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## Microorganism-derived extracellular vesicles: emerging contributors to female reproductive health

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Extracellular vesicles (EVs) are cell-derived nanoparticles that carry small molecules, nucleic acids, and proteins long distances in the body facilitating cell–cell communication. Microorganism-derived EVs mediate communication between parent cells and host cells, with recent evidence supporting their role in biofilm formation, horizontal gene transfer, and suppression of the host immune system. As lipid-bound bacterial byproducts, EVs demonstrate improved cellular uptake and distribution *in vivo* compared to cell-free nucleic acids, proteins, or small molecules, allowing these biological nanoparticles to recapitulate the effects of parent cells and contribute to a range of human health outcomes. Here, we focus on how EVs derived from vaginal microorganisms contribute to gynecologic and obstetric outcomes. As the composition of the vaginal microbiome significantly impacts women's health, we discuss bacterial EVs from both healthy and dysbiotic vaginal microbiota. We also examine recent work done to evaluate the role of EVs from common vaginal bacterial, fungal, and parasitic pathogens in pathogenesis of female reproductive tract disease. We highlight evidence for the role of EVs in women's health, gaps in current knowledge, and opportunities for future work. Finally, we discuss how leveraging the innate interactions between microorganisms and mammalian cells may establish EVs as a novel therapeutic modality for gynecologic and obstetric indications.

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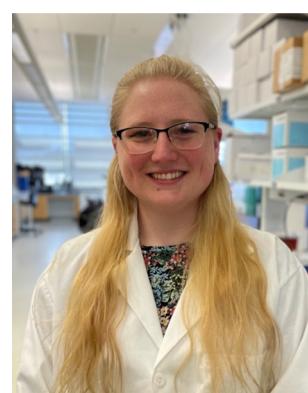
## Significance

Extracellular vesicles are an emerging contributor to obstetric and gynecologic health. This review details recent work done to understand the role of microorganism-derived EVs in the female reproductive tract, with important implications for engineering next generation therapies for women's health.



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## Introduction

The human body contains more bacterial cells than mammalian cells.<sup>1</sup> This “human microbiome” plays a role in a range of health outcomes across several organ systems, from the gastrointestinal tract to the central nervous system. The female reproductive tract, too, is home to a unique microenvironment which significantly impacts gynecologic and obstetric outcomes. Unlike the diversity seen in the gastrointestinal environment, an optimal vaginal microbiome is one dominated by a single *Lactobacillus* spp.<sup>2</sup> High-throughput screening studies have determined five primary community state types (CSTs), four of which are dominated by a single species of *Lactobacillus* (CST I by *L. crispatus*, CST II *L. gasseri*, CST III *L. iners*, CST V *L. jensenii*).<sup>3</sup> Conversely, CST IV describes a poly-microbial environment marked by an overgrowth of facultative anaerobes. Clinically, CST IV is referred to as bacterial vaginosis (BV), a condition which affects ~30% of women in the United States and is associated with an increased risk for pelvic inflammatory disease, urinary tract infections, infertility, acquisition of sexually transmitted infections, and preterm birth.<sup>4–9</sup> The mechanisms that facilitate these disease manifestations have been explored through both clinical and pre-clinical studies, but are still not well understood.

Communication between microbes and mammalian cells is facilitated through the production of small molecules and proteins which elicit host immune responses and trigger functional changes in host tissues.<sup>10–13</sup> *Lactobacillus* spp. are considered protective in the vaginal environment, as they produce lactic acid and hydrogen peroxide, both thought to prevent the growth of pathogenic microbes.<sup>14–16</sup> *Gardnerella vaginalis*, *Mobiluncus mulieris*, and *Prevotella bivia*, on the other hand, produce mucus degrading enzymes, shed lipopolysaccharide, and secrete cytolsins, contributing to the onset of adverse health events.<sup>17–19</sup> In recent studies, the microbiology field has turned its attention to extracellular vesicles (EVs) as a mode of microbe-host communication.<sup>20–22</sup> Here, we use the term “EVs” to describe the biological particles produced by both Gram negative and Gram positive bacteria, as well as fungal species and parasites which inhabit the vaginal microenvi-

ronment.<sup>23</sup> This term is used to encompass all naturally released particles from a cell, including membrane vesicles, outer membrane vesicles, outer-inner membrane vesicles, cytoplasmic membrane vesicles, and explosive outer membrane vesicles that, according to the International Society for Extracellular Vesicles (ISEV), “are delimited by a lipid bilayer and cannot replicate.”<sup>24</sup> EVs are nano-sized (50–1000 nm, see Table 1) particles with different classifications used to describe their biogenesis (Fig. 1).<sup>25</sup> With a thick cell wall, EVs produced by Gram positive bacteria are called membrane vesicles or cytoplasmic membrane vesicles and are thought to be produced *via* bubbling cell death.<sup>26,27</sup> Gram negative bacteria have an inner and outer membrane, resulting in the production of several classes of EVs. Outer membrane vesicles are the result of stress causing blebbing of the outer membrane. Explosive outer membrane vesicles and outer-inner membrane vesicles are produced after weakening of the peptidoglycan layer.<sup>26,27</sup> These varied mechanisms of EV production result in differences in EV cargoes, including small molecules, nucleic acids, and proteins.<sup>28</sup> These cargoes are protected from degradation and dilution *in vivo* by a lipid membrane, often decorated with surface proteins which facilitate tissue targeting and cellular uptake.<sup>29,30</sup> EVs have innate barrier crossing abilities, which further enhance their ability to communicate with host cells.<sup>31</sup> For example, previous work from our group demonstrates that the cervicovaginal mucus barrier has a mesh size of ~300 nm, which is too small for whole microbes to efficiently penetrate.<sup>32,33</sup> In contrast, EVs from vaginal microbes are 100–300 nm and may be able to evade steric and adhesive interactions with mucins, permitting them to reach female reproductive tract tissues to deliver differential signals to host cells.<sup>31</sup> The growing interest in microbial EVs has highlighted the many unanswered questions regarding the role and use of microbial EVs in the context of female reproductive health.

In this review, we examine recent studies which provide evidence for the role of bacterial EVs in mediating female reproductive health. We discuss bacterial EVs from both healthy and dysbiotic vaginal microbiota, and EVs from common vaginal bacterial, fungal, and parasitic pathogens, highlighting evidence for their role in gynecologic health, as well as gaps in current knowledge and opportunities for future work. Finally, we discuss how leveraging the innate interactions between microorganisms and mammalian cells may establish EVs as a novel therapeutic modality for gynecologic and obstetric indications. It is worth noting that a growing body of work details how mammalian cell-derived EVs participate in female reproductive tract physiology, ranging from infertility and miscarriages, to gynecologic cancers and homeostasis during pregnancy.<sup>34–38</sup> There are several thorough and recent reviews which detail how EVs from cervical, placental, uterine, and endometrial cells contribute to important gynecologic and obstetric function.<sup>39–41</sup> While EVs produced by mammalian cells likely enable host-microbe communication, and likely serve as a response to signaling from microorganisms in the female reproductive tract, the focus of this review is on EVs produced by microorganisms. We hope that by understanding



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**Table 1** EVs are produced by microorganisms in the female reproductive tract. Differences in physical characteristics and function may be artifacts of differences in isolation and handling of EVs. As work in the EV field continues to grow, careful consideration should be taken to follow the minimal reporting guidelines as defined by the International Society of Extracellular Vesicles<sup>45,50</sup>

Parent cell source	Parent cell strain	Isolation method	Storage method	Size	Concentration	Cargo analysis	Function	Ref.
<i>L. crispatus</i> Patient-derived isolates	BC5	Ultracentrifugation	PBS at -80 °C	89.3 ± 49.2 nm	34.70 ± 2.31 × 10 <sup>9</sup> particles per mL	Total protein analysis, nucleic acid analysis	Affected bacterial adhesion to cervical cells	69
<i>L. gasseri</i> Patient-derived isolates	BC12	Ultracentrifugation	PBS at 4 °C	129.1 ± 51.9 nm	30.60 ± 1.05 × 10 <sup>9</sup> particles per mL	3.26 ± 0.11 × 10 <sup>10</sup> particles per mL	Suppressed HIV-1 replication	56
<i>L. crispatus</i> Patient-derived isolates	BC3	Ultracentrifugation	PBS at 4 °C	133.14 ± 2.90 nm	1.18 ± 0.32 × 10 <sup>10</sup> particles per mL	1.18 ± 0.32 × 10 <sup>10</sup> particles per mL	No protection against HIV-1 infection	
<i>L. gasseri</i> Patient-derived isolates	BC5			141.26 ± 9.78 nm	5.87 ± 0.20 × 10 <sup>10</sup> particles per mL	5.87 ± 0.20 × 10 <sup>10</sup> particles per mL	Suppressed HIV-1 replication	
<i>S. aureus</i>	ATCC	BC12	Ultracentrifugation	~140 nm	~140 nm	1.32 ± 0.44 × 10 <sup>11</sup> particles per mL	No protection against HIV-1 infection	
<i>E. faecium</i>	ATCC	BC13	Ultracentrifugation	~140 nm	3.52 ± 1.79 × 10 <sup>11</sup> particles per mL	3.52 ± 1.79 × 10 <sup>11</sup> particles per mL	Suppressed HIV-1 replication	77
<i>E. faecalis</i>	ATCC	19434		~200 nm	3.78 ± 1.61 × 10 <sup>11</sup> particles per mL	3.78 ± 1.61 × 10 <sup>11</sup> particles per mL		
<i>G. vaginalis</i>	ATCC	19433		~190 nm	2.27 ± 0.88 × 10 <sup>12</sup> particles per mL	2.27 ± 0.88 × 10 <sup>12</sup> particles per mL		
<i>L. gasseri</i>	ATCC	14018	Ultracentrifugation, filtration	179.07 ± 17.22 nm	5.42 ± 1.65 × 10 <sup>11</sup> particles per mL	5.42 ± 1.65 × 10 <sup>11</sup> particles per mL		
<i>G. vaginalis</i>	ATCC	9857		85 nm	1.6 × 10 <sup>10</sup> to 2.2 × 10 <sup>11</sup> particles per mL	1.6 × 10 <sup>10</sup> to 2.2 × 10 <sup>11</sup> particles per mL	Total protein analysis, nucleic acid analysis, proteomics	55
<i>G. vaginalis</i>	ATCC	14018		115 nm	3.21 × 10 <sup>10</sup> to 1.5 × 10 <sup>11</sup> particles per mL	3.21 × 10 <sup>10</sup> to 1.5 × 10 <sup>11</sup> particles per mL	Enhanced <i>T. vaginalis</i> cytoadhesion	
<i>L. gasseri</i>	ATCC	19992	Ultracentrifugation	PBS at -20 °C	295–458 nm	—	Total protein analysis	72
<i>L. crispatus</i>	BCRC	14618	Ultracentrifugation, filtration	PBS	110.8 ± 3.2 nm	—	Total protein analysis	67
<i>A. muciniphila</i>	ATCC	BAA835	Ultracentrifugation	PBS at -80 °C	119.2 ± 3.4 nm	—	Total protein analysis, RT-qPCR, 16S rDNA gene sequencing, RNA sequencing	68
<i>G. vaginalis</i>	ATCC	14019	Ultracentrifugation	PBS at -20 °C	120–260 nm	—	Total protein analysis, proteomics	49
<i>G. vaginalis</i>	ATCC	14019	Ultracentrifugation	PBS (pH 7.4) at -20 °C	190–250 nm at pH 6.5, 190–615 nm at pH 3.5	—	Disrupted <i>G. vaginalis</i> and <i>S. aureus</i> biofilm formation	
<i>C. albicans</i>	ATCC	90028	Centrifugation	PBS (pH 7.4)	135 nm; 100–250 nm, 300–350 nm, 420–480 nm	61.63 × 10 <sup>6</sup> particles per mL	Decreased oxidative stress-induced trophoblast death	66
	ATCC	10231			195 nm; 100 to 400 nm	195 nm; 100 to 400 nm	Decreased viability of and increased IL-8 production in vaginal epithelial cells	68
	ATCC	SC5314			175 nm; 100 to 400 nm	175 nm; 100 to 400 nm	Decreased biotfilm formation	71

Table 1 (Contd.)

Parent cell source	Parent cell strain	Isolation method	Storage method	Size	Concentration	Cargo analysis	Function	Ref.
<i>C. albicans</i> —	SN152	Size exclusion chromatography	PBS (pH 7.2)	EVs: 30–200 nm, 200–100 nm; biofilm EVs: 30–200 nm	~1 × 10 <sup>6</sup> particles per mL in biofilms	Proteomics, carbohydrate analysis, lipidomics	Enhanced biofilm formation and antifungal drug resistance	75
<i>S. agalactiae</i> Patient-derived isolates (GBS)	GB00020, sequence type 1, serotype cpsV	Ultracentrifugation	PBS at -80 °C	—	~0.05–0.10 particles per CRU	Proteomics	—	88
	GB00037, sequence type 1, serotype cpsV	—	—	~0.05–0.10 particles per FU	—	—	—	—
	GB00112, sequence type 17, serotype cpsIII	—	—	~0.05–0.15 particles per FU	—	—	—	—
	GB00411, sequence type 17, serotype cpsIII	—	—	~0.05–0.15 particles per FU	—	—	—	—
	GB00653, sequence type 12, serotype cpsII	—	—	~0.05–0.15 particles per FU	—	—	—	—
	GB01455, sequence type 12, serotype II	—	—	~0.15–0.20 particles per FU	—	—	—	—
<i>S. agalactiae</i> Patient-derived isolates (GBS)	A909-derived ΔcovR	Ultracentrifugation	PBS	—	—	Total protein analysis	Caused hemolysis in human neutrophils, decreased neonate survival in mice Non-hemolytic	89
	A909-derived ΔcovRΔcylE	—	—	—	—	—	—	—
	A909 (serotype Ia)	Ultracentrifugation	PBS (pH 7.2)	<50 nm; 150–300 nm	1.7 × 10 <sup>4</sup> particles per mL	Total protein analysis, proteomics, nucleic acid analysis, lipidomics	Degraded collagen and reduced fetal membrane stiffness preterm birth and intrauterine demise	90
<i>C. trachomatis</i>	serovar F/Cal-1-13	Nitrogen cavitation, density gradient ultracentrifugation	PBS at 4 °C or -80 °C	700–2000 nm	—	western blot	Immune modulating	95
<i>N. gonorrhoeae</i>	FA1090, F62, MS11, and 1291	Ultracentrifugation	PBS	—	—	Proteomics	—	100
<i>N. gonorrhoeae</i>	WS1, F62 (serum-susceptible)	Ultracentrifugation, filtration	—	—	—	Electrophoresis	Serum-resistant EVs protected serum-susceptible strains	101
<i>N. gonorrhoeae</i> Reference Unit	K454	Differential ultracentrifugation	PBS at -80 °C	100 ± 20 nm	—	—	Immunosuppressive; Reduced T-cell proliferation	104
<i>T. vaginalis</i> —	B7RC2	Density gradient ultracentrifugation	PBS at -20 °C	95 nm; 50–150 nm	—	—	Immune modulating, increased parasite adherence to cells	106
<i>T. vaginalis</i> —	B7RC2 ((PA) and Δ wild type)	Ultracentrifugation	PBS	105 nm	2.74 × 10 <sup>12</sup> particles per mL	Total protein analysis, proteomics, total RNA analysis	—	107
<i>T. vaginalis</i> —	B7RC2 ((PA) and Δ wild type)	Centrifugation	PBS + 1X HALT protease inhibitors	MVs: 380 nm; Exosomes: 63 nm	—	Proteomics	Taken up by parasites and cervical cells	54

Table 1 (Contd.)

Parent cell source	Parent cell strain	Isolation method	Storage method	Size	Concentration	Cargo analysis	Function	Ref.
<i>T. vaginalis</i> isolate	Patient-derived	GT-21	Centrifugation	PBS (pH 7) + protease inhibitors	50–100 nm	—	Total protein analysis, nucleic acid analysis	108
<i>T. vaginalis</i>	ATCC 50167	B7RC2	Density gradient ultracentrifugation	PBS (pH 7.4) at -20 °C	—	—	Total protein analysis	109
<i>C. albicans</i>	ATCC MYA-2876	SC5314	Centrifugation	PBS	≤50 nm; 50–100 nm; 450–850 nm	—	Lipidomics	112
<i>C. albicans</i>	ATCC	90028	Centrifugation	PBS (pH 7.4)	50–100 nm; 450–850 nm	—	Proteomics, total protein analysis, lipidomics	114
Patient-derived isolate		11	Ultracentrifugation	PBS + TEAB buffer	50–100 nm; 350–450 nm; 50–450 nm; 100–200 nm	—	Proteomics, total protein analysis, lipidomics	115
<i>C. albicans</i> isolate	Patient-derived	SC5314 (71)	Ultracentrifugation	PBS + TEAB buffer	50–100 nm; 350–450 nm; 50–450 nm; 100–200 nm	—	Total protein analysis, proteomics	116

EVs produced by microorganisms and their effect on the female reproductive tract, future studies may begin to ask more complicated questions regarding host–microbe crosstalk facilitated by EVs.

### Considerations for isolation and characterization of extracellular vesicles

Throughout EV research, variability in isolation, storage, and characterization protocols makes it difficult to compare across studies (Table 1).<sup>42,43</sup> Even the tubes used to store EVs throughout experiments may alter observed results.<sup>44</sup> ISEV has up-to-date recommendations for isolation procedures and reporting guidelines that will help in comparing results across future studies.<sup>45</sup> Nonetheless, here we make an effort to highlight experimental procedures as we assess differences in EVs from vaginal microbes that may contribute to differential clinical outcomes.

Several emerging techniques are used to efficiently isolate pure populations of EVs.<sup>46</sup> First, it is necessary to remove parent cells from cultured media or biological matrix, often done *via* low-speed centrifugation (2000 to 15 000 rcf) or filtration (0.2 or 0.45 µm). Sequential ultracentrifugation (100 000 to 200 000 rcf) or density ultracentrifugation is used to isolate particles in the size range of EVs. Ultracentrifugation typically generates high-purity, but low-recovery EV samples. Size exclusion chromatography is used to separate the components of conditioned media based on size, with large particulates eluting from the column in earlier fractions than small particulates. Chromatography methods suffer from impurity issues, where lipoproteins and other small non-EV cell byproducts may elute in the same fractions as EVs. Given their lipid membrane and net negative surface charge, EVs are amenable to isolation using anion exchange chromatography. Ultrafiltration, as well as precipitation with a number of commercially available reagents, allows for concentration of isolated EVs. Differences in isolation protocols may account for variability in experimental observations regarding EV function, so it is typically necessary to optimize isolation protocols for EVs from different parent cells (Table 1).

After isolation, the physical characteristics of EVs can be measured using nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS).<sup>47</sup> NTA and DLS measure the diffusivity of particles in solution and calculate a hydrodynamic radius of individual particles based on principles of Brownian motion. NTA presents an improvement over DLS, which may overestimate the size of large particles. Transmission electron microscopy (TEM) is commonly used to visualize and measure EVs, specifically assessing structure and morphology. Protein concentration is also part of the ISEV recommended reporting guidelines, with techniques such as western blot and flow cytometry being used to detect specific surface markers. *Escherichia coli*-derived EVs have been detected using the surface marker OmpA.<sup>48</sup> While less work has been done to determine surface markers of EVs from vaginal microbes, future work could use surface layer proteins or something more specific, such as the cytolytic protein vagi-

BiogenesisFunction

Bubbling Cell Death



Explosive Cell Death



Blebbing



100-300 nm

Induce Cell Death

Inhibit Biofilm Formation

Promote/Prevent Pathogen Colonization

Mediate Viral Infectivity

Modulate Host Immune Response

**Fig. 1** Bacteria and microorganism-derived EVs have demonstrated effects on host cells. Vaginal microbes generate different classifications of EVs, with Gram positive bacteria producing EVs via bubbling cell death and Gram negative bacteria producing outer membrane vesicles, explosive outer membrane vesicles, and outer–inner membrane vesicles via cell blebbing. Different classes of EVs have different biological functions ranging from inducing host cell death, inhibiting biofilm formation, decreasing viral infectivity, and eliciting host immune responses.

nolysin which may be found on the surface of *G. vaginalis* EVs.<sup>45,49,50</sup> Generally, studies report size, concentration, morphology, and protein content of EVs, with some also reporting the surface charge ( $\zeta$ -potential).

Where the size,  $\zeta$ -potential, and morphology of EVs may affect stability in the vaginal environment, membrane composition (lipids) and targeting proteins may impact EV uptake into female reproductive tract cells (Fig. 1). Proteomics and lipidomic studies can give insight into membrane compositions that affect uptake. EVs are taken up *via* several mechanisms: they may bind to specific cell receptors; they may enter host cells by direct fusion with the cell membrane; or they may enter *via* an endocytic uptake (clathrin-mediated or caveolin-mediated endocytosis, phagocytosis, micropinocytosis).<sup>51–53</sup> Studies demonstrate that route of uptake may vary based on size, as well as macromolecules within the EV membrane. Specific to female reproductive tract cells, *G. vaginalis*-derived EVs adhere to the cell surface and are internalized by vaginal epithelial cells.<sup>49</sup> *T. vaginalis* EVs are taken up by mammalian cells *via* caveolae-dependent endocytosis.<sup>54</sup> By further exploring mechanisms of cellular uptake that facilitate microbe-host communication by EVs, future work may advance our understanding of the biological effects of EVs, as well as their use as therapeutic agents.

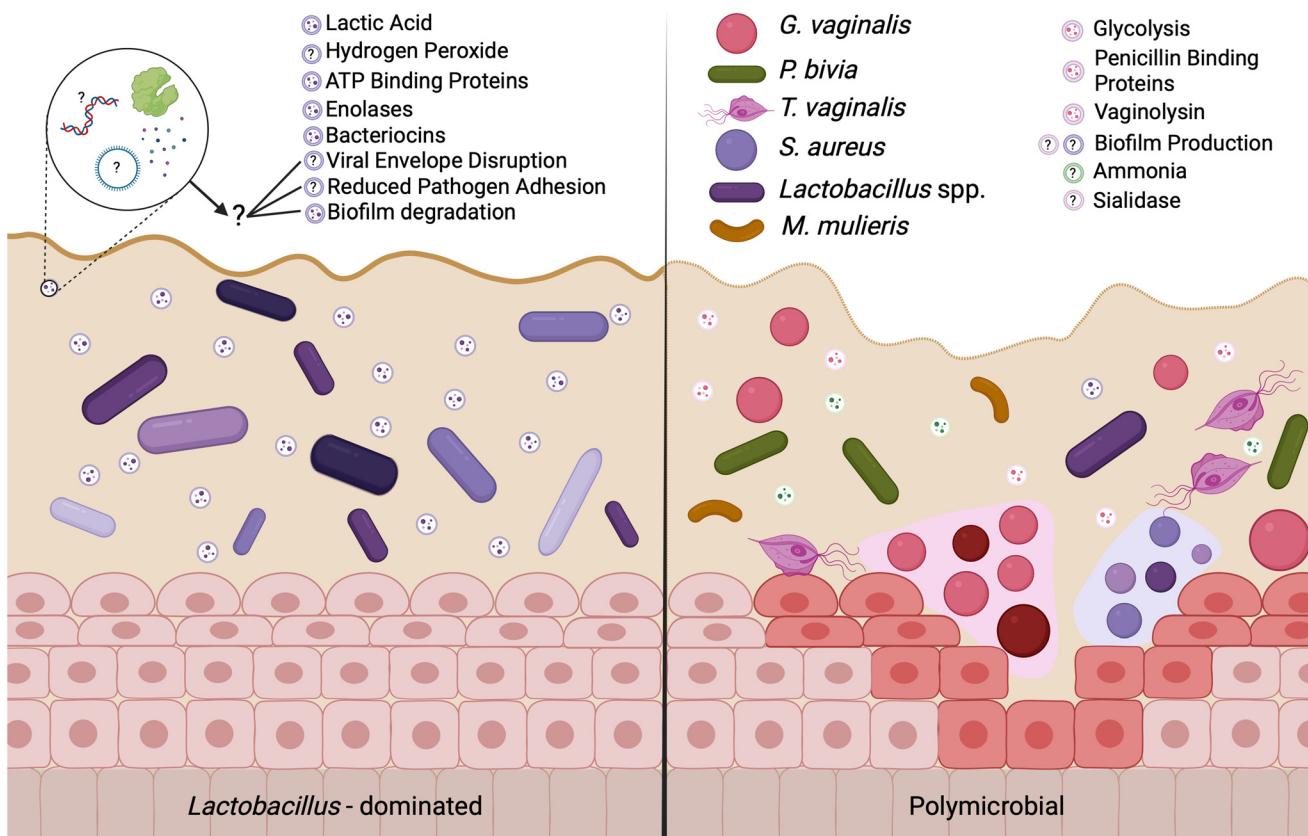
Once EVs reach a target host cell, internal cargoes are released into the cytoplasm, allowing for unique biological cargoes to be delivered to host cells.<sup>49,55,56</sup> These cargoes have

immunomodulatory properties, both in and outside the context of gynecologic and obstetric health. RNA cargoes (mRNA, siRNA, miRNA) can activate host immune response, regulate bacterial growth, and weaken host biological barriers (Table 1). DNA cargoes may enable horizontal gene transfer with the potential to increase antibacterial resistance (Fig. 2).<sup>57</sup> Protein cargoes may mediate bacterial virulence and immune system evasion.<sup>49,58</sup> In addition to nucleic acids and proteins, *Lactobacillus*-derived EVs were found to carry metabolites with antiviral activity, including lactic acid.<sup>56</sup> Understanding differences in EV cargoes will not only reveal differences in the biological function of EVs, but may help to identify therapeutic opportunities using EVs or mimicking EV interactions with host tissues using synthetic lipid nanoparticles.

#### Vaginal bacterial extracellular vesicles mediate female reproductive outcomes

A *Lactobacillus* spp. dominated vaginal microbiome is considered protective, playing an important role in maintaining a healthy environment (Fig. 2). *Lactobacillus* spp. produce lactic acid, which lowers the pH of the vagina, preventing the growth of pathogenic bacteria and reducing inflammation in the reproductive tract. These microbes also produce antimicrobial compounds, including hydrogen peroxide, bacteriocins, and enzymes. In contrast, dysbiosis in the vaginal microenvironment is associated with an increased risk for a broad spectrum of gynecologic and obstetric disease.<sup>2–4,59</sup> The pathogen-





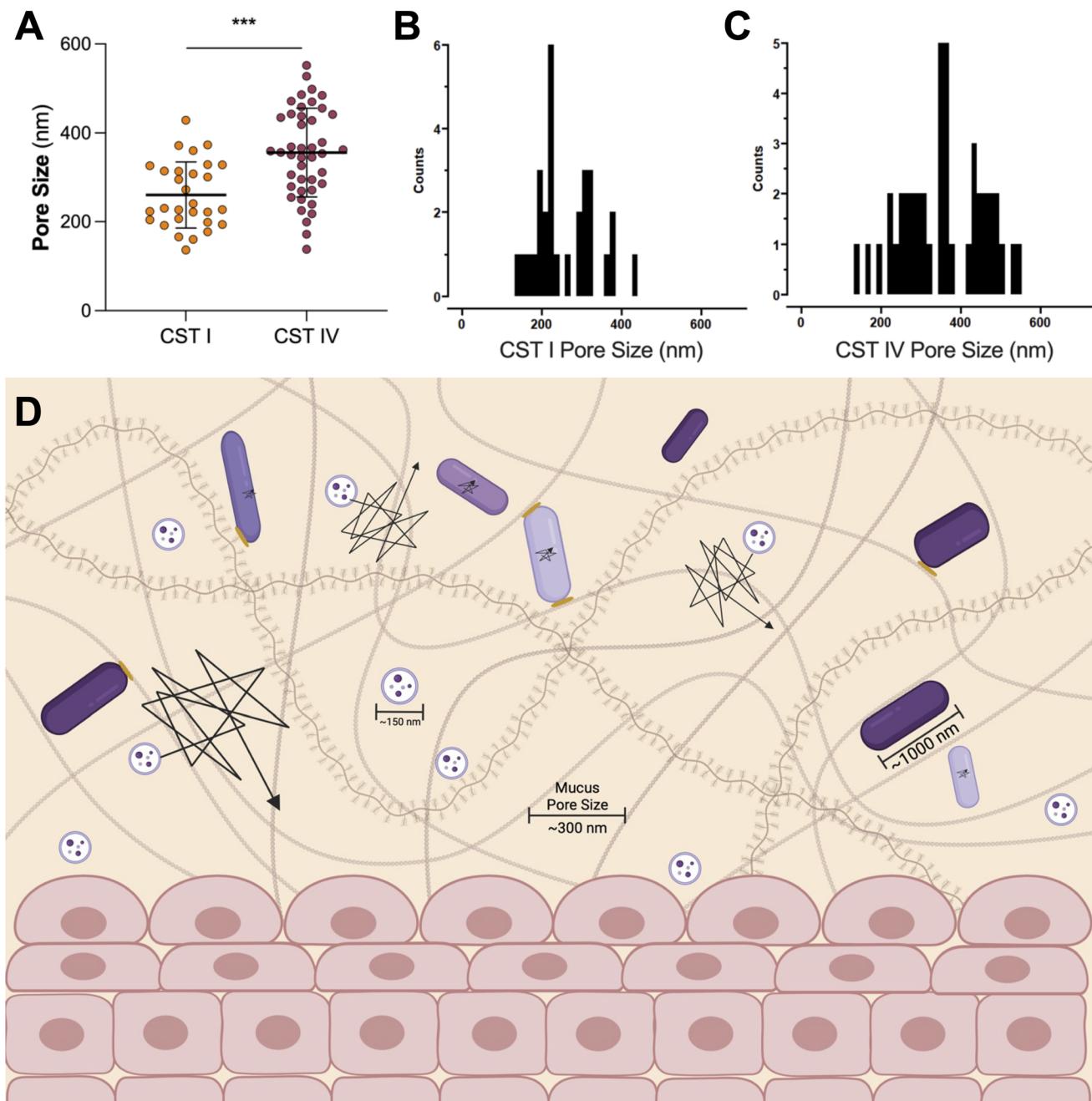
**Fig. 2** EVs may mediate differences in female reproductive outcomes are associated with the composition of the vaginal microbiome. The composition of the vaginal microbiome is associated with differential gynecologic and obstetric outcomes, where a lactobacilli-dominated microenvironment is clinically associated with healthy outcomes and a polymicrobial environment is associated with adverse outcomes including preterm birth, infertility, urinary tract infections, and increased risk for sexually transmitted infections. Recent work suggests that some of these outcomes are facilitated by microorganism-derived EVs. EVs from probiotic bacteria carry antimicrobial factors, including lactic acid, hydrogen peroxide, and antimicrobial peptides to protect against pathogens and promote the growth of healthy-associated microbes. In contrast, pathogen-derived EVs carry cytolytic proteins and mucin degrading enzymes, allowing for evasion and suppression of the host immune system. The current evidence supports further efforts in understanding EV cargoes and EV-mediated outcomes in the female reproductive tract.

esis of BV begins with the introduction of *G. vaginalis*, *P. bivia*, and *A. vaginalae* into the vaginal environment, synergistically displacing lactobacilli (Fig. 2). As *G. vaginalis* forms a biofilm, the secretion of proteolytic enzymes and production of amino acids promotes the growth of *P. bivia* which produces ammonia, thereby continuing the cycle of biofilm formation.<sup>17,18</sup> Sialidase produced by these microbes degrades the mucus barrier, allowing bacterial invasion into the vaginal epithelium.<sup>60–62</sup> Despite improved fundamental understanding of the vaginal microbiome, promoting a healthy vaginal microbiome and healthy reproductive outcomes remains clinically challenging. It has been proposed that bacteria may ascend from the vaginal environment into upper levels of the female reproductive tract, causing inflammation and adverse health outcomes. However, our recent work demonstrates that the pore size of the cervicovaginal mucus barrier is too small for whole bacteria to penetrate (Fig. 3A–C).<sup>32,33</sup> Given their small size, it may be more likely that EVs penetrate cervicovaginal mucus to deliver proteins, nucleic acids, or small molecules that initiate reproductive outcomes (Fig. 3D). Supporting

this hypothesis, separate work suggests that first-pass meconium may contain bacterial EVs from the maternal microbiome, indicating that bacterial EVs may be able to ascend from the vaginal environment, reach the uterine cavity, and traverse the placental barrier to impart differential signals that may influence pregnancy and neonatal outcomes.<sup>63,64</sup> Another study suggests that EVs may be a source of microbial DNA in the placenta, with evidence of *in vitro* uptake of bacterial EVs by trophoblasts.<sup>65</sup> In this section, we present recent evidence to support the role of vaginal bacterial EVs in female reproductive outcomes (Tables 1 and 2).

BV is associated with increased reproductive tract inflammation. One recent study demonstrated the pro-inflammatory and cytotoxic activity of *G. vaginalis* EVs in vaginal epithelial cells *in vitro*.<sup>49</sup> Cellular uptake of EVs induced cell blebbing and a dose-responsive decrease in cell viability. EV surface proteins associated with pathogenesis, virulence, antibiotic resistance, and protection against the host immune system caused functional changes in vaginal epithelial cells *in vitro*. Virginolysin, a pore forming toxin associated with the virulence





**Fig. 3** The pore size of cervicovaginal mucus may be permissive of EVs. (A) The pore size of cervicovaginal mucus differs based on the composition of the vaginal microbiome, where a polymicrobial environment has an increased pore size. (B) The distribution of pore sizes in CST I samples, and (C) CST IV samples shows a range of pore sizes for pathogens or EVs to move through mucus. Data republished with permission from ref. 33. (D) Even the increased pore size observed in CST IV samples is not large enough for whole bacteria to penetrate. In contrast, even the less permissive mucus barrier observed in the case of CST I samples (pore size  $\sim 300$  nm) is large enough for EVs  $\sim 100$ – $200$  nm in size to move through mucus without significant steric hindrance. *In vitro* and *ex vivo* evidence suggests that EVs may mediate female reproductive tract disease, potentially by penetrating the vaginal mucus barrier and ascending into upper levels of the female reproductive tract. This proposed mobility makes EVs useful as carriers for vaginally delivered therapeutics. Black lines represent the trajectories of particles or bacteria through mucus, where a larger trajectory is indicative of a more mobile particle. Bacteria are hindered through steric interactions with mucus pores, represented by yellow ovals.

of *G. vaginalis*, was also detected in the isolated EVs.<sup>49</sup> As a follow-up, the same group investigated the effects of a decreased pH on *G. vaginalis* growth and EV production as a proxy for understanding how a lactobacilli-dominated vaginal

environment may change the effects of *G. vaginalis* EVs on epithelial cells.<sup>66</sup> In addition to decreased viability of *G. vaginalis*, the size, morphology, and protein content of *G. vaginalis* EVs were significantly altered at pH 3.5. Of note, vaginolysin was

**Table 2** Bacteria-free supernatants demonstrate effects on host cells. Bacteria-free supernatants have been used to examine the effects of bacterial byproducts on host cells. While these studies do not explicitly investigate the contribution of EVs to host responses, the supernatants used throughout the experiments undoubtedly contain EVs. The results of these studies support further investigation into the effects of EVs on female reproductive tract cells

Parent cell	Parent cell source	Parent cell strain	Function	Ref.
<i>L. crispatus</i>	Patient-derived isolates	BC3 BC5	Decreased HIV-1 replication and infectivity in <i>ex vivo</i>	76
<i>L. gasseri</i>	Patient-derived isolates	BC12 BC13	—	
<i>G. vaginalis</i>	Patient-derived isolates	BC16 BC17	—	
<i>L. crispatus</i>	ATCC	33197	Decreased <i>C. trachomatis</i> infectivity	78
	ATCC	33820		
	ATCC	55844		
	PTA	10138		
<i>L. jensenii</i>	ATCC	25258	Did not alter <i>C. trachomatis</i> infectivity	
<i>L. iners</i>	ATCC	55195	Decreased <i>C. albicans</i> growth and propagation in a vulvovaginal candidiasis murine model	
<i>L. crispatus</i>	Patient-derived isolates	—	Decreased growth of <i>C. albicans</i> <i>in vitro</i>	79
<i>L. gasseri</i>	Patient-derived isolates	—	Significantly increased tight junctions in cervicovaginal epithelial cells	80
<i>L. jensenii</i>	Patient-derived isolates	—	Mildly increased tight junctions in cervicovaginal epithelial cells	
<i>L. crispatus</i>	ATCC	33197	Did not alter tight junctions in cervicovaginal epithelial cells	
<i>L. jensenii</i>	ATCC	25258	Immune modulating, decreased ectocervical cell permeability	
<i>L. iners</i>	ATCC	55195	Increased cervical cell permeability, pro-inflammatory	81
<i>G. vaginalis</i>	ATCC	14018	Decreased matrix metalloproteinases in female reproductive tract cells	82
<i>L. crispatus</i>	Patient-derived isolates	—	Increased matrix metalloproteinases in female reproductive tract cells	
<i>L. iners</i>	ATCC	55195	Decreased cervical cell permeability	84
<i>G. vaginalis</i>	ATCC	14018	Increased cell death permeability	
<i>L. crispatus</i>	ATCC	33197	Increased cell permeability and cytokine secretion	
<i>G. vaginalis</i>	ATCC	14019		
<i>L. crispatus</i>	ATCC	33197		
<i>G. vaginalis</i>	ATCC	14018		
<i>M. mulieris</i>	ATCC	35243		

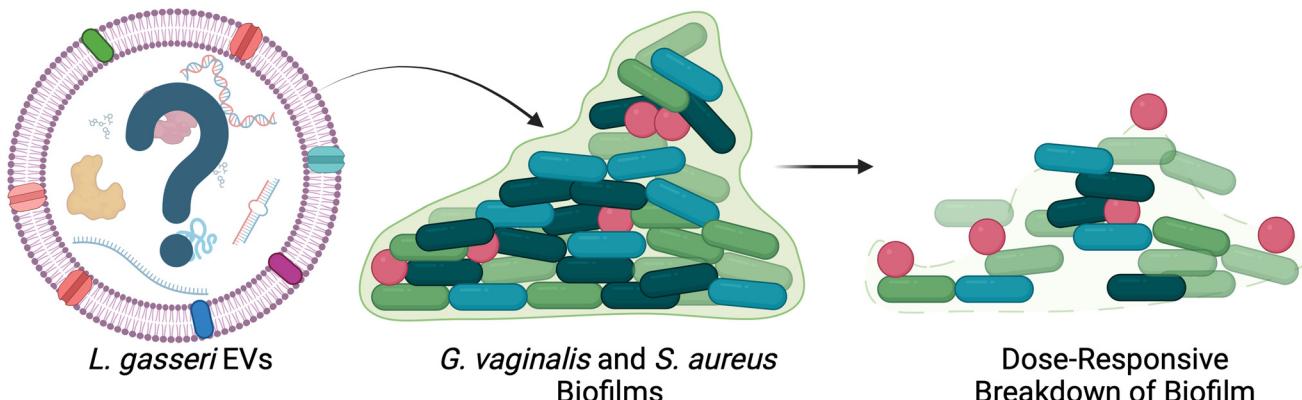
no longer found in the EVs from *G. vaginalis* cultured at pH 3.5. Authors attributed this change to a degradation of vaginolysin prior to EV release. Interestingly, EVs from *G. vaginalis* cultured at pH 3.5 still increased IL-8 production from vaginal epithelial cells, indicating a potential role for *G. vaginalis*-derived EVs in modulating the host immune system in an acidic vaginal environment.<sup>66</sup> Artuyants, *et al.* similarly reported vaginolysin as the most significantly enriched protein found in EVs from *G. vaginalis* cultures.<sup>55</sup> However, in this study, treatment with *G. vaginalis* EVs did not alter human ectocervical cell viability. Future work could evaluate mechanisms of cellular uptake that differ based on culture conditions and strain-level differences in *G. vaginalis* cultures.

A *Lactobacillus*-dominated vaginal environment is associated with healthy pregnancy outcomes. Recent work investigated the effects of *L. crispatus* EVs on placental function in response to oxidative stress.<sup>67</sup> While oxidative stress in the first trimester initiates important adaptations to pregnancy, chronic or severe oxidative stress may result in pregnancy loss, preterm birth, preeclampsia, fetal growth restriction, or hypertension. Given the association of *L. crispatus* with healthy pregnancies, Wang and coworkers investigated the role of *L. crispatus* EVs on mitigating the effects of hydrogen peroxide-induced oxidative stress on placental cells *in vitro*. *L. crispatus* EVs rescued cells from hydrogen peroxide-induced death by

recovering Akt phosphorylation and inhibiting mitochondrial fission.<sup>67</sup> Another preclinical study investigated *Akkermansia muciniphila*-derived EVs as prophylactic treatment of preeclampsia.<sup>68</sup> *A. muciniphila* is a gut microbe found in lower levels in patients who develop preeclampsia. In mice, daily oral administration of *A. muciniphila* EVs prevented intrauterine growth restriction, lowered blood pressure, and alleviated placental dysfunction.<sup>68</sup> The mechanisms underlying this attenuation of disease remain unknown, so future work may investigate the protective properties of EVs by assessing protein and nucleic acid cargoes.

In addition to microbe-host communication, bacterial EVs mediate microbe-microbe communication in the female reproductive tract. *Lactobacillus* spp. EVs have been found to promote the colonization of *Lactobacillus* spp., while reducing the adhesion of pathogenic species.<sup>69</sup> By pretreating cervical cells with *L. crispatus*- and *L. gasseri*-derived EVs, Croatti, *et al.* demonstrated increased adhesion of lactobacilli *in vitro*, accompanied by reduced adhesion of *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Enterococcus faecalis*.<sup>69</sup> Beyond bacterial adhesion, some evidence suggests that EVs may contribute to high recurrence rates of BV by facilitating the formation of biofilms in the vagina (Fig. 4).<sup>70–75</sup> Khan, *et al.* investigated the role of *L. gasseri* derived EVs in disrupting biofilms formed by





**Fig. 4** *L. gasseri* EVs disrupt biofilm formation *in vitro*. *G. vaginalis* and *S. aureus* biofilms were disrupted after treatment with *L. gasseri* EVs in a dose-responsive manner. This work presents a potential therapeutic avenue for bacterial EVs in the female reproductive tract. Adapted with permission.<sup>72</sup>

*G. vaginalis* and *S. aureus*.<sup>72</sup> Following 48 h of biofilm formation, *G. vaginalis* and *S. aureus* cultures were treated with *L. gasseri*-derived EVs. The authors observed a dose responsive decrease in biofilm thickness and integrity.<sup>72</sup> Further work could be done to determine which specific cargoes may play a role in these observations.<sup>69</sup>

A *Lactobacillus* spp.-dominated vaginal microbiome is associated with a decreased risk of sexually transmitted infections, including HIV-1. These protective properties are facilitated through several biological mechanisms: maintenance of the mucosal barrier, inactivation of HIV-1 *via* acidification of the vagina, and induction of host immune response against HIV-1. Building upon prior work using bacteria-free supernatants from cultures of lactobacilli,<sup>76</sup> a recent study investigated the ability of EVs from two strains of each *L. crispatus* and *L. gasseri* to inhibit HIV-1 infection both *in vitro* and *ex vivo*. Authors observed strain-specific reduction of HIV-1 replication in T cells after incubation with EVs.<sup>56</sup> The authors found similar results in human tonsillar and cervicovaginal tissues *ex vivo*. As there were no physical differences in EVs (similar size and production rates), the authors hypothesized their findings may be attributed to differences in cargo. Indeed, amino acids, lactic acid, and several proteins were hallmarks of the EVs which inhibited HIV-1 replication.<sup>56</sup> Interestingly, the authors found similar results using EVs from *G. vaginalis*, *S. aureus*, *Enterococcus faecium*, and *E. faecalis*.<sup>77</sup> As pretreatment of cells with EVs did not prevent HIV-1 replication *in vitro*, these findings suggest that bacterial EVs inhibit infection through direct interactions with HIV-1 virions rather than indirect interactions with viral target cells.<sup>56</sup> In a separate study, Artuyants, *et al.* evaluated microbe-pathogen interactions, specifically studying the effects of *L. gasseri* and *G. vaginalis* EVs on *T. vaginalis* cytoadhesion. Consistent with clinical observations, EVs from *L. gasseri* inhibited *T. vaginalis* cytoadhesion, whereas *G. vaginalis* EVs enhanced cytoadhesion.<sup>55</sup> While the exact mechanisms behind these observations remain unclear, together these studies are suggestive of the

role of lactobacilli-derived EVs in supporting a healthy vaginal microenvironment.

As the EV-field continues to grow, we can look to studies which investigate the effects of bacteria-free supernatants on female reproductive tract cells (Table 2). Bacteria-free supernatants are typically generated by centrifugation at a relatively low speed to remove whole cells without removing other byproducts, similar to the first step of EV isolation. While other bacterial byproducts are certainly present in bacteria-free supernatants, these samples undoubtedly also contain EVs. Despite these studies not explicitly mentioning EVs, we believe they support the idea that EVs play a role in the microbe-host communication that underlies microbiome-associated diseases. Edwards, *et al.* investigated *Lactobacillus* spp. supernatants on *Chlamydia trachomatis* infectivity and observed a decrease in human cervical epithelial cell infection after pre-treatment with lactic acid, or *L. crispatus* or *L. jensenii*, but not *L. iners*, bacteria-free supernatants.<sup>78</sup> The authors observed more persistent protection with bacteria-free supernatants than with lactic acid alone, suggesting that there may be important bacterial byproducts which contribute to protection against *C. trachomatis*.<sup>78</sup> Jang, *et al.* observed that *L. crispatus* bacteria-free supernatants reduced the growth of *Candida albicans*.<sup>79</sup> Certainly more work should be done to investigate the mechanisms underlying these observations, but the current evidence supports that a bacterial byproduct, perhaps EVs, may contribute to increased protection against vaginal infections.

Investigating the effects of bacterial byproducts on cervical remodeling, Delgado-Diaz, *et al.* tested the effects of lactic acid on tight junctions in ectocervical cells, demonstrating increased barrier integrity and altered gene expression related to epithelial barrier properties.<sup>80</sup> Interestingly, when treated with bacteria-free supernatants tight junction genes were further increased (Table 2). While the commercially available lactic acid and bacteria-free supernatants were not directly compared, the apparent increase in expression of barrier func-



tion genes associated with supernatant treatment may be attributed to other secreted factors in the conditioned media. In a similar study, Anton and coauthors investigated the effects of microbial supernatants on cervical cell permeability, as a representation of how bacteria may contribute to premature cervical remodeling and preterm birth (Table 2).<sup>81</sup> The authors demonstrated that *L. crispatus* bacteria-free supernatants protected *in vitro* cervical barriers from degradation by lipopolysaccharide (LPS). The supernatants reduced IL-6 and IL-8 secretions, and were protective against *G. vaginalis*-induced changes relevant to cervical remodeling.<sup>81</sup> Interestingly, *L. crispatus* supernatants elicited a stronger anti-inflammatory response than live bacteria across all three cell types, indicating that a byproduct of the parent cells may be responsible for the protective nature of *L. crispatus* in the female reproductive tract.<sup>82</sup> In a follow-up study, authors observed differing effects of *L. crispatus* and *G. vaginalis* supernatants on vaginal epithelial, ectocervical, and endocervical cells.<sup>82–84</sup> Where *L. crispatus* supernatants were protective of barrier function, *G. vaginalis* supernatants caused degradation of the cervical barrier *in vitro*, accompanied by an increase in cytokine production and release of matrix metalloproteinases (Table 2).<sup>81,84</sup> In a later study, the group investigated bacteria-free supernatants from *M. mulieris*, another bacterium highly associated with preterm birth.<sup>83</sup> *M. mulieris* supernatants significantly increased cervical cell degradation and production of inflammatory cytokines IL-6 and IL-8, as compared to non-conditioned media.<sup>83</sup> While these studies did not specifically investigate the role of EVs on cervical remodeling and preterm birth, and the mechanisms linking bacterial EVs and preterm birth need to be further explored, there is convincing evidence for investigating how bacterial byproducts (EVs, in particular) may mediate microbe-host communications in the context of gynecologic and obstetric health. As lipid-bound bacteria-produced nanoparticles, EVs may aid in the transport and uptake of cargoes that contribute to adverse gestational outcomes. We propose that, in addition to small molecules and secreted proteins, the bacteria-free supernatants evaluated in the aforementioned studies contained bacterial EVs and suggest that future studies utilize isolated particles to directly probe the effects of EVs on epithelial integrity, as well as cervical remodeling and related mechanisms of parturition.

Many questions remain regarding the role of bacterial EVs in the vaginal microenvironment and reproductive outcomes, including infertility, recurrent pregnancy loss, miscarriage, pelvic inflammatory disease, and more. With clinical evidence linking *Lactobacillus* spp. with improved fertility, gestational, and gynecologic outcomes, and pathogenic species with adverse outcomes, there are likely many pathways in which EVs potentiate clinical outcomes. We can look to work done using EVs from other species to inform hypotheses regarding EV function, but it is clear that species- and strain-level differences must be evaluated to fully understand microbe-host communication facilitated by bacterial EVs in the vaginal environment.

## Extracellular vesicles enable pathogen:host communication

Beyond the adverse health outcomes associated with BV, the female reproductive tract is susceptible to infection by other pathogenic bacteria, parasites, and fungi. Recent studies examined the role of EVs in promoting pathogenesis of these microorganisms in the vaginal environment and female reproductive tract.

### Group B streptococcus

*Streptococcus agalactiae*, or group B streptococcus (GBS), resides in the rectovaginal area of up to 30% of adults.<sup>85–87</sup> While generally considered harmless, GBS can cause severe infection and disease in neonates. For this reason, pregnant people are screened for GBS at around 36 weeks of pregnancy. If positive, patients are given antibiotics at time of delivery. As all the serotypes of GBS are known to produce EVs (Table 1), the effects of GBS may persist even after antibiotic treatment.<sup>88</sup> These biological nanoparticles may also be able to ascend into the uterine compartment, leading to adverse obstetric outcomes prior to antibiotic treatment. Several studies have investigated the production of EVs across different, clinically relevant strains of GBS.<sup>88–90</sup> One study systematically evaluated EVs from six strains of GBS, demonstrating differences in production, physical properties, and protein cargoes, where nearly 30% of protein cargoes were detected across all six strains of GBS.<sup>88</sup> Variable abundance of peptidase, hyaluronidase, and sialidase were observed across the three sequence types analyzed in this study, highlighting the potential for a strain-specific EV contribution to GBS virulence.<sup>88</sup>

A separate study evaluated the hemolytic activity of EVs from hemolytic and nonhemolytic GBS.<sup>89</sup> Authors demonstrated that the EVs protected GBS against host defenses, specifically reactive oxygen species. *In vivo* work demonstrated that intraperitoneal injection of hemolytic and nonhemolytic EVs into neonatal mice caused morbidity and mortality due to neonatal lung injury, consistent with clinical manifestations of GBS disease in human neonates.<sup>89</sup> A third study evaluated EVs from GBS in their ability to cause preterm birth and intrauterine fetal demise.<sup>90</sup> The authors found that EVs caused a dose-dependent decrease in cervical cell viability *in vitro*, led to a breakdown of murine chorio-decidual membranes *ex vivo*, and were able to ascend into the murine uterus within 6 h of vaginal administration. Intraamniotic injections of GBS EVs caused breakdown of the chorio-decidual epithelium, an influx of neutrophils and lymphocytes into the amniotic sacs (reflective of chorioamnionitis), and a significant and dose-dependent induction of preterm birth.<sup>90</sup> These *in vivo* studies are certainly interesting and suggestive of the ability of GBS-derived EVs to lead to adverse gynecologic outcomes. Future work could better mimic the human case by investigating fetal response to EVs after maternal vaginal administration.

### Sexually transmitted bacterial infections

*Chlamydia trachomatis* and *Neisseria gonorrhoeae*, two microbes which cause sexually transmitted infections, produce EVs

(Table 1).<sup>91</sup> *C. trachomatis* is an intracellular bacterium that causes chlamydia, the most common sexually transmitted infection. Chlamydia can lead to cervicitis, pelvic inflammatory disease, and infertility in females. Neonates exposed to *C. trachomatis* may develop pneumonia or conjunctivitis.<sup>92</sup> Infection occurs when elementary bodies are phagocytosed and use cellular nutrients to develop reticulate bodies, eventually producing more elementary bodies.<sup>93</sup> As cell nutrients are taken for *C. trachomatis* growth, the host cell will rupture, releasing new elementary bodies to infect other cells. As the pathogen develops into reticulate bodies, it communicates with the host cell, in part, through the production of EVs.<sup>94–96</sup> EVs from *C. trachomatis* were first described based on observed changes to the bacterial membrane in response to stressors, such as antibiotics, nutrient deprivation, and co-infections.<sup>97</sup> Physical perturbations in the outer membrane of the bacteria led to the production of EVs blebbing from the membrane of the inclusions. TEM images of *C. trachomatis*-infected human endocervical cells revealed EVs present within inclusion bodies within infected cells, and Frohlich and coworkers were able to isolate *C. trachomatis* EVs from infected mouse fibroblast cells.<sup>94</sup> EV cargo analysis revealed cytotoxic proteins present in the isolated vesicles, supporting the hypothesis that EVs play a role in modulating the host immune response. Albeit mild, EVs from *C. trachomatis* caused an increase in IL-6 from mammalian cells *in vitro*, suggesting that EVs may aid in evasion of the host immune system.<sup>95</sup> Understanding EVs from intracellular bacteria is challenging, as it is difficult to differentiate between EVs produced by host cells and those produced by infectious bacteria. However, as technology in the EV field develops there will be more opportunities to understand how EVs play a role in infection and how they may be harnessed to treat and prevent infections.

*N. gonorrhoeae* causes gonorrhea, another potentially asymptomatic sexually transmitted infection that can lead to significant complications in neonates, usually corneal infection that can progress to blindness.<sup>98</sup> Outer membrane blebs from *N. gonorrhoeae* were first described by Dorward and Judd in 1989.<sup>99</sup> EVs were observed to be released during the log-phase of growth and to have similar immunogenic properties as parent microbes. More recently, Zielke and coworkers assessed the protein composition of *N. gonorrhoeae* cell envelopes and EVs.<sup>100</sup> Authors found differences across strains, but identified a large fraction of proteins responsible for biogenesis and cellular metabolism. While the authors did not investigate the function of specific proteins, follow-up studies have built upon this work to develop vaccines and therapies for preventing gonorrhea (see more in section “Extracellular vesicles as a therapeutic platform”).

A 1992 study investigated the role of *N. gonorrhoeae* EVs in protecting bacteria against human serum immunoglobulins.<sup>101,102</sup> Pettit and Judd found differences in host recognition of EVs produced by serum-resistant and serum-susceptible strains of *N. gonorrhoeae*.<sup>102</sup> Hypothesizing that the EVs may be able to remove antibodies against the parent microbe from serum, authors demonstrated that incubating serum-susceptible bacteria with EVs from resistant strains increased the viability of the bacteria.<sup>101</sup> Similarly, a 2007 study by Lee and coworkers demonstrated that opacity-associated (Opa) proteins on the surface of *N. gonorrhoeae* EVs mediated binding to human carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs).<sup>103,104</sup> CEACAM-specific EVs caused a reduction in T-cell proliferation and inhibited expression of CD69 in T-cells. These studies highlight the immunosuppressive nature of EVs and help to identify design criteria for effective vaccines using bacterial EVs.

### Trichomonas vaginalis

*Trichomonas vaginalis* is a protozoan parasite which causes trichomoniasis.<sup>105</sup> A sexually transmitted disease, trichomoniasis is usually asymptomatic in males, but causes itching, discharge, and painful urination and vaginal intercourse in females. The first study to report on *T. vaginalis* EVs identified cup-shaped particles between 50–100 nm in size that transferred both RNA and protein cargoes to ectocervical cells.<sup>106</sup> Nievias, *et al.* found different cargo signatures in different sizes of EVs, demonstrating the heterogeneity of EV biogenesis, loading, and function.<sup>54</sup> Artuyants, *et al.* isolated *T. vaginalis* EVs and identified small RNA cargoes with high abundance of tRNAs that were delivered to host cells.<sup>107</sup> Twu, *et al.* observed a proinflammatory response in ectocervical cells caused by *T. vaginalis* EVs comparable to the response induced by ectocervical cell exposure to *T. vaginalis* parasite.<sup>106</sup> Furthermore, treatment with EVs led to a dose-responsive increase in adherence of parasite to ectocervical cells.<sup>106</sup> In a separate study, Olmos-Ortiz and coworkers observed that mice vaginally pre-treated with *T. vaginalis* EVs had significantly more viable trophozoites than mice that did not receive pre-treatment with EVs.<sup>108</sup> Authors observed a significant increase in IL-10 secretion in vaginal lavage, and a significant decrease in IL-13 and IL-17 secretion, after exposure to *T. vaginalis* EVs, suggesting that EVs may reduce the host immune response to allow for persistence of the parasite.<sup>108</sup>

Specifically investigating host cell uptake of *T. vaginalis* EVs into mammalian cells, Rai and Johnson identified heparan sulfate proteoglycans on the surface of host cells as critical mediators of EV internalization.<sup>109</sup> Inhibitors for clathrin-mediated endocytosis, phagocytosis, and micropinocytosis showed no effect on EV uptake, whereas inhibition of caveolae-dependent endocytosis significantly reduced EV uptake.<sup>109</sup> Understanding mechanisms of uptake, as well as immunomodulatory properties of EVs may guide the development of therapeutics for female reproductive tract disease.

### Candida albicans

*Candida albicans* is a commensal fungus found in the skin, mouth, gastrointestinal tract, and vagina.<sup>110</sup> In the female reproductive tract, *C. albicans* causes vulvovaginal candidiasis, which causes vaginal itching, soreness, pain during sexual intercourse and urination, and abnormal discharge. Recurrent vulvovaginal candidiasis, in particular, severely affects the quality of life of women.<sup>111</sup> Albuquerque, *et al.* first isolated

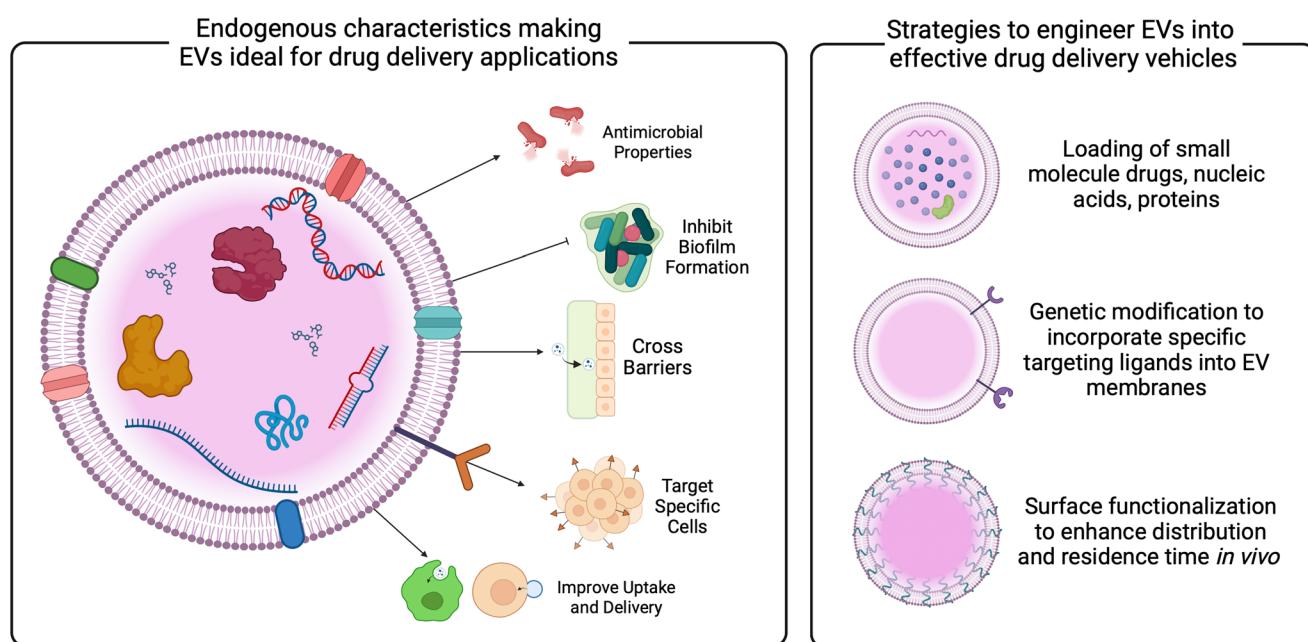
EVs from *C. albicans*, and used TEM analysis to measure particles in the 100–200 nm range.<sup>112</sup> Vargas and coworkers later identified differences in EV populations from different strains of *C. albicans*, where cargo proteins were involved in pathways including entry and adhesion into host cells, gluconeogenesis, glycolysis, and biofilm formation.<sup>113,114</sup> As biofilms are a primary mechanism of fungal pathogenesis, several studies have evaluated the role of EVs in biofilm adhesion and matrix biogenesis. Building upon work identifying phospholipids as a primary component of *C. albicans* biofilms, Zarnowski and coworkers hypothesized that EVs may contribute to biofilm formation.<sup>74,75</sup> As the endosomal sorting complexes required for transport (ESCRT) pathway is responsible for the production of EVs in other eukaryotes, authors developed mutants with deletion of ESCRT subunits. In addition to observing a decrease in the number of EVs produced by this library of mutants, authors reported increased biofilm susceptibility to antifungal treatments.<sup>74</sup> By reintroducing EVs, the susceptibility was reversed to near-control levels, indicating that the observed effect was due to a lack of EVs produced by the fungus. In a follow-up study, the same group evaluated EV cargoes for specific contributions to drug susceptibility, biofilm adhesion, and biofilm dispersion, and highlighted specific protein cargoes as potentially useful therapeutic targets.<sup>115</sup>

In addition to EVs secreted from *C. albicans* biofilms, Martínez-López, *et al.* identified differences in EVs secreted from yeast compared to EVs secreted from hyphal cells.<sup>116</sup> The yeast to hypha transition is critical for *C. albicans* virulence,

where hyphal cells are better equipped for invasion of host tissues and yeast typically allow for dispersion and adhesion. EVs secreted by yeast were larger than those secreted by hyphal cells and carried different protein cargoes which contributed to differences in host cytotoxicity, where hyphal EVs increased cell death and TNF $\alpha$  secretion. The virulence factors that affect cellular immune response have been observed in other studies, where in an invertebrate model of *C. albicans* infection, pre-treatment with EVs reduced fungal colony forming units, suggesting that EVs may prime the host system to prevent fungal growth.<sup>116</sup>

#### Extracellular vesicles show potential as biomarkers and therapeutics

Given their role as communicators *in vivo*, EVs have gained interest as a modality for reporting and treating disease (Fig. 5).<sup>20</sup> Reflective of parent cell function, EVs derived from human samples may give insight into cellular function and, in the case of bacteria, important strain level differences. Advances must be made in isolation protocols for this to be a clinical reality, but at least one other study isolated EVs from respiratory mucus.<sup>117</sup> Similarly, characterization of EVs from human samples is challenging based on the heterogeneity of EVs represented in a given biofluid, as well as changes to bacteria based on host pressures (*i.e.*, hormones, stress, environmental exposures). Engineering technologies to enhance the detection of surface proteins and single-particle technologies will advance our use of EVs as biomarkers of female reproductive tract disease.



**Fig. 5** Improving the understanding of EVs will enable the translation of next generation EV therapeutics for gynecologic and obstetric indications. EVs present an interesting modality for therapeutic delivery. With the ability to cross biological barriers, target specific cells, and improve cellular uptake, EVs may display benefits over synthetic nanoparticles. Established methods demonstrate the ability for EVs to be loaded with small molecule drugs, nucleic acids, and proteins either pre- or post-isolation. Technological developments may help to improve targeting and residence time *in vivo*.



As a therapeutic platform, EVs from bacteria present benefits over mammalian cell-derived EVs. Bacteria are easily scalable and rapidly proliferate. Further, there are established and easy gene editing protocols for cargo loading. Clinical studies have used bacterial EVs as vaccines, and pre-clinical work highlights EV functionality as carriers for small molecules, nucleic acids, and proteins.<sup>118–122</sup> These cargoes may help to prime a host immune system to ward off infections by parent pathogens, making them especially interesting as vaccine agents. Other reviews describe bacterial EVs as vaccines in more detail, but briefly, a meningococcal B EV-based vaccine was used with varying efficacy in Cuba, Norway, and New Zealand to vaccinate against meningitis.<sup>121–123</sup> Using cultures of single strains of *N. meningitidis* may have limited the efficacy, as multiple strains are typically behind epidemic outbreaks. A retrospective study suggested that this vaccine may also protect against gonorrhea, given the overlap in genetic homology between *N. meningitidis* and *N. gonorrhoeae*; however, it is difficult to make assessments based on the longitudinal data.<sup>118,123</sup> Future studies could evaluate vaccine effectiveness *a priori* designed trials, and expand EV-based vaccines to prime the host immune system against BV-associated bacteria, for example.

In addition to vaccines, bacterial EVs have been proposed as novel drug delivery vehicles. Compared to synthetic carriers, EVs demonstrate advantages, including enhanced biocompatibility, reduced immunogenicity, innate barrier-crossing abilities, and specific targeting.<sup>22,29,31,124–127</sup> Studies have demonstrated the loading of bacterial EVs with both hydrophobic and hydrophilic small molecule drugs, as well as nucleic acid and protein cargoes.<sup>128–130</sup> These data reveal the role of EV membrane composition on loading efficiencies, indicating the need for tailored protocols for EVs from different parent cells. Loading techniques vary, and are described in more detail elsewhere,<sup>131–133</sup> but have not been widely studied in EVs relevant to the female reproductive tract. Given the significant role of the vaginal microbiome in gynecologic outcomes, as well as the growing evidence of EVs mediating these outcomes discussed above, vaginal bacterial EVs may be useful as carriers of therapeutics for gynecologic and obstetric health (Fig. 4 and 5).

Innovation in genetic modification of bacteria, as well as methods for enhancement of EVs post-production may improve targeting and residence time *in vivo* (Fig. 5). Described in more detail elsewhere,<sup>20,134,135</sup> current work has investigated surface modification *via* PEGylation of EVs as a method for improving circulation and penetration through biological barriers,<sup>136,137</sup> as well as the addition of targeting ligands to enhance specific cell uptake.<sup>138,139</sup> Based on studies of synthetic particles for gynecologic indications, PEGylation may enhance EV mobility through cervicovaginal mucus.<sup>140–142</sup> Similarly, EV membranes could be fused with anti-oxytocin receptor antibodies for targeting myometrial contractions,<sup>143</sup> or with chondroitin sulfate A-binding peptides to target trophoblast cells for placental drug delivery.<sup>144</sup> Additional concerns for the translation of bacterial EVs lie with the pro-

duction and manufacturing of EVs. Advances in scalable manufacturing must be made to enable the clinical translation of EV therapeutics. From other parent cells, media components and stressors have been investigated for increasing the production of EVs.<sup>145–148</sup> Recent work demonstrated that genetic manipulation of both Gram positive and Gram negative microbes could increase EV secretion.<sup>149</sup> Similarly, improvements in isolation procedures that increase both recovery and purity are needed and currently being studied. While ultracentrifugation is an industry standard,<sup>150,151</sup> advances in tangential flow filtration, anion exchange chromatography, and immunocapture assays may increase yield, purity, and reproducibility of EV isolations.<sup>152,153</sup> Specific work should focus on the use of vaginal bacterial EVs as therapeutics and how to improve EV isolation from relevant parent cells.

Based on their endogenous interactions with host cells in the female reproductive tract, bacterial EVs may prove to be an optimal platform for vaginally delivered therapies. Vaginal drug delivery is optimal for targeting female reproductive tract tissues.<sup>141,142</sup> By directly exposing target tissues to therapeutic cargoes, treatment efficacy is improved. Vaginal drug delivery bypasses the harsh gastrointestinal environment and the hepatic first pass, which are challenges to traditional oral and systemic formulations.<sup>140,141,154</sup> However, vaginally administered formulations must evade adhesive and steric interactions with the protective cervicovaginal mucus barrier. Recent work demonstrated that the pore size of cervicovaginal mucus is ~300–400 nm, with a significant increase in the average pore size of polymicrobial (CST IV) samples.<sup>32,33</sup> While EVs are small enough to penetrate the mucus mesh, it is unclear how their surface properties may affect adhesive interactions with carboxyl and sulfate groups and hydrophobic regions of the mucus mesh. Future studies should investigate the adhesive interactions between bacterial EVs and mucus. However, given preclinical *in vivo* evidence of particle ascension and the proposed functionality of EVs in mediating gynecologic and obstetric outcomes, it seems possible, if not likely, that EVs are able to penetrate the cervicovaginal mucus barrier to reach the underlying epithelium. Taken together, there is strong support for utilizing EVs as a vaginal drug delivery platform, however, as bacterial byproducts, regulatory studies must be done to understand the use of EVs in the clinic. For example, it is important to understand how culture-derived EVs differ from endogenously produced EVs, and how these differences may impact EV uptake and function *in vivo*.

## Concluding remarks

EVs enable communication between cells across all forms of life. Given that the human microbiome so significantly impacts human health outcomes, recent interests have been directed towards understanding the functionality of bacterial EVs, with specific attention to EVs from the gut microbiome. A smaller, but no less important, body of work examines bacterial and pathogen-derived EVs relevant to the female repro-



ductive tract. Whether these biological nanoparticles are protective against the invasion of pathogenic bacteria and viruses, as in the case of *L. crispatus*-derived EVs, or are immunosuppressive and aid in the pathogenicity of BV or other sexually transmitted infections, there is clear evidence for the role of bacteria- and pathogen-derived EVs in women's health. Further study of microorganism-derived EVs as mediators of disease and as a novel therapeutic platform for treating gynecologic and obstetric indications are needed and warranted. With the many unanswered questions surrounding mechanisms that contribute to female reproductive tract disease, and the significant need for new therapies to treat these indications, EVs may prove to be an important modality in understanding and improving women's health outcomes.

## Author contributions

All authors contributed to manuscript drafting, editing, and revision, and approved the submitted version.

## Conflicts of interest

There are no conflicts to declare.

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