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Writing Assignment

Advanced Technologies for Studying Microbiome-Female Reproductive Tract Interactions: Organoids, Organoids-on-a-Chip, and Beyond

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Layman Summary

Within the complexity of the human body, the female reproductive tract (FRT) is a bustling hub of tiny living organisms known as microbiomes. Far from being just simple passengers, these microscopic beings actively influence aspects of reproductive health such as fertility, and they're either our allies or enemies in fighting diseases depending on the type of microbe. The quest to truly understand their relationships within the FRT is a journey filled with opportunities to unlock insights into common and devastating conditions like infertility, endometriosis, and even cervical cancer. However, this journey hasn't been easy. Traditional tools and approaches have often fallen short, leaving us with an incomplete picture. Enter our research, where we investigate groundbreaking technologies like organoids and microfluidic devices, painting a much more detailed and intimate portrait of organoids' use case for studying the FRT's microscopic residents.

What makes organoids so unique? They are essentially miniature organs grown in the lab. Organoids come from stem cells found in different tissues and are developed in specialised gels. They can be derived from normal or diseased tissues, so personalised medicine strategies like patient-specific drug testing are possible. Organoids of various regions of the FRT have already been made successfully, but the addition of microbes to these FRT organoids has not yet become a standard practise because there is no single best approach for this yet. We investigated the techniques to do this so that, when combined with microbes, organoid models will allow for the development of novel pathology research, personalised medicine strategies, and patient-specific drug testing. These co-culture models are opening doors to tailored medical treatments, revolutionising how we approach individual reproductive health issues. Microfluidic devices are another key player in our research. They deftly manage minuscule amounts of fluid, simulating the FRT's dynamic environment. The goal is to discover whether the synergy between organoids and microfluidics offers us a life-like representation of the female reproductive system that is unparalleled in its accuracy.

Our paper's goal is clear: investigate ways for these cutting-edge techniques to craft realistic models of the FRT, inhabited by both friendly and harmful microbes. This includes incorporating the hormonal cycle into the models as well as oxygen gradients and facets of the immune system.
This approach will help us answer critical questions, such as whether conditions like endometriosis cause the microbiome to become unbalanced or if it's the other way around. Why does this matter? Simply put, these discoveries could reshape how we treat infections, support fertility, and even detect cancer at its earliest stages. We're transitioning from merely observing connections to actively uncovering causes and crafting targeted therapies. Imagine our research as forging a potent lens, bringing into focus the incredible complexity of the world inside the FRT. Though we have room to grow, this exciting combination of organoids and microfluidic devices is already a transformative step in reproductive health.

Abstract

The female reproductive tract (FRT) is home to diverse microbial communities that play a pivotal role in reproductive health and disorders such as infertility, endometriosis, and cervical cancer. To understand the complex host-microbiome interactions within the FRT, models that authentically replicate the FRT's environment, including the interplay between the microbiome, mucus layer, immune system, and hormonal cycle, are key. Recent strides in organoid and microfluidic technologies are propelling research in this domain, offering insights into FRT-microbiome interactions and potential therapeutic avenues. This review delves into the current state of FRT organoid models and microbe integration techniques, evaluating their merits and challenges for specific research objectives. Emphasis is placed on innovative approaches and applications, including integrating organoids with microfluidics, and using patient-derived biobanks, as this offers potential for deeper mechanistic insights and personalised therapeutic strategies. Modelling various FRT properties in organoids is explored, from encompassing age-related epithelial features, oxygen levels, and hormonal effects to mucus layers, immune responses, and microbial interactions, highlighting their potential to transform reproductive health research and predict possible outcomes.

Key Words: female reproductive tract, microbiota, organoids, *in vitro* models, mucus, microbiome, reproductive health, host-microbe interactions, organ-on-a-chip, microfluidic, organoid-on-a-chip

1. Introduction

Understanding the complex interplay between the human body and its resident microorganisms, known as the microbiome, is a crucial pursuit in scientific exploration. Over the past decade, extensive research has illuminated the intricate dance between the microbiome and various aspects of human health, including nutrition, immunity, and disease susceptibility [1]. Within this paradigm, the female reproductive tract (FRT) emerges as a critical arena where microbial interactions profoundly influence reproductive health and fertility. The FRT is composed of different anatomical regions, each with its unique physiological and microbial characteristics [2]. The vagina, fallopian tubes (FT), endometrium, cervix, and placenta all harbor distinct microbial communities that play a significant role in maintaining reproductive health and fertility [2]. However, alterations in these microbial communities, known as dysbiosis, can lead to various reproductive disorders, ranging from bacterial vaginosis and pelvic inflammatory disease to infertility and pregnancy complications [2].

The vagina is home to a distinct microbial community, predominantly composed of *Lactobacillus* species such as *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, and *Lactobacillus iners*. These bacteria promote a low pH environment ($pH < 4.5$), which is unfavorable for the growth of pathogenic microbes, thus maintaining vaginal health [3]. However, dysbiosis in the vaginal microbiome can lead to bacterial vaginosis, characterized by overgrowth of anaerobic bacteria like *Gardnerella vaginalis*, *Prevotella spp*., and *Mobiluncus spp*. [4]. The presence of these pathogenic microbes increases the pH, causing a range of symptoms from unpleasant odors to itching and burning [2]. Studies have also linked vaginal dysbiosis to higher susceptibility to sexually transmitted infections (STIs), including human immunodeficiency virus 1 (HIV-1) [5].

Traditionally, the upper reproductive tract (URT), including the FT, has been considered sterile. However, emerging studies have challenged this concept, identifying a unique and less diverse microbial community in the FT compared to the lower reproductive tract (LRT) [2]. Predominant microbes identified include *Pseudomonas*, *Acinetobacter*, and *Prevotella* species. The role of these microbes in health and disease is still not completely understood [3]. However, some studies suggest that microbial dysbiosis in the FTs may be associated with diseases like hydrosalpinx and pelvic inflammatory disease (PID), which can lead to infertility and ectopic pregnancy [6].

Similar to the FT, the endometrium was previously considered sterile, but recent studies have identified a unique endometrial microbiome composed mainly of *Lactobacillus spp*., with less representation from *Gardnerella*, *Streptococcus*, and *Bifidobacterium spp*. [2]. Dysbiosis in the endometrial microbiome, often characterized by reduced *Lactobacillus* and increased anaerobic bacteria, has been associated with reproductive disorders such as endometriosis and recurrent pregnancy loss [2]. Moreover, studies indicated that the endometrial microbiome composition influences the outcomes of *in vitro* fertilization (IVF), with a *Lactobacillus*-dominant microbiome associated with successful implantation and pregnancy [2].

The cervix, like the vagina, is dominated by *Lactobacillus* species, but dysbiosis can lead to conditions such as cervicitis [2]. More importantly, studies have shown that cervical dysbiosis, characterized by reduced *Lactobacillus* and increased diversity of anaerobic bacteria, is associated with an increased risk of cervical intraepithelial neoplasia and cervical cancer [7]. Some studies also suggest a potential link between the cervical microbiome and Human Papillomavirus (HPV) infection, the primary cause of cervical cancer [8].

The existence of a placental microbiome has been debated, with early studies identifying a unique microbial community including phyla like *Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes*, and *Fusobacteria* believed to influence immune regulation and metabolic function for the foetus [2]. However, recent research with stricter contamination controls challenges this notion, while some studies still link changes in the purported placental microbiome to adverse pregnancy outcomes, including preterm birth and preeclampsia [9], [2]. The nature and role of these microbes in the placenta remain undetermined.

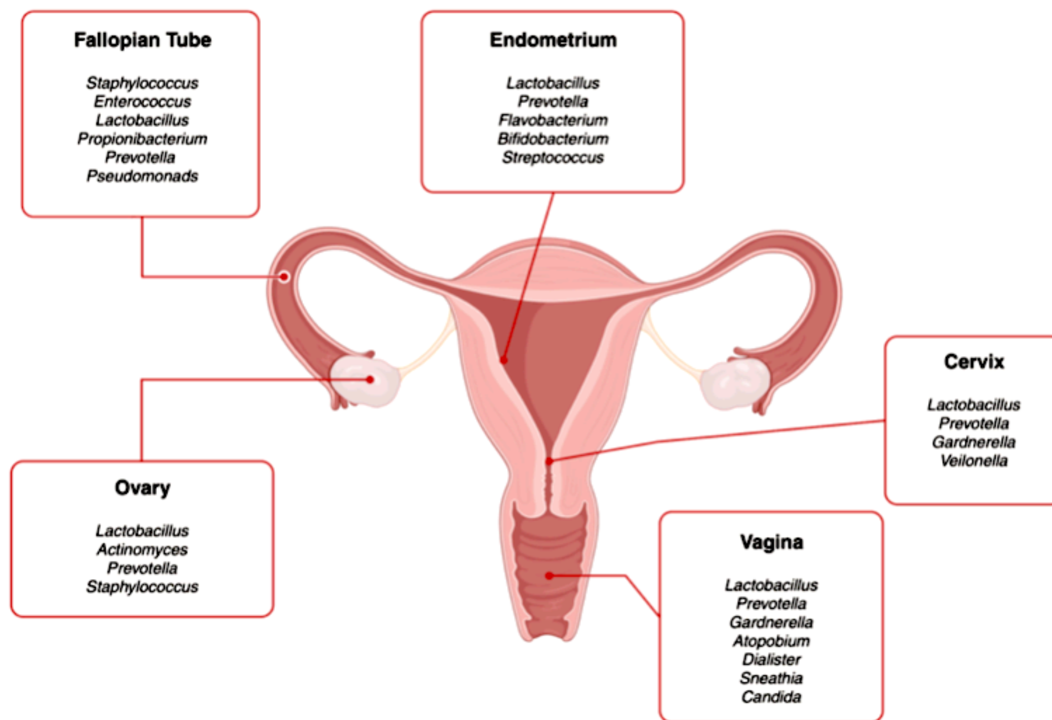

Figure 1. An overview of the composition of a microbiota in the reproduce tract of healthy reproductive-age woman

Understanding these complex host-microbe interactions in the FRT is crucial for developing effective strategies for preventing and treating reproductive disorders. However, studying these interactions *in vivo* is challenging due to the complex and dynamic nature of the FRT environment. Recognising these complexities, research has turned towards innovative *in vitro* methodologies that

more accurately reflect the native conditions. Hence, organoid models have emerged as a powerful tool for studying host-microbe interactions in the FRT [10]. Organoids are three-dimensional cell culture systems that mimic the structure and function of the tissue of origin, providing a more physiologically relevant environment for studying these complex interactions [11]. Organoids provide various advantages for investigating host-microbe interactions, including the ability to match the tissue's natural architecture and cellular composition, offering a physiologically relevant model for investigating complicated interactions [10]. Organoids with specific genetic changes can be generated, permitting the investigation of the genetic basis of host-microbe interactions as well as mechanisms controlling susceptibility or resistance to infections [10]. Moreover, organoids' three-dimensional structure simulates the *in vivo* environment, enabling precise regulation of variables such as microbial strains, nutritional factors, and hormonal impacts [12]. Organoids can also provide personalised insights into disease processes, guiding personalised treatment methods [12]. They provide an ethical alternative to animal models by fitting more closely with human biology and allowing for the investigation of cellular and subcellular responses using advanced microscopy and single-cell analysis methods [12]. They also aid in the development of molecular and omics technologies such as transcriptomics and proteomics, which provide insights into host-microbe dynamics and disease aetiology, host defence mechanisms, and the delicate balance of commensal microorganisms under physiological settings [12].

The majority of FRT organoids are derived from adult stem cells (ASC) located within epithelial tissue fragments isolated from surgical samples or biopsies [10]. The tissue is enzymatically digested, and the fragments containing stem cells are embedded in extracellular matrix (ECM) gels, predominantly Matrigel, and overlaid with defined media to promote the proliferation of stem cells and their self-organisation into organoids [13], [14]. The specific media formulations vary based on the source of the FRT tissue but typically contain niche factors like Wnt, BMP, TGF-

inhibitors, and mitogens like EGF, RSPO1, and FGF10 to support stem cell growth while preventing differentiation (Table 1) [15]. For instance, endometrial organoids require Wnt activation and BMP/TGF inhibition for robust expansion [15], while FT organoids rely on Wnt and Notch signalling [13]. Pluripotent stem cells like ESC or iPSC have also been differentiated into FRT organoids, but this approach is less common at present [16]. In general, FRT organoids are cultured using similar approaches as other epithelial organoids, relying on Matrigel for 3D structure and defined niche factors for stem cell growth. However, culture conditions for each FRT organoid need further optimisation to improve efficiency and maturity [10].

Table 1. Culture conditions and niche factors used for FRT organoids

Organoid	Key Niche Factors
Ovarian	Noggin, RSPO1, WNT3A, FGF10, Nicotinamide
Fallopian tube	WNT3A, RSPO1, EGF, FGF10, Noggin
Endometrial	WNT3A, RSPO1, EGF, FGF10, Noggin, and A83-01
Cervical	RSPO1, Noggin, EGF, and Jagged-1
Trophoblast	EGF, FGF2, CHIR99021, A83-01, RSPO1, Noggin

This review paper will investigate the current state of organoid (derived) technologies for culturing FRT organoids together with microbes in order to provide insights into host-microbe interactions and their impact on reproductive health and fertility [12]. The evolution of organoid (derived) models for studying host-microbe interactions will be outlined, starting from basic 3D organoid structures and their 2D equivalents before advancing to more intricate microfluidic-based systems. The aim is to divulge which of these models have the potential to mimic the physical and mechanical properties of the FRT, enhancing *in vitro* study capabilities [17]. The ideal system ought to be able to model the complex interplay between mucus, mucins, and host-microbe interactions within the FRT as this is essential for reproductive health and immunity [18]. The review will

conclude by discussing what features need to be integrated to create more representative *in vitro* models, highlighting their main applications, and discussing anticipated future trends. Ultimately, our goal is to move from correlation to causation, leveraging organoids to unravel the underlying mechanisms that define the critical role of the microbiome in FRT health and disease.

2. Established Organoid Models of the FRT

Organoid technology has enabled the development of *in vitro* models of the FRT, where most of these organoids recapitulate many features of the native tissues [11]. Established organoid models of the FRT include endometrial organoids that form gland-like structures and respond to hormones, enabling the study of the menstrual cycle and infertility-related defects [10]. FT organoids, containing both secretory and ciliated cells, model oviduct physiology and support fertilization [10]. Cervical organoids mimic both squamous and columnar epithelium, providing insights into cervical biology [10]. Trophoblast organoids are valuable for studying placental development and maternal-foetal interactions [10]. While models for the uterus are well established, robust human vaginal tissue models are still in development. These organoids are genetically stable and can be derived from both normal and diseased tissues, enabling personalised medicine approaches such as patient-specific drug testing [10]. Ovarian organoids, derived from normal and cancerous tissues, display *in vivo*-like characteristics, though optimisation is needed for modelling ovulation [10]. Nonetheless, ovarian organoids, or “ovaroids," containing oocyte progenitor cells and supporting granulosa cells have been generated from human induced pluripotent stem cells (iPSCs) [19]. These ovaroid cells model follicle development, oocyte maturation, and hormone secretion. The ovaroid permits the investigation of human ovarian biology without requiring tissue samples from patients. Hence, this approach has the potential to facilitate the creation of novel therapies for issues such as infertility and ovarian cancer, as well as be used for studying host-microbe interactions by culturing the ovaroid

with select microbes to study the effects of different microbial populations on ovarian biology and function.

3. Introducing Bacteria to the Organoid System: Selecting the Best Technique

Choosing an accurate method for introducing microbes to organoids is vital for effective *in vitro* modelling of host-microbe interactions within the FRT [20]. This choice must consider the type of microbes involved, compatibility with natural conditions like pH and oxygen levels, as well as study requirements such as duration, scalability, and throughput. The ideal method should explore the dynamic relationship between microbes and host cells, covering aspects like immune responses and disease progression while also considering factors like cost, technical complexity, and reproducibility [20]. By evaluating these variables, researchers can select the method that best aligns with their goals, ensuring consistency and a robust understanding of complex relationships within the FRT (Table 2). By considering these technical variables, the most effective method (Figure 2) that aligns best with the research objectives can be determined from the following information:

3.1. Suspension Culture: A Common Approach with Limitations

Suspension culture is a widely utilised and nuanced technique in host-microbe interaction studies [20]. This approach involves the growth of organoids in a liquid medium with microbial additions, where they remain suspended [63]. The microbial additions include a variety of possibilities: the incorporation of live bacteria for direct interaction, the inclusion of bacterial products to study specific metabolic effects, or even using the medium wherein the bacteria were cultured to examine subtler interactions [20]. This approach does present challenges, such as restricting bacterial access to the apical side of the organoid, which may limit the authenticity of the simulated environment. Despite these limitations, suspension culture has played a pivotal role in

understanding host-microbe interactions within the FRT. There are two significant studies employing the suspension technique to investigate host-pathogen interactions:

Yu *et al*. utilized this method for inoculating patient-derived FT organoids with *Lactobacillus crispatus* and *Fannyhessea vaginae* [21]. The inflammatory response within the organoids was assessed by analysing the expression profile of 249 inflammatory genes. Results showcased significant differences in the expression of inflammatory genes in organoids cultured with either bacterial species, with *Lactobacillus crispatus*-infected organoids showing distinct differences from

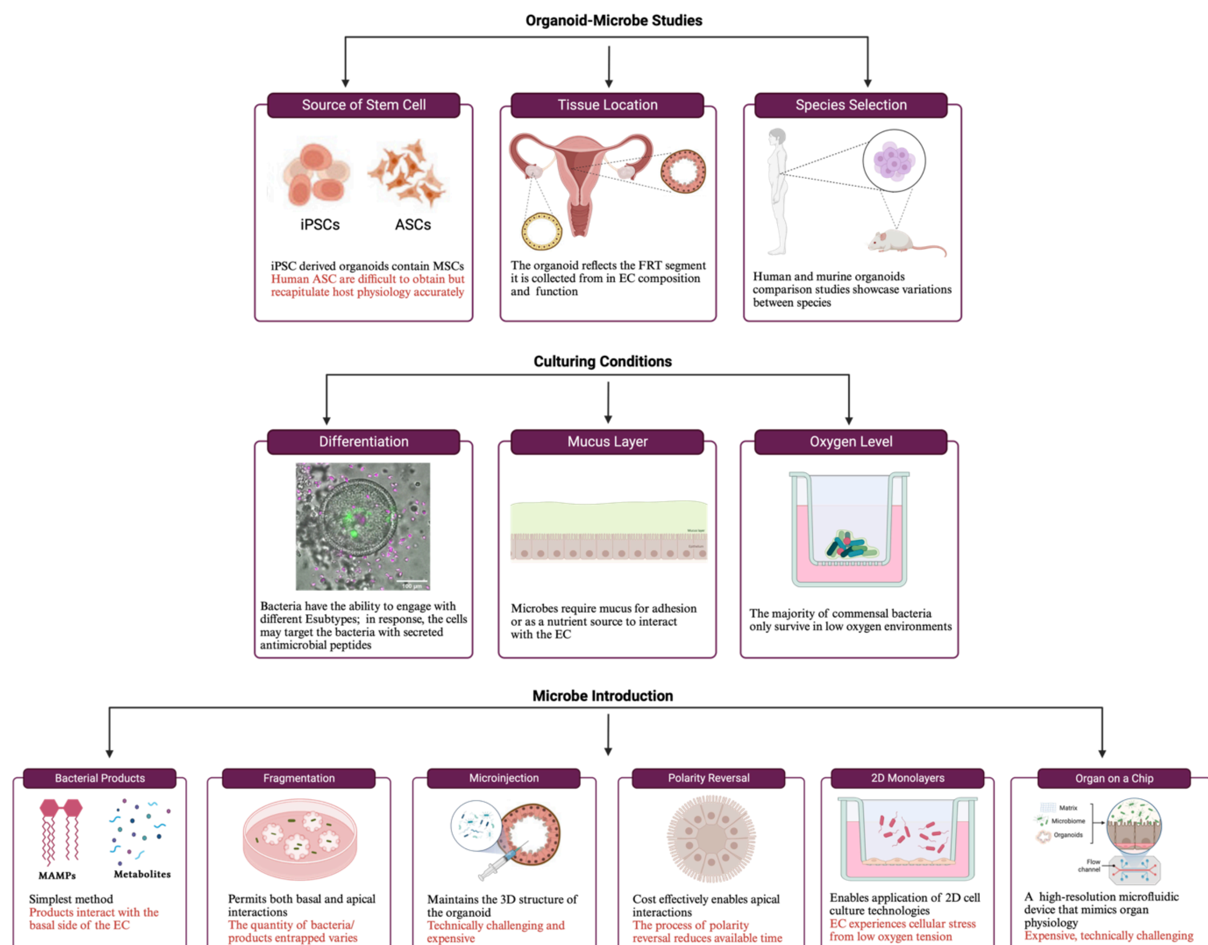

Figure 2. 3D organoids and organoid derived models for organoid-microbe studies. Organoids need to be created from the most relevant and accessible stem cell source, FRT region, and appropriate host. Prior to microbe introduction, organoid models should aim to model specific *in vivo* FRT conditions: differentiation into specific tissue subtypes, formation of a functional mucus layer, and the maintenance of gradient oxygen levels. These conditions enable the *in vitro* addition, attachment, and multiplication of microbes, which can be incorporated through different methods, each with its own set of advantages and disadvantages.

those infected by *Fannyhessea vaginae*. Flow cytometry analysis indicated that the immune cells initially found in the FT tissue decreased in the culture, suggesting the observed inflammatory response was generated by epithelial cells (ECs) within the organoids [21]. This highlights the utility of FT organoids as a model system for understanding host-pathogen interactions during bacterial infections.

Koster *et al*. investigated the role of pathogenic microbes in cervical mucosa co-infections using patient-derived ectocervical organoids [22]. The organoids were genetically modified to introduce HPV-16 E6E7 oncogenes, leading to precancerous lesions. Infection was carried out by incubating organoids with *Chlamydia trachomatis* non-replicative elementary bodies (EBs) for 2 hours, followed by reseeding into Matrigel. The study found that HPV16 E6E7 interferes with *Chlamydia* development, inducing persistence and leading to a reprogramming of host cell processes. Additionally, *Chlamydia* was found to hinder HPV-induced mechanisms that maintain cellular and genome integrity. The findings highlight the danger of multiple infections and the unique cellular environment created, which may contribute to neoplastic progression. The study emphasises the potential of patient-derived ectocervical organoids as a tool for better understanding co-infections and their role in disease development, thereby suggesting future avenues for research and potential therapeutic interventions.

The insights derived from Yu *et al*. and Koster *et al*. emphasize the method's relevance for exploring host-microbe interactions within the FRT and prompt the need for more sophisticated models to offer a comprehensive view of host-microbe interactions in the FRT. Despite some inherent challenges, the method's versatility allows for detailed investigation and contributes to the understanding of intricate disease mechanisms. These findings guide potential therapeutic advancements and pave the way for further research in the field.

3.2. Fragmentation: A Double-Edged Sword

Fragmentation is a technique that involves breaking down organoids into fragments and mixing them with bacteria or bacterial products before reseeding them into the ECM [20]. This method allows bacteria to interact with both the apical and basal sides of the ECs, and it has been used to study both homeostatic and pathogenic bacteria-organoid interactions. However, this technique's physiological relevance has been questioned due to its limitations, such as variations in the quantity of bacteria or bacterial products entrapped per organoid and the possibility of bacterial interactions with both sides of the ECM [20]. Despite this, two exemplary studies have used this method to study the long-term effects of *Chlamydia* trachomatis infections in the FRT:

In the research conducted by Kessler *et al*., human FT organoids were paired with *Chlamydia serovars* D, K, and E for an exhaustive long-term *in vitro* analysis to investigate FT scarring and infertility as consequences of chronic *Chlamydia* infections [23]. The organoids were released from Matrigel, mechanically disrupted, and infected with different *Chlamydia* strains, maintaining them in a controlled environment for four months. The study observed lasting effects of *Chlamydia* on the FT epithelium, including less differentiation and DNA hypermethylation, which could contribute to tubal pathologies like high-grade serous ovarian cancer, thus offering insights into long-term co-cultures and tubal pathologies. The use of organoids in this study enhances understanding of the long-term effects of *Chlamydia* on FTs and underscores the potential of organoids in studying reproductive health disorders.

Bishop *et al*. took a distinctive approach by developing murine endometrial organoids (EMOs), which were created by mechanically fragmenting endometrial glands and infecting them with mCherry-expressing *Chlamydia trachomatis* [24]. This method allowed the EMOs to undergo a full

developmental cycle, offering novel insights into *Chlamydia*trachomatis infection and endometrial interactions with other microbes and paving the way for therapeutic interventions.

The studies by Kessler *et al*. and Bishop *et al*. underline the fragmentation technique's flexibility and promise in organoid research, demonstrating its capacity for chronic analysis of *Chlamydia*'s effects and application in new model systems. Yet, some consideration for improving fragmentation consistency and physiological relevance may be needed. Collectively, these works suggest the fragmentation technique as a vital tool with potential for refinement in decoding complex organoid-bacteria interactions in the FRT and guiding future therapeutic advancements.

3.3. Microinjection into Organoid Lumens: A Precise but Challenging Technique

Microinjection is a technique that allows bacteria to contact the apical side of the epithelium while preserving the organoid's 3D structure as microbes are directly injected into organoid lumens [12]. This technique has been optimised using optimal injection volumes, fluorescent bacteria visualisation, repeated microinjections, and high-throughput microinjection platforms [12]. Both commensal and pathogenic bacteria have been microinjected into organoids, with commensal bacteria maintaining species diversity for over 96 hours [25]. Pathogens like *Salmonella enterica* stimulate cellular secretions in organoids only when applied to the apical side [20]. Repeated microinjections of genotoxic *Escherichia coli* led to mutational changes similar to colorectal cancer signatures, emphasising the carcinogenic effect of this strain [20]. Microinjection offers advantages such as precise dosing of bacteria, hypoxic conditions, and longer experimental durations for studying organoid proliferation and epithelial cell subtypes [20]. However, it can cause structural damage to organoids, lack of oxygen in the organoid lumen means that a stable co-culture with anaerobic bacteria cannot be maintained for long period of time, and it may not be ideal for beginners or high-throughput applications due to its high cost and technical challenges [20].

Despite these limitations, microinjection has proven to be a high-potential technique, as demonstrated by the following two studies by Dolat *et al.*:

Dolat *et al.* utilised an EMO to investigate the interactions between *Chlamydia* and epithelial and immune cells in the UGT [26]. The model, which replicated the complex structure of endometrial tissue, was constructed with hormone expression, sex hormone responsiveness, and fluorescent dextran microinjection to confirm barrier function. The study revealed that *C. trachomatis* affected the epithelial barrier and explored immune cell recruitment, particularly neutrophils. Despite limitations such as individual variability and a focus on EMOs, the study's innovative techniques and insights into hormonal interactions provide promising directions for future research, potentially transforming our understanding of *Chlamydia* infections and leading to new methodologies and treatments.

In subsequent research, Dolat *et al.* examined how *C. trachomatis* disrupts epithelial tight junctions by microinjecting EMO with specific bacterial strains [27]. The study revealed that *Chlamydia trachomatis* disrupts epithelial barriers by using the effector protein TepP to disassemble tight junctions early during infection. This disrupts host proteins like EPS8, promoting secondary invasion events. Genetic deletion of EPS8 makes cells and organoids resistant to TepP-mediated tight junction remodeling. The findings have far-reaching implications for understanding infection mechanics and pathways, setting a precedent for related research.

The investigations highlight the efficacy of microinjection as a method to scrutinize bacterial interactions with a unique combination of accuracy and flexibility, unveiling vital mechanisms. Notably suited for brief analyses involving strict anaerobes, it facilitates a direct interface between microbial and epithelial cells, enabling the assessment of transcriptional gene expression [12]. A

further proposition suggests the potential use of microinjection to implant specific immune cells or their products into reproductive tract organoids. Such an approach would offer a controlled environment for examining the interplay between immune cells, epithelial tissue, and microbes. Consequently, this could deepen the understanding of the immune system's role in reproductive tract disorders like endometriosis and recurrent implantation failure, where immune regulation is known to be a critical factor.

3.4. Organoid Polarity Reversal: A Novel Technique with Potential

A novel technique has been developed to reverse the polarity of 3D organoids, allowing them to grow in suspension for three days until they invert, which has created a new paradigm for studying interactions between microbes and host cells [12]. This method ensures proper epithelial barrier integrity and nutrient uptake without the need for microinjection, letting microbes interact directly with the organoid's outward-facing apical surface. In some studies, invasive pathogens such as *S. Typhimurium* and *L. monocytogenes* exhibited distinct invasion strategies for polarised epithelium, while enteropathogenic *E. coli* attached primarily to the apical side of mucin-secreting cells on inverted organoids [28].

Despite these advancements, the method has notable limitations, including the time required for reversal, increased cell death, and a tendency for inverted organoids to adhere to each other in the absence of an ECM [20]. Moreover, a particular challenge with this technique is that changing media can lead to unrestricted bacterial growth and toxins, disrupting the carefully balanced ecosystem that controls bacterial proliferation and toxin neutralisation, thereby affecting the integrity and function of the organoids themselves [29]. The challenge of controlling bacterial growth explains why the organoid reversal technique is mainly suited for short-term studies, as long-term control over interactions and the maintenance of equilibrium becomes increasingly complex and unpredictable,

limiting its application in sustained research [29]. This ultimately highlights the need for precision in long-term studies focused on homeostatic host-microbe interactions. Future research is needed to understand the impact of polarity inversion on apical-out organoids' phenotype, metabolism, and microbial response, providing insights into similarities and differences between this model and self-organized organoids. Once these fundamentals have been established, more intricate investigations can be conducted, such as where both immune cells and microbes are added to the medium of apical-out organoids to study how immune cells and microbes influence the function and behaviour of epithelial tissue.

3.5 Organoids cultured as 2D monolayers

The use of organoids transformed into 2-dimensional (2D) monolayers and air-liquid interface (ALI) cultures has provided a substantial leap forward in the study of host-microbe interactions because it makes the apical side more accessible [20]. Hence, these methods enable us to bypass some of the complexities associated with 3D organoid cultures while still maintaining relevant modelling of the biological system.

3.5.1. 2D Monolayers

The transformation of 3D organoids into 2D monolayers has emerged as a recognised method for introducing bacteria or their byproducts into host-microbe interaction studies by simply adding them to the culture media [20]. Organoids are linearized by fragmenting 3D structures into small cell clusters or individual cells, which are then plated onto extracellular matrix-coated surfaces to form a monolayer [12]. While sacrificing the more naturalistic 3D structure, this approach offers a simplified yet highly relevant model that accommodates traditional cell culture techniques, such as the assessment of EC functionality, tight junction integrity, cell differentiation, and immune responses [20]. One of the major advantages of 2D monolayers lies in their compatibility with

existing lab equipment and protocols. By employing transwells to segregate the apical and basal compartments, this technique can further refine EC differentiation and allow for the inclusion of additional host factors like mesenchymal cells or immune cells, more closely mimicking the *in vivo* environment of the FRT [20]. In practical applications, 2D monolayers have proven their utility for studying epithelial integrity upon exposure to microbes [30]. The described model facilitates the simultaneous cultivation of organoids along with aerobic bacteria and bacteria-generated metabolites for limited time periods (less than 24 hours for bacteria and under 48 hours for metabolites) [12]. This system, designed for easy application, allows the examination of various conditions within a single experiment [12]. Despite its advantages, this model does not support co-culture with strictly anaerobic bacteria due to the necessity of maintaining aerobic conditions to ensure the survival of the organoids [12].

However, the shift from a 3D to a 2D model in studying host-microbe interactions has drawbacks, such as potentially overlooking vital spatial and morphological characteristics of *in vivo* conditions [12]. This can limit understanding of 3D cellular behaviours and lead to an oversimplified representation that might not fully capture the complexity of the FRT. Despite these limitations, 2D monolayers serve as a useful bridge between cell culture techniques and complex 3D models and continue to evolve, offering insights into infections, immune responses, and host-pathogen dynamics. They remain a prominent tool, likely to be refined and expanded in future biological research.

3.5.2. Air-Liquid Interface

The Air-Liquid Interface (ALI) technique, wherein cells are cultured on a permeable support exposed to both air and liquid medium, offers a physiologically relevant environment more akin to natural bodily conditions than traditional methods [12]. Especially, when studying anaerobic bacteria, which demand controlled oxygen levels, conventional transwell cultures falter. ALI's

capability to create low-oxygen conditions thus emerges as a pivotal tool for investigating these bacteria's relationships with host tissues [20]. In groundbreaking research by Zhu *et al*., a polarized vaginal epithelium was recreated using 3D ALI culture, leading to a novel Herpes simplex virus-2 (HSV-2) infection model. This model, besides showcasing HSV-2 susceptibility, offers deeper insights into its infection mechanisms, drug target identification, and therapeutic efficacy evaluations [31].

However, while ALI cultures and 2D organoid-derived monolayers present immense advantages, they bear limitations. For instance, the need for abundant ECs for initialization, and potential inaccuracies in representing epithelial cell dynamics, pose challenges [12]. Nonetheless, evolving 3D-centric technologies, such as light-sheet, 2-photon microscopy, and microfluidic chips forming tubular organoid structures, signify a likely amalgamation of 2D and 3D methods [32], [33]. This synergy promises enhanced *in vitro* models, capturing nuances like mucin release from the host. The fusion of 3D organoids into 2D monolayers, combined with ALI and emerging technologies, augments *in vitro* study potential. Such integrative methods are set to redefine our comprehension of microbe-epithelial cell interplay in the FRT and open new avenues for therapeutic interventions [12].

Tabel 2. Advantages and disadvantages of organoid models to study host-microbiota interactions in the FRT

Model	Advantages	Limitations	References
Suspension Culture	**Simplicity**: Easy to set up and handle. **Scalability**: Suitable for large-scale cultivation. **Uniformity**: Can provide homogeneous cell distribution and exposure to nutrients. **Cost-Effective**: Generally less expensive in terms of equipment and materials.	**Limited Complexity**: May not accurately mimic in vivo conditions or interactions. **Shear Stress**: Cells can be damaged due to agitation. **Lack of Structure**: Absence of tissue architecture may not allow for realistic host-microbe interactions.	[20], [21], [22]

Model	Advantages	Limitations	References
Fragmentation	**Preservation of Structure**: Maintains some of the in vivo architecture. **Versatility**: Can be applied to various tissues. **Accessibility**: Does not require highly specialized equipment.	**Inconsistency**: Fragment size and shape can be variable, affecting results. **Limited Longevity**: May not support long-term studies. **Potential Damage**: Risk of harming cells during the fragmentation process.	[20], [23], [24]
Microinjection	**Precision**: Allows for targeted delivery of microbes or substances. **Control**: Enables control over the quantity and location of the injection. **Applicability**: Strict anaerobes can be introduced for shorter assays.	**Technically Challenging**: Requires specialized skills and equipment. **Time-Consuming**: Not suitable for high-throughput studies (if done manually) **Risk of Damage**: Potential harm to cells at the injection site. Cannot sample microbiota during co-culture	[12], [25], [20], [26], [27]
Polarity Reversal	**Access to Apical Surface**: Reversing the polarity exposes the apical surface, providing a more realistic platform for studying host-microbe interactions.Can sample microbiota during co-culture **Enhanced Study of Specific Interactions**: Offers a novel perspective to explore specific cellular interactions, including those involving luminal microorganisms. **Integration with Other Models**: Can be combined with other *in vitro* methods, expanding the range of possible studies. **Potential for Personalized Medicine**: May allow for individualized analyses based on patient-derived organoids, leading to more tailored treatments.	**Complex Procedure**: Reversing polarity might require advanced techniques and expertise, making it more challenging to implement. **Potential Loss of Structural Integrity**: The procedure might disrupt the integrity of the organoid, affecting the accuracy of the model. **Limited Applicability**: May not be suitable for all types of organoids or tissues, restricting its universal use. Can only sustain the growth of facultative anaerobes for short-term assays. **Cost and Resource Intensive**: Might require specialized equipment and reagents, adding to the overall cost and complexity of the study. **Potential for Artefacts**: Manipulating organoid polarity might induce artificial conditions that do not accurately reflect the *in vivo* situation, leading to potential misinterpretations of the results.	[12], [28], [29]
2D Monolayers	**Controlled Environment**: Facilitates the study of specific interactions. **Accessibility**: Easier to image and manipulate compared to 3D models. **Standardization**: Allows for more consistent and replicable conditions.	**Limited Complexity**: Lack of 3D structure might not fully represent *in vivo* conditions. **Potential for Artificial Interactions**: Might not accurately mimic host-microbe relationships.	[12], [20], [30]

Model	Advantages	Limitations	References
Air-Liquid Interfaces	**Mimics *In Vivo* Conditions**: Represents natural barrier interfaces like mucosal surfaces. **Flexibility**: Can be applied to different cell types and tissues. **Suitable for Long-Term Culture**: Supports differentiated and polarized cells.	**Complexity**: Requires careful control of conditions and handling. **Cost**: May be more expensive due to specialized equipment.	[12], [20], [31]
HuMix	**Human-Microbe Interaction Modeling**: Specifically designed to study human-microbe interactions. Strict anaerobes can be introduced. **Controlled Environment**: Precise control over various factors like pH, temperature. **Integration**: Allows for integration with other methods and technologies.	**Specialized Requirements**: Needs unique expertise and equipment. **Cost**: Can be expensive to set up and maintain. **Limited Availability**: May not be accessible to all researchers.	[12]
Organ-on-a-Chip	**Highly Realistic**: Mimics *in vivo* structure, function, and dynamics. **Precision Control**: Allows for control over physical and biochemical conditions. **Versatility**: Can model various organs and systems.	**Complexity**: Requires specialized knowledge, skills, and equipment. **High Cost**: Initial setup and ongoing maintenance can be expensive. **Scalability**: May not be suitable for large-scale studies.	[42], [44], [45], [46], [47]

4. Integrating Organoids into Microfluidic Devices

The intersection of microfluidics and cell culture has led to the evolution of OOC technology [34]. By mimicking human organ microarchitecture and functions on a microfluidic platform, these devices enable precise, controlled cellular behavior studies, leading to innovations in personalized medicine and understanding complex biological processes. The merger of organoids with microfluidic culture apparatuses heralds the genesis of organoids-on-a-chip (OdOC) [17]. In these, minute networks of fluid channels meticulously dictate the organoid's cellular environment [17]. This precise control ensures: spatiotemporal modulation of morphogens and nutrients for organoid growth and organisation; the introduction of physiological forces (such as fluid shear stress and strain; vascularization by co-culturing with endothelial cells); high-throughput drug testing by simultaneously nurturing diverse organoids; and continuous surveillance of organoid dynamics using

embedded sensors [35]. These features render the OdOC a versatile and potent tool to assess the influence of certain aspects, such as the microbiome, on physiology. In OdOC, microfluidic channels and chambers are thoughtfully engineered to guide fluid flow and house the organoids, connecting them to other compartments (Figure 3) [17]. These organoids can be formed directly within the device or be incorporated from pre-formed organoids [17]. What makes the microfluidic platform remarkable is its capacity to precisely govern biochemical factors like nutrients, growth factors, oxygen, and even microbes and microbial products through fluid flow. However, what potentially makes the microfluidic platform necessary is the fact that fluid flow is able to more accurately mimic *in vivo* conditions, preventing microbial overgrowth and cell damage while facilitating physiological interactions [12]. By ensuring a consistent supply of nutrients and oxygen and the efficient removal of waste, fluid flows contribute to the health and functionality of the organoid cultures, enabling a more realistic and sustainable model for studying host-microbiome relationships. Furthermore, it can apply mechanical forces to organoids via stretchable membranes and flow-induced shear stress, promoting their maturation and facilitating more life-like tissue organisation [17]. Microfluidic devices, an integral component of the OOC paradigm, grant unparalleled benefits for organoid cultivation [36]. Given the low volume requirements, they're economically viable, consuming fewer growth factors [36]. Overall, these devices tackle and transcend the constraints of traditional static organoid cultures, optimising control, reproducibility, and functionality. This integration not only permits precise control over the organoid's biochemical and biophysical microenvironment but also opens up new opportunities to enhance organoid maturation and incorporate vascularization and mechanical forces, ultimately allowing for the development of meticulously controlled studies of host-microbe interactions [36].

4.1. The Current State of Organoid-on-a-Chip Models

Currently, OdOC is a nascent field where much of the findings are based on organ systems other than the FRT, but the core concepts and techniques can be adopted to create FRT OdOCs. OdOC for different organs such as the brain, liver, intestine, and kidney have been successfully developed [17]. For instance, brain organoids have been developed using high-throughput micropillar array chip design and a perfusable chip system with parallel multichannels, enabling *in situ* neural differentiation and brain organoid formation on a single device [17]. These organoids have been able to replicate various aspects of neuronal differentiation and cortical organisation. Liver organoids have been similarly advanced with controlled EB formation using a perfusable micropillar chip system to demonstrate enhanced hepatic-specific functions [17]. Intestinal organoids have been cultivated using a multilayer chip design, generating polarised intestinal folds with multiple epithelial subtypes for modelling biological responses [17]. Kidney organoids have been cultured on platforms like the three-lane OrganoPlate, allowing the construction of polarised kidney tubules and modelling various kidney diseases [17].

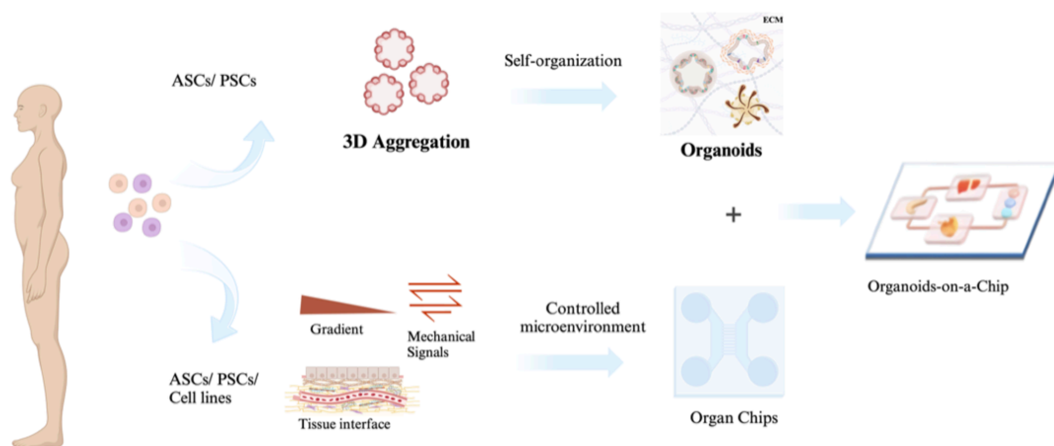

Figure 3. Diagram of engineered organoids-on-chips illustrates the combination of organs-on-chips with organoids. Organoids are 3D structures made up of multiple cells, originating from human pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), or adult stem cells (ASCs) through self-organisation. Organ chips are employed to engineer organoids by directing the differentiation, organisation, and formation of organoids within a regulated microenvironment. This enhances the functionality and maturity of the organoids, furthering their potential in biomedical research. Alternatively, organoid derived cells are seeded onto organ chips where they continue to develop in a microfluidic environment [17].

A notable example by Kasendra *et al*. involved creating a primary human small intestinal chip containing ECs from healthy intestinal biopsies [37]. Organoids were cultured, fragmented, treated enzymatically, and seeded onto a specially designed microfluidic OOC. Over 12 days, the fragments grew into continuous epithelium displaying villus-like structures, co-cultured with primary human intestinal microvascular endothelial cells (HIMECs), emulating the motions of a living human intestine. This model allowed for the investigation of various cell types and structural features within an *in vivo*-relevant culture microenvironment. Transcriptomic analysis revealed that the intestinal chip closely mimicked the whole human duodenum *in vivo*, and sequential analysis of fluid samples quantified various *in vitro* functions such as nutrient digestion and intestinal barrier function. These OdOC devices demonstrate significant promise for research in areas like metabolism, infection, drug pharmacokinetics, personalised medicine, and microbiome and host-microbe interaction studies, showcasing the potential of this nascent field in advancing our understanding of complex biological systems.

4.2. Host-Microbe Interaction Studies Utilising Organ-on-a-Chip and Organoid-on-a-Chip

The burgeoning field of OdOC technologies is realising the potential to study complex organ structures and functions, especially the interaction between host and microbes. Notably, Tovaglieri *et al*. used colon organoids in a gut-on-a-chip system to explore species-specific responses to E. coli metabolites [38]. The Colon Chip, featuring two parallel microchannels separated by a 7μm porous ECM membrane, simulated the large intestine's internal environment, offering a model for future host-microbe studies [38]. The findings revealed the role of gut microbiome products in enhancing epithelial injury and influencing key virulence pathways. This approach could be applied to study host-microbe interactions in other organ systems, providing insights into species-specific susceptibilities and responses and guiding targeted treatments or prevention strategies.

Similarly, Gazzaniga *et al*. employed mouse colon chips to investigate the host's response to *Salmonella typhimurium infection*, enhancing our grasp of both the infection process and the corresponding host reactions [39]. This was achieved through the utilization of mouse intestine organoids, which were isolated from matrigel, fragmented, resuspended in mouse organoid media, and then seeded onto the basal channel of ECM-coated chips. Through the modelling of symbiotic relationships between various bacteria and the intestinal epithelium, the effectiveness of the colon chip in replicating the complex dynamics of the gut environment was demonstrated. This system's ability to visualise bacteria, evaluate pathogenic responses, and explore colonisation dynamics provides a valuable instrument for comprehending host-microbiome symbiosis. The depth of understanding and flexibility shown in this study may be applied to investigating host-microbe interactions within the FRT. This approach has the potential to aid in the examination of FRT-specific pathogens or symbiotic relationships, subsequently leading to the development of targeted interventions or therapies. Such models demonstrate the OdOC's capability to culture complex microbiomes, simulating dynamic flow and thereby creating an organ-level context that can be precisely manipulated [17].

4.3. Utilising microfluidic models to study the FRT

The complexities of the FRT, including menstrual cycles and fertilisation, necessitate sophisticated models [10]. Recent strides have been made in developing organoids from reproductive components like ovary and FT organoids, which are pivotal for folliculogenesis and embryo transport [40]. Microfluidic platforms have allowed the emergence of ovary and FT OOCs, illuminating folliculogenesis, ovulation, and fertilisation processes [41]. These could be further enhanced by introducing microbes relevant to the reproductive tract to study microbial impacts on ovarian function.

Recent technological strides have shed light on FRT physiology and pregnancy through tissue-specific 3D geometry, dynamic fluid flow, and intricate cellular structures [36]. Notably, research has simulated a 28-day menstrual cycle and delved into the placental barrier [42-43]. Blundell *et al.* devised a device echoing the human placental barrier's architecture and functions [43]. This innovation enables the integration of specific microbial strains into its maternal compartment, closely simulating *in vivo* microbial scenarios and illuminating microbes' impact on placental roles. Additionally, microfluidic systems have enhanced *in vitro* fertilisation-embryo transplantation (IVF-ET), addressing challenges like suboptimal fertilisation rates [44]. Wei-Xuan *et al.*'s "uterus-on-a-chip" emerges as a compelling substitute for traditional IVF-ET techniques, promising the inclusion of microbes to probe their effects on conditions such as infertility and endometrial cancer [45]. Likewise, dual-chamber microfluidic devices facilitate the emulation of intricate *in vivo* scenarios, enriching our grasp on endometrial functions [46].

Lastly, multi-organ reproductive system models such as Jang, K.-J. *et al.'s,* and Xiao *et al.*'s complete FRT models provide more holistic studies [47], [42]. The EVATAR model, developed by Xiao *et al.*, is a groundbreaking tool in studying FRT health. It uses advanced microfluidic systems to simulate the human menstrual cycle and pregnancy, allowing for comprehensive exploration of hormonal signals, follicle maturation, and organ interactions [42]. The model can mimic *in vivo* conditions and offers promising insights for future research. It's worth investigating if organoids can be used as substitutes for tissues in the original study, but the EVATAR model holds potential for various future applications. Microbes could be integrated into a controlled microfluidic environment, allowing for authentic study of host-microbe interactions. The EVATAR model could study microbial colonization and dynamics in the FRT, elucidating how microbes colonize different organs and how microbial populations change over time in response to hormonal signals. Real-time monitoring and

safety protocols ensure accurate observations and compliance. Overall, emerging techniques show great potential for reproductive health research and studying host-microbe interactions in OdOC.

5. Modelling specific properties of the FRT

Many microbes require specific structural and functional features to interact with their host epithelium, which could be impacted if the *in vitro* organoid model does not recreate relevant *in vivo* conditions [18]. As revealed in this section, this can involve mimicking an appropriate developmental stage, the mucus layer, the immune system, and proper oxygen levels.

5.1. Modelling Age-Specific Properties of the FRT Epithelium

Modelling age-specific properties of the FRT epithelium is a key component for understanding fertility, particularly as ageing profoundly affects the FRT's physiology [48]. Endometrial organoids derived from postmenopausal women exhibit altered morphology and reduced hormone responsiveness, highlighting the utility of organoids in modelling age-related properties of the FRT epithelium [49]. This also entails that when generating ASC-derived organoids, features like the age of the donor need to be accounted for, especially when conducting host-microbe studies, as age-specific properties have been shown to affect microbial communities. For instance, the ovulatory cycle's influence on microbial composition, with oestrogen and progesterone causing changes in epithelial thickness and glycogen deposition, leads to different community state types (CST) with various *Lactobacillus* species [50]. Menopause results in a reduction in *lactobacilli*, associated with higher follicle-stimulating hormone (FSH) levels and lower oestrogen levels, linked to vaginal dryness and atrophy [49]. A study conducted in Brazil revealed that postmenopausal oestrogen deficit affects the vaginal microbiome, reducing *lactobacilli* and correlating with decreased serum oestrogen levels [51]. The relationship between the microbiome and postmenopausal vaginal symptoms was found to be related to the bacterial vaginal population, though more robust studies are needed for

confirmation. These insights into the age-specific properties of the FRT epithelium and their relationship with microbial interactions are vital for fertility studies. The ability to model these complex dynamics through organoids and other methods offers promising avenues for understanding and potentially addressing fertility challenges across different stages of a woman's life. By taking into account the age of the donor, more accurate and representative models ought to be created that reflect the physiological conditions of the FRT at different life stages.

5.2. Modelling Oxygen Levels

Oxygen levels, which are not uniform across different bodily environments, play a crucial role in influencing microbial localization and various biological processes in the reproductive tract [51]. Low oxygen levels prevalent in reproductive tissues foster stem cell maintenance, while anaerobic or microaerophilic conditions favor the growth of specific reproductive tract microbes [50]. Conversely, changes in local oxygen levels during inflammation and disease may dramatically alter host-microbe dynamics [52]. However, replicating these conditions within organoid models is complex, as the standard oxygen concentration in organoid cultures (around 20%) contrasts sharply with the *in vivo* reality of less than 5% oxygen [11], [51]. Creating stable and reproducible oxygen gradients across organoid cultures presents a technical challenge, but recent advances such as microfluidic organoid culture devices and oxygen-permeable scaffolds offer promising tools for maintaining controlled oxygen levels [17]. The co-culture of obligate anaerobes with organoids, along with computational modelling, marks significant milestones in refining oxygen-controlled organoid culture systems [17].

The human-microbial cross-talk module (HuMiX) represents a significant advancement in this field, allowing anaerobic bacteria to be maintained in an almost anoxic compartment [12]. Established in gastrointestinal host-microbe interaction systems, HuMiX consists of three parallel microfluidic chambers separated by semipermeable membranes with a modular design that facilitates

disassembly and cell collection. Successfully co-cultured with anaerobic bacteria like *B. caccae* and *L. rhamnosus*, as well as immune cells, the system is being further developed to become the immuno-HuMiX, allowing the coexistence of patient-derived microbiota, ECs, and immune cells, although the mucus-coated membrane currently prevents direct host-microbe contact [12].

Mastering the incorporation of oxygen within organoids will significantly enhance their utility for modeling symbiotic and pathogenic host-microbe interactions in reproductive tissues. Future co-culture systems must strive to incorporate realistic oxygen gradients, reflecting true *in vivo* conditions more accurately. By defining the optimal oxygen conditions for organoids and associated microbes, invaluable insights into reproductive diseases can be gleaned, illuminating the path to understanding the complex role of oxygen in shaping host-microbe interactions in the reproductive tract.

5.3. Modelling the Influences of the Hormonal Cycle

The FRT is a dynamic system where the endometrium, ovaries, and cervix are influenced by hormonal changes that occur throughout the menstrual cycle, affecting cellular and molecular events, resident microbiota, and thereby host-microbe interactions [48]. These hormonal shifts, like rising oestrogen levels, induce remarkable remodelling in the endometrium, affecting aspects like the proliferation of glands and the maturation of functional layers [48]. Turco *et al.,* used organoids to model how glandular endometrial organoids respond to ovarian sex hormones, revealing specific hormonal effects on differentiation and showing the potential for studying the FRT microbiome's role in these processes [49]. Additionally, a microfluidic ovary-FT co-culture device has demonstrated oestrogen-mediated inter-organ signalling [42]. Modelling the hormonal cycle *in vitro* is vital as it would enable the study of dynamic changes such as pH, nutrient availability, and immune factors that influence host-microbe interactions throughout the cycle. Traditional studies often overlook

these temporal dynamics, but emerging bioengineering approaches, such as engineering cyclic hormone exposure in organoids and microfluidic devices, offer ways to recreate this complex and changing environment *in vitro*. This modelling is essential to understanding cyclical susceptibilities to infections, interactions between the microbiome and mucosal immunity, and probiotics' ability to persist across the cycle.

5.4. Modelling the Mucus Layer

5.4.1. Composition and Function of Mucus in the Female Reproductive Tract (FRT)

The mucus layer in the FRT is a complex and dynamic entity, playing a crucial role in various reproductive functions. Its composition and physical properties fluctuate with hormonal changes, regulating and maintaining a healthy environment for fertilisation, gestation of the embryo, and menstruation [18]. The cervico-vaginal tract, the lower part of the FRT, serves as an anatomical barrier, striking a delicate balance between facilitating sperm passage, supporting healthy commensal bacteria, and eliminating harmful dysbiotic bacteria and pathogens [53]. The mucus, predominantly secreted by the cervix, is key to maintaining tissue homeostasis [53]. However, disturbances such as bacterial vaginosis can lead to adverse health conditions including preterm birth, miscarriage, and a higher risk of HIV-1 infection [18]. Hormonal cycles significantly influence the FRT, with hypertrophic changes during the menstrual cycle and pregnancy altering mucus amount, composition, and glycosylation patterns [18]. Oestrogen dominance during ovulation results in highly hydrated mucus, while progesterone induces thicker, more acidic mucus [18]. Healthy mucus composition and mucin-binding proteins increase the adherence of *Lactobacillus* species and downregulate pathogens like *Candida albicans* [18]. Dysbiosis, however, can lead to significant changes in the mucosal barrier, impaired fertility, increased infections, and the emergence of pelvic inflammatory disease and endometritis [18].

5.4.2. Glycosylation and its Influence on Implantation

Endometrial tissue glycosylation is a vital aspect of reproductive success, influencing implantation [18]. Protein glycosylation, a post-translational modification involving the attachment of carbohydrates to proteins, is essential for endometrial receptivity and embryo implantation [18]. Glycoconjugates on the uterine surface influence embryo implantation, and osteopontin and proteoglycans help endometrial cells migrate and communicate during implantation [18]. The dynamics of uterine microbiota and glycosylated macromolecule interactions with host cells are becoming active areas of research. Commensal and pathogenic microorganisms affect FRT glycosylation, and glycans are being studied in endometrial receptivity and host-microbiota interactions in fertility and infertility-related dysbiosis. The glycome, microbiota, and host uterine tissue are being examined in conditions like endometriosis and endometrial polyps, which cause uterine infertility. Endometriosis may result from changed microbiota and glycosylation, but further research is needed to gain insights into these pathological processes [18].

5.4.3. Challenges and Innovations in Modelling Cervico-Vaginal Mucus

Despite the growing recognition of cervico-vaginal mucus, the underlying mechanisms of homeostasis, disease initiation, and adverse reproduction outcomes are poorly understood [53]. Current knowledge relies on clinical samples or mouse models, which differ significantly from humans. There is a need for human *in vitro* models that accurately represent mucus composition, structure, and function. Research has utilized various *in vitro* models, such as conventional cultures, Transwell inserts, 3D engineered constructs, and organoid models, to tackle these challenges. However, these models have limitations, and human OOC technology has been applied to overcome these challenges and model mucus biology more physiologically [53]. One notable advancement was made by Izadifar *et al.*, who developed a two-channel cervical chip lined with primary human cervical ECs [56]. This innovation, along with the human vaginal chip, has provided insights into

mucus physiology and pathophysiology [18]. A simplified alternative, the Mucus Chip, has also emerged, offering insights into mucus penetration for drug delivery [18]. OOC technology's potential extends beyond modelling, facilitating simultaneous characterization of mucus biochemical, structural, and biophysical properties [18].

For future research, it would be of useful to see if such FRT OOC models can be made using organoid derived cells or linearised organoids in order to have an accessible mucus layer. Then the properties between the differently constructed models' mucus layers could be assessed, both with microbial cultures and without. In summation, human OOC models represent a cutting-edge platform for conducting mechanistic studies of mucus formation, functions, and underlying influences of tissue biology, holding great promise for shaping the future of studies on host-microbe interactions in the FRT.

5.5. Modelling the Immune System

The mucus layer in FRT shapes the microbial community, acting as a selective barrier, influencing local immune responses, and affecting reproductive health through a complex interplay [56]. When any one of the reproductive tract microorganisms, metabolites, or immunity is out of balance, it will affect the other two, leading to the occurrence and development of diseases [53]. Organoid microbe co-cultures are emerging as powerful *in vitro* models to study host-microbe interactions, with techniques being developed to integrate immune cells into these systems. Incorporating immune cells into organoids enhances their ability to model infection, inflammation, and other disease processes driven by immune-microbe interactions.Incorporating immune cells into organoid structures entails methods like direct lumenal injection, addition to culture medium, or leveraging organoids with inherent immune cells, the latter highlighted by Yu et al. [21]. However, achieving uniform incorporation, phenotype maintenance, and accounting for variability in immune

cell sources present challenges, necessitating standardised protocols for organoid construction, microbe co-culture, and immune cell amalgamation.

Microfluidic platforms are emerging as game-changers, addressing pitfalls observed in traditional static models, which grapple with microbial overgrowth leading to cell demise. Such devices champion long-term co-cultures, ensuring an equilibrated environment through continuous nutrient replenishment and waste management [53]. Their utility has been validated in OOCs, and extending these principles to OdOC models seems a logical trajectory. Evidently, a microfluidic chip, furnished with primary vaginal epithelium, demonstrated barrier preservation when co-cultured with *L. crispatus*, while *G. vaginalis* elicited increased permeability and inflammation [55]. These avant-garde microfluidic setups emulate the body's dynamic milieu, fostering explorations into how parameters like pH, nutrient presence, and hormonal fluctuations modulate microbial activities and immune responses. Through mirroring authentic in-body conditions, such models promise unparalleled insights into immune functionalities in conditions like endometriosis, pelvic inflammatory disease, and cervical cancer. Moreover, their potential isn't limited to comprehension but extends to pioneering therapeutic avenues like targeted drug dispatch and bespoke medical care, enhancing our grasp on intervention efficacies within the FRT's complex framework.

5.6. Modelling the Interplay of Microbial Influences

The interaction of male and female reproductive microbiomes and their influence on reproductive health is an emerging area of research. The seminal microbiome, once believed to be sterile, has been found to contain diverse bacterial communities, including specific taxa like *Lactobacillus*, *Pseudomonas*, and *Prevotella*, which are associated with semen quality parameters such as sperm count, motility, and morphology [56]. Additionally, the testicular microbiome may influence spermatogenesis and fertility, and disruptions in these microbiomes have been linked to

male infertility [57]. Evidence of bi-directional transmission between the seminal and vaginal microbiomes in monogamous couples suggests a complex interplay that may either promote optimal health or introduce dysbiosis [58].

The gut microbiome further adds complexity to this system, influencing reproductive microbiomes and fertility through mechanisms such as microbial translocation and inflammation, modulation of hormone levels, and production of specific metabolites [59]. Animal studies have shown that gut microbiome changes can affect sperm quality and testicular gene expression, pointing to a significant gut-reproductive axis [60]. Studying these intricate interactions could be advanced through the use of OdOC, where organoids from different systems are co-cultured with microbes (the gut and the FRT) whilst they are linked. This approach would allow for real-time observation of how changes in one microbial community might influence another and how these changes might affect overall reproductive health. It would also enable controlled experimentation to determine cause-and-effect relationships and test potential therapeutic interventions such as probiotics, prebiotics, or microbiota transplantation.

In conclusion, the interplay of reproductive microbiomes in both males and females, as well as between organ systems, is vital to understanding reproductive health and fertility. The integration of organoids on a chip offers a promising avenue for exploring these complex microbial communities and their interactions. Continued research in this direction holds the potential to open new possibilities for diagnosing, preventing, and treating reproductive conditions and infertility.

9. Concluding Remarks

Organoid-derived technologies have unequivocally propelled the study of the FRT to an unparalleled frontier, deftly navigating the chasm between traditional cell cultures and animal models. This evolution allows for the demystification of intricate FRT interactions, revealing nuanced microbial relationships, maternal-foetal dynamics, and pathogenic implications. Yet, despite advancements in maturation and model standardization, there's an undeniable need for meticulous model refinement and standardization [10].

A structured trajectory for research is paramount. Priorities must encompass standardizing FRT organoid-microbe co-cultures with variables like donor age, hormonal cycles, and disease statuses in mind. By achieving this, crafting advanced models with both aerobic and anaerobic functionalities becomes an attainable milestone, indicating a more accurate representation of *in vivo* conditions suitable for both short and long term co-cultures. Subsequently, the progression to interconnected multi-organoid systems, integrated with their respective microbiomes and enhanced through microfluidic technologies, would represent a pinnacle in replicating the cellular intricacies of the FRT. Then models can be made which aim to have vascularization, immune, stromal, and other essential cell types incorporated. Nevertheless, the technical challenges, from achieving optimal flow rates to creating compatible media compositions, will need redoubled research focus to nurture interactions with other factors such as different cell types, microbes, and hormones.

Embracing hypotheses like probing conditions such as endometriosis through patient-specific organoid models is essential. Moreover, with the ongoing convergence of organoid technology with genomics, proteomics, and interdisciplinary insights, we're ushered into a new era of understanding FRT health [17]. While existing models grapple with complexities such as vascularization and *in vivo* condition replication, the momentum in the field is palpable. Advancements in co-culture

systems, biomaterials, and integrated screenings are promising avenues towards mimicking the *in vivo* microenvironment. In conclusion, as organoid based models continually evolve, Their synthesis with microfluidic technologies, despite inherent challenges, holds the potential to revolutionize our grasp on the role microbes play in reproductive health by allowing for multi-faceted culture conditions. This will redefine therapeutic interventions and bring personalised medicine to reproductive healthcare.

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