made in studies of gut samples, thus warranting questions as to the extent at which this data can be extrapolated to other microbiomes. For example, similar to the primer issues experienced in studies of the vaginal microbiome, studies of respiratory microbiome samples also experience a lack of primer standardisation (López-Aladid *et al.*, 2023). The selection of primer sets can have a significant effect on results, and may offer an explanation for the conflicts in results from different studies of the vaginal microbiome (Graspeuntner *et al.*, 2018).

Developments in high-throughput sequencing techniques has allowed for DNA and RNA taken directly from microbial samples to be analysed at unparalleled levels, providing a far broader and exhaustive description of vaginal microbial communities. Currently, there are three different community sequencing techniques primarily used: amplicon sequencing, shotgun sequencing and metatranscriptomic sequencing (Berman et al., 2020). Amplicon sequencing typically utilises PCR amplification of the 16S rRNA gene of vaginal microbes, and sequences these amplified genes, or 'amplicons', to provide an inventory of the vaginal microbiota present. Shotgun sequencing sequences all of the DNA extracted from a vaginal sample, both human and microbial. It permits an insight beyond what is permissible by amplicon sequencing; it shows which microbial species are present and reveals the functional genetic potential of vaginal microbiota. Metatranscriptomic sequencing sequences all of the metagenomic mRNA extracted from a vaginal sample. It provides a snapshot of the genes being actively transcribed in the vaginal environment, both human and microbial, at the time of sequencing. Although mRNA expression is a more powerful indicator of the functional activity than DNA, metatranscriptomics cannot truly be considered to give a thorough description of a vaginal microbial community as some community members may not be adequately active at the time sampling occurred.

Despite the variety of community sequencing techniques available to researchers, sequencing of the 16S rRNA gene remains the most commonly used in research (Ravel *et al.*, 2011; Borgdorff *et al.*, 2017). The 16S rRNA gene is typically the target of next-generation sequencing platforms (Sroka-Oleksiak *et al.*, 2020). The 16S rRNA gene is a prokaryote-specific molecular marker consisting of highly conserved regions that can be targeted for primer binding during NGS (Figure 2.). Between these conserved regions are

nine hypervariable regions, V1-V9. These hypervariable regions have a species-specific level of sequence diversity, allowing for the identification and characterisation of bacterial species in a sample. The lack of standardisation in microbiome research techniques, in particular in choosing primer pairs during 16S rRNA sequencing and DNA isolation techniques again proves problematic here. Currently, no universal primers are used in the field, resulting in different studies targeting different hypervariable regions of the 16S rRNA gene during sequencing. This affects the ability to accurately compare the findings of different studies. While some studies, including those conducted during the Human Microbiome Project, use primers to target the V1-V3 regions, others target the V3-V4 regions (The Integrative HMP (iHMP) Research Network Consortium, 2019). The impact of these differences can be far from subtle. While primers for the V1-V3 regions can successfully distinguish between different species within the *Lactobacillus* genus, they fail to separate the *Enterobacteriaceae* from other genera and may underestimate the presence of genera such as *Staphylococcus* (Sroka-Oleksiak *et al.*, 2020). Recent endeavours have been made to establish a standard primer set for 16S rRNA gene sequencing. Primers targeting the V4 region proved to capture high levels of microbial diversity (Van Der Pol *et al.*, 2019), although this may be done at the expense of accurately differentiating between *Lactobacillus* species (Qing *et al.*, 2024). Primers targeting the V3-V4 region seem superior to those targeting the V1-V2 region in accurately identifying taxa, diversity and detecting pathogens (Graspeuntner *et al.*, 2018).

While 16S rRNA gene based sequencing fails to specify direct data on the functional capabilities of the bacterial species present, this technique remains in favour due to its cost effectiveness. To overcome this shortfall, 16S rRNA amplicon sequencing is typically used in combination with computational tools in order to perform functional analysis on a sample. Programs such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) use large databases of gene families and reference genomes to predict the functionality of bacterial communities from any 16S rRNA sequence (Douglas *et al.*, 2020). This technique, however, is not without its shortcomings. The reliability of programs such as PICRUSt2 are dependent on the quality of the reference databases they use and are restricted by their completeness, which in turn creates difficulty in detecting discrete strain specific differences. Additionally, these programs currently are not suitable to detect subtle health related differences from functional data when tested using real-world data (Matchado *et al.*, 2024).

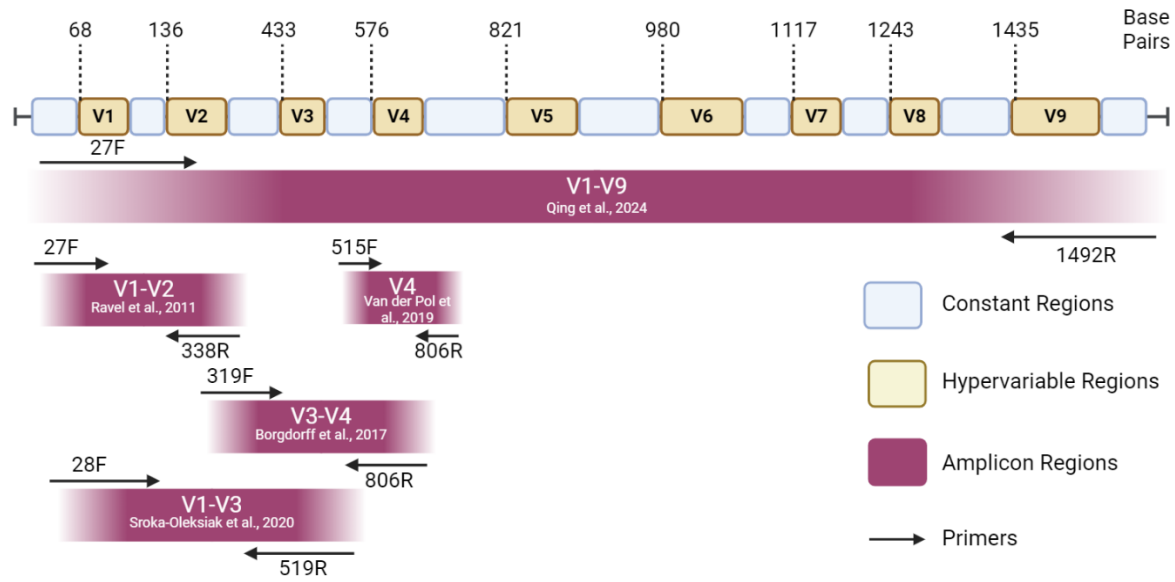

Figure 2: The bacterial 16S rRNA gene consists of nine hypervariable regions (*in orange*) flanked by constant regions (*in blue*), making it an ideal target for amplification and sequencing. Some of the most commonly used amplicons of the 16S rRNA gene and their associated primer targets have been highlighted (*in purple*), as described by the authors of the studies mentioned in the figure. Figure created by the author using *biorender.com*

3.3. Sample Collection and Technical Bias.

The majority of studies on the vaginal microbiome have addressed how microbial composition has varied between samples, without necessarily scrutinising the exact source of this variation. Variation in data between studies of the vaginal microbiome can be externally introduced at any stage of the research pipeline, even from as early on as sample collection. The full potential of 16S rRNA sequencing in community sequencing is highly dependent on how the samples are processed. Although easy to acquire, vaginal microbiota sample collection lacks a gold standard (Schoenmakers and Laven, 2020). Factors such as sampling frequency, storage methods, choice of collection tools, etc can introduce technical biases which may impede comparability between studies. Oxygen exposure may trigger cellular death in strictly anaerobic bacterial species while multiple sampling events may create difficulties in sample comparison due to fluctuations in levels of diversity at different time points (Sharma *et al.*, 2021).

Recently, several attempts have been made to address these constraints. In one pilot study, researchers proposed the use of either AssayAssure Genelock (a commonly used urine sample buffer) or 95% ethanol for the effective preservation of vaginal swab samples (Kumar *et al.*, 2024). They also determined that the composition of the vaginal microbiome is only minimally affected by consecutive sampling, with no significant

differences in β-diversity between original and successive swabs. Another study aimed to simplify vaginal sampling methods showed that the cold chain step associated with Copan swabs, a commonly used vaginal swabbing device, was redundant (Kero *et al.*, 2023). Samples taken using Puritan swabbing, which used shield fluid reagents allowing samples to be stored at room temperature, actually resulted in higher DNA yields and comparable alpha and beta diversity measurements, without the need for a cold chain. This would be a major cost benefit both in the clinic and academia. Vaginal samples can be taken using fewer resources and transported further, for less. Further research using larger study cohorts testing are needed in order to develop on these findings and to standardise vaginal sample collection techniques.

Altogether, these novel findings further promote opportunities for potential at-home sample collection, thus allowing the benefits of vaginal microbiome screening to be accessible to a wider demographic than would otherwise be possible. Screening the vaginal microbiome for health applications requires patient participation. Therefore, patient acceptance levels should be taken into account when considering which sample collections to use. Self-reported patient 'embarrassment' is a factor proven to cause many eligible people to miss their cervical-screening appointments in the United Kingdom (Logan and Mcilfatrick, 2011). The potentials to expand on at-home sampling, as well as, the introduction of novel collection methods, such as the use of menstrual tampons (Turner *et al.*, 2023) and menstrual cups (Short *et al.*, 2023), can be argued in favour of feasibly adopting vaginal microbiome screening into routine sexual and reproductive health practices.

3.4. Reemergence of culturing methods.

Historically, studies into the microbiome relied on the cultivation of microbes directly from samples. However, microbiome research, and indeed the whole field of microbiology has been restricted by the inability to culture the vast majority of known microbes (Steen *et al.*, 2019). As advancements were made in molecular techniques, particularly metagenomics, it was believed that culturing was to become superfluous. Nonetheless, the limitations of metagenomics soon became apparent. The power of metagenomics as a characterisation technique is reduced by several factors including: its depth bias, its inability to provide information on bacteria viability, its reliance on oftentime incomplete genetic databases as well as its failure to produce bacterial strains for further downstream analysis. Thus, interest has been regenerated in the reemergence of culture, particularly in the culturing of bacteria that have so far only been characterised on a molecular level. Refinement of past culture methods and the development of new techniques is essential if

the vaginal microbiome is to be comprehensively analysed. Culturomics was developed in order to address this desire. Culturomics is a technique that combines a wide variety of culture conditions with a high-throughput microbial identification methodology, typically via Matrix-Assisted Laser Desorption Ionisation-Time of Flight mass spectrometry (MALDI-TOF MS) (Lagier *et al.*, 2012). MALDI-TOF MS is a highly sensitive tool that generates a species-specific mass spectrum fingerprint based on the detection of bacterial ribosomal proteins and peptides.

Culturomics has exposed the gaps in the known human microbial repertoire. When comparing the analyses of stool samples by 16S rRNA amplicon sequencing to culturomics. Lagier et al. noted dramatic differences between the two techniques. Only 15% of the total bacterial strains identified via culturomics were also identifiable via sequencing alone (Lagier *et al.*, 2012). This discrepancy may be due to the fact that culturomic techniques are far less impacted by depth bias than sequencing and can detect microorganisms present at far lower concentrations than is possible with sequencing alone. The revival of culture in vaginal microbiome studies has allowed us to view beyond the surface of the microbial world and allows us to further challenge the low diversity dogma of the healthy vaginal microbiome. Indeed, a recent study using culturomics isolated 206 bacterial species from the vagina of a single healthy human patient, 65 of which had never previously been isolated from human vaginas, including one novel bacterial species, *Porphyromonas vaginalis* (Chacra *et al.*, 2024). The advent of culturomics will surely revolutionise studies of the vaginal microbiome, and provide further support to the theory that a healthy human vagina is far more polymicrobial than previously thought.

Although culturomics combined with MALDI-TOF MS grants researchers the ability to study the vaginal microbiome at a depth previously not possible, its clinical applications are limited due to the burdensome techniques involved. Culturomics requires extensive culture conditions and resources to isolate and accurately identify the full spectrum of microbial species present in a sample, making it a rather time-consuming and labour-intensive process. A failure to include specific sets of growth conditions may lead to certain species remaining undetected. Despite these problems, the MALDI-TOF MS approach to the identification of bacteria in a sample has been shown to decrease costs five-fold (Lagier *et al.*, 2012). Although typically restricted to the analyses of single colonies, advancements have been made in mass spectra fingerprint analyses and machine learning applications to facilitate the characterisation of whole microbiota (Chen *et al.*, 2023). Classification of these microbiota mass spectra fingerprint profiles was found to be highly reproducible and MALDI-TOF MS could competently characterise different microbiota profiles. As knowledge of the

vaginal microbiome increases and our understanding of key players which contribute to human disease expands, MALDI-TOF MS represents a promising technique for the clinical prediction of disease and health risks using the vaginal microbiome.

4. THE VAGINAL MICROBIOME IN REPRODUCTIVE HEALTH.

The vaginal microbiome plays an undeniable role in the sexual and reproductive health of humans. As previously mentioned, a vaginal microbiome dominated by *Lactobacillus* has been regarded as a marker for prime vaginal health due to the protective qualities associated with *Lactobacillus* vaginal colonisation. In recent years, our understanding of normalcy has begun to shift, with various studies reporting dysbiosis in otherwise asymptomatic, healthy people. A Dutch study revealed the prevalence of dysbiosis in reproductive-age people to be as high as 38.5% (Borgdorff *et al.*, 2017). As a result, it may be time to reconsider the rigid association of a loss of *Lactobacilli* dominance with an unhealthy vaginal microbiome. It has been made evident from multiple studies that species dominance of vaginal microbiomes varies between different ethnic and biogeographical groups (Ravel *et al.*, 2011; Borgdorff *et al.*, 2017; Callahan *et al.*, 2017; Holm *et al.*, 2023). Although how a normal and healthy human vaginal microbiome is defined remains up for debate, what is clear is that the composition of vaginal microbial communities can contribute to human disease and welfare, and that the maintenance of a healthy vaginal microbiome is especially crucial in the sexual and reproductive health of humans. The vaginal microbiome is of additional interest in reproductive health as it is considered one of the easiest microbiomes to obtain contamination-free samples from (Schoenmakers and Laven, 2020) and thus more permissible for screening.

4.1. Infertility and Assisted Reproduction.

According to the World Health Organisation, infertility is an issue affecting around 17.5% of the global adult population (Cox *et al.*, 2022). In an analysis of global trends in fertility, fertility rates have declined in all 204 countries and territories surveyed, with future fertility rates projected to fall below the replacement-rate in 76% of nations by 2050 (Bhattacharjee *et al.*, 2024). This is due to a variety of factors such as increased access to contraceptives, a lack of pro-natal policies and an increase in the mean childbearing age. This has led to an increased demand for assisted reproductive technology, one of the most popular methods being in vitro fertilisation (IVF) (The European IVF-monitoring Consortium (EIM)‡ for the European Society of Human Reproduction and Embryology (ESHRE) *et al.*, 2020). Although the advent of IVF revolutionised human infertility treatment and

reproduction, the financial associations of this technology can have major implications. Economically, infertility is estimated to cost policy makers on average 70 million euro per 10,000 women (Bourrion *et al.*, 2022). The cost association of assisted reproduction stretches far beyond actual expenses and affordability. The perceived financial burden of undergoing fertility treatment can lead to a decrease in quality of life and can contribute to diminished levels of sexual satisfaction between couples, regardless of household income (Allsop *et al.*, 2023). Therefore, there is a growing requirement for strategies to determine optimal IVF success which in turn will help to limit the cost of assisted reproduction. Subsequently, there has been increased interest in studying the vaginal microbiome and whether or not there lies an association between its composition and IVF success rates. Such an association would grant clinicians the use of vaginal microbiome data as a predictive tool for this often costly procedure and may help to increase IVF-related pregnancy rates.

4.2. The Vaginal Microbiome as a Biomarker for IVF Success.

The role of vaginal microbiota as a potential biomarker for IVF success has received more attention in recent years. At the genus level, it has been shown that IVF pregnancy rates are associated with the presence of *Lactobacillus* along with a decreased abundance of pathobionts such as *Gardenerella*, *Prevotella*, etc (Kong *et al.*, 2020). Although research in this area is novel, it shows promise. Using Nugent scoring as a reference for setting threshold levels, a Danish study defined an ‘abnormal’ vaginal microbiome as one that had high bacterial loads of the pathobionts *Atopobium vaginae* and *Gardnerella vaginalis* (Haahr *et al.*, 2016). When employing BV as a measure of abnormality, 21% of IVF patients in the group were determined to have an abnormal vaginal microbiome. However, if qPCR was used to detect relative loads of pathobionts and *Lactobacillus* species, this figure rose to 28%. Furthermore, only 9% of people with a qPCR-defined abnormal vaginal microbiome went on to achieve pregnancy, which highlights the importance of a healthy vaginal microbiome in reproductive success. When these researchers further studied the vaginal microbiome of IVF patients using high-throughput 16S rRNA sequencing, they found no significant association between CSTs and pregnancy success (Haahr *et al.*, 2019). Though 16S rRNA sequencing allowed more in-depth analysis of the vaginal microbiota, it failed to outperform qPCR as a means of defining and detecting an abnormal vaginal microbiome. These results point to the potential of vaginal microbiome screening via qPCR as a method to enhance IVF success, especially considering that the more modest and cost-effective qPCR methodology outperformed high-throughput sequencing in screening capacity. Corroboration of these results in larger scale studies would give clinicians a very practicable

strategy to pre-screen IVF patients for vaginal microbiome abnormalities in order to enhance the success of their treatment.

4.3. The impact of vaginal dysbiosis.

The exact causes of infertility remain elusive and thus neither BV nor dysbiosis can be fully considered a direct cause of infertility. It remains difficult to determine whether infertility arises due to a dysbiotic vaginal microbiome or whether dysbiosis is a result of infertility. In one study, people presenting to clinics with fertility issues were more likely to have asymptomatic BV than people without fertility issues and were less likely to have a *Lactobacillus* dominated vaginal microbiome (Babu *et al.*, 2017). However, utilising BV as a predictive tool for fertility and pregnancy success, as the aforementioned study suggests, may be a misguided approach, as other factors such as genetic predisposition may have a larger influence. Furthermore, recent reports opposingly concluded that a person's BV status or vaginal CST had no significant effect on their ability to conceive (Van Den Tweel *et al.*, 2024).

The lack of consensus between these studies here is a perfect example of the consequences of a lack of standardisation in the field. The former study solely relied on culturing methods and microscopy to detect BV while the latter used molecular methods such as qPCR and 16S rRNA sequencing. While it may be expected that different methodologies will vary in accuracy, the relevance of these differences can be debated. The ability of each method to detect BV is relatively similar. Meta-analysis of infertility and IVF research revealed that studies relying on microscopy detected BV in 17% of infertile IVF patients compared to an average of 19% when molecular methods were used (Skafte-Holm *et al.*, 2021). In conclusion, the overall levels of variation in reported BV levels amongst studies further emphasises the unsuitability of using BV status alone as a predictor for IVF outcomes.

There are many documented cases of vaginal community members who appear phenotypically identical but differ at minute sequence levels and therefore exhibit different functional capacities. Additionally, there is evidence that the host's vaginal environment may select for strains with these sequence variations as these differences may offer functional advantages to these bacteria. Vaginal dysbiosis can be coupled with a pro-inflammatory response and thus a dysbiotic vagina is a niche under immunological pressure (Borgdorff *et al.*, 2017). As a result, some bacterial species or subspecies may utilise immune evasion mechanisms as a survival strategy which aids their growth and colonisation during dysbiosis.

A recent study analysed genetic differences in *L. jensenii* strains isolated from preterm birth (PTB) and full term birth (FTB) samples and identified a PTB genetic signature in certain *L. jensenii* strains (Nori *et al.*, 2023). They recorded two gene clusters that were most commonly found in *L. jensenii* strains isolated from PTB samples. One cluster contained ten genes with predicted roles in biosynthesis of cell surface glycans which may allow for immune evasion. The second gene cluster was deemed to have a function in carbon-source utilisation. Typically, bacteria of the vaginal microbiome have been considered to indirectly utilise host epithelial cell glycogen as an energy source. However, evidence from this study suggests direct utilisation. This second gene cluster contained two genes with unknown functions. A homology search revealed their closest homologs to be α- and β-amylase genes respectively. This cluster contained genes encoding an α-rhamnosidase which is involved in the hydrolysis of polysaccharides. In addition, this cluster was theorised to permit adhesion of *L. jensenii* to cervical mucins found in the vagina and allow for the utilisation of these mucins as a carbon source. This supports previous findings showing that breaches to the structural integrity of cervical mucus are a common feature in people who experience PTB (Critchfield *et al.*, 2013). Additionally, the preterm *L. jensenii* strains contained variations in the *ldhD* gene involved in the production of the D-lactate isomer. The gene clusters carried by the preterm *L. jensenii* strains provides them with a unique ability to directly utilise host sugars and alter their cell surface proteins as a means to surviving a dysbiotic vaginal environment. A separate study also noted *L. crispatus* strains isolated from healthy vaginal microbiome samples that had genetic, but not phenotypic, differences from strains isolated from dysbiotic vaginal microbiome samples (Van Der Veer *et al.*, 2019). Strains isolated from dysbiotic samples were more likely to possess gene fragments associated with phase variation and thus immune evasion than strains from healthy microbiomes. Such variation potentially allows *L. crispatus* to survive at low abundances during dysbiosis. This study was also the first to provide evidence that *L. crispatus* can grow on extracellular glycogen due to the presence of a pullanase type I gene (*pulA*). Strains exhibiting poor growth on glycogen all contained amino acid deletions within the N-terminal sequence of this gene.

Together, these studies show that simply looking at the microbial composition and relative frequencies of vaginal microbiome community members serve as a poor biomarker for vaginal health, given the intricate yet functionally relevant differences that can be present between strains of species we would typically consider markers of good health. The identification of these genetic signatures associated with adverse health outcomes such as PTB serve as interesting lines of inquiries for the identification of biomarkers and could allow for the development of cost-effective PCR strategies to identify risk-associated strains, as has been previously demonstrated to monitor *Lactobacillus* species in probiotic and dairy

products (Kim *et al.*, 2021). Validation of the existence of *L. crispatus* strains that can directly utilise glycogen and even alter their glycome also opens the potential for the development of new probiotics. These strains have a competitive survival advantage over other *L. crispatus* strains. Van der Veer, et al. revealed during the course of their research that people undergoing metronidazole treatment for BV who positively responded to treatment and had a *L. crispatus* dominated microbiome post-treatment contained the gene fragments associated to phase variation (Van Der Veer *et al.*, 2019). This suggests that the vaginal microbiome could be used to predict responsiveness to antibiotic treatment for BV and that further insights into strain specific variations that promote colonisation and persistence could pave the way for the development of new treatments and interventions.

4.4. The Vaginal Microbiome's role in Pregnancy.

One role vaginal microbiota may play in fertility is via their impact on blastocyst implantation in the endometrium. Once considered a sterile site, the endometrium is now increasingly believed to be colonised by bacteria from the vagina and cervix which are able to ascend up into the uterine cavity (Mitchell *et al.*, 2015). During early pregnancy, a mild level of endometrial inflammation is required to promote proper blastocyst implantation into the endometrial tissue and to activate tissue repair mechanisms. During this process, the Th1/Th2 balance in the endometrium shifts towards Th1 dominance which is coupled with the production of proinflammatory cytokines. After implantation has occurred, the balance shifts back towards Th2 dominance in order to promote an anti-inflammatory environment promoting growth of the placenta and the developing foetus. Patients suffering from recurrent implantation failure (RIF), which is characterised by a series of unsuccessful implantations after numerous embryo transfers, have significantly higher levels of α-diversity compared to healthy controls (Fu *et al.*, 2020), and increased abundances of BV-associated bacteria (Haahr *et al.*, 2019; Fu *et al.*, 2020; Kong *et al.*, 2020). People with RIF also have lower abundances of *Lactobacillus* compared to IVF patients with successful implantations (Diaz-Martínez *et al.*, 2021). One early study showed that embryo transfer tips coated in *Lactobacillus* increased the likelihood of live-births in people undergoing IVF treatment (Moore *et al.*, 2000). Thus, it seems likely that a *Lactobacillus* dominated vagina prior to and during implantation could predict a higher chance of success. One theory to support this claim is that the presence of *Lactobacillus* helps promote a tolerogenic environment for the blastocyst. In the gut microbiome, the presence of antigen-specific T-regulatory cells (Tregs) has been documented, allowing for immune tolerance to certain gut commensals (Lathrop *et al.*, 2011). These Tregs are selected for and regulated by the commensals in order to promote a favourable environment that prevents the elimination of these symbionts.

Therefore, it is plausible that *Lactobacillus* helps promote a favourable immunological environment in the lower and upper reproductive tract. A diminishment in *Lactobacillus* abundance due to the overgrowth of pathobionts may thus disrupt the delicate Th1/Th2 balance of the implantation process. This could cause the maternal innate immune system to reject the blastocyst, resulting in implantation failure or spontaneous early abortions. The impact of the vaginal microbiome on maternal immune tolerance to the implanting blastocyst requires further investigation in order to fully open the potential of vaginal microbiome screening or manipulation as an intervention for RIF and a strategy to optimise IVF success.

The predictive capacity of the vaginal microbiome on IVF success has been previously demonstrated. In a study cohort undergoing fresh embryo transfers, low *Lactobacilli* dominance was predictive of low embryo transfer success, with a predictive accuracy of 94% (Koedooder *et al.*, 2019). Using vaginal microbiome data in this way will be pivotal in increasing the success of assisted reproduction technology and will allow those undergoing such procedures to make better, more informed decisions before proceeding with treatment.

Complications arising from PTB are the leading cause of mortality in infants under the age of five (Perin *et al.*, 2022). PTB often occurs as a consequence of poor reproductive tract health. In a clinical setting, medical professionals regard a person with a CST-4 type vaginal microbiome as having poor vaginal health due to this state type's association with BV. This in turn is associated with an increased risk for PTB. However, a notable cohort of seemingly 'healthy' individuals with a CST-4 vaginal microbiome has been described (Ravel *et al.*, 2011; Babu *et al.*, 2017). While some studies have linked CST-4 to PTB (DiGiulio *et al.*, 2015), others have failed to determine a clear association between vaginal CSTs and the risk of PTB (Feehily *et al.*, 2020). The apparent difficulty to relate CSTs to PTB and the lack of consensus between studies suggests it is due time to turn our attention away from the strict margins of CSTs and instead focus more on identifying other risk markers.

Furthermore, the incidence of euploid miscarriages has been linked to dysbiotic vaginal microbiomes (Grewal *et al.*, 2022). Sporadic, chromosomally normal miscarriages had a greater association to dysbiotic vaginal samples than aneuploid miscarriages, underlying different aetiologies of these events. Despite the increased incidence of *Lactobacilli* depletion in euploid miscarriage patients, the vaginal microbiomes of patients who had a viable term pregnancy did not significantly differ in measures of richness or diversity. Instead, a loss of *Lactobacilli* dominance was associated with increased levels of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α which may trigger an improper

host immune response leading to poor pregnancy outcomes. This further highlights the impracticality of using relative measures of diversity and CST types as a predictive tool and suggests that the importance of vaginal community members and their associated host interactions should be held to a higher regard. The use of CSTs, which rely on relative frequencies of the most abundant bacterial species in a microbiome, may inaccurately represent the real landscape of the vaginal environment, as depicted in Figure 3.

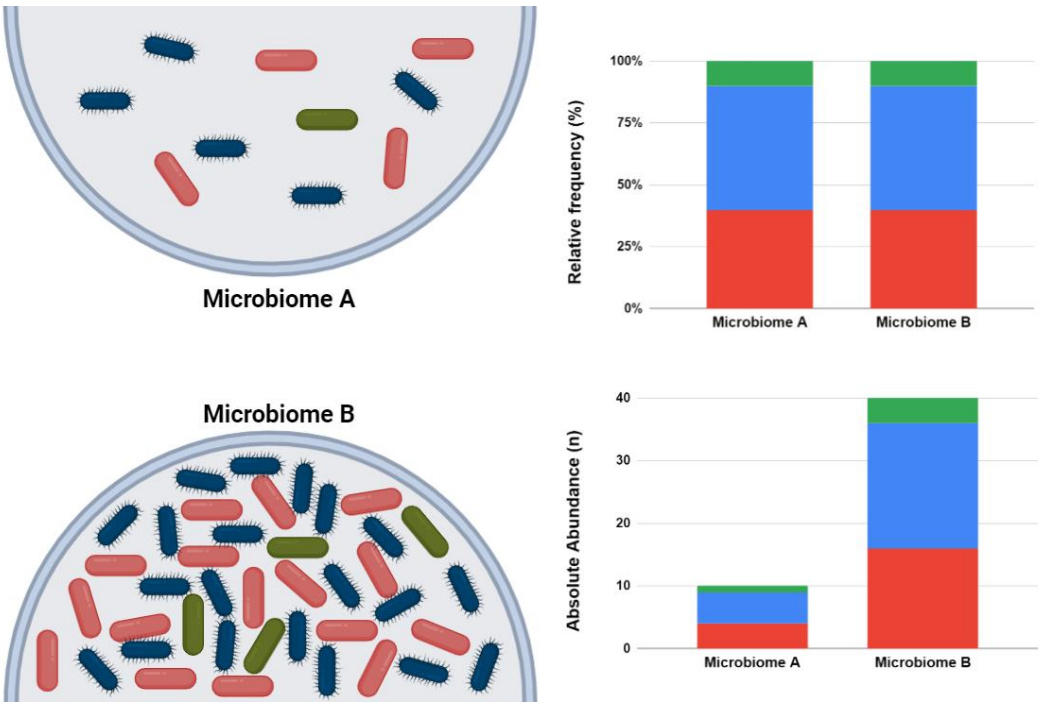

Figure 3: Depiction of differences of measuring relative (*top*) vs absolute (*bottom*) abundances. Using relative frequency would lead to the assumption that both microbiomes have the same landscape. Using absolute abundance gives a more accurate representation of the state of the microbiome. Created by the author using *biorender.com*. Adapted from Shanahan & Hill, 2019.

4.5. Reconsidering Community State Types

A simpler ecological representation of vaginal microbiomes based on the quantitative frequencies of key taxa has been proposed in favour of traditional CSTs. By measuring frequencies of *G. vaginalis*, *L. iners*, and *L. crispatus*, researchers were able to identify a microbial signature for PTB (Callahan *et al.*, 2017). Increased concentrations of *G. vaginalis* in a sample were associated with PTB, as was a low abundance of *L. crispatus*. The study was split between two cohorts. Cohort-1 mainly consisted of Caucasian people with a low risk of PTB while cohort-2 predominantly consisted of African American people with a high risk of PTB. From 16S rRNA sequencing data, three major sequence variants of *G. vaginalis* that differed in sequence by only one or two nucleotides were identified, labelled G1, G2 and

G3. Interestingly, despite the high similarity between sequence variants, the association of *Gardnerella* with PTB was solely driven by a single variant of *G. vaginalis*: G2. This is suggestive of a causative agent and thus warrants further study into *G. vaginalis* sequence variants. This study also revealed an exclusionary relationship between *L. crispatus* and *G. vaginialis,* in which abundance ratios were largely skewed in favour of one or the other. Meanwhile, *L. iners* and *G. vaginalis* were typically found to coexist at similar abundances. This does not necessarily suggest that *L. iners* itself contributes to adverse health effects. Rather, it suggests that *L. iners* is more permissive of pathogenic growth than *L. crispatus* and thus offers less protective qualities, a theory supported by the observation of *L. iners* colonisation post-antibiotic treatment in recurrent BV (Joag *et al.*, 2019).

Additionally, in cohort-2 but not cohort-1, lower abundances of *L. gasseri* and *L. jensenii* were also associated with an increased risk of PTB. This is an interesting observation considering the lower reported instances of *L. crispatus* predominance in African American people (Ravel *et al.*, 2011; Borgdorff *et al.*, 2017). Additionally, the mgCST most reported in African American people was a *G. vaginalis* predominated mgCST (Holm *et al.*, 2023). This difference in species' association between cohorts is possibly explained by the different metabolic capabilities of *Lactobacillus* species. As previously mentioned, different *Lactobacillus* species differ in their abilities to produce lactic acid isomers. *L. crispatus* and *L. gasseri* can produce both L- and D-lactic acid. *L. jensenii* can only produce D-lactic acid while *L. iners* can only produce the L-lactic acid isomer. Previous studies have linked D- to L-lactic acid ratios to the production of the extracellular matrix metalloproteinase inducer membrane protein (EMMPRIN) (Witkin *et al.*, 2013). L-lactic acid levels are positively associated with EMMPRIN levels while an increase in the ratio of D-lactic acid relative to L-lactic acid was found to significantly decrease EMMPRIN levels in vaginal epithelial cells. One function of EMMPRIN is to induce the expression of a collagen cleaving enzyme, MMP-8, which is involved in the degeneration of the cervical plug (Figure 4). A loss of cervical plug integrity allows for the passage of bacteria from the vagina to the upper reproductive tract, which may contribute to PTB. Indeed, the same study documented the ability of *Gardnerella* species to produce low levels of L-lactic acid. The microbial signature of PTB defined by Callahan et al. aligns with this hypothesis, in that the signature species associated with PTB would result in an increased L-lactic acid concentration relative to D-lactic acid and subsequently an increased amount of EMMPRIN (Callahan *et al.*, 2017). The loss of the protective qualities of the D-lactic acid isomer associated with this PTB-microbial signature may offer insight into the microbial aetiology of PTB, although this theory requires further scrutiny. Overall, as well as this microbial signature of PTB being of value to clinicians, direct

measurements of D- to L-lactic acid ratios as well as EMMPRIN concentrations possibly have a predictive potential for reproductive health.

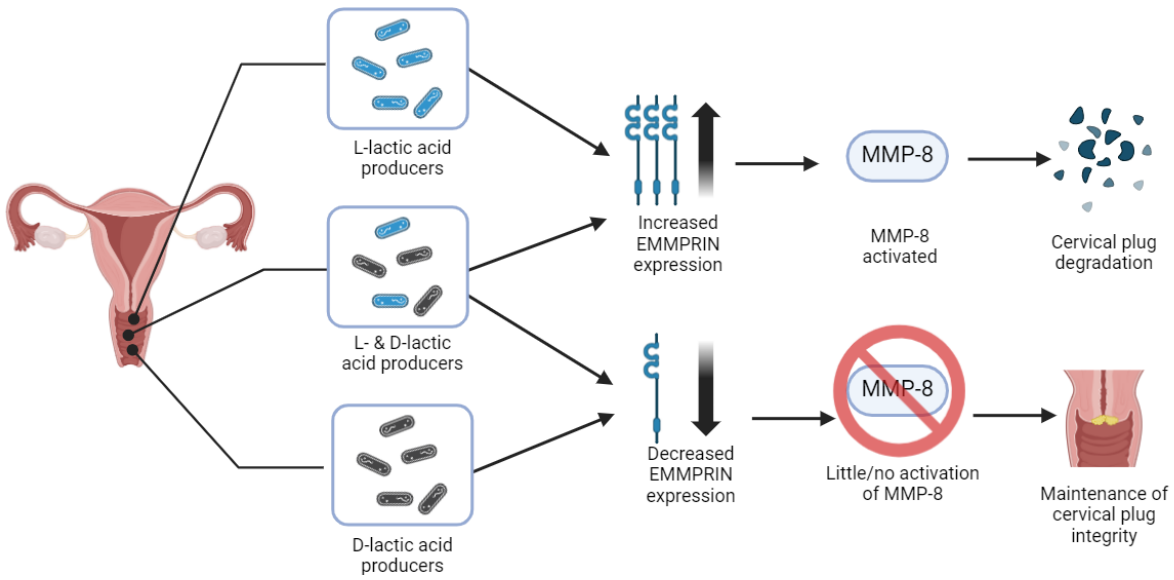

Figure 4: Different vaginal microbiota species have different lactic acid isomer production capabilities. L-lactic acid and D-lactic acid have positive and negative associations with EMMPRIN respectively. The overall L-lactic acid to D-lactic acid ratio can have downstream impacts on reproductive tract health and the risk of preterm birth. While *Lactobacilli* such as *L. crispatus* and *L. gasseri* produce both lactic acid isomers, the influence of less abundant vaginal microbiota may contribute to imbalances in this ratio. For example, low levels of Gardnerella may increase L-lactic acid ratios in *L. crispatus*- and *L. gasseri*-predominant vaginal microbiomes. Figure created by the author using *biorender.com*.

5. THE VAGINAL MICROBIOME IN OTHER CONDITIONS.

5.1. Genitourinary Syndrome of Menopause.

The vaginal microbiome is not solely of importance to people of reproductive age. The vaginal microbial landscape of postmenopausal people has repercussions on health outcomes and in turn quality of life. Many postmenopausal people will experience genitourinary syndrome of menopause (GSM), a chronic and progressive condition. The term was initially defined in 2014 to describe the collection of symptoms associated with the decreases in oestrogen during menopause such as vaginal dryness, sexual discomfort, dysuria, and general vaginal and urogenital atrophy. Though the exact prevalence of GSM is difficult to estimate, it is thought to affect anywhere from 27-84% of postmenopausal people, although many of these will only experience mild manifestations (The NAMS 2020 GSM Position Statement Editorial Panel, 2020). The reason why only some, but not all, postmenopausal people experience GSM has elucidated researchers, however recent

developments show that the answers may be found from analyses of the vaginal microbiome. The most commonly observed CST in postmenopausal people was the polymicrobial CST-4, which was reported in almost 66% of the study population (Waetjen *et al.*, 2023).

Despite this association of CST-4 with menopause, only one symptom of GSM, sexual pain, had an association with this CST. Interestingly, in the Waetjen et al. study, Black postmenopausal people were twice as likely to have the *L. crispatus* dominated CST-1 compared to other racial groups, a finding that has been corroborated in other studies (Hudson *et al.*, 2021; Waetjen *et al.*, 2023). This differs from what has been reported in premenopausal, reproductive-age Black populations where CST-4 and non-Lactobacillus dominance is the norm (Ravel *et al.*, 2011; Borgdorff *et al.*, 2017). One possible explanation for this difference may be confounding variables. In the Waetjen et al. study, Black people had the highest average BMI. People with higher BMIs were more likely to have CST-1 than people with lower BMIs, which may be due to the increased oestrogen levels associated with obesity. Increased oestrogen would result in higher amounts of glycogen deposition within the vaginal microenvironment for *L. crispatus* to utilise and survive. This in itself provides evidence for the use of exogenous oestrogen as an intervention for vaginal dysbiosis during menopause. Indeed people using exogenous oestrogen had a greater prevalence of *Lactobacillus* dominance compared to those who were not (Waetjen *et al.*, 2023). Though there remains a lack of direct correlation between vaginal microbiota and the majority of GSM symptoms, the association of GSM as well as *L. crispatus* depletion with decreased levels of oestrogen is enough to warrant further investigation here. To this effect, a randomised controlled trial found that oestrogen used in combination with probiotics was superior in relieving GSM symptoms than oestrogen alone (Ribeiro *et al.*, 2019). However, the probiotics used in this trial contained gut-associated *Lactobacilli*. Further studies should be conducted testing the same effect using vagina-associated *Lactobacilli*.

5.2. HIV-1 Acquisition.

In addition to the predictive capacity of the vaginal microbiome in reproductive health, the vaginal microbiome has shown promise in other areas of healthcare. For instance in infection biology, a polymicrobial vaginal microbiome is associated with an increased risk for HIV-1 acquisition, which is of particular interest given that over 50% of newly acquired HIV-1 infections occur in females. Identifying a vaginal microbiome landscape that poses a heightened risk for HIV-1 acquisition would be incredibly valuable in disease prevention for the Global HIV Programme (UNAIDS, 2021). It has been shown that the rate of HIV-1 infection is over sevenfold higher in people with a polymicrobial vaginal microbiome

compared to those with a *L. crispatus* dominated vaginal microbiome (Wang *et al.*, 2023). For example, *Prevotella timonensis*, a vaginal microbe associated with dysbiosis, exacerbates host susceptibility to HIV-1 infection (Van Teijlingen *et al.*, 2022). *P. timonensis* is unique among dysbiosis-associated microbes in that they can convert HIV-1 destroying Langerhans cells into HIV-1 reservoirs, by permitting HIV-1 entry via an alternative, CD4- and CCR5-independent pathways, thus allowing the virus to escape autophagy. This allows HIV-1 viral cells to exist undetected for several days before being transmitted to susceptible CD4+ T-cells. Revealing this vaginal microbiome community member that increases host risk to HIV-1 infection is a valuable target for future intervention. Detecting this microbe in at-risk communities may allow clinicians to target and predict high-risk individuals for treatment in order to decrease their chance of acquiring HIV-1, assisting in the goal to limit the spread of HIV globally.

Typically, healthy vaginal microbes and their associated metabolites are the first line of defence along the lower reproductive tract. Studies on vaginal epithelial cell lines have shown that lactic acid concentrations associated with a healthy vaginal microbiome reinforce vaginal epithelial cell barrier integrity while dysbiosis-associated metabolites, such as acetic acid, succinic acid and butyric acid had an opposing effect, even after exposure to HIV-1 (Schwecht *et al.*, 2023). These short-chain fatty acids are associated with BV. The condition's increase in anaerobic bacterial species and have been shown to activate the NFκB proinflammatory signalling pathway. Interestingly, lactic acid suppressed NFκB activation in dysbiotic conditions, even at low concentrations (Schwecht *et al.*, 2023). Healthy vaginal metabolites were further shown to contribute to a protective vaginal environment by increasing the expression of the genes involved in tight junction formation and cell-to-cell adhesion (Delgado-Diaz *et al.*, 2022; Schwecht *et al.*, 2023). Overall, this data shows the promise of measuring vaginal metabolites in order to determine HIV-1 risk and additionally opens the possibility of investigating the use of lactic acid as an intervention to prevent HIV-1 infection in 'at-risk' populations. Although it is alluring to determine 'at-risk' groups as solely polymicrobial and 'protected' groups as those with a *Lactobacillus* dominated vaginal microbiome, this may be an oversimplification. This would wrongly attribute equal HIV-protective capacities to all vaginal *Lactobacillus* species. While various studies show this is not the case, their results are conflicting. While some studies conclude that *L. crispatus* dominance offers superior protection to HIV-1 infection than *L. iners* dominance (Borgdorff *et al.*, 2017), others have attributed high levels of *L. iners* to increased HIV-1 protection (McClelland *et al.*, 2018). Differentiating HIV susceptibility risks of *Lactobacilli* will grant a deeper predictive value to vaginal microbiome data and will allow for more thorough identification of 'at-risk' individuals and permit earlier intervention.

5.3. Cancer.

Considering the intricate relationship between vaginal microbiota and host health, it is logical that the vaginal microbiome may also serve as a predictive tool in oncology. This is particularly true for cancers of the reproductive tract. Uterine corpus cancer, more colloquially referred to as endometrial cancer, is one of the few cancers who's mortality rate has increased in the last decade (Giaquinto *et al.*, 2022). This may be a factor of a lack of a standardised routine screening for endometrial cancer. Although incidence of this cancer is roughly equal between racial groups, the mortality rate of endometrial cancer in Black people is almost double that of White people, the largest racial disparity of any surveyed cancer in the U.S.A.. Though some explanations for this imbalance may be due to sociocultural reasons, it is perhaps of interest to future studies to investigate these differences in the context of vaginal microbiome variation between races.

Presently, the ability of vaginal microbiome patterns to be used as a predictor of endometrial carcinoma disease stage and tumour histologic grade has been verified. Using supervised clustering models and machine learning, it was possible to make predictions on whether gynaecological disease was benign or cancerous based on the detection of key vaginal microbiome biomarkers (Hakimjavadi *et al.*, 2022). Similarly, using 16S rRNA sequencing data, researchers detected key vaginal microbial signatures in the vaginal microbiomes of cervical cancer patients compared to healthy controls (Wu *et al.*, 2023). Taxa highlighted in these signatures were associated with clinically relevant functional alterations that were theorised to contribute to carcinogenesis, such as increased fatty acid, carbohydrate and vitamin biosynthesis. Not only could the vaginal microbiome serve as a biomarker for cancer, but it also had a strong predictive capacity for carcinoma grade. For example, CST1 had a significant association to benign disease, CST2 to low-grade endometrial carcinoma and CST3 and CST4 associated with high-grade endometrial carcinoma. As previously reported, CST4 is the most commonly observed state type in Black people, which may offer an insight into increased mortality in this demographic. Furthermore, key species were identified with significantly different abundances between disease states. In particular, *Fusobacterium ulcerans* was more abundant in the vaginal microbiomes of patients with high-grade tumours relative to both benign disease and low-grade tumours. This bacterial species is known to produce high levels of butyric acid, which as previously mentioned has a negative effect on epithelial cell integrity and thus may help contribute to tumour progression. Meanwhile, *Lactobacillus* species, whose lactic acid producing and

bacteriocin producing capabilities are known to inhibit pathogen growth had reduced abundances in high-grade tumours. Manipulating microbiomes in order to improve cancer therapies is a novel yet emerging area of clinical research. In clinical trials of advanced melanoma patients with resistance to checkpoint inhibitor immunotherapy, altering patient gut microbiomes via faecal microbiota transplants (FMTs) facilitated tumour sensitivity to immunotherapy as resistance was overcome (Davar *et al.*, 2021). Therefore, knowledge of vaginal microbiota's role in cancer should be further scrutinised so that its ability to improve efficacy of cancer therapies and reduce mortality rates can be studied.

The predictive potential of the vaginal microbiome extends beyond diagnostics, as it could also play a role in patient outcome and quality of life. Regimes such as chemotherapy and radiation therapy typically used in the treatment of gynaecological cancers are associated with vaginal microbiome instability (Tsementzi *et al.*, 2021). Consequently, survivors of gynaecological cancers are often plagued with a series of undesirable side-effects such as sexual discomfort, vaginal pain, vaginal bleeding and other related toxicities, which have an impact on their quality of life. While community typing showed poor associations to symptoms, individual species served as better biomarkers. For example, *Delftia* species showed significant associations to high vaginal pain scores in cancer patients. Monitoring changes to the vaginal microbiome during and post-treatment may be a method of alleviating these symptoms. To this effect, a recent clinical trial attempted to alleviate vaginal dysbiosis in gynaecological cancer patients undergoing radiation therapy by randomly treating these patients with a commercially available probiotic containing *Lactobacillus delbrueckii* subspecies *lactis* (Bi *et al.*, 2023). Patients who received the probiotic treatment in conjunction with their radiation therapy had diminished vaginal microbiota disruption as the probiotic proved to maintain *Lactobacillus* abundance while simultaneously limiting the growth of pathobionts such as *Prevotella*. Identification of key species associated with microbiome instability and vaginal toxicities calls for future investigation of new interventions, druggable targets and the use of probiotics. Future research should evaluate vaginal microbiome biomarkers associated with these symptoms in order to allow clinicians to potentially intervene at the vaginal microbiome level to enhance patient outcome.

6. EMERGING APPLICATIONS OF VAGINAL MICROBIOME DATA IN REPRODUCTIVE HEALTH.

6.1. Using the vaginal microbiome to diagnose endometriosis.

An increased appreciation for the value of the vaginal microbiome in medicine could help to reshape the field of reproductive medicine. Using vaginal microbiome data may help to make reproductive medicine and diagnostics more accessible and routine to patients across the world. The diagnosis of endometriosis is an example of an area that may benefit from these advancements. Endometriosis is a chronic inflammatory disease affecting nearly 10-15% of people of reproductive age (Parasar, Ozcan and Terry, 2017). The current gold standard for diagnosing this condition typically involves laparoscopic surgery. Thus, there is a call for the development of tools to facilitate a non-invasive, cost-effective method for confirmatory diagnosis of endometriosis. To this effect, pilot studies have demonstrated the ability of the vaginal microbiome to be used as a tool to predict endometriosis and disease severity. One study noted the differential abundance of non-*Lactobacillus* species between endometriosis patients and healthy controls (Yang *et al.*, 2023). A separate study conducted on a cohort of South American patients used machine-learning methods based on 16S rRNA sequencing of vaginal microbiomes to predict the severity of endometriosis. The genus *Anaerococcus* most accurately distinguished between mild and severe stages of disease (Perrotta *et al.*, 2020). These advancements could allow for the development of a standardised set of biomarkers to diagnose endometriosis. The extrapolation of such findings could facilitate easier, cheaper and thus more accessible diagnostics of a range of reproductive tract diseases and conditions.

6.2. Vaginal microbiota transplants.

The development and use of FMT has emerged in recent times as a promising and acceptable means to manage disease at the gut microbiome level. Currently, FMT is used as an intervention to treat cancers, Crohn's disease, recurrent *Clostridioides difficile* infection, etc (Feng *et al.*, 2023). FMT involves transplanting faecal microbiota from a healthy individual into a diseased individual in order to recolonise an unhealthy gut microbiome. Thus, it is plausible to conceive a similar mechanism of action being used with vaginal microbiota. Though evidence of this is lacking, recent attempts have been made to investigate the practicality of vaginal microbiota transplants. A proof-of-concept study in Denmark successfully performed an antibiotic-free vaginal microbiota transplant on a patient with a clinical history of vaginal dysbiosis and pregnancy loss (Wrønding *et al.*, 2023). The transplant resulted in a successful natural pregnancy four months post-transplant as well as

donor engraftment and subsequent *L. crispatus* dominance up to one year after the initial transplant. Though these results are tightly limited by the size of the study, they offer a glimpse at the potential of this technique. Experiments in endometriosis murine models have also suggested promise for the role of vaginal microbiota transplants in this area (Lu *et al.*, 2022). Thus, vaginal microbiota transplants pose as an auspicious intervention for not only a history of unsuccessful pregnancy, but a range of vaginal dysbiosis associated conditions and perhaps will transform the future of reproductive tract medicine.

6.3. Improvements in studying the vaginal microbiome's interaction with the reproductive tract.

Advancements in studying the interactions between host and vaginal microbiota will facilitate novel and pioneering research into this area that will ultimately further cement the use of the vaginal microbiome as a predictive tool in the future. While organoid models have been established for the study of the upper reproductive tract, organoid models of vaginal tissue are still in development (Kaya, De Zoete and Steba, 2023). To this extent, an organ-on-a-chip model of the human vagina was successfully developed, facilitating the study of *L. crispatus* and *G. vaginalis* colonisation of vaginal epithelial cells (Mahajan *et al.*, 2022). Organ-on-a-chip systems are microengineered microfluidic 3D models mimicking the structure and function of human tissues. These devices, including the newly developed vagina-on-a-chip model have applications in drug development, disease modelling, pathogenesis analysis, etc that will assist in studies of the future clinical applications of the vaginal microbiome.

The advent of artificial intelligence (AI) and machine learning has entered the world into a new era of innovation and discovery, and the field of vaginal microbiome research has not escaped its reach. Using data from five independent studies, researchers trained a deep-neural network that could predict the occurrence of PTB with an accuracy of over 84%, outperforming all other traditional computational prediction models (Chakoory *et al.*, 2024). According to this study, overall vaginal diversity rather than the contribution of individual species was a better predictor of PTB. Furthermore, in 2022 there was an open-call to computational scientists across the world to compete in a challenge to design the best machine-learning model to predict PTB based on publicly available vaginal microbiome data (Golob *et al.*, 2024). This open call is testament to the increasing appreciation to the contribution of the vaginal microbial landscape to reproductive health and helps to lay the groundwork for further advancements and developments of these predictive models. One interesting note from this open call was that across the hundreds of models submitted to this

challenge, species and strain level variation of *Lactobacillus* and *Gardnerella* stood out as particular players in the association of the vaginal microbiome to PTB, supporting further investigation into these community members.

7. FUTURE OUTLOOKS AND CONCLUSIONS

We do not have to wait for the future in order to witness the adoption of the vaginal microbiome as a predictive tool. Indeed since 2017 in The Netherlands, the Dutch Health Authority has approved the use of ReceptIVFity, a vaginal microbiome analysis test, in several clinics performing assisted reproduction (ReceptIVFity study group *et al.*, 2018). This test uses self-collected vaginal mucus samples to predict the success of pregnancy after IVF based on the microbial composition of the vagina. Success of this test in Dutch clinics has led to its uptake in certain clinics in Germany and the United Kingdom, a testament to the increased acknowledgment of the vaginal microbiome as an important tool in a clinician's repertoire. Additionally, researchers have developed and proposed the use of a disposable paper-based detection tool for the diagnosis of BV (Avila-Huerta *et al.*, 2023). They did so by creating a monoclonal antibody conjugated to a fluorophore to detect the presence of sialidase, a metabolic product of BV-associated microbes such as *Gardnerella* and *Prevotella*. Wide-spread adoption of this device would grant clinicians rapid, cheap and on-site diagnosis of BV and thus expand their diagnostic capabilities to a wider demographic.

Overall, the potential of the vaginal microbiome as a predictive tool is promising. A better understanding of the vaginal-microbial interactions underlying reproductive tract diseases and pregnancy-related complications will help to solidify the widespread uptake of data from this microbial niche as a predictive tool and is set to revolutionise the field of reproductive health care. Though we may still be at the tip of the proverbial iceberg in regards to our knowledge of the vaginal microbiome, the advantages it offers over many conventional tools and diagnostic methods implores researchers to persevere and further study the impact of the vaginal microbiome on human health. Deeper understanding and appreciation of the power of the vaginal microbiome is set to improve patient outcomes, facilitate personalised therapeutics to intervene at a patient's vaginal microbiome level and reduce the economic, financial and personal burden of undergoing assisted reproduction.

8. SEARCH STRATEGY

The search for relevant literature was performed between February and March of 2024. Key terminology was identified and adapted for database searching on PubMed. When necessary, additional relevant search terms were used in support of the key terminology for the area being researched at the time. The search was restricted to articles published in the English language. The database search was supplemented by a manual citation search of articles and all literature was selected for or excluded based on a manual review of their study objectives and relevance. Filters such as publication date and article type were applied as required.

Key words identified:

Vaginal microbiome, Vaginal microbiota, genetic diversity, bacterial vaginosis, PCR, metagenomics, primers, fertility, infertility, implantation, IVF, menopause, *Lactobacillus cripsatus*, preterm birth.

Databases searched:
pubmed.ncbi.nlm.nih.gov

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