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# Gardnerella vaginalis promotes group B Streptococcus vaginal colonization, enabling ascending uteroplacental infection in pregnant mice

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## OBSTETRICS

# *Gardnerella vaginalis* promotes group B *Streptococcus* vaginal colonization, enabling ascending uteroplacental infection in pregnant mice

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**BACKGROUND:** Group B *Streptococcus* is a common vaginal bacterium and the leading cause of invasive fetoplacental infections. Group B *Streptococcus* in the vagina can invade through the cervix to cause ascending uteroplacental infections or can be transmitted to the neonate during vaginal delivery. Some studies have found that women with a "dysbiotic" polymicrobial or *Lactobacillus*-depleted vaginal microbiota are more likely to harbor group B *Streptococcus*. *Gardnerella vaginalis* is often the most abundant bacteria in the vaginas of women with dysbiosis, while being detected at lower levels in most other women, and has been linked with several adverse pregnancy outcomes. Mouse models of group B *Streptococcus* and *Gardnerella vaginalis* colonization have been reported but, to the best of our knowledge, the two have not been studied together. The overarching idea driving this study is that certain members of the dysbiotic vaginal microbiota, such as *Gardnerella vaginalis*, may directly contribute to the increased rate of group B *Streptococcus* vaginal colonization observed in women with vaginal dysbiosis.

**OBJECTIVE:** We used a mouse model to test the hypothesis that vaginal exposure to *Gardnerella vaginalis* may facilitate colonization and/or invasive infection of the upper reproductive tract by group B *Streptococcus* during pregnancy.

**STUDY DESIGN:** Timed-pregnant mice were generated using an allogeneic mating strategy with BALB/c males and C57Bl/6 females. Dams were vaginally inoculated at gestational day 14 with group B *Streptococcus* alone (using a 10-fold lower dose than previously reported models) or coinoculated with group B *Streptococcus* and *Gardnerella vaginalis*. Bacterial titers were enumerated in vaginal, uterine horn, and placental tissues at gestational day 17. The presence (Fisher exact tests) and levels (Mann-Whitney *U* tests) of bacterial titers were compared between mono- and coinoculated dams in each compartment. Relative risks were calculated for outcomes that occurred in both groups. Tissue samples were also examined for evidence of pathophysiology.

**RESULTS:** Inoculation of pregnant mice with $10^7$ group B *Streptococcus* alone did not result in vaginal colonization or ascending infection. In contrast, coinoculation of group B *Streptococcus* with *Gardnerella vaginalis* in pregnant mice resulted in a 10-fold higher risk of group B *Streptococcus* vaginal colonization (relative risk, 10.31; 95% confidence interval, 2.710—59.04; *P*=.0006 [Fisher exact test]). Ascending group B *Streptococcus* infection of the uterus and placenta occurred in approximately 40% of coinoculated animals, whereas none of those receiving group B *Streptococcus* alone developed uterine or placental infections. Immunofluorescence microscopy revealed group B *Streptococcus* in both the maternal and fetal sides of the placenta. Histologic inflammation and increased proinflammatory cytokines were evident in the setting of group B *Streptococcus* placental infection. Interestingly, placentas from dams exposed to group B *Streptococcus* and *Gardnerella vaginalis*, but without recoverable vaginal or placental bacteria, displayed distinct histopathologic features and cytokine signatures.

**CONCLUSION:** These data suggest that *Gardnerella vaginalis* vaginal exposure can promote group B *Streptococcus* vaginal colonization, resulting in a greater likelihood of invasive perinatal group B *Streptococcus* infections. These findings suggest that future clinical studies should examine whether the presence of *Gardnerella vaginalis* is a risk factor for group B *Streptococcus* vaginal colonization in women. Because *Gardnerella vaginalis* can also be present in women without bacterial vaginosis, these findings may be relevant both inside and outside of the context of vaginal dysbiosis.

**Key words:** ascending infection, bacterial vaginosis, *Gardnerella vaginalis*, group B *Streptococcus*, health disparities, microbiota, placenta, uterus, vagina, vaginal microbiome

## Introduction

Group B *Streptococcus* (GBS) is a Gram-positive bacterium associated with

multiple adverse pregnancy outcomes and life-threatening neonatal infections.[1,2] In the United States, GBS colonization rates during pregnancy range from 15% to 60%, depending on the study population.[3–5] Vaginal colonization with GBS occurs in approximately 18% of pregnant women worldwide[6] and has been linked with preterm birth (PTB),[2,7–9] preterm premature rupture of membranes (PROM),[10] and neonatal intensive care unit (NICU) admission.[11] GBS vaginal colonization is a risk factor for neonatal

transmission during delivery[12,13] and to infants during the postnatal period[14] and is associated with a 3-fold increased rate of NICU admission.[11] GBS in the vagina during pregnancy can also invade through the cervix to cause uteroplacental and fetal infections.[12,15,16] Mouse models of GBS vaginal colonization during pregnancy display several of these adverse pregnancy outcomes, including invasive ascending infection and placental inflammation,[17,18] neonatal transmission,[19] and PTB or intrauterine fetal demise (IUFD).[17,18,20] Despite the

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**Why was this study conducted?**
This study aimed to develop a murine pregnancy model to test whether *Gardnerella vaginalis* (*G. vaginalis*)—an abundant vaginal bacterium in certain settings, especially bacterial vaginosis (BV)—facilitates vaginal group B *Streptococcus* (GBS) colonization or invasive intrauterine infection.

**Key findings**
Coinoculation with *G. vaginalis* promoted sustained GBS vaginal colonization and enhanced the likelihood of uterine and placental GBS infection associated with a strong inflammatory signature in pregnant mice. Sustained vaginal detection of *G. vaginalis* was dispensable for GBS colonization or invasion. Even in mice without detectable ascending infection or histologic inflammation, placentas from those coexposed to GBS and *G. vaginalis* displayed other histopathologic features and a distinct cytokine signature.

**What does this add to what is known?**
*Gardnerella* may be a causal factor that engenders susceptibility to GBS colonization and invasive infections during pregnancy. These findings might help explain persistent disparities in invasive GBS infections because BV is more common among Black women.

adverse outcomes arising from GBS in the vagina, host or microbial factors that promote GBS vaginal colonization during pregnancy are poorly understood.

A *Lactobacillus*-dominated vaginal microbiome is regarded as beneficial and is believed to ward off colonization by potential pathogens such as GBS. Although results vary, some clinical findings have suggested that GBS colonization is more likely in women with a polymicrobial, "dysbiotic" vaginal microbiota or those with decreased *Lactobacillus* (Supplemental Table 1).[3,21–24] In addition, there is a notable parallel in the epidemiology of GBS colonization and the composition of the vaginal microbiota. Black[3,25–30] and African American[21,31,32] women have higher rates of GBS vaginal colonization and are more likely to have a polymicrobial vaginal microbiota, defined either as bacterial vaginosis (BV) or as community state type (CST)-IV, than White women. The factors driving the association between the vaginal microbiota and GBS colonization and infection are not clear. We hypothesized that certain members of the dysbiotic vaginal microbiota may facilitate vaginal colonization or ascending uterine and placental infection by GBS.

*Gardnerella vaginalis* (*G. vaginalis*) is frequently the most abundant member of the dysbiotic vaginal microbiota and has historically been associated with the clinical syndrome now defined as BV.[33–38] Some studies have implicated BV in spontaneous abortion and miscarriage,[39] PROM,[40] PTB,[40,41] low birthweight and clinical chorioamnionitis,[42] and adverse neonatal outcomes including NICU admission and neonatal sepsis.[43] *G. vaginalis* has itself been associated with adverse pregnancy outcomes including spontaneous PTB,[44] chorioamnionitis,[45,46] and intraamniotic infection.[47] Importantly, we previously demonstrated in nonpregnant mice that *G. vaginalis* can trigger features of BV on its own (eg, clue-like cells, sialidase activity, mucus degradation, epithelial exfoliation) and also encourages pathogenesis, including ascending uterine infection, by other urogenital pathogens.[48–50] In a pregnant mouse model, *G. vaginalis* triggered a local proinflammatory cytokine response and cervical remodeling.[51] Here, we developed a mouse model of vaginal coinoculation during pregnancy to test the hypothesis that *G. vaginalis* may enhance GBS colonization and/or invasive infection during pregnancy.

## Methods

### Bacterial strains and growth conditions

All experiments used a spontaneous streptomycin-resistant strain of *G. vaginalis* JCP8151B-Sm[R] derived from a strain isolated from a woman with BV,[52] and a serotype III GBS strain COH1 isolated from a case of systemic neonatal infection,[53] expressing a plasmid containing an erythromycin resistance cassette. Antibiotic-resistant bacteria were used because bacteria endogenous to the mice may be able to grow on nonselective plates with no antibiotic agents. The use of antibiotic-resistant strains of GBS and *G. vaginalis* in conjunction with selective plates containing those antibiotic agents provided confidence that the colonies observed and counted to monitor infection were indeed the inoculated strains.

*G. vaginalis* was grown statically in NYCIII media at 37°C in an anaerobic chamber (Coy Laboratory, Grass Lake, MI) and GBS was grown statically in Todd Hewitt (TH) media supplemented with 100 $\mu$g/mL erythromycin at 37°C aerobically overnight. After growth, the bacterial cultures were centrifuged, and each pellet was resuspended in phosphate-buffered saline (PBS). The optical density (OD) of the bacterial suspensions were measured. The bacterial suspensions were centrifuged and resuspended in the appropriate volume of PBS according to the equation ($[OD_{600}$ of PBS suspension]×[volume of PBS suspension centrifuged])/(inoculum target $OD_{600}$). The target ODs were determined empirically to be GBS to either OD=8 (approximately $10^7$ colony-forming units [cfu] per 10 uL) or OD=40 (approximately $10^8$ cfu per 10 uL) and *G. vaginalis* to OD=10 (approximately $10^7$ cfu per 10 uL). The actual doses of bacterial inocula were confirmed by serial dilution and plating immediately after performing mouse inoculations.

### Generation of timed-pregnant mice

Female C57BL/6NCR mice were obtained from the National Cancer Institute (now Charles River Laboratories International, Inc, Frederick, MD), and male BALB/c mice were obtained from

Jackson Laboratory between January 2015 and April 2018. Four days before mating (day 0), some urine-soaked bedding from a BALB/c male's cage was added to the bedding of the females' cages. The female mouse's cycle is 4 to 5 days in length, but group-housed females often develop cycles that are more irregular and longer. The pheromones in the male urine will cause most of the group-housed females to begin a new estrus cycle by the third day of exposure. At day 0, females were weighed, and their stage of the estrus cycle was determined visually. Females that were determined to be in estrus were placed in the cage of a Balb/c male (1:1) late in the afternoon of day 0. The following morning (day 0.5-day 1), females were removed from the male cages, checked for the presence of a vaginal plug, and returned to their original cage. Females that were both visibly rounded by gestational day (E)13 and weighed a minimum of 120% of their original weight were classified as pregnant and used for infection experiments.

### Mouse vaginal coinoculation model

A schematic of the mouse model timeline is shown in Figure 1A. At E14, dams were restrained and inoculated vaginally with 2 immediately successive 10 $\mu$L inoculations as follows: PBS and PBS (mock vehicle controls), G. vaginalis then PBS, PBS then GBS, and G. vaginalis then GBS. This small volume of liquid was entirely taken up into the vagina (it did not pool at the introitus). To further ensure that the inoculum was maintained in the vagina, the mouse was restrained stationary with its tail end raised up for approximately 30 seconds before being returned to its cage. An initial experiment confirmed that the order of inoculation of the 2 bacteria did not affect colonization or infection. Previous studies reported that a $10^8$ cfu inoculum of GBS strain COH1 resulted in vaginal colonization and ascending intrauterine infection in >90% of pregnant mice and IUFD and/or PTB in 16% to 40% of pups.[18,54,55] As a positive control, we included a small number of mice inoculated with $10^8$ cfu of GBS (GBS$^{high}$) but used a 10-fold lower $10^7$ dose of GBS (GBS$^{low}$) in our

coinoculation model. The dose of G. vaginalis was approximately $10^8$ cfu, and the dose of GBS was either $10^7$ or $10^8$ cfu. Dams convalesced undisturbed (so as to not trigger adverse pregnancy outcomes) until they were sacrificed at E17 (as explained in the following paragraphs).

Dams were sacrificed by cervical dislocation under isoflurane anesthesia at E17 to evaluate aseptically dissected tissues from all animals at 1 time point for bacterial cfu and histologic analysis (Supplemental Material). In contrast to studies using higher doses or more invasive strains of GBS, we saw no evidence that any dam delivered before E17 in 13 independent experiments (although we did not continuously monitor the dams with video surveillance). Furthermore, there was no difference in fetal weight and incidence of IUFD between mono- and coinfected groups (Figure 1, B–D), and there was no GBS recovered from the amniotic fluid or fetuses.

### Tissue collection

At E17, dams were sacrificed by cervical dislocation under isoflurane anesthesia to evaluate aseptically dissected tissues from all animals at 1 time point. Vaginas were collected and bisected longitudinally; one-half was fixed in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid), and the other half was homogenized for bacterial cfu determination (as explained in the following text). A piece of uterine tissue was collected and weighed from each horn immediately adjacent to the cervix and surrounding the most proximal fetus. All placentas and fetuses from both horns were collected and weighed. The uterine tissue and the first 2 placentas proximal to the cervix from the left horn were homogenized in sterile PBS. The remaining placentas were fixed in methacarn.

### Bacterial colony-forming unit determination

To distinguish GBS and G. vaginalis from endogenous mouse vaginal bacteria, cfu were determined by serial dilution in 96-well plates in PBS and plating on selective agar media (supplemented with streptomycin for G. vaginalis and

erythromycin for GBS). GBS plates were incubated at 37°C aerobically overnight. Our previous experiments determined that the plasmid conferring erythromycin resistance in GBS is maintained during in vivo vaginal colonization experiments over extended time periods (data not indicated). G. vaginalis plates were grown at 37°C anaerobically for 48 hours. Colonies were counted and reported as cfu per gram of tissue or cfu per placenta.

### Histologic analysis and placental pathology score

Histologic slide preparation and hematoxylin and eosin staining of the fixed vaginal and placental tissue were performed by the Department of Developmental Biology Histology Core at Washington University. Placentas were visualized with a ZEISS ApoTome microscope (ZEISS, Oberkochen, Germany) using a 20× objective. A blinded observer scored the pathophysiology based on the presence or absence of placental cellular damage and vascular lesions characterized by dark eosin staining and fragmented hematoxylin signals as follows: 0=absent or barely seen, 1=occasional, 2=moderate, and 3=abundant. Vaginas were visualized on an BX61 microscope (Olympus, Tokyo, Japan) using a 10× objective and scored by a blinded observer for epithelial keratinization and exfoliation as follows: 0=absent, 1=mild, and 2=severe.

### Placenta cytokine and chemokine analysis

Placenta homogenates were centrifuged for 5 minutes at 12,000$g$ at 4°C, and the supernatant was transferred (taking care to avoid the pellet and any fat) to a fresh Eppendorf tube and stored at −20°C until analysis. Supernatants were thawed on ice, centrifuged again at 4°C to remove any remaining particulates, and collected. Cytokine and chemokine levels were measured using the Bio-Plex Pro Mouse Cytokine 23-Plex Panel multiplex cytokine bead kit (Bio-Rad Laboratories, Hercules, CA), which quantifies 23 different cytokines and chemokines. The assays were performed according to the manufacturer's

## FIGURE 1
### Coinoculation with *G. vaginalis* facilitates GBS vaginal colonization in pregnant mice
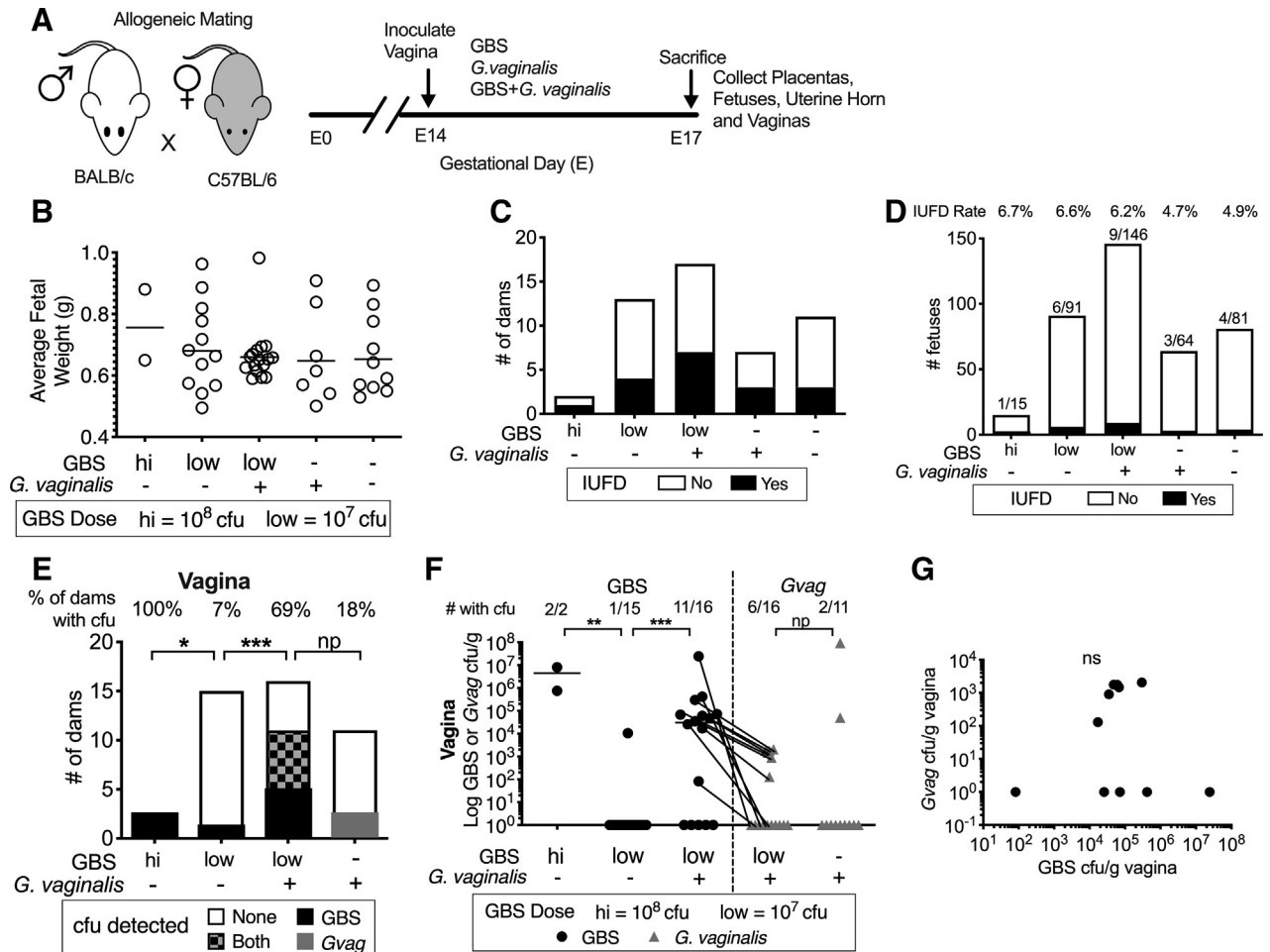


**A,** Schematic of allogeneic timed-pregnancy vaginal coinoculation model. **B,** Fetal weights at E17. Each *dot* represents the average fetal weight for an individual dam. **C,** Number of dams with 0 (*white bars*) or 1 or more (*black bars*) instance of intrauterine fetal demise (IUFD) at E17. **D,** Fetal outcomes: alive (*white bars*) or IUFD (*black bars*). The fraction of fetuses that died in utero (indicated above each bar) were used to calculate the IUFD rate indicated across the top of the graph. **E,** The number of dams without and with bacterial cfu in vaginal homogenates. The percentage of dams with detectable cfu indicated across the top of each bar. Fisher's exact test, Triple asterisk indicates $P<.001$; single asterisk indicates $P<.05$. **F,** Bacterial titers in vaginal tissue homogenates. Data points for GBS (*circles*) and *G. vaginalis* (*triangles*) cfu from the same tissues are connected with *lines*. The fraction of vaginas with detectable cfu is indicated across the top of graph. *Bars* denote geometric mean; 11 independent experiments. Mann-Whitney *U* test; double asterisks indicate $P<.01$; triple asterisks indicates $P<.001$. **G,** Titers of *G. vaginalis* and GBS present in vaginas were plotted for organs from which cfu was detectable. No significant (ns) correlation (Spearman) was detected in either organ, even if the samples with only 1 of the 2 organisms were excluded from the analysis.

*cfu*, colony-forming unit; *E17*, gestational day 17; *G. vaginalis*, Gardnerella vaginalis; *GBS*, group B *Streptococcus*; *IUFD*, intrauterine fetal demise; *np*, not powered; *ns*, not significant.

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instructions, except using 10-fold less standard and half the number of coupled beads and detection antibodies indicated in the protocol.

**Immunofluorescence microscopy**
Slides were stained for GBS (rabbit polyclonal antibody 1:200, ab 53584;

Abcam, Cambridge, United Kingdom) and the cytoskeleton (rabbit monoclonal antivimentin antibody 1:200, ab 92547; Abcam, Cambridge, United Kingdom). After 3 PBS washes at room temperature, antigen-antibody complexes were detected with species-specific Alexa Fluor 488 and 594—conjugated secondary antibodies (1:500; Invitrogen,

Carlsbad, CA). Slides were counterstained with 4′,6-diamidino-2-phenylindole (1:1000) for 10 minutes to visualize the nuclei and mounted with ProLong Gold (Life Technologies, Carlsbad, CA). Images were obtained with a ZEISS ApoTome microscope using ×40 or ×60 oil immersion objectives.

## FIGURE 2
### Coinoculation with *G. vaginalis* results in increased GBS invasive ascending infection
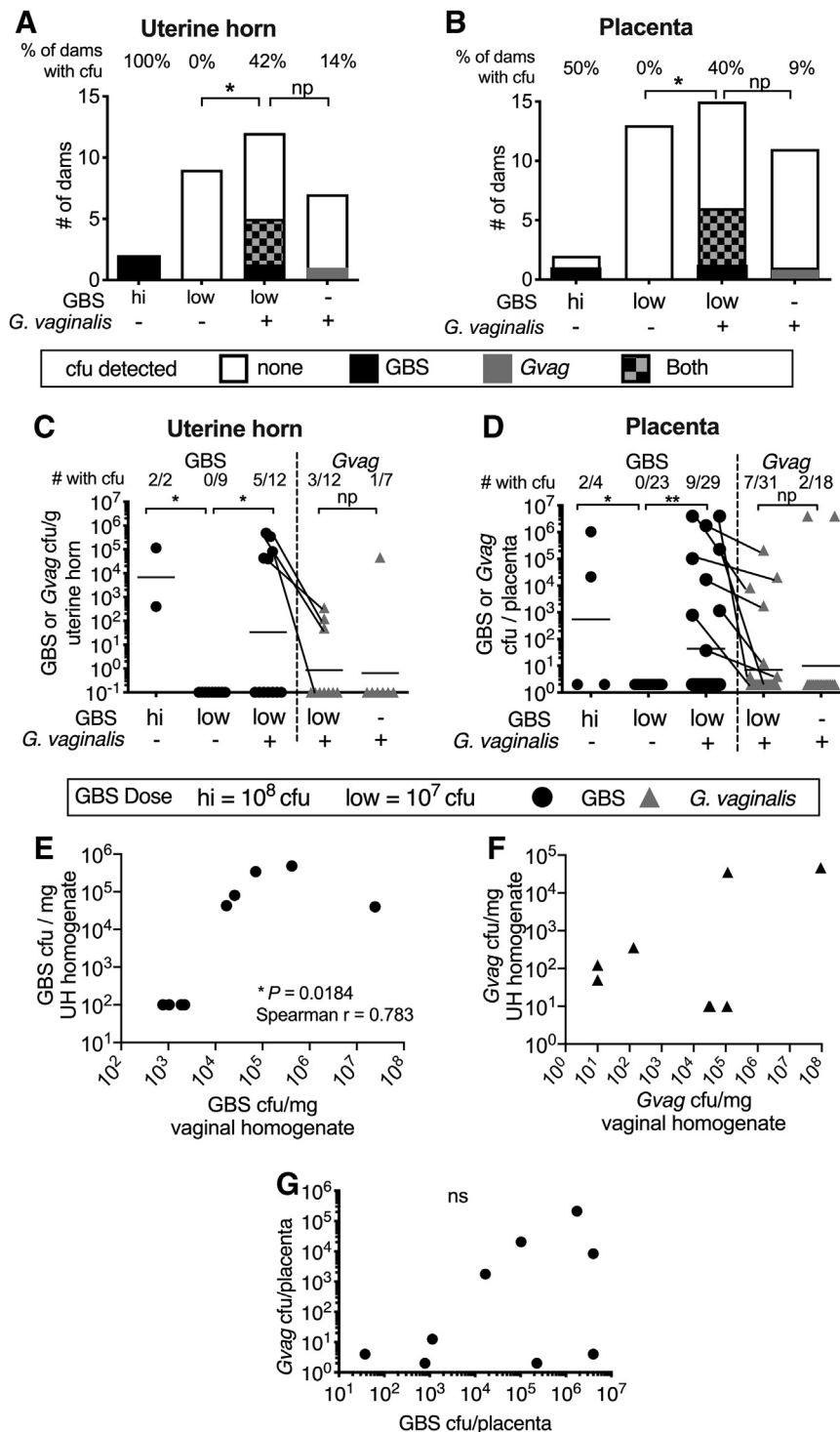


**A** and **B,** The number of dams without or with bacterial cfu in uterine horn **(A)** or placental **(B)** tissue homogenates. The percentage of dams with detectable cfu indicated across the top of each bar. Fisher's exact test; single asterisk indicates $P<.05$. **C,** Bacterial titers in uterine tissue homogenates. The fraction of dams with detectable cfu is indicated across the top of the graph; 11 independent experiments. Uterine tissue was not collected from every dam; therefore, data are from a subset of animals included in Figure 1. **D,** Bacterial cfu in placental homogenates. The fraction of placentas
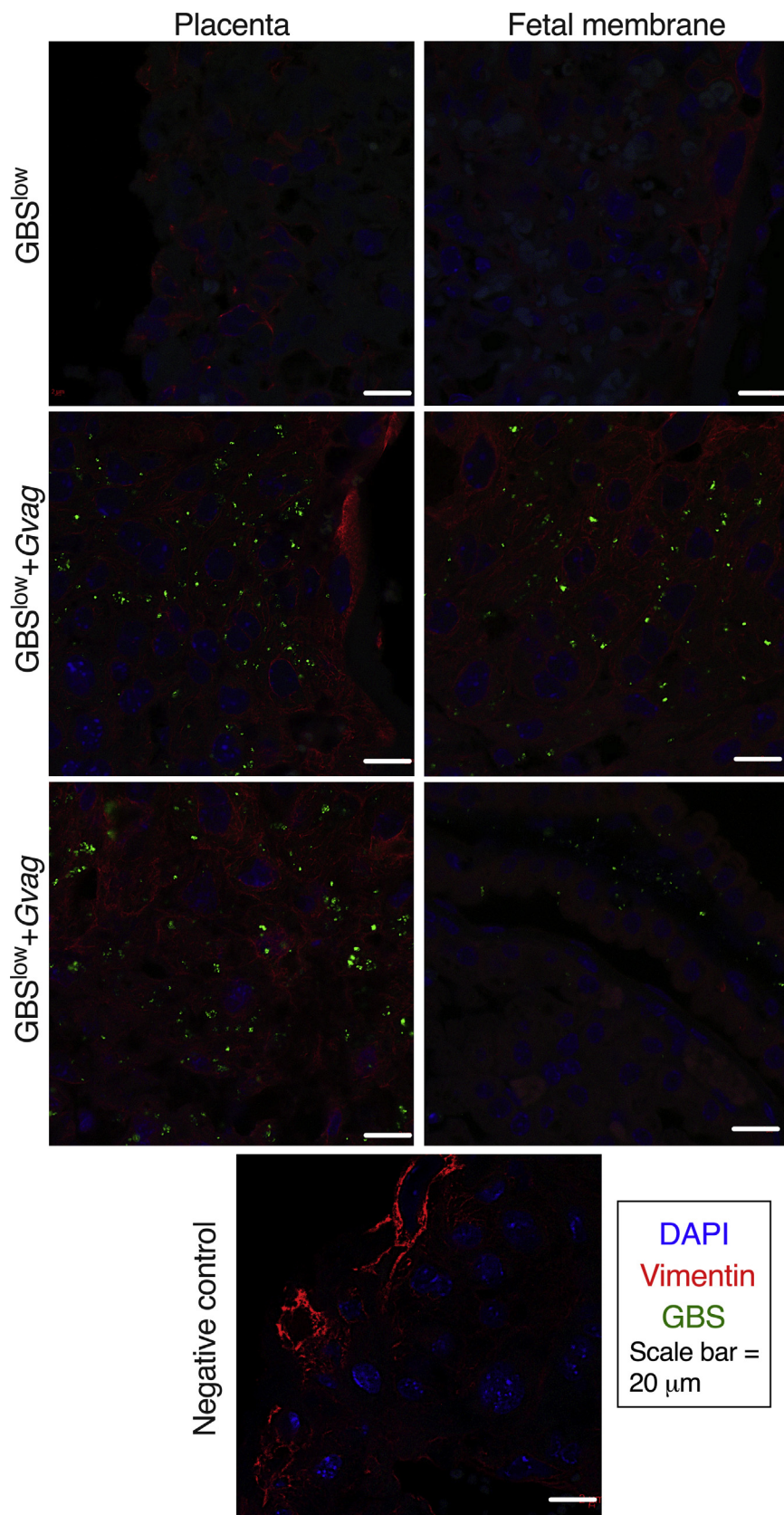
### Statistics

Our primary outcome of interest was vaginal colonization by GBS. To determine sample sizes, we anticipated 10% of dams would be colonized vaginally with GBS alone. We required a minimum of 11 mice in each group to detect a significant difference (alpha, 0.05; beta, 0.2; power, 80%) if 60% of coinoculated mice became colonized with GBS (ClinCalc.com). We used 15 and 16 mice in the GBS and GBS+*G. vaginalis* groups, respectively, which powered us to detect a significant difference if 52% of coinoculated dams became colonized with GBS. We used a minimum number of animals in the high-dose GBS group because we anticipated, based on previous studies, that 100% of dams would become colonized. Thus, fewer animals were required to detect a significant difference between high-dose GBS and low-dose GBS groups. Our study was underpowered (at only 18%) to detect a significant effect of GBS on *G. vaginalis* because we only had 11 mice in the *G. vaginalis*—alone group. Based on the observed rates of vaginal colonization by *G. vaginalis* (*G. vaginalis* alone, 18%; *G. vaginalis*+GBS, 38%), we would require 78 animals per group to detect significance with alpha of 0.05, beta of 0.2, and

with detectable cfu is indicated across the top of the graph; 13 independent experiments. **C** and **D,** *Bars* denote geometric mean. Data points for GBS (*circles*) and *G. vaginalis* (*triangles*) cfu from the same tissues are connected with lines. Mann-Whitney *U* test; double asterisks indicate $P<.01$ and single asterisk indicates $P<.05$. Mann-Whitney test comparing *G. vaginalis* titers in placentas from mono- vs coinoculated dams was $P=0.07$ if the 2 outliers were excluded from the analysis. **E** and **F,** Correlation of GBS (E) and *G. vaginalis* (F) cfu in UH and vaginal homogenates. **G,** Titers of *G. vaginalis* and GBS present in placentas were plotted for organs from which cfu were detectable. No significant correlation (Spearman) was detected in either organ, even if the samples with only 1 of the 2 organisms were excluded from the analysis.

*cfu*, colony-forming unit; *G. vaginalis*, *Gardnerella vaginalis*; *GBS*, group B *Streptococcus*; *np*, not powered; *ns*, not significant; *UH*, uterine horn.

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## FIGURE 3
### Coinoculation with *G. vaginalis* facilitates GBS maternal-fetal unit invasion



power of 80%, which is well beyond the scope of mouse pregnancy models. Prism 8.0 software (GraphPad, San Diego, CA) was used for all statistical analyses; tests used to analyze each data set are indicated in the figure legends. Relative risk (RR) was calculated using the Koopman asymptotic method.

### Ethics statement

The mouse experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals and approved by the Animal Studies Committee of Washington University School of Medicine (protocol #20140114 and #20170081).

### Results

Consistent with previous reports,[18,51] a high dose (approximately $10^8$ cfu) of GBS or *G. vaginalis* alone in our model (Figure 1, A) resulted in vaginal colonization (Figure 1, E and F). Vaginal colonization was only rarely achieved upon a 10-fold reduction of the GBS inoculum (GBS^low=$10^7$ GBS cfu, hereafter referred to as "GBS alone") (Figure 1, E and F). In contrast, coinoculation with *G. vaginalis* resulted in a 10-fold increased risk of dams becoming vaginally colonized by GBS compared with animals inoculated with GBS alone (69% vs 7%; RR, 10.31; 95% confidence interval [CI], 2.710−59.04; $P=.0006$ [Fisher exact test]) (Figure 1).

Representative images of immunofluorescence microscopy of the maternal-fetal interface from placentas isolated from dams inoculated with GBS^low or GBS^low+Gvag. GBS bacteria were detected with a monoclonal antibody (*green*). Sections were counterstained with DAPI (nuclei [*blue*]) and vimentin (vasculature [*red*]). Similar robust GBS staining was observed in placentas that were collected from GBS^low+Gvag dams that had placental infection evident by detectable cfu. The negative control panel (*bottom*) is a section from a GBS^low+Gvag placenta stained in parallel but omitting the GBS 1° antibody.

*cfu*, colony-forming unit; *DAPI*, 4′,6-diamidino-2-phenylindole; *Gvag*, *Gardnerella vaginalis*; *G vaginalis*, *Gardnerella vaginalis*; *GBS*, group B *Streptococcus*.

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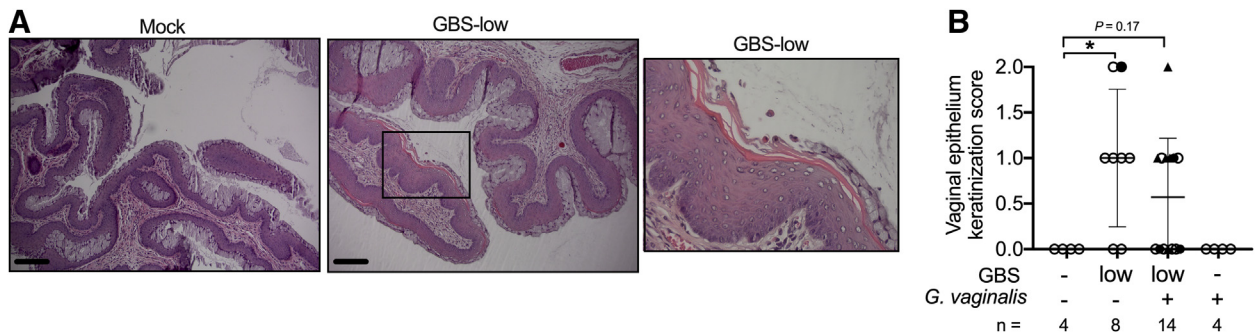
**A,** Representative images of H&E-stained vaginal sections collected on E17. Area of keratinization and exfoliation in boxed region is shown at higher magnification. Phenotype was present in 0 of 4 mock, 6 of 8 GBS alone, 7 of 14 GBS+*G. vaginalis*, and 0 of 4 *G. vaginalis* dams. **B,** Vaginal keratinization score. Each *dot* represents an individual dam. *Filled circles* denote tissues with detectable GBS cfu, and *filled triangles* denote detectable GBS and *G. vaginalis* cfu in the vaginal homogenates. *Lines* denote the mean±standard deviation. Mann-Whitney *U* test, asterisk indicates *P*=.05.

*cfu*, colony-forming unit; *E17*, gestational day 17; *G vaginalis*, *Gardnerella vaginalis*; *GBS*, group B *Streptococcus*; *H&E*, hematoxylin and eosin.

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Infection by GBS in utero is known to be a progressive infection that starts with GBS ascending from the vagina to the cervix, followed by placental invasion, causing chorioamnionitis and bacterial invasion into the amniotic sac. Mice receiving GBS alone never developed ascending infection of uterine (0 of 9 dams) or placental tissues (0 of 23 dams) (Figure 2, A and B). Note that RR was ∞ because there were zero events of uteroplacental infection in the GBS-alone group. Nevertheless, compared with GBS alone, significantly more of the GBS+*G. vaginalis* coinoculated dams had detectable bacterial infections in uterine (42%; *P*=.0451 [Fisher exact test]) and placental tissues (40%; *P*=.0178) (Figure 2, A−D). Immunofluorescence microscopy of placentas collected in parallel revealed that GBS was present in both maternal and fetal sides of the placenta (Figure 3); however, GBS cfu were not detected in the amniotic fluid or fetus (data not indicated). The absence of detectable GBS in amniotic fluid suggests (1) GBS infection had not progressed to the point of invading the amniotic sac, (2) GBS invaded earlier but was cleared from this niche by the time we sacrificed the animals, or (3) GBS was present in amniotic fluid, but at a level lower than our limit of detection. We think the first possibility is the most likely. When GBS cfu were detected in the placenta or uterus of coinoculated dams, GBS cfu were also detected in the vagina (Supplemental Table 2). Levels of GBS in the vagina and uterine tissues were significantly correlated (Figure 2, E). Some dams from both the GBS-alone and coinoculated groups showed evidence of keratinization and exfoliation of the vaginal epithelium at E17, but this phenotype did not correlate with vaginal colonization or ascending infection (Figure 4).

Consistent with previous studies using high GBS doses or more invasive GBS strains,[17,55,56] placentas examined for pathology revealed inflammatory infiltrates at the decidual compartment by the junctional zone specifically in coinoculated dams with detectable placental infections (Figure 5, A). Consistent with the presence of inflammatory cells, there were high levels of interleukin (IL)-1 beta, macrophage inflammatory protein-1 alpha, and granulocyte colony−stimulating factor exclusively in placentas from coinoculated dams that had detectable titers (triangle symbols), suggesting this response depended on active infection (Figure 5, B).

Other histopathologic phenotypes were observed within the junctional zone and labyrinth in placentas from coinoculated mice that did not have signs of histologic inflammation and irrespective of whether live placental bacteria were detected (Figure 6, A). In a blinded analysis, compared with the mock or GBS-alone groups, placentas from coinoculated dams were significantly more likely to exhibit histopathology (Figure 6, B). Interestingly, certain cytokines (different from those elevated in infected placentas presented in Figure 4, B) were elevated in mice vaginally inoculated with bacteria, independent of detectable placental infection (Figure 6, C). Placental IL-2, IL-6, and macrophage inflammatory protein-1 beta were higher in mice inoculated with bacteria (*G. vaginalis*, GBS alone, or both) than PBS controls. Mice inoculated with GBS alone (with or without *G. vaginalis*) had significantly higher levels of regulated upon activation, normal T cell expressed, and presumably secreted (RANTES). Eotaxin, interferon gamma, IL-12p70, and keratinocytes-derived chemokine (KC) (mouse functional homolog of IL-8) were significantly higher in the GBS+*G. vaginalis* group. IL-6, RANTES, and KC were highest in placentas with active infection (triangle symbols), but the significant difference between the mock and GBS+*G. vaginalis* groups remained even if the data from infected placentas were excluded from

## FIGURE 5
### Placental inflammation is evident in coinoculated dams with detectable GBS placental infection
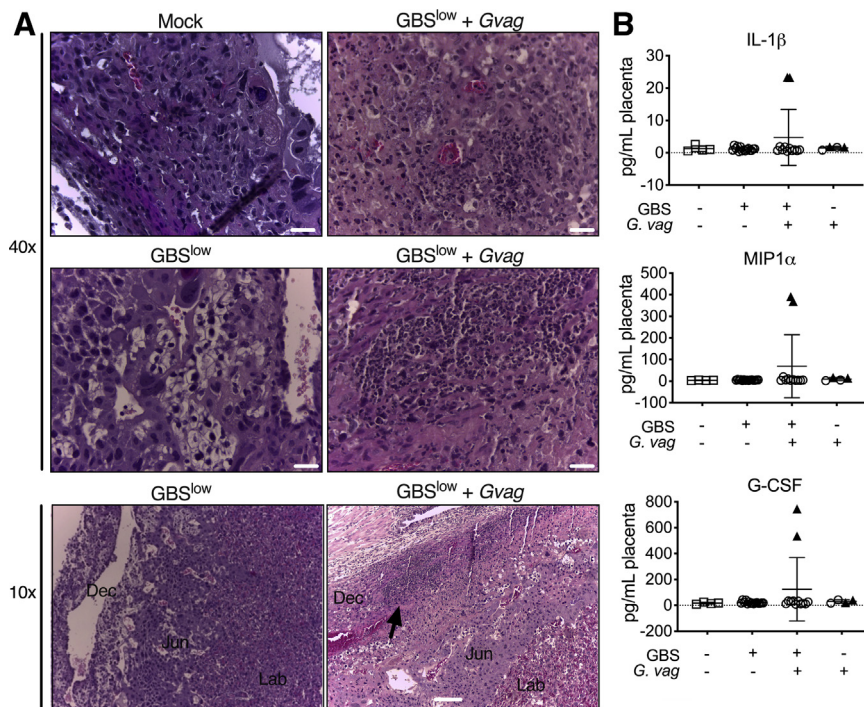


**A,** H&E staining reveals prolific PMNs, cell debris, and monocytes in the decidual compartment by the junctional zone (*black arrow*) in placentas from the GBS[low]+Gvag dams that had detectable infection. Scale bars, 20 μm (top, 40× images) and 100 μm (bottom, 10× images). **B,** Cytokine/chemokine levels in placenta homogenates. *Black triangles* denote placentas that had detectable bacterial cfu.

*cfu*, colony-forming unit; *Dec*, decidua; *G-CSF*, granulocyte colony—stimulating factor; *Gvag*, *Gardnerella vaginalis*; *GBS*, group B *Streptococcus*; *H&E*, hematoxylin and eosin; *IL-1β*, interleukin 1 beta; *Jun*, junctional zone; *Lab*, labyrinth; *MIP1α*, macrophage inflammatory protein-1 alpha; *PMNs*, polymorphonuclear leukocytes.

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the analysis. Together, these data suggest that vaginal exposure to bacteria can trigger histopathology and a placental cytokine response that overlaps with, yet is distinct from, the response present during active placental infection.

To examine whether uterine infection was evident at earlier time points, 10 dams (GBS alone, n=5; GBS+*G. vaginalis*, n=5) were sacrificed at 24 hours after infection (E15). Dams inoculated with GBS alone cleared the bacteria rather rapidly, with only 1 of 5 having detectable GBS cfu in the vagina at 24 hours. In contrast, *G. vaginalis* and GBS were detectable in the vagina of 3 of 5 coinoculated dams. However, none of these dams had detectable ascending infection by either bacterium at 24 hours (data not indicated), supporting the conclusion that ascending infection by

both organisms required longer than 24 hours to occur in this model. These data also indicate that the inoculation procedure did not introduce the bacterial inoculum into the upper reproductive tract.

Although the experiments were not originally designed to investigate the effect of GBS on *G. vaginalis*, we note that 38% of coinoculated dams had detectable vaginal colonization with *G. vaginalis* compared with 18% of dams inoculated with *G. vaginalis* alone (Figure 1). *G. vaginalis* and GBS cfu were not significantly correlated in the vagina (Figure 1, G). A previous study using a different strain of *G. vaginalis* inoculated at E12—13 did not detect uterine or placental infection at E15.[51] In our model, the rate of *G. vaginalis* placental infection at E17 was 9% in mice

inoculated with *G. vaginalis* alone and 30% in mice coinoculated with GBS (Figure 2, B). Although these findings are potentially interesting, we were underpowered to detect significant differences between the proportions for *G. vaginalis* vaginal colonization and placental infection. Unlike GBS, *G. vaginalis* titers in vaginal and uterine horn tissues were not significantly correlated (Figure 2, F). Moreover, 7 placentas had both GBS and *G. vaginalis* cfu, but the titers were not significantly correlated (Figure 2, G). Nonetheless, among the coinoculated dams, 2 with no detectable vaginal titers of *G. vaginalis* had *G. vaginalis* cfu in uterine horn tissue, one of which also had placental titers (Supplemental Table 2). Similarly, 5 coinoculated dams with no detectable *G. vaginalis* in the vagina nonetheless had evident ascending GBS infection (Supplemental Table 2).

## Comment
### Principal findings
Coinoculation of pregnant mice with *Gardnerella* led to a 10-fold higher RR of vaginal colonization with GBS. In this model, invasive infections of uterine and placental tissue by GBS only occurred in coinoculated animals. These data are consistent with our stated hypothesis that *G. vaginalis* may enhance GBS colonization and invasive infection. Levels of invasive GBS in uterine tissue were proportional to the GBS vaginal burden. Ascending *G. vaginalis* uterine and placental infection also occurred, including in mice that did not have detectable vaginal titers. This suggests that after ascension of *G. vaginalis*, the mouse was able to clear *G. vaginalis* from the vagina, but not from the upper reproductive tract. Sustained vaginal colonization by *G. vaginalis* was also not required to enhance GBS ascending infection. Finally, coinoculation with GBS and *G. vaginalis* resulted in placental histopathology even in the absence of placental infection.

### Clinical implications
Our results provide causal information regarding the effect of *G. vaginalis* on

## FIGURE 6
## Coinoculation with GBS and *G. vaginalis* adversely affects the placenta independent of sustained ascending infection
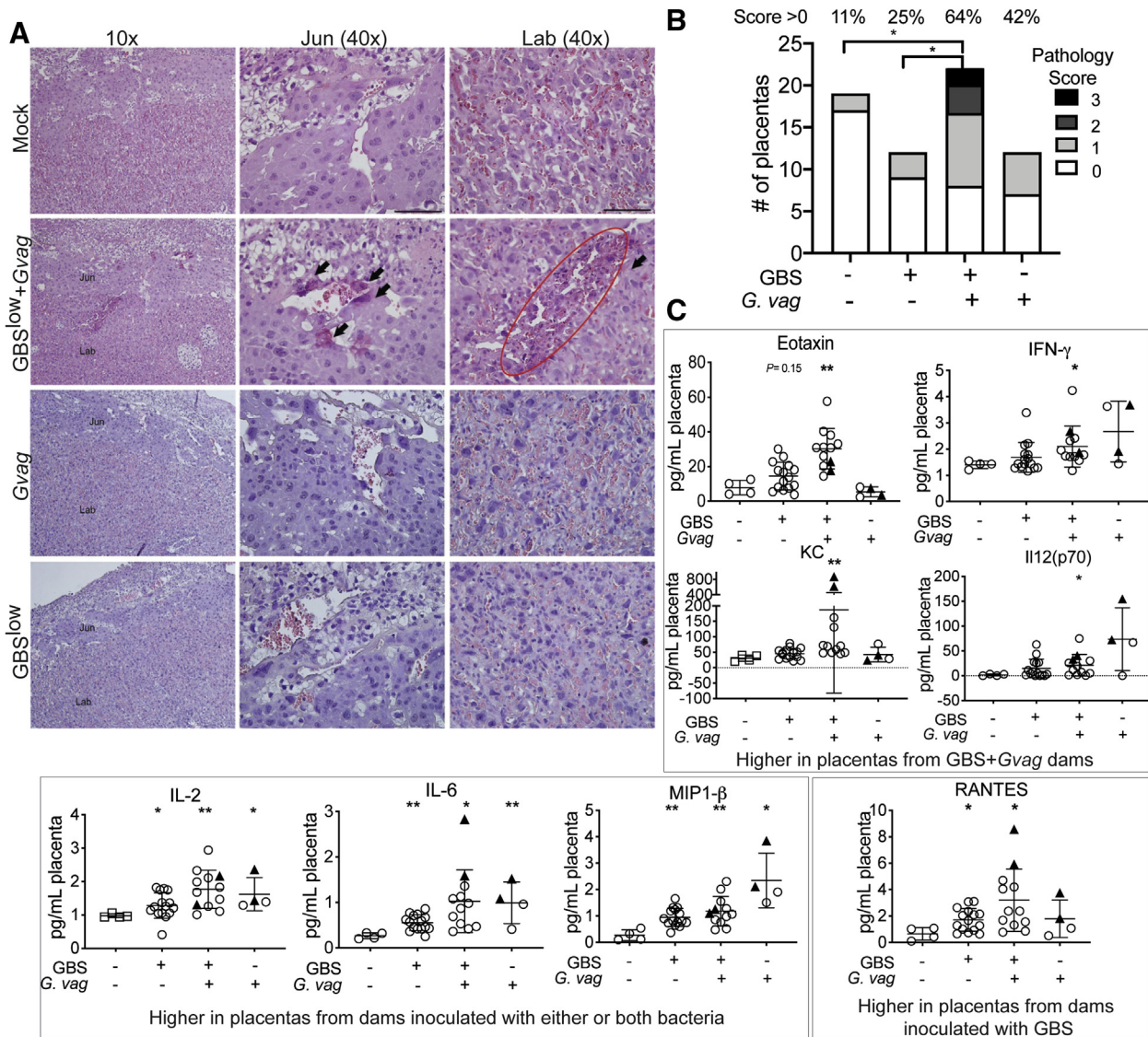


**A,** Representative images of H&E-stained fixed placental sections from dams inoculated with bacteria, but without detectable placental infection, or mock controls. Placentas from GBS$^{low}$+*G. vaginalis* show cellular damage, characterized by dark eosin staining and fragmented hematoxylin signals (*arrows*), exclusively within the Jun and Lab layers. Scale bars, 50 $\mu$m. **B,** Blinded scoring of the placental pathologic features shown in panel **A**; 2 independent experiments, 3 dams per group. **C,** Cytokine and chemokine levels in placenta homogenates. A Kruskal-Wallis test was performed, followed by Dunn's multiple comparisons test comparing each bacterial group with the PBS control group. Single asterisk indicates $P<.05$; double asterisks indicate $P<.01$.

*Gvag*, *Gardnerella vaginalis*; *G vaginalis*, *Gardnerella vaginalis*; *GBS*, group B *Streptococcus*; *H&E*, hematoxylin and eosin; *IFN-γ*, interferon gamma; *IL-2*, interleukin 2; *IL-6*, interleukin 6; *IL12(p70)*, interleukin 12 p70; *Jun*, junctional zone; *KC*, keratinocyte-derived chemokine; *Lab*, labyrinth; *MIP-1β*, macrophage inflammatory protein-1 beta; *RANTES*, regulated upon activation, normal T cell expressed, and presumably secreted.

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GBS vaginal colonization that generates a specific and testable question for clinical research application. Namely, is *G. vaginalis* a risk factor in women that contributes to GBS colonization and neonatal GBS disease? Two observations from the clinical literature already suggest that this could be true. First, some data in women show an association between GBS colonization and BV and intermediate Nugent score, both of which have high levels of *G. vaginalis*.[3,21] Second, Black and African American women have disproportionately high rates of GBS colonization and neonatal infections[3,21,25–27,31] and are also more likely to have a polymicrobial vaginal microbiota or BV.[28–30,32] As of 2016, the rate of severe GBS infections within the first week of life in the United States was 300% higher in Black infants than their

White counterparts.[57] Furthermore, a recent study showed that African American women had a higher rate of conversion from GBS-negative to GBS-positive status between the time of routine screening and presentation at the labor and delivery unit (RR, 2.0; 95% CI, 1.02−3.8).[58] Understanding the factors that contribute to racial disparities in GBS colonization is necessary for the development of measures to limit the disproportionate burden of GBS disease. Our findings warrant future clinical studies to determine whether vaginal colonization by *G. vaginalis* is a risk factor for GBS colonization, becoming GBS positive after antenatal screening, or for invasive GBS disease. The potential for a relationship between *G. vaginalis* and GBS colonization and infection may be relevant even outside of the context of vaginal dysbiosis because *G. vaginalis* has been detected, albeit at lower levels than during BV, in the majority of women examined. If *G. vaginalis* proved to be a risk factor for GBS colonization in women, this could translate to improved screening for vaginal colonization and treatment strategies in specific at-risk populations.

### Research implications

Further studies are needed to delineate the mechanisms by which *G. vaginalis* encourages GBS colonization, define the determinants of ascending infection, and examine how exposure to these bacteria adversely affects the placenta independent of active infection and whether other aspects of pathogenesis are affected, such as PTB or vertical transfer to the neonate.[17,19] Our data suggest that even transient vaginal exposure to *G. vaginalis* may promote GBS colonization. Furthermore, transient vaginal exposure to GBS or *G. vaginalis* resulted in distinct placental cytokine signatures and histopathology, even in the absence of detectable placental infection. The findings are consistent with other models in which *Gardnerella* has been described as a "covert pathogen," a microbe that can have pathologic effects despite being absent at the time and place disease features manifest.[49,50] The presence of *G. vaginalis* in mouse placental tissue in our model is

consistent with studies that have found *Gardnerella* in human placenta[59] and provide a tool for investigating the impact of this bacteria on tissues of the upper reproductive tract during pregnancy. Similar studies in mice could be performed to examine whether additional strains of GBS and *G. vaginalis* yield similar results. Studies could be expanded to test whether other bacteria associated with BV or CST-IV could have a similar effect as *G. vaginalis* on GBS colonization and infection. Understanding how different strain backgrounds of GBS or *G. vaginalis*, or even other members of the vaginal microbiota, contribute to findings in women or in mouse models could also yield insights important for future research efforts aimed at treatment and prevention.

### Strengths and limitations

Key strengths of our study were the development of a GBS/G. vaginalis coinoculation model during allogeneic pregnancy.[60] The limitations necessitated by the labor and cost-intensive nature of the mouse pregnancy model include that only 1 strain each of *G. vaginalis* and GBS were tested, multiple doses or variations in timing of bacterial inoculation were not thoroughly examined, and observations were primarily at a single time point. We used a 10-fold higher dose of *G. vaginalis* than GBS; therefore, coinoculated dams received a higher total bacteria dose than those inoculated with GBS alone. The bacterial load in the vagina is significantly higher in the context of BV than without BV.[61] Therefore, even if the effect of *G. vaginalis* on GBS infection in pregnant mice is caused by the heightened overall bacterial load of the inoculum used, this is directly relevant to the situation of BV in women. Our primary focus was to examine the effect of *G. vaginalis* on GBS; thus, our studies included fewer dams inoculated with *G. vaginalis* alone and were underpowered to detect significant effects of GBS on *G. vaginalis*. Vaginal inoculation models in pregnant mice have proven valuable,[17−19,51,62] but they do not recapitulate all features of human pregnancy. Mice have a different endogenous vaginal microbiota than

humans, and not all studies on women concur with the interpretation that dysbiotic communities are more likely to harbor GBS (Supplemental Table 1). Interestingly, a recent study showed that the composition of the endogenous vaginal microbiome can affect GBS ascending uterine infection in nonpregnant mice.[63] As with previous GBS and *G. vaginalis* mouse models in pregnancy, it is unknown whether or how the endogenous microbiota in mice may influence the observed phenotypes in our model.

### Conclusions

These data suggest that *G. vaginalis* plays a causal role in promoting GBS colonization of the vaginal niche during pregnancy. ∎

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**SUPPLEMENTAL TABLE 1**
**Summary of the clinical literature examining relationship between GBS vaginal colonization and the vaginal microbiota**

| Study | Total, N GBS (+), n | Pregnancy status | Country/region | Method of vaginal microbiome analysis | Method GBS detection | Significant findings |
|---|---|---|---|---|---|---|
| Meyn et al (2009)[1] | N=1248 (+), n=814 | Nonpregnant | United States (White, 2496 visits; Black, 1361 visits) | Nugent score | Culture (Columbia agar+5% sheep blood and selective broth) identified by colony morphology, $\beta$-hemolysis, and catalase reaction | GBS+ status positively associated with intermediate (adjusted OR, 2.1 [1.7−2.6]; $P<.001$) and BV (adjusted OR, 1.7 [1.4−2.0]; $P<.001$)[a] |
| | | | | No BV score, 0−3 | | |
| | | | | Intermediate score, 4−6 | | |
| | | | | BV score, 7−10 | | |
| Meyn et al (2002)[2] | N=1248 (+), n=365 | Nonpregnant | United States (White, 61.2%; African American, 34.8%) | Nugent score (see above) | Culture (Columbia agar+5% sheep blood and selective broth) identified by colony morphology, $\beta$-hemolysis, and catalase reaction | GBS+ status more likely in women with intermediate ($P<.05$)[a] |
| Mu et al (2019)[3] | N=66 (+), n=22 | Nonpregnant | China | 16S (V1−V2) | GBS nucleic acid detection kit | GBS+ group had higher betadiversity and lower *Lactobacillus* and higher *Prevotella*, *Megasphaera*, and *Streptococcus* relative abundance ($P<.01$)[b] |
| Rosen et al (2017)[4] | N=428 (+), n=92 | Nonpregnant | United States ("racially and ethnically diverse") | 16S (V3−V4) CST IV-A microbiota dominated by *Streptococcus* and *Prevotella* | RT-PCR | Significantly higher proportion of GBS+ samples in CST IV-A ($P=.001$ [Fisher exact test]) *Prevotella bivia* and *Staphylococcus* associated with GBS+ (LDA score, >2.5)[b] |
| | | | | | For samples with Ct 41−50, recorded as positive only if typical (mauve) colonies were present on CHROMagar StrepB | |
| Kubota et al (2002)[5] | N=4025 (+), n=408 | Pregnant | Japan | Culture (aerobic, Trypticase Soy Agar II+5% sheep blood, Chocolate II Agar, and Drigalski Agar, modified; anaerobic, Brucella Agar medium with hemin and vitamin K) | Culture (details not reported) | GBS+ samples decreased $\alpha$-diversity, higher likelihood of *Klebsiella pneumoniae* and MRSA, and lower likelihood of *Lactobacillus*, coagulase-negative *Staphylococcus*, and *Prevotella*[b] |

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*(continued)*

## SUPPLEMENTAL TABLE 1
### Summary of the clinical literature examining relationship between GBS vaginal colonization and the vaginal microbiota (continued)

| Study | Total, N GBS (+), n | Pregnancy status | Country/region | Method of vaginal microbiome analysis | Method GBS detection | Significant findings |
|---|---|---|---|---|---|---|
| Cools et al (2016)[6] | N=430 | Nonpregnant (n=229), pregnant (n=30), adolescents (n=60) | Sub-Saharan Africa (Kenya, Rwanda, and South Africa) | Nugent score (see above) | qPCR | GBS+ status negatively associated with BV (crude OR, 0.29 [0.12−0.70]; P=.006; adjusted OR, 0.43 [0.21−0.88]; P=.022) |
| | | | | | GBS PCR−positive samples confirmed with Lim Broth culture | |
| Hillier et al (1992)[7] | N=7918 | Pregnant | United States | Nugent score (see above) | | GBS+ status associated with no BV or intermediate negative association with BV |
| Brzychczy-Włoch et al (2014)[8] | N=42 (+), n=15 | Pregnant | Poland | Nugent score | Culture (Columbia+5% sheep blood agar) | No significant differences in the numbers of $H_2O_2$ positive/negative *Lactobacillus* spp or *Bifidobacterium* spp between GBS+ and GBS samples |
| | | | | Excluded samples with BV score (7−10) | qPCR | |
| | | | | MRS agar for *Lactobacillus* | | |
| | | | | Bifidobacterium agar for *Bifidobacterium* | | |
| Rochetti et al (2011)[9] | N=405 (+), n=103 | Pregnant | Brazil | Nugent score | Culture (Todd Hewitt, subcultured in blood agar; colonies confirmed by catalase and CAMP test) | Logistic regression analysis did not detect significant association between GBS status and intermediate or BV GBS positively associated with cytolytic vaginosis |
| | | | | Intermediate (8.1%) | | |
| | | | | BV (10.9%) | | |
| Thorsen et al (1998)[10] | N=3174 (+), n=278 | Pregnant | Denmark (all White) | Amsel criteria | Culture (blood agar, GBS confirmed by Gram stain and coagulation test) | No significant association between GBS status and BV |
| Honig et al (2002)[11] | N=250 (+), n=50 | Nonpregnant | The Netherlands | Nugent score (see above) | Culture (blood agar and chocolate agar, colonies grouped using Streptex test) | No significant association between GBS status and BV |

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(continued)

**SUPPLEMENTAL TABLE 1**
**Summary of the clinical literature examining relationship between GBS vaginal colonization and the vaginal microbiota** *(continued)*

| Study | Total, N GBS (+), n | Pregnancy status | Country/region | Method of vaginal microbiome analysis | Method GBS detection | Significant findings |
|---|---|---|---|---|---|---|
| Altoparlak et al (2004)[12] | N=150 (+), n=41 | Pregnant | Turkey | Nugent score (see above) | Culture (details not reported) | *Lactobacillus* detected less frequently in GBS− group (*P*<.01) |
| Rick et al (2017)[13] | N=896 (+), n=155 | Pregnant | Guatemala | 16S (V3−V4) | Cultured (CHROMagar StrepB and in BBL Lim Broth; mauve or dark pink colonies confirmed by GBS latex agglutination) | GBS+ samples significantly higher abundance of *Corynebacterium* and lower abundance in *Aerococcus* |

*BV*, bacterial vaginosis; *CAMP*, Christie-Atkins-Munch-Peterson; *CST*, community state type; *GBS*, group B *Streptococcus*; $H_2O_2$, hydrogen peroxide; *LDA*, linear discriminant analysis; *MRS*, de Man, Rogosa, and Sharpe; *MRSA*, methicillin-resistant *Staphylococcus aureus*; *OR*, odds ratio; *qPCR*, quantitative polymerase chain reaction; *RT-PCR*, reverse transcription polymerase chain reaction.

[a] Those who found a significant positive association between GBS vaginal colonization and BV or intermediate Nugent score; [b] Those who found a significant association between GBS vaginal colonization and lower *Lactobacillus* or the presence of a non-*Lactobacillus* dominated microbiota (CST IV-A).

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**SUPPLEMENTAL TABLE 2**
**Organ infections in GBS + *G. vaginalis* coinfected dams**

| Mouse ID | Placenta | Uterus | Vagina |
|---|---|---|---|
| 32 | No cfu | ND | GBS and *G. vaginalis* |
| 14 | GBS and *G. vaginalis* | ND | GBS and *G. vaginalis* |
| 39 | GBS and *G. vaginalis* | ND | GBS and *G. vaginalis* |
| 44 | GBS and *G. vaginalis* | ND | GBS and *G. vaginalis* |
| 41 | ND | GBS and *G. vaginalis* | GBS and *G. vaginalis* |
| 85 | GBS and *G. vaginalis* | GBS and *G. vaginalis* | GBS |
| 255 | GBS | GBS | GBS |
| 95 | No cfu | GBS and *G. vaginalis* | GBS |
| 78 | No cfu | GBS | GBS |
| 224 | GBS | No cfu | GBS |
| 10 | No cfu | No cfu | GBS and *G. vaginalis* |
| 70 | No cfu | No cfu | No cfu |
| 89 | No cfu | No cfu | No cfu |
| 92 | No cfu | No cfu | No cfu |
| 235 | No cfu | No cfu | No cfu |
| 252 | No cfu | No cfu | No cfu |

*cfu*, colony-forming unit; *G. vaginalis*, *Gardnerella vaginalis*; *GBS*, group B *Streptococcus*; *ID*, identification; *ND*, not determined.

*Gilbert et al. Group B Streptococcus and the vaginal microbiota. Am J Obstet Gynecol 2021.*

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