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Review

Host–Pathogen Interactions during Female Genital Tract Infections

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Dysbiosis in the female genital tract (FGT) is characterized by the overgrowth of pathogenic bacterial, fungal, or protozoan members of the microbiota, leading to symptomatic or asymptomatic infections. In this review, we discuss recent advances in studies dealing with molecular mechanisms of pathogenicity factors of *Gardnerella vaginalis, Mycoplasma genitalium, Mycoplasma hominis, Neisseria gonorrhoeae, Streptococcus agalactiae, Chlamydia trachomatis, Trichomonas vaginalis,* and *Candida* spp., as well as their interactions with the host and microbiota in the various niches of the FGT. Taking a holistic approach to identifying fundamental commonalities and differences during these infections could help us to better understand reproductive tract health and improve current prevention and treatment strategies.

Highlights

The microbiota of the FGT consists of a complex population of aerobic and anaerobic microorganisms, typically dominated by *Lactobacillus* spp. However, lactobacilli can be outnumbered by pathogenic microbial members causing an infection of the vagina or cervix.

The causative agents of infection can be bacteria, yeasts, or protozoa, thus presenting a very diverse group of pathogens that have developed different mechanisms to adapt to a highly challenging environmental milieu.

Despite their evolutionary distance, these agents share similar strategies of adhesion and biofilm formation, invasion into host cells, nutrient acquisition, evasion of host defenses, as well as secretion of multiple factors required for survival and epithelial damage.

The Highly Dynamic Nature of the FGT Ecosystem

The FGT is a complex and physiologically dynamic system of organs which undergo continuous and profound changes during the reproductive years [1]. The mucosal lining of the upper FGT (endometrium, endocervix, and Fallopian tubes) is made up of a single layer of columnar epithelial cells, while the mucosa of the lower FGT (vagina and ectocervix) is comprised of a stratified squamous nonkeratinized epithelium [2]. The FGT mucosal surfaces are coated with a mixture of cervical mucus and vaginal transudate called cervicovaginal secretion (CVS) and colonized by a complex microbial community – the **microbiota** (see Glossary), where most of its members exist in a **mutualistic** relationship with the host [3]. Therefore, epithelial cells, CVS and microbiota are all key components responsible for maintaining FGT health and serve as a physical, biochemical, and biological barrier against invading microorganisms [4,5].

FGT physiology and its microbiota are strongly influenced by multiple factors [6–9]. For example, in the vagina, hormone levels influence the microbiota composition and, due to low levels of estrogen, the vaginal microbiota resembles the microbial composition of the skin and the gut in the premenarchal period and during menopause [10] (Figure 1, Key Figure). In contrast, estrogen and progesterone shape the vaginal environment during the reproductive age by promoting the growth of **lactobacilli** and their dominance over other microorganisms. Estrogen stimulates the proliferation of vaginal cells and glycogen production, while progesterone stimulates the lysis of cells and glycogen release. Glycogen and its breakdown products are then metabolized by lactobacilli to produce lactic acid, which contributes to the normal vaginal pH of <4.5 [3]. Lactobacilli maintain a healthy vaginal environment by acidification, production of hydrogen peroxide and antimicrobial substances, enhancement of epithelial barrier function, and stimulation of host innate immune responses that prevent proliferation of potentially pathogenic species [11] (Figure 1).

Besides the temporal dynamic composition of the vaginal microbiota within a single individual, the microbial community also significantly varies between different individuals, making it difficult to define a single concept of 'healthy vaginal microbiota'. For example, although lactobacilli have long been thought to be the keystone species of vaginal communities [3] and represent the 'healthy microbiota', this is not necessarily the norm, since a significant proportion (~30%) of healthy women do not possess a *Lactobacillus*-dominated microbiota [12]. Apart from the vagina, the microbiota of other FGT anatomical sites is also critical. The cervix, for example, has a distinct epithelial structure and specific immune and physiological environments (including different oxygen levels and alkaline pH), which all impact the microbial composition [13,14].

A dysbiosis in the FGT can lead to infections, which are associated with an increased risk for several reproductive and obstetric disorders (reviewed in [6]). Besides poor pregnancy outcomes, FGT

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 https://doi.org/10.1016/j.tim.2019.07.006

infections can be associated with tubal infertility, outcome of *in vitro* fertilization [15,16], progression of cervical dysplasia, and development of pelvic inflammatory disease [6,17].

The majority of microbes causing FGT infections are natural members of the vaginal microbiota and are thus considered opportunistic pathogens and **commensals.** They can outnumber *Lactobacillus* spp. or other protective bacteria and can cause symptomatic or asymptomatic vaginal infections called bacterial vaginosis, aerobic vaginitis, or vulvovaginal candidiasis, depending on the causative agent (Table 1). Other pathogens can be introduced from the outside (exogenous), mostly during sexual contact, causing sexually transmitted infections (STIs) of vagina or cervix (Table 1) [18].

Although FGT pathogens represent a highly heterogeneous group of evolutionary very distant members, they share similar strategies to secure colonization and to cope with the challenges existing in the FGT microenvironment, such as the presence of lactobacilli (Box 1) and the CVS (Box 2). However, it is important to note that different anatomical regions of the FGT differ in their characteristics and can be infected by different pathogens.

In this review, we aimed to identify fundamental commonalities and differences in pathogenicity mechanisms of microbes infecting the FGT, such as adhesion and biofilm formation, invasion into host cells, secretion of virulence factors, nutrient acquisition, and evasion of host defenses. We focused on common bacterial, protozoan and fungal FGT pathogens, such as *G. vaginalis*, *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis* and *Candida* spp. (Box 3). Additionally, we included microbes which are often associated with FGT infections and vaginal dysbiosis, but potentially not the cause of FGT infections (e.g., *M. hominis and M. genitalium*), or which originate from the FGT but which are responsible for neonatal infections (e.g., *S. agalactiae*). We believe that the presence of some of these microbes within the FGT and their interactions with members of the FGT microbiota and/or other FGT pathogens (Box 4) may not only be linked to FGT infections but also that knowledge about these species may provide us with valuable insights into microbial strategies used to persist in the FGT milieu.

Adhesion and Biofilm Formation

Although epithelial surfaces are normally protected by mucus (Box 2), pathogens can pass through this layer and can attach to epithelial cells to prevent their elimination through natural mucus clearance mechanisms. Therefore, adhesion to epithelial cells is an initial step and important prerequisite for the onset of infection [19,20].

Microbial adhesion to other cells (microbial or human) or to abiotic surfaces can be unspecific (hydrophobic interactions), but is mostly based on specific protein ligand (adhesin)–receptor interactions [21]. For example, the lipophosphoglycan of *T. vaginalis* can bind to galectin-1, a host protein belonging to the family of lectins that specifically binds galactose-containing carbohydrate structures, thus facilitating the adhesion to cervical epithelial cells [22]. Similarly, initial adherence of *N. gonorrhoeae* to host epithelial cells is mediated by type IV pili (T4P), which bind to I-domain-containing integrins on primary cells derived from cervical epithelia [23]. This is followed by tight adherence via opacity proteins (Opa) in the bacterial outer membrane which bind to carcinoembryonic antigen-related cell adhesion molecules (CEACAM) and heparan sulfate proteoglycans on epithelial cells along the FGT [24]. Specifically, Islam *et al.* showed that Opa–CEACAM5 binding on vaginal epithelia facilitates the long-term colonization of the lower genital tract [24].

Besides integrins, host extracellular matrix (ECM) proteins can also be a target for the binding of microbial adhesins. For example, major nonpili *S. agalactiae* adhesins are the fibrinogen-binding protein A (FbsA) [25] and the secreted laminin-binding protein Lmb [26]. Microarray analysis detected a significant upregulation of the adhesin genes *dpsA* and *glnQ* at a low pH [27,28], indicating another adaptation of this pathogen to the acidic vaginal environment. Interestingly, PbsP, although previously described as a plasminogen-binding protein, was recently discovered to be a dual adhesin that can also interact with human vitronectin through its streptococcal surface repeat (SSURE) domains, leading to invasion (discussed later) [29].

Glossary

Antimicrobial peptides (AMPs): host-derived defense peptides that act against microbes; they are found in a wide variety of life forms from microorganisms to humans.
Biofilms: complex microbial communities attached to an inert or living surface, held together by a self-produced matrix.
Commensalism: a relationship in which one species benefits from the host species, which is unaffected.
Lactobacilli: Gram-positive, facultatively anaerobic or microaerophilic, rod-shaped bacteria that convert sugars to lactic acid; they are important members of the microbiome of the gut and the urogenital system.
Microbiota: a community of bacteria, archaea, protists, fungi, and viruses found in and on a given multicellular organism.
Mutualistic: refers to the relationship between two species in which each benefits from the other.
Th17 T cell immune response: an immune response with the main role of maintaining mucosal immunity that involves T helper 17 cells, defined by their production of interleukin 17.

Key Figure

Dynamics of the Vaginal Microenvironment

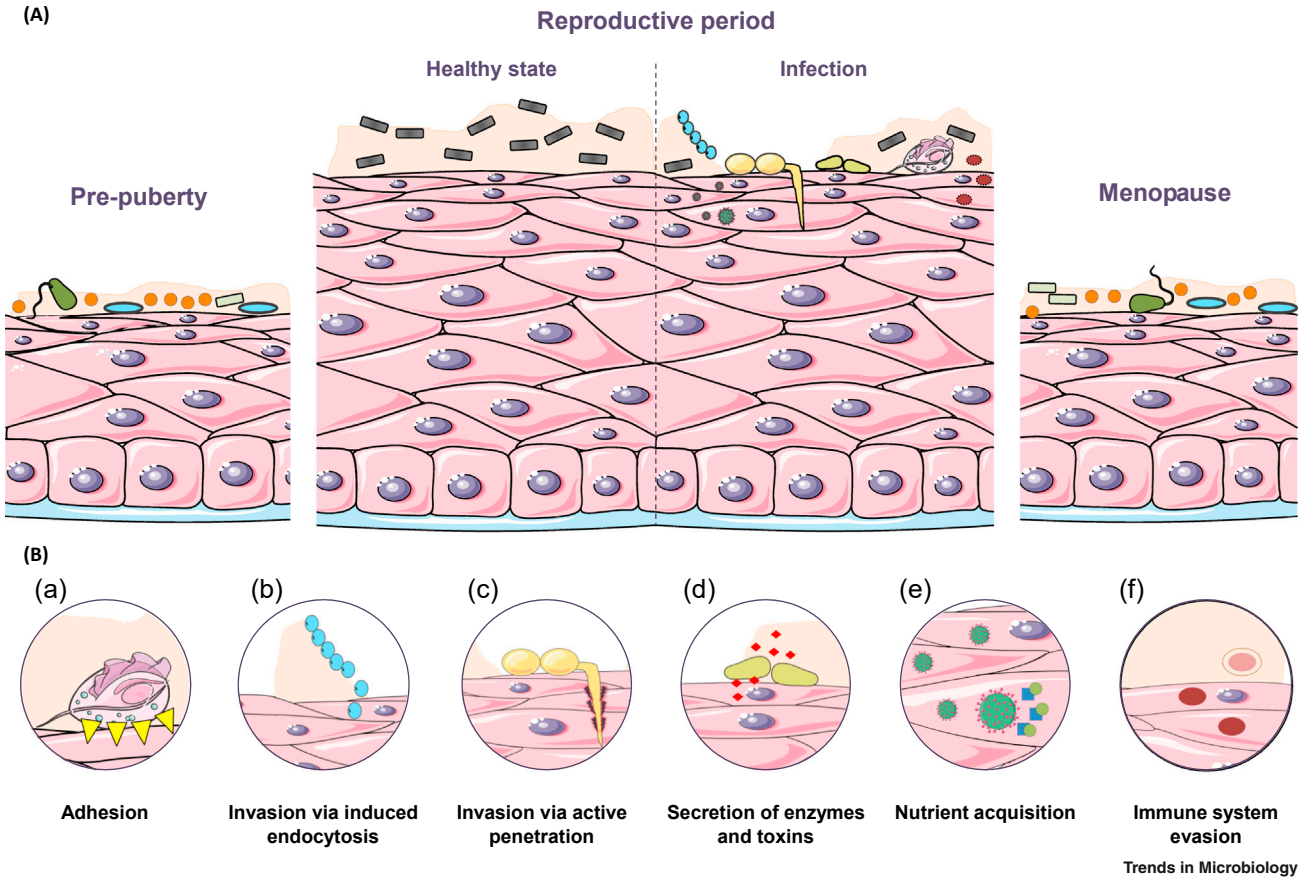

Figure 1. (A) Overview of the temporal dynamics of the female reproductive tract (FGT) microbiota composition showing the vaginal mucosa, stratified squamous nonkeratinized epithelium coated with mucus as an example. In pre-puberty and menopause, the vaginal microbiota is mostly composed of enteric bacteria, pH is neutral or alkaline, and the level of glycogen is low. In the reproductive age, the epithelium becomes thicker, pH is acidic, and the level of glycogen rises. The vaginal environment oscillates between a healthy state (usually dominated by lactobacilli) and an infection, where different pathogens can dominate and cause the infection. (B) Overview of different virulence strategies of pathogens covered in this review. In order to secure colonization and promote the infection, these pathogens have to: (a) express adhesion molecules in order to attach to host cells; (b) invade host cells by induced endocytosis, or (c) carry out active penetration; (d) secrete different virulence factors, such as enzymes and toxins; (e) acquire nutrients for their survival, and (f) successfully evade the host defenses. Note: Apart from invasion, all of these mechanisms are characteristic for all pathogens discussed; however, *Trichomonas vaginalis, Streptococcus agalactiae, Candida albicans, Gardnerella vaginalis, Neisseria gonorrhoeae*, and *Mycoplasma hominis* (respectively) are shown here as illustrative examples. This figure was created with images adapted from Servier Medical Art by Servier. Original images are licensed under a Creative Commons Attribution 3.0 Unported License.

A dual role of the same protein in both adhesion and invasion has also been observed in *Candida albicans*. The gene encoding the major hypha-associated adhesin Als3 (agglutinin-like sequence 3) is strongly upregulated during epithelial infection *in vitro* [30]. However, this protein also acts as an invasin by binding to host cell receptors such as E-cadherin and N-cadherin, thereby inducing endocytosis by host cells [31,32]. Additionally, Als3 plays an important role in biofilm formation, and facilitates iron acquisition from host cell ferritin [33,34]. Other adhesins of *C. albicans*, including Hwp1, Hwp2, and Rbt1, also have multiple functions, mostly related to biofilm formation [35]. Genomic

Table 1. Female Genital Tract (FGT) Infections: Types, Main Characteristics, and Causative Agents

Type of FGT infection	Main characteristics	Main causative agents	Refs
Bacterial vaginosis (BV)	Increased vaginal pH (≥4.5); white adherent discharge with exfoliated epithelial cells and Gram-variable polymorphic rod-shaped bacteria attached to their surfaces (clue cells)	Often polymicrobial, mainly anaerobic microorganisms including *Gardnerella vaginalis*, *Mycoplasma genitalium* and *Mycoplasma hominis*, *Prevotella* spp. and *Mobiluncus* spp.	[17,120]
Aerobic vaginitis (AV)	Increased vaginal pH (>4.5), associated with more severe inflammation than in BV	Aerobic enteric commensals or pathogens, such as *Streptococcus agalactiae*, *Enterococcus faecalis*, *Escherichia coli*, and *Staphylococcus aureus*	[17]
Vulvovaginal candidiasis (VVC)	Itching, burning, pain, redness of the vulva and vaginal mucosa, vaginal discharge; immunopathology associated with recruitment of polymorphonuclear neutrophils into the vaginal lumen that are unable to reduce the fungal burden	*Candida* spp.	[100,121–123]
Sexually transmitted infections (STIs)	Immunopathological state, where immune responses trigger inflammation and/or influx of immune cells into the lower FGT but in most cases do not eradicate the infection	*Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Trichomonas vaginalis*	[91]

data indicate that adhesin families described for *C. albicans* also exist in other pathogenic *Candida* species, such as *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. lusitaniae*, and *C. guilliermondii*, but not in *C. glabrata*, the second most common cause of candidiasis. Since *C. glabrata* is an evolutionarily distant relative of *C. albicans*, it has been suggested that they evolved their virulence independently [36], which is also demonstrated by the lack of homology between adhesion proteins. Instead, *C. glabrata* has another large epithelial adhesin (Epa) family, encoded by subtelomeric gene clusters [37].

Even though they all slightly differ, the majority of fungal adhesins maintained a similar structure as glycosylphosphatidylinositol (GPI)-proteins that are covalently bound to β-1,6-glucan of the cell wall or integrated in the cell membrane via GPI-anchors [35]. Their primary structure is conserved, a fact that can be used to identify putative adhesins by bioinformatic analysis [35].

Box 1. Challenges of the FGT Microenvironment – Lactobacilli

FGT microbial communities reside in an ecosystem that is shaped by the characteristics of the host, local environment, and microbial composition [124]. As discussed previously, in most of the cases, a healthy vaginal mucosa is colonized by one or two dominant *Lactobacillus* species (mainly *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus jensenii*, and *Lactobacillus gasseri*) [125], which strongly influence the function and structure of this ecosystem by producing lactic acid and maintaining a low pH (usually below 4.5). They also produce hydrogen peroxide (H_2O_2) that inhibits the growth of catalase-negative anaerobic organisms by the production of hydroxyl free radicals, as well as antimicrobial peptides (AMPs) such as bacteriocins, bacteriocin-like substances, and biosurfactants [5]. Moreover, they can bind to the surface of the vaginal epithelium and competitively prevent other microbes from adhesion and infection [5,126]. Vaginal lactobacilli have different adhesins and display increased adhesiveness relative to lactobacilli that exist in other environments, such as the gut [127–130]. Lactobacilli can also colonize the cervix [131], and experiments with human cervical cells showed a competition for adherence sites of lactobacilli with *N. gonorrhoeae* [132]. Obligate intracellular pathogens, like *C. trachomatis*, use host integrins to attach and enter host cells, and it has been shown that *L. crispatus* BC5 interferes with this process by reducing the α5 integrin subunit exposure on the plasma membrane of cervical cells [133]. It is important to point out that these adhesion studies were done using vaginal or cervical epithelial cells *in vitro*, where the CVS is absent, meaning that those microorganisms would not have such a direct access to host epithelium in healthy conditions *in vivo*. However, the findings are still significant considering that the CVS can be degraded and/or modified in structure and porosity, mostly during the infection (Box 2).

Box 2. Challenges of the FGT Microenvironment – Mucus

The barrier between potential pathogens and epithelial cells in FGT is the CVS. It is a mucous layer composed of mucins – large, highly glycosylated proteins that consist of up to 16% sialic acids by weight and form a viscoelastic gel that physically traps pathogens in a thick gel phase [4,87,124]. Besides mucins, other secreted proteins are present in the CVS, including AMPs and secretory immunoglobulin A (sIgA) and IgG antibodies, involved in the recognition and neutralization of antigenic microbial products [5,134].

Degradation of mucus components might be an important factor in the etiology of FGT infections. For example, *G. vaginalis* secretes a sialidase that cleaves sialic acid from glycoproteins, thus leading to the degradation, foraging, and depletion of protective host mucus barriers [87]. A significant depletion of mucus sialic acids is seen in women with vaginosis compared with healthy controls [87]. Numerous other FGT pathogens, including *N. gonorrhoeae, T. vaginalis* and *C. albicans*, are known to produce glycosidases and/or proteases that could degrade mucins and host defense components within the mucus [135,136].

In a 3D human organotypic endocervical epithelial cell model, microbial products significantly upregulated expression of MUC1, MUC4, and MUC16 mucins in the endocervix and lead to the differential induction of host AMPs [137]. Mucin and AMP expression levels were each distinctively modulated by a panel of bacterial and viral products tested, suggesting that endocervical cells selectively respond to specific microbes [137]. Mucins were shown to adhere directly to *C. albicans*, through α(1,2)fucosylatedglycans [138], and to inhibit hyphal formation, which is essential for epithelial invasion [139]. However, considering the importance of the mucus layer for preventing pathogenicity, not much is known about the interaction of pathogens with proteins of the CVS and their influence on CVS protein expression, structure, and function, especially in the human host.

Mediators of adhesion by *Mycoplasma* species are surface-associated structures such as membrane proteins, lipids, and lipoproteins [38]. The major adhesins of *M. genitalium* are P140 and P110, which form a surface adhesion complex known as NAP [39–41] that is essential for adhesion and gliding motility of the organism [40]. Both P140 and P110 are immunodominant proteins which constitute the main target of host antibodies during infection [42]. Not much is known about specific interactions with host proteins, but crystal structures of the extracellular region of P110 identified the binding domain of this adhesin to sialylated receptors, essential for *Mycoplasma* adherence [42]. The binding pocket of the P110 adhesin is conserved, but the antigenic regions exposed to the surface are highly variable [42,43], leading to immune evasion (discussed later). Other adhesins seem to have multiple functions. For example, AP65 appears to be a major adhesin, together with AP23, AP51, and AP33, of *T. vaginalis* [44]. However, besides its adhesin role, AP65 can act as a hydrogenosomal NAD-dependent decarboxylating malic enzyme, which is released into cultures during growth [45]. Similarly, this protozoan possesses many cysteine proteases (CPs) (described later) that also have an important role in cytoadherence, such as TvLEGU-1, a novel virulence factor upregulated by iron [46]. This functional diversity is not completely understood, but it demonstrates that adhesins can be proteins that possess a variety of roles and may not have evolved as adhesins *per se*.

Apart from adhering to host cells, vaginal pathogens can use adhesins to attach to each other and to form **biofilms,** complex microbial aggregates held together by a self-produced ECM [47]. Biofilm formation by pathogenic microbes is an effective strategy for survival in their hosts, either by preventing full exposure to antimicrobials and/or by escaping host defense mechanisms [48,49]. This plays an important role in infection, since the incomplete eradication of biofilms leads to a high recurrence rate of infection [50], a common problem in FGT infections. The ability to form biofilms is common for many bacterial and fungal vaginal pathogens, but this varies among the different clinical isolates of the same species [51–53]. Additionally, some species, such as *G. vaginalis*, are able to incorporate other bacterial vaginosis-associated species, yielding a polymicrobial biofilm [11] which further complicates the appropriate treatment and increases the risk of recurrence.

Collectively, this shows that adherence presents a crucial step in pathogenesis, enabling persistence of extracellular microorganisms and biofilm formation. Additionally, in the case of intracellular pathogens, adhesion is an initial step for invasion into intracellular compartments of host cells.

Box 3. Main Characteristics of Pathogens Discussed in This Review

Bacteria

M. genitalium and *M. hominis* are important urogenital pathogens, causing STIs. Both species have been detected along the FGT and are associated with FGT infections, but their contribution to the pathological process is unknown [140,141]. *Mycoplasma* spp. are the smallest prokaryote organisms capable of self-replication. With a very small genome and the absence of a cell wall, they adopted a parasitic lifestyle, relying on the host for providing the nutrients needed for survival [104].

G. vaginalis is a Gram-variable facultative anaerobic bacterium with remarkable virulence factors, such as production of the pore-forming toxin vaginolysin, sialidase secretion, and the ability to produce polymicrobial biofilms. Due to the high genotypic and phenotypic diversity, these virulence-related characteristics significantly differ between different isolates [51].

S. agalactiae (Group B *Streptococcus*, GBS) is a Gram-positive, β-hemolytic, chain-forming commensal coccus. This facultative anaerobe can be vertically transmitted from mother to neonate and cause neonatal sepsis, pneumonia, and meningitis, making maternal vaginal colonization a key risk factor for neonatal disease [142]. Since it encounters different niches, GBS possesses a precise regulation of virulence factor expression to coordinate the transcription of pore-forming toxins and factors that promote adherence, invasion, and immune evasion [143].

N. gonorrhoeae is a Gram-negative diplococcus and obligate human pathogen that is a major cause of STIs worldwide. A repertoire of virulence factors has been identified, such as phase and/or antigenic variation, expression of pili and opacity-associated proteins, efflux pumps, and nutrient-acquisition systems [88,107,144,145].

C. trachomatis is an obligate intracellular Gram-negative pathogenic bacterium, causing STIs worldwide. It has a unique biphasic developmental cycle alternating between the extracellular infectious elementary body and the intracellular reticulate body [146]. Infections are often asymptomatic, thus remaining untreated, but they can significantly complicate the reproductive outcomes [133].

Fungi

Candida spp. are yeasts normally found on the human mucosa. Although more than 200 *Candida* species have been identified, *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* remain the main causative agents. They all evolved different virulence strategies and adapted to interact with a wide range of host cells and molecules, both during commensal colonization and various disease manifestations [32,36].

Protozoa

T. vaginalis is an extracellular parasite, with the biggest protist genome currently sequenced [147]. *T. vaginalis* lacks mitochondria and peroxisomes, but it produces hydrogenosomes in which the fermentative oxidation of pyruvate and ATP production take place [148]. It also secretes exosomes and hydrolytic enzymes which favor its survival, influencing the vaginal microbiota and modulating the host response [147,148].

Invasion into Host Cells

Entry into host cells and a transient intracellular lifestyle can have advantages for FGT pathogens: it protects them from the immune system, antimicrobials, and mucus clearance, and can provide access to intracellular nutrients [54,55]. Entry is secured by invasion, and the most common mechanism for bacteria is induced endocytosis: the ability of bacteria to induce their own uptake by nonprofessional phagocytic host cells [56]. Since invasion is the step following adhesion, many adhesins are also involved in the process of invasion (discussed above). Induced endocytosis using 'zipper' mechanisms slightly varies based on the protein partners involved, but basically means that the binding of microbial adhesins to the host cell receptors initiates cytoskeletal reorganization to induce membrane ruffling and subsequent microbial uptake. As a consequence, the microorganisms are internalized, followed by the induction of different signaling pathways for intracellular survival and trafficking [57]. Since cytoskeletal rearrangements are important for cellular invasion, microbial cells often target integrins – host transmembrane proteins that link ECM proteins to the actin cytoskeleton. *C. trachomatis* has developed several strategies to enter host cells, and recently the adhesin and

Box 4. Microbial Interactions within the FGT Microenvironment

FGT pathogens are constantly interacting with other members of the microbiota. A substantial number of synergistic or antagonistic associations have been described for different microbial partners in different niches; however, not much is known for the FGT environment. Besides the competition for the same spatial and nutritional resources, recent data show that such interactions could play an important role in cocolonizations or coinfections.

In some cases, FGT pathogens can take advantage of others and ensure their survival. For example, *C. trachomatis* is a tryptophan auxotroph and relies on host sources which are limited due to a protective effect of human interferon gamma (IFNγ) via tryptophan catabolism. However, IFNγ exposure induces an upregulation of chlamydial genes that can synthesize tryptophan through indole salvage [149]. This salvage pathway is possible during coinfections since other FGT pathogens (such as *Prevotella* spp., Clostridiales and *T. vaginalis*) can express a functional tryptophanase to produce indole from tryptophan [150].

Interestingly, *S. agalactiae* and *C. albicans* may promote their cocolonization: When incubated together, adhesion of both is significantly increased [142]. The adhesin Als3 expressed on *C. albicans* hyphae and the *S. agalactiae* antigen I/II family adhesins are responsible for this synergistic interaction by ensuring direct physical contact [142]. However, another recent study showed that *S. agalactiae* significantly attenuated hyphal formation of *C. albicans* [151]. This coinfection was associated with the **Th17 T cells immune response** and decreased host immune clearance, which may promote *C. albicans* colonization in a mouse model of vulvovaginal candidiasis [151].

T. vaginalis is known to carry *M. hominis* intracellularly (detection rate 25–89%) and transmit it to mycoplasma-free recipient *T. vaginalis* cells, but also to human epithelial cells *in vitro*, suggesting a potential role of the protozoan in transmitting the bacterial infection to the human host [152]. This is the first symbiosis described in which two obligate human mucosal pathogens are able to invade and infect the same anatomical region, with both agents capable of producing independent diseases [153]. *M. hominis* benefits from the intracellular location and supply of putrescine, essential for normal cellular growth and multiplication. However, the energy metabolism of both pathogens during symbiosis is increased, leading to an increased growth rate of *T. vaginalis* [152]. Despite the wide range of species known to be members of the FGT microbiota, their ecological functions are largely undetermined, but are most likely shaped by coevolutionary processes between the human host and specific microbial partners [3].

invasin Ctad1 has been shown to interact with the integrin β1 subunit and induce clustering of integrins at the attachment sites, leading to invasion of HEp-2 epithelial cells [58]. Indirectly, pathogens can also bind to components of the ECM that are bound to integrins, such as the previously mentioned *S. agalactiae* PbsP that binds vitronectin and mediates invasion of epithelial cells by exploiting the host vitronectin/αv integrin axis [29].

Endocytosis can also be triggered by binding to cadherins – host proteins that form cell–cell interactions. For example, *C. albicans* Als3 mimics host cell cadherins and induces endocytosis by binding to N-cadherin on endothelial cells and E-cadherin on oral epithelial cells [31]. It is likely that similar processes also cause *C. albicans* invasion into vaginal epithelia, since E-cadherin seems to play an important role during vaginosis [59]. The levels of soluble E-cadherin (a by-product of proteolytic cleavage of E-cadherin) are in fact a molecular marker of cervical epithelial barrier disruption and are significantly increased in mice colonized with *G. vaginalis* [59]. Alternatively, invasion by induced endocytosis using a 'trigger' mechanism involves bacterial secretion systems that inject proteins that often mimic or hijack specific host cell factors to manipulate a variety of signaling networks. This includes the activation of small Rho GTPases and cytoskeletal reorganization that triggers bacterial uptake, and has been described for *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis* [60]. Interestingly, *N. gonorrhoeae* can also target membrane rafts and signaling pathways of cervical cells that depend on host caveolin-1 phosphorylation and cause bacterial internalization [60]. This invasion mechanism is mediated by gonococcal protein PorB subtype A and is independent of pili and Opa proteins [61].

Fungi and parasites do not depend on induced endocytosis as an invasion mechanism but can also directly invade by physical forces. Hyphae from *C. albicans* can invade host cells by two different routes: induced endocytosis, predominantly via the invasin Als3 and E- or N-cadherin (see above),

and active penetration [32]. While induced endocytosis only requires the invasin on the fungal surface, but no viability of the fungal cell, active penetration requires hyphal extension, causing physical forces at the hyphal tip, and is supported by hydrolytic activity, such as production of secreted aspartic proteases (Saps; discussed later).

In summary, the invasion mechanisms described here indicate that FGT pathogens evolved several strategies to reach the host intracellular milieu and secure proliferation by having access to nutrients and by avoiding confrontation with the host defense.

Secreted Factors for Survival and Epithelial Damage

Microbial pathogens secrete numerous compounds in order to obtain nutrients, disrupt epithelial barrier function, damage cells, and activate or modulate host immune responses [62–65]. In most cases, pathogens secrete extracellular hydrolytic enzymes, and despite their variety, proteinases are by far the enzymes that are most commonly associated with virulence [66–68]. *T. vaginalis* possesses ~440 proteinase-coding genes, showing one of the most complex degradomes described, half of which (~220) belong to the subtype of CPs [69,70]. CPs are involved in diverse processes such as adhesion, invasion, cytotoxicity, cytoskeleton disruption of red blood cells, hemolysis, and evasion of immune responses, and their expression is differentially modulated by environmental changes [70], especially by host iron levels [71].

The predominant extracellular hydrolases produced by *C. albicans* are Saps, phospholipases, and lipases. Of these, Saps have been the most comprehensively studied as key virulence determinants [66]. Their main biological function may be the enzymatic digestion of complex extracellular proteins for nutritional purposes, and their secondary (perhaps unintended) function is involved in virulence, including innate immune signaling, damage, and enhanced colonization [72,73]. The *SAP* genes are highly expressed during both rat and mouse vaginal infections, and have varied levels of expression in clinical samples [74,75]. However, overexpression of *SAP2* or *SAP5* in a murine model of vaginitis was insufficient to induce robust vaginitis immunopathology, which suggests that the physiological contributions of Saps to vaginal immunopathology require additional factors, such as hypha formation (and toxin production, see below), in order to cause a symptomatic infection [72].

Pore-forming toxins (PFTs) and proteins are further examples of secreted factors commonly found in the microbial world [76]. *G. vaginalis* secretes vaginolysin (VLY), a PFT belonging to the family of cholesterol-dependent cytolysins which generally recognize and bind to membrane cholesterol to create pores and lyse target cells. VLY is selective for human cells, and host specificity occurs through recognition of the complement regulatory molecule CD59 [77]. The CAMP factor is a PFT secreted by *S. agalactiae* that binds GPI-anchored host proteins [78] and causes heterogeneous circular membrane lesions of up to 12–15 nm in diameter. Further analysis by protein truncation and site-directed mutagenesis indicated that the N terminal 5-helix bundle is responsible for membrane permeabilization, whereas the C terminal 3-helix bundle is likely responsible for host receptor binding [79]. The cytolytic peptide toxin of *C. albicans*, candidalysin, the first peptide toxin discovered in a human fungal pathogen [65], directly damages epithelial membranes, triggers a danger response signaling pathway, and activates epithelial immunity via the epidermal growth factor receptor [80–82]. The exact mechanism of membrane disruption is unclear, but biophysical experiments with lipid bilayers composed of dioleoylphosphatidylcholine (DOPC) showed that candidalysin efficiently intercalates and permeabilizes DOPC membranes. Lesions were heterogeneous, transient, and rapidly formed, causing an inward current associated with calcium influx, similar to lesions caused by other PFTs [65].

Finally, besides enzymes and toxins, FGT pathogens secrete other molecules such as nucleic acids and proteins with unclear functions that have been associated with virulence. *N. gonorrhoeae* possesses a unique type IV secretion system which secretes single-stranded DNA (ssDNA) into the extracellular milieu, which can serve to transform highly competent *Neisseria* species, thereby increasing the transfer of genetic information. Additionally, this secreted ssDNA is involved in initial stages of biofilm formation and intracellular survival during infection [83]. *N. gonorrhoeae*, and some other

Gram-negative pathogenic bacteria, can also secrete outer-inner membrane vesicles (O-IMVs) carrying DNA, cytoplasmic and inner membrane proteins, and ATP. The functionality of O-IMVs remains unclear but they could be involved in lateral gene transfer, transfer of cytoplasmic proteins, or inter- and intraspecies communication [84].

T. vaginalis produces and secretes microvesicles similar to mammalian exosomes, characterized by the presence of RNA, conserved exosomal proteins and parasite-specific proteins. These exosomes fuse with and deliver their contents to host cells and modulate host–parasite interactions, but also promote host cell colonization by increasing the adherence and potentially play a role in parasite–parasite communication [85].

Thus, FGT pathogens secrete a large variety of molecules, from proteins to nucleic acid, using different systems for their delivery. The best described are hydrolytic enzymes with the main role of nutrient acquisition. However, many further secreted compounds seem to be associated with virulence, but their roles remain poorly understood in many cases.

Nutrient Acquisition

A fundamental requirement for pathogenicity is the ability of a microorganism to assimilate sufficient nutrients for growth within the host [86]. Virulence strategies discussed above, especially secretion of hydrolytic enzymes and invasion, lead to degradation of mucus, ECM, and entry into host cells, which provides access to nutrients, such as carbohydrates, amino acids, or fatty acids [87]. Their biosynthesis is costly because of the energy-metabolite resources needed, which explains why many pathogens and symbionts have evolved the luxury of reliance upon host resources for preformed compounds to conserve energy [88]. Besides these, transition metals (iron, zinc, and manganese), necessary for a variety of biochemical reactions and energy generation, must also be actively scavenged from infected host tissues [86,89]. Iron is one of the most abundant trace metals in organisms and arguably the one with the most diverse roles in cellular processes, including central metabolic pathways, mostly through the incorporation of iron, or the iron-containing prosthetic group heme, into the active centers of key enzymes [90]. The host binds and transports iron using different proteins, such as hemoglobin, transferrin, lactoferrin, and ferritin, while free levels are maintained at extremely low levels, thus limiting availability to potential microbial pathogens [86,88]. Increased endogenous human lactoferrin has been found in the CVS of women suffering from *N. gonorrhoeae, C. trachomatis*, and *T. vaginalis* infections [91], which functions to sequester the iron necessary for microbial survival and growth, and could represent a marker of unhealthy conditions [92]. These sophisticated sequestration mechanisms evolved by the host to limit microbial access to micronutrients is termed 'nutritional immunity' [93]. In order to counteract host iron-binding proteins and enable iron uptake, microorganisms have developed different strategies, such as secretion of siderophores, a heterogeneous class of small molecules, to bind extracellular ferric iron with extremely high affinity [90]. However, some species, such as *G. vaginalis*, are unable to produce siderophores, so they acquire iron by lysing erythrocytes through hemolysin production, utilizing hemoglobin as an iron source [92], which is consistent with the observation that *G. vaginalis* levels increase during menses [94]. Other species (such as *N. gonorrhoeae, C. albicans*, and *C. glabrata*) rely on xenosiderophores, that is, siderophores produced by other microbes, or use these as an additional iron source [88,90]. *Neisseria* species overcome nutritional immunity not only by using xenosiderophores, but also host metal-sequestering proteins as metal sources, without the deployment of siderophore intermediates [88]. The neisserial transferrin receptor system is specific for human transferrin [95] and exclusively binds to and extracts iron from the C-lobe [96]. Similarly, *C. albicans* can use heme as an iron source [97], and the hyphae of this fungus have been shown to bind host ferritin at the hyphal cell surface, both *in vitro* and during coincubation with human epithelial cells [34]. This process is mediated by Als3, and is followed by a reductive iron-assimilation pathway for its utilization [34].

All in all, nutrient acquisition strategies are essential to enable survival and to promote the infection in highly competitive microbial environments. They present a complex interplay of different

mechanisms to access host cell nutrients, perform their degradation, and secure their uptake, despite counteracting host sequestration mechanisms.

Evasion of Host Defenses

Host epithelial cells employ different mechanisms to control potentially pathogenic microorganisms and prevent infection, for example, by producing **antimicrobial peptides (AMPs)** and employing metal-sequestration strategies, as discussed above. In case of infection, microbial compounds are recognized by pattern-recognition receptors (PRRs) leading to the production of proinflammatory cytokines, chemokines, alarmins, and AMPs [98]. This activates the immune response that ultimately results in leukocyte recruitment to the site of infection in an attempt to eliminate pathogens from the FGT [99]. For example, Toll-like receptor 2 (TLR2) signaling has recently been found to be important for the recognition and progression of local response in a murine model of vaginal candidiasis [100]. However, in the case of commensals, these defense mechanisms need to be finely tuned in order to avoid overstimulation of the immune response and unnecessary tissue damage. How the host distinguishes between the commensal and pathogenic state of the same species is poorly understood. For *C. albicans*, morphology seems to be the key. Both yeast and hyphae activate the same signaling pathways, but with a different intensity: in the presence of yeast cells, these are insufficient to induce immune activation, while hyphae, associated with candidalysin production and the pathogenic state, induce a far stronger response [101,102]. Similarly, a recent transcriptome study, using RNA sequencing, showed that *G. vaginalis* biofilms, in order to secure persistence, maintain low metabolic rates and do not produce VLY, thus avoiding the elicitation of a host immune response [103]. Considering that many strict pathogens cause asymptomatic infections, they have to adopt a spectrum of strategies to avoid immune defenses and ensure their persistence. *M. genitalium* possesses such an ability, leading to chronic urogenital infections [104]. Lacking any specific toxins or secreted virulence factors, stimulation of the immune response by *M. genitalium* is believed to be primarily mediated by surface-exposed lipoproteins, which probably evade immune recognition through antigenic and phase variation [104].

Host AMPs target different microbial structures, such as the cytoplasmic membrane or cell wall, but also interfere with microbial cell processes, such as DNA and protein synthesis, protein folding, and cell wall synthesis [105,106]. To counteract AMPs, *N. gonorrhoeae* possesses the MtrC–MtrD–MtrE efflux pump system that expels AMPs such as human cathelicidin LL37 [107]. The absence of this efflux system causes decreased bacterial survival in a mouse model of *N. gonorrhoeae* lower FGT infection, supporting its role in protecting against host defenses [108]. This pathogen also produces SilC and Ng_1981 that inhibit lysozyme, a host antimicrobial enzyme that causes bacterial wall degradation primarily of Gram-positive bacteria [109].

Pathogens are able to affect innate immunity not only by modulating host cytokine signaling [110], but also by triggering host cell death by induction of apoptosis or pyroptosis of macrophages. *N. gonorrhoeae* secretes PorB via outer membrane vesicles (OMVs) and targets it to mitochondria to induce apoptosis [111], while *T. vaginalis* and *C. albicans* induce NLRP3 inflammasome activation and pyroptosis [112,113]. For *T. vaginalis*, it is not yet clear which factors are involved, but for *C. albicans*, candidalysin seems to be required for triggering the NLRP3 inflammasome, but not pyroptosis [112]. Another very recent study showed that, during the early stage of epithelial cell invasion, *N. gonorrhoeae* is sequestered into double-membrane autophagosomes that subsequently fuse with lysosomes for destruction. However, *N. gonorrhoeae* is able to inhibit the autophagy pathway by activating the autophagy repressor mammalian target of rapamycin (mTOR) complex 1, thus allowing intracellular survival and exocytosis [114].

Apart from evading detection by innate immunity, some of these pathogens developed strategies to evade adaptive arms of the immune response, thus preventing clearance and securing the chronicity of infection. For example, *C. trachomatis* can modulate the cell-mediated adaptive immune response by suppression of both MHC class I and class II antigen expression in the different infected cells lines, including cervical cells [115,116]. For *N. gonorrhoeae*, one immune evasion strategy is its extraordinary

capacity for antigenic variation of major surface components [117]. However, Liu *et al.* proposed that *N. gonorrhoeae* avoids the generation of specific antibodies in the first place, since it has been shown that *N. gonorrhoeae* strongly induced TGF-β, immunosuppressive IL-10, and type 1 regulatory T cell (Tr1) production which leads to the inhibition of Th1/Th2 development in mice [117–119].

To sum up, immune evasion mechanisms are crucial for FGT pathogens not only to establish infection and secure persistence, but also to survive as commensals. Many FGT pathogens developed sophisticated strategies to modulate immune responses on different levels, from the initial recognition by innate immunity to specific responses of adaptive immunity.

Concluding Remarks and Future Perspectives

The FGT is a dynamic environment shaped by hormonal changes and different inflammatory stimuli which dictate the composition of its microbial community. The FGT pathogens evolved a variety of strategies to adapt to such an environment, despite the presence of various pHs and oxygen levels, the limited availability of metals and other nutrients, and sophisticated host defenses.

This huge variability in the FGT environment presents a challenge for diagnostics since the basic concepts of a healthy microbiota are still not well defined. Following the same line, therapeutic challenges are driven not only by microbial resistance and inappropriate diagnostics, but also by the fact that any applied treatment will rarely affect only the target organism but rather influence other microbial partners as well, which are possibly responsible for maintaining a healthy environment, such as lactobacilli. More research is needed in order to establish reliable models to study and fully understand the composition and function of FGT microbiota, but more importantly, how their constituent members interact with one another to form a dynamic ecosystem that responds to external factors (see Outstanding Questions) [3].

The challenge of treating FGT infections could, in theory, be tackled by considering more specific or more general approaches, for example, by targeting specific or common virulence determinants, thus attacking more pathogens at the same time, but without harming beneficial members of the microbiota. Supporting colonization by lactobacilli is a strategy that has already been proven effective. Another strategy could, for instance, support nutritional immunity. However, focusing on species-specific factors (such as VLY for *G. vaginalis*, or candidalysin for *C. albicans*) or a single factor with multiple functions (such as Als3 in *C. albicans*, or PbsP in *S. agalactiae*) would direct the applied prevention/therapeutic approach and at least partially avoid the influence on other members of the vaginal microbiota and its dynamics. Having a holistic approach to identifying similarities and differences during FGT infections could help us to better understand FGT health and improve current prevention and treatment strategies.

Outstanding Questions

Which approaches could help us to define a healthy microbiota of different anatomical sites of the FGT?

What could be improved in the current *in vitro/ex vivo/in vivo* models of vaginal and cervical infections in order to consider all the factors influencing these microenvironments, such as lactobacilli and other microorganisms, mucus, pH level, and hormonal changes?

Which infection-specific factors could be used as diagnostic markers, which would exclude the detection of commensals?

Having in mind the complexity of host–pathogen interactions, could the ideal solution for prevention and treatment be to target both host (e.g., nutritional immunity) and pathogen (e.g., secretion of toxins, expression of adhesins/invasins) features at the same time?

Acknowledgments

M.P. and B.H. received funds from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 642095 for the OPATHY consortium. S.M. and B.H. received funds from Leibniz Association Campus InfectoOptics SAS-2015-HKI-LWC. B.H. is further supported by the Deutsche Forschungsgemeinschaft CRC/TR FungiNet Project C1 and the H2020–Marie Skłodowska-Curie Actions–European Training Networks grant agreement No 812969 - FunHoMic. J.N. is supported by grants from the Wellcome Trust (214229_Z_18_Z), Biotechnology and Biological Sciences Research Council (BB/N014677/1) and the National Institutes of Health (NIH) Research at Guys and St Thomas's National Health Service (NHS) Foundation Trust and the King's College London Biomedical Research Centre (IS-BRC-1215-20006).

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