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ORIGINAL ARTICLE

Intrauterine colonization with *Gardnerella vaginalis* and *Mobiluncus mulieris* induces maternal inflammation but not preterm birth in a mouse model

Andrea Joseph[1] | **Emma L. Lewis**[2] | **Briana Ferguson**[2] | **Yuxia Guan**[2] | **Lauren Anton**[2] | **Michal A. Elovitz**[1]

[1]Department of Obstetrics, Gynecology and Reproductive Sciences, Icahn School of Medicine, New York, New York, USA

[2]Center for Research on Reproduction and Women's Health, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Correspondence
Andrea Joseph, Department of Obstetrics, Gynecology and Reproductive Sciences, Icahn School of Medicine, New York, NY, USA.
Email: andrea.joseph@mssm.edu

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Abstract

Problem: Preterm birth (PTB) remains a leading cause of childhood mortality. Recent studies demonstrate that the risk of spontaneous PTB (sPTB) is increased in individuals with *Lactobacillus*-deficient vaginal microbial communities. One proposed mechanism is that vaginal microbes ascend through the cervix, colonize the uterus, and activate inflammatory pathways leading to sPTB. This study assessed whether intrauterine colonization with either *Gardnerella vaginalis* and *Mobiluncus mulieris* alone is sufficient to induce maternal-fetal inflammation and induce sPTB.

Method of study: C56/B6J mice, on embryonic day 15, received intrauterine inoculation of saline or 10^8 colony-forming units of *G. vaginalis* ($n = 30$), *M. mulieris* ($n = 17$), or *Lactobacillus crispatus* ($n = 16$). Dams were either monitored for maternal morbidity and sPTB or sacrificed 6 h post-infusion for analysis of bacterial growth and cytokine/chemokine expression in maternal and fetal tissues.

Results: Six hours following intrauterine inoculation with *G. vaginalis*, *M. mulieris*, or *L. crispatus*, live bacteria were observed in both blood and amniotic fluid, and a potent immune response was identified in the uterus and maternal serum. In contrast, only a limited immune response was identified in the amniotic fluid and the fetus after intrauterine inoculation. High bacterial load (10^8 CFU/animal) of *G. vaginalis* was associated with maternal morbidity and mortality but not sPTB. Intrauterine infusion with *L. crispatus* or *M. mulieris* at 10^8 CFU/animal did not induce sPTB, alter pup viability, litter size, or maternal mortality.

Conclusions: Despite inducing an immune response, intrauterine infusion of live *G. vaginalis* or *M. mulieris* is not sufficient to induce sPTB in our mouse model. These results suggest that ascension of common vaginal microbes into the uterine cavity alone is not causative for sPTB.

KEYWORDS
intrauterine infection, intrauterine inflammation, mouse model, preterm birth, vaginal microbiome

1 | INTRODUCTION

Preterm birth (PTB), defined as a preterm delivery earlier than 37 weeks of gestation, affects more than 15 million births globally. Approximately 1 million infants and children die due to direct complications of PTB and many more develop lasting disabilities including diverse neurobehavioral disorders, persistent medical complications, and decreased lifespan.[1–4] The majority of PTB occur spontaneously. Multiple molecular pathways are theorized to cause spontaneous PTB (sPTB) including uteroplacental ischemia, decidual hemorrhage, cervical injury, stress, infection, and inflammation.[5–7] Of these, a strong body of evidence over the past two decades supports the association of sPTB with inflammation, particularly from host-microbial interactions within the cervicovaginal (CV) space.[8–14]

Microorganisms in the vagina are a first line of defense against invading pathogens. Predominance of *Lactobacillus* subspecies (spp.) in the CV space is believed to play a key role in the prevention of reproductive and urogenital diseases through the maintenance of an acidic microenvironment, production of anti-microbial and anti-inflammatory factors, and competitive exclusion of other microbes.[15] In contrast, vaginal communities characterized by a decrease in *Lactobacillus* spp. and an abundance of diverse anaerobic species are associated with adverse reproductive outcomes.[16–19] These anaerobes include *Gardnerella vaginalis*, *Mobiluncus* species, *Prevotella* species, *Mycoplasma hominis*, and *Ureaplasma urealyticum*.[20] Bacterial vaginosis (BV), characterized by *Lactobacillus*-deficient, anaerobe-rich vaginal communities, is present in up to 15%–20% of pregnant individuals and is a known risk factor for sPTB.[20–29] Even in the absence of clinical symptoms of BV, anaerobe-dominated vaginal communities have been associated with an increased risk of sPTB.[30–40] A recent large, well-characterized human cohort identified seven bacterial species significantly associated with sPTB.[33] In particular, *Mobiluncus curtsii/mulieris* had the most profound effect on sPTB risk, a probability which increased with its increasing relative abundance in the microbial community.[33] Further, *Mobiluncus* species and *G. vaginalis* have both specifically been identified in amniotic fluid from individuals who go on to deliver preterm, suggesting an essential role of vaginal microbes in sPTB.[41–44]

To mechanistically link these findings, an accepted paradigm proposes that sPTB is initiated by ascension of vaginal microbes to the uterus and the subsequent inflammation triggers myometrial contractility followed by preterm parturition.[45–47] However, many of the vaginal microbes associated with sPTB, including *G. vaginalis* and *M. mulieris*, are known to be non-motile microbes.[48–50] As such, the mechanistic contributions of anaerobic vaginal bacteria to sPTB remain unclear. If ascension of CV anaerobes and the consequent intrauterine inflammation is sufficient to trigger sPTB, then direct colonization of the uterus with these microbes should be sufficient to induce sPTB.

To advance our understanding of sPTB and reveal pathways for optimal therapeutics, it is necessary to identify mechanisms by which vaginal microbes induce sPTB. Therefore, the objectives of this study were to determine whether intrauterine inoculation with vaginal microbes *G. vaginalis*, *M. mulieris*, and *Lactobacillus crispatus* in pregnant C57/BL6 mice can induce immune responses across the maternal-fetal interface and whether activation of inflammatory processes by these microbes is sufficient to induce sPTB.

2 | METHODS

2.1 | Animal experiments and ethics statement

C57/BL6 timed-pregnant mice were purchased from Jackson Laboratories (Bar Harbor, ME). The day of mating was considered embryonic day 0 (E0) and E1 was determined based on presence of copulatory plug. Animals were shipped on day E12 and housed individually in our facilities. These animals were acclimated for 2 days before performing experiments on E15. All the experiments were performed in accordance with the National Institutes of Health Guidelines on Laboratory Animals and with approval from the University of Pennsylvania's Institutional Animal Care and Use Committee (IACUC #805513).

2.2 | Bacterial cultivation

Bacteria were grown at 37°C in an anaerobic glove box (Coy Labs, Grass Lake, MI). *G. vaginalis* (ATCC 14018), *L. crispatus* (ATCC 33197), and *Mobiluncus mulieris* (ATCC 35243) were grown in New York City III broth as previously described.[51–53] All media were supplemented with 5% horse serum (Gibco, Thermo Fisher Scientific). Efficient bacteria growth was measured and quantified by colony forming unit (CFU) assays. Bacteria were centrifuged twice to remove the growth media and the final pellet was resuspended in sterile phosphate buffered saline (PBS) for use in animal experiments. An aliquot of the same PBS (saline) was used as the control in all animal trials.

2.3 | Mouse model of intrauterine inoculation and tissue harvest

These studies utilized a well-established mouse model of intrauterine inflammation in late gestation.[54,55] On E15, pregnant mice underwent a mini-laparotomy while under isoflurane anesthesia. Each mouse received an infusion into their right uterine horn, between the first and second amniotic sacs proximal to the cervix, as previously described.[56–58] Each infusion was either 100 μL sterile PBS (saline control group), 10^2–10^8 CFUs/animal of *G. vaginalis* (n = 8-11 per dose), or 10^8 CFUs/animal of *L. crispatus* (n = 16) or *M. mulieris* (n = 17). Surgery sites were closed by surgical staples. At each dose, a set of dams were monitored for maternal morbidity and PTB every 6–12 h for the first 48 h and then every 24 h until delivery. Morbidity was noted by piloerection, lethargy, and ill appearance; dams with severe symptoms were euthanized. After delivery, the number of total pups was recorded. PTB was defined as delivery prior to E18. At 24 h post-delivery, the number and weight of live pups was recorded.

In a separate set of dams receiving saline ($n = 8$) or 10^8 CFU/animal of *G. vaginalis* ($n = 10$), dams were euthanized by carbon dioxide/cervical disloaction and tissues were harvested 6 h post-surgery. Maternal blood was collected by cardiac puncture and amniotic fluid was removed from each gestational sac with a 25 gauge needle on a 1 mL syringe. To assess for live bacteria, approximately 50 μL of each fluid was spread on Tryptic Soy Agar supplemented with 5% rabbit blood and incubated for 48 h under the anaerobic conditions mentioned above. The remaining fluid was centrifuged at 10,000×g for 10 min at 4°C and serum, amniotic fluid supernatant, and pelleted amniotic fluid cells were stored separately at −80°C. The uterus was harvested after removal of placentae. Fetal liver and placentae were collected from the first two fetuses proximal to the cervix in both uterine horns. All tissues were flash frozen in liquid nitrogen and stored at −80°C.

A similar experiment was performed to collect identical tissues from dams receiving saline, *L. crispatus*, or *M. mulieris* ($n = 3$/treatment group at 10^8 CFU/animal). In these experiments, aliquots of maternal blood and amniotic fluid from *L. crispatus*-exposed dams were spread on De Man, Rogosa and Sharpe agar (Fisher Scientific) while fluids from *M. mulieris*-exposed dams were spread on Brucella blood agar (Fisher Scientific). Fluids from saline dams were spread on both types of agar. All other tissues were harvested as above.

2.4 | Bacterial DNA isolation and qPCR

Genomic DNA (gDNA) was isolated and purified from the spleen, uterus, placenta, fetal liver, amniotic fluid, and vagina using the ZR fecal MiniPrep DNA extraction kit (Zymo Research, Irvine, CA). qPCR was performed on the 7900HT Real-Time PCR System (Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturers' protocols. We used TaqMan probes specific to *G. vaginalis* (Assay ID Ba04646236_s1), *L. crispatus* (Assay ID Ba04646245_s1), and *M. mulieris* (Assay ID Ba04646246_s1). Standard curves were created from serially diluted gDNA from each bacteria to quantify the amplification. The results were analyzed using the RQ manager software v2.4 (Applied Biosystems).

2.5 | Luminex assay

Tissue from the uterus, placenta, fetal liver, and cervix were homogenized in 1 mL of RIPA Lysis and Extraction Buffer (G-Biosciences, St. Louis, MO) with cOmplete Mini Protease Inhibitor Cocktail (Roche, Penzberg, Germany) by TissueLyser II (Qiagen, Germantown, MD) for 6 min at 30/s with a 5 mm steel bead. Homogenized tissues were rested on ice for 1 h and then centrifuged at 12,000 rpm for 15 min at 4°C. Tissue homogenate supernatants, serum, and amniotic fluid samples were stored at −80°C until processed by the University of Pennsylvania Human Immunology Core. Samples were assayed with the 32-plex Milliplex Mouse Cytokine/Chemokine Magnetic Bead Assay (Millipore Sigma, Darmstadt, Germany). All samples were run in duplicate per the manufacturer's protocol on the FLEXMAP3D Luminex platform (Luminex, Austin, TX). Absolute quantification in pg/mL was obtained using a standard curve generated by a five-parameter logistic (5PL) curve fit using Xponent 4.2 software (Luminex). Fold change values were calculated between treatment groups and saline controls and presented in a heat map created using GraphPad Prism as described below.

2.6 | Statistical analysis

Statistical analyses were performed for all experiments with the GraphPad Prism Software (Version 9.0, San Diego, CA). For data that were normally distributed, one-way analysis of variance (ANOVA) was performed. If statistical significance was reached ($p < .05$), then pairwise comparison with a Tukey post hoc test was performed for multiple comparisons. If data were not normally distributed, then the nonparametric Kruskal-Wallis test was used and pairwise comparison was done using Dunn's multiple comparison test. Analysis of Luminex cytokine data was performed using a one-way ANOVA followed by post-hoc tests comparing treatment groups to saline controls using a Dunnett's multiple comparison test.

3 | RESULTS

3.1 | Vaginal microbes localize to both maternal and fetal tissues after intrauterine inoculation

Following intrauterine infusion of *G. vaginalis* (10^8 CFU/animal) or saline on E15, microbial DNA was identified in the uterus of exposed dams but not in the spleen, fetal liver, and placenta of either bacteria- or saline-treated dams (Figure 1A). Similarly, DNA from *L. crispatus* (Figure 1B) and *M. mulieris* (Figure 1C) was present in the uterus of dams treated with each microbe ($p < .0001$ for both). DNA from *L. crispatus* was also significantly increased in the fetal liver compared to saline controls ($p < .00002$).

As DNA cannot distinguish live versus dead bacteria, the presence of live bacteria at the maternal-fetal interface was assessed by bacterial growth on agar plates in maternal blood and amniotic fluid (Figure 2). *L. crispatus*, *G. vaginalis*, and *M. mulieris* live bacterial colonies, identified by qPCR, were observed in maternal blood and amniotic fluid 6 h after intrauterine infusion with the specific microbes.

3.2 | Maternal immune response to intrauterine inoculation with vaginal microbes

After confirming the presence of live microbes in multiple tissues after intrauterine infusion, we sought to assess whether bacteria induced an immune response across the maternal-fetal interface. We measured expression of 32 cytokines/chemokines by Luminex array using tissues harvested 6 h after intrauterine inoculation. Analyte expression after

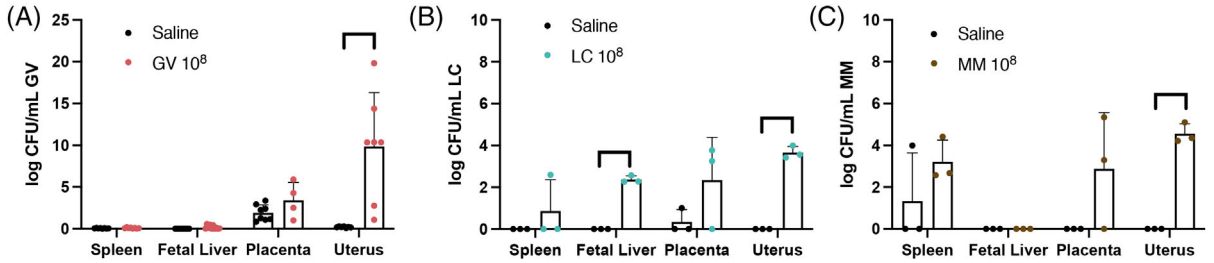

FIGURE 1 Confirmation of bacterial localization by qPCR. DNA from (A) *G. vaginalis*, (B) *L. crispatus*, and (C) *M. mulieris* was identified in the uterus of dams treated with each microbe respectively. *L. crispatus* was also identified in the fetal liver.

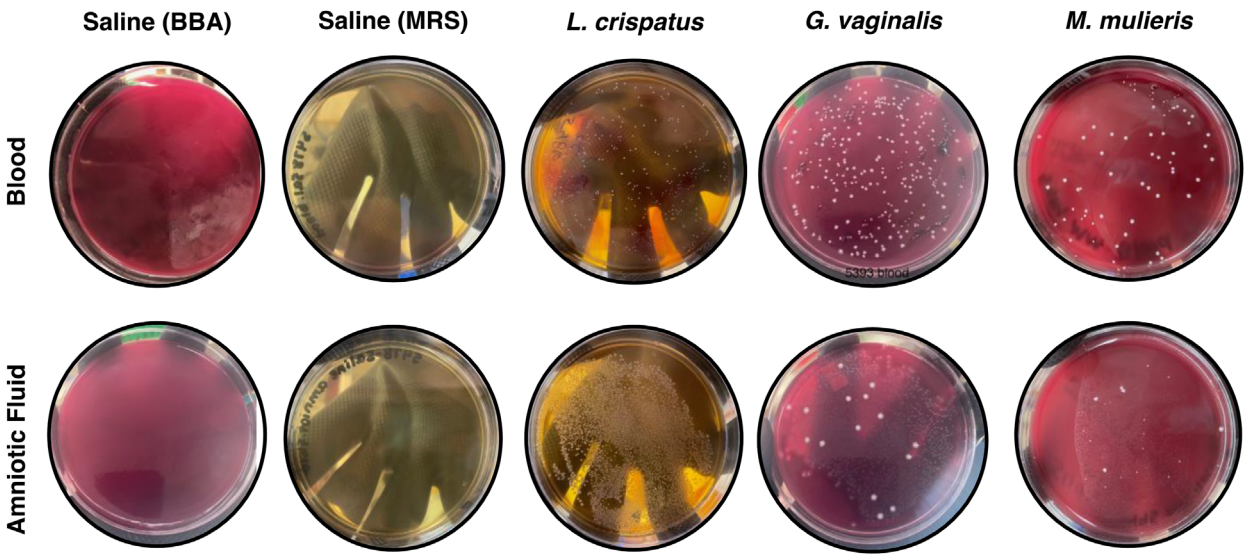

FIGURE 2 Confirmation of live bacterial load by growth on agar plates. Representative images of blood and amniotic fluid smears from saline-, *L. crispatus*-, *G. vaginalis*-, and *M. mulieris*-treated dams indicate live bacterial colonization in the treated dams but not saline controls.

intrauterine infusion with each microbe is shown in a heat map as a fold-change relative to saline controls (Figure 3A). In the uterus, *G. vaginalis* inoculation resulted in overexpression of nine cytokines compared to saline controls: G-CSF, eotaxin, IFNγ, IL-1β, KC, LIX, MIP-1α, MIP-1β, and TNFα ($p < .047$ for all). Seven cytokines were significantly increased after inoculation with *L. crispatus* (G-CSF, GM-CSF, IL1β, LIF, MCP1, MIP1α, and MIP1β; $p < .045$ for all) while one cytokine (G-CSF, $p = .019$) exhibited increased expression after intrauterine inoculation with *M. mulieris*. In maternal serum, intrauterine inoculation with *G. vaginalis* increased expression of five cytokines relative to saline controls: G-CSF, IL-10, MIP-1α, MIP-1β, and TNFα ($p < .023$ for all). Inoculation with *M. mulieris* or *L. crispatus* altered expression of only one cytokine each, IL-1α ($p = .028$) and IL-9 ($p = .031$), respectively. In the cervix, inoculation with *L. crispatus* increased expression of 6 cytokines (G-CSF, GM-CSF, LIF, KC, MIP2, and TNFα; $p < .049$ for all) while inoculation with *M. mulieris* increased expression of 4 cytokines (GM-CSF, IL-15, IL-17, and VEGF; $p < .036$ for all). No cytokines were altered in the cervix following intrauterine inoculation with *G. vaginalis*.

3.3 | Fetal immune response to intrauterine inoculation with vaginal microbes

Next, we determined the immune response to each live microbe in the fetal compartments: placenta, amniotic fluid, and fetal liver (Figure 3B). In the placenta, three cytokines were overexpressed after intrauterine inoculation with *L. crispatus* (G-CSF, KC, and MIP-2, $p < .038$ for all) and two cytokines were overexpressed after intrauterine inoculation with *M. mulieris* (G-CSF and eotaxin; $p < .048$ for both). In the fetal liver, two cytokines were overexpressed after intrauterine inoculation with *M. mulieris* (MIG and VEGF, $p < .037$ for both). No immune response was identified in the amniotic fluid after inoculation with any microbe, and intrauterine inoculation with *G. vaginalis* did not result in an immune response in any fetal compartment (selected cytokines shown in Supplemental Figure 1). The full list of all cytokines/chemokines included in the Luminex assays, their average values (pg/mL), standard deviation, and significance values can be found in Supplemental Tables 1–3.

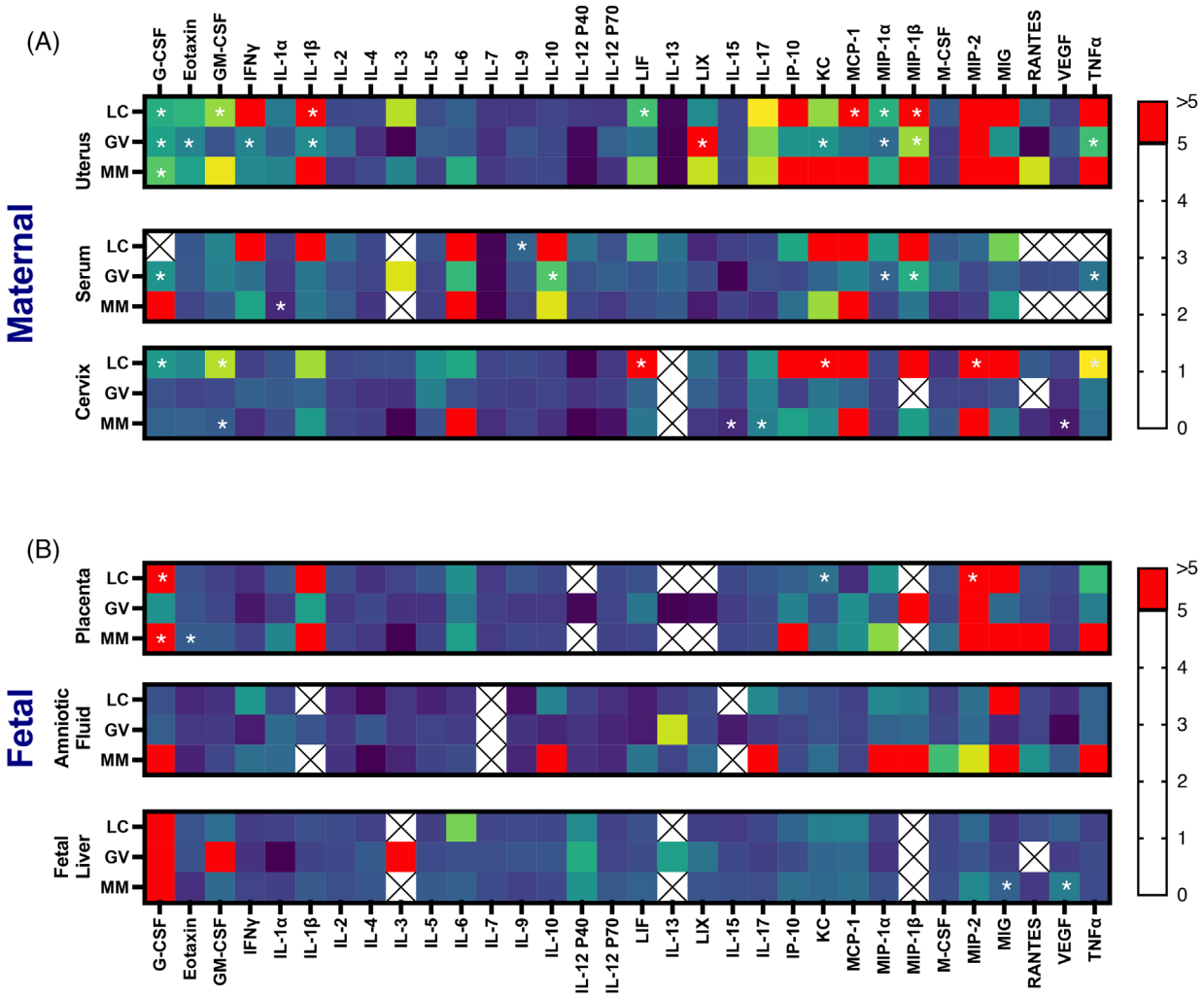

FIGURE 3 Assessment of maternal and fetal immune responses following intrauterine inoculation with vaginal microbes. Immune cytokines/chemokines released in (A) maternal compartments of the uterus, serum, cervix, and (B) fetal compartments of the placenta, amniotic fluid, and fetal liver were measured by Luminex from tissues harvested 6 h after bacterial inoculation. Heat map depicts fold change versus saline-treated dams by color and *p*-value by asterisks.

3.4 | Intrauterine *G. vaginalis or M. mulieris* does not consistently induce PTB

Finally, *G. vaginalis* (10^2 to 10^8 CFUs/animal) was infused into the uterine horn at E15, resulting in 1.5% PTB (n = 1/68) (Table 1). There was a positive correlation (p = .0064) between *G. vaginalis* dose and neonatal death by 24 h (Supplemental Figure 2). Neither intrauterine infusion of *M. mulieris* (n = 14) nor *L. crispatus* (n = 13) resulted in PTB at 10^8 CFU/animal. No significant differences in litter size, pup weight, or pup mortality were observed after intrauterine inoculation of 10^8 CFU/animal of *M. mulieris* and *L. crispatus* compared to 10^7 CFU/animal of *G. vaginalis* (Table 1). At 10^8 CFU/animal of *G. vaginalis*, dams appeared ill with decreased movement and piloerection by 6 h. Within 24 h, all dams receiving this bacterial load (n = 20) exhibited significant morbidity requiring euthanasia (Table 1). No maternal morbidity was observed after intrauterine infusion of *M. mulieris* or *L. crispatus*.

4 | DISCUSSION

In this study, we demonstrate the activation of immune responses across the maternal-fetal interface by intrauterine delivery of vaginal microbes associated with adverse reproductive outcomes including sPTB. Interestingly, infusion of vaginal microbes into the uterine cavity resulted in migration of these live microbes to the feto-placental unit. A potent cytokine response was induced in the uterus and maternal serum but not from the placenta, amniotic fluid, or fetal liver. Neither the presence of microbes in the uterine and fetal compartment nor their corresponding immune responses were sufficient to trigger preterm parturition. Increasing doses of *G. vaginalis* was correlated with reduced pup viability and a high bacterial burden of *G. vaginalis* in the uterus resulted in significant maternal morbidity while *L. crispatus* and *M. mulieris* had no significant effect on maternal morbidity or PTB.

While vaginal anaerobes have been consistently linked to adverse pregnancy outcomes,[16–19,33] limited studies have investigated

TABLE 1 Summary of preterm birth, maternal mortality, and pup viability outcomes after uterine inoculation of *G. vaginalis*, *L. crispatus*, and *M. mulieris*.

Dose	*n*	PTB	Litter size Median (IQR)	Average pup weight (g) Median (IQR)	% dead pups per litter
Median (IQR)					
Saline	18	0	4 (3-5)	1.17 (1.14-1.24)	0% (0%–40%)
GV 10^2	8	0			
GV 10^3	8	0			
GV 10^4	8	0	7 (6.25-9.75)	1.19 (1.18-1.30)	0% (0%–0%)
GV 10^5	11	1	6.5 (5.75-7.25)	1.20 (1.13-1.22)	17% (10%–47%)
GV 10^6	7	0	5 (5-6.5)	1.14 (1.08-1.21)	10% (0%–27%)
GV 10^7	6	0	2.5 (2-4.25)	1.23 (1.17-1.32)	50% (28%–68%)
GV 10^8	20	N/A – 100% maternal mortality			
LC 10^8	13	0	1 (0-4)	1.15 (1.10-1.22)	35% (0%–70%)
MM 10^8	14	0	5 (3.25-6.75)	1.19 (1.16-1.21)	17% (0%–42%)

whether uterine colonization by these microbes is a necessary intermediary. In a mouse model, intra-amniotic injection of the vaginal anaerobe *Ureaplasma parvum* (10^3-10^5 CFU/animal) resulted in PTB rates of 0%—50%, depending on clinical isolate, as well as elevated cytokine expression in amniotic fluid.[59] Similarly, intra-amniotic injection of *U. parvum* (10^7 CFU/animal) or *Mycoplasma hominis* (10^5-10^7 CFU/animal) in rhesus macaques resulted in elevated cytokine expression in amniotic fluid and 100% PTB.[60] While intra-amniotic delivery is a useful model for fetal infection, ascending vaginal infection would theoretically require intrauterine infection to precede infection of the amniotic cavity. Therefore, the present work investigated the effects of vaginal anaerobes after intrauterine infusion.

Many prior studies have focused on intrauterine infusion of *Escherichia coli* ($10^{3\text{-}5}$ CFU/animal) which reliably induces PTB in many species including mice and rhesus macaques.[61–63] The high and consistent rate of sPTB is not surprising given the known pathogenicity of *E. coli* and its ability to recruit a strong host immune response.[64] In contrast, intrauterine infusion of a common less pathogenic vaginal anaerobe, *Prevotella bivia* ($10^{5\text{-}8}$ CFU/animal), resulted in much lower rates of PTB (21%–33%).[65] Similarly, intrauterine infusion with *G. vaginalis* in rabbits has previously induced poor fetal outcomes (death and low pup weight) without PTB.[66–68] The ability of microbes, common to the lower reproductive tract, to induce PTB appears to be complex and depends on many factors including the pathogenicity of the organism, bacterial load, inoculation site, host species, and the host immune response. This study confirms that *G. vaginalis* can induce maternal and fetal immune responses but suggests that intrauterine colonization of this microbe alone is unable to induce sPTB. Our finding does not eliminate the potential role of *G. vaginalis* in the pathogenesis of sPTB but it does bring into question whether ascension of this microbe and intrauterine colonization are a critical mechanism by which vaginal anaerobes contribute to the outcome of sPTB.

The induction of inflammatory pathways in the uterus is believed to be a trigger for sPTB. The results from our study are interesting in that they demonstrate, in a mouse model, that activation of inflammatory pathways in the uterus may not be sufficient to induce sPTB. At a short time period after IUI, we observed significantly elevated chemokine and cytokine levels in the maternal serum and uterus after infusion with *G. vaginalis*, *M. mulieris*, and *L. crispatus*, consistent with other reports of animal models with intrauterine infection.[69,70] Further, our results indicated that live bacteria can migrate from the uterus to the fetal compartment, as noted by live bacteria isolated from the amniotic fluid. However, despite the presence of the live vaginal microbes in the fetal compartment, there was minimal response in the placenta, amniotic fluid, or fetal liver. The limited fetal inflammatory response is surprising given the known presence of bacteria-responsive Toll-like receptors in placental and fetal tissues.[71] One possible explanation is that amniotic fluid contains several anti-microbial factors, preventing long-term bacterial colonization and a sustained inflammatory response.[72] Other mechanisms of bacterial death or clearance, including by neutrophils or macrophages, may also explain low levels of fetal inflammation in our model. As a fetal inflammatory response (FIRS) is believed to be a trigger for preterm partition, the absence of a FIRS in our study may be a reason why no PTB was observed.[73,74] Further, it is possible that inflammation was transient in nature and that a prolonged immune response is required to initiate parturition.

Importantly for understanding microbial-host immunity for pregnancy health, our study revealed a microbe-dependent effect in pregnancy outcomes and immune response. Intrauterine colonization by *G. vaginalis* at a high bacterial burden resulted in maternal morbidity while equivalent doses of *L. crispatus* or *M. mulieris* were tolerated by the host, demonstrated by no maternal morbidity after intrauterine inoculation with high doses of these microbes. In terms of a maternal immune response, high bacterial loads of intrauterine *G. vaginalis* resulted in a significant 3.5-fold increase of TNFα expression in maternal serum, while dams infused with *L. crispatus* or *M. mulieris* did not have significantly altered TNFα levels. TNFα, a cytokine known to be involved in fever and sepsis, may have therefore contributed to the

G. vaginalis-specific maternal morbidity observed.[75] Prior work in a pregnant rabbit model also noted evidence of systemic inflammation after intrauterine infusion of *G. vaginalis*: positive cultures were found in maternal blood and a low proportion of dams developed fever.[67,68] The consequences of intrauterine *L. crispatus* or *M. mulieris* have not previously been studied. In our work, microbe-specific differences in immune response and outcome are not attributable to the abundance of bacterial cell wall since equivalent doses were used for all inoculations. It is possible that *L. crispatus* and *M. mulieris* have increased immune regulation or clearance compared to *G. vaginalis*, which was not captured by our single assessment. Another possibility of the observed maternal morbidity specific to GV would be from liberation of vaginolysin, a cytotoxic compound released specifically by strains of *G. vaginalis*.[76,77] Beyond toxicity to vaginal epithelial cells, vaginolysin also induces toxicity in red blood cells, potentially explaining maternal morbidity and elevated cytokine levels observed in maternal serum.[78]

Our study has several notable strengths. First, our mouse model is well-established and commonly used to assess immune response and bacterial effects across the maternal-fetal dyad.[54,55,79,80] We validated our model by confirming intrauterine bacterial colonization in multiple methods, by detection of bacterial DNA as well as growth on agar plates. Additionally, we took an unbiased approach to assessing the immune response in maternal and fetal compartments. We rigorously evaluated the effect of intrauterine colonization by *G. vaginalis* on PTB with a sufficiently large sample size at the highest dose ($n = 20$), supported by large groups treated with lower doses ($n = 48$ across six lower doses). Further, our study included *L. crispatus* and *M. mulieris* as microbes associated with reproductive health and sPTB respectively, providing insight on microbe-specific effects.

One limitation of our study is that effects of a polymicrobial infection were not evaluated. The objective of this work was to investigate the independent consequences of intrauterine inoculation of *G. vaginalis* and *M. mulieris*, as these organisms are individually implicated in sPTB and other adverse reproductive outcomes.[32,33] However, the potentially cooperative effects of intrauterine colonization by two or more anaerobes must be further understood. Another limitation our study is the lack of multiple time points to assess temporal changes in the immune response. Additional time points could assess the time course of immune activation across the maternal-fetal interface and how bacterial clearance in maternal and fetal compartments may differ. While these types of investigations are not feasible in humans, the differences between mouse and human pregnancy including gestational length, role of progesterone withdrawal in parturition, and placental biology are noted.[81,82] Although mouse models are well established to investigate reproductive biology and immunological responses in mice share many similarities with humans, our findings may not be fully translatable to human pregnancy.

Our study invites further investigation to define the conditions by which bacteria-associated inflammatory activation can trigger sPTB. For example, although a single infusion in late gestation is insufficient to induce sPTB, the prolonged effect of multiple infusions or a single infusion earlier in pregnancy may produce a different outcome. Existing mouse and other animal models can be modified for these studies. In parallel work, microbe-specific effects observed in our study must be further explored, including enhanced immune regulation or clearance of *L. crispatus* and *M. mulieris* or systemic entry of *G. vaginalis*-specific vaginolysin. Finally, our study calls for increased investigation of microbial effects in their natural residence, the cervix and vagina. As supported by our laboratories and others, the interaction of vaginal microbes with the CV epithelium and the potential of those interactions to induce premature cervical remodeling should be a focus for advancing our understanding of the pathogenesis of sPTB.[51–53,83,84] Through understanding specific mechanisms by which vaginal microbes modulate the function of reproductive tissues, we can begin to appropriately target therapeutic strategies to meaningfully reduce sPTB.

Collectively, our results demonstrate that intrauterine colonization by *G. vaginalis* or *M. mulieris* is not sufficient to induce sPTB in a mouse model. The contributions of these anaerobes to sPTB may result from microbial-host interactions in the reproductive tract prior to pregnancy and/or their role in sPTB may depend on their actions within the CV space. Recent work on CV epithelial responses to common vaginal anaerobes has revealed functional roles of bacteria in immune activation and cervical remodeling, implicated in sPTB.[52,53] Further studies must investigate the propagation of a CV response to the uterus, in the absence of microbial ascension, in order to fully elucidate the molecular and cellular mechanisms underlying sPTB.

AUTHOR CONTRIBUTIONS

Andrea Joseph: Investigation, Formal Analysis, Writing Original Draft; Emma L. Lewis: Investigation, Formal Analysis; Briana Ferguson: Investigation, Formal Analysis; Yuxia Guan: Investigation; Lauren Anton: Investigation, Writing Review & Editing, Supervision; Michal A. Elovitz: Investigation, Writing Review & Editing, Supervision, Funding Acquisition.

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CONFLICT OF INTEREST STATEMENT

MAE receives compensation and has equity interest in Mirvie.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Andrea Joseph https://orcid.org/0000-0001-9419-9812
Emma L. Lewis https://orcid.org/0000-0002-2572-1002

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SUPPORTING INFORMATION

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