EXPLORING HOST FACTORS THAT INFLUENCE GROUP B *STREPTOCOCCAL* VAGINAL COLONIZATION IN PREGNANCY

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ABSTRACT

Group B *streptococcal* (GBS) infections are a leading cause of neonatal morbidity and mortality worldwide: a recent metanalysis reveals that the annual global GBS burden contributes up to 3.5 million preterm births, 320,000 cases of neonatal infection, and 50,000 stillbirths. Third trimester rectovaginal colonization is the primary risk factor for developing invasive GBS infection, however, our understanding of host factors for that modulate colonization during pregnancy is limited. In this dissertation, I developed a novel mouse model to characterize vaginal GBS colonization dynamics in pregnancy and use it to identify host factors unique to pregnancy that regulate vaginal GBS colonization. Chapter 2 discusses a mouse model of GBS vaginal colonization in pregnancy, where nonpregnant females maintain persistent vaginal GBS carriage. However, persistently colonized females clear GBS from the vaginal tract soon after mating. Chapter 3 seeks to identify factors in early pregnancy that restrict GBS colonization and persistence. We hypothesized that exposure to seminal fluid components and increases in ovarian hormones contribute to GBS clearance. Removal of seminal vesicle fluid from male ejaculate led partially restored GBS carriage post-copulation, suggesting that seminal fluid components contribute to GBS clearance in pregnancy. Further, exogenous progesterone treatment led to substantial GBS clearance from the vaginal tract and this correlated with neutrophil influx, suggesting that progesterone is a key driver of GBS clearance in early pregnancy which may be driven by increases in vaginal neutrophils. The findings discussed in this dissertation reveal prospective novel risk factors and therapeutic interventions to treat GBS disease.

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CHAPTER 1: LITERATURE REVIEW

GROUP B *STREPTOCOCCAL* DISEASE BURDEN AND PATHOGENESIS

Global Health Burden

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae*, is a Gram-positive commensal bacterium that colonizes the lower gastrointestinal and vaginal tract of ~18-20% of healthy adults[1]. GBS causes severe disease in a small subset of individuals, including pregnant women, neonates, and the elderly. Of these subgroups, neonates and pregnant women exhibit the greatest burden of GBS disease[1]. GBS only recently emerged as a leading cause of neonatal infection, where extensive use of tetracycline post-world war II led to the selection and global dissemination of resistant clones that cause a majority of GBS neonatal infections today[3–5]. The global health burden of GBS disease remains significant: up to 3.5 million preterm births, 50,000 stillbirths, and 320,000 cases of neonatal infection are attributable to GBS annually[1].

Neonatal GBS infection is clinically subcategorized into early-onset and late-onset disease, which are distinguished by symptom onset and presentation. Early-onset disease (EOD) occurs within 7 days of the post-natal period and primarily manifests as sepsis or bacterial pneumonia. Late-onset disease (LOD) occurs between one week to three months of the post-natal period, and is primarily associated with meningitis and bacteriemia[3]. While specific GBS strains are strongly associated with neonatal infection in general[1,4,5], it is unclear what drives differences between EOD and LOD manifestation. EOD likely arises via vertical transmission in utero or during delivery from mothers with extensive recto-vaginal colonization. The mode of transmission for LOD is less clear: vertical transmission during birth and breast feeding from colonized mothers, as well as environmental sources like hospital care are all considered potential exposure routes[6–8].

Risk factors for GBS disease

GBS is a highly diverse species, and colonization with specific GBS strains is a risk factor for developing invasive disease[1,5]. Historically, GBS strains were classified by their capsular polysaccharide (CPS), or serotype, where 10 distinct serotypes have been described. When robust sequencing methods became available, GBS strains could also be identified by sequencing 7 genes conserved across serotypes, also known as multi-locus sequence typing (MLST), which allowed more nuanced categorization of GBS isolates by sequence type (ST).

Certain GBS strains are associated with invasive disease outcomes, while others are more frequently associated with asymptomatic colonization. Serotypes I-V are most prevalent, comprising >93% of patient isolates[9], where serotypes Ia and III are strongly associated with invasive disease, especially meningitis, and accounts for 60-85% of invasive neonatal disease cases[9]. However, it is important to note that regional variations in strain prevalence and associations with invasive disease outcomes exist, especially in specific regions of South America and Asia[4,5]. As with serotype, specific STs are frequently associated with invasive disease outcomes: ST-17s are almost exclusively serotype IIIs and are associated with 82% of neonatal meningitis cases[12–14]. Compared to other GBS strains, ST-17s generally encode virulence factors that facilitate invasive disease, such as enhanced attachment and invasion of neonatal tissues[15], immune evasion[16], and stress resilience[8,17,18]. Indeed, type III expressing ST-17 and ST-19 strains are more likely to persist in women, even under antibiotic stress, compared to other STs and phenotypes[16], and serotypes III and Ia are more likely to persist longer in women than in men[17], possibly indicating these strains are better adapted to the vaginal niche.

In addition to colonization with specific GBS strains, several clinical risk factors associated with invasive infection have been identified, including maternal rectovaginal

colonization, premature birth, low birth weight, prolonged rupture of membranes, black or African race, gestational diabetes and previous pregnancies with GBS disease[8,18,19]. Of these, rectovaginal colonization is the primary risk factor for invasive GBS infection, where approximately 50% of colonized women vertically transmit to GBS to the neonate if left untreated[20,21]. Vaginal colonization is a stronger predictor of neonatal EOD than LOD; this discrepancy is likely due to unique modes of transmission[18]. Despite being the best available risk factor, third trimester GBS rectovaginal colonization can be an unreliable prognosticate for invasive disease outcomes; up to 40% of women who test positive at 34-37 weeks are no longer positive at birth, and 1-12% of colonized infants are born to non-colonized mothers[22,23]. As such, additional factors that identify patients at greatest risk for invasive infection are needed.

Treatment and Prophylactic Interventions

In high-resource countries, women are routinely screened for GBS in the third trimester (34-37 weeks gestation) using standard culture-based methods. GBS-positive women receive intrapartum antibiotic prophylaxis (IAP), or antibiotics during delivery, which has reduced neonatal EOD incidence by up to 80%[3]. Despite IAP's efficacy in preventing EOD, IAP does not improve LOD incidence, nor does it prevent preterm or still births[3,19]. There are additional drawbacks with IAP use. For instance, emerging antibiotic resistance is a concern: while most GBS remains susceptible to penicillin, clindamycin and erythromycin resistance is increasing in countries that routinely use antibiotics to treat GBS[26–29], increasing treatment risk in women with penicillin allergies. Moreover, perinatal exposure to antibiotics carries long-term implications, as early life exposure to antibiotics disrupts post-natal microbiome development[30–34], which is associated with metabolic, autoimmune, and allergic disease development later in life[35].

A GBS vaccine has the potential to target every clinical outcome of GBS disease, mitigate the need for IAP, and provide a more feasible intervention for low- and middle-income countries where screening and IAP are unavailable. As such, several GBS vaccine candidates have been developed over the last four decades. A majority target GBS capsular polysaccharide (CPS), a key virulence factor that is exposed to the external environment and thus has the potential for immune recognition. Indeed, maternal and neonatal anti-CPS IgG antibodies confer protection against GBS infection in neonates[31]. Original CPS vaccines developed in the 1980's consisted of purified CPS from invasive serotypes, but proved ineffective as polysaccharide antigens alone are poor elicitors of memory B cell responses necessary for long-term protection[36]. To enhance CPS immunogenicity, more recent vaccine generations conjugate CPS to a protein carrier, also known as glycoconjugate vaccines[36], which allow for enhanced recognition and response to bystander polysaccharide antigen. Current formulations are multivalent meaning they contain multiple CPS serotype antigens which allows for broad spectrum protection against GBS strains associated with invasive disease. Also in development are recombinant protein subunit vaccines that target GBS virulence proteins commonly expressed amongst invasive GBS strains[37]. Protein subunit vaccines are attractive as they mitigate concern over GBS capsule switching as opposed to CPS vaccines[38]. Despite multi-decade efforts toward GBS vaccine development, the low incidence of GBS disease has precluded licensure, as randomized controlled efficacy trials would require enrollment of large numbers of pregnant women. In response, the World Health Organization and other global stakeholders are reevaluating approaches to achieve vaccine licensure and has set a target for 2026[39].

HOST-GROUP B *STREPTOCOCCAL* INTERACTIONS

GBS Colonization of the Female Reproductive Tract and Virulence Factor Expression

GBS must adapt to various host niches, which differ in pH, metabolite availability, commensal organisms, and immune environment. GBS senses and responds to the external environment through two-component systems that enable its adaptation to various niches and their stressors. The GBS genome encodes many virulence factors, many of which are regulated by the well-studied CovR/S two component system. Virulence factors encoded by CovR/S enable GBS to transition from asymptomatic colonizer to pathogen[40].

To colonize the host tissues GBS expresses surface-associated adhesins to bind to extracellular matrix components including fibrinogen, fibronectin, and laminin. Adhesin expression is tightly regulated to adapt to distinct host niches, and many are differentially expressed between invasive and colonizing isolates and thus dictate virulence[39,41,42]. Furthermore, adhesins play unique roles to aid different stages of GBS pathogenesis, from cervicovaginal colonization to distal tissue attachment like microvascular endothelial cells of the blood brain barrier[42]. While most are important for all phases of pathogenesis, some adhesins like Ssr2[14,43] and HvgA[44] are strongly associated with invasive GBS isolates and likely allows these strains to invade distal tissues.

As an opportunistic pathogen, GBS has evolved mechanisms to evade innate immune detection and activation that aid host colonization and invasive pathogenesis. The GBS capsular polysaccharide (CPS) is a great example of this, where specific modifications to the capsule gives GBS the ability to evade immune detection. GBS decorates CPS with sialic acid residues, which engages Siglec receptors on host innate immune cells to evade their detection and suppress inflammation[43]. Sialic acid limits complement deposition and subsequent

opsonophagocytic uptake by macrophages, dendritic cells, and neutrophils[45]. Despite the importance of sialic acid in GBS pathogenesis, GBS serotype- and ST-specific sialic acid composition is not well-defined. One study shows GBS type II CPS does not strongly engage any Siglec receptors on leukocytes, whereas type III CPS strongly engages Siglec-9[46,47], suggesting that invasive isolates are better equipped to evade host recognition via unique sialic acid composition. Another cell wall modification employed by GBS is D-alanylation of CPS lipoteichoic acid (LTA) to change the surface charge of the cell wall. D-alanylation mutants are more susceptible to phagocytic killing by neutrophils and macrophages due to enhanced binding of cationic antimicrobial peptides[47].

In addition to capsule, GBS secretes virulence factors that promote ascending invasive infection and immune evasion in the reproductive niche. The mechanism by which GBS secretes virulence factors is not fully understood, however, recent evidence implicates GBS membrane vesicles as a vehicle to deliver several virulence factors[48] that worsen pregnancy outcomes in mice[49]. The most well-known secreted GBS virulence factor is β-hemolysin/cytolysin (βH/C) , due to its strong association with invasive infection in neonates. Hemolysin is a pigmented pore-forming toxin that promotes injury to host cells and evades phagocytic defenses by targeting neutrophils and macrophages[50,51].

GBS has evolved several additional secreted virulence factors that target different innate immune signaling pathways to facilitate immune evasion. Most target neutrophil and macrophage responses that directly target GBS and amplify inflammation. For instance, hyaluronidase is highly expressed by invasive GBS isolates and dampens anti-GBS responses from by degrading pro-inflammatory hyaluronan fragments into disaccharides that antagonize toll-like receptor 2/4 (TLR 2/4) in macrophages[12,52]. Similarly, C5a peptidase, a conserved

surface protease, diminishes immune responses by cleaving complement protein, C5a, necessary for neutrophil and macrophage opsonophagocytic killing[48]. While GBS penicillin-binding protein 1a (PBP1a) and pilus protein (PilB) promote resistance to cathelicidin and defensin killing[59], the primary antimicrobial peptides (AMP) produced by neutrophils and macrophages. GBS carotenoid pigment acts as an antioxidant to limit ROS-mediated killing and thus enhances intracellular survival in phagocytes[53–57], while GBS cell wall-anchored b protein promotes survival by engaging siglec-5 on neutrophils to decrease phagocytosis, oxidative burst, neutrophil extracellular trap (NET) formation, and IL-8 production[58,59]. Altogether, these examples highlight the multipronged system that GBS has evolved to adapt to host environments and evade innate immune cell targeting, especially neutrophils and macrophages.

Cervicovaginal Immune Recognition and Response to GBS

GBS induces robust immune responses from the host. This is evident in humans and in animal models of intrauterine and systemic infection, where GBS induces a range of pro-inflammatory cytokines and leukocytic infiltration of infected tissues[60]. The host response to GBS colonization of the lower reproductive tract is less well-defined and is likely different from in-utero and systemic infections, as the trajectory of an immune response is heavily influenced by route of infection or immunization due to tissue specific immunity. Indeed, the vagina, cervix, and uterus are morphologically and immunologically distinct. The lower reproductive mucosa with its closer proximity to the external environment has a unique microbiome dominated by *Lactobacillus spp.* that play a homeostatic role in the cervicovaginal niche, to include modulation of the local immune environment[60].

To tolerate endogenous microbes, the vaginal and cervical epithelium express fewer innate immune receptors that recognize pathogen-associated molecular patterns (PAMPs)[61] and

thus has reduced capacity to respond to pathogenic insults. Nevertheless, the lower reproductive tract shows robust innate capacity to respond to pathogen signals. For instance, the lower reproductive mucosa expresses TLR2/6 that detect Gram-positive pathogens, including endogenous *Lactobacillus spp.*[62]. Under homeostatic conditions, *Lactobacillus spp.* prevents constitutive activation of TLR2/6 by secreting lactic acid that inhibits vaginal and cervical epithelial pro-inflammatory signaling[63]. *Lactobacillus spp.* also secrete antimicrobial compounds[64,65] to directly inhibit competing bacteria in the vaginal niche[66]. Evidence in humans shows a strong association between the endogenous vaginal microbial composition and susceptibility to infection with pathogenic microbes, including GBS. Supporting mechanistic evidence from mouse studies show that *Lactobacillus spp.* inhibit GBS vaginal colonization by modulating the immune response[67], while certain disease states, like gestational diabetes, alter vaginal microbiota composition and increases GBS disease burden[68]. These examples illuminate the lower reproductive mucosa's distinct immune-microbe signaling network and the importance of endogenous microbes in the vaginal mucosa's recognition and response to GBS.

Existing mouse models of persistent GBS vaginal colonization allow us to elucidate host-GBS interactions that confer GBS persistence or clearance. Female mice that are artificially synced to estrus exhibit long-term vaginal GBS carriage post infection, a method spurred by the observation that female mice infected during estrus show persistent vaginal GBS carriage compared to mice infected at other stages of the estrus cycle[67,68]. Previous studies suggest changes to the vaginal and cervical epithelium during estrus promote GBS attachment and persistence due to the ability of GBS to use glycogen produced by the vaginal epithelial cells as a fuel source for persistence[69].

Cervicovaginal GBS immunity is largely driven by epithelial cells that secrete a range of pro-inflammatory cytokines and chemokines[70] that amplify innate immune cell recruitment and responses. Evidence suggests that neutrophils, mast cells, and macrophages, are the major innate populations that respond to GBS vaginal infection, each employing unique effector mechanisms to target GBS[68,70–72]. Neutrophils release extracellular traps that contain antimicrobial compounds and reactive species[68], while mast cell degranulation releases histamine and other effectors molecules[72]. All three effector cell types can in turn release pro-inflammatory cytokines, like IL-6, IL-1β, and TNFα to further amplify anti-GBS immune responses[68,70–73]. Responses appear to be GBS strain-dependent; rapidly-cleared GBS strains elicit robust pro-inflammatory responses while persistent colonizing GBS strains are significantly less immunogenic[70]. This suggests that persistent strains employ unique mechanisms to evade host recognition or immune activation. One study revealed that IL-17 production is strongly correlated with GBS clearance, implicating effector cells producing this cytokine, such as Th17, γδ T cells, or neutrophils, as key modulators of GBS clearance[70].

Notably, the previously discussed findings were from non-pregnant mice, and our understanding of GBS-immune dynamics in the vaginal tract under pregnant conditions is limited. One study investigating the impact of gestational diabetes on GBS disease pathogenesis revealed that neutrophil depletion in the third trimester led to increased GBS burden in vaginal and placental tissues, suggesting that neutrophils provide protection against GBS vaginal colonization, ascension, and placental invasion during pregnancy[75,76]. Pregnancy has significant impacts on immune system function, due to dramatic increases in ovarian hormones that directly communicate with leukocytes and the reproductive mucosa to alter immune responsiveness. Given GBS vaginal colonization is the primary risk factor for maternal and neonatal invasive

infection, it's imperative to understand how GBS carriage is affected by physiological changes associated with pregnancy.

OVARIAN HORMONES

Progesterone in Pregnancy Establishment and Maintenance

Pregnancy is a complex, tightly coordinated process that is regulated by the reproductive, neuroendocrine and immune systems. In rodents, copulation is the first signal received by the female post-ovulation that initiates physiological responses to prepare for pregnancy establishment. During copulation, male penetration stimulates the cervix to induce a neuroendocrine response that leads to hypothalamic-pituitary release of gonadotropic hormones[77–79]. Pituitary prolactin (PRL) and luteinizing hormone (LH) target the recently ovulated follicle in the ovary; LH promotes granulosa to luteal cell transition, the cells that produce massive levels of steroid hormones[80]. PRL prevents degradation of the corpus luteum (luteolysis) to maintain its production of progesterone and estrogen[81].

Embryo implantation serves as the second critical signal to maintain pregnancy and occurs during a specified "implantation window" when the endometrium is receptive to embryo. Prior to implantation, estrogen and progesterone induce changes to the endometrial environment that facilitate successful embryo implantation. Progesterone engages nuclear and membrane progesterone receptor expressed by endometrial stromal cells to induce their proliferation and alter their gene expression profile to accommodate implantation[80]. Estrogen promotes endometrial epithelial cell proliferation through ERα[80,82] and alters epithelial gene expression to enhance embryo attachment and invasion[75,77,80].

In mice, implantation occurs on day 4.5 to induce endometrial decidualization, where endometrial stromal cells phenotypically and functionally shift to further aid embryonic

trophoblast cell invasion of endometrium[77,83]. Prolactin is a critical component that arises from the functional and metabolic decidual cell shift as it maintains corpus luteum function[78,79,84], specifically progesterone production. In mice, progesterone is sustained by decidual prolactin until about 9 days post-coitus, whereafter the trophectoderm becomes the predominant source of PRL-like proteins for the remainder of pregnancy[85]. In the absence of implantation and decidual prolactin, the CL produces 20α-Hydroxysteroid Dehydrogenase (20α-HSD) that degrades progesterone and allows decidual production of prostaglandin-F2α that initiates luteolysis[74].

Pseudopregnancy is a physiological state that mimics the early stages of pregnancy. In rodents, pseudopregnancy can be achieved by mating an estrus female to a vasectomized male, and the resulting pseudopregnancy is confirmed by increase in serum progesterone which peaks at day 6.5 pc and decreases to baseline by day 8.5pc[86,87]. Mating causes a neuroendocrine reflex via cervical stimulation that induces pituitary release of PRL to transiently prolong CL progesterone production[88]. However, without a fertilized embryo and implantation, further PRL production from the decidua is absent and the CL undergoes luteolysis.

As pregnancy progresses, progesterone concentrations peak at day 16 of mouse pregnancy[89,90]. Progesterone is critical to pregnancy maintenance; administration of progesterone antagonist RU486 (mifepristone) leads to abortion in mice and humans[91–93]. Eventually, a decrease in progesterone concentration or activity is required to induce labor. Towards the end of mouse pregnancy, proinflammatory cytokines induce PGF2α production by the decidua, initiating CL degradation and subsequent P4 reduction[94], resulting in a rapid decline two days prior to labor onset that transitions the myometrium from quiescent to contractile. In humans, progesterone levels do not decline prior to labor onset, rather, a functional withdrawal from progesterone activity at the receptor level is thought to occur. Progesterone receptor (PR) has

two isoforms, PR-A and PR-B, where PR-A suppresses. Evidence from human studies show myometrial PR-A/PR-B ratio is increased at labor, and is associated with increased ERα expression, rendering smooth muscle cells insensitive to progesterone while increasing sensitivity to estrogen to enhance myometrial contractility[95]. Further investigation is needed to define the exact mechanism by which progesterone and estrogen facilitate human parturition.

Effects of Ovarian Hormones on Cervicovaginal Mucosal Function

The female reproductive tract is a unique mucosal environment because of its responsiveness to steroid sex hormones: estrogen and progesterone act through their receptors to induce profound physiological changes to the vaginal, cervical, and uterine mucosa in a cyclical fashion. While much research has focused on progesterone and estrogen's impact on the endometrium and pregnancy outcomes, ovarian hormones equally impact cervicovaginal mucosa morphology and function[96].

The mucosal surface of reproductive tract serves as a barrier to protect against the outside environment. The lower tract consists of the vagina and ectocervix and is lined with stratified squamous epithelium, which undergoes distinct morphological changes during the estrogen-dominant phase of the estrous and menstrual cycles, when there is a significant increase in epithelial thickness due to proliferation[94]. In contrast to the vagina and ectocervix, the endocervical epithelium does not proliferate in response to estrogen; rather, these cells alter the composition and abundance of secreted mucus to benefit sperm transport[59,96–100]. During the progesterone-dominant secretory phases, the vaginal epithelium thins, allowing increased leukocyte trafficking to the vaginal lumen which, in mice, are measurable through vaginal cytology[101]. The cervix secretes mucus components that favor protection from pathogens, but is less agreeable to sperm survival and transport[102]. Thus, the epithelium responds to hormones to

potentiate changes in barrier composition and integrity that support the physiological requirements for copulation and pregnancy establishment.

Changes in mucus viscosity across the cycle are achieved by altering the composition of the gel-forming mucins, which are large, negatively charged, highly glycosylated proteins. Four gel-forming mucins comprise cervicovaginal mucus: MUC2, MUC5AC, MUC5B, and MUC6[103–106]. MUC5B regulates mucus viscosity, as its concentrations fluctuate across the menstrual and estrus cycles and are inversely correlated to progesterone levels[107]. In addition to facilitating sperm transport, cervicovaginal mucus prevents attachment and colonization of microorganisms to the vaginal epithelium and slows their diffusion, providing opportunity for epithelial and local immune cells to mount a protective inflammatory response when needed[103]. Many vaginal pathogens have evolved mechanisms to counteract or use gel-forming mucins to persist in the vaginal tract[104–107]. This includes GBS, which upregulates surface pili expression in response to MUC5B to enhance its ability to bind MUC5B and outcompete commensals that depend on mucin-binding for survival[108]. The cervicovaginal mucus contains other secreted factors, including cytokines, chemokines, and antimicrobial peptides (AMPs) that fluctuate cyclically and are hormonally regulated[59,97–100,109–111]. Since steroid hormones influence the overall antimicrobial environment in the lower reproductive tract, it is important to consider how hormonal changes during pregnancy may impart vulnerability to GBS infection.

Neutrophils as Key Mediators of Cervicovaginal Immunity

Nearly all human and mouse immune cells express estrogen receptors, progesterone receptors, or both[112–114], which permits hormonal immunomodulation and integration of the immune, endocrine, and reproductive systems[103]. Most of what we understand about estrogen and progesterone's effects on the cervicovaginal immune populations is garnered from studies

investigating hormonal fluctuations across the rodent estrus cycle. Estrogen-dominant proestrus and estrus are characterized by limited to no leukocytic influx into the vaginal lumen[117], while progesterone-dominant metestrus and diestrus are characterized by substantial leukocytic influx, especially polymorphonuclear (PMN) granulocytes, which can be observed in vaginal cytology smears[94,103,117]. During mouse pregnancy, vaginal cytology shows persistent PMN presence, similar to metestrus, again implicating hormones in CVL immune cell composition and abundance[118]. Neutrophils are the predominant leukocyte found in vaginal lavage: flow cytometric analysis of cervicovaginal lavage confirms vaginal cytology observations, where neutrophils account for 95% of the CD45+ leukocyte population during diestrus. Macrophages, dendritic cells, and eosinophils constitute the remaining 10% of CD45+ cells, however, due to their limited abundance in CVL little is understood about their homeostatic function and how ovarian hormones affect their composition and function across the estrus cycle.

Multiple studies demonstrate that progesterone and estrogen modulate neutrophil chemotaxis through antagonistic mechanisms. Estrogen signaling through estrogen receptor (ER) appears to have an inhibitory effect, where vaginal epithelium specific deletion of ER results in excess neutrophils in the vaginal epithelium and lumen, regardless of estrus cycle stage[115,119]. Further, ovariectomized mice supplemented with exogenous estrogen lack neutrophils in the vaginal lavage, while those supplemented with progesterone have an abundance of neutrophils in vaginal lavage. In mice, both progesterone treatment and genetic deletion of the ER promoted neutrophil trafficking into the vaginal lumen and clearance of *Candida albicans*[119], further highlighting the inverse role these hormones play in neutrophil trafficking.

Neutrophil migration to the cervicovaginal epithelium is regulated by ovarian hormones through a variety of mechanisms. Expression of neutrophil adhesion molecules, CD44 and

CD47, are modified by estrogen and progesterone in the vaginal epithelium, where estrogen downregulates and progesterone upregulates expression[115,120]. Likewise, estrogen inhibits and progesterone enhances production of neutrophil chemoattractant, CXCL1, to drive chemotaxis and transepithelial migration into the vaginal lumen[120]. Together, these studies portray antagonistic roles for estrogen and progesterone in vaginal immunity, where estrogen impairs while progesterone promotes clearance of pathogen through altered neutrophil trafficking to the vagina.

Neutrophils are important to GBS immune responses: they are the first innate immune effector to infiltrate and found in significantly greater numbers than other immune cell populations in GBS-infected tissues[53,54,68,121]. Neutrophils target pathogens through mechanisms such as phagocytosis, release of extracellular traps and antimicrobials, and production of soluble inflammatory mediators. During GBS infection, neutrophils release extracellular traps (ETs), that are often accompanied by an assortment of antimicrobial compounds, including metal-sequestering proteins that starve GBS of metals essential for survival[53,122]. Calprotectin, a metal-sequestering protein that comprises ~45% of neutrophil cytosolic protein content[123,124], is released in NETs[125] and found in large quantities at sites of GBS infection[122]. In response, GBS evolved systems to sense and respond to metal abundance to adapt to niches with varying ionic environments. GBS thwarts neutrophil calprotectin-mediated stress through the two-component regulatory system, SaeRS, which assembles metal transport systems to survive in metal-restricted environments[122,126,127]. Neutrophils release pro-inflammatory mediators, like IL-1β, in response to GBS infection to amplify their own recruitment[49,127,128]. In return, GBS hyaluronidase degrades hyaluronan into fragments that inhibit TLR2/4 signaling and dampens neutrophil activation[49,121,128], while GBS hemolysin induces neutrophil cell death to subvert

killing by NETs[130,131]. In non-pregnant mice, persistent vaginal colonization is associated with chronic neutrophil influx into the vaginal lumen[68]. It's likely that GBS subverts neutrophils in the vaginal tract by employing some of the previously described tactics. However, it's unknown how pregnancy-level concentrations of ovarian hormones affect neutrophil immunity to GBS in the vaginal tract and whether the same observations would be observed in pregnancy.

SEMINAL FLUID: IMMUNOMODULATORY AND ANTIMICROBIAL FUNCTIONS

GBS carriage during pregnancy is variable. Studies point to GBS strain-specific differences that facilitate carriage during pregnancy. For example, women colonized with invasive GBS serotypes are more likely to exhibit persistent colonization between trimesters than women colonized with non-invasive serotypes, and GBS strain-specific virulence factor expression contributes to invasive disease pathogenesis. However, it's less well understood what host factors dictate GBS acquisition, persistence, or clearance, especially during pregnancy. Studies of college students show that GBS colonization is associated with sexual activity; in nonpregnant college-aged women, any sexual activity and number of sex partners were positively associated with GBS carriage, and sex partners tended to be colonized with identical GBS strains. Interestingly, neither penile vaginal penetration nor condom use affected risk for GBS carriage, and oral sex and intercourse were equally implicated in risk of GBS carriage[133]. These observations suggest that GBS is likely transmitted through intimate skin contact and not necessarily in semen like other sexually transmitted bacterial pathogens. For instance, *Chlamydia trachomatis, Neisseria gonorrhoae*, and others are able to persist in seminal fluid[134–136] despite a slew of antimicrobial and immune components[133–135]. Given seminal fluid's abundance of bioactive compounds, it's important to explore how GBS responds to seminal fluid components and how it impacts vaginal colonization.

Seminal plasma has several functions beyond transporting spermatozoa through the female reproductive tract for oocyte fertilization, many of which could have bystander effects on GBS survival and colonization. Seminal plasma contains bioactive compounds that serve to optimize fertility and pregnancy outcomes[133] – from immunomodulatory proteins that elicit endometrial responses critical for pregnancy establishment[136–138] to antimicrobial compounds that limit microbial ascension during copulation and insemination[134,135,139,140]. Seminal fluid components originate from the male accessory sex glands including epididymis, prostate, seminal vesicles, and bulbourethral glands. The seminal vesicles are the largest contributor to seminal fluid, comprising ~60% of seminal plasma volume[141].

Seminal vesicle fluid (SVF) in particular, is critical to pregnancy establishment: it envelops sperm in adhesive and coagulating proteins that maintain quiescence until capacitation is needed in the female reproductive tract, it provides energy metabolites, antimicrobials and prostaglandins to aid sperm transport through the cervix, and it contains an abundance of signaling compounds that play essential roles in establishing the peri-conception environment to ensure productive implantation, maternal immune tolerance, and placentation [139,142,143]. As such, seminal-vesicle excised (SVX) male mice have significantly reduced fertility[144] and men with diseases of the seminal vesicles are infertile[145]. In mice, SVF induces inflammatory responses that propagate changes to maternal endometrium that allow for embryo implantation[136,146] and establish maternal immune tolerance to fetal tissue[137,138,147]. These processes in turn drive productive placentation, fetal, and neonatal development. These seminal fluid functions are highly conserved across invertebrate and vertebrate species, suggesting an important evolutionary role to maximize offspring viability and fitness[133,148].

In mice, SVF-derived TGF-β has been identified as a primary driver of the endometrial immune response[136,149,150]. The uterus produces pro-inflammatory cytokines including TNFα, IL-6, IL-1β, CSF2, and CSF1, as well as chemotactic factors CXCL1, CXCL2, and CCL3, which recruit dendritic cells, macrophages, eosinophils, and neutrophils to further remodel the endometrium for implantation[136,146,149–154]. In the human cervix, seminal fluid elicits soluble pro-inflammatory mediators IL-6, IL-8, TNFα, CSF-2, GM-CSF, and MCP-1 that recruit leukocytic infiltrates, including neutrophils, macrophages, dendritic cells, natural killer cells, and $CD8^+$ T cells[151,155,156]. Leukocytic infiltration of the cervix is thought to remove non-viable sperm and limit microbial ascension during sperm transport to the uterus[157]. Interestingly, GBS elicits similar soluble inflammatory mediator production and immune cell recruitment in other tissues. Thus, immune responses elicited by seminal vesicle fluid in the cervix may collaterally target GBS.

The vaginal epithelium appears less responsive to seminal fluid when compared to ectocervical epithelial cells and the uterus. *In vitro* studies using human cell lines show that vaginal epithelium releases IL-6 and MCP-1 in response to seminal fluid treatment, which are important for T cell and macrophage recruitment, respectively[156], while neutrophil chemotactic factors and other pro-inflammatory cytokines were not expressed. However, these studies were performed without steroid sex hormones, which may alter immune responses. Indeed, studies show that the endogenous hormonal environment takes precedent over external stimuli in dictating immune responses in the vaginal tract[157], thus it is possible vaginal epithelium has an altered response to SVF in different hormonal contexts. In general, seminal fluid modifies the immune environment across the entire female reproductive tract which may impact responses to pathogenic stimuli like GBS.

In addition to cell-mediated immunity, human and mouse SVF directly target vaginal pathogens[135,140], including one study that shows human seminal plasma inhibits GBS growth[135]. SVF's antimicrobial activity is attributable to its abundance of antimicrobial peptides (AMPs), which are thought to limit pathogen ascension to the uterus during copulation and sperm transport. SVF-specific AMPs, including lactoferrin, lysozyme, phospholipase A2, SLPI, and β-defensins, exhibit antimicrobial activity through a variety of mechanisms and some have known activity against GBS. For instance, lactoferrin binds free iron and limits GBS growth through iron sequestration[161] and can also enhance immune complement targeting of GBS[162]. While lysozyme targets the peptidoglycan layer of the cell wall[163] and its transgenic expression in mouse lung epithelium decreased GBS burden and dissemination to distal tissues[164].

Post-coitus, the vaginal tract is exposed to seminal fluid components for up to several days. Our understanding of how such components impact GBS survival is limited. Further exploration is warranted to determine how seminal fluid exposure affects risk of GBS carriage in pregnancy and whether its components contain therapeutic potential.

CURRENT CHALLENGES AND GAPS IN KNOWLEDGE

GBS uniquely afflicts pregnant women and neonates. Vaginal carriage is the strongest predictor of risk for GBS disease, yet only ~1% of colonized women will go on to develop invasive disease outcomes. Additional factors to better profile patients at high risk for GBS disease are urgently needed.

Ovarian hormones drastically change across pregnancy. While cyclical hormonal fluctuations alter immunity to other reproductive pathogens, no studies have thoroughly investigated how hormones impact GBS disease pathogenesis. In particular, neutrophil immunity to reproductive pathogens, like *C. albicans* are strongly influenced by fluctuations in

progesterone and estrogen[162]. Exploring how the endocrine environment affects GBS vaginal colonization may identify factors that affect individual risk for GBS disease during pregnancy, which will hopefully improve screening efficacy and patient care. Further, these studies will expand our knowledge of how the endocrine system regulates cervicovaginal immunity, which may translate to other reproductive infectious diseases and inform sex-differences in vaccine efficacy.

Antibiotics are the only current available intervention to treat vaginal GBS colonization. Given the risks associated with widespread antibiotic use, including antibiotic resistance and negative impacts to the fetal microbiome, novel therapeutic interventions to treat vaginal GBS colonization are needed. Previous studies reveal that male ejaculate components contain a wide range of compounds that can directly target or enhance immunity to reproductive pathogens, including a study that shows human seminal plasma contains antimicrobial activity against GBS. However, a more in-depth assessment of whether seminal fluid components can target GBS in vivo is warranted to justify whether specific compounds within seminal fluid can be used as a therapeutic intervention to effectively target vaginal GBS colonization.

This dissertation seeks to address outstanding questions about host factors that dictate GBS vaginal colonization during pregnancy. 1) How does pregnancy impact vaginal GBS carriage in females that exhibited persistent carriage prior to pregnancy onset? 2) How do male seminal fluid components introduced during coitus impact vaginal GBS carriage? 3) How does the endocrine environment affect vaginal immunity and GBS colonization of the vaginal tract? Findings from this dissertation will deepen our understanding how seminal fluid components and the endocrine environment influence vaginal GBS colonization in pregnancy, and lay the foundation for future mechanistic studies.

In Chapter 2, we aimed to develop a mouse model that would allow us to investigate GBS vaginal colonization dynamics across pregnancy. We hypothesized that pregnancy would alter GBS carriage in female mice that were persistently colonized with GBS prior to mating. In Chapter 3, we explored host factors that dictate GBS vaginal colonization in pregnancy using the mouse model we established in Chapter 2. We hypothesized that ovarian hormones and male seminal fluid components affect GBS vaginal carriage dynamics in early pregnancy.

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CHAPTER 2: ESTABLISHING A MOUSE MODEL OF PERSISTENT GROUP B *STREPTOCOCCUS* VAGINAL COLONIZATION AND SUBSEQUENT PREGNANCY

ABSTRACT

Group B *streptococcus* (GBS) infections are a leading cause of neonatal morbidity and mortality. A recent metanalysis reveals that the annual global GBS burden contributes up to 3.5 million preterm births, 320,000 cases of neonatal infection, and 50,000 stillbirths. Third trimester rectovaginal colonization is the primary risk factor for developing invasive GBS infection, but our understanding of host factors for that modulate colonization during pregnancy is limited. Persistently colonized females clear GBS from the vaginal tract soon after pregnancy is established, but what drives this clearance is unknown. We sought to identify factors in early pregnancy that restrict GBS colonization and persistence. We hypothesized that exposure to seminal fluid components and increases in ovarian hormones contribute to GBS clearance. Removal of seminal vesicle fluid or sperm from male ejaculate led partially restored GBS carriage post-copulation, suggesting that seminal fluid components can contribute to GBS clearance in pregnancy. Further, exogenous progesterone treatment led to substantial GBS clearance from the vaginal tract and this correlated with neutrophil influx, suggesting that progesterone is a key driver of GBS clearance in early pregnancy. Our findings reveal novel prospective risk factors and therapeutic interventions to treat GBS disease.

INTRODUCTION

As an opportunistic pathogen, GBS is highly adaptive, allowing it to colonize and invade multiple host niches. Existing mouse models probe distinct phases of GBS pathogenesis – from asymptomatic colonization to ascension and intra-amniotic infection during pregnancy. Like most pregnancy pathogens, GBS causes complications predominantly in the third trimester of human pregnancy[1]. As such, a majority of mouse models perform GBS infection in the late third trimester to mimic preterm birth and neonatal infection in humans. For instance, dams inoculated with GBS intravaginally or injected directly into the amniotic sac after gestational day 13.5 (human third trimester equivalent) exhibit a severe preterm birth phenotype[73,163], allowing researchers to identify GBS-host dynamics that permit bacterial ascension and intrauterine invasion, the inflammatory pathways that drive preterm birth, and investigate routes of neonatal transmission that correspond with EOD or LOD manifestation. While these models serve clinically relevant purposes, vaginal colonization is the primary risk factor for developing invasive GBS infection, yet little is understood about GBS vaginal colonization dynamics across pregnancy. Vaginal colonization with specific GBS strains is strongly associated with invasive infection[5], however, not all women vaginally colonized with invasive GBS isolates develop invasive infection. Moreover, evidence from human pregnancy studies reveal that GBS carriage across trimesters is often transient and that invasive GBS serotypes are only weakly associated with persistent carriage[165]. Together, these observations tell us that invasive disease pathogenesis is only partially determined by GBS strain types, and that variations in the host environment likely also determine invasive disease development. As such, additional animal models that mimic human GBS carriage dynamics during pregnancy are needed, and will allow us to identify

risk factors unique to pregnancy that determine GBS vaginal colonization and women at greatest risk of invasive infection.

Current mouse models of persistent GBS vaginal colonization use estrogen treatment to maintain GBS in the vaginal tract for up to several months. This method originated from the observation that females infected intravaginally during estrus carry GBS significantly longer than those infected at other estrous cycle stages[67,68]. Using this method, researchers have identified several host that regulate GBS colonization dynamics. For instance, vaginal IL-17 production correlates with GBS clearance, implicating specific immune cell populations, such as Th17 cells or neutrophils, as important effectors for GBS clearance[70]. Further, this model has also identified GBS factors that facilitate colonization, like β-hemolysin which counteracts host immune defenses to facilitate persistent colonization in the vaginal tract[68]. Because this model is performed in nonpregnant mice, it is a useful tool for specific lines of inquiry, like GBS-immune dynamics within the nonpregnant vaginal niche relevant to vaccine development. However, this is also its major limitation, as it is unclear how findings from nonpregnant females translate to pregnancy, especially given the dramatic shifts in immune composition and responsiveness that occurs in the reproductive niche during pregnancy.

In this study, we sought to develop a mouse model that would allow us to investigate GBS colonization dynamics during pregnancy and identify host factors that either permit or restrict GBS carriage. Our model consists of two distinct phases: the first phase establishes pre-pregnancy persistent GBS carriage, followed by the second phase where persistently colonized females are paired with males to become pregnant. We observe distinct carriage dynamics between the pre-pregnancy and pregnancy phases, suggesting there are certain factors unique to pregnancy that alter the vaginal tract's susceptibility to GBS colonization in mice. This model

will enable identification of prospective risk factors that determine GBS carriage in human pregnancy and novel therapeutic approaches for GBS-positive pregnant women.

MATERIALS AND METHODS

Animal Housing and Care

Mouse studies were performed with 6-10 week old C57Bl/6 mice from Jackson Laboratories. Mice were fed a standard breeding diet, allowed free access to food and water, and housed in stable conditions (12h:12h light-dark cycle, 22-24°C). Mice were acclimated to the facility for 7 days prior to introduction to experiments.

Bacterial Strains and Culture Conditions

GB653 and GB411 were cultured by inoculating glycerol stocks into 10mL of Todd Hewitt broth (THB) and incubating at 37°C, 5% CO_2 overnight. 24 hours later, cultures were diluted 1:100 in fresh 10mL of THB, grown to $OD_{600} = 0.13$ (approximately $1x10^8$ CFU/mL). GBS was prepared for infections by centrifuging 1mL of inoculum at 17000g for 1 minute, washed in 1mL of PBS, and resuspended in PBS to a final concentration of $1x10^9$/mL for vaginal infections. Inoculums were serial diluted 10-fold and spot plated on selective and differential Chromagar to enumerate CFUs and determine inoculating concentrations.

Mouse Model

We established persistent vaginal colonization in nonpregnant females as previously described[67,68]. Briefly, females were given subcutaneous 17β-estradiol (1 mg/mL) in sesame oil and the following day were intravaginally infected with $1x10^7$ colony forming units (CFUs) of GBS in 10uL of PBS. Successful mating was determined by visual confirmation of a copulatory plug the morning after pairing. Once mating was confirmed, GBS vaginal burden was quantified across pregnancy by intravaginal swabbing on gestational days (GD) 3.5, 6.5, 10.5, and 15.5.

GBS burden for reproductive tissues and newborn pups was quantified one day post-partum by thoroughly swabbing the luminal side and external surfaces, respectively. Swabs were performed with Puritan hydraflock swabs that were wetted with PBS prior to swabbing tissues and subsequently placed in a 1.5mL Eppendorf tube containing 100uL of PBS. Tubes were then vortexed for 15 seconds to release GBS into suspension. Suspensions were serial diluted 10-fold and spot plated on selective and differential Chromagar to enumerate CFUs.

Statistical Analysis

Statistical analysis was performed as stated in the legend of each figure. Kruskal-Wallis test was used for multiple comparisons of non-parametric data. Mann-Whitney U test was used to compare two groups of non-parametric data. One-way ANOVA with Tukey HSD was used to compare multiple groups of normally distributed data. Statistical analyses were perfomed using an online calculator resource, Statistics Kingdom (www.statskingdom.com)[69,167,168], that uses R codes to run statistical tests and calculate p-values.

RESULTS

Persistent colonization of nonpregnant females

We established persistent GBS vaginal colonization in nonpregnant females by giving a subcutaneous injection of 17β-estradiol prior to inoculating females intravaginally with $1x10^7$ colony forming units (CFUs) of GBS. We tested two genetically and clinically distinct patient isolates: GB411, which is an ST-17 serotype III and was isolated from a septic neonate, and GB653, which is an ST-12 serotype II and was isolated from a colonized pregnant woman without invasive infection. Importantly, these sequence types exhibit differences in stress resilience, persistence in humans, and elicit unique immune responses from the host. Using these two distinct isolates in our model allows us to investigate any strain-dependent effects on vaginal

carriage during pregnancy. Consistent with previous findings, nonpregnant females carried GBS long term, where GB411- and GB653-infected females exhibited vaginal carriage across 21 days A higher percentage of females carried GB411 compared to females inoculated with GB653 (Fig. 2.1), suggesting GBS strain-dependent differences in vaginal persistence.

Examining the impact of pregnancy on persistent GBS carriage

To determine the impact of pregnancy on vaginal carriage, a cohort of colonized females were paired with males at 21 days post-infection. In stark contrast to nonpregnant females, pregnant females rapidly cleared GBS (Fig. 2.2). By day 3.5 post-copulation (pc) 60% and 25% of GB411- and GB653-infected dams were negative for GBS, respectively. By day 6.5 pc 100% of GB411-infected dams were negative for GBS, and by day 10.5 pc all females in both groups were negative for GBS and this was maintained at day 15.5 pc. These data suggest that pregnancy establishment leads to GBS clearance from the vaginal tract.

While all dams were vaginally-negative for GBS after day 10.5 pc, it is possible that GBS ascended to the endocervix or uterus, anatomical regions that would be unreachable by vaginal swab. Indeed, an estimated 10% of women who are GBS-negative at their 37-week screening test positive for GBS in amniotic fluid at delivery[169–171], suggesting that the upper reproductive tract can potentially serve as a reservoir for GBS persistence. As such, we also quantified colonization in the vagina, cervix, uterus at parturition to determine whether GBS persisted elsewhere (Fig. 2.3). Pups were also swabbed to determine if vertical transmission occurred. GBS-infected dams exhibited post-partum colonization that was strain- and tissue-dependent. Of GB411-infected dams (N=11), 45% were positive in the vaginal tract, 9% were positive in the cervix, and 9% were positive in the uterus post-partum. Of GB653-infected dams (N=11), none were positive in the vaginal tract, 9% were positive in the cervix, and 18% were positive in the uterus post-

partum. Despite detectable GBS quantities in maternal tissues post-partum, no pups born from GBS-infected dams tested positive for GBS (data not shown).

Because we observed some GBS clearance from the vaginal tract in the nonpregnant phase, we wanted to confirm whether the loss we observed in pregnancy truly due to pregnancy status. Therefore, we paired females with males at 7 days post-infection when greater than 90% of females were still colonized with GBS and compared colonization rates to nonpregnant females. Similar to females paired at 21 days post-inoculation, pregnant females rapidly cleared GBS from the vaginal tract post-coitus compared to nonpregnant females (Fig. 2.4). These results confirm our findings that pregnancy is nonpermissive to vaginal GBS carriage.

Pregnancy outcomes

To determine whether GBS colonization prior to pregnancy affected pregnancy outcomes, we compared GBS- to PBS-inoculated mice. Variables indicative of pregnancy health were measured, including gestational weight gain, gestation length, and percent fetal loss per litter (Fig. 2.5). We observed no significant difference in gestational weight gain nor gestation length between groups. Similarly, although some GBS-inoculated females exhibited fetal loss, there was no difference in fetal loss per litter compared to PBS-inoculated dams, suggesting that loss of GBS across pregnancy was protective against fetal loss and transmission of GBS to pups.

DISCUSSION

In this study, we aimed to develop a mouse model that emulates GBS carriage in human pregnancy. To do so, we adopted a model of persistent GBS vaginal colonization to include a pregnancy phase, with the goal of understanding GBS colonization dynamics during pregnancy and its impact on pregnancy outcomes. Consistent with previous findings, non-pregnant estrus-induced females exhibited persistent GBS carriage, with a small percentage showing continuous

carriage lasting longer than 50 days. Carriage in non-pregnant females was strain-dependent, where those infected with the invasive strain, GB411 (ST-17, serotype III), exhibited longer carriage than females infected with the colonizing strain, GB653 (ST-12, serotype II). This could be due to differences in virulence gene expression. Genetic studies showed that the deletion of GBS virulence gene(Δ*covR*) resulted in strains that are less persistent than WT due to increased host inflammatory responses[71], indicating that any virulence gene expression, likely those that aid immune evasion, is important for persistence in the vaginal tract.

While GBS carriage is transient across human pregnancy, it was unclear what factors drove variability in carriage. Invasive GBS strain types (i.e. ST17, serotype III) are associated with persistent carriage during pregnancy, however, not all women colonized with an invasive isolate experience adverse pregnancy outcomes, and approximately 1-7% of neonatal invasive disease cases are caused by colonizing isolates not commonly associated with invasive infection[170–172]. This again underscores the importance of the host environment in determining an individual's susceptibility to invasive disease.

In our model, colonized females rapidly cleared GBS once pregnancy was established, regardless of infecting strain type. Similarly, no pups born to GBS-inoculated dams were positive for GBS at birth, to include those born to dams that exhibited post-partum colonization in the vaginal tract, uterus, or cervix. Pregnancy outcomes were not impacted in GBS-inoculated females compared to PBS-inoculated dams, suggesting that progressive loss of GBS from the vaginal tract across pregnancy protected against vertical transmission of GBS to neonates and against fetal loss.

The successive decrease in vaginal GBS across pregnancy compared to nonpregnant females suggests that factors unique to the pregnant vaginal niche are able to eliminate or disrupt

GBS colonization. One of these factors could be steroid sex hormones, whose concentrations increase significantly throughout pregnancy until labor onset. During the first few days of mouse pregnancy, steroid sex hormones increase dramatically to orchestrate structural, secretory, and immunological changes to the reproductive tract that facilitate implantation. The immunological environment is heavily influenced by hormones, as most leukocyte populations express steroid hormone receptors rendering them susceptible to hormone signaling[103]. Similarly, progesterone and estrogen alter innate immune responsiveness and signaling of uterine, cervical, and vaginal epithelial and stromal cell populations. As such, it is plausible that increases in sex hormones seen in early pregnancy modify the vaginal tract's immune composition and response to GBS.

Another major factor that could influence GBS colonization is seminal fluid. Prior to hormonal changes in early pregnancy, copulation and introduction of seminal fluid are major homeostatic disruptors to the female reproductive niche. Seminal fluid contains many bioactive compounds with functions beyond spermatozoa transport[133,148]. Immunomodulatory proteins, like TGF-β, potentiate long-term changes to female reproductive immunity that facilitate embryo implantation and maternal immune tolerance to fetal alloantigen[136,137,156]. Seminal fluid also contains a plethora of antimicrobial compounds that are thought to prevent microbial ascension during copulation and fertilization[135,140]. These other unique seminal fluid functions could directly or indirectly target GBS in the vaginal tract.

Taken together, the dynamic state of the female reproductive tract in early pregnancy may explain the inability of GBS to persist in the vaginal tract once pregnancy is established. In the remainder of this thesis, I use our model to probe prospective host factors that drive GBS clearance from the vaginal tract in early pregnancy. Understanding the biological pathways

within the host niche that limit GBS colonization can be used to identify novel therapeutic interventions and risk factors for GBS invasive disease.

Study/Model Limitations

Previous studies showed that GBS persistence differs between inbred and outbred mice, and this difference may be attributable to more robust innate immune responses in outbred mice. Importantly, outbred strains are considered more immunologically/genetically reflective of the human population and in our studies we use inbred mice. Additionally, the mouse vaginal microbiome is distinct from humans. The mouse vaginal microbiome is dominated by *Staphylococcus and Enterococcus spp.*[176–178], whereas healthy human vaginal microbiome is *Lactobacillus spp.* dominant, which play significant homeostatic roles in the cervicovaginal niche, primarily through immunomodulatory and antimicrobial action. These differences may explain discrepancies in results between mouse and human studies and should be taken into consideration when interpreting study results using our model.

FIGURES

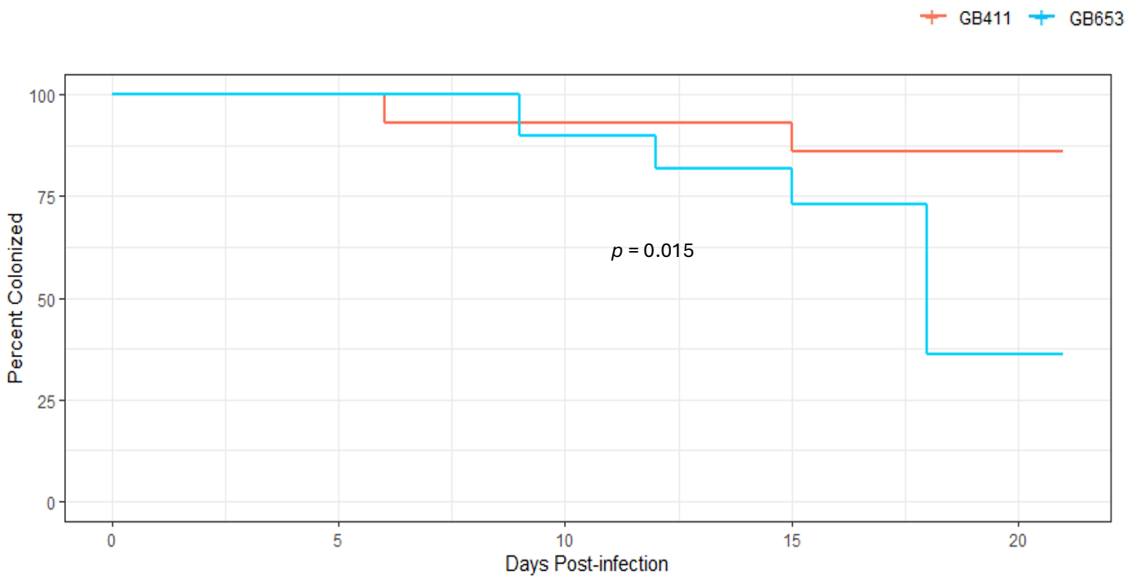

Figure 2.1: Pre-pregnancy GBS vaginal carriage across 21 days

Female mice were intravaginally infected with $1x10^7$ CFU of GB653 or GB411 (N=11, each group). GBS vaginal load was quantified by intravaginal swab every three days prior to pairing with males on day 21 post-infection. Swabs were eluted into PBS and 10-fold serial dilutions were spot plated on Chromagar. GBS colony forming units (CFU) are shown. Statistical analysis was performed using Kaplan Meier survival curve with log rank test P-values are shown.

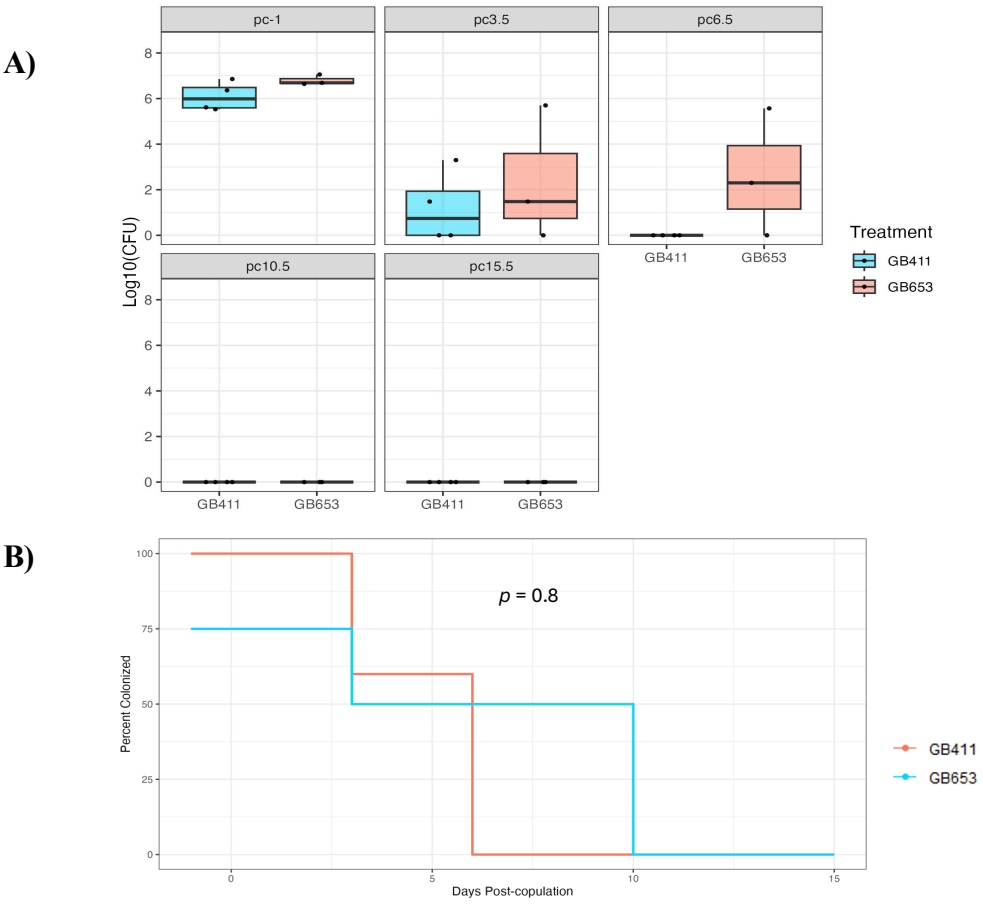

Figure 2.2: GBS vaginal carriage across pregnancy

GBS vaginal carriage in GB411-infected (N=5) and GB653-infected (N=4) females was quantified across pregnancy by intravaginal swab at 3.5, 6.5, 10.5, and 15.5 days post-copulation. Swabs were eluted into PBS and 10-fold serial dilutions were spot plated on Chromagar and GBS colonies were counted the following day. **A)** GBS CFU and **B)** percentage of females colonized are shown. The median CFU is represented in each box. The black dots represent biological replicates. Red dots indicate outliers. Statistical analysis was performed using Mann-Whitney U test on CFU data and Kaplan Meier curve with log rank test on percent colonized data. Comparisons with p-values < 0.05 are indicated with an asterisk or reported.

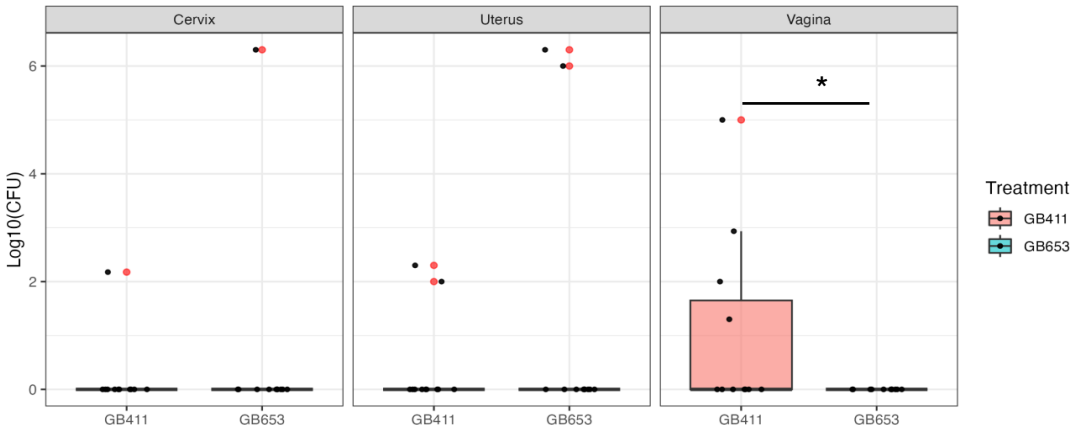

Figure 2.3: Post-Partum GBS colonization in the vagina, cervix, and uterus

Post-partum GBS CFU load in the vagina, cervix, and uterus was quantified in GB411- and GB653- infected (N=11, each group) dams on the day of delivery. To quantify GBS CFU load in tissues, dams were sacrificed after delivering litters and tissues were harvested and swabbed. Swabs were eluted into PBS and 10-fold serial dilutions were spot plated on Chromagar and GBS colonies were counted the following day. The median CFU is represented in each box. The black dots represent biological replicates. Red dots indicate outliers. Statistical analysis was performed using Mann-Whitney U test on CFU data. Comparisons with p-values < 0.05 are indicated with an asterisk.

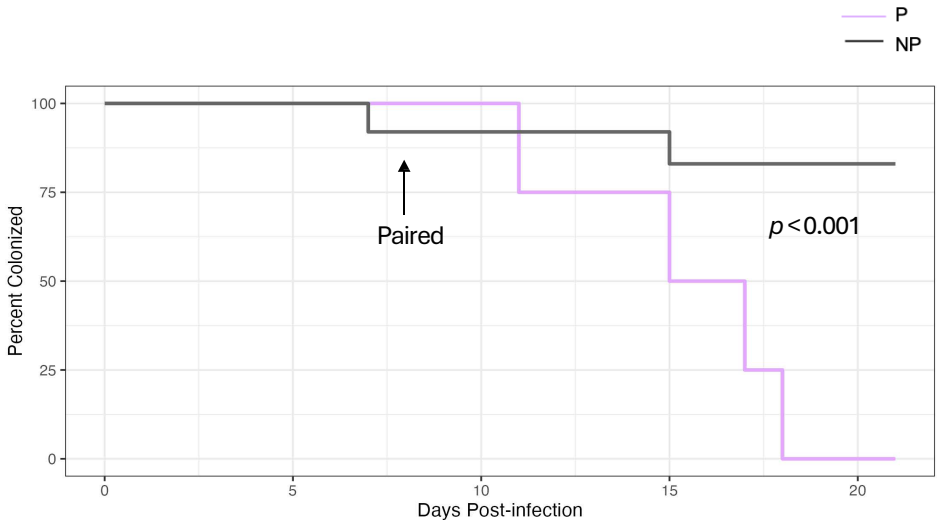

Figure 2.4: GBS vaginal colonization in pregnant and non-pregnant females

Vaginal GBS carriage in GB411-infected females either paired with males at 7 days post-infection (pregnant cohort, P, (N=4) or unpaired (non-pregnant cohort, NP, N=). Pregnant females were swabbed every three days until pairing, then at 3.5, 6.5, 10.5 and 15.5 post-copulation, while non- pregnant females were swabbed every three days continuously. Statistics determined using Kaplan-Meier survival curve with log rank test. P-value shown.

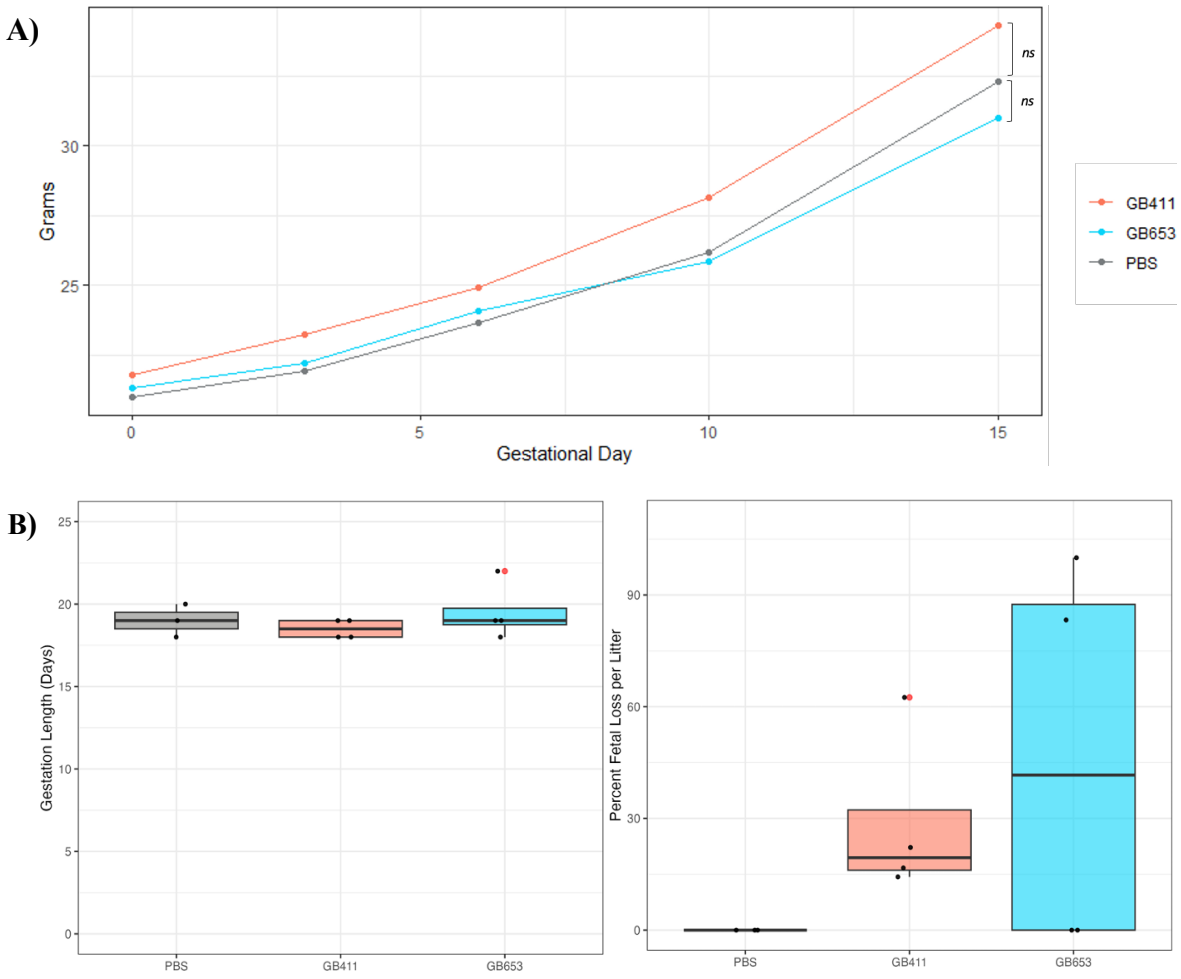

Figure 2.5: Pregnancy outcomes in GBS-infected females

A) Gestational weight gain and B) gestational length and percent fetal loss per litter in PBS (N=3),GB653 (N=4), and GB411 (N=4). Fetal loss included the number of pups born still or that died in-utero. Female weight was measured on the day of pairing and on gestational days 3.5, 6.5, 10.5, and 15.5. Statistical analysis was performed using one-way ANOVA with Tukey's HSD. P-values < 0.05 are indicated with an asterisk.

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CHAPTER 3: MALE AND FEMALE FACTORS THAT MODULATE GROUP B *STREPTOCOCCUS* VAGINAL COLONIZATION DURING PREGNANCY

ABSTRACT

Group B *streptococcus* (GBS) infections are a leading cause of neonatal morbidity and mortality. A recent metanalysis reveals that the annual global GBS burden contributes up to 3.5 million preterm births, 320,000 cases of neonatal infection, and 50,000 stillbirths. Third trimester rectovaginal colonization is the primary risk factor for developing invasive GBS infection, but our understanding of host factors for that modulate colonization during pregnancy is limited. Persistently colonized females clear GBS from the vaginal tract soon after pregnancy is established, but what drives this clearance is unknown. We sought to identify factors in early pregnancy that restrict GBS colonization and persistence. We hypothesized that exposure to seminal fluid components and increases in ovarian hormones contribute to GBS clearance. Removal of seminal vesicle fluid or sperm from male ejaculate led partially restored GBS carriage post-copulation, suggesting that seminal fluid components can contribute to GBS clearance in pregnancy. Further, exogenous progesterone treatment led to substantial GBS clearance from the vaginal tract and this correlated with neutrophil influx, suggesting that progesterone is a key driver of GBS clearance in early pregnancy. Our findings reveal novel prospective risk factors and therapeutic interventions to treat GBS disease.

INTRODUCTION

Group B *Streptococcus* (GBS) is a gram-positive opportunistic pathogen that can cause severe disease in pregnant women and neonates: annually an estimated 3.5 million preterm births, 50,000 stillbirths, and 120,000 cases of neonatal infection are attributed to GBS[1]. In high-income countries, women are routinely screened for GBS at their third trimester perinatal visit, and those who test positive are given intrapartum antibiotic prophylactic (IAP) treatment[1,3,18,23]. IAP has effectively reduced neonatal early onset infection (>80%) in geographical regions that have screening and antibiotics available. Despite IAP's efficacy in treating early onset infection, IAP does not target GBS-associated preterm birth, nor has it effectively reduced neonatal meningitis incidence typically associated with late-onset GBS infection. In addition to IAP's inefficacy against neonatal late onset disease, in-utero and early-life antibiotic exposure increases an individual's risk for allergic and metabolic disease development[180], and antibiotic resistant GBS is growing concern in geographical regions that routinely use IAP to treat GBS infection[181,182]. As such, there is a critical need to develop alternative therapeutic approaches to treat GBS disease.

Maternal GBS vaginal colonization is the primary risk factor for developing invasive infection and an attractive therapeutic target for preventing GBS-associated adverse outcomes. Mouse models of persistent vaginal GBS colonization in nonpregnant females have identified host and GBS virulence factors that contribute to GBS persistence[67,68,70,71,108,168,169]. However, pregnancy is a dynamic physiological state that dramatically impacts reproductive mucosal biology, thus findings from nonpregnant mice may not translate to pregnancy.

Prior to fertilization and embryo implantation, intromission and ejaculate exposure induce major physiological changes in female reproductive tract. Recent studies identified

seminal fluid constituents, specifically those originating from the seminal glands, that activate endometrial immune pathways important for implantation and establishing maternal immune tolerance to fetal alloantigens[136–138,154,170]. Seminal vesicle fluid also harbors a variety of antimicrobial components that are thought to limit pathogen ascension during copulation and sperm transport[134,135,140]. Together, these seminal vesicle fluid functions are key to optimal pregnancy outcomes.

Post-copulation, ovarian hormone concentrations rise steeply to orchestrate structural, metabolic, and immune changes to the reproductive mucosal landscape necessary for pregnancy. This is evident by gestational day 3 of mouse pregnancy, when serum progesterone has increased 5-fold from pre-pregnancy levels[69,171,172]. Several studies point to ovarian hormones as major gatekeepers of cervicovaginal immunity, and thus impact susceptibility to common reproductive pathogens, including *Candida albicans*[119,158,162,171–173], *Gardnerella vaginalis*[69,174,175], Herpes Simplex Virus[176–178], HIV[174,178–181], and others. Surprisingly, no studies have evaluated how pregnancy-level concentrations of ovarian hormones affect GBS colonization, even though GBS disproportionately affects pregnant women and infants.

The examples highlighted here illustrate dynamic physiological changes that coordinate pregnancy establishment and the importance of investigating GBS vaginal colonization in a pregnancy model. To address this need, we established a mouse model persistent GBS vaginal colonization and subsequent pregnancy, where we show that persistently colonized females rapidly clear GBS post-copulation. In this study, we use our mouse model to investigate factors unique to the early pregnancy reproductive niche that restrict GBS vaginal colonization. We find that male seminal fluid components and female ovarian hormones impact GBS vaginal colonization, and likely work together to suppress GBS colonization during pregnancy. Our

findings identify novel pathways by which GBS is targeted in the reproductive niche that have implications for GBS disease risk management and treatment in pregnant women.

MATERIALS AND METHODS

Mice

Mouse studies were performed with C57Bl/6 mice from Jackson Laboratories. Mice were fed a standard breeding diet, allowed free access to food and water, and housed in stable conditions (12h light dark cycle, 22-24°C). Mice were acclimated to the mouse facility for 7 days prior to introduction to experiments. Mouse studies were performed in accordance with protocols approved by the Michigan State University Institutional Care and Use Committee (Protocol no. 202200021).

Bacterial Culture and Preparation

Two genetically and clinically distinct GBS patient isolates were used in this study. GB411 is an ST-17 serotype III and was isolated from a septic neonate. GB653 is an ST-12 serotype II and was isolated from a colonized pregnant woman without invasive infection. These strains exhibit differences in stress resilience, persistence in humans, and elicit unique immune responses from the host, which allowed investigation of any strain-dependent effects on vaginal carriage in our pregnancy model. GBS and DH5α *Escherichia coli* was cultured by inoculating glycerol stocks into 10mL of Todd Hewitt broth (THB) or LB broth, respectively, and incubating at 37°C, 5% CO_2 overnight. The next morning, cultures were diluted 1:100 in fresh 10mL of THB, grown to OD_{600} = 0.13 (approximately $1x10^8$ CFU/mL). GBS was prepared for infections by centrifuging 1mL of inoculum at 17000 × *g* for 1 minute, washed in 1mL of PBS, and resuspended in PBS to a final concentration of $1x10^9$/mL for vaginal infections (~$1x10^7$ CFU per 10uL intravaginal inoculum).

Establishing Persistent GBS Vaginal Colonization in Female Mice

To establish persistent vaginal GBS infection in female mice, 6-8 week old C57Bl/6 females were given 0.1mg of subcutaneous 17-βestradiol (Manufacturer, location) in 100uL sesame oil, as previously described. 16-24 hours after estradiol administration, females were anaesthetized with 3% isoflurane and intravaginally inoculated with $1x10^7$ colony forming units (CFU) of either GB411 or GB653 in 10uL of PBS. Vaginal GBS was quantified by intravaginal swabbing every three days post-infection, where vaginal swabs were serially diluted 10-fold and plated on CHROMagar™ Strep B (CHROMagar, Saint-Denis, France). Bacterial plates were incubated overnight at 37□C. GBS colonies were counted following incubation. followed by dilution plating on Chromagar

Vasectomy and Seminal Vesicle Excision Surgery and Mating

Males (8-10 week old C57Bl/6) were prepared for surgical excision of seminal vesicles (SVX), vasectomy (VAS), or both (SVX/VAS) by anaesthetizing with 3% isoflurane. Males were kept on 37□C heating pads to maintain body temperature while under anesthesia, eyes were coated with ophthalmic ointment to protect from scratches/abrasion during surgery, and subcutaneous meloxicam (5mg/kg) and topical bupivicane (0.25%) were administered to manage pain. Mice were prepped by shaving around the incision site using electric shave clipper, the incision site was cleaned using alternating sterile swabs of chlorohexidine and 70% ethanol. An incision was made in the lower abdomen. For bilateral seminal vesicle excisions, 5-0 polypropylene suture (ETHICON, Raritan NJ) was used to ligate seminal vesicle at the base of the gland. After ligating, the coagulating gland was carefully blunt dissected away from the seminal gland before excising the seminal gland above the ligation. For bilateral vasectomies, the vas deferens was located and, using forceps, the vas deferens was excised using the crush and tear method at each

end of the tube. The body wall was closed with 5-0 PDO violet absorbable suture (Covetrus, Portland ME), and the dermis was closed using wound clips. Meloxicam was given 48- and 72-hours post-operation for continued pain management and recovery. Males were allowed to recover for two weeks before further experimental use. Each mouse underwent two test matings to confirm that SVX and SVX/VAS males do not form copulatory plugs as expected before introduction to experimental matings.

To observe vaginal colonization post-coitus, females were intravaginally infected with GBS and subsequently paired with either intact, SVX, VAS, or SVX/VAS C57Bl/6 males 6 days post-infection. For matings with intact and VAS males, coitus was confirmed by presence of a copulatory plug the next morning. For matings with SVX males, coitus was confirmed by presence of sperm in the vaginal tract the next morning. For SVX/VAS matings, coitus was confirmed visually by observation of videos recorded for 1 hour after pairing. GBS vaginal colonization was quantified post-coitus by intravaginal swab on post-coitus (PC) day 3.5, 6.5, 10.5, and 15.5.

Ex vivo Seminal Vesicle Fluid Bacterial Growth Assay

Seminal vesicles were harvested from 8-12-week old C57Bl/6 male mice for *ex vivo* bacterial growth assays. Seminal vesicle fluid (SVF) was harvested by careful blunt dissection of the seminal gland from the coagulating gland, ensuring the coagulating gland was left intact to prevent inadvertent coagulation of SVF. Seminal glands were excised at the base and immediately transferred to 1.5 mL Eppendorf tubes into which gland fluid was squeezed using forceps. Two glands rendered approximately 100uL of SVF. Tubes were then placed at 37□C for 10 min to maintain SVF viscosity while bacterial tubes were prepared. In a separate tube, $1x10^2$ CFU GBS or *E. coli* were suspended in 50uL of room temperature PBS. Using a cut pipette tip,

100uL of SVF or 100uL of PBS (control) was transferred to the tubes containing GBS, mixed, and incubated at 37□C for 10 minutes. After 10 minutes, the entire 150uL GBS and *E.coli* mixturse were plated on Chromagar and LB agar, respectively, and incubated overnight at 37□C. Colonies were counted the next morning. For protease experiments, SVF was preincubated with proteinase K (Zymo Research, Irvine CA) (1 mg/ml) or PBS (control) for 2 hours prior to bacterial incubation.

Alzet Implant Surgeries – Exogenous Progesterone Treatment

6-8 week old C57Bl/6 females were vaginally colonized with GBS as previously described. On day 6 post-infection, mice began supplementation with PBS (vehicle) or 250ug/day of water-soluble progesterone delivered via surgically implanted subcutaneous osmotic pumps (Model 1002, Alzet, Cupertino CA). Briefly, surgeries were performed under 3% isoflurane anesthesia and on 37□C heating pads to maintain body temperature, eyes were coated with ophthalmic ointment, and subcutaneous meloxicam (5 mg/kg) and topical bupivicane (0.25%) were administered to manage pain. To prep the incision site, the mouse was shaved and sanitized using chlorohexidine and 70% ethanol. A unilateral dorsal incision was made and the dermis was separated from the body cavity using forceps to create a subdermal "pocket" for the implant. After implants were placed the incision was closed using wound clips. Meloxicam was given 48- and 72-hours post-operation for continued pain management and recovery. Females were vaginally swabbed on day 3.5, 6.5, 10.5, and 15.5 post-surgery to quantify GBS load and neutrophil abundance via dilution plating and flow cytometry, respectively.

Flow cytometry

Cervicovaginal lavage (CVL) was collected by swab from mice receiving exogenous progesterone or PBS via Alzet osmotic pumps. Swabs performed one day prior to implant

surgery and on day 3.5, 6.5, 10.5 and 15.5 post-implant. CVL was eluted from the swab into 100uL of PBS by vortexing for 15 seconds. Swabs were removed and CVL-containing tubes were spun at 400g for 5 min to pellet cell populations. The supernatant was used to quantify GBS by 10-fold serial dilutions and subsequent spot plating on Chromagar. The cell pellet was resuspended in 100uL of PBS for flow staining and analysis. Flow staining was performed by incubating pellet with Fixable Viability Dye (Invitrogen) for 30 min on ice (1:1000 in PBS). After 30 minute incubation, cells washed three times using flow staining buffer, and resuspended in the antibody cocktail listed below (Table 3.1) for 45 minutes on ice. Cells were washed 3 times in flow staining buffer before fixing in 1% PFA for 15 minutes at room temperature. Cell were washed three times and resuspended in flow staining buffer prior to running on the Cytek Aurora spectral flow cytometer.

Table 3.1: Flow Cytometry Staining Antibody Panel

Antibody	Dilution	Manufacturer, Catalog #
Fc Block	1:100	BD Pharmigen, 553142
Anti-GR1-BV711	1:400	BioLegend, 108443
Anti-CD11c-BV650	1:200	BioLegend, 117339
Anti-F4/80-APC/Cy7	1:200	BioLegend, 123118
Anti-I-A/I-E(MHCII)-PE/Cy7	1:200	BioLegend, 107629
Anti-CD45-BV421	1:400	BioLegend, 103133
Anti-CD11b-APC/Fire 810	1:200	BioLegend, 101287

Statistics

Statistical analysis was performed as stated in the legend of each figure. Kruskal-Wallis test was used for multiple comparisons of non-parametric data. Mann-Whitney U test was used to compare two groups of non-parametric data. One-way ANOVA with Tukey HSD was used to compare multiple groups of normally distributed data. Statistical analyses were perfomed using an online calculator resource, Statistics Kingdom (www.statskingdom.com)[179], that uses R codes to run statistical tests and calculate p-values.

RESULTS

Seminal vesicle fluid inhibits GBS growth in vitro

As established in Chapter 2, a significant percentage of females cleared GBS by day 3.5 post-coitus (Fig. 2.2). As such, we speculated that factors associated with early pregnancy were impacting GBS growth. Intromission and insemination majorly disrupt the female reproductive niche, and seminal vesicle fluid, in particular, contains an abundance of antimicrobial compounds. Therefore, we hypothesized that seminal vesicle fluid (SVF) contains direct bactericidal/bacteriostatic activity against GBS. To test this, we compared GBS growth on agar when supplemented with SVF or PBS as a negative control. Previous work shows DH5α *E. coli* is sensitive to SVF and was used as a positive control in this experiment. Compared to PBS control, incubation with SVF significantly inhibited growth of both GB411 and GB653, and the level of inhibition was similar to DH5α (Fig. 3.1, A). Because SVF contains an abundance of antimicrobial proteins, we hypothesized that pretreatment of SVF with proteinase K would inhibit SVF's antimicrobial activity against GBS and restore GBS growth. Indeed, samples pretreated with proteinase K had partially restored GB411 growth (Fig. 3.1, B), suggesting that SVF contains antimicrobial proteins capable of targeting GBS.

Seminal vesicle fluid contributes to GBS clearance from the vaginal tract post-insemination

Given that SVF exhibited direct antimicrobial activity against GBS *in vitro*, asked whether this effect could be mimicked *in vivo*. To do so, we surgically excised seminal vesicles from males prior to mating, allowing us to determine how presence of SVF in the male ejaculate affected GBS colonization post-coitus. Because we observed no strain-dependent differences in response to SVF antimicrobial activity *in vitro* nor in our pregnancy model as described in chapter 2, we decided to only use the more clinically relevant strain, GB411, in the remainder of our *in vivo* experiments in order to limit the number of animals used.

Intact- and SVX-mated females exhibited similar GBS carriage at 3.5 pc. By 6.5, 10.5, and 15.5 pc, however, SVX-mated females sustained higher amounts of GBS in the vaginal tract compared to intact-mated females (Fig. 3.2), suggesting that SVF contributes to the reduction in vaginal GBS carriage post-coitus. Of note, SVX males exhibited reduced fecundity, where only 33% of SVX-mated females were pregnant and delivered litters. Because approximately 50% of SVX-mated females cleared GBS similar to intact-mated controls, we considered whether pregnancy status in SVX-mated females was an additional variable affecting colonization rates. To address this, we stratified the SVX-mated females by pregnancy status, which revealed that a majority of the vaginal GBS burden exhibited in the SVX-mated group was in nonpregnant females (Fig. 3.3). Nonpregnant SVX-mated females exhibited greater GBS carriage post-coitus compared to intact-mated females, while pregnant SVX-mated females exhibited no difference compared to intact-mated females, except at 10.5 pc. Together, these results suggest that pregnancy status affected GBS carriage in SVX-mated females which prompted us to further

investigate the relative contributions of seminal vesicle fluid and pregnancy status on post-coital GBS vaginal colonization.

Impact of seminal fluid components and pregnancy status on GBS carriage

To further isolate the contribution of SVF and pregnancy status in GBS clearance post-coitus, we created vasectomized (VAS) males and vasectomized/seminal vesicle excised (VAS/SVX) males. VAS male ejaculate lacks sperm but maintains SVF; as such, VAS-mated females will not become pregnant but are still exposed to SVF during coitus. VAS/SVX male ejaculate lacks both sperm and SVF, thus VAS/SVX-mated females serve as negative controls for both pregnancy status and SVF exposure. Together, these two additional sets of males allowed us to individually probe the role of SVF and pregnancy status in GBS clearance post-coitus.

At 3.5 days pc, all females exhibited a similar level of GBS clearance from the vaginal tract (Fig. 3.4). At 6.5 days pc SVX- and VAS/SVX-mated females maintain greater GBS in the vaginal tract compared to intact- and VAS-mated females, indicating that SVF exposure impacts to GBS colonization levels at earlier timepoints post-coitus. At 10.5 and 15.5, only intact-mated females sustained GBS clearance from the vaginal tract, while VAS-, SVX-, and VAS/SVX-mated females had significantly greater CFUs at these later time points. Both VAS- and intact-mated females were exposed to SVF, yet only intact-mated females maintained clearance, while 50% of VAS-mated females experienced rebound, carrying high levels of GBS at 10.5 and 15.5 days post-coitus. These findings suggest that pregnancy status, in addition to SVF exposure, plays a role in maintaining low GBS vaginal levels at later time points post-coitus.

Progesterone reduces GBS carriage in the vaginal tract

Because seminal vesicle fluid did not fully restore GBS following mating, we next considered whether hormones play a role in vaginal GBS carriage post-coitus. During pregnancy,

progesterone rises dramatically between days 0-6.5 days post-coitus and continues to increase as pregnancy progresses. Unlike pregnancy, serum progesterone in pseudopregnant mice peaks at post-coital day 6.5 and rapidly returns to baseline levels by day 8.5 p.c.[178]. Mating females to vasectomized males is a common method for inducing pseudopregnancy in females. Strikingly, vaginal GBS carriage in VAS-mated females appeared to inversely correlate with what would be predicted serum progesterone levels in pseudopregnancy: GBS is undetectable at day 6.5 pc when serum progesterone would be at its peak during pseudopregnancy, but then rebounds at days 10.5 and 15.5 pc, when serum progesterone levels would return to baseline (Fig. 3.4). This pattern of GBS carriage in the VAS-mated cohort prompted us to further investigate whether progesterone directly impacts GBS's capacity to colonize the vaginal tract.

To test whether progesterone influences GBS vaginal colonization, we administered exogenous progesterone (250ug/day) or PBS (vehicle control) to GBS-colonized females via surgically implanted Alzet osmotic pumps. Intravaginal GBS was quantified at 3.5, 6.5, 10.5 and 15.5 days post-implant (Fig. 3.5). Indeed, progesterone-treated females clear GBS significantly faster from the vaginal tract than PBS-treated females (Fig. 3.6). At day 6.5 post-implant, 50% of progesterone-treated females cleared GBS, compared to 0% of PBS-treated females. At day 10.5 post-implant this trend continues, where 87.5% of progesterone-treated females cleared GBS, compared to only 20% of PBS-treated females. These results indicate that progesterone alone decreases GBS carriage in the vaginal tract and likely plays a role in GBS clearance during pregnancy observed in our model.

Progesterone-mediated GBS clearance correlates with neutrophil influx into the vaginal lumen

Progesterone induces neutrophil influx into the vaginal lumen. This is demonstrated across the mouse estrus cycle, where progesterone-dominant diestrus is identified by increased leukocyte

numbers in vaginal cytology smears, of which ~95% are neutrophils. Previous studies investigating the impact of cyclical ovarian hormone fluctuations on sexually transmitted infection show that exogenous progesterone supplementation directly induces neutrophil influx into the vaginal lumen to target *Candida albicans*. We next asked whether exogenous progesterone influenced neutrophil abundance in the vaginal lumen of females. GBS CFUs and neutrophils were quantified in the vaginal lavage of PBS- (N=5) and P4-(N=5) treated females using dilution plating and flow cytometry, respectively (Fig. 3.7). Indeed, we found increased neutrophils in P4-treated females compared to PBS controls at day 6.5 and 10.5 post-implant, and neutrophil numbers inversely correlated with GBS load.

DISCUSSION

GBS vaginal colonization is a strong risk factor for developing infection during pregnancy. Previous studies have identified several host and GBS factors that either permit or restrict persistent vaginal GBS carriage in nonpregnant female mice. Because GBS is a pregnancy pathogen, in Chapter 2 we sought to develop a mouse model of vaginal colonization in pregnancy, where we observed that, compared to nonpregnant females, pregnant females rapidly clear GBS from the vaginal tract soon after pregnancy was established. This suggested that early pregnancy is nonpermissive to vaginal GBS colonization. Thus, in Chapter 3 we used our mouse model to identify multiple factors unique to the early pregnancy cervicovaginal niche that regulate GBS colonization. In particular, we found that male seminal vesicle fluid contributes to GBS clearance post-coitus and can target GBS through direct antimicrobial activity *in vitro*. We then further identified that the ovarian hormone, progesterone, modulates key immune populations in the vaginal tract and drives sustained GBS clearance from the vaginal tract.

Using seminal vesicle excised males, which lack SVF in their ejaculate, we were able to show that SVF helps eliminate GBS post-coitus, as SVX-mated females exhibited partially restored GBS vaginal carriage. Previous studies implicate seminal fluid-derived antimicrobial peptides and immunomodulatory compounds as functionally important for optimal pregnancy outcomes. Of the various seminal plasma components, seminal vesicle fluid constitutes ~60% of the total seminal plasma volume and is the primary source of its immunomodulatory and antimicrobial activity. Several AMPs exist in human seminal plasma and in mouse male reproductive tract secretions[134,135,140]. Proteins identified in mouse SVF have microbicidal activity against other reproductive pathogens like *E. coli*[140], and one study found that human seminal plasma inhibits GBS growth[135]. Thus, we hypothesized that SVF may exert its effects through direct antimicrobial activity against GBS. Indeed, our study is the first to demonstrate that mouse SVF directly inhibits GBS growth. Intriguingly, SVF's antimicrobial effects were exerted equally across GBS strain types, which could be due to the wide variety of antimicrobial components founds in SVF, endowing it with broad spectrum protection against invasive and colonization GBS isolates.

Because removing seminal vesicle alone only partially restored GBS, we sought to identify additional factors unique to pregnancy that drive clearance post-coitus. Consistent with previous findings, only 33% of SVX-mated females in our study were pregnant. We noted GBS carriage in SVX-mated cohort was different between pregnant and nonpregnant females, where GBS was less abundant in pregnant compared to nonpregnant females. Thus, we hypothesized that another factor unique to pregnancy, independent of SVF, was contributing to GBS reduction in the vaginal tract. To determine whether other pregnancy factors separate from seminal fluid exposure contribute to post-coital GBS vaginal clearance, we added two sets of males to our

repertoire: VAS males, which are infertile but produce SVF in the ejaculate, as well as SVX/VAS males, that are both infertile and lack SVF in the ejaculate. Our full repertoire of males allowed us to probe how SVF exposure and pregnancy status were independently impacting vaginal colonization post-coitus. This allowed us to determine whether other factors of pregnancy independent of seminal fluid exposure contributed GBS clearance. To our surprise, VAS-mated females prompted our most interesting line of inquiry, as their carriage appeared to mimic pseudopregnancy and serum progesterone levels. As such, we investigated whether progesterone alone dictated GBS vaginal carriage. Using osmotic pumps to deliver steady state progesterone to nonpregnant females we showed that progesterone alone significantly reduces GBS vaginal colonization.

Previous studies reveal that ovarian cyclicity impacts GBS persistence in the vaginal tract, where females infected during estrus carry GBS significantly longer than females infected at other estrous cycle stages[183–185]. These observations suggest that ovarian hormone levels likely dictate GBS colonization dynamics. Progesterone and estrogen have pleiotropic activity due to broad but highly regulated expression of their receptors across cell types and tissues, including the vaginal epithelium and most immune cell populations[103]. Within the cervicovaginal niche this becomes evident with estrous cycle staging: using vaginal cytology each stage can be characterized by distinct immune cell composition and abundance that correspond with estrogen and progesterone fluctuations. Estrogen-dominant estrus is identified by abundant anucleated keratinocytes with few to no leukocytes, while progesterone-dominant diestrus is characteristically abundant in leukocytes, especially neutrophils[187,188]. Because neutrophils are the predominant leukocyte found in cervicovaginal lavage and are hormonally regulated, we quantified neutorphils in progesterone and PBS treated females. Our results reflect hormone-

driven fluctuations in the vaginal immune cell populations throughout the estrous cycle, where exogenous progesterone treatment led to significantly increased neutrophil numbers compared to PBS controls. While we show that neutrophil influx correlates with GBS clearance, studies in nonpregnant female mice show that neutrophils accumulate in cervicovaginal lavage with continuous GBS carriage[68], but are unable to effectively clear GBS. Future studies should investigate whether progesterone-mediated GBS clearance is dependent on neutrophils using antibody-mediated depletion methods.

Beyond immunomodulation, it's important to note that ovarian hormones affect other facets of cervicovaginal physiology that could impact GBS colonization, including epithelial thickness, keratinization, and glucose abundance. During the luteal phase, serum progesterone induces apoptosis and inhibits vaginal epithelial proliferation leading to epithelial thinning and reduced keratinization[183–185], while increases in estrogen during estrus cause vaginal epithelial cell proliferation that leads to epithelial thickening and increased epithelial. GBS uses adhesins to bind keratins and colonize highly keratinized surfaces, like lung and vaginal epithelium[186,187]. Furthermore, highly keratinized vaginal epithelia store large amounts of glycogen that is metabolized by *Lactobacillus spp.* into lactic acid. Similar to *Lactobacillus spp.,* GBS ferments glycogen to lactic acid in low oxygen environments[186], like the vaginal tract, to enhance its persistence. Therefore, high progesterone levels observed in our model may decrease available keratin and glycogen stores to further restrict GBS.

Future investigation of alternative hormonal contexts should also be considered. Our study demonstrates that progesterone alone leads to elimination of GBS from the vaginal tract, however, estrogen is also present at high concentrations in the third trimester (~5-fold increase from ovulation)[187] and likely impacts cervicovaginal epithelial structure, immunity and microbial

composition. Indeed, decreases in microbial alpha diversity during pregnancy, indicative of vaginal microbiome stability, is attributed to increases in estrogen[188,189] and estrogen inhibits neutrophil trafficking into the cervicovaginal space. Moreover, models of ascending GBS infection clearly demonstrate that mice are susceptible to GBS infection in the third trimester, when progesterone and estrogen levels are at their highest. Future studies should evaluate how progesterone and estrogen together impact GBS colonization dynamics, a hormonal context that better reflects that of pregnancy. Furthermore, our model can be used as a comparator to ascending infection models to identify first and third trimester differences that determine susceptibility to GBS infection.

We conclude that progesterone and SVF are key modulators of GBS colonization during pregnancy in mice. Future studies should investigate how our findings translate to risk for GBS disease in humans. Observational studies of college-aged nonpregnant women indicate sexual activity is positively associated with GBS carriage, however, no pregnancy studies of GBS infection have documented whether seminal fluid exposure during pregnancy impacts GBS carriage and risk for invasive infection. Furthermore, it is not clear how progesterone activity affects GBS carriage in human pregnancy or risk of GBS-associated adverse outcomes. For instance, as at least 40% of preterm births are associated with intrauterine infection[190,191]. Our results suggest that progesterone can directly impact susceptibility to GBS infection. It is not clear whether progesterone activity plays any role in an individual's risk for infection during pregnancy or infection-associated preterm birth.

FIGURES

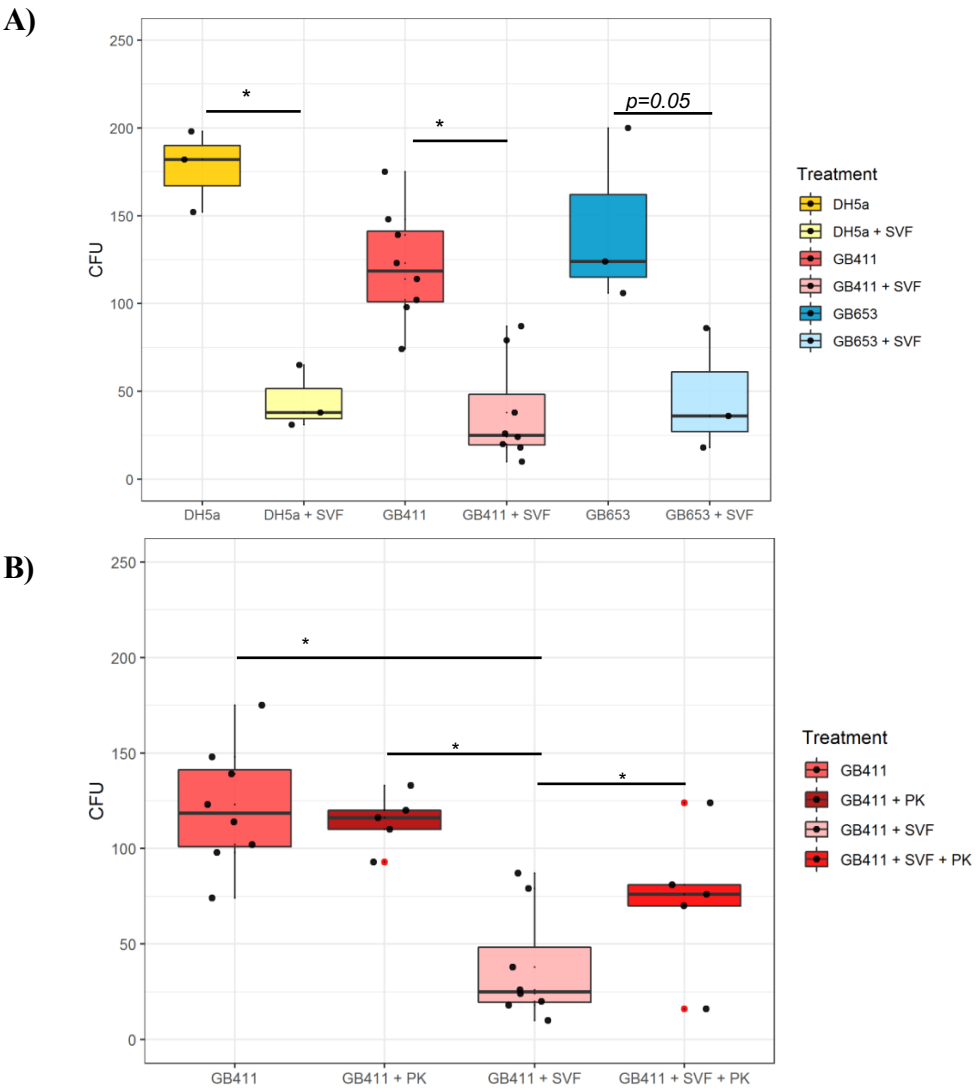

Figure 3.1: GBS incubated with seminal vesicle fluid

A) DH5a *E.coli,* GB411, or GB653 grown on agar with PBS or freshly harvested seminal vesicle fluid (SVF). **B)** GB411 grown on agar with PBS, SVF, or SVF + proteinase k (PK). The median CFU is represented in each box. The black dots represent biological replicates. Red dots indicate outliers. Statistical analysis was performed using one-way ANOVA test with Tukey HSD. Comparisons with p-values < 0.05 are indicated with an asterisk.

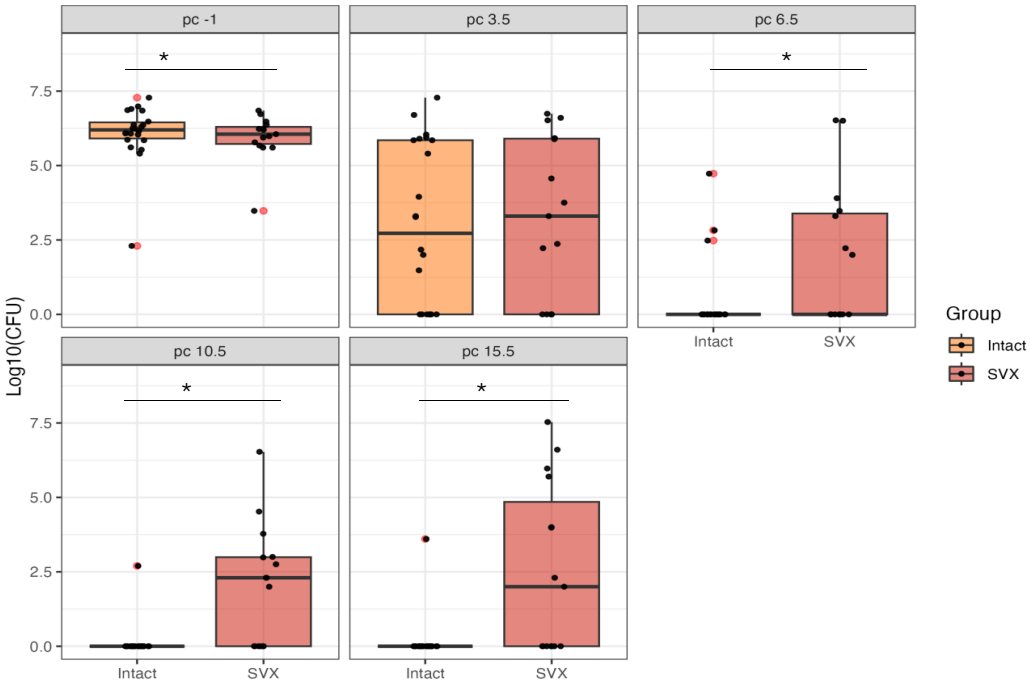

Figure 3.2: Impact of seminal vesicle fluid on GBS vaginal carriage post-coitus

Females were vaginally infected with GB411 and at 6 days post-infection were paired with intact or seminal vesicle excised males. Vaginal GBS CFUs of SVX-mated (SVX, N=14) and intact-mated (N=22) females were quantified immediately prior to pairing (pc -1) and at 3.5, 6.5. 10.5, and 15.5 days post-coitus (pc). The median CFU is represented in each box. The black dots represent individual vaginal swabs. Red dots indicate outliers. Statistical comparisons were performed using Mann-Whitney U test. Comparisons with p-values < 0.05 are indicated with an asterisk.

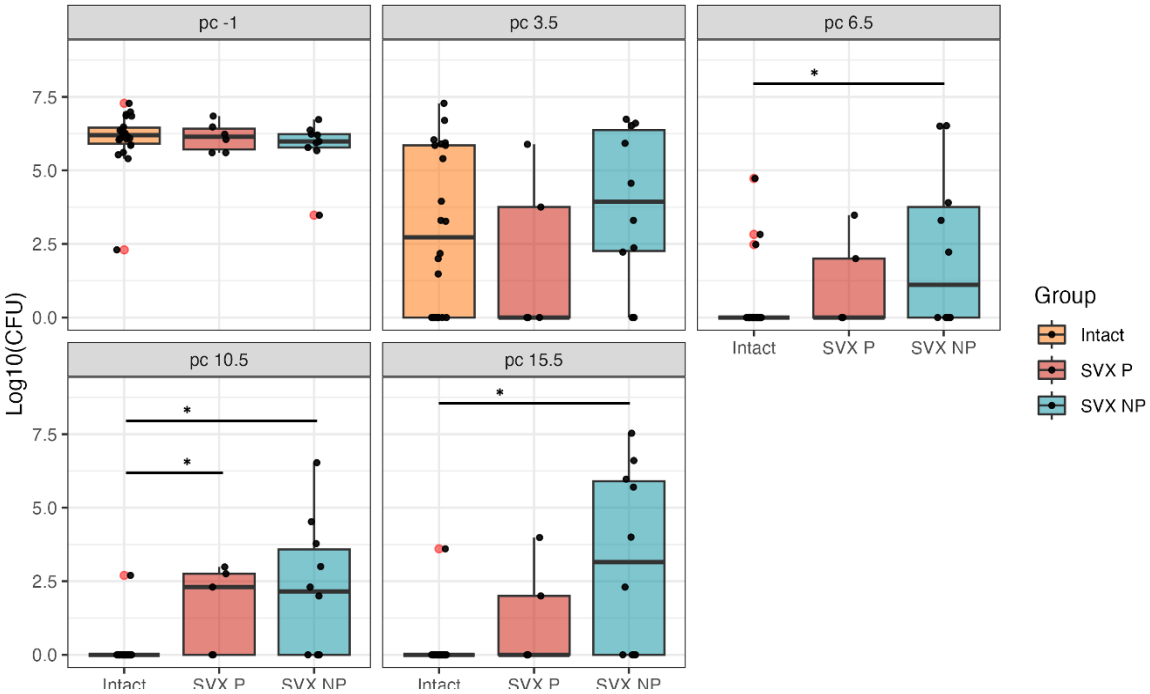

Figure 3.3: GBS vaginal colonization in intact-, pregnant SVX- and non-pregnant SVX-mated females

Females were vaginally infected with GB411 and paired with intact or seminal vesicle excised males at 6 days post-infection, where the SVX-mated cohort data is stratified by pregnancy status. Vaginal GBS CFU of pregnant SVX-mated (SVX P, N=5), non-pregnant SVX-mated (SVX NP, N=9), and intact-mated (N=22) females at indicated times post-coitus are shown. The median CFU is represented in each box. The black dots represent individual vaginal swabs. Red dots indicate outliers. Statistical comparisons were performed using Kruskall-Wallis with post-hoc Dunn's test for multiple comparisons. Comparisons with p-values < 0.05 are indicated with an asterisk.

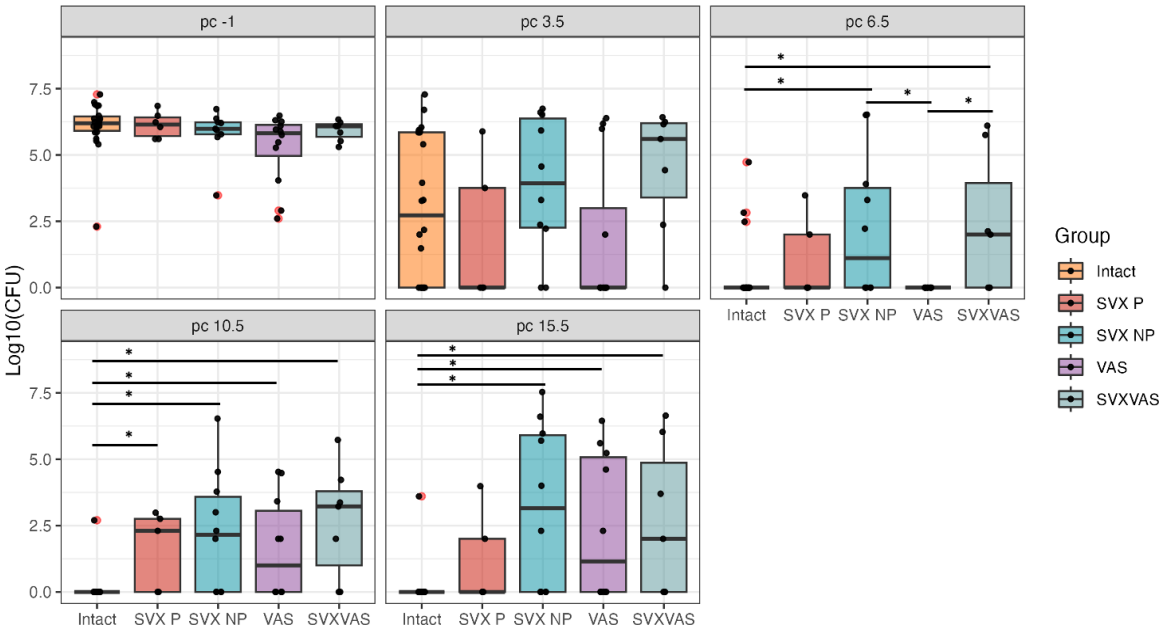

Figure 3.4: GBS vaginal colonization in intact-, SVX-, VAS-, and SVX/VAS-mated females

Quantification of GBS CFUs in the vaginal tract of intact-mated (N=22), pregnant SVX-mated (N=5), nonpregnant SVX-mated (N=9), VAS-mated (N=10), and SVXVAS-mated (N=7) females immediately prior to pairing (pc -1) and at 3.5, 6.5, 10.5, and 15.5 days post-copulation (pc). The median CFU is represented in each box. The black dots represent individual vaginal swabs. Red dots indicate outliers. Statistical comparisons were performed using Friedman test with Nemenyi post- hoc test for multiple comparisons. Comparisons with p-values < 0.05 are indicated with an asterisk.

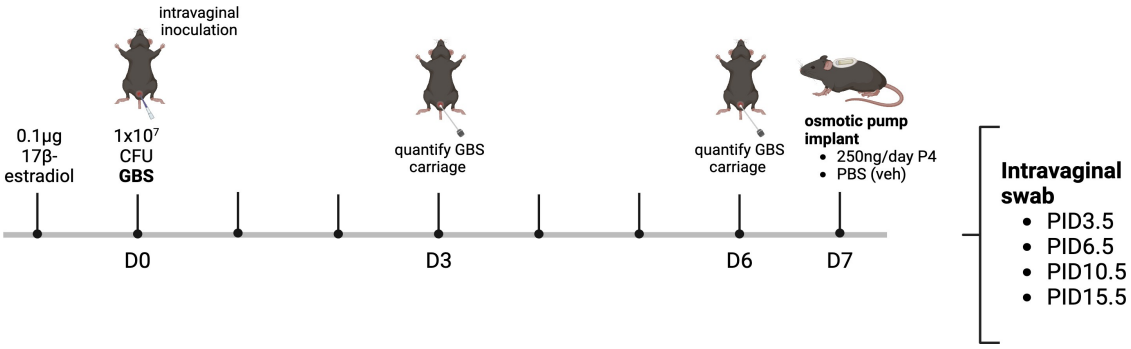

Figure 3.5: Experimental timeline for assessing the impact of progesterone treatment on GBS vaginal colonization

Females were intravaginally infected with $1x10^7$ CFU of GB411 one day after subcutaneous estrogen treatment. On day 3 and 6 post-infection intravaginal GBS was quantified via swab to ensure females were colonized prior to receiving surgical implants. Progesterone (P4)- or PBS-filled osmotic pumps were surgically implanted on day 7 post-infection. GBS vaginal load was quantified by intravaginal swab on post-implant day (PID) 3.5, 6.5, 10.5, and 15.5.

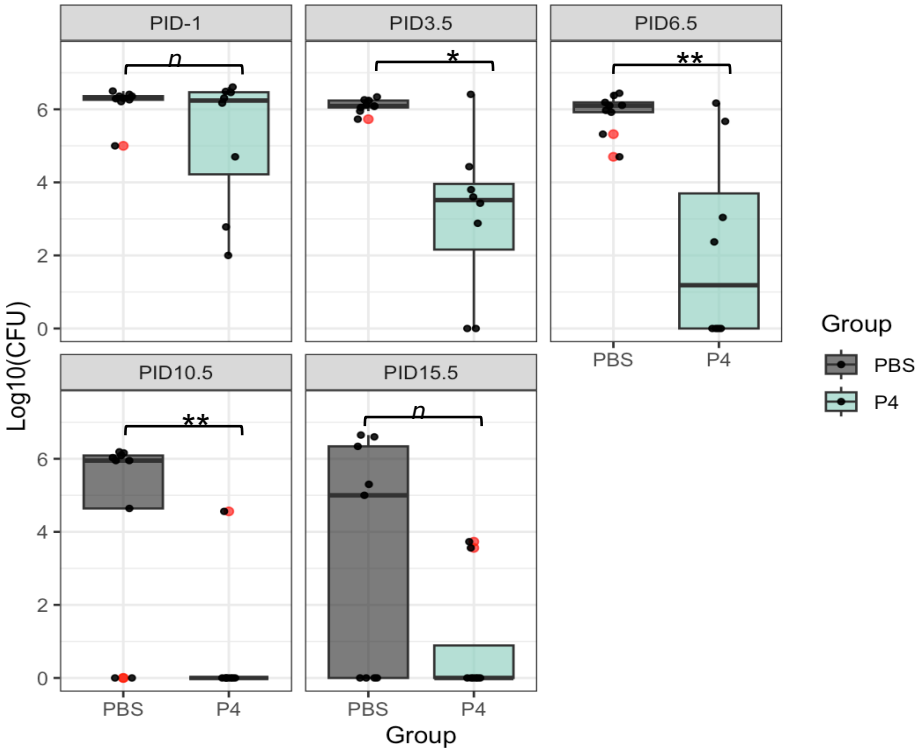

Figure 3.6: GBS Vaginal Colonization in Progesterone- and PBS-treated females

GB411-colonized females were administered 250ug/day of exogenous progesterone (P4) or PBS (N=8, each group) via surgically implanted Alzet osmotic pumps. Intravaginal GBS was quantified via swab at 3.5, 6.5, 10.5 and 15.5 days post-implant (PID). The median CFU is represented in each box. The black dots represent individual vaginal swabs. Red dots indicate outliers. Statistical comparisons were performed using Mann-Whitney U test. Comparisons with p-values < 0.05 are indicated with an asterisk.

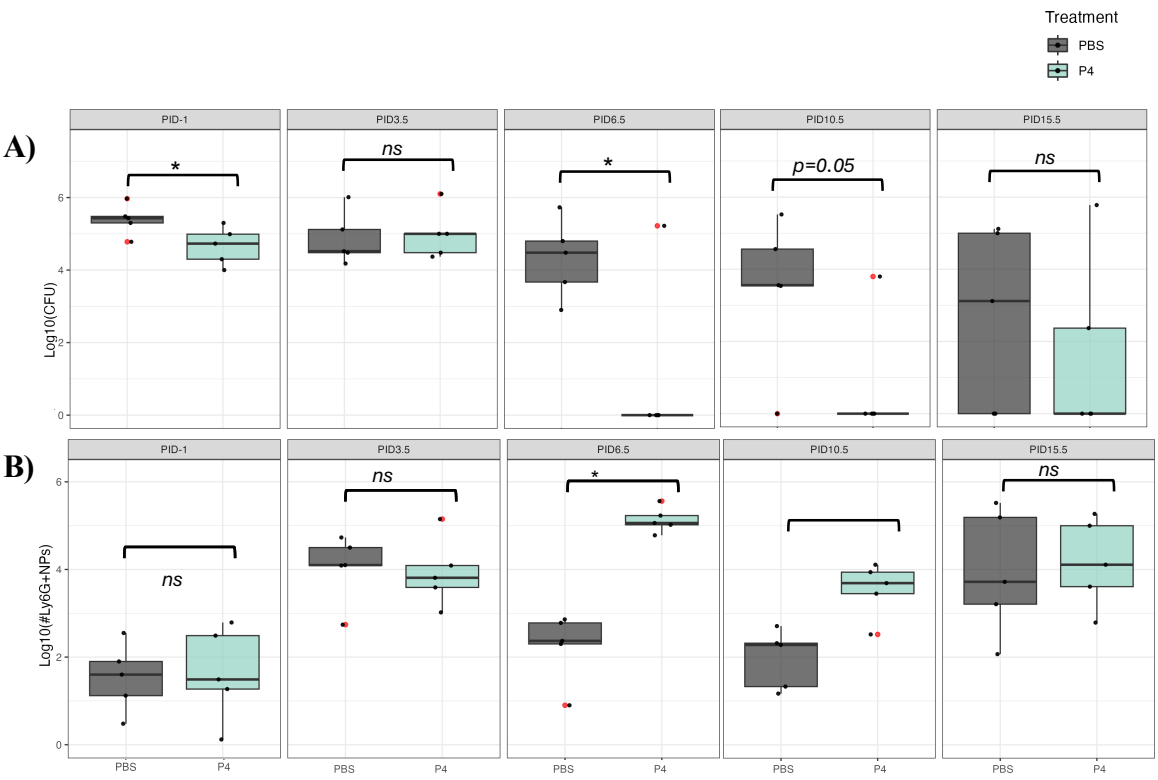

Figure 3.7: Quantification of vaginal GBS and neutrophils in progesterone- and PBS-treated females

GB411-colonized females were administered 250ug/day of exogenous progesterone (P4) or PBS (N=5, each group) via surgically implanted Alzet osmotic pumps. **A)** Intravaginal GBS was quantified via swab at 3.5, 6.5, 10.5 and 15.5 days post-implant (PID). **B)** Neutrophils were quantified in vaginal swabs by flow cytometry. The median CFU or number of neutrophils is represented in each box. The black dots represent biological replicates. Red dots indicate outliers. Statistical comparisons were performed using Mann-Whitney U. Comparisons with p-values < 0.05 are indicated with an asterisk.

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CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

GBS infections remain a leading cause of neonatal mortality and a significant global health burden. Vaginal colonization is the primary risk factor for developing invasive disease outcomes, yet only a small percentage (~1%) of vaginally colonized women will develop GBS invasive disease pregnancy complications. While intrapartum antibiotic prophylaxis (IAP) has effectively reduced early-onset neonatal infections, IAP does not prevent preterm birth nor does it reduce late-onset neonatal infections that typically manifest as meningitis[1]. Thus, there is a crucial need to improve patient screening with additional risk factors to better identify women at greatest risk of developing invasive GBS infection, and as well as alternative therapeutic approaches to GBS disease treatment. The focus of this dissertation was to identify host factors uniquely present in pregnancy that impact GBS vaginal colonization. Previous work identified specific immune populations and soluble inflammatory mediators as important for eliminating GBS from the vaginal tract[2,3]. However, these studies were performed in nonpregnant mice, and how the pregnant niche impacts vaginal immunity to GBS not well understood. The studies discussed in this dissertation sought to address this gap in knowledge and identify the factors that influence vaginal immunity and GBS colonization post-coitus.

In the first study, we sought to develop a mouse model that would allow us to evaluate GBS colonization dynamics in the vaginal tract across pregnancy. Previous studies identified methods to induce long-term vaginal GBS carriage in nonpregnant mice. We adapted this model this model by adding a pregnant phase, where long-term colonized females were paired with males 7 days post-infection. Interestingly, pregnant females lost vaginal GBS colonization early in pregnancy. This suggested that factors unique to early pregnancy were causing loss of GBS from the vaginal tract. Despite early loss of detectable vaginal GBS, 45% of GB411-infected females were positive for GBS post-partum, while no GB653-infected females were positive in

the vaginal tract. This is consistent with findings in humans that show serotype III, ST-17/ST-19 GBS strains are more likely to persist after delivery than other GBS strains4. A detailed genomic and transcriptomic analysis of GBS colonies isolated across pregnancy and post-partum could yield more information about how GBS senses and adapts to pregnant and nonpregnant environments in our model.

The goal of the second study was to further investigate observations from our first study and identify factors that could be driving GBS clearance from the vaginal tract in early pregnancy. We specifically asked whether exposure to seminal fluid components or changes to the endocrine environment could impact GBS carriage, since both occur in early pregnancy.

Removal of seminal vesicle fluid from the male ejaculate significantly increased the amount of GBS found in the vaginal tract post-coitus, and ex vivo, seminal vesicle fluid significantly inhibited GBS growth on Chromagar. Taken together, these experiments demonstrated that seminal vesicle fluid can target GBS and partially contributes to GBS clearance from the vaginal tract observed in early pregnancy. While removal of seminal vesicle fluid from male ejaculate partially restored GBS vaginal colonization post-coitus, we did not test whether exogenous intravaginal treatment with seminal vesicle fluid could target GBS. Future studies should investigate whether treatment with freshly harvested seminal vesicle fluid can reduce GBS vaginal colonization as this has implications for novel therapeutic development.

A low percentage of SVX-mated females were pregnant (~33%), and when we stratified the data by pregnancy status, it was clear that nonpregnant females carried a greater amount of GBS in the vaginal tract compared to pregnant females post-coitus, suggesting that other factors in the pregnant cervicovaginal niche affect GBS colonization. Given that the steroid hormone, progesterone, increases immediately after mating and continues to increase until parturition, we

hypothesized that progesterone could decrease vaginal GBS carriage. Indeed, our study is the first to show that exogenous progesterone significantly decreases the amount of GBS in the vaginal tract and this reduction GBS density corresponds with increases in neutrophil numbers. These results demonstrate that progesterone has a suppressive effect on GBS in the vaginal tract and may orchestrate GBS clearance via increased neutrophil trafficking to the vaginal lumen.

Previous studies show that progesterone-mediated clearance of other pathogens from the vaginal tract is dependent on neutrophils5. Using antibody-mediated depletion methods, future studies should investigate a direct role for neutrophils or other immune cell populations in progesterone-driven GBS clearance. Interestingly, in nonpregnant mice, neutrophils accumulate in the vaginal tract with long-term GBS colonization, indicating that neutrophils traffic to the vaginal lumen but are unable to eliminate GBS once they arrive[8]. Given that progesterone reduces GBS bacterial density and is associated with neutrophil accumulation in our model, it would be worthwhile to explore how distinct hormonal environments (i.e. pregnant versus nonpregnant) impact vaginal immune cell effector responses to GBS infection.

Progesterone's suppressive effect on vaginal GBS colonization might explain findings from our first study, where GBS rebounded in a percentage of females post-partum. Evidence from women suggests this occurs with intrapartum antibiotic prophylactic (IAP) treatment, where IAP only temporarily eliminates GBS and rebounds in a fraction of women within days after delivery[4,6]. Performing a genetic and transcriptomic analysis of GBS colonies isolated before, during, and after progesterone treatment could render more information about how GBS adapts to this host stressor. Moreover, we only performed progesterone treatment in GB411-infected females. Future studies should compare other clinically distinct GBS patient isolates to determine any strain-dependent differences in adaptation to progesterone.

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