THE IMPACT OF *LACTOBACILLUS* AND BACTERIOPHAGE ON GROUP B *STREPTOCOCCUS* AND THE PLACENTAL MEMBRANES

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ABSTRACT

THE IMPACT OF *LACTOBACILLUS* AND BACTERIOPHAGE ON GROUP B *STREPTOCOCCUS* AND THE PLACENTAL MEMBRANES

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The human microbiota encompasses the microbes that live on and in the human body. While some body sites have been studied extensively for their role in human health, other body sites including the extraplacental membranes (EPM) have been historically considered sterile and are less studied. The EPM surrounds the fetus during pregnancy and serves as an important protective barrier during pregnancy. Several studies have established which bacteria are found in this site, but few studies have been conducted to characterize their impact on the EPM *in vitro* or their potential to impact pathogens that invade the EPM during pregnancy. Further, our knowledge of the viral component of the microbiome in human health remains incomplete.

In this dissertation, *Lactobacillus*, a well-studied probiotic in other body sites, was evaluated for its effect in the placental membranes and the opportunistic pathogen Group B *Streptococcus* (GBS). We confirmed the ability of four *Lactobacillus* strains representing three species to colonize a cell line model of the outermost layer of these membranes, the decidual cells. Further, *Lactobacillus* dampens a known cell signaling pathway, the Mitogen Activated Protein Kinase (MAPK) pathway, which is associated with inflammation and host cell death by reducing the total protein level of p38. Collectively, these data suggest that *Lactobacillus* could maintain a commensal interaction in the placental membranes as described in other body sites. The ability of the same *Lactobacillus* strains to impact two GBS strains was also examined as GBS can ascend from the vaginal tract to infect placental membranes, triggering premature birth

or neonatal infection. We found live *Lactobacillus* does not affect GBS growth or biofilm production. *L. gasseri* increased association of both strains of GBS to the decidual cells but did not result in increased invasion of the cells. Instead, co-culture with *Lactobacillus* reduced host cell death. Secreted products of *Lactobacillus* drastically reduced growth in 35 GBS strains that broadly represent GBS diversity and could prevent biofilm formation; this inhibition was strain dependent. Unfortunately, increased GBS-induced host cell death with *Lactobacillus* supernatants was also observed. Collectively, these data suggest that both live *Lactobacillus* and its supernatant could impact GBS interactions with the placental membranes.

Bacteriophage are one of the most abundant members of the microbiome but their impact on opportunistic pathogens such as GBS remains unknown. As GBS can be isolated from gastrointestinal tract, we hypothesized fecal phage communities (PC) would inhibit the growth of GBS *in vitro*. Approximately 6% of the tested communities inhibited the growth of GBS. To examine GBS host range, we examined capsule, sequence and clinical types of 35 strains with three inhibitory PCs. As no significant differences were found with these traits, we examined Clustered Regularly Interspaced Palindromic Repeats (CRISPR), which serve as an adaptive immune system against invading foreign DNA by the acquisition of spacer sequences. GBS strains with fewer than nine spacers were less likely to be lysed by a phage community than strains with more than sixteen spacers. Further, sensitive strains of GBS were significantly more likely to be inhibited by PCs with a lower abundance of GBS in the corresponding bacterial component of each PC. Collectively, these data suggest that the phage component of the intestinal microbiome could impact GBS colonization. The work described in this dissertation helps elucidate the impact of *Lactobacillus* and bacteriophage as members of the microbiota on GBS and the placental membranes and their impact on adverse pregnancy outcomes.

For Maui and my boo.

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KEY TO ABBREVIATIONS

A595	Absorbance 595nm
ANOVA	Analysis of variance
BCA	Bicinchroninic acid
BHI	Brain heart infusion
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
cAMP	cyclic AMP
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CI	Confidence interval
CPS	Capsular polysaccharide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CV	Crystal violet
dT-HESCs	decidualized human endometrial stromal cells
EPM	Extraplacental membrane
GBS	Group B Streptococcus
HESCs	Human endometrial stromal cells
HMP	Human microbiome project
IAP	Intrapartum antibiotic prophylaxis

IBD	Irritable bowel disease
IgA	Immunoglobulin
Lc	Lactobacillus cirspatus
Lg	Lactobacillus gasseri
LPS	Lipopolysaccharide
Lr	Lactobacillus reuteri
mTHB	Modified Todd Hewitt broth
MAPK	Mitogen activated protein kinase
MLST	Multilocus sequence types
MOI	Multiplicity of infection
MRS	deMan, Rosoa and Sharpe
NCBI	National Center for Biotechnology Information
OD	Optical density
PBS	Phosphate buffered saline
PC	Phage community
PEG	Polyetheleneglycol
PTB	Preterm birth
PVDF	Polyvinyldene difluoride
RVC	RVC
ST	Sequence type
STI	Sexually transmitted infection
TBS	Tris-buffered saline
TSA	Tryptic soy agar

TSB	Tryptic soy broth
TSBd	Tryptic soy broth with 1% dextrose
THA	Todd Hewitt agar
UTI	Urinary tract infection

CHAPTER 1

LITERATURE REVIEW: THE HUMAN MICROBIOME AND ITS EFFECTS ON HEALTH AND GROUP B *STREPTOCOCCUS*

The microbiota, or the microorganisms that live on and in the body, have been shown to play large roles in human health. These organisms contribute fundamentally to health by aiding in the extraction of nutrients from food, education of the immune system and as a barrier to invading pathogens. Studies including the Human Microbiome Project have begun to establish the members of the microbiota and have even pushed into examining the role the microbiome may play in complex disease states. Despite these advances in our understanding, some components of the microbiome have been less examined. First, the colonization of certain body sites, including the upper reproductive tract, remains controversial, and our understanding of the impact of individual organisms within the microbiota on health in these body sites remains incomplete. Specifically, the impact of the microbiota on opportunistic pathogens of the reproductive tract, including Group B *Streptococcus*, remains unknown. Finally, viruses are a generally under-appreciated residents of the human body that play large roles in the ecology of the microbiota but remain understudied in the reproductive tract. Herein, current knowledge on these topics will be reviewed and gaps in current understanding will be assessed.

The Human Microbiome and Its Effects on Human Health

Microorganisms, including bacteria, eukaryotes, archaea and viruses, live on and in the human body and are collectively called the microbiota. The genetic component of the microbiota is called the microbiome. Collectively, these organisms have a large impact on human health. While much work was initially done in culture-based methods, it was found that 80-95% of the microbiota were unculturable,[1] leading to increased interest in culture independent methods, such as metagenomic sequencing. Both approaches have contributed to our understanding of the impacts of the microbiome in different ways. Metagenomic sequencing has given a better

understanding of which organisms are present, but it is limited because the presence of DNA does not always denote that the organism is living or active in the community. While this limitation is being actively combated by the use of other non-culture based methods, including proteomics and transcriptomics, culturing bacteria from a given body site provides insight into individual contributions of bacteria through *in vitro* assays and therefore the mechanisms behind the correlations observed in non-culture based methods. Continued efforts to culture complex communities through techniques including cultureomics, or the application of massive parallel culturing methods, will continue to increase our understanding of how the microbial communities that live on and in us impact our health.

The bacterial component

The Human Microbiome Project (HMP) has pushed our understanding of the bacterial component of the microbiome, identifying the unculturable component through the use of metagenomics. This project found that body sites contain distinct microbiomes, or genetic signatures of the microbiota, but that the potential function of that signature was still maintained between sites.[2] Other work has found that stability of the microbiome is also determined by the site.[2]For example, the gut microbiome is stable over time, while the vaginal microbiome changes with menstrual cycle and pregnancy. Our microbiomes are acquired early in life and differ with age, geographical location, ethnicity, and health.[3-7] To date, the microbiome has been associated with a wide variety of health topics including but not limited to obesity, cancer, vitamin production, pathogen invasion and education of the immune system and complex disease states including Irritable Bowel Disease (IBD), diabetes and preterm birth (PTB).[8–11] In the first phase of HMP, the study largely focused on the bacterial component of the microbiome as 16S rRNA

sequencing technology was used to assess the composition of the bacterial community at different sites, but shotgun metagenomics was also used to determine the microbial pathways that were present in each site.[12]

Other research, including the second phase of the HMP (iHMP or HMP2), widened the scope of the research to include gene expression and protein profiles of the microbiome and host-specific properties such as genetic, epigenomics, and antibody and cytokine profiles, which collectively allow for better understanding of how the microbiome is affecting the host.[13] Another often overlooked portion of the microbiome – the viral community – was also included in iHMP.

The viral component

The viral community is composed of both eukaryotic viruses that can themselves directly impact the host and bacteriophage that can impact the host through the bacterial component of the microbiome. The genetic signature of viruses is referred to as the virome. Similar to the bacterial communities, the viral community is seeded at an early and develops with over time.[14] Additionally, the viral component also appears to be vertically transferred from mother to infant as the mode of birth impacts the composition of the fecal virome at one year of life.[15] In contrast to the bacterial microbiome, the viral community is highly individualized.[16] While research has found that the viral community can directly impact the immune system,[17] many studies focus on the impact of bacteriophage – viruses that infect bacteria – on the bacterial component of the microbiota.

Bacteriophage (phage) fall into two broad categories: lytic phage and lysogenic (temperate) phage. Lytic phages infect a bacterial host, replicate and obligately lyse the bacteria,

releasing viral particles and killing the host. Lysogenic phage can follow two lifestyles. First, a lysogenic phage will infect a host and integrate into the host genome. Its genome will replicate with the host until a trigger – typically a stress that causes DNA damage such as reactive oxygen species, antibiotics or ultraviolet light – causes the phage to enter a lytic life cycle, releasing viral progeny back into the environment. As bacteria are the natural host of bacteriophage, it is easy to see how the composition of the bacteriophage could affect the composition of the bacteria of the microbiota. Indeed, metagenomic research has found inverse correlations between bacteriophage and bacteria in the microbiome.[18–20] For example, in IBD patients, increases in *Caudovirales* phage inversely correlated to its host *Bacteroidaceae* bacterial families in the disease state.

The stability of bacteriophage in the human body is thought to be the result of ecological dynamics, in which the resident bacteriophages use resident bacteria to maintain numbers. This has been partially determined in gnotobiotic, or sterile, mouse models that have examined the bacteriophage-host dynamics in the gut.[21] This stability is also maintained by the ability of phage to attach to mucosal surfaces.[22] Barr *et al.* found that bacteriophage accumulate in the mucosa 10x more than bacteria.[22] This research also found that these bacteriophages serve as a protective barrier to pathogen invasion. Most of the research done on the human virome has been conducted in the gut using fecal samples; therefore, more research is needed to fully examine if these dynamics apply to other body sites including the reproductive tract.

The Microbiota of the Reproductive Tract

The bacterial microbiota of the lower reproductive tract and its impact on human health

The lower reproductive tract, or the vaginal tract, is the best studied region of the female urogenital tract. It maintains a high abundance, low diversity microbiome and is considered one

of the simplest in the human body.[23] Traditionally, *Lactobacillus* has been considered the dominant member of the vaginal microbiome;[23] however, variation has been observed across races as non-white women were shown to have lower levels of *Lactobacillus*.[24] Despite differences between races, the presence of *Lactobacillus* is considered a marker of health, while a reduction in *Lactobacillus* abundance is a marker of dysbiosis, or a perturbed state in the microbiome, which is often linked to bacterial vaginosis.[25,26] This dysbiosis is correlated to an increased risk of a variety of infections including sexually transmitted infections (STI),[27] urinary tract infections[2829] and yeast infections.[29] This is in part due to *Lactobacillus*'s contribution to the acidification of the vaginal tract.

A high abundance of *Lactobacillus* is also correlated to better pregnancy outcomes. Women with *Lactobacillus*-dominated vaginal microbiomes are less likely to deliver prematurely.[30–33] Indeed, vaginal dysbiosis, which is characterized by depletion of *Lactobacillus* spp., was significantly increased prior to premature rupture of membranes.[33] While certain species including, Group B *Streptococcus* and *Sneathia* spp., have been found to be increased, a general increase in diversity has mostly been observed.[24,33] The importance of the vaginal microbiome extends beyond the mother's health and pregnancy outcomes. The infant is seeded with the vaginal microbiome during vaginal birth, and this transfer has been shown to have short-term and long-term effects on outcomes in the child.[34–3738] Indeed, the use of antibiotics during birth to prevent vertical transfer of pathogens such as Group B *Streptococcus*, and disruption of this vertical transfer by cesarean delivery can disrupt the gut microbiome of the infant for an extended period of time.

The microbiota of the upper reproductive tract and its impacts

While the microbiota of the vaginal tract has been well studied, the upper reproductive tract, consisting of the uterus, fallopian tubes, ovaries, and placenta, is traditionally considered sterile. This sterility was thought to be maintained by the cervix, especially during pregnancy. Cervical cells not only secrete a thick mucus that physically restricts access to the upper reproductive tract, but they also secrete other products including defensins, lysozyme and nitric oxide.[39] In spite of this barrier, research has demonstrated that bacteria are still capable of passing through the cervix to the uterus.[40] Studies establishing the microbiota are limited in part because of the increased difficulty of sampling these areas compared to other body sites. However, current studies have reported that the microbiota is distinct from the vaginal tract and contains higher diversity than the *Lactobacillus*-dominated vaginal tract[41] and a lower total abundance of bacterial DNA.[42] Reported genera include *Acinetobacter*, *Escherichia*, *Enterobacter* and *Lactobacillus*. These studies remain controversial for several reasons. First, sampling of healthy women is mostly done by sampling through the vaginal tract, which may contaminate the sample with the vaginal microbiota. While some studies have examined women undergoing surgeries, these studies are severely limited and do not give an accurate picture of a "healthy" microbiome since the women have various health conditions requiring surgical intervention.[41,43] Another common argument against studies that claim a low abundance microbiome is the existence of a "kit-ome," or the DNA present in reagents used in metagenomic processing.[44] In respect of these considerations, studies in these sites have provided negative controls[45] or assessed the sampling method itself.[41]

Recent papers on the microbiota of the upper reproductive tract have been reviewed by Peric *et al*.; therefore, this chapter will focus on publications involving the placenta and placental membranes.[39] The placental microbiome was initially described by Aagaard *et al*. in 2014.[46] Containing Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria and Tenericutes phyla, it was found to more closely resemble the oral microbiome than that of the vagina.[46] While *Lactobacillus* was identified, it was at a lower abundance compared to other organisms, which is in direct contrast to what has been observed in the vaginal tract.[47] Since this initial publication, some metagenomic studies have also reported bacteria in the placental membranes,[48–50] while others assert that no microbial sequences were present after comparison to the kit-ome.[51–53]With this disagreement, it is important to note that observational and culture-based studies have claimed the presence of bacteria in the placenta and its membranes without visible damage to the tissue as early as 1982.[54] Indeed, this study cultured vaginally associated bacteria including *Lactobacillus* from the placenta after cesarean delivery, which counters the argument of contamination from the vaginal tract. Confirmation of a placental microbiome would greatly benefit from the use of additional techniques beyond sequencing. Seferovic *et al.* utilized *in situ* hybridization with 16SrRNA probes to support the initial report of a low load of bacterial colonization without signs of tissue damage.[55] This report has the benefit of visual evidence of a live bacterial component and contributes to structural knowledge of the microbiota.

In addition to the assertion that a microbiome exists in the placental tissues, some studies have also found variation in the placental microbiome composition based on traits of the mother such as maternal obesity and gestational diabetes, the health of the pregnancy and neonatal outcomes.[56–59] For example, *Acinetobacter*, *Escherichia coli*, *Enterobacter* and *Lactobacillus*

were increased in healthy, term pregnancies in comparison to preterm births with chorioamnionitis, or inflammation of the placental membranes.[50] Higher abundance of *Lactobacillus spp., Propionibacterium spp.* and members of the *Enterobacteriaceae* family in healthy states were also confirmed in other studies.[30,60,61] The weight of full-term infants also varied depending on the composition of the placental microbiome, with normal birth weight being associated with the presence of *Lactobacillus.*[59]

Group B *Streptococcus* is an important opportunistic pathogen in the reproductive tract

The microbiota can also be a source of infection. One in every three preterm infants can be associated with intra-amniotic infection.[62] The bacteria isolated from placental membranes are in many cases those that normally maintain an asymptomatic colonization of the vaginal tract.[63,64] Such organisms that cause disease in only specific circumstances are called opportunistic pathogens. One such organism capable of ascending from the vaginal tract is Group B *Streptococcus.*

Group B *Streptococcus* presents a global human health burden

Group B S*treptococcus* (*Streptococcus agalactiae*, GBS) is an asymptomatic colonizer of the rectal vaginal tract of 18% of women globally, with regional variation ranging from 11-35%.[65] It also contributes to maternal infections, stillbirth, preterm birth and neonatal infections. In the United States, GBS is a leading cause of neonatal sepsis. GBS infections are lethal for 4-6% of neonates and can result in other long-lasting effects, including deafness and developmental disabilities.[66] GBS caused a total of 31,850 cases of invasive disease in 2017 in the United Stated, resulting in 2,030 deaths.[67] GBS neonatal infections can be divided into two types based on time of onset. Late onset disease occurs between one week of life and three

months, and the route of infection remains unknown. Early onset disease occurs within the first week of life and is thought to occur when GBS is aspirated by the infant while passing through the vaginal tract or by ascending infection and invasion of the extraplacental membranes (EPM). Ascending infection is also thought to contribute to GBS's ability to trigger premature birth and stillbirth. GBS can ascend from a commensal state in the vaginal tract, through the cervix and associate with the EPM.

Group B *Streptococcus* strain variation and disease outcome

GBS diversity can be examined in multiple ways and examining this diversity can reveal correlations between GBS types and disease outcome. First, GBS can be grouped phenotypically according to serotype based on the salicylic acid capsular polysaccharide (CPS).[68–70] This capsule has also been found to be important in immune evasion, with serotype III being most commonly associated with disease.[71,72] As CPS is not closely linked to phylogenetic lineage, capsule typing cannot be used to determine the relatedness of strains.

A genotypic method, called multilocus sequence typing (MLST), examines sequence variation in seven conserved genes to group strains into different sequence types (STs).[71] MLST is useful for examining genetic relatedness and evolutionary relationships among strains from different sources and serotypes. In GBS, MLST uses variation within seven conserved genes to group strains. Epidemiological studies have found that ST-17 strains are highly associated with neonatal disease;[68,69,73] however, there is even strain variation within these groupings in phenotypes that may impact virulence. For example, our research group has found that ST-17 strains vary in: ability to attach to several human cell types, mechanisms of phagosomal stress survival, biofilm production, provocation of cytokine responses in macrophages, and induction

of host responses from decidual cells.[74–79] We and others have also shown that ST-17 strains also have unique genetic characteristics that may impact pathogenesis, and that ST-17 strains are more likely to persist in pregnant women following antibiotic prophylaxis during childbirth.[74,75,79–81] Enhanced persistence of ST-17 GBS following pregnancy likely contributes to the high incidence of late onset disease in infants; little is known, however, about other host factors that contribute to persistence.

GBS strains can also be grouped according to where they were isolated from. In this way, a strain can be noted as invasive (isolated from an active infection) or colonizing (isolated from a mother who never had disease symptoms). Similarly, a strain's resistance to antibiotics given during pregnancy can also be noted by grouping strains that were lost after antibiotic treatment together versus those that persisted. The CDC recently reported that while GBS remains sensitive to penicillin, the most commonly used antibiotic used for preventative measures, it has developed resistance to clindamycin and erythromycin. Very recently, rare instances of vancomycin resistance have also been detected.[82]

Group B *Streptococcus* has multiple routes of infection

GBS infections begin with the ability to asymptomatically colonize the vaginal tract. The first step of this colonization is attachment. GBS cell surface proteins, ScpB, FbsA and laminin binding protein (Lmb), can bind to host extracellular matrix proteins fibronectin, fibrinogen and laminin, respectively.[83–86] Distinct pili structures and serine-rich repeat proteins (*srr*-1/2) also interact with the epithelial surface of different cell types in the vagina, decidual layer of the placental membranes, and lung tissue.[87–90] After establishing colonization, GBS must persist using multiple methods to avoid immunological clearing. First, host mimicry allows GBS to hide

from the immune system. The capsule (*cps*) of GBS is coated in sialic acid, which mimics the residues found on host cells.[91,92] Another evasion and mimicry mechanism is the use of CspA to break down fibrinogen to fibrin, which coats and "hides" the cell.[93] Secondly, GBS can also actively dampen the immune response using a serine protease (ScpB) that cleaves C5a, which is an important factor in the complement cascade of the immune system for recruitment of neutrophils.[94–96] GBS can also avoid phagocytic uptake through a variety of mechanisms including the capsule, β-protein, Complement Interfering Protein (CIP) and BibA.[97] This ability to persist in the presence of immune stressors is crucial to GBS survival and can contribute to disease during pregnancy.

GBS can also cause disease by ascending from the vaginal tract into the upper reproductive tract, but little is known about what triggers ascending infections. Most studies focus on epidemiology of ascending infection in cohort studies, which only provide correlations, not molecular mechanisms. As described above, such studies link disruptions in the vaginal microbiome and increased risk of ascending infections. One molecular mechanism study in GBS did find that bacterial strains isolated from premature birth cases maintained active hyaluronidases, which could contribute to disease by cleaving hyaluronic acid in the cervix where it contributes to epithelial barrier function.[98] This role was confirmed by reduced rates of ascending infection of GBS hyaluronidase (*hylB*) mutants in a murine model.[98] Once ascending to the uterus, GBS can attach to the outermost layer of the placental membranes, the decidual cells, using the same attachment proteins (pili, ScpB, LMB and FbsA) described above. This association is thought to trigger an inflammatory cascade that leads to premature rupture of the membranes and premature birth.[97] GBS is also capable of invading the placental membranes to access the infant during pregnancy. This can occur through at least three mechanisms. First,

GBS can secrete a cytolysin (ß-hemolysin) that lyses host cells, breaking down the host barriers.[99–101] Secondly, it can invade transcellularly by hijacking host cell machinery to provoke cytoskeleton rearrangement to enter host cells.[102] Finally, GBS can interact with gap junctions to utilize a paracellular route that does not result in tissue damage.[103] After gaining access to the amniotic cavity, GBS can actively replicate in amniotic fluid,[104] thereby allowing it to infect the fetus *in utero* via the lungs or gut.[105,106] Once GBS enters the fetus, it can effectively enter the bloodstream, causing sepsis, and continue from the blood across the blood-brain barrier, resulting in meningitis.

Current therapies for GBS and potential alternatives

Current therapies and their success

Public health policies in regard to GBS preventative therapies vary from country to country.[107] While some countries utilize risk factors to guide risk assessment, the consensus guidelines in the United States test for GBS colonization of each mother using a rectal vaginal swab at 37 weeks of pregnancy.[108] It is recommended that women with GBS colonization or who have tested positive in previous pregnancies receive intrapartum antibiotic prophylaxis (IAP). Successful IAP requires at least four hours of intravenous antibiotic treatment prior to the birth of the infant.[108] IAP has successfully reduced early onset neonatal infections, but the timing of IAP during labor restricts its usefulness to early onset disease, which is only one of the many ways GBS can affect maternal and fetal health. Unfortunately, IAP has no effect on premature birth, stillbirth, maternal infections, or late onset disease. In addition to its limited use, IAP has known drawbacks, including disruption of the microbiome. As described above, the vaginal microbiome of the mother is determinant of infant microbiome and alterations in it can have

adverse effects on the mother and the infant.[34–36] Additionally, the reduction of GBS-associated neonatal sepsis has occurred with a simultaneous increase in antibiotic resistant *E. coli* sepsis cases in very low birth weight infants, resulting in no overall reduction of neonatal sepsis cases.[109,110] These shortcomings of IAP and the continued disease burden highlight the need for alternative therapies and continued research of this pathogen.

Vaccination

Alternative therapies for GBS should continue to focus on reducing maternal colonization but also need to be safe for earlier, more frequent use during pregnancy. During development of IAP, studies have examined the success of early and repeated use of antibiotics, but these treatments were associated with increased risk of infection and adverse pregnancy outcomes, resulting in the recommendation of a single dose during labor.[108] One of the earliest suggested alternatives was maternal vaccination. This could be used to reduce colonization of the mother and pass protective antibodies to infants through breastmilk, thereby reducing late-onset disease.[68,111] GBS vaccine development began after the observation of protective antibodies to GBS in mice.[112] Initial rounds of vaccines used purified CPS as the antigen, but have also included conjugate polysaccharides.[113] Rounds of vaccine testing have each had variable success in producing immune responses, but clinical trials with the most up to date vaccines are still on-going and have not been published.[113]

Phage therapy

Another alternative therapy that has gained traction is the use of virulent bacteriophage to infect and kill unwanted bacteria, which is termed phage therapy. Phage therapy was examined soon after their discovery in the early 1900's but was ignored in the United States and much of

Europe with the advent of antibiotics.[114,115] With the rise of antibiotic resistance and the realization of the negative effects of antibiotics on the microbiome, phage therapy has been revisited for a number of different applications in human health and the food industry.[116] In spite of early application of phage therapy and its continued use in Eastern Europe, few large trials that meet current standards have been performed to validate phage therapy or to ensure its safety.[117] Instead, bacteriophage therapy is typically applied in single cases when antibiotics fail and patients are left with no other option.[118–120]

Phagoburn, which examined the use of bacteriophage on burn wound infections, became the first completed randomized, controlled and double-blinded study to pass all requirements for good clinical practices.[121] In addition to testing the efficacy of the treatment, Jault *et al.* revealed previous unrecognized difficulties with production and administration of bacteriophage that will contribute to future studies.[121] While the bacteriophage treatment was successful in reducing infective bacteria in the burn wounds, the trial was terminated early because the standard practice of antibiotic treatment was more successful. This outcome could have been due to the difficulties with instability of the phage cocktail, resulting in a drastically lower multiplicity of infection (MOI) than intended. In addition to this study, more studies with similar levels of controls have recently finished, are currently being executed or are recruiting patients.[122]

Use of bacteriophage during pregnancy is even less studied as little research has been conducted with pregnant women; however, the potential importance of phage therapy during pregnancy has been discussed.[115] It is of particular interest to apply phage therapy to opportunistic pathogens that asymptomatically colonize the vaginal tract but cause issues during pregnancy, as this would allow for their selective removal without disrupting the microbiome.[115]

Bacterial targets include *Ureaplasma* spp. and GBS, which are associated with preterm birth, and *Pseudomonas aeruginosa* and *E. coli,* which are associated with urinary tract infections (UTIs). Indeed, UTIs are common during pregnancy and are typically treated with antibiotics, which affect the entire microbiome potentially leading to dysbiosis. As dysbiosis itself is associated with preterm birth and could potentially alter the transfer of a beneficial microbiome to the fetus, a more targeted approach is preferred. Sybesma *et al.* documented the success of a cocktail of bacteriophage in clearing UTIs from 50 patients with either *E. coli* or *Klebsiella pneumoniae* infections.[123] In another study using a mouse model, a single oral dose of a phage cocktail was able to clear a UTI caused by uropathogenic *E. coli.*[124] Studies on *Ureaplasma* spp. are limited as traditional phage isolation techniques are difficult; therefore, there is no available information on phages capable of targeting this species.

Currently, no clinical trial have been conducted examining the ability of a live phage to reduce colonization of GBS; however, in 2005, Cheng *et al.* examined the ability of a phage lysin to reduce GBS colonization in a mouse model.[125] This phage treatment successfully targeted multiple strains of varying serotypes *in vitro* and eradicated GBS colonization in the vaginal tract with a single dose. Although several *in vitro* studies have examined the relationship between human GBS isolates and phage, these phages were mostly lysogenic, meaning they incorporate into the genome of the bacteria instead of obligately lysing the bacteria, as is the goal with phage therapy.[115]

Recently, the first virulent bacteriophage, HN48, that targeted GBS was isolated from pond water, but the GBS was associated with tilapia, not human infection.[126] GBS colonizes a wide variety of animals including camels, crocodiles, cows and tilapia, but the host range of GBS

strains is species-specific.[127] This host specificity raises the question of whether findings for one type of GBS are extendable to GBS infection in humans, but the discovery of the lytic HN48 is still exciting as all isolated phage for human associated GBS are lysogenic or temperate. Characterization of this *Caudovirales* phage demonstrated that it had a relatively wide host range (67%) when evaluated against GBS from a variety of fish sources. Moreover, HN48 was highly specific to GBS, suggesting it would be useful in controlling disease in tilapia.[126] Additional experiments demonstrated that HN48 prevented disease in tilapia when co-infected with GBS; bacterial load was significantly decreased 12 hours post-infection and survival rate increased from 33% to 66%. It is also interesting to note that HN48 was sensitive to low pH (3-5), which would be important if applied in the vaginal tract.[126] These finding underscore the importance of characterizing individual phage to examine traits that will help it be effective in the system of interest.

Use of probiotic Lactobacillus against GBS

The use of probiotics – or "live organisms which when administered in adequate amounts confer a health benefit on the host" – has also been suggested to reduce GBS colonization. Because *Lactobacillus* is a major contributor to health in the vaginal tract, and it has been found in the upper reproductive tract, it represents a useful bacterial population to study for probiotic effects. Metagenomic studies, for instance, have shown that GBS colonization is negatively correlated with *Lactobacillus* colonization in the vagianl tract.[27,128] Studies on both *Lactobacillus* and GBS are mostly limited to the vaginal tract. DeGregorio *et al.* demonstrated that *L. reuteri* could modulate the immune response and reduce GBS colonization in a murine model.[129,130] Recently, another group showed a reduction in rectal GBS colonization of 72% of women and in vaginal colonization of 68% of women.[131] Additionally, studies testing oral dosing of murine

models have also demonstrated the ability of *Lactobacillus* to significantly reduce GBS colonization in the vaginal tract.[129] By contrast, some studies have had less success in reducing GBS colonization, suggesting that the strain of *Lactobacillus*, the dose or timeframe may be important.[132,133] Additional *in vitro* studies have examined the interactions between *Lactobacillus* and GBS, however, no human trials have been conducted to determine the effects of *Lactobacillus* beyond rectal vaginal colonization. *In vitro* studies have examined phenotypic effects such as GBS growth inhibition,[134] reduction in attachment,[135,136] effects of *Lactobacillus* bactericidins,[137,138] and biosurfactants.[139,140]

Many different *Lactobacillus* traits could be beneficial for use as a probiotic, though significant strain variation has been observed. *Lactobacillus* is thought to establish association with the vaginal tract by binding fibronectin using fibronectin binding proteins. *Lactobacillus* strains with surface layers (S-layer) can also attach to host fibronectin using S-layer proteins, such as SlpA.[141] As fibronectin is also commonly used by invading pathogens to establish infections, the presence of *Lactobacillus* at those sites could serve as a barrier to preventing pathogen colonization.[142]

Beyond barrier function, different strains of *Lactobacillus* also vary in the ability to directly inhibit pathogens. This inhibition could be due to the production of hydrogen peroxide, lactic acid and bactericidins. Lactic acid is one of the end products of the metabolism of all lactic acid bacteria, including *Lactobacillus*, and results in a general reduction of pH that can inhibit a range of Gram-positive and -negative bacteria. Indeed, production of this byproduct by *Lactobacillus* is credited with the acidic pH of the vaginal tract. Hydrogen peroxide is a reactive oxygen species that can lead to peroxidation of membrane lipids and increased membrane

permeability of other bacteria.[143,144] Studies in the vaginal tract of women have found variation in the ability of vaginal *Lactobacillus* strains to produce hydrogen peroxide, which was correlated with differential risk of bacterial vaginosis.[143,144] Bactericidin production vary between strains of *Lactobacillus* and the range of susceptible organisms for each bactericidin also varies.[145,146] The considerable differences in host attachment and pathogen inhibition demonstrate the importance of examining a variety of strains for probiotic use, but also the importance of not generalizing the effect of one strain to other strains.

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CHAPTER 2

LACTOBACILLUS STRAINS VARY IN THEIR ABILITY TO INTERACT WITH HUMAN ENDOMETRIAL STROMAL CELLS

ABSTRACT

While previously thought to be sterile, the placental membranes that surround the fetus during pregnancy are now thought to contain a low abundance microbiome. Specifically, abundance of *Lactobacillus*, a probiotic and dominant member of the microbiome of the lower reproductive tract, has been shown to correlate to with healthy, term pregnancies. We sought to access if four different *Lactobacillus* strains are able to associate with a model of the outermost cells of the placental membranes (dT-HESCs). Further, we examined the outcomes of that interaction. Herein, we found that all four *Lactobacillus* strains were capable of interacting with dT-HESCs; however, *L. crispatus* was statistically more successful ($p < 0.00005$), with 10.6% of bacteria attaching to host cells, while only an average of 0.8% of the other strains associated. The four strains also varied in their ability to form biofilms. Dependent on media type, *L. reuteri* 6475 forms the strongest biofilms *in vitro*. To examine the potential impact of *Lactobacillus* association with these cells on immune responses, total and phosphorylated protein levels of p38 of the Mitogen Activated Protein Kinase (MAPK) pathway was examined. Total levels of p38 were reduced to an average of 44% that of the cells without *Lactobacillus* ($p < 0.05$). While we observed a trend towards reduction of the phosphorylated form of p38, this difference was not significant ($p > 0.05$). Further, we found that association with *Lactobacillus* did not result in increased host cell death. Collectively, these data suggest that *Lactobacillus* attaches to the outermost cells of the placental membranes and that this association would not induce the MAPK pathway which has been associated with inflammation and host cell death.

INTRODUCTION

The placental membranes surround the fetus during pregnancy and are made of two layers, the amnion and choriodecidua, held together by connective tissue and fibroblast cells. These layers serve as the final barrier between the fetus and ascending pathogens from the vaginal tract. Pathogens such as Group B *Streptococcus* that cause adverse pregnancy outcomes, including preterm birth and *in utero* infections, must interact with these membranes in order to cause infection. Infection of these membranes is thought to cause weakening of the membranes, leading to miscarriage, preterm birth or neonatal sepsis.[1,2] While previously considered sterile, some metagenomic studies have found that the placenta and placental membranes may have a small load of commensal bacteria, including the genera *Acinetobacter*, *Escherichia coli*, *Enterobacter* and *Lactobacillus*.[3] Some bacteria, including *Lactobacillus* species, have been detected in healthy, term pregnancies,[3] yet few live culture studies have been performed to determine whether *Lactobacillus* can interact with the host cells in the placental membranes.

Lactobacillus is a commensal bacterium that dominates the healthy vaginal tract of most women.[4] Its role in human health has been heavily studied in the gut and urogenital tract. Mechanisms of health promotion include modulation of host immune responses and by direct or indirect inhibition of pathogens.[5] *Lactobacillus* can indirectly inhibit pathogens by occupying host attachment sites or by competing for nutrients or other growth factors. Direct inhibition includes production of lactic acid or other secondary metabolites. Immune modulation by *Lactobacillus* can be localized or systematic. Mechanisms include increased production of immunoglobulins (IgA), stimulation of cytokines and increased phagocytosis by immune cells.[6] As *Lactobacillus* is generally considered to be safe, its use as a probiotic during pregnancy in the urogenital tract is also being accessed.

In the urogenital tract, *Lactobacillus* has been found to inhibit a variety of individual pathogens including HIV, Uropathogenic *Escherichia coli* (UPEC), Group B *Streptococcus* (GBS), *Neisseria gonorrhoeae* and *Garnella vaginalis*.[7] It has also been shown to counteract more complex disease states such as bacterial vaginosis.[8] Modulation of the immune system has been more heavily studied in the gut, but recent studies suggest that *Lactobacillus* may also play a role in the urogenital tract. Specifically, supernatants from lactobacilli were shown affect the cytokine response to lipopolysacchadide (LPS) in the decidua, reducing a potentially harmful inflammatory response during pregnancy.[9]

To better understand the interactions between live *Lactobacilli* and the placental membranes, we sought to characterize the interactions of four *Lactobacilli* strains with a cell line model of the outermost layer of cells of the placental membranes: decidualized human endometrial stromal cells (dT-HESCs). Using this model, we assessed whether *Lactobacillus* maintains a beneficial interaction with these cells to provide similar protective functions as has been described in other body sites. Due to known variation between *Lactobacilli* strains, we also characterized growth and biofilm phenotypes of the strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus strains were selected to represent species that have been found in both the vagina and extraplacental membranes via metagenomics.[3,10] Additionally, previous work on *Lactobacillus* has focused on certain species including *L. reuteri* (Lr), *L. gasseri* (Lg) and *L. crispatus* (Lc); therefore, strains were selected to represent these species. Lr6475 was isolated from breast milk (MM4-1A PTA-6475), Lg33323 from the vagina (DSM 20243 [63 AM]), and Lc19390 from stool. Lr17938 is the daughter strain of *L. reuteri* ATCC 55730 which was isolated from breast milk but also carried potentially transferable resistance traits for tetracycline and lincomycin.[11] Lr17938 no longer carries this plasmid. *Lactobacillus* strains were cultured in deMan, Rososa and Sharpe (MRS) broth (Difco 288130) or agar at 37°C with 5% CO_2. Growth curves were performed by diluting an overnight culture of *Lactobacillus* 1:10 and observing growth by OD_{595} for 8 hours in a BioTek Cytation 3 Imager.

Biofilm Production

Over-night cultures of *Lactobacillus* were inoculated into fresh Tryptic Soy Broth (TSB) + 1% dextrose (TSBd) or MRS in a 96 well plate. Each biological replicate contains at least four technical replicates and media controls. Plates were incubated for 48 hours and subsequently washed twice with PBS to removal non-adherent cells. 100µl of 3% Crystal Violet was added to each well and incubated for 10 minutes. Wells were washed four times with PBS, and 200µl of ethanol was added to solubilize the crystal violet. 50µl was moved to a fresh plate to read by 595 absorbance. To analyze, the media control was subtracted and each resulting number was multiplied by four. Technical replicates were averaged within a plate. At least three biological

replicates were performed. Significance was determined using an unpaired analysis of variance (ANOVA). Cutoff values were established as preciously described.[13] Briefly, an OD_{595} cut-off value (ODc) was assigned based on the negative control and its standard deviations across three biological replicates (ODc = negative control + 3 standard deviations = 0.25). Non-biofilm producers fall below the ODc, while weak biofilm formers fall below 2 x ODc (0.50), moderate biofilm formers are below 3 x ODc (.75), and strong biofilm formers are above 4 x ODc (1.0).

Cell Culture

Telomerase-immortalized human endometrial stromal cells (T-HESC, ATCC CRL-4003)[12] were cultured in DMEM/Nutrient Mixture Ham's F-12 with l-glutamine (Sigma) supplemented with 1% ITS+ Universal Culture Supplement Premix (BD), 1.5 g/liter sodium bicarbonate, 2% pen/strep, and 10% charcoal-treated FBS (HyClone), which is referred to as HESC medium herein. For the cell experiments, T-HESCs were decidualized (dT-HESCs) as previously described[14,15] by incubating the cells with 0.5 mM 8-bromo-cyclic amp (cAMP) (Sigma Aldrich, St. Louis, MO) for three to six days. Completely confluent monolayers were used for all experiments to ensure that only decidual cell surfaces were available for bacterial interactions.

Association of *Lactobacillus* strains with decidual cells. Association assays were performed as previously described.[15,16] Briefly, monolayers of decidual cells were washed thrice with phosphate buffered saline (PBS) before infection with bacterial cultures. Overnight bacterial cultures of *Lactobacillus* were washed once with PBS and re-suspended in infection media (T-HESC medium without ITS+ or antibiotics and with 2% charcoal-treated FBS). dT-HESC cells were infected at a multiplicity of infection (MOI) of ten *Lactobacillus* cell per host cell

(MOI=10). Following a two-hour incubation at 37°C in atmospheric conditions, samples were taken from each well to determine the final colony forming units (CFU) of *Lactobacillus*. To quantify the number of associated cells, wells were washed three times to remove unattached cells. Host cells were disrupted using Triton-X, and wells were scraped thoroughly. Repeated pipetting and vortexing ensured even resuspension before plating. The percent of associated cells was calculated by dividing the CFU of associated cells by the final CFU of each well. These experiments were performed in three to four biological replicates of technical triplicates. Significance was determined using an unpaired ANOVA.

Cytotoxicity assays

Monolayers of dT-HESCs were cultured in 24-well plates and cells were infected with GBS (MOI=10) using a protocol described by Korir, et al.[17] After incubation for four hours, cells were washed twice with PBS, treated with 4μM ethidium homodimer 1 (Molecular Probes, Eugene, OR), suspended in PBS, and incubated at room temperature for 30 mins without light. Fluorescence was measured at 528-nm excitation and 617-nm emission using a plate reader (Beckman Coulter, Inc). The total number of cells in each well was calculated by adding 0.1% (wt/v) Saponin (Sigma) and incubating for at least 20 minutes before repeating the fluorescence reading. The percent permeability (cell death) was calculated by dividing the initial reading by the second and multiplying by 100. These experiments were performed in three to four biological replicates of technical triplicates. Significance was determined using an unpaired ANOVA.

Detection of IL-10 by ELISA:

dT-HESCs were infected with *Lactobacillus* (MOI=10). Cell supernatants were collected following a three-hour incubation, spun down at 28,000 rpm for 20 minutes to remove bacteria

and cellular debris, and stored at -20°C. Concentration of IL-10 was determined using the IL-10 Human ELISA kit: ab100549 (Abcam;Cambridge, MA) according to manufacturer's instructions and comparisons were made relative to mock infection. Data from three biological replicates were pooled to quantify the average cytokine concentrations (pg/mL). Significance was determined by unpaired ANOVA and post-hoc Dunnett's tests relative to mock infection.

Western Blotting for p38

dT-HESC were infected with *Lactobacillus* (MOI=10) as described above and protein lysates were collected following a three-hour infection to quantify the abundance of p38. Cells were washed twice with PBS and lysed with 300µl of lysis buffer for ten minutes at 4°C. Each well was scraped, and the lysate was collected. Samples were spun at 28,000 rpm for 20 minutes to pellet cellular debris, transferred to a fresh tube, and stored at -20°C. Protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce) using Bovine Serum Albumin (BSA) immediately prior to separation on a 4-15% polyacrylamide gel (BioRad). Samples were transferred to a polyvinylidene difluoride (PVDF) membrane and the membrane was blocked in 5% BSA (Fisher Scientific) + 0.1% Tween 20 (Sigma) in Tris-Buffered Saline (TBS) for at least two hours. The membranes were exposed to primary antibodies (1:1000) for phospho MAPK-p38 (T180+Y182; #4511S, Cell Signaling Technology), total MAPK p38 (1:1000; #8690S, Cell Signaling), or beta-tubulin (1:500; Santa Cruz Biotechnology) overnight at 4°C. Membranes were washed five times over an hour in TBS+0.1% Tween 20 and incubated with goat-anti-rabbit IgG-HRP secondary antibodies (1:5000, Life Technologies) at room temperature for 1.5 hours followed by another set of wash steps in TBS+0.1% Tween 20. The membranes were incubated with ECL chemiluminescence reagent (Pierce) prior to developing using an Amersham

Imager 600 (GE Life Sciences). Relative abundance of the proteins was determined using Image J and values from three independent replicates were pooled. Beta tubulin was used as a loading control.

RESULTS

Lactobacillus strains vary in growth in various media types

To determine growth phenotypes of these *Lactobacillus* strains in the various media types required for experiments, growth curves were performed. deMan, Rososa and Sharpe (MRS) broth is a standard growth broth for *Lactobacillus*. All the strains grew in MRS media and demonstrated similar lengths of lag phase and growth rates (Figure 2.1A). However, they did differ in the length of exponential phase and maximum OD_{595}. *Lr*6475 entered stationary phase earliest at 10 hours and reached a maximum OD_{595} of 1.5 at 12 hours. *Lg* 33323 reached a similar maximum OD_{595} of 1.5 but entered stationary phase three hours later. *Lr*17938 reached the lowest OD_{595} of 1.14 after 14 hours of growth. *Lc*19390 reached the highest OD_{595} of 1.73 but also entered stationary phase later, about 20 hours into growth. TSB containing 1% dextrose (TSBd) added has been used previously to assess biofilms in GBS; therefore, we sought to characterize *Lactobacillus* biofilms in this media as well.[15] All the *Lactobacillus* strains grew poorly in the media. *Lc*19390 and *Lg*33323 grew similarly, each reaching a maximum OD_{595} of 0.02. *Lr*6475 grew slightly better, reaching an OD_{595} of 0.04, while *Lr*17938 grew the best, reaching 0.06 in 48 hours (Figure 2.1B). Human Endometrial Stromal Cells (HESC) 2/0 media is a minimal media, which contains 2% per volume fetal bovine serum and 0% antibiotics and antimycotics. None of the *Lactobacillus* strains grew in the cell culture media, never reaching above 0.005 OD_{595} (Figure 2.1C).

L. reuteri strains form significantly better biofilms

Bacterial biofilms have been shown to play a role in adherence to surfaces and persistence in hosts; therefore, we assessed these strains differ for the ability to form biofilms.

Weak versus strong biofilms were differentiated as described in the methods. Biofilm formation was dependent on media type (Figure 2.2). *L. reuteri* 6475 and *L. gasseri* 33323 formed significantly better biofilms in MRS media ($p < 0.05$; $p < 0.00005$) compared to TSBd. Conversely, *L. reuteri* 17938 formed the strongest biofilm in TSBd compared to MRS ($p < 0.05$). While there was not a statistically significant difference in biofilm formation for *L. crispatus* between media types, it did form a higher biofilm in TSBd, increasing from 0.039 (non-biofilm former) to 0.37 (weak biofilm former).

There were also differences between *Lactobacillus* strains within media types. In the MRS media, biofilms formed by *L. reuteri* 6475 were significantly higher than *L. reuteri* 17938 ($p < 0.05$) and *L. crispatus* ($p< 0.005$) but were not different from those formed by *L. gasseri* ($p > 0.05$). Biofilms formed by *L. gasseri* were also significantly higher than *L. reuteri* 17938 ($p < 0.005$) and *L. crispatus* ($p< 0.005$). Biofilm formation was not statistically different between *L. reuteri* 17938 and *L. crispatus* in the MRS media type ($p > 0.05$). In TSBd both *L. reuteri* strains formed strong biofilms, reaching ODs of 1.74 and 2.71, respectively (Figure 2.2B). *L. crispatus* formed a weak biofilm of 0.37. *L. gasseri* was classified as a non-biofilm former in TSBd, falling below the ODc at 0.18. In this media, biofilms formed by *L. reuteri* 17938 were significantly stronger than both *L. gasseri* and *L. crispatus* ($p < 0.05$, 0.005; unpaired ANOVA). *L. reuteri* 6475 was only significantly stronger than *L. gasseri.* ($p < 0.05$, unpaired ANOVA). There was no statistical difference in biofilm production between *L. gasseri* and *L. crispatus* ($p > 0.05$).

L. crispatus associates with decidual cells significantly better than other *Lactobacillus* strains

A key step to establishing an interaction with a host cell is attachment. To determine if different *Lactobacillus* strains can interact with dT-HESCs, association assays were performed. As this is the first assay with *Lactobacillus* using this cell type, we compared association levels with those published for an organism known to colonize decidual cells, Group B *Streptococcus* (GBS). Our lab has previously used this experimental design to establish GBS association from approximately 0.02 to 0.6% (Korir, 2014). This level of association is similar to what we observe with *Lactobacillus*, suggesting *Lactobacillus* is capable of associating with this cell type. Nonetheless, variation between strains was observed (Figure 2.3). Of the strains tested, *L. crispatus* associated significantly better than the other strains with 10.6% of the total bacteria in the well establishing a stable association with the cells ($p < 0.0005$, unpaired ANOVA). The other strains were not significantly different from each other in percent association ($p > 0.05$); 1.11% of *L. reuteri* 6475, 0.52% of *L. gasseri* and 0.77% of *L. reuteri* 17938 associated with the dT-HESCs.

Lactobacillus does not produce an inflammatory response in dT-HESCs

As *Lactobacillus* associated with dT-HESCs, we next sought to assess if they triggered an immune response in this cell line. p38 is a key player in the MAPK pathway that promotes cell death and inflammation.[18] As previous studies have found that *Lactobacillus* can affect levels of this protein and protect against invading pathogens,[19-23] we wanted to determine if these strains have a similar affect and if strains vary in that effect. Western blots were used to determine the levels of both the phosphorylated (active) form and total p38 after a three-hour infection with *Lactobacillus*. In comparison to a mock infection with cell culture media, each *Lactobacillus*

strain lowered the levels of total p38, but did not affect the phosphorylated form of p38 (Figure 2.4A). Compared to the mock infection, total p38 was significantly reduced to 42.7%, 44.3%, 46.6% and 41.5%, respectively, for *L. reuteri* 6475, *L. gasseri, L. reuteri* 17938 and *L. crispatus* ($p < 0.05$). There were no significant differences between the *Lactobacillus* strains ($p > 0.05$). While levels of phosphorylated p38 were also decreased with the addition of *Lactobacillus* in comparison to the mock infection, these results were not statistically significant ($p > 0.05$). *L. reuteri* 6475, *L. gasseri, L. reuteri* 17938 and *L. crispatus* reduced levels of phosphorylated p38 to 66.5%, 56.7%, 52.0% and 69.4%, respectively (Figure 2.4B). There were no differences between strains. To further assess if *Lactobacillus* had any anti-inflammatory effects, we also utilized an ELISA to detect changes in IL-10 secretion. We were unable to detect any changes in IL-10 production as very low production of this cytokine was observed in both the mock and *Lactobacilli* treatments (data not shown).

Lactobacillus does not trigger dT-HESC death

Reduction in total p38 and maintenance of the phosphorylated form, led us to assess downstream effects of the mitogen-activated protein kinase pathway to determine the effect of the reduction in total levels of p38. Because this pathway is associated with cell death,[18] we compared host cell permeability during incubation with *Lactobacillus* to that of a mock infection as a marker of host cell death (Figure 2.5). No significant differences in the host cell permeability were observed with *Lactobacilli* infection ($p > 0.05$, unpaired ANOVA). The media control contributed to a 24.9% cell death rate, while *L. reuteri* 6475, *L. gasseri, L. reuteri* 17938 and *L. crispatus* caused 29.1%, 26.93%, 30.2% and 27.0% cell death, respectively. Further, we observed no differences between the individual strains ($p > 0.05$, unpaired ANOVA, Figure 2.5).

DISCUSSION

Lactobacillus has been studied as a probiotic in the gut and urogenital tract, but its effects in the placental membranes remain uncharacterized. As this bacterium is being proposed as a probiotic during pregnancy,[24,25] it is important to understand the potential effects of *Lactobacillus* colonization of the placental membranes should it reach them. Previous work has found vast differences in probiotic capacity between species of *Lactobacillus* and stains within the same species;[26] therefore, we first sought to characterize traits that may affect their ability to interact with host cell membranes including growth and biofilm formation. Growth differences were observed between strains and between medias. As HESC 2/0 media lacks critical nutrients, it is not surprising that all four of the *Lactobacillus* strains were unable to grow. The inability to grow in this model system may affect some traits such as the production of secondary metabolites.

To colonize the placental membranes, *Lactobacillus* must interact with the outermost layer of cells in the membranes, decidualized stromal cells. Indeed, we have demonstrated that each of the *Lactobacillus* strains was capable of attaching to dT-HESCs, though variation was observed in the percent association across strains. The overall level of association to dT-HESCs was similar to that described for GBS, an opportunistic pathogen that can attach to and invade dT-HESCs even though variation across GBS strains was also observed.[14,15,21] Notably, the *L. crispatus* strain was significantly better at associating with the decidualized stromal cells, which provides support for the recovery of *L. crispatus* from the placental membranes of healthy, term pregnancies using metagenomics.[3] As *L. acidophilus* was the other *Lactobacilli* species found in this study,[3] future work should also examine if it is more capable of associating with decidual cells. The ability to attach to the placental membranes suggests that these strains may be able to

serve as a barrier to invading pathogens as their presence may remove potential attachment sites for those bacteria. Further work coculturing *Lactobacillus* with different pathogens known to colonize the placental membranes should be completed to assess this potential.

The ability to form a biofilm has been associated with persistence in many environments, and strains of *Lactobacillus* have been found to persist in other host sites including the intestines for up to a week.[27] We found that the *Lactobacillus* biofilms were dependent on growth media, suggesting nutrients are an important factor in the ability of *Lactobacillus* to form biofilms. Indeed, *L. reuteri* 6475 and *L. gasseri* 33323 formed significantly better biofilms in MRS media ($p < 0.05$; $p < 0.00005$), while *L. reuteri* 17938 formed a stronger biofilm in TSBd ($p < 0.05$). Further, we found differences in biofilm formation between individual *Lactobacillus* strains. *L. crispatus* did not form strong biofilms in either media, while *L. reuteri* 6475 formed strong biofilms in both medias. *L. gasseri* and *L. reuteri* 17938 were more drastically impacted by media type as the growth media determined if each was a low or high biofilm former. These differences in biofilm production could impact a given strain's ability to persist in a given environment.

Premature birth and preterm premature rupture of the membranes (pPROM) are associated with inflammation and host cell death in the placental membranes;[28] therefore, it is important to assess whether *Lactobacillus* has an effect on immune signaling and host cell death. The MAPK pathway responds to stress and is involved in inflammatory signaling and cell death.[18] We observed significantly reduced levels of total p38 among all four strains. The lower amount of total protein could reduce the overall output of the pathway, potentially stunting the ability of this pathway to promote host cell death. Though not significant, we also found a trend

toward reduced levels of phosphorylated or active form of p38 in response to *Lactobacillus* compared to the mock infection, suggesting that *Lactobacillus* is not inducing this pathway. While not previously studied in this cell type, other studies have found that *Lactobacillus* can induce the MAPK pathway in macrophages, while other studies observed reduced MAPK induction with *Lactobacillus* in the gut, intestinal epithelial cells and the liver.[19–23] Still other work in a nematode model of *E. coli* sepsis, only certain strains of *Lactobacillus* further increased MAPK activation during infection, resulting in better survival outcomes.[29] Further work will be needed to more fully understand the effect of *Lactobacillus* on this pathway and its downstream effect on pregnancy outcomes. The reduced activation of the MAPK pathway in addition to no changes in host cell death (Fig 2.4) suggests that colonization of *Lactobacillus* would not likely damage the placental membranes through this cell type. As there are other cells types in the placental membranes, including macrophages, it would also be important to access the effects of *Lactobacillus* in these other cell types as well or in a more complex model. Indeed, there were no significant differences in host cell permeability after four hours of incubation. In this same experimental design and conditions, known pathogen GBS can cause about 70% host cell death (Chapter 3, Figure 3.4), suggesting that *Lactobacillus* does not induce host death in these cells.

In other systems, *Lactobacillus* modulates the immune system to reduce inflammation or prime immune cells for invading pathogens.[21] Production of IL-10 is a marker of an anti-inflammatory response to *Lactobacillus.* This interleukin has previously been shown to be induced by *Lactobacillus* in other cell types including macrophage cell lines (RAW264.7 (mouse) and THP-1(human)), cervical tumor cells (HeLa), and colonic epithelial cells (Caco2).[21,30,31] However, we observed very little production of IL-10 in mock or following

infection with all four strains of *Lactobacillus* in our study. Because it is possible that this cell line does not produce higher levels of cytokines in general, other models, such as whole decidua that produce higher cytokine levels, may serve as a better model of studying this interaction.[9,32]

Collectively, these data suggest that different strains of *Lactobacillus* sustain a commensal relationship with cells of the decidua. We found variation among strains of *Lactobacillus* in growth, biofilm production and association with dT-HESCs, suggesting that this relationship will be strain-dependent, as suggested in the literature for other body sites. Further research needs to be done to examine if these characteristics allow *Lactobacillus* to perform the barrier function against pathogen invasion as seen in the vaginal tract. These strains should also be studied in more complex models to examine the effect of the other cell types in the decidua, as they may alter the relationship. Since the placental membranes play such an important role in maintaining a healthy pregnancy, increasing our understanding of how traditional commensal bacteria affect these membranes could enhance knowledge of premature birth and other adverse pregnancy-related outcomes.

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APPENDIX

Figure 2.1. *Lactobacillus* growth varies by media type. *Lactobacillus* strains were grown overnight, washed in PBS, and inoculated into fresh A) MRS media; B) TSA + 1% Dextrose; or C) HESC 2/0 media. The Optical Density 595 (OD_{595}) was measured every 30 minutes for 48 hours using a spectrophotometer.

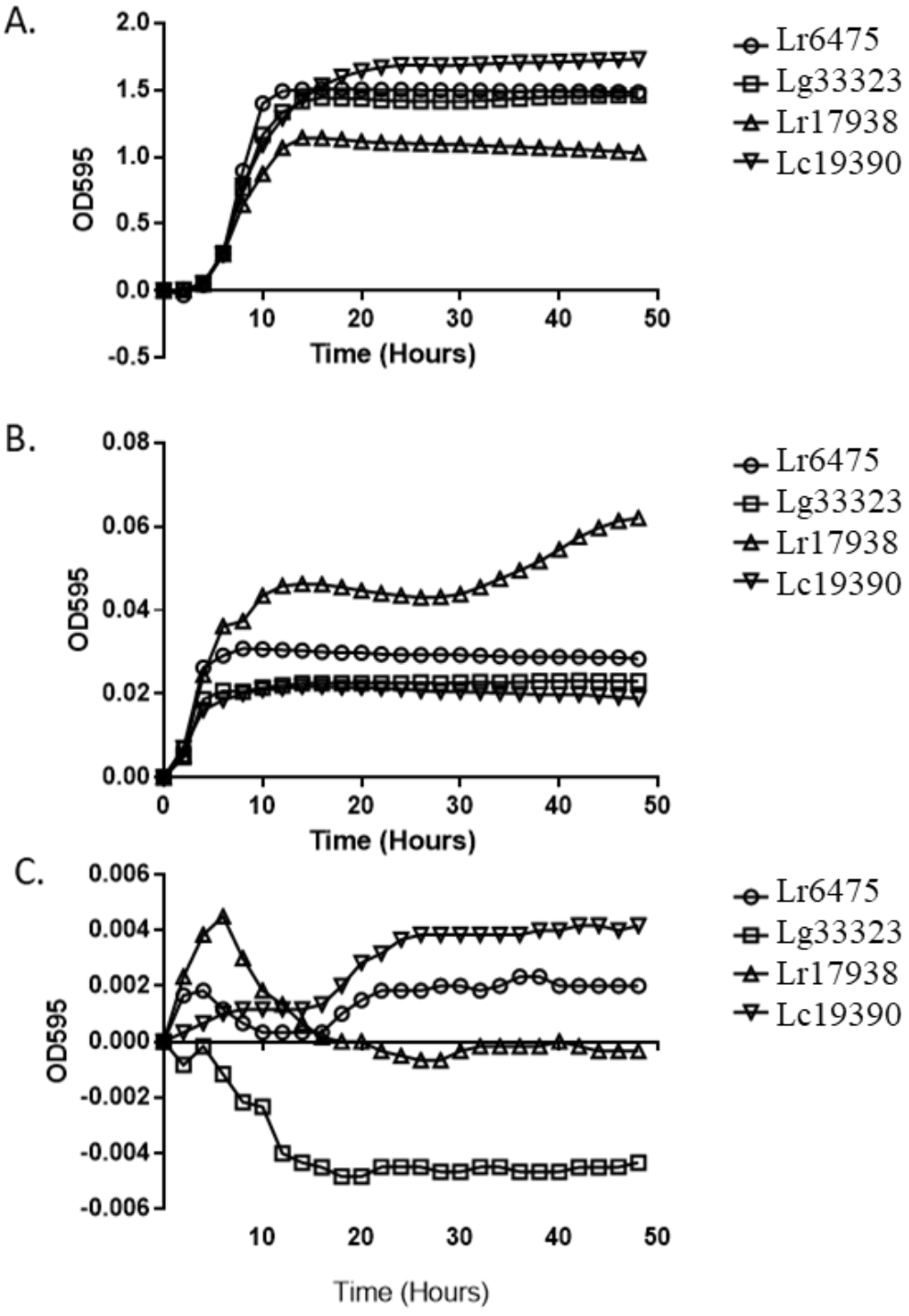


Figure 2.2. *Lactobacillus* species differ in the ability to form biofilms depending on media type. *Lactobacillus* was grown overnight, washed in PBS and incubated in: A) TSB with 5% dextrose; or B) MRS media for 48 hours in a 96-well plate. Biofilms were assessed via crystal violet staining. Normalized absorbance is calculated by taking the OD_{595} subtracting that of the control and multiplying by four to account for dilutions. Experiments were performed in biological triplicate of technical quadruplicates. Error bars represent standard deviation between biological trials. Significance was determined by an unpaired ANOVA.

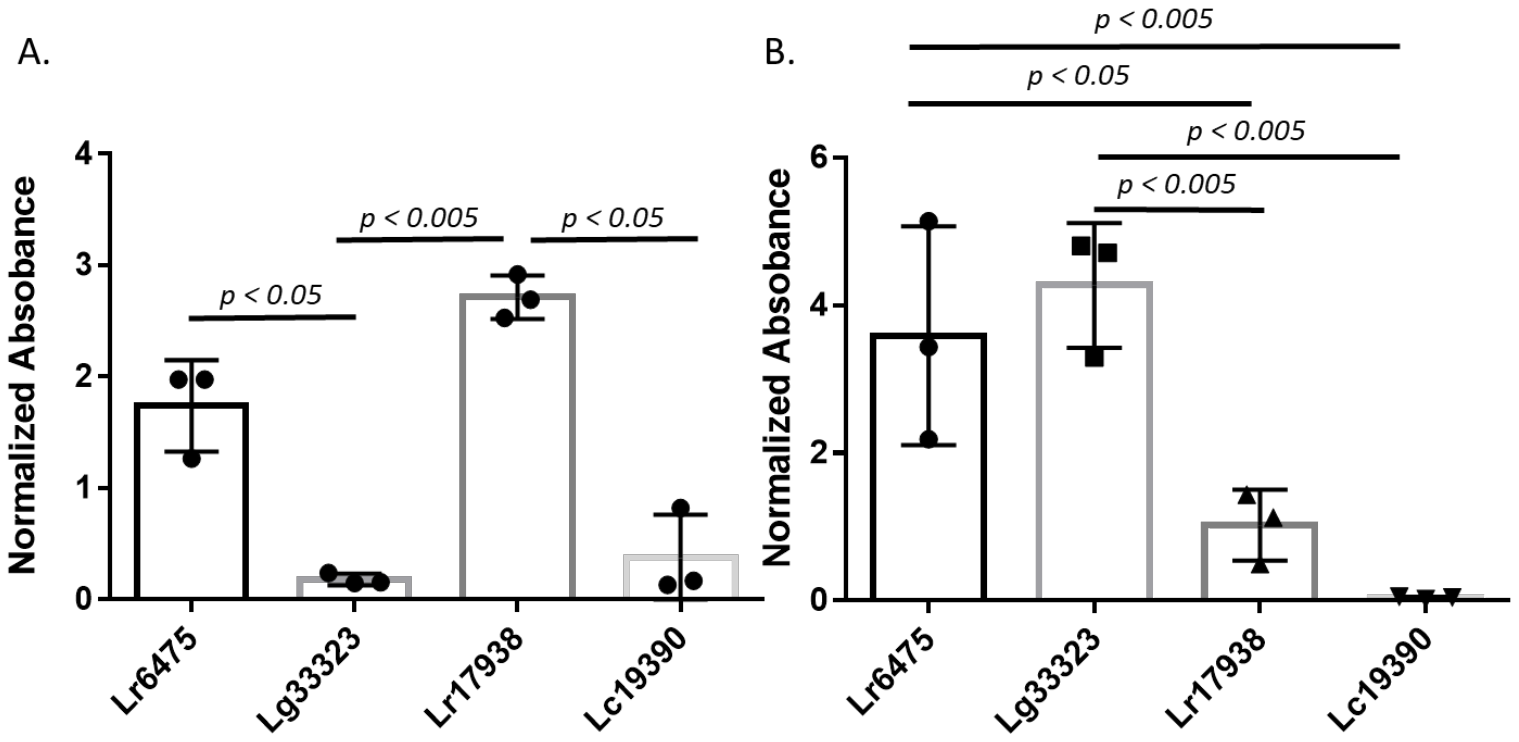


Figure 2.3. *Lactobacillus* associates with decidualized Human Endometrial Stromal Cells (dT-HESC). dT-HESCs were infected with *Lactobacillus* at a MOI of 10 for two hours. The percent of associated bacteria was calculated relative to the total number of bacteria in the well. Experiments were completed in biological quadruplets of technical triplicates. Error bars represent standard deviation between biological trials. Significance was determined by an unpaired ANOVA.

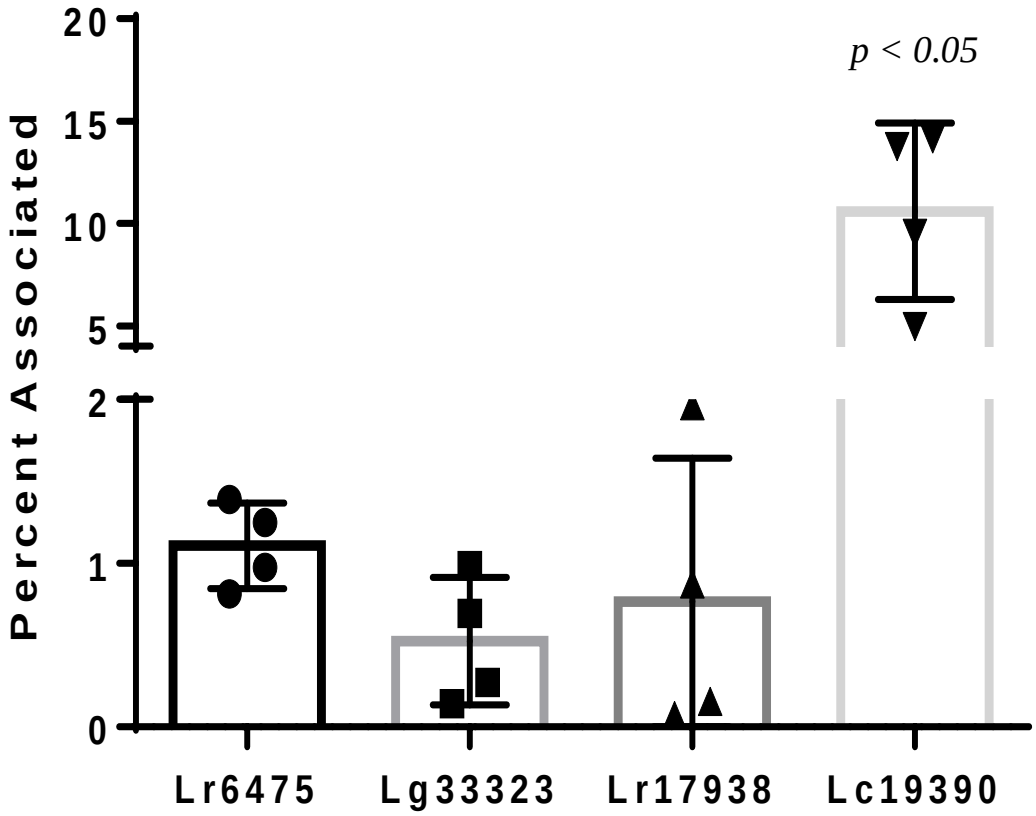


Figure 2.4. *Lactobacillus* affects total p38 and phosphorylated levels of p38 (Pp38). dHESCs were infected with *Lactobacillus* at a MOI of 10 and incubated for three hours. Protein lysates were collected and analyzed via Western Blotting using densitometry in Image J. A β-tubulin internal loading control was used to account for loading differences. Graphed densitometry data is presented as a percent of the mock control for each of three biological replicates. Error bars represent the standard deviation of the data. Significance was determined using an unpaired ANOVA.

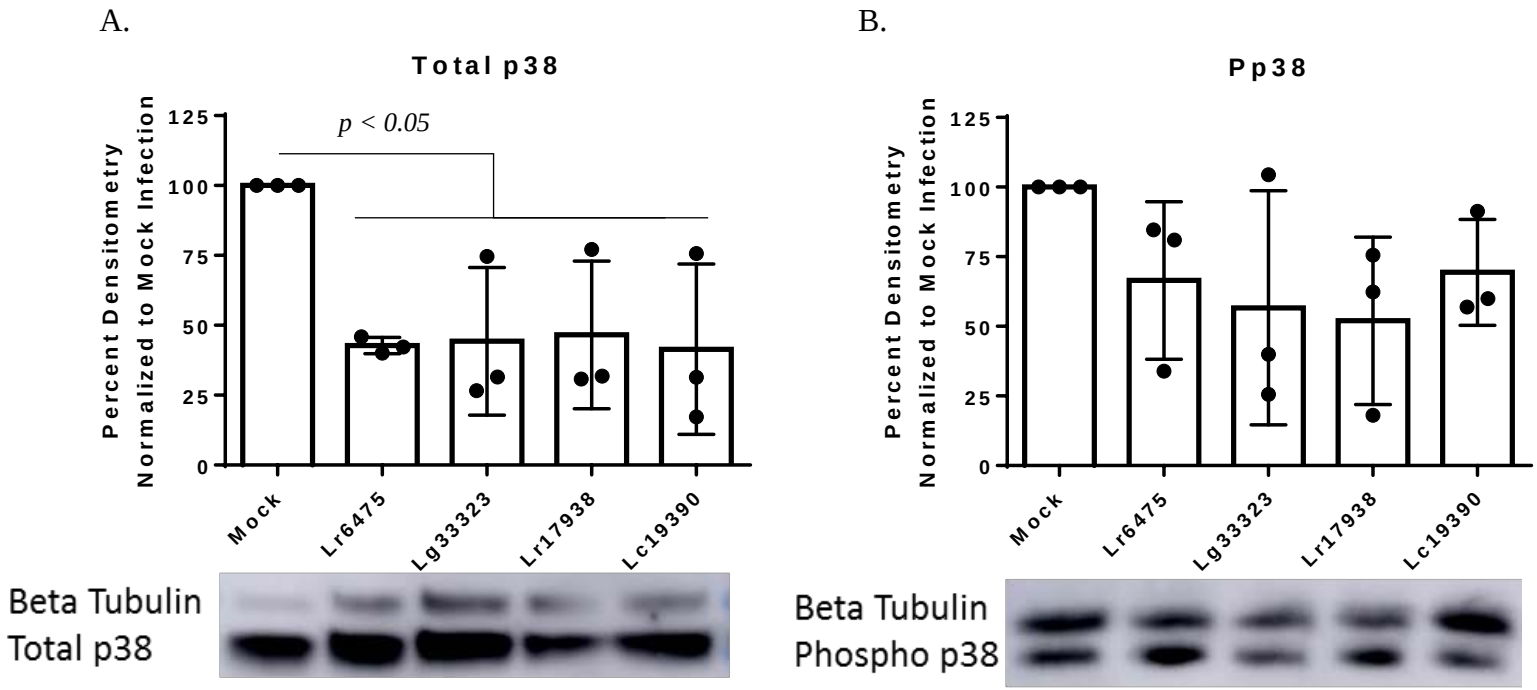
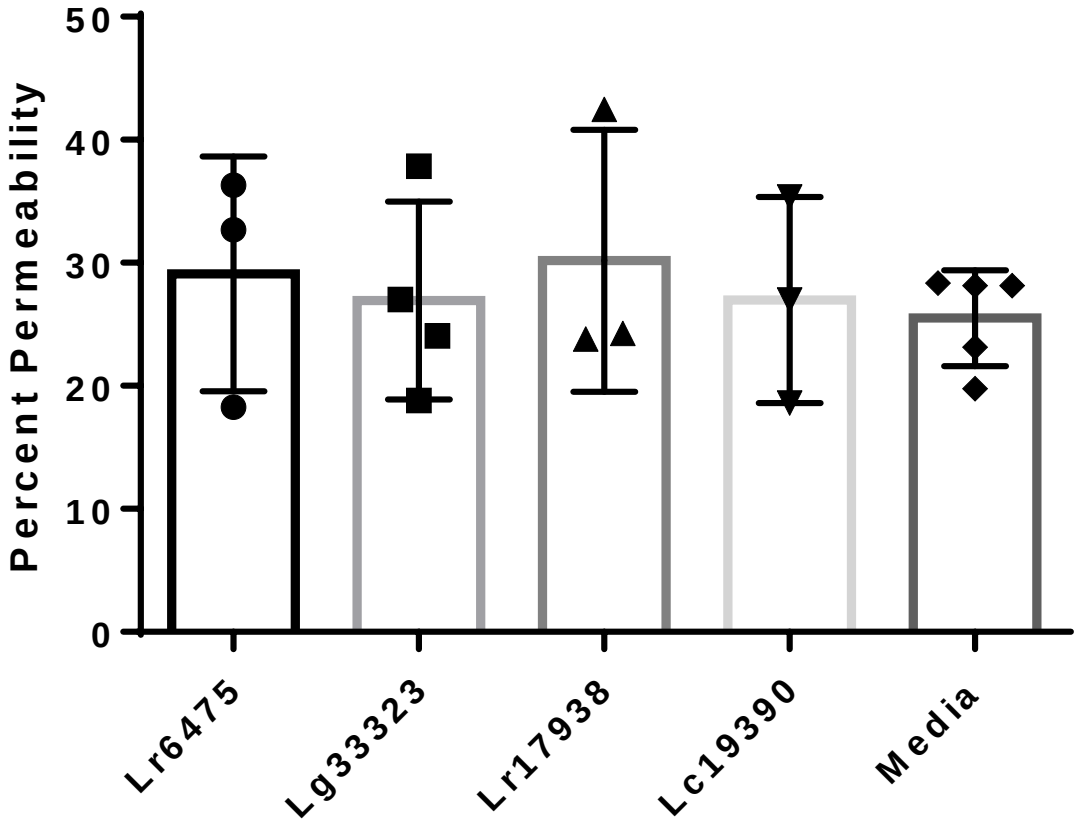


Figure 2.5. *Lactobacillus* does not induce dT-HESC death. dT-HESCs were infected with *Lactobacillus* at a MOI of 10 and incubated for four hours. Cell permeability was detected using an ethidium homodimer assay, and percent permeability was calculated by lysing the remaining cell in each well. Graphed data represents three biological replicates, and the error bars represent the standard deviation of the data. Significance was determined using an unpaired ANOVA.



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CHAPTER 3

THE IMPACT OF *LACTOBACILLUS* ON GROUP B STREPTOCOCCAL INTERACTIONS WITH PLACENTAL MEMBRANES

ABSTRACT

Group B S*treptococcus* (*Streptococcus agalactiae*, GBS) contributes to the global human disease burden through adverse pregnancy outcomes and neonatal disease. Currently, the only preventative measure implemented in the United States is intrapartum antibiotic prophylaxis (IAP), which has only impacted one form of neonatal disease and does not improve pregnancy outcomes. *Lactobacillus* is the dominant member of the microbiota of the lower reproductive tract and has recently been identified in the upper reproductive tract including the placental membranes during healthy, term pregnancies. Previous work has shown that *Lactobacillus* reduces rectal vaginal colonization of GBS, but no studies have examined how *Lactobacillus* might impact the ability of GBS to cause adverse pregnancy outcomes by associating with and invading the placental membranes. Herein, we characterized interactions using two GBS strains (colonizing and invasive) and four *Lactobacillus* strains that had been previously characterized for their ability to interaction with a model of the outermost cells of the placental membranes, dT-HESCs. We found that live *Lactobacillus* does not affect growth or biofilm production of GBS *in vitro*, but that *L. gasseri* 33323 increases GBS association with dT-HESCs in both GBS strains by 4 - 6% ($p < 0.005$). Increased association did not result in increased invasion ($p > 0.05$) or increased host cell death. Indeed, a statistically significant reduction in host cell death was observed with certain combination of GBS and *Lactobacillus* ($p < 0.05$). As *Lactobacillus* is known to secrete many inhibitory compounds, we also sought to characterize the effect of *Lactobacillus* supernatants on GBS. We found that these supernatants were able to inhibit growth ($p < 0.00005$) and biofilm formation ($p < 0.005$) of GBS, though this was strain dependent. We also observed increases in GBS-induced host cell death with *Lactobacillus* supernatants in the invasive strain of GBS. Finally, we assessed whether the supernatant from one strain, *L. reuteri*

6475, could broadly inhibit growth of GBS. Indeed, we found that growth was reduced to an average of 46.6% of each GBS strain alone. Collectively, these data suggest that both live *Lactobacillus* and its supernatant could impact GBS interactions with the placental membranes.

INTRODUCTION

Group B S*treptococcus* (*Streptococcus agalactiae*, GBS) is a global human health burden , contributing to neonatal infections and deaths and adverse pregnancy outcomes, including premature birth and stillbirth. In the United States alone, GBS is thought to cause 12% of stillbirths[1] and is the leading cause of neonatal pneumonia, sepsis and meningitis.[2,3] 4-6% of infected newborns succumb to the infection, while others have long-lasting effects including deafness and developmental disabilities.[3] GBS is thought to contribute to premature birth and stillbirth by accessing the fetus via an ascending infection. While GBS vaginal colonization is asymptomatic, GBS association or invasion of the placental membranes has been linked to inflammation and subsequent fetal infection.[4–7]

Currently, consensus guidelines in the United States recommend that mothers who are culture positive for GBS at 37-39 weeks of pregnancy should receive intrapartum antibiotic prophylaxis (IAP). Proper administration of IAP requires four hours of antibiotics during labor. This practice has reduced the rate of early onset neonatal disease number of early onset cases but has not affected late onset disease or adverse pregnancy outcomes.[8] Additionally, the antibiotic treatment is known to affect both the maternal vaginal microbiome and the neonatal gut microbiome, which are important for neonatal development.[9–13] These shortcomings of IAP combined with growing concern of antibiotic resistance have resulted in increased interest in alternative therapies for the prevention of GBS-associated neonatal disease and adverse pregnancy outcomes. As maternal colonization is the primary risk factor for neonatal infection, therapies should reduce maternal colonization.[2] These therapies should be more specific than the

antibiotic course; however, so that multiple doses could be used to combat adverse outcomes in the mother and baby and the microbiomes of the mother and neonate could remain unaltered.

Lactobacillus, a dominant member of the vaginal and cervical microbiota in most women, has been examined as a probiotic against a variety of bacteria including GBS. Human trials using *Lactobacillus* alone or in combination with other probiotic species, for instance, have shown variable, but promising success in reducing rectal-vaginal colonization in pregnant mothers.[14–16] Reasons for variability include differences in the length of intervention, strains and dosage.[14] There is less research available on how *Lactobacillus* may affect GBS and ascending infection; however, some studies have found that *Lactobacillus* can reduce the risk of premature birth triggered by inflammation.[17–19] To our knowledge, no prior studies have been conducted to determine how *Lactobacillus* affects GBS ascension to the vagianl tract or virulence phenotypes.

As *Lactobacillus* has been identified in the human placental membranes using metagenomic techniques,[20] we aimed to assess how *Lactobacillus* alters the ability of GBS to associate with and invade this important barrier to the fetus. To further characterize the interaction, we examined changes in GBS growth and host cell death. These *in vitro* studies reveal the mechanisms behind *Lactobacillus*'s ability to affect colonization and premature birth, allowing for better understanding of its use as a probiotic and of its limitations.

MATERIALS AND METHODS

Bacterial strains and growth conditions

GBS strains were selected based on multilocus sequence type (ST) designation, capsular serotype, and source. Two strains belonged to ST-17, which has been most associated with severe disease in neonates.[21,22] One strain, GB00112, was isolated using a rectal-vaginal swab from a colonized pregnant woman[23] and the second from the blood of a septic newborn (GB00411)[24] GBS was cultured in Todd-Hewitt broth (THB) or half concentrated THB with agar (THA) at 37°C with 5% CO_2. The extended strain set that was used to determine the effect of *Lactobacillus* supernatant on GBS growth is detailed in Table 1. *Lactobacillus* strains were selected to represent species that have been found in both the vaginal tract and extraplacental membranes via metagenomic techniques.[20] Since previous work on *Lactobacillus* has focused on certain species including, *L. reuteri* (Lr), *L. gasseri* (Lg) and *L. crispatus* (Lc), which commonly inhabit the genitourinary tract, strains representing these species were selected. Lr6475 was isolated from breast milk (MM4-1A PTA-6475), Lg33323 from the vagina (DSM 20243 [63 AM]), and Lc19390 from stool. Lr17938 is the daughter strain of *L. reuteri* ATCC 55730, which was isolated from breast milk but also carried potentially transferable resistance traits for tetracycline and lincomycin.[25] Lr17938 no longer carries this plasmid. All strains were cultured in deMan, Rososa and Sharpe (MRS, Difco 288130) broth or agar at 37°C with 5% CO_2. Plating of a co-culture of *Lactobacillus* and GBS was performed on sheep's blood agar plates (Tryptic Soy broth supplemented with 5% sheep's blood, Northeast Lab Services) at 37°C and 5% CO_2. *Lactobacillus* colonies were given 48 hours to grow as colonies were small.

Cell Culture

Telomerase-immortalized human endometrial stromal cells (T-HESC; ATCC CRL-4003)[26] were cultured in DMEM/Nutrient Mixture Ham's F-12 with L-glutamine (Sigma) supplemented with 1% BD ITS+ universal culture supplement premix, 1.5 g/liter sodium bicarbonate, 2% penicillin / streptomycin, and 10% charcoal-treated FBS (HyClone), which is referred to as HESC medium herein. For all cell experiments, the HESC line was decidualized (dT-HESCs) as previously described[27] by incubating with 0.5 mM 8-bromo-cyclic amp (cAMP) (Sigma) for three to six days. The resulting cells are referred to as decidual cells in this work. Assays were only performed when cells reached a 100% confluent monolayer so that only decidual cell surfaces were available for bacterial interactions.

Isolation of *Lactobacillus* supernatants

Lactobacillus was loop-inoculated into 10mL of MRS broth with or without 5mM glycerol in a 15mL conical tube and incubated at 37°C for 18-20 hours with the cap slightly loosened. After incubation, cultures were vortexed, centrifuged to pellet the bacteria, and filter sterilized with a 0.22 micron filter. Supernatants were used immediately.

Bacterial growth curves

To examine the effect of live *Lactobacillus* and its supernatants, growth curves were generated by serial plating or using a plate reader (Beckman Coulter, Inc). To examine the effect of co-culture on GBS growth, overnight cultures of GBS and *Lactobacillus* were washed once with PBS and diluted to an equivalent optical density $(OD)_{600}$ of 0.1 in HESC infection media. Each culture was inoculated 1:10 to have a starting culture with a 1:1 ratio of each bacteria. Samples were collected hourly for six hours and differentially plated on tryptic soy agar (TSA)

supplemented with 5% sheep blood. To examine the effect of supernatants, 100µL of 0.1OD_{600} culture in HESC infection media was added to a 96-well plate with 25µL of supernatant or additional infection media. Time points were collected by plate reader every 15 minutes for eight hours (BioTek Cytation 3 Imager). The Area Under the Curve (AUC) was calculated using GraphPad Prism 6. Significant differences were determined by comparing the AUC of three biological replicates by unpaired ANOVA.

Biofilm assays

Biofilm production was assessed as previously described in Chapter 2.[28] Briefly, overnight cultures of GBS and *Lactobacillus* were diluted to an equivalent OD_{600} of 0.1 and resuspended in tryptic soy broth supplemented with 1% Dextrose (TSAd); 50µl of each culture was added to a 96-well plate for co-culture biofilms. Mono-culture wells contained 50µl of culture and 50µl of media. *Lactobacillus* supernatant was added to GBS in mono-culture at the beginning of the incubation period at 10% v/v of the total volume of the well. Plates were incubated for 48 hours at 37°C and 5% CO_2. After incubation, wells were washed twice and 100µl of crystal violet was added. After a ten-minute incubation, crystal violet was removed, and the wells were washed four times with 150µl of PBS. Remaining crystal violet was solubilized with 100% ethanol; 50µl was taken from each well, and absorbance at OD_{595} was determined using a plate reader (Beckman Coulter, Inc). The total absorbance was calculated by subtracting the average of the media controls and multiplying by four. Significance was determined by unpaired ANOVA of at least three biological replicates of technical quadruplicates.

Association with and invasion of decidual cells

Monolayers of decidual cells were washed thrice with phosphate buffered saline (PBS) before infection with bacterial cultures. Overnight bacterial cultures of GBS and *Lactobacillus* were washed once with PBS and re-suspended in HESC infection media. dT-HESC cells were infected at a multiplicity of infection (MOI) of ten bacterial (GBS) cell per host cell (MOI = 10) for both mono-culture and co-culture wells to assure the same number of GBS cells were available to affect decidual cells.

Co-culture wells had an additional MOI = 10 of *Lactobacillus*. Experiments with *Lactobacillus* supernatant 10% v/v of supernatant was added to each well at the beginning of the two-hour incubation. An equivalent volume of HESC infection media was added to each control well to control for total volume between wells. Following a two-hour incubation at 37^{o}C in atmospheric conditions, samples were taken from each well to quantify the final colony forming units (CFU) of GBS.

To calculate the number of associated cells (attached and invaded), wells were washed three times to remove unattached cells and host cells were disrupted using Triton-X as described previously.[28] Wells were scraped and thoroughly re-suspended before plating for CFU. To enumerate intracellular bacteria, extracellular bacteria were killed with 100 μg/ml of gentamicin (Gibco) and 5 μg/ml of penicillin G (Sigma) for one hour prior to continuing the Triton-X treatment and enumeration steps described above. The percent associated cells were determined by dividing the associated cells by the final CFU of each well. The invasion frequencies were calculated by dividing each well by the average of the three technical replicates of the final CFU. All presented data represent the average of three biological replicates of three technical replicates.

Cytotoxicity assays

Monolayers of dT-HESCs were cultured in 24-well plates. Cells were infected as described above and/or treated with 10% v/v of *Lactobacillus* supernatant. After incubation, cells were washed twice with PBS and treated with 4μM ethidium homodimer 1 (Molecular Probes, Eugene, OR) suspended in PBS as previously described.[30] Plates were incubated at room temperature for 30 minutes without light, and fluorescence was measured at 528-nm excitation and 617-nm emission using a plate reader. The total number of cells in each well was calculated by adding 0.1% (wt/v) Saponin (Sigma) and incubating for at least 20 minutes before repeating the fluorescence reading. The percent permeability (cell death) was calculated by dividing the initial reading by the second and multiplying by 100. Significance was determined by unpaired ANOVA of at least three biological replicates of technical triplicates.

RESULTS

Lactobacillus does not impede GBS growth in T-HESC infection media

To evaluate growth of each strain in the media used for each experiment, growth curves were performed with bacteria in mono- and in co-culture. None of the *Lactobacillus* strains grew in the T-HESC infection media (shown previously in Figure 2.1) yet both GB112 and GB411 grew in the media (Figure 3.1A). To determine if there was a difference in growth, Area Under the Curve (AUC) was calculated. Co-culturing GBS with *Lactobacillus* did not affect the growth of GBS ($p > 0.05$, Figure 3.1B). Colonies of *Lactobacillus* were not detectable in co-culture after the first time point due to the higher concentration of GBS; therefore, we were unable to calculate its growth in co-culture.

Lactobacillus does not affect GBS biofilm formation

As biofilms are thought to be important for colonization and persistence,[31,32] we sought to determine if co-culturing GBS with live *Lactobacillus* would affect the ability of GBS to form biofilms. Biofilm production at 48 hours was compared between each GBS strain in mono-culture and to each GBS strain in co-culture with all four *Lactobacillus* strains (Figure 3.2). The colonizing GB112 strain formed a weak biofilm of 0.86 in mono-culture (Figure 3.2A). Although co-culture of GB112 with *Lactobacillus* increased biofilm production to absorbance values of 1.36, 1.37, 1.49, and 0.97 for *L. reuteri* 6475, *L. gasseri* 33323, *L.reuteri* 17938, and *L. crispatus* 19390, respectively, the difference was not statistically significant ($p > 0.05$). The invasive GB411 strain formed a stronger biofilm than GB112 with an absorbance value of 1.22 ($p > 0.05$) but there were not statistical differences between GB112 and GB411 with a given *Lactobacillus* strain (Figure 3.2B). Co-culturing with *L. reuteri* 6475, *L. gasseri* 33323, *L.reuteri* 17938, and *L.*

crispatus 19390 increased biofilm production to 1.44, 1.50, 1.80 and 1.25, respectively, but this change was also not significant ($p > 0.05$).

Lactobacillus variably affects GBS association with dT-HESCs

As we have demonstrated previously, dT-HESCs serve as a model of GBS attachment to and invasion of the outermost layer of the placental membrane.[28–30] We have also previously established that *Lactobacillus* is capable of associating with this cell line (Figure 2.3). Hence, the goal of this experiment was to determine whether live *Lactobacillus* affects the ability of GBS to interact with this important barrier. The colonizing (Figure 3.3A) and invasive (Figure 3.3B) strains of GBS did not significantly differ in the ability to associate with dT-HESCs, associating at 0.025% and 0.021%, respectively ($p > 0.05$). Both GBS strains also demonstrated similar responses to the addition of *Lactobacillus*, with no observed statistical differences between each co-culture condition. Notably, co-culture with *L. gasseri* 33323 significantly increased association of the colonizing GBS strain to 6.4% (Figure 3.3A; $p < 0.005$) and of the invasive strain to 4.8% (Figure 3.3B; $p < 0.05$). Association to dT-HESCs by *L. gasseri* 33323 plus both GBS strains was significantly greater when compared to all other *Lactobacillus*/GBS combinations. Additionally, no statistical differences were observed between each GBS strain in mono-culture compared to co-culture with *Lr* 6475, *Lr* 17938, or *Lc* 19390 (Figure 3.3A&B, $p > 0.05$).

GBS invasion of the dT-HESCs was also examined. Although there was no significant difference in percent invasion between the colonizing (0.003%) and invasive (0.00076%) strains of GBS ($p > 0.05$), a trend of greater invasion in the colonizing strain relative to the invasive strain was observed (Figure 3.3C&D). The addition of each of the *Lactobacillus* strains did not

affect the ability of either GBS strain to invade the dT-HESCs ($p > 0.05$). It is interesting to note, however, that if calculating the percent of associated cells that invaded, there was a reduction for all cells when co-cultured with *Lactobacillus*. An average of 11.05% of the colonizing GBS cells invaded, while 5.75%, 0.10%, 0.94% and 1.04% of GBS invaded while in co-culture with *Lr* 6475, *Lg* 33323, *Lr* 17938 and *Lc* 19390, respectively. A similar reduction was observed with the invasive strain of GBS, with 3.68% of GBS invading in mono-culture compared to 1.00%, 0.03%, 0.10% and 0.13% when co-cultured with each *Lactobacillus* strain, respectively. Because these experiments were conducted separately, we cannot determine if these differences are significant as they cannot be paired in a way to allow for biological replicates.

Co-culture with *Lactobacillus* variably affects host cell death

GBS is known to lyse host cells using the β-hemolysin, CylE, and this hemolysis could influence the ability of GBS to cross barriers such as the placental membranes.[5] To determine if *Lactobacillus* is capable of preventing from GBS-mediated cell lysis, we performed host cell permeability assays. We have previously established that *Lactobacillus* alone does not induce dT-HESC death (Figure 2.5). Both the colonizing and invading GBS strains significantly damaged the host cells, causing 71.16% ($p < 0.00005$) and 70.78% ($p < 0.00005$) cell death in the four-hour period compared to the mock infection (24.85%) (Figure 3.4). No significant difference in cell damage was observed between the colonizing and invasive strains ($p > 0.05$). Certain strains of *Lactobacillus* significantly reduced cell death when co-cultured with GBS. These effects, however, were dependent on the GBS strain. For the colonizing GBS strain, *L. reuteri* 17938 and *L. crispatus* 19390, reduced host cell death from 71.16% to 58.21% ($p < 0.05$) and 56.59% ($p < 0.005$),

respectively (Figure 3.4A). Conversely, in the invasive GBS strain, only *L. reuteri* 6475 significantly reduced host cell death from 70.78% to 53.25% ($p < 0.05$; Figure 3.4B)

Lactobacillus supernatants inhibit GBS growth

Because *Lactobacillus* is unable to grow in the infection media, we hypothesized that it may not be producing the secondary metabolites that could inhibit GBS. To assess secreted metabolites or other inhibitory compounds, we grew *Lactobacillus* overnight and collected the supernatant. Supernatants were added at 10% v/v of supernatant to GBS, and differences in growth were assessed by calculating the Area Under the Curve (AUC). Supernatants from *Lr* 6475, *L. gasseri* 33323 and *Lr* 17938 reduced the AUC of the colonizing strain from 1.44 to 0.46 ($p < 0.00005$), 0.25 ($p < 0.00005$) and 0.53 ($p < 0.00005$), respectively (Figure 3.5A). Similarly, the same strains reduced the AUC of the invasive strain from 1.21 to 0.39 ($p < 0.00005$), 0.37 ($p < 0.00005$), and 0.68 ($p < 0.0005$), respectively (Figure 3.5B). The supernatant of *L. crispatus* 19390 did not affect GBS growth ($p > 0.05$; 1.44 to 1.39 & 1.21 to 1.50) and no statistical difference was observed between the colonizing and invasive GBS strains.

One characterized secreted compounds is As reuterin is created by altering glycerol,[33] we also assessed the growth effects of supernatants created by *Lactobacillus* grown with glycerol. The addition of glycerol, however, did not significantly reduce the AUC for either GBS strain upon co-culture with *L. reuteri* 6475, *L. gasseri* 33323, or *L. crispatis* 19390 ($p > 0.05$; Figure 3.5). Conversely, addition of glycerol did increase the inhibitory effect of *Lr* 17938 ($p < 0.05$). For the colonizing strain of GBS, the addition of glycerol further reduced the AUC to 0.15 ($p < 0.05$), while it further reduced the AUC of the invasive strain to 0.29 ($p < 0.005$).

Lactobacillus supernatants prevent GBS biofilm formation

Since we found that supernatants affected growth of GBS, we hypothesized they may also impact GBS biofilm formation. Given that only one of the two *L. reuteri* strains was affected by glycerol addition, we only examined the effect of supernatant without glycerol, which was added (10% v/v) at the beginning of the 48-hour incubation period. Statistical differences in GBS biofilm formation were observed for all the supernatants compared to the control except for the invasive strain with *L. reuteri* 6475 supernatant (Figure 3.6). Supernatants of *L. reuteri* 6475, *L. gasseri* 33323, *L.reuteri* 17938, and *L. crispatus* 19390 decreased biofilm formation in the colonizing strain from 1.72 to 0.16 ($p < 0.00005$), 0.31 ($p < 0.00005$), 0.21 ($p < 0.00005$) and 0.70 ($p < 0.0005$), respectively. The same supernatants also decreased biofilm production in the invasive strain from 1.26 to 0.72 ($p > 0.05$), 0.29 ($p < 0.005$), 0. 15 ($p < 0.0005$) and 0.43 ($p < 0.005$). No significant differences were found between how a given supernatant affected the colonizing versus invasive strains ($p > 0.05$).

Lactobacillus supernatants variably affect association of GBS, but do not affect invasion

To further assess the effects of *Lactobacillus* supernatants, we characterized how the addition of this supernatant would affect association and invasion of dT-HESCs. As this experimental design accounts for growth within the time span of the experiment, differences would be independent of the growth changes observed in Figure 3.7. Because *Lactobacillus* is a lactic acid producing bacteria, we were concerned that the supernatant may damage the host cells, which are sensitive to pH changes. We confirmed there was no significant effect of supernatant on host cell permeability for all supernatants, except that of *L. gasseri* ($p < 0.05$; Figure 3.7). *L. gasseri* supernatant significantly increased the host cell permeability from 27.27%

to 59.84% in three hours ($p < 0.05$; Figure 3.7); therefore, the following results using this supernatant in combination with dT-HESCs must be interpreted carefully as results may be due to supernatant-induced host cell permeability.

Generally, the addition of all *Lactobacillus* supernatants, with the exception of *L. reuteri*, reduced association of GBS with dT-HESCs. 10% v/v of supernatant was added at the beginning of the incubation period and percent association was examined as a percentage of each GBS strain alone to account for variation between trials. When *Lactobacillus* was grown without glycerol, the supernatant of *L. reuteri* 6475 increased association of the colonizing strain to 169.90% of the strain alone. Conversely, *L. gasseri* 33323, *L.reuteri* 17938, and *L. crispatus* 19390 reduced the association of the colonizing strain to 35.85% ($p > 0.05$), 29.74% ($p < 0.05$) and 4.41% ($p < 0.005$), respectively (Figure 3.8A). The addition of glycerol during *Lactobacillus* growth also resulted in reduced association for the colonizing strain, but this decrease was not statistically significant. Indeed, supernatants with glycerol from *L. reuteri* 6475, *L. gasseri* 33323, *L.reuteri* 17938, and *L. crispatus* 19390 reduced association to 41.85% ($p > 0.05$), 14.16% ($p < 0.005$), 72.27% ($p > 0.05$) and 50.00% ($p > 0.05$) of the invasive strain without supernatant added.

Association of the invasive strain was also reduced by *Lactobacillus* supernatants (Figure 3.8B). Supernatants without glycerol from *L. reuteri* 6475, *L. gasseri* 33323, *L.reuteri* 17938, and *L. crispatus* 19390 reduced association of the colonizing strain to 44.50% ($p < 0.00005$), 21.36% ($p < 0.00005$), 38.17% ($p < 0.00005$) and 30.49% ($p < 0.00005$). Similarly, supernatants made with glycerol also significantly reduced association. Association of the invasive strain was reduced to 21.95% ($p < 0.00005$), 34.38% ($p < 0.00005$), 27.36% ($p < 0.00005$) and 23.99% ($p <$

0.00005) by *L. reuteri* 6475, *L. gasseri* 33323, *L.reuteri* 17938, and *L. crispatus* 19390, respectively. There were no significant differences between how the colonizing and invasive strains interacted with a given supernatant, with the exception of *L. reuteri* grown without glycerol, which increased association of the colonizing strain but reduced association of the invasive strain ($p < 0.00005$).

Invasion of the placental membrane is an important step in *in utero* infection for GBS; therefore, we also sought to assess the effect of these supernatants on intracellular invasion. As with the association experiments, supernatant was added at 10% v/v, and the percent invasion was normalized to the invasion of the GBS strain alone to account for variation between replicates. We found that even controlling for variation between the GBS strain alone between biological replicates did not account for all the variation observed in these experiments (Figure 3.8C&D). The effect of supernatants from *L. crispatus* 19390 were particularly variable (Figure 3.8C). Given this high variability, we did not observe any statistical differences with the addition of *Lactobacilli* supernatants ($p > 0.05$, Figure 3.8 C&D).

<u>*Lactobacillus* supernatants increase host cell death in the invading GBS strain</u>

As maintenance of the placental membranes is important for a successful pregnancy, we examined if the supernatants altered the amount of host cell damage GBS does. To this end, we examined host cell death in the presence of both GBS and *Lactobacillus* supernatants five hours post-infection. Indeed, we observed an increase in host cell death that was dependent on the GBS strain. The colonizing strain of GBS caused 60.5% cell death alone, but, with the addition of supernatants from *L. reuteri* 6475, *L. gasseri* 33323, *L.reuteri* 17938, and *L. crispatus* 19390, cell death increased to 74.8%, 77.0%, 75.1% and 81.0%. These increases were not statistically

significant ($p > 0.05$). The invasive strain of GBS also increased host cell death in the presence of *Lactobacillus* supernatant. Percent host cell death increased from 53.6% alone to 77.8% ($p < 0.05$), 79.9% ($p < 0.005$), 83.6% ($p < 0.005$) and 77.1% ($p < 0.05$) with *L. reuteri* 6475, *L. gasseri* 33323, *L.reuteri* 17938, and *L. crispatus* 19390, respectively. No statistical differences were observed between host cell death with the colonizing strain versus the invasive strain with the same supernatant.

Supernatant from *Lactobacillus reuteri* 6475 broadly inhibits GBS strains

To assess if growth inhibition of GBS was specific to the two strains used in the experiments above, an extended list of strains was used that spanned ST, capsule and clinical type (Table 3.1). We found that GBS growth was broadly inhibited, averaging 46.6% of each GBS strain alone and ranging from 39.2% to 54.7% (Figure 3.10). We did not observe any statistical differences between the strains' growth inhibition (unpaired ANOVA). Further, we observed no differences based on sequence, capsule or clinical type.

DISCUSSION

Efforts to reduce the burden of GBS through antibiotic treatment have not been fully successful.[3] While reducing early-onset disease, there has been no effect on late-onset disease or adverse pregnancy outcomes. Further, the use of antibiotics is not without their own negative effects including effects on maternal and neonatal microbiomes, increased risk of *E. coli*-associated disease and antibiotic resistance.[10] For this reason, alternative therapies, like probiotics, have been suggested. Herein, we sought to characterize the effects of *Lactobacillus* and its secreted factors on GBS and key components of its interactions with dT-HESCs. To determine if GBS-*Lactobacillus* interactions could play a role in differences in disease outcome between different strains of GBS, we also compared interactions following exposure to two strains of GBS from different sources, a neonate with infection and a colonized pregnant woman.

Surprisingly, we found that live *Lactobacillus* does not affect the growth or biofilm production of either GBS strain (Figure 3.1&2). This result is likely due to the inability of *Lactobacillus* to grow in both the infection media and the biofilm media (Figure 2.1). It would be interesting to determine the relative composition the *Lactobacillus* and GBS within the biofilm as previous work has found that *Lactobacillus* can inhibit biofilm formation in other species, including *Pseudomonas fluorescens* and *Bacillus cereus* in biofilms.[34] Further, *Lactobacillus* did not greatly alter GBS interactions with dT-HESCs, which serve as a model of the outermost layer of the placental membranes. While being able to attach (Figure 2.3), live *Lactobacillus* cannot inhibit the association of GBS (Figure 3.3). The MOI of this experiment may be too low to be able to assess whether these two organisms would have to compete for binding sites on the cells. While most strains had no effect, *L. gasseri* significantly increased association of both the colonizing and invasive GBS, suggesting that the *L. gasseri* and GBS may interact in some way

to increase the attachment. It has been previously observed that this strain of *Lactobacillus* can aggregate with other pathogens, which may increase the colonization of both *L. gasseri* and GBS if the aggregate is secured to the host cells.[35] This increased association may not be negative as it did not also lead to an increase in invasion (Figure 3.3) or host cell death (Figure 3.5).

Because *Lactobacillus* is not capable of growing in the medias used for the experiments, we were not assessing the impact of any secreted factors; therefore, we isolated the supernatants of *Lactobacillus* grown over-night and challenged GBS with it. We first found effects on growth and biofilm production. Biofilms are a congregation of cells surrounded by a polysaccharide matrix.[36] We hypothesize that the reduction in biofilm production may be due to the limited growth of GBS. Previous work has demonstrated the importance growth conditions for GBS biofilm production.[31] It is interesting to note that though supernatant from *L. crispatus* did not significantly inhibit growth, it did significantly inhibit biofilm formation ($p < 0.005$). While this growth deficiency may explain the effects on biofilm production, it does not explain the effects on association. Because the experimental design accounts for growth, the reduction observed is due to another factor. Further characterization of the supernatants and the effects of association to determine how the association in being lowered.

We also observed high variation in the impact of the supernatant on GBS invasion. While the host cell death assay time points are three hours later than those for the invasion assay, these results could suggest that the host cells are becoming more permeable within the time points of the invasion assay. If this cell death is increased or decreased during the first two hours of the experiment, it may explain the high variability between biological replicates, as the assay requires the host cell to be non-permeable to protect the invaded GBS from antibiotic treatment.

It is also interesting that host cell death is increased with the combination of GBS and supernatant (Figure 3.9) as the supernatant itself did not affect host cell death, with the exception of *L. gasseri* (Figure 3.7). This suggests that the GBS alters its interactions in some way in response to the supernatant, which was more pronounced in the invasive strain of GBS compared to the colonizing strain (Figure 3.9). Hence, these data suggest that there may also be differences in how these two strains respond. Previous research has found that stressors, including antibiotics and reactive oxygen species, alter GBS interaction with host cells including increasing macrophage uptake.[37] Further experiments will be needed to access how GBS alters its transcription or protein profile to better understand these changes. This increase in virulence in response to *Lactobacillus* supernatants is of particular importance because the placental membranes are key to maintaining a health pregnancy.

Lactobacillus is known to secrete a large number of factors that could be affecting GBS. We hypothesized that reuterin may be one. Reuterin is created by altering glycerol;[33] therefore, we hypothesized that glycerol addition would result in increased GBS inhibition. However, we only observed increased inhibition by *L. reuteri* 17938, suggesting that it is the only strain capable of producing it. Since we observed effects on growth and association, it would be interesting to determine what components of the supernatant are responsible for each phenotype. Further work is needed to examine the different components of the supernatants. The supernatants could be fractionated and examined individually for the effects observed on association and growth. This fractionation could also allow for separation of positive effects of the supernatant, such as inhibition of GBS growth (Figure 3.5), from negative effects including increased GBS-induced host cell death (Figure 3.9). Identification of the growth inhibitory

compound would be of particular interest due to its broad impact on GBS growth across strains (Figure 3.10).

Collectively, these data suggest that different strains of *Lactobacillus* have variable effects on GBS strains and that these effects may be further complicated by the GBS strain. While current literature examines the effect of *Lactobacillus* on vaginal-rectal colonization, we sought to determine if this organism could also impact GBS phenotypes that are known to be important for GBS ascending infections, including growth, biofilm production, association with and invasion of decidual cells and host cell death. We found that while live *Lactobacillus* has minimal impact on GBS, its supernatant could impact growth, biofilm production, association and induced host cell death. Some of these effects, namely increased association and induced host cell death, are concerning when considering the importance of the placental membranes in maintaining a healthy pregnancy, demonstrating the importance of thorough examination of the effects of any potential alternative therapy. Further examination of the complex supernatants may reveal an individual compound that could serve such a purpose.

ACKNOWLEDGEMENTS

We would like to thank Dr. Cindy Arvidson and Dr. Poorna Visvanathan for donation of *Lactobacillus* strains. We would also like to thank Dr. Neal Hammer and Dr Arvidson for their careful editing of the chapter.

APPENDIX

Table 3.1. Extended GBS strain list. ST: Sequence Type; CPS: capsule type, VRC: vaginal-rectal swab, EOD: early onset disease, LOD: late onset disease

GBS Strain Information				
Strain	Clinical Type	ST	CPS	Colonization Site
GB00012	Colonizing	ST-1	cpsV	VRC
GB00020	Colonizing	ST-1	cpsV	VRC
GB00033	Invasive	ST-23	cpsIa	EOD/sepsis
GB00036	Invasive	ST-19	cpsIII	EOD/sepsis
GB00037	Invasive	ST-1	cpsV	EOD/sepsis
GB00045	Invasive	ST-17	cpsIII	EOD/sepsis
GB00066	Invasive	ST-19	cpsIII	EOD/sepsis
GB00079	Invasive	ST-19	cpsIII	EOD/sepsis
GB00084	Colonizing	ST-1	cpsVIII	VRC
GB00097	Colonizing	ST-17	cpsIII	VRC
GB00121	Invasive	ST-26	cpsV	Unknown
GB00279	Colonizing	ST-23	cpsII	VRC
GB00285	Colonizing	ST-12	cpsIb	VRC
GB00291	Colonizing	ST-12	cpsII	VRC
GB00305	Colonizing	ST-12	cpsIa	VRC
GB00310	Invasive	ST-1	cpsV	EOD/sepsis
GB00374	Invasive	ST-12	cpsIb	EOD/sepsis
GB00377	Invasive	ST-19	cpsIII	EOD/sepsis
GB00390	Invasive	ST-23	cpsIa	EOD/sepsis/meningitis
GB00418	Invasive	ST-17	cpsIII	EOD/sepsis
GB00438	Invasive	ST-12	cpsIb	LOD/sepsis
GB00555	Colonizing	ST-12	cpsIb	VRC
GB00557	Colonizing	ST-17	cpsIII	VRC
GB00561	Colonizing	ST-19	cpsV	VRC
GB00571	Colonizing	ST-19	cpsIII	VRC
GB00590	Colonizing	ST-19	cpsIII	VRC
GB00620	Colonizing	ST-1	cpsIa	VRC
GB00651	Colonizing	ST-19	cpsIb	VRC
GB00653	Colonizing	ST-12	cpsII	VRC
GB00663	Colonizing	ST-19	cpsIII	VRC
GB00686	Invasive	ST-1	cpsV	Stillbirth
GB00910	Invasive	ST-12	cpsII	EOD/sepsis
GB01007	Invasive	ST-19	cpsIII	Stillbirth
GB01454	Invasive	ST-1	cpsV	Stillbirth
GB01455	Invasive	ST-12	cpsII	Stillbirth
NEM316	Invasive	ST-23	cpsIII	EOD/sepsis

Figure 3.1. *Lactobacillus* does not impact GBS growth. Individual GBS and *Lactobacillus* strains were added at a 1:1 ratio in co-culture. Growth was observed by colony-forming units for six hours. The Area Under the Curve (AUC) was calculated with PRISM 6. Statistical analysis was performed using an unpaired ANOVA to evaluate the AUC. Error bars represent standard deviation between three biological trials.

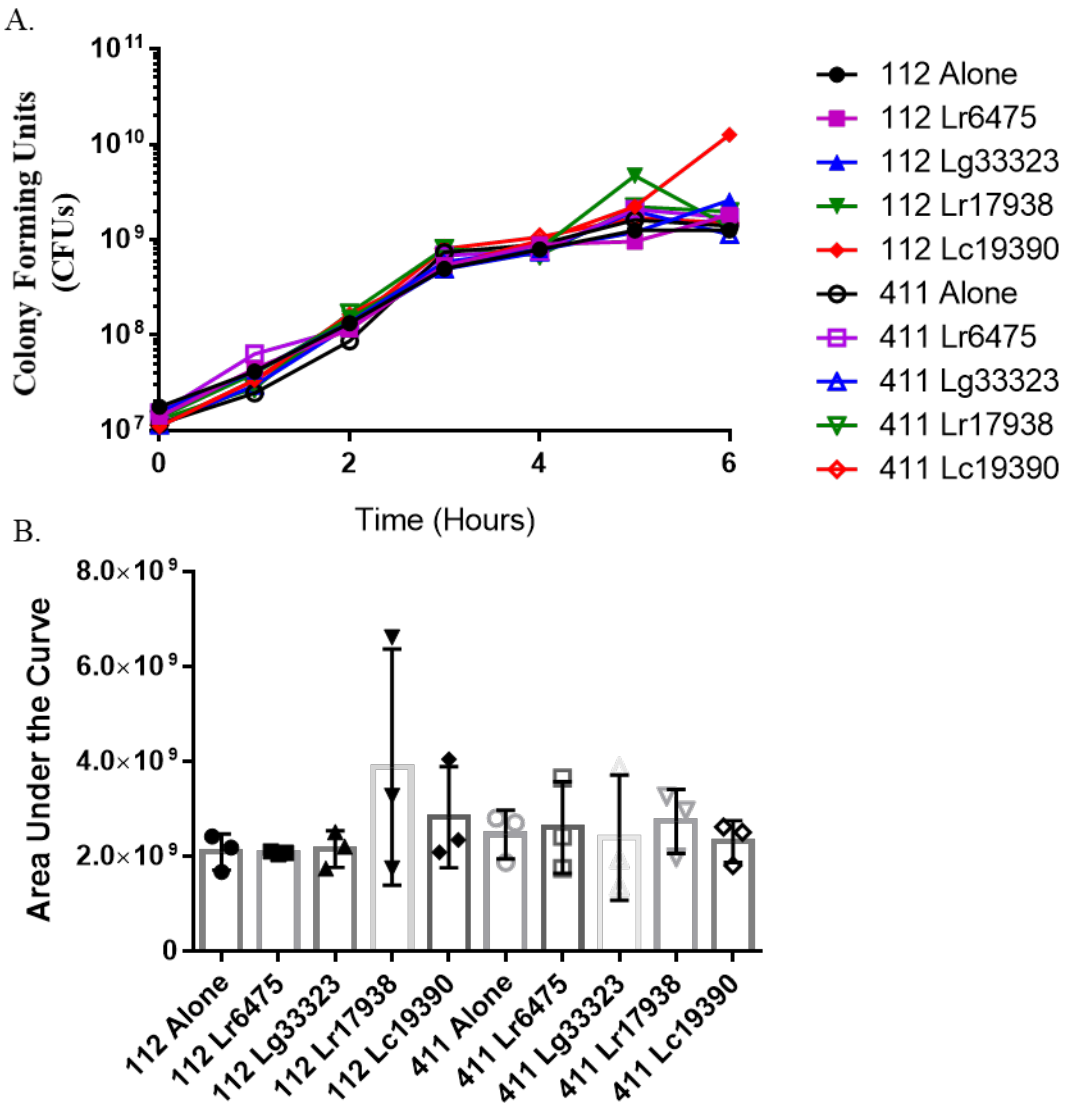
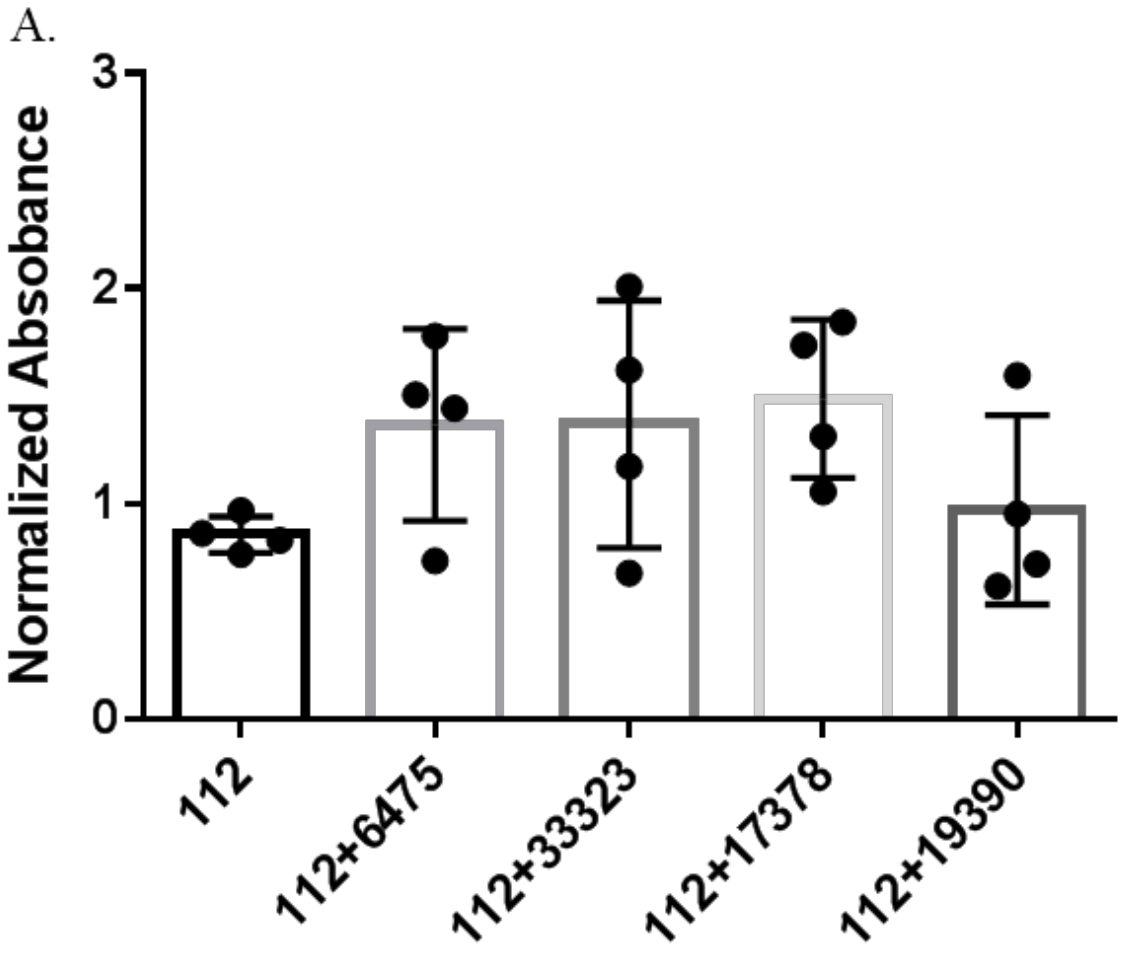


Figure 3.2. *Lactobacillus* does not affect GBS biofilm formation. GBS and *Lactobacillus* were added in a 1:1 ratio to a 96-well plate. After 48 hours, wells were washed to remove non-adherent cells and stained with crystal violet. Normalized absorbance was calculated by taking OD_{595}, subtracting the media control and multiplying by four. Error bars represent standard deviation between biological trials. No statistical differences were found by unpaired ANOVA.



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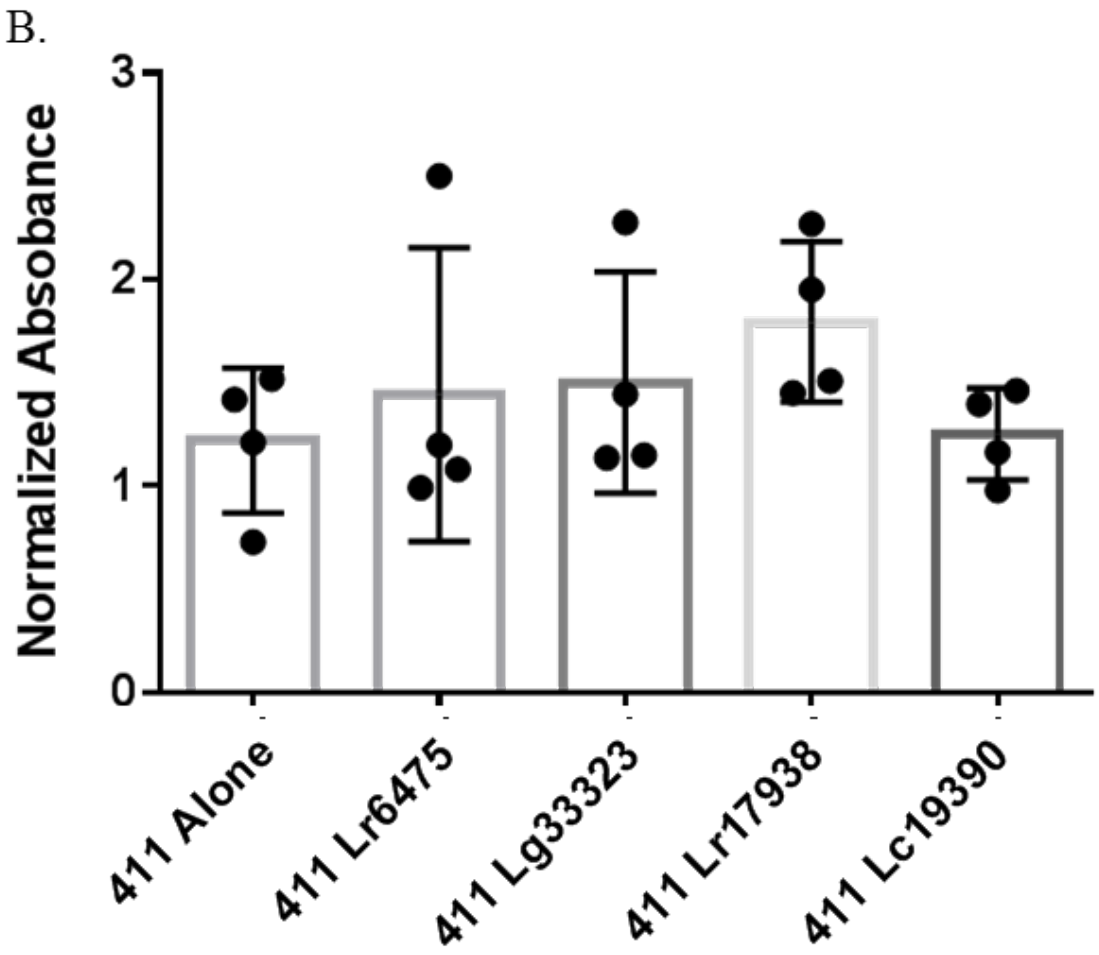


Figure 3.3. *Lactobacillus* variably affects GBS association with dT-HESCs. dT-HESCs were infected with GBS at a MOI of 10 for two hours with or without an equivalent amount of *Lactobacillus*. The percent of associated and invaded bacteria were calculated relative to the total number of bacteria in the well. Experiments were completed in biological quadruplets of technical triplicates. Error bars represent standard deviation between biological trials. Significance was determined by an unpaired ANOVA. $p < 0.005$ **

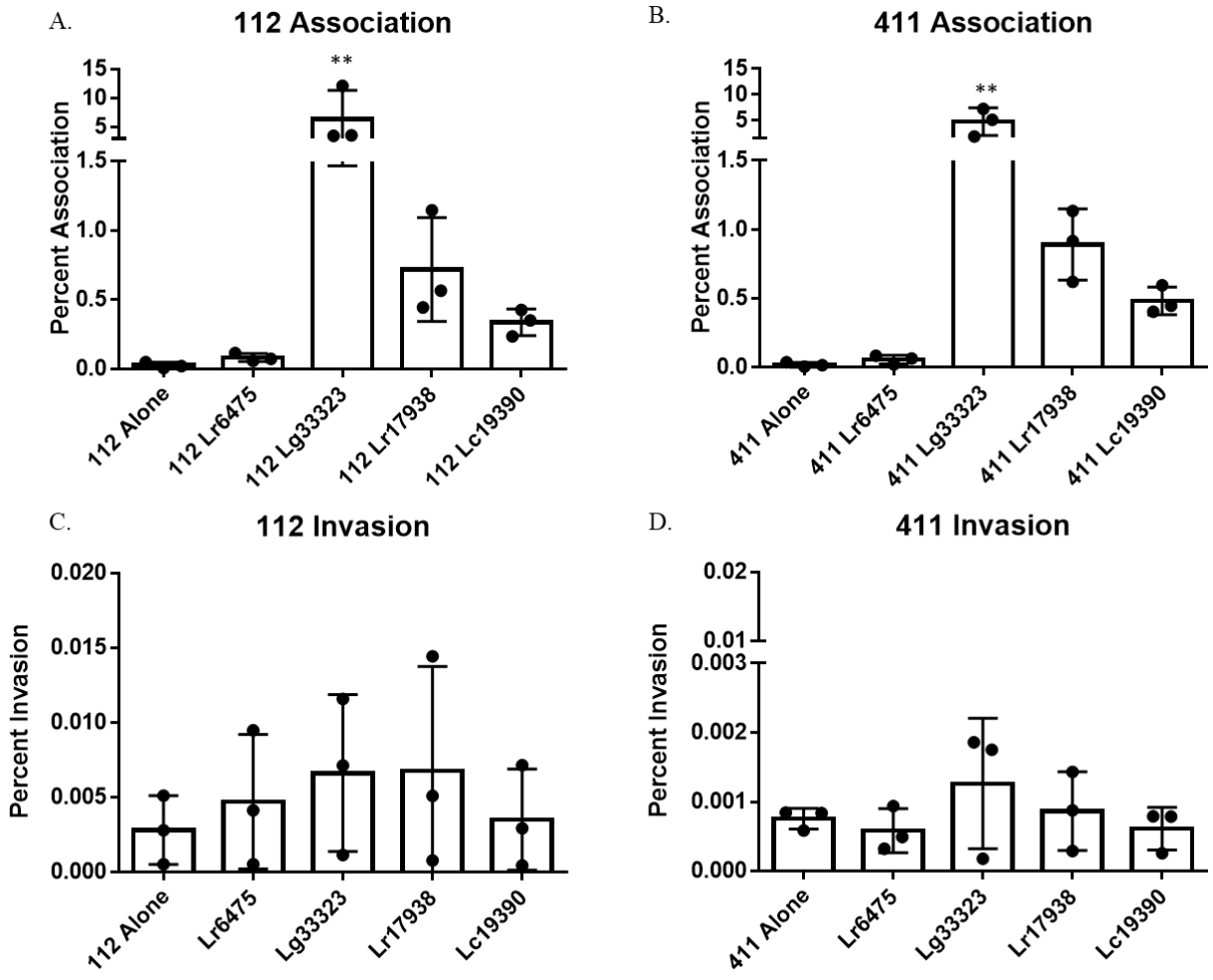


Figure 3.4. *Lactobacillus* variably affects host cell death. dT-HESCs were infected with GBS at a MOI of 10 with or without the equivalent amount of *Lactobacillus* and incubated for four hours. Cell permeability was detected using an ethidium homodimer, and percent permeability was calculated by lysing the remaining cell in each well. Graphed data represents three biological replicates, and the error bars represent the standard deviation of the data. Significance was determined using an unpaired ANOVA between mono-culture and co-culture.
$p < 0.05$ *; $p < 0.005$ **; $p < 0.00005$ ####All other conditions were significantly higher than the media control.

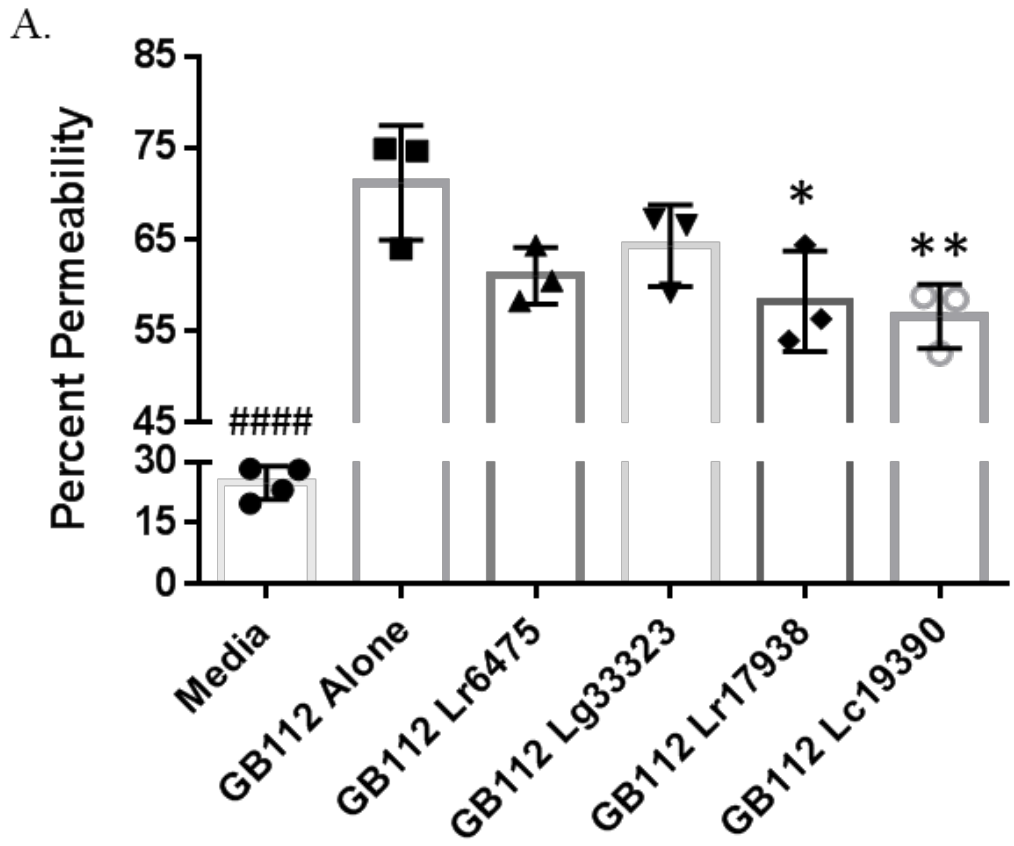


Figure 3.4 (cont'd)

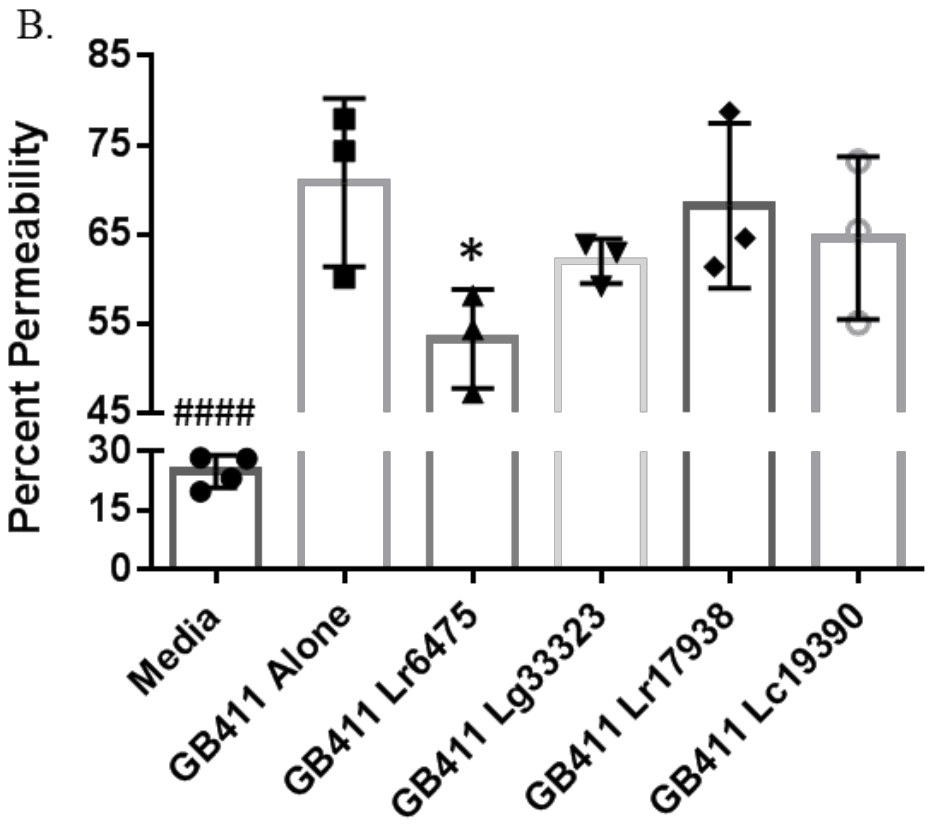
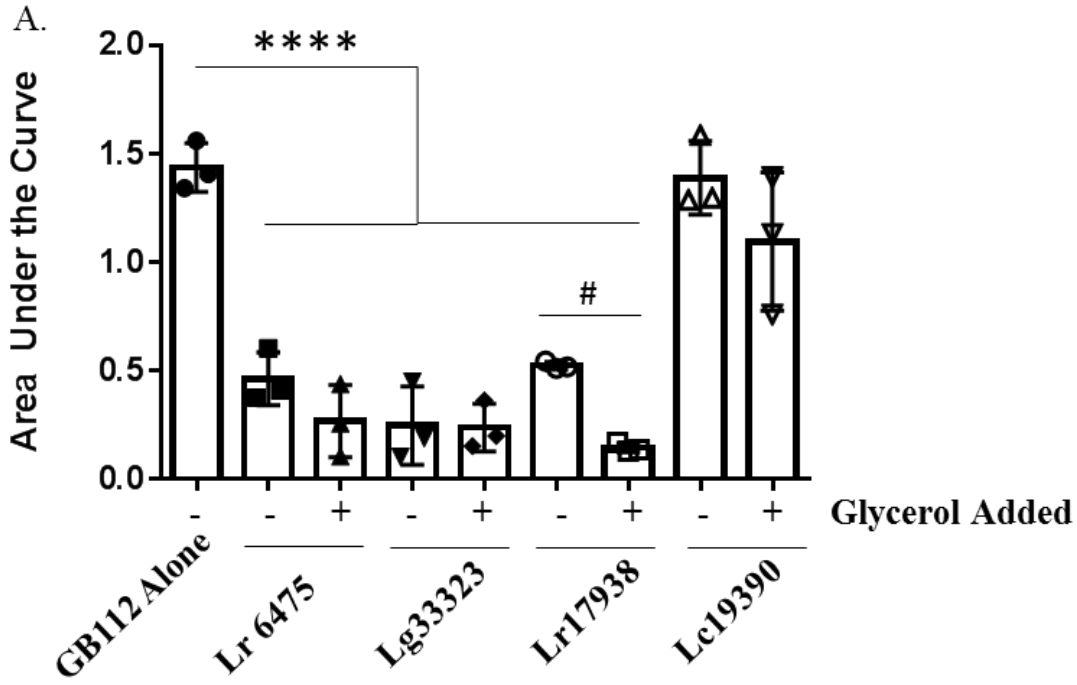


Figure 3.5. *Lactobacillus* supernatants inhibit GBS growth. GBS growth was monitored for eight hours by plate reader (OD_{595}) with or without *Lactobacillus* supernatant (25% v/v). The Area Under the Curve (AUC) was calculate with PRISM 6. Error bars represent standard deviation between three biological trials. A Student's T-test was used to evaluate differences between the addition of glycerol. $p < 0.05$ # An unpaired ANOVA was used to assess differences between each supernatant condition and GBS alone. $p < 0.00005$ ****



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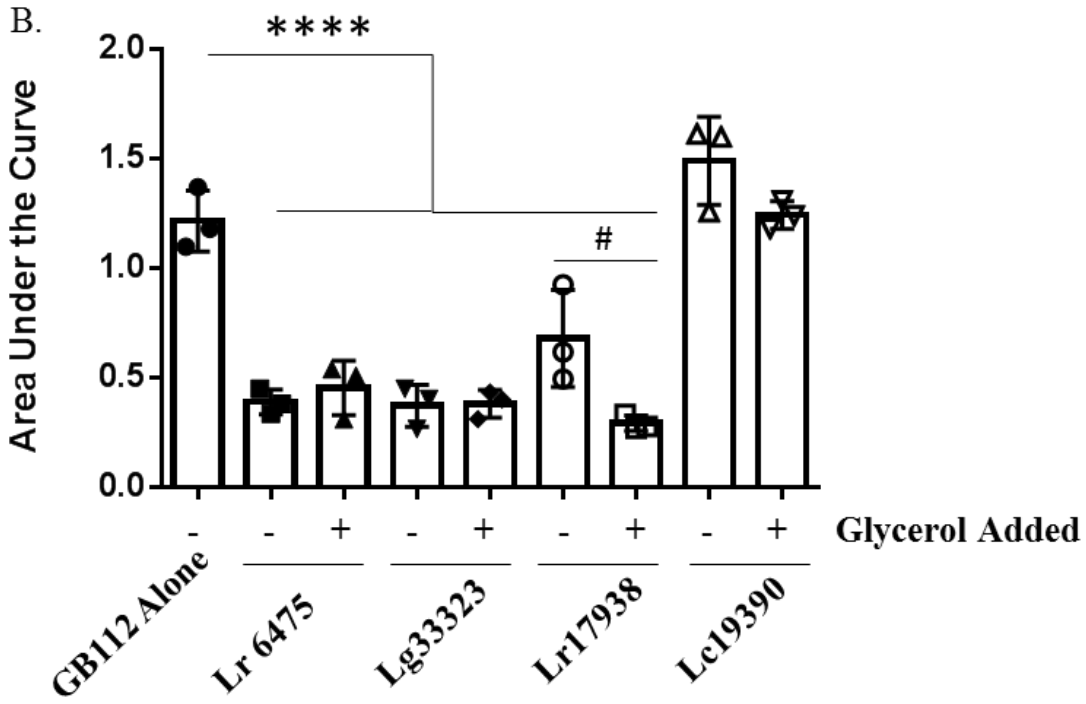


Figure 3.6. *Lactobacillus* supernatants prevent GBS biofilm formation. GBS wase added to a 96-well plate with or without 10% v/v *Lactobacillus* supernatant without glycerol added. After 48 hours, wells were washed to remove non-adherent cells and stained with crystal violet. Normalized absorbance was calculated by taking OD_{595}, subtracting the media control and multiplying by four. Error bars represent standard deviation between biological trials. Statistical differences between GBS alone and with supernatant added were calculated in PRISM 6 with unpaired ANOVA. $p < 0.005$ ** $p < 0.0005$ *** $p < 0.00005$ ****

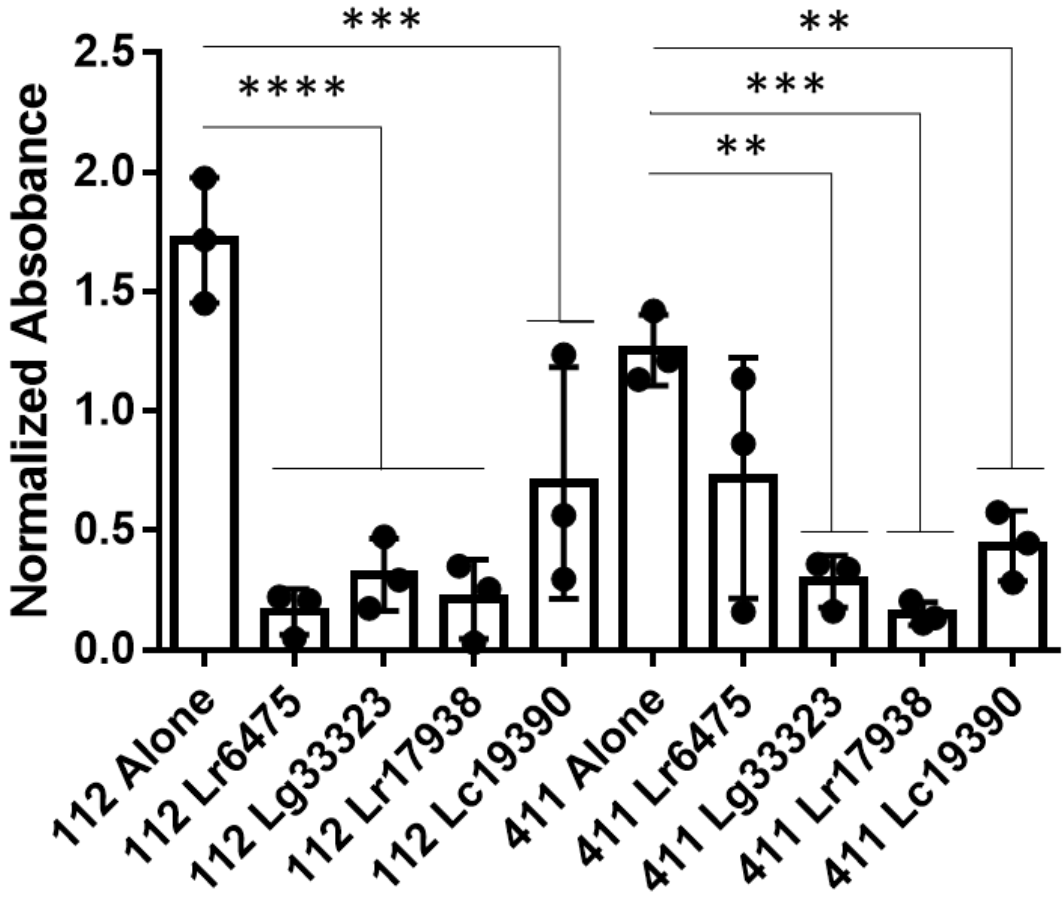


Figure 3.7. *Lactobacillus* supernatants variably affect host cell permeability. dT-HESCs were incubated with *Lactobacillus* supernatants for four hours. Cell permeability was detected using an ethidium homodimer assay, and percent permeability was calculated by lysing the remaining cell in each well. Graphed data represents three biological replicates, and the error bars represent the standard deviation of the data. Significance was determined using an unpaired ANOVA to compare each condition to the media alone. $p < 0.005$ **

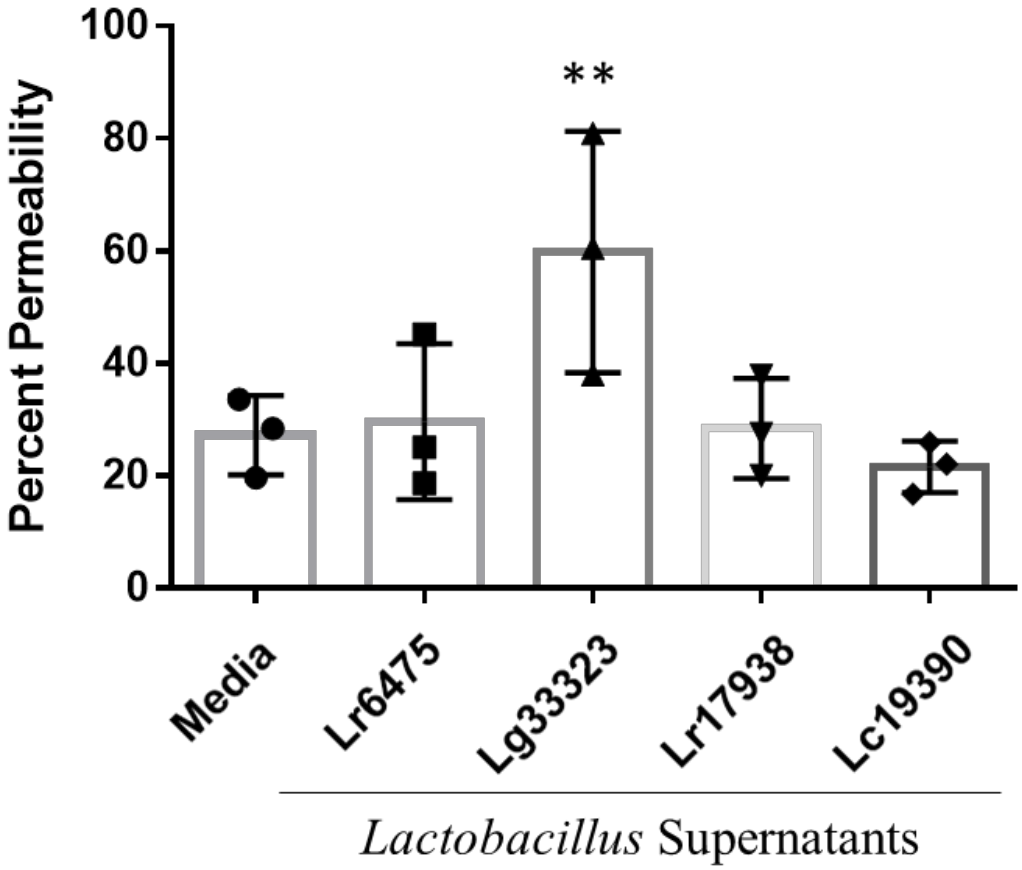


Figure 3.8. dT-HESCs were infected with GBS at a MOI of 10 for two hours with or without 10% v/v *Lactobacillus* supernatant. The percent of associated and invaded bacteria were calculated relative to the total number of bacteria in the well. Experiments were completed in biological quadruplets of technical triplicates. Error bars represent standard deviation between biological trials. Significance between GBS alone and with supernatant was determined by an unpaired ANOVA. $p < 0.05$ *

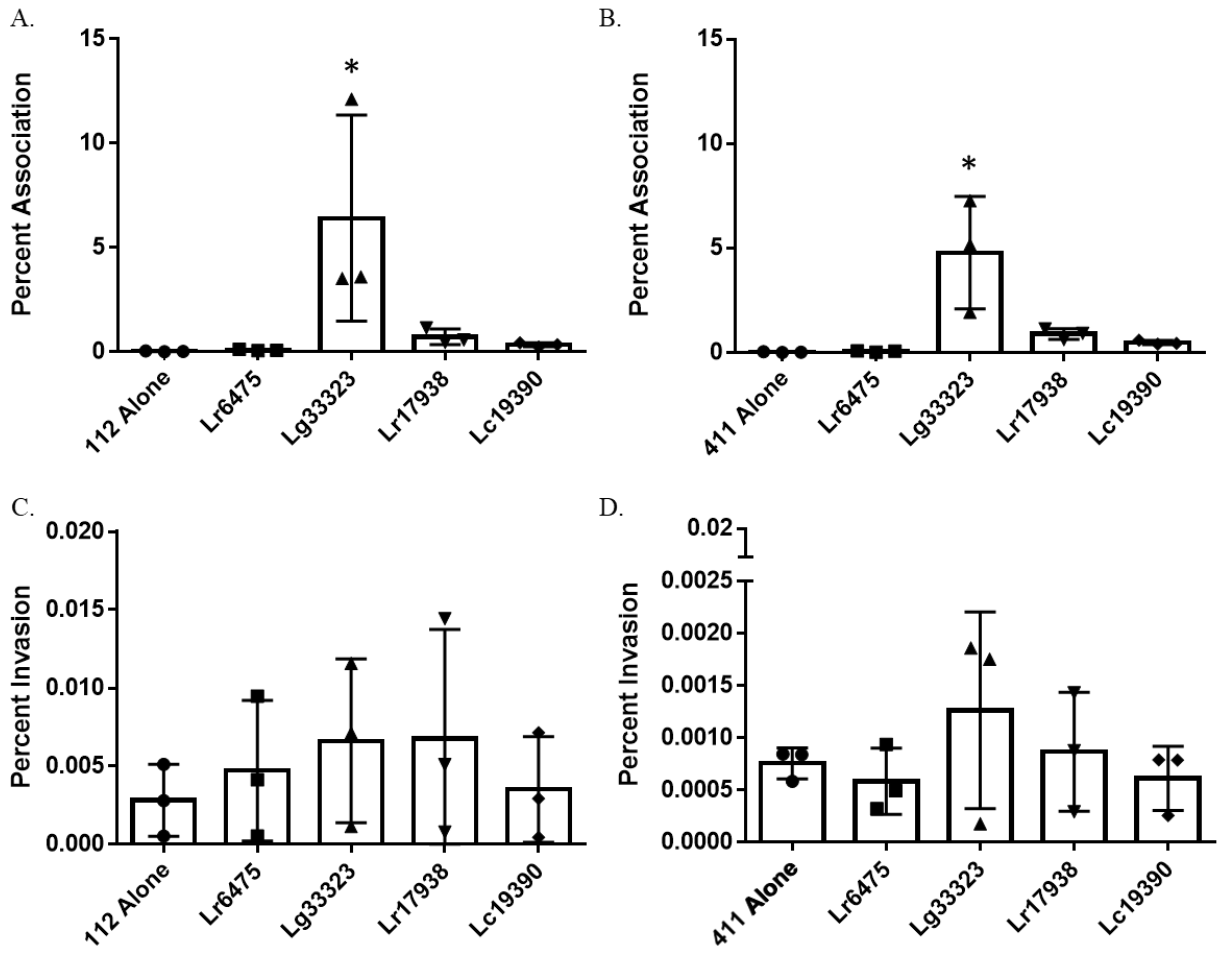


Figure 3.9. *Lactobacillus* supernatants increase GBS-induced host cell death. dT-HESCs were infected with GBS at a MOI of 10 with or without 10% v/v *Lactobacillus* supernatant and incubated for four hours. Cell permeability was detected using an ethidium homodimer, and percent permeability was calculated by lysing the remaining cell in each well. Graphed data represents three biological replicates, and the error bars represent the standard deviation of the data. Significance was determined using an unpaired ANOVA between GBS alone and with supernatant. $p < 0.05$ *; $p < 0.005$ **

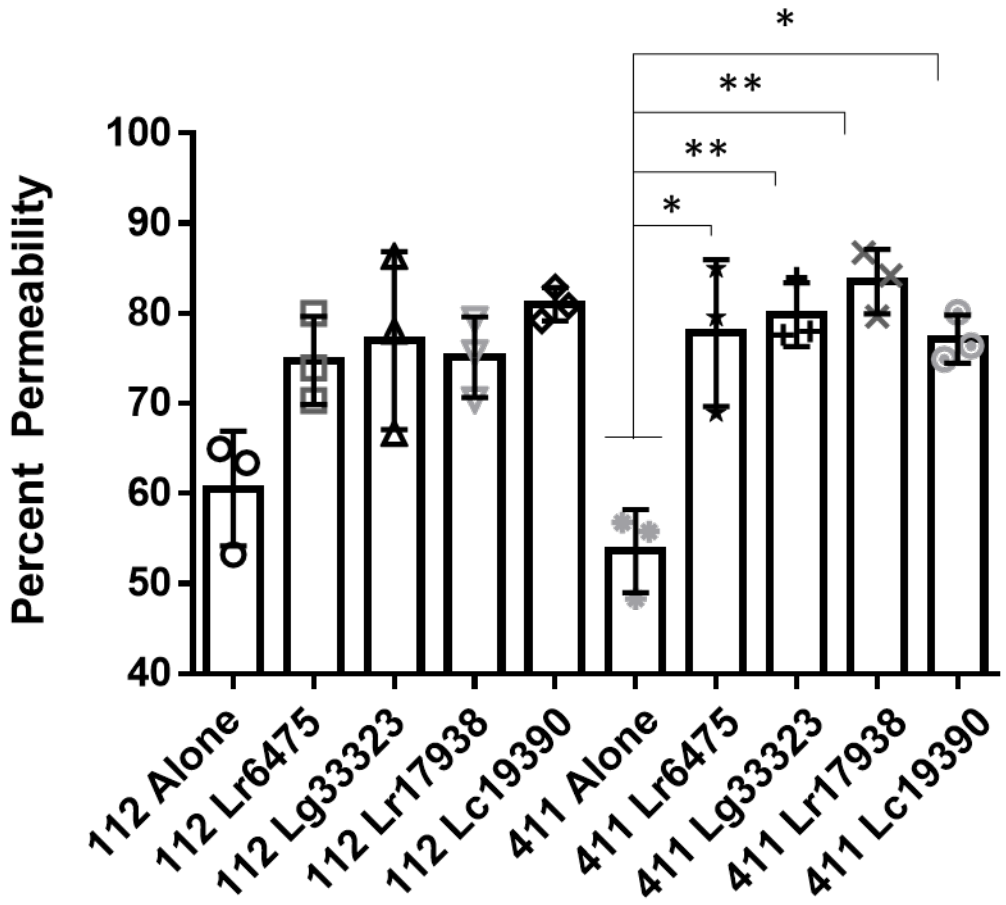
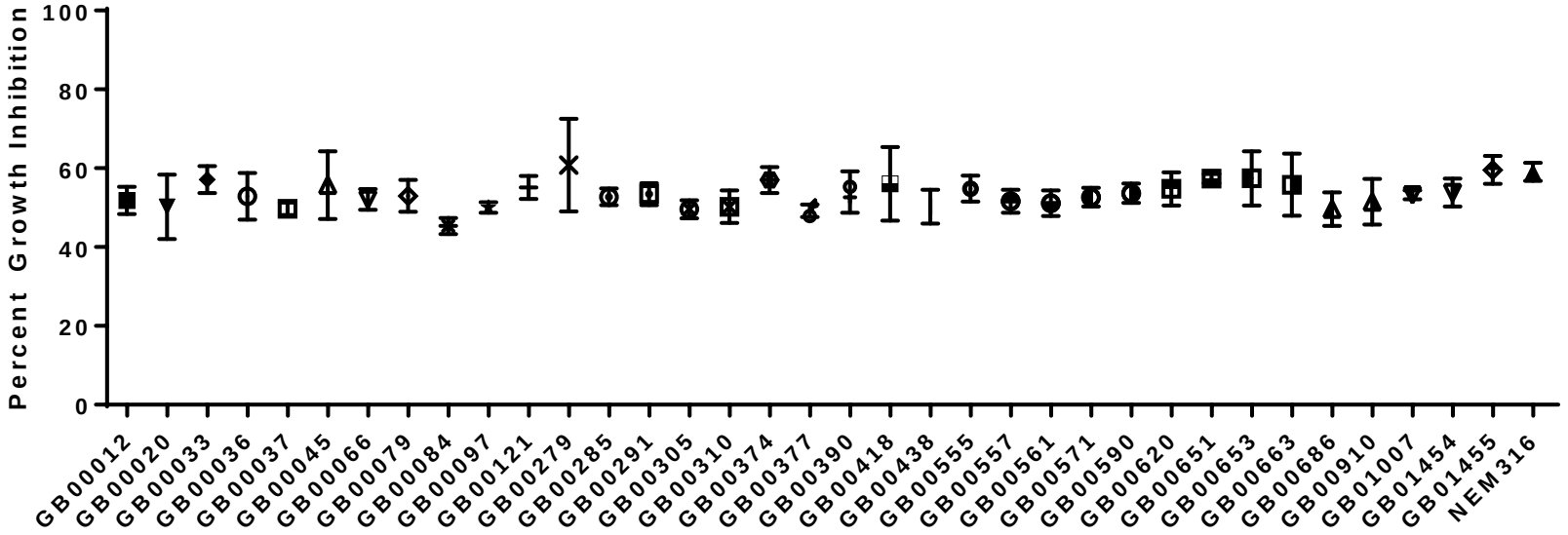


Figure 3.10. Supernatant from Lr6475 broadly inhibits GBS growth. GBS growth was monitored for eight hours by plate reader (OD_{595}) with or without Lr6475 supernatant (25% v/v). The Area Under the Curve (AUC) was calculate with PRISM 6. Data is presented as the percent of growth with the supernatant compared to without. Error bars represent standard deviation between three biological trials. No statistical difference was found between strains by unpaired ANOVA.



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CHAPTER 4

THE EFFECT OF PHAGE COMMNUNITIES ISOLATED FROM HUMAN FECAL SAMPLES ON GROUP B *STREPTOCOCCUS*

ABSTRACT

Group B *Streptococcus* colonizes the rectal-vaginal tract of approximately 30% of women but is simultaneously a leading cause of neonatal disease and a major contributor to adverse pregnancy outcomes. Maternal colonization is the greatest risk factor in neonatal infections; however, determinants of this colonization are not fully understood. The bacteriophage component of the microbiome has been shown to preferentially lyse invading pathogens in the gut and is thought to be important in maintaining a healthy bacterial microbiota. Because GBS can be frequently isolated by rectal swab, we hypothesized that phage communities isolated from fecal samples could contain phage capable of inhibiting GBS as GBS is commonly isolated using fecal swabs. Indeed, we found that 6% of phage communities isolated from fecal samples were capable of inhibiting GBS. We further characterized the interactions between complex phage communities and GBS hosts and found that capsule, sequence and clinical types of the strains did not affect lysis patterns. However, the number of spacers in Clustered regularly interspaced palindromic repeats (CRISPR) did correlate to sensitivity to a phage community. As we had observed the ability of these communities to inhibit GBS *in vitro*, we hypothesized that we would observe a difference in GBS genetic presence in the metagenomic reads of the associated phage community. While this correlation did not exist across all GBS strains tested, strains that were generally sensitive to phage communities were significantly more likely to be inhibited by phage communities with a lower abundance of GBS. Collectively, these data indicate the importance of both the GBS strain and the phage community in this interaction and suggest that the phage component of the intestinal microbiome could impact GBS colonization. To further examine this interaction, we sought to isolate and

characterize an individual phage from these communities; however, we were unsuccessful in doing so.

INTRODUCTION

Group B S*treptococcus* (*Streptococcus agalactiae*, GBS) is a leading cause of neonatal infections and deaths in the United States and contributes globally to adverse pregnancy outcomes, including premature birth and stillbirth. While GBS is less recognized for its ability to negatively affect maternal health, studies have found that GBS is responsible for 20% of bacteremia in Ireland[1] and 25% of puerperal bacteremia in the United States.[2] A recent meta-analysis also estimated that the global incidence of GBS maternal effects is 0.38 per 1,000 pregnancies.[3] Combined with the increased risk of preterm birth (Risk Ratio = 1.21), maternal GBS colonization continues to be a major health concern worldwide.[4] GBS colonization is assessed in the United States by the combination of a vaginal and rectal swab at 37 weeks gestation. Swabs from both areas are taken because GBS is thought to colonize the vaginal tract in a rectal-vaginal route and rectal colonization has been shown to be a good predictor of vaginal colonization.[5]

In spite of the known risk of GBS colonization for both the mother and the fetus, the only preventative measure currently employed is Intrapartum Antibiotic Prophylaxis (IAP), which is only effective against early onset neonatal disease. This antibiotic treatment is known to negatively impact both the maternal vaginal microbiome and the neonatal gut microbiome, which are important for neonatal development.[6–10] The shortcomings of IAP combined with growing concern of antibiotic over-use have resulted in increased interest in alternative therapies for the prevention of GBS-associated neonatal disease and pregnancy outcomes. As maternal colonization is the most important risk factor,[11] therapies should ideally reduce maternal colonization; however, they should be more specific than the antibiotic course so that multiple

doses could be used to prevent adverse pregnancy outcomes and neonatal disease while keeping the microbiomes of the mother and neonate intact.

Phage therapy is the use of a virulent bacteriophage to preferentially kill an undesirable bacterium and is of particular interest for GBS because bacteriophage are more specific than antibiotics.[12] The earliest successful publication of phage therapy was in 1917, when Félix d'Hérelle published on the use of a virus to treat bacilli dysentery.[13] While largely overlooked in the United States, phage therapy has been studied and implemented in France, Russia, Poland and Georgia.[13] In spite of this history, few well-structured, accessible studies have been conducted. However, a recent insurgence of interest has produced several key studies that support the efficacy of phage therapy as a therapeutic and alternative to antibiotics[14–18] Most recently, a large, well-controlled study examined the use of bacteriophage in burn wounds.[19] While the trial was canceled because the bacteriophage treatment was not as effective as the standard care of antibiotics, it was able to reduce infections in the burns, suggesting adjustments in the dosing may lead to better results. Though phage therapy has only recently been applied to GBS,[20] the use of phage-derived lysins has been researched. Success with such lysins has been demonstrated against GBS in two different mouse models that represent vaginal colonization in humans and bovine mastitis, which is also caused by GBS.[21,22] While lysins are more specific than antibiotics, allowing for minimal effect on the microbiota, an added advantage of a phage would be self-propagation. Self-propagation allows for phage amplification only in the presence of host bacteria, resulting in limited amplification.[12] Research on lysins combined with the recent study on tilapia validates that phage may be used to specifically attack GBS, but more work is needed to isolate phage and validate them for use as a therapeutic during pregnancy. Phage

capable of infecting GBS have not been extensively studied, though first described by Russell et al. in 1969.[23] Available research has focused on phage isolated from cow milk samples or induction of lysogenic phage residing in GBS strains.[24–26] These phage are typically *Siphoviridae*, which has a double-stranded DNA genome and a non-contractile tail.[27] Additionally, studies that investigated host range found a limited host range that is restricted to Streptococci, for example, Group D *Streptococcus* or Group B *Streptococcus*.[24,28]

Another remaining gap in our understanding of GBS-specific phage is their role in GBS colonization. GBS is estimated to colonize one of every five women worldwide,[29] but we do not fully understand why some women are colonized while others are not. Additionally, this colonization is often transient, but the reason for this transience is also unknown. While a generally overlooked component of the human microbiome, phage outnumber human cells and rival bacteria in the human body and are thought to greatly contribute to the stability and diversity of the human microbiota.[30,31] To date, no studies have examined the prevalence of GBS-specific phage in the human microbiome; however, a recent study has demonstrated that there is a relevant relationship between phage and GBS in the vaginal tract by examining the Clustered regularly interspaced short palindromic repeats (CRIPSR) regions of GBS isolates. CRISPR is thought to act as an adaptive immune system against invading phage and foreign DNA by storing small complementary segments of phage or invading DNA so that it can be used to guide targeted degradation of the invading DNA.[32] Across two studies, Beauruelle *et al.* have demonstrated that GBS strains are actively incorporating CRISPR spacers during vaginal carriage, supporting that GBS is interacting with bacteriophage in the vaginal tract.[33,34]

As GBS is thought to colonize the vaginal tract from a rectal route,[5] we utilized our large collection of phage communities that were previously isolated from fecal samples (unpublished, Nohomovich) to identify GBS-specific phage in these communities. In all, we examined 130 phage communities for their ability to reduce the growth of a GBS strain isolated from a neonate with sepsis. A subset of these communities was then examined using a larger GBS strain subset to examine if any GBS characteristics were determinant of the phage community's ability to reduce GBS growth. Further, the abundance of GBS-assigned reads in certain fecal metagenomic samples was examined to determine if the infectivity of a phage community is associated with GBS abundance in the gastrointestinal tract. Finally, we attempted to isolate lytic phage from three communities of interest, but were unsuccessful to in isolating an individual phage at high enough titer to characterize.

MATERIALS AND METHODS

Bacterial strains and growth conditions

GBS strains were selected based on multilocus sequence type (ST) designation, capsular serotype (CPS), and source. A total of 38 GBS strains were used and are detailed in Table 1; all strains were characterized in prior studies.[35,36] Unless otherwise noted, GBS strains were grown up overnight in Todd Hewitt Broth (THB) with 5% CO_2 at 37°C. The area under the curve (AUC) was determined from individual growth curves using PRISM 6. Growth inhibition was calculated as the percent of AUC with the phage community (PC) over the AUC of the GBS strain alone. [(AUC_{PC} / AUC_{Alone}) * 100].

GBS genome sequencing and extraction of GBS CRISPR spacers

Genomes were available for 11 GBS strains and were sequenced in prior studies (Table 1) as described previously.[37] Sixteen additional strains were sequenced as part of this study, resulting in a total of 27 strains. Briefly, Isolates were grown in THB overnight DNA was isolated using the Wizard® Genomic DNA purification kit and subsequently prepped for sequencing using the Nextera XT kit (Illumina, San Diego, CA, USA) following manufacturer's instructions. Libraries were sequenced. Spades, 3.10.1 was used to *de novo* genome assemblies following trimming and quality checking with Trimmomatic and FastQC, respectively. Multiple k-mers (21, 33, 55, 77, 99, 127) were used and k-mers that passed quality control were cross-assembled to generate the assembly used for downstream analyses. Error correction was performed during the assembly process to minimize the number of mismatches present in the assembled contigs. (www.bioinformatics.babraham. ac.uk/projects/fastqc/). Contigs that contained both genes that flank the CRIPSR repeats (*csn2* and *ndk*) were extracted and analyzed in Geneious.[38] to identify the CRISPR spacers. Any strain that did not have complete loci

containing both genes flanking the CRISPR spacers/repeats was not included in the analysis. Spacers were matched to annotated sequences available at the National Center for Biotechnology Information (NCBI) using the following criteria: <15% difference in alignment length and 85% identity.

Isolation of phage communities from human fecal samples

Phage communities were isolated from stool samples collected from otherwise healthy individuals. These samples were collected as part of a larger study to examine the intestinal microbiome in patients with enteric infections and was described previously.[39] Following collection, all samples were transported in Cary Blair medium, homogenized and centrifuged. The supernatant was collected and filtered with a .22μm filter (Nohomovich, unpublished). The resulting supernatant is referred to as a phage community (PC) herein.

Metagenomics of fecal samples and estimation of GBS abundance in the microbiome

Fecal samples were previously sequenced using the Illumina HighSeq 2500 Rapid Run platform in a 1x150 or 2x250 paired-end format as described (Nohomovich, unpublished) In brief, the output was demultiplexed and converted to FastQ format using Bcl2Fastq v.1.8.4 (Illumina), and reads were quality checked using Trimmomatic.[40] All reads passing quality control were compared to human RefSeq genome, GRCh38_1118, downloaded November 2018 from NCBI with Bowtie 2.[41] SAMtools was used to remove the humans reads.[42] Reads that could be annotated as *Streptococcus agalactiae* at the species level using Kaiju[43] with default parameters were extracted. The percent GBS in the metagenomic sample was calculated as the number of GBS reads divided by the total number of reads per sample and multiplied by 100 to

calculate percent GBS in a sample. Only samples with greater than 80x coverage were used, resulting in 26 samples for analysis.

GBS growth inhibition by Phage Communities (PCs)

In all, 130 PCs were initially screened for the ability to negatively affect the growth of two ST-17, serotype III GBS strains, GB00411 and/or GB00112. These two strains were recovered from a neonate with sepsis and an asymptomatically colonized mother, respectively.[35,36] (Manning 2009, Manning 2008). Early log phase cultures of GBS were mixed with different PCs and added to a 96-well plate. The OD_{595} was monitored over 8 hours in a plate reader (BioTek Cytation 3 Imager). PCs that caused a decrease in growth of these two strains were of interest and were targeted for downstream experiments aimed at isolating individual phage and for examining PC host range using 36 additional GBS strains for a total of 38 GBS strains.

PC sensitivity was determined by calculating the AUC of a GBS strain grown alone and with a PC. GBS strains that had at least a 10% reduction in growth compared to the strain alone [$(AUC_{PC} / AUC_{Alone}) * 100 < 90$] were considered sensitive to inhibition by the PC. Combinations of PCs and GBS strains were coded into 1's (inhibition) and 0's (no inhibition) based on GBS sensitivity to each PC. Uncorrected Chi square tests were used to examine associations between GBS sensitivity to a given PC and the number of spacer sequences present within the GBS genome. GBS strain source was also taken into consideration. We also examined associations between GBS sensitivity and the percent of GBS within 14 of the 26 fecal metagenomes from which each PC originated.

Assessment of infectivity by spot plating

To determine if a given PC and bacterial host were compatible, a spot plating method was employed. Briefly, soft agar was overlaid on a plate of the same media. After allowing the soft agar to solidify for 10 minutes, and 10µl of phage sample was pipetted on top without piercing the soft agar. The plates were left to solidify for at least 30 minutes and were incubated overnight at 37°C with 5% CO_2. This version of spot plating was attempted with a range of soft agar concentrations (.3% to .7%) and media types including Brain Heart Infusion (BHI), Tryptic Soy Agar (TSA), Todd Hewitt Agar (THA), TSA + 5% Sheep blood, Mueller-Hinton Agar and modified THA (mTHA). A liter of mTHA consists of 30 g of THB, 2 g of yeast extract, 12 mg of CaCl2, and 10 mg of l-tryptophan (Domelier, 2009). After troubleshooting, BHI with 0.7% agar was selected for remaining experiments because it resulted in the most confluent bacterial lawns.

Plaque assays

To isolate individual phage from the PCs showing the greatest level of GBS inhibition, we attempted to perform plaque assays. The same media types and agar concentrations were attempted. Briefly, soft agar was prepared and cooled to 50°C. 300-500µl of log-phase host bacteria were added to 10-50µl of phage community or enriched phage sample that has been previously diluted in phage buffer containing Tris (10 mM), pH 7.5, MgSO4 (10 mM), NaCl (68 mM), and CaCl2 (1 mM). After an incubation period of 10-15 minutes, soft agar (3-5mL) was added to the mixture and poured over agar plates that matched the soft agar; multiple media types were evaluated.

Enrichment of GBS-specific phage

To enrich a GBS specific phage, PCs were added to actively growing GBS strain GB411 when it reached 0.2 OD_{595}. During the trouble-shooting phase, a range of temperature (25-37°C),

shaking speeds (0 – 200), atmospheric or 5% CO_2 and lengths of incubation (two to sixteen hours) were tried. Final conditions were 28°C, atmospheric CO_2, 50rpm shaking and a four-hour incubation. Afterwards, samples were centrifuged to pellet bacteria, and the resulting supernatant was collected and filter sterilized with a 0.45µm filter. The resulting enriched phage sample was given the original number of the phage community followed by the name of the GBS strain used. For example, if PC 561 was grown with GB112, it was termed PC561GB112. Three different concentration methods were also tested. First, an additional Polyetheleneglycol (PEG) precipitation step was included after filter sterilization for portions of the trouble shooting. Briefly, PEG was added to precipitate the phage, and the sample was incubated overnight at 4°C. The sample was then centrifuged and resuspended in phage buffer. Second, a 100kd centricon was used to remove any particles less than 100kd. Finally, a cesium chloride gradient was used to separate samples based on size as previously described.[44] Visible bands were isolated via needle extraction, and 500µl fractions of the remaining gradient were collected. Individual fractions or bands were then tested for infectivity on GB112 or GB411. The success of each of these steps was examined by spot plating, a growth screen in broth or both.

Microscopy of enriched phage samples

Phage samples were added to continuous carbon TEM grids, which had been plasma cleaned for 20 seconds prior to sample application. They were be negatively stained using 1% uranyl acetate and imaged using a JEOL 2200FS TEM operating at 200 keV.

RESULTS

Screening phage communities for GBS-specific phage isolation

We hypothesized that phage communities that greatly impacted GBS growth would contain GBS-specific phage that could be isolated for further examination. A total of 130 communities were screened using two GBS strains of interest, GB112 and GB411. Approximately 6% (n=8) were considered sensitive to the phage communities (PCs) based on at least a 10% reduction in Area Under the Curve (AUC). The average reduction across these samples was 52%, ranging from an 11 to 86% reduction in AUC. Of the eight PCs of interest, three were selected for further analysis based on substantial growth reduction in the growth curves and the amount of sample available. PC561 reduced the maximum OD_{600} of GB411 from 0.233 alone to 0.155 with PC561 (Figure 1). PC801 and PC895 reduced the growth of GB411 by approximately 90%. Notably, some communities increased the growth of GBS, suggesting other components of the phage community may affect GBS growth. The infectivity of PC561, 801 and 895 was also confirmed via spot plating. All three communities resulted in large spots, or zones of clearing, in both GB112 and GB411.

Host range of select communities

To assess if certain strain characteristics of GBS made them more likely to be lysed by the communities, the host range of PC801 and PC895 communities was assessed using 38 GBS strains of various clinical types, CPS types and STs as well as three commensal *Streptococcus salvarius* strains. PC561 was not examined for inhibition in any of the GBS strains due to low sample volume. Similarly, the number of GBS strains tested with PC801 was reduced (n=17 GBS, 20 total) due to a lower sample volume compared to PC895. The impact of PCs on all GBS

strains was examined using both spot plating and growth inhibition in broth. PC801 lysed 15 of the 17 GBS strains (Table 2), while PC895 lysed 36 of the 38 GBS strains tested. It is notable that the two PCs had different lysis profiles. For example, both strains that PC895 could not lyse, GB00121 and GB00310, are CPS type V, while PC801 did not lyse GB00079 and GB910 which are CPS III and II, respectively. Due to the low number of strains that were resistant to phage, we could not determine if strains with specific capsules or sequence types were more or less likely to be lysed. The commensal strains of *Streptococcus salvarius* (n=3) were less frequently lysed than GBS. PC801 lysed two of the three strains, while PC895 lysed two. We did not assess differences because sample size of the commensal strains is drastically lower.

CRISPR spacer regions differ across GBS strains

Since we did not observe an effect of capsule or sequence type, we sought to examine CRISPR spacers regions as they have been shown to act as a adaptive immune system in bacteria. The CRISPR regions of 27 of the 38 GBS strains with genome sequencing data available were examined for CRIPSR spacers (Figure 2). In all, 192 unique spacers were observed across the 27 genomes. Most of the spacer sequences were unique, but 39 (20.3%) of the spacers were found in at least two strains. Spacer 13 predominated and was found in 48% (13/27) of the GBS strains. Notably, 65 (33.9%) of these spacers were homologous to different phage and plasmid sequences available in the NCBI database (Table 3). Many of the annotated spacers (57/62; 92%) match to known *Streptococcus* phage, including JX01 (n = 15), LF2 (n = 14) and LSSO9 (n = 16). Additionally, six spacers matched plasmids. These include: pCBU1 from *Clostridium butyricum*, pSRC1 from *Selenomonas runinantium*, pNCT2 from *Bacillus megaterium*, and pSAL813 from *Streptococcus salvarius* as well as a cloning vector from

Streptococcus mutans and a plasmid from *Legionella adelaidensis* strain NCTC12735. Interestingly, two of the four strains that could not be lysed by either PC801 and PC895, each strain had spacers that are homologous to *Streptococcocus* phage LYGO9. We do not have associated metagenomic data for these two PCs; therefore, we cannot examine these communities for the presence or abundance of this LYGO9 phage.

GBS strains also varied in the number of CRISPR spacer repeats, ranging from 3 to 24. The number of spacer sequences was previously linked to clonal complexes, which represent closely related strains defined by multilocus sequence typing.[45] In our dataset, we also found differences in the number of CRISPR spacers across sequence types (ST), with the lowest average number of spacers in ST-17 strains (7.8 spacers) and ST-1 (12.8 spacers). Other common STs including STs 12, 19 and 23, had similar numbers of spacers with 16.7, 15.1 and 14.5, respectively (Figure 3A).

When stratifying strains by clinical type (colonizing versus invasive), colonizing strains averaged 15.4 spacers, while invasive strains averaged 11.5 (Figure 3B); this difference was not statistically significant. We also examined differences between capsule type (Figure 3C). While capsule type III, which is commonly associated with ST-17, had a significantly lower average number of spacers (10.6) than the other capsule types (16.6 (CPS I), 17.5 (CPS II) and 14.0 (CPS V)), these differences were not significant by ANOVA. Because this result could be due to the unbalanced distribution of samples, we grouped CPS types I, II and V together for comparison to CPS type III. Importantly, CPS III strains had significantly less spacers on average (10.6) compared to other capsule types (17.0) (Figure 3D; Mann-Whitney T-test, $p = 0.0101$). To further examine this association, strains were defined as having a low or high number of spacers; seven strains had fewer than nine spacers (first quartile), while seven strains had greater than 16

spacers (fourth quartile). Indeed, six of the 12 CPS III strains had a low number of spacers as calculated by the 25% quartile (≤ 9 spacers) compared to only one of the 14 strains representing the other CPS types (Fishers exact p = 0.047).

The number of CRISPR spacers significantly impacts the ability of phage communities to inhibit growth

Since CRISPR acts as an adaptive immune system in bacteria, we hypothesized that a reduced number of spacers would result in a higher frequency of growth inhibition by PCs. Fourteen of the 27 GBS strains were selected to test this hypothesis based on number of spacers identified in the genome analysis using the previously defined quartiles. Strains were grown in the presence of fourteen different phage communities and reduction in AUC was calculated as a percent of growth without the PC (Figure 4). We found that 67 of the 196 combinations resulted in growth inhibition (34.2%). We observed that certain PCs were also more likely to affect GBS strains, including ER0788. Likewise, there were certain GBS strains were more likely to be sensitive, including GB000390 and GB000910, which were inhibited by all the PCs. Combinations that resulted in at least a 10% reduction in growth were considered sensitive (0) while those above the cutoff were considered resistant (1) to the PC. GBS strains in the lower quartile that possess the lowest number of spacers, were 2.0 times less likely to be sensitive to inhibition by the PCs (95% CI: 1.09, 3.63). Within this smaller data set, however, there was not a high enough or even enough distribution to account for CPS or ST-type.

GBS presence in the microbiome is significantly correlated with GBS inhibition

A selection of the fecal samples used in this study were previously used for metagenomics (Nohomovich, unpublished). Using these data, we calculated the percent of GBS

within the total bacterial microbiome. The abundance of GBS ranged from 0.9% to 3.1%, with an average of 2.1% in reads with detectable GBS. Samples with undetectable (0%) GBS were removed from the analysis because the metagenomic data did not reach a high enough read redundancy to confidently say that a zero result is in fact negative. Eight of the sequenced samples had ≤1.8% (25% quartile), while another eight had ≥2.4% (75% quartile). These quartiles were selected for further analysis (Figure 4).

As previous research determined that the phage community in the gut determines the bacterial component, we hypothesized that presence of GBS in the microbiome may be dictated by the phage community (PC). We found no correlation between the percent of GBS in the microbiome and the sensitivity of GBS strains to the PCs. We further hypothesized that this observation may be due to the resistance of the GBS strains to a given community, therefore, we examined only strains that were sensitive to PCs as defined in the methods. Within this subset of 14 strains, we found that if GBS abundance was high (≥2.4%), then the associated PC was 2.7 times less likely to be able to reduce the growth of GBS (95% CI: 1.00, 6.46). Indeed, the average growth inhibition across the phage communities with lower percent GBS-associated reads was 79.9%, while the average for those with high percent GBS associated reads was 85.1%. Further, the percent of GBS found in the microbiome significantly correlated to the percent growth reduction in sensitive strains (Pearson correlation = 0.88).

Recovery of phage from PCs capable of inhibiting GBS

A common technique used to isolate individual phage is picking an isolated plaque from a titer plate; however, this technique is known to be difficult for many phages, similar to difficulties with unculturable bacteria.[46] The ability to form plaques is reliant on several

components, which we systematically attempted to troubleshoot in attempt to isolate individual phage. In spite of troubleshooting, we were unable to observe plaques. Titer assays resulted in either a clear plate or a confluent lawn, even with 1:2 dilutions (Figure 5).

1. Media type and agar concentration: Media can greatly affect the ability of a phage to successfully infect a host.[47] As there was little available research on phage in GBS, we started in the most commonly used media for GBS, Todd Hewitt Broth (THB). This is a general-purpose broth used for *Streptococcus* that contains peptones, dextrose, and salts (VMR). With this broth, we observed minimal growth of the bacterial host. While lawns grew, they were translucent and easy to see through. We observed similar problems with Tryptic Soy Agar (TSA) and Mueller-Hinton Agar. The concentration of agar in the soft agar overlay can affect migration of the phage and affect the growth of the bacteria[48] We used a range of 0.2 to 1% agar but did not observe improvement in the lawn or the appearance of plaques. We hypothesized that the poor lawn may be due to GBS being unable to grow in a soft agar overlay; therefore, we tried using the standard technique used to assess antibiotic resistance in GBS, which results in a confluent lawn on the surface of an agar plate. A bacterial culture is spiral streaked on TSA + 5% Sheep blood and allowed to grow. While this did result in confluent growth over the top of the plate, it was difficult to adapt this method to titer as such a small amount of the bacteria and phage mixture was being added to a plate. A recent publication on GBS phage in tilapia used Brain Heart Infusion (BHI) with 0.7% agar to pick isolated phage plaques (Luo, 2018). Use of this media resulted in more confluent lawns; therefore, this media was used for all other assays. The use of this media also led to better spot tests revealing the hazy ring around the spot from PC801 and 895 that is indicative of a lysogenic phage, which was not visible on the other media types (Figure 6).

2. Incubation conditions: As the physiology of the host bacteria affects the ability of a phage to form plaques, we tried different temperatures, periods of exponential phase, shaking conditions and CO_2 concentrations. GBS is typically grown in 5% CO_2 at 37°C; therefore, we began by incubating plates in these conditions; however, we observed more success with atmospheric CO_2. Incubation at atmospheric CO_2 resulted in more confluent lawns and an increased enrichment; therefore, future experiments were preformed at atmospheric CO_2. To successfully infect, phage must have an actively replicating host; however, the period of exponential phase (early OD_{595} 0.2, middle 0.3 or late 0.4) may have difference results. Early exponential phase cultures seemed to result in better lawns and a more concentrated enrichment; therefore, experiments were preformed with cultures at an OD_{595} of 0.2-0.3.

Different temperatures also affect how the host is growing and the ability of the phage to attach to the host.[47] We assessed varying temperatures including 28, 30, 32 and 37°C, and found the most success with 28°C as phage recovery was highest at this temperature. Finally, for liquid enrichment, shaking the culture allows for increased contact between the phage and bacterial host, but high amounts of aeration affects growth of GBS and could shear phage, rendering them incapable of infecting. Gentle shaking at 50 rotations per minute (rpm) resulted in the greatest reduction of growth of GBS during enrichments in comparison to each control.

3. Concentration steps: Polyetheleneglycol (PEG) precipitation can be used to concentrate phage by clumping the phage together so that they can be pelleted and concentrated. We did not observe a corresponding increase in infectivity, suggesting this procedure did not help concentrate out phage. Next, we tried using 100 kilodalton (kd) centricons to reduce the volume. This was performed before Cesium Chloride gradients and microscopy. The results from these experiments suggest that the samples were still too dilute.

Enriching GBS-specific phage

Due to the difficulty getting an isolated plaque from the PCs, we attempted to enrich for the phage of interest multiple times, thereby reducing unwanted components from the fecal sample, such as nutrients or inhibitory compounds, while maintaining the desired phage. The addition of a suitable host allows a bacteriophage to infect and propagate, thereby preferentially increasing concentrations of the phage that are specific to the bacteria of interest. We attempted to enrich PC561, 801 and 895 using GB112 and GB411. As a control for these experiments, we also added the community to an equivalent amount of broth without a bacterial host to confirm that the agent responsible for GBS growth inhibition needed to propagate in order to have an effect on GBS (Figure 6). We found that these controls did not spot, confirming that any inhibition observed with the enrichment samples is due to a phage. Samples from the attempted enrichments had a smaller zone of clearance in spot plates (Figure 6). We confirmed a reduced infectivity of the resulting sample by measuring growth reduction in broth (data not shown). We hypothesized this reduction could be the result of a lysogenic phage. Indeed, the hazy perimeter observed on the spot plates is indicative of lysogenic phage (Figure 6). For PC561, the enriched sample maintained its killing ability of both GBS strains (Figure 7A), but also demonstrated the ability to inhibit a variety of other bacteria including *Lactobacillus gasseri* and *Escherichia coli* (Figure 7B&C). Because an individual phage is unlikely to infect Gram-positive and negative bacteria, it is likely that the enrichment step was not sufficient and other methods need to be applied in order to isolate the GBS-specific phage in these samples.

Cesium Chloride Gradient

Cesium chloride gradients allow for separation of particles based on density. When phage samples are added an observable band can be often observed and removed by needle puncturing the band and withdrawing the band. We hypothesized this would allow for isolation of phages of interest from other contaminants in the PC resulting from the fecal matter. No bands were observed for PC801 and PC895, but two bands were observed for PC561. The bands and individual fractions of the gradient were taken and tested for infectivity. Fractions C through G maintained infectivity, while fractions A, B, H as well as the pellet had no effect on GBS growth (Figure 8, representative PC895); however, these fractions were not capable of forming spots on soft agar. Additional attempts to enrich the phage from the gradients were unsuccessful.

DISCUSSION

GBS remains a significant health burden in the United States and globally; however, preventive measures are limited to antibiotic treatments with known flaws. As GBS can be isolated from rectal swabs, we hypothesized that fecal phage communities would contain GBS-specific phage. Indeed, we found that many samples inhibited the growth of GBS strains and further examined three communities of interest. We found broad host range for two of these communities, PC801 and PC895, as they inhibited 88% and 94% of the strains tested, respectively (Table 2). While there were no GBS traits associated with the ability of PC801 or PC895 to lyse GBS, a larger data set that includes resistant strains of GBS may reveal more about the host range of these specific communities. This broad host specificity could be due to the presence of multiple phage types or inhibitory compounds present in the phage communities.

The phage characterization portion of this study is severely limited by the inability to isolate an individual phage by plaque isolation; however, this is also known to be a problem outside of this work. It was estimated that less than 1% of phage will develop a plaque, or zone of clearing in a bacterial host that allows for recovery of individual viruses.[46] Due to this limitation, we are unable to determine if the host range of a given PC is due to a single phage or a combination of phages in the sample. The complex nature of a phage community, as it is isolated directly from fecal samples, further complicates data interpretation since other proteins could be present in the phage communities. Indeed, we found that some communities increased growth, which we hypothesized may be due to extra carbon sources present in the fecal matter. Hence, it was important to confirm that the inhibitory compound is a bacteriophage. As the inclusion of a bacterial host was required for continued lysis during enrichment, it is unlikely that the inhibitory agent is a protein or antibiotic. In spite of this, we do not know how the fecal

sample is contributing to bacterial-phage interactions. For example, a component of the complex community may play a role in increasing the virulence of the bacteriophage in PC 801 or 895.

To reduce the complexity of the samples, we enriched PCs with two GBS hosts, GB112 and GB411, but still observed a broad host range including other species such as *Lactobacillus* and *E. coli* (Figure 8). Since this was an enriched sample, it is unlikely that this broad specificity is due to a challenge that is generally inhibitory to bacteria such as pH as the sample had been significantly diluted. It is more likely that another phage was still present, which is not surprising given that the feces contain a broad range of phage. Indeed, these communities have been used to lyse a variety of enteric pathogens including *E. coli* strains, and *Lactobacillus* phage have been previously observed in fecal samples via metagenomics. (Nohomovich, unpublished).

Phage characterization is also limited by the low titer of this phage. While we were unable to properly titer these samples by plaque-forming units, we estimate that the titer is low based on the clearance of 10^{-2} plate. This low titer limited our work by making microscopy and sequencing difficult. Typically, a sample needs to be at least 10^4 PFU in order to continue with downstream methods, which we were never able to accomplish. Both of these methods would have contributed greatly to our understanding of the bacteriophage residing within these communities.

Despite these limitations, troubleshooting for optimal conditions for these GBS phage helped to develop spot plating and growth inhibition assays to examine how traits of both metagenomes of the fecal samples and the GBS host strains affect interactions. This study is unique in its examination of the impact of complex phage communities on GBS isolates. There are still fundamental gaps in our understanding of GBS colonization and to date, there have not been studies conducted to examine the role of the phage community in the gut in GBS carriage.

Herein, we examined whether different GBS traits affected the ability of a complex phage community to inhibit the growth of GBS. While CPS, ST and clinical type have been previously shown to affect a number of traits in GBS,[33,49–52] but did not affect the ability of the phage community to lyse, we demonstrated that the number of CRISPR spacers present within the genome may be linked to infectivity. Specifically, we found that a lower number of spacers correlated to a lower rate of infectivity suggesting that strains with higher numbers of CRISPR spacers could be more resistant to removal by the phage community. Unfortunately, two of the four strains that did not lyse with PC801 and 895 have yet to be sequenced so we cannot assess this idea well in the current data set. The idea that strains with a lower number of spacers would be more likely to be cleared from the gut is interesting given that strains belonging to ST-17, which had the lowest average number of spacers, are more frequently associated with sepsis and meningitis. It would also be interesting to evaluate the CRISPR regions of strains isolated in longitudinal studies to examine if persistence correlates with the number of CRISPR repeats. Although Beauruelle *et al.* followed women with GBS longitudinally and examined CRISPR spacers, no details were provided showing that continued carriage was correlated to the number of CRISPR spacers. [33]

We also hypothesized that if the phage community was having an *in vivo* effect on GBS, then a reduced percent of GBS in the microbiome would correlate to a higher infectivity of GBS *in vitro*. We found that this was not true when all the strains were examined together; however, examination of just the sensitive strains showed such a correlation. This finding suggests that the phage community might impact GBS carriage, but only if the strain itself is less resistant to bacteriophage. As phage sensitivity is also correlated to the number of spacers, it may be

important to continue a more comprehensive examination of CRIPSR spacers when considering GBS disease globally.

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APPENDIX

Table 4.1. GBS Strain List. ST: sequence type; CPS: capsule type; VRC: vaginal-rectal colonization; EOD: early onset disease; LOD: late onset disease.

Streptococcus Strain Information

Strain Number	Clinical Type	ST	CPS	Isolation Site
GB00012	Colonizing	ST-1	V	VRC
GB00020	Colonizing	ST-1	V	VRC
GB00033	Invasive	ST-23	Ia	EOD/sepsis
GB00036	Invasive	ST-19	III	EOD/sepsis
GB00037	Invasive	ST-1	V	EOD/sepsis
GB00045	Invasive	ST-17	III	EOD/sepsis
GB00066	Invasive	ST-19	III	EOD/sepsis
GB00079	Invasive	ST-19	III	EOD/sepsis
GB00084	Colonizing	ST-1	VIII	VRC
GB00097	Colonizing	ST-17	III	VRC
GB00112	Colonizing	ST-17	III	VRC
GB00121	Invasive	ST-26	V	Unknown
GB00279	Colonizing	ST-23	II	VRC
GB00285	Colonizing	ST-12	Ib	VRC
GB00291	Colonizing	ST-12	II	VRC
GB00305	Colonizing	ST-12	Ia	VRC
GB00310	Invasive	ST-1	V	EOD/sepsis
GB00374	Invasive	ST-12	Ib	EOD/sepsis
GB00377	Invasive	ST-19	III	EOD/sepsis
GB00390	Invasive	ST-23	Ia	EOD/sepsis/meningitis
GB00411	Invasive	ST-17	III	EOD/sepsis
GB00418	Invasive	ST-17	III	EOD/sepsis
GB00438	Invasive	ST-12	Ib	LOD/sepsis
GB00555	Colonizing	ST-12	Ib	VRC
GB00557	Colonizing	ST-17	III	VRC
GB00561	Colonizing	ST-19	V	VRC
GB00571	Colonizing	ST-19	III	VRC
GB00590	Colonizing	ST-19	III	VRC
GB00620	Colonizing	ST-1	Ia	VRC
GB00651	Colonizing	ST-19	Ib	VRC
GB00653	Colonizing	ST-12	II	VRC
GB00663	Colonizing	ST-19	III	VRC
GB00686	Invasive	ST-1	V	Stillbirth
GB00910	Invasive	ST-12	II	EOD/sepsis
GB01007	Invasive	ST-19	III	Stillbirth
GB01454	Invasive	ST-1	V	Stillbirth
GB01455	Invasive	ST-12	II	Stillbirth

Table 4.1 (cont'd)

NEM316	Invasive	ST-23	III	EOD/sepsis
19232	Commensal *Streptococcus salvarius*			Saliva
19233				Unknown
19234				Oral Cavity

Table 4.2. PC 801 and 895 have broad host range within *Streptococcus*. 10 microliters of PC was added to a lawn of bacteria (0.7% BHI agar overlay). A total of 41 strains were tested for PC895, while 20 were tested for PC801. Grayed boxes represent strain combinations that were not performed. ST: sequence type; CPS: capsule type; VRC: vaginal-rectal colonization; EOD: early onset disease; LOD: late onset disease ST: sequence type; CPS: capsule type; VRC: vaginal-rectal colonization; EOD: early onset disease; LOD: late onset disease.

Streptococcus Strain Information					Spot Plate	
Strain Number	Clinical Type	ST	CPS	Isolation Site	801	895
GB00012	Colonizing	ST-1	V	VRC		Yes
GB00020	Colonizing	ST-1	V	VRC		Yes
GB00033	Invasive	ST-23	Ia	EOD/sepsis		Yes
GB00036	Invasive	ST-19	III	EOD/sepsis	Yes	Yes
GB00037	Invasive	ST-1	V	EOD/sepsis	Yes	Yes
GB00045	Invasive	ST-17	III	EOD/sepsis		Yes
GB00066	Invasive	ST-19	III	EOD/sepsis		Yes
GB00079	Invasive	ST-19	III	EOD/sepsis	No	Yes
GB00084	Colonizing	ST-1	VIII	VRC		Yes
GB00097	Colonizing	ST-17	III	VRC	Yes	Yes
GB00112	Colonizing	ST-17	III	VRC	Yes	Yes
GB00121	Invasive	ST-26	V	Unknown	Yes	No
GB00279	Colonizing	ST-23	II	VRC		Yes
GB00285	Colonizing	ST-12	Ib	VRC	Yes	Yes
GB00291	Colonizing	ST-12	II	VRC		Yes
GB00305	Colonizing	ST-12	Ia	VRC	Yes	Yes

Table 4.2 (cont'd)

GB00310	Invasive	ST-1	V	EOD/sepsis	Yes	No
GB00374	Invasive	ST-12	Ib	EOD/sepsis		Yes
GB00377	Invasive	ST-19	III	EOD/sepsis		Yes
GB00390	Invasive	ST-23	Ia	EOD/sepsis/meningitis		Yes
GB00411	Invasive	ST-17	III	EOD/sepsis	Yes	Yes
GB00418	Invasive	ST-17	III	EOD/sepsis	Yes	Yes
GB00438	Invasive	ST-12	Ib	LOD/sepsis		Yes
GB00555	Colonizing	ST-12	Ib	VRC		Yes
GB00557	Colonizing	ST-17	III	VRC		Yes
GB00561	Colonizing	ST-19	V	VRC		Yes
GB00571	Colonizing	ST-19	III	VRC	Yes	Yes
GB00590	Colonizing	ST-19	III	VRC	Yes	Yes
GB00620	Colonizing	ST-1	Ia	VRC	Yes	Yes
GB00651	Colonizing	ST-19	Ib	VRC		Yes
GB00653	Colonizing	ST-12	II	VRC	Yes	Yes
GB00663	Colonizing	ST-19	III	VRC		Yes
GB00686	Invasive	ST-1	V	Stillbirth		Yes
GB00910	Invasive	ST-12	II	EOD/sepsis	No	Yes
GB01007	Invasive	ST-19	III	Stillbirth		Yes
GB01454	Invasive	ST-1	V	Stillbirth		Yes
GB01455	Invasive	ST-12	II	Stillbirth	Yes	Yes
NEM316	Invasive	ST-23	III	EOD/sepsis		Yes
19232	Commensal *Streptococcus salvarius*			Saliva	No	Yes

Table 4.2 (cont'd)

19233	Unknown	No	No
19234	Oral Cavity	Yes	Yes

Table 4.3. CRISPR spacers can be annotated using NCBI.

Spacer Number	Spacer Sequence	NCBI Annotation
1	ACCCTTGTATGTTAAATCCGCAAGATTTTA	
2	ACTAAAATCTAGATTTGAATAATAGTATAG	
3	AATACCATTTTCCACCCAATCAAATCCAAC	
4	ACCTCTTAAAATTTAAGTAAATCCTCTTGA	
5	AACATTAGCCTTTTCTAACTCTTCAGCTGT	
6	TATGTCTTCTAACAGTTGCTTCTTGTGCTT	
7	CCGTCAAACAAGAGCGACAGCGAAACAAGC	
8	TTTTATTGGTTTTCTAAGTGCTCGACCATC	
9	AGTTACTTCTGCTTGGGTTTGATAAGGGTC	
10	GAAATGTGGAGTCATTCAGGTTGATGATGG	Streptococcus phage LF2
11	AAAAAATAAATGACTTTAAAGCACTTGGAG	
12	CTGTAAGCGGTAGACTTGACAAAATTAAAA	
13	AAGCTAATTCTCATCTCACCGAGATGGATA	
14	GGATGATTTCGATTATGCGGCGGTGGTTGA	
15	AAATGTTAATTTCATATCTACATCTTGTTC	
16	ACATAACGTTCAAAAGTTTCCACTAATAGC	
17	TCAAACAACATTGGTGATCTTATTGCGGTA	
18	TTATTTAAGACGTGATTTAATGTTAAAACT	
19	CAAACTATCTTGATTATAAGTGGACTGATA	Streptococcus phage JX01 Streptococcus phage LYGO9
20	ATTTTTACACGAGTGCTAGAAAACGGGGCA	Streptococcus phage JX01 Streptococcus phage LYGO9
21	ACATTTGTTAAATTTGAACTTACTGGAAAT	Bacillus virus PBS1 Bacillus phage AR9
22	TCCAAAACAAATACGAATGCTTGAGCGATA	
23	GATTACCTTAGATGATGTTCTAATCGGTAA	
24	TTAACAGTTTCAAGTCTGTCTTGTTACTTA	

Table 4.3 (cont'd)

25	ACTCTAAATGATAGTTATGAGTTAAATGTT	
26	CAAATTACAGTTTCGACTGATTATGGAAAT	
27	ATATGTTCCACTCTATGAATTTAGGCTCAT	Enterococcus phage vB_EfaP_Zip Enterococcus phage vB_EfaP_IME199
28	TTTTTACCAATGCTTCCATATCGCTTATAT	
29	TACTTGACGAATTGAAGATGACGGAATTTA	
30	ACATATCAGGAATATAAAAATCGTTCAAAT	
31	TGCCTTTTAATTCAGCTCCCACTTTTTATC	
32	CGGCGACTGATTAGGTAACCGTTAGCTCGT	Streptococcus phage PhiNIH1.1
33	AGATGGTTATAACGCTTGGGCTATATCAAT	
34	AAGGTACTTTTAGCTTGTTCTTGTGGTGTT	
35	ACAGCTACTGTAAATTCTGCTTTTACGGTT	
36	TAGTGCAGTTGTCAAGGAGATTGTGAGCGA	Streptococcus phage IPP35/34
37	TTAAAAGATTTAAACTATCAAGCGTCAATT	
38	TTCTAAATGCTGGTGACTGCTTTGCATAAA	Streptococcus phage LF2
39	AACATATTAGACCTACTTCACTTCAATATC	
40	TGGTTATACATTTACTAATCCATCAGCATT	
41	CGTCACCTATTGTTTTATTATTTTTACTA	
42	GTATTTGCCCATTTTCTAAATAAGTATATT	
43	CCGTTCAATCTGTTCTTGCTTTTGGTCATC	Streptococcus phage LYGO9
44	GCGATGATGGTAAGTCATCATGGACAGCGT	Streptococcus phage JX01 Streptococcus phage LYGO9
45	TCCTTTTTTAGATAATGTGCGATCACGGAC	
46	TTAATAACCTTATGTGATAGATAATCATTT	
47	ACTGCTGGCGCTGTTTGTTCAACTGCTGGT	
48	ACGACCCCAGGTTCTAATACAAATCAACCA	
49	TTAATCAATAACAAACGCCAACGACCTAAA	
50	AGACAAGATAAAAAGACCTTGTATTGTGAT	Streptococcus phage SOCP Streptococcus phage Cp-1

Table 4.3 (cont'd)

		Bacillus phage vB_BthM-Goe5
51	TTATCAATTTGTTTCACAAGAATTTGATAA	Streptococcus phage Cp-7
		Streptococcus phage SOCP
		Streptococcus phage Cp-1
52	CGTACTTTGTTATAAACCCTGTTTCCAGCA	
53	GAAGCTATTGTCTTAAACTCAAAAACTTT	Bacillus phage vB_BsuM-Goe3
54	CGATTTGCTCGTACATTATAAATACCACCA	
55	TTGTATATGACCCGTTACCGCTATCAAATA	
56	TACCTGGAATGTTAAATTTACCTTTTAGCA	Streptococcus phage LF2
		Streptococcus phage JX01
		Streptococcus phage LYGO9
57	ATGTTGCCACTAGTTGCTCTTTATGAACAG	
58	AGTTTCTTTTAAGTTCAATGTTTGTCAAAT	
59	AGTCCCTCCTTAACTTTTTGAGCGTAAATT	Streptococcus phage LF2
60	TTTATTTTTTCTCAGTTCCTTGATTTTAGA	
61	GATGATGGCCGAACAAGTTACACCAGATGA	
62	TTAAGCAACATATTTTGGATGATTAAAACA	Streptococcus phage T12
		Streptococcus phage PhiNIH1.1
63	ATAATTGATGTTAAATCATCAATATCGATA	Streptococcus phage LF1/4
64	TCTGCATCATGCGGAACTCTGATTGTTTTG	Streptococcus phage LF1/4
65	TAGTATAATGCTCGAGGACTATCCGTCTGA	
66	TCAAAATATCACACATAAATCATCCTCCTT	
67	AATGGAATTATCACAAGCGGGGCATATGGT	Streptococcus phage LF1/4
68	AGACTGCTTGCGCTTTGGTGGATTGTATGT	Streptococcus phage T12
69	ATCGGGTCTATATGCCCCCTAAAATCAATT	Streptococcus phage LF1/4
70	AGTCTAATCGCCTATTATTATCCAGATAAA	
71	TGAAAGCGAATTGACGATAAGCGAAAATGA	
72	TCATTACTTGTATTTATAAATGATTTAGCA	Streptococcus phage LF2
		Streptococcus phage LYGO9

Table 4.3 (cont'd)

73	ATAGACCCATCACGAATCGAACGTGATTAA	Streptococcus phage LF1/2/4 Streptococcus phage LYGO9 Streptococcus phage JX01
74	ATCGAGCTTTCACTTTGTTTAACATGGCTT	Streptococcus salivarious strain LAB813 plasmid pSAL813
75	TCAAATCATCGACGGTTCAAAAGTAAAAGA	
76	TAAGTGAGTGACTTCTTCGGTTGAATAGTC	
77	AGAGCGGTCAGCATGTTGTGCAACGCTGG	Streptococcus phage JX01 Streptococcus phage LYGO9
78	ACGTCTTCAGGTGTTATTCTTGGGTCTTCT	
79	AAGACTTAAAATCGATTAGAATTGATTTTA	Streptococcus phage LF1/4
80	CCAGTTTAGAAACAGAACAAAACTAACGAA	Streptococcus phage PhiNIH1.1
81	AGACAAAGAAGATGGCAAGTCTATCAACAA	
82	AGTAGAAACATAACGATAATTCCATGAATA	
83	ATGTTGTAGAATCATATCGACCATATAACC	
84	GCTTGGTCTCGAAGAATTACAGAAGAACAT	
85	CAATTGATTGCCGTTAAAACCGATAGAGGA	Streptococcus phage LF1/2/4 Streptococcus phage LYGO9 Streptococcus phage JX01
86	AAATTGCTCTATCAGTCAATAAAGCAAGAT	
87	TCATATTGAGTGGTTGTTTAATTAAATTGA	
88	TGAATTCCACGCCACCAAGTAAACCTGTGA	Streptococcus phage LYGO9 Streptococcus phage LF2
89	ACAAGAAACAAACAGCCTTGATGACTTAAT	Streptococcus phage LYGO9 Streptococcus phage LF2
90	AAAAAGCAATGGAAGCTAACGAGATTGCTA	Streptococcus phage LYGO9 Streptococcus phage LF2 Streptococcus phage JX01
91	CAACCCTATGTTTGATAATATTTTAGACGT	

Table 4.3 (cont'd)

92	AAGTGAAGTTGAATTTTATTTGAGATACTA	
93	TAATACTTTTACAATATGTGTTTTCACTAC	Streptococcus phage LF1/2/4
94	TTCTATCTTCTGAAGATATTTCACAAGTGA	
95	TCAGCGAGATGCTCTAAGTAAGCATGTTGA	
96	TCTTCTTTTTAATTCTTCTAACACTCCATC	
97	ATCTTCTTTTGACCTAACAAAAGGATATGT	Selenomonas ruminantium subsp. Lactilytica TAM6421 plasmid pSRC1
98	AAGTCCAAAGCTTTTTTCACTGTTGCTGGA	
99	TAATTCCTTAAGCTTGATTATAGTATAACA	Streptococcus phage JX01
100	CAACTTTTCTATGAAATTAAAATGGCTTCT	Streptococcus phage DCC1738
101	GAAGTATACTACGGTTAGAGATTGGCTCAA	Streptoccocus phage LF2
102	GAAACTTCGATTAGTTTGCGTACTCGCTCA	Streptococcus phage LF1/4
103	ATTAGTCCTGTTGTTATGATGGATGATTAT	
104	GCAAACCTCTAATGGATAATATAGAACAAA	
105	ACACAATTAAACATAAAGAAGTGCTTATTT	Legionella adelaidensis strain NCTC12735 plasmid
106	TCAGGAGATTGTGTGTACTCACGAATTTTT	
107	AAATTGTTTTTGTTGTAATATAAAGTCGAC	Streptococcus phage phi3396
108	AACCTTGATAGGCGTACTGTATGGATTCCA	Erysipelothrix phage phi1605
109	CTACTGTATTATCTGAAATAGTATCGTTTT	
110	TCTCGGGAAATGCAACTGATGCTGCAATCA	
111	GCTAGCATGGCACAAAAATAGCGTTGGAAT	Streptococcus phage JX01
112	ACCTTGCTCCGATGACACCATCGCGAACCT	Streptococcus phage LYGO9 Streptococcus phage LF1/2/4
113	TTAATCGGATTGGTCAAAATATCAATCAGC	
114	GAAGTCGAAAAAGATGACTTCTATTACTGG	
115	AATGAAGATATTCTAGCTCGTGTAAAAATA	
116	TTGGAAAAAACACGAAAGTGATATTACTTT	
117	TAAGAATTTTAGATACTCTACTTGAATGCT	

Table 4.3 (cont'd)

118	AAGTGCCACAGTTTGTGGCTGATTGGATTG	Streptococcus phage 20617
		Streptococcus phage IPP42/41/5/54
		Streptococcus phage 23782
		Streptococcus phage phi ARI0131-2
		Streptococcus phage phiBHN167
119	CATTCAAGGACTACCCTCAACAGTAACTCT	Streptococcus phage 20617
		Streptococcus phage SW27/19/18/14/4/24/7
		Streptococcus phage CHPC1033/1148/1073/ 1027/927/877/572/1151/1005/676/640
		Streptococcus phage P9854/9851/8921/7601/ 7574/7151/7134/7133/5651/3681/0094/0093/0092
		Streptococcus phage D4276
		Streptococcus phage SWK1/2
120	TCAATTTAAATATTATTTAGCCTTCTCTAA	
121	TCATTTAAATCAGACTTGTAAGTCTCGACT	Streptococcus phage T12
122	CACTATCAGCCTGACTTCTACGTTTAAGGT	
123	ATTGCTTCGTGAGCTTCAGGACTATCCAAT	
124	ATGATTATATCAAAAAAGCCCGACGGGAAT	Streptococcus phage P9
		Streptococcus phage PhiNIH1.1
125	GCCTGCCCCACAAAAAAGTATATTATTATT	Streptococcus phage LF1/4
126	TTAATTAAGGTATTTATACCACCTTTTTGT	Streptococcus phage LF2
127	AGAGTGTGTCCAAGACCAGAGTTACTGTTT	Streptococcus phage LF1/4
128	CTATTGGTTAGAATTTTTTTACAGGAAGAA	
129	CCACCTCTAGGTCCACGTAGAGTCTTATGT	
130	CCACCCCCTTTCTGTGGTATAATTGAAATA	
131	AACCCTCATAGCCTCATTTTTATTAGTCGT	Streptococcus phage LF3
		Streptococcus phage Str03
		Bacillus megaterium NCT-2 plasmid pNCT2_1
132	ATTTATTTTTTTATGCATAGCAATTTGACT	

Table 4.3 (cont'd)

133	CGCTCGATTGATGCTATCAACTATATTGAA	
134	TTCTTCAAGAGAACTTGTAGAACAGCTTCA	Clostridium butyricum NBRC 13949 plasmid pCBU1
135	TTTAACCTTTGAAAATGTGAAAGGCTCGTA	
136	TTTTACACACGATGTCAGATATAATGTCAA	Streptococcus phage LF1/4
137	AGTACTGCACTAGGAATTGTAGAGATCAAA	
138	CTAAAAATAAACTGTTTGGGTCCAGCAGCAA	
139	ACGGTGTTGCACACTCTATCACTTATAAAA	Streptococcus phage LYGO9 Streptococcus phage LF2 Streptococcus phage JX01
140	ACCACTAGCAGGATTTTCTATGATGAAATA	
141	GCATATAGTCATAGACATCTTGAAAGTAAT	
142	TAGAAATGTACATTCTAGGAAAAGACATTA	
143	AAAATTACCGTCAAACGTTACAAGTTCGCC	Streptococcus phage LF2
144	CACAAGTATTCCCACAATCACAATGACATA	
145	GAGTAAACATGATATTATTCAAAATTAAAC	
146	AAGATGAGAAACCATATAGCATTGATAACT	Streptococcus phage LF1/4
147	ACATTGGCAATTGTTTTCGTCTCGTAGATA	
148	CTACTAGGGATAAAACAAAATACTTATAGT	
149	AGTAAAGAACCAGATGCGCCTAAGCCTATT	
150	AAAACCAAAGGAAGATATGATAACACACTT	
151	AAATTGTCCTGGATTATTGTGCAAATCGTT	
152	TGAAATGGCTGGTTATGTCGACGGCGAGGA	
153	AAAACCATCTGCACAAACTATTTCAATATT	
154	TAGATATATCCCTTTCGAGGAAGCTGTGTT	
155	AAAGGTTCGAAAGTCATGAAAGCTAGTATG	Streptococcus phage LYGO9 Streptococcus phage LF2 Streptococcus phage JX01
156	TCAGAATGATCATCTTGTAGAAATTATTGA	
157	CGCCGTTTGTAATGGTTTGCCAGTAAGAGT	

Table 4.3 (cont'd)

158	ACAACCTCACCAATAATTCTAAAGTCGCTA	Streptococcus phage T1 2Streptococcus phage JX01 Streptococcus phage T12 repressor (excisionase)
159	AGATGCAACTAAAAACGGTGCAGACTTCAT	Streptococcus phage Str01 Streptococcus phage A25 Streptococcus phage JX01 Bacteriophage PSA
160	GACAAACTTTCCATCTTAACATCTTTACAA	Streptococcus phage P9
161	TATGGTCATCTTCTTTGATAACTTTGGGG	Bacillus clarkii bacteriophage BCJA1c Streptococcus phage IPP66/65/55/54/53/52/48/39/14 Streptococcus phage SpGS-1 Streptococcus phagephiARI0639b/0468- 4/0285-2/0378 Streptococcus phage phiBHN167 Streptococcus phage MM1
162	TATTTCTATATTTATTATATAATATATTAT	
163	ATTTTTAGCATAGATTGCTGATTTGGACCC	
164	AAGATGGGACTGATGGAAAAGACGGGTTAC	
165	ACGACTTCGTTTTCTTCGATTTCTGACCAT	
166	AAGAAGCGGAGAGACGGTTAAAAGTGCCGT	
167	CATACTGGGCTTTCTTGACCGCTTCCAGAT	
168	AAATACACTCTATAAGTTGAAAACTCAAAA	Streptococcus phage phiBHN167
169	ACAACTAATATTGCAAGAACTCCCATAAG	
170	CTGCGTTAACCCCTCTGCCATCTTTCCAA	
171	ACAAAATAAGGGACGTCTTCCCAAAGGCAA	
172	TGAAGGCTTGTGTGATTATGCTGAAAGCAG	
173	CAATGAAACCAAGTCTCAACATCATGGAGT	
174	CACGATGGAGCGAACAGTGGTTTTTACCTT	
175	TAATGTGTTCTAGCCTATGAAAAGAGCATA	

Table 4.3 (cont'd)

176	ACTGCCTTGTCCTGTTGATTCAAGTCAGTT	
177	ATCCGCATTCGTTACCGCCCCAATAGTCTC	
178	AATCAGAGTTTTTAGCCGACAAACCAGATG	
179	ACCACGAGCGAACGACTAACGTTAGCTTTA	
180	CAAACGTATAGAAGATGAAGATTTTAAATT	Streptococcus phage JX01
		Streptococcus phage LYGO9
181	AAAAATCGAAAAATAGATGTGCGTCCAGCA	
182	AAGGGTGTTAGATGATAATACCTTTTTTAA	
183	ACACCGTTGCGGTTGTTGTCGGTCACTCAA	Streptococcus phage LF2
184	TCTATTAACAATAGTTTTATCCAATTGTTT	
185	TGAAAACAAGCGCAAAGCTGTCAGAAAACA	
186	CGTACCATCTATCAATTTACCGCAAGCTGT	Streptococcus phage LF1/2/4
187	CCTCAACATAGTAATAGCTCTTTCCCATAG	
188	TTTCATTTTTAATAACCTGCTGGCTCATAT	
189	CTATCAACGGCTTTTTCAATTAGTGAGATA	
190	GTCATGTTATAATTTTCTTGCAAAAAAAAT	shuttle/cloning vectors Streptococcus mutans strain MD plasmid
191	TTAGTTTTGCTGATTTAAGAAAAGGGGGGT	
192	TAGTCGACATAAAACCATTCTTACCACCTC	Streptococcus phage IPP62

Figure 4.1. Phage communities (PCs) can affect the growth of GBS. 10%v/v of three PCs was added to actively growing GBS in a 96-well plate. Growth was observed by OD_{595} for eight hours.

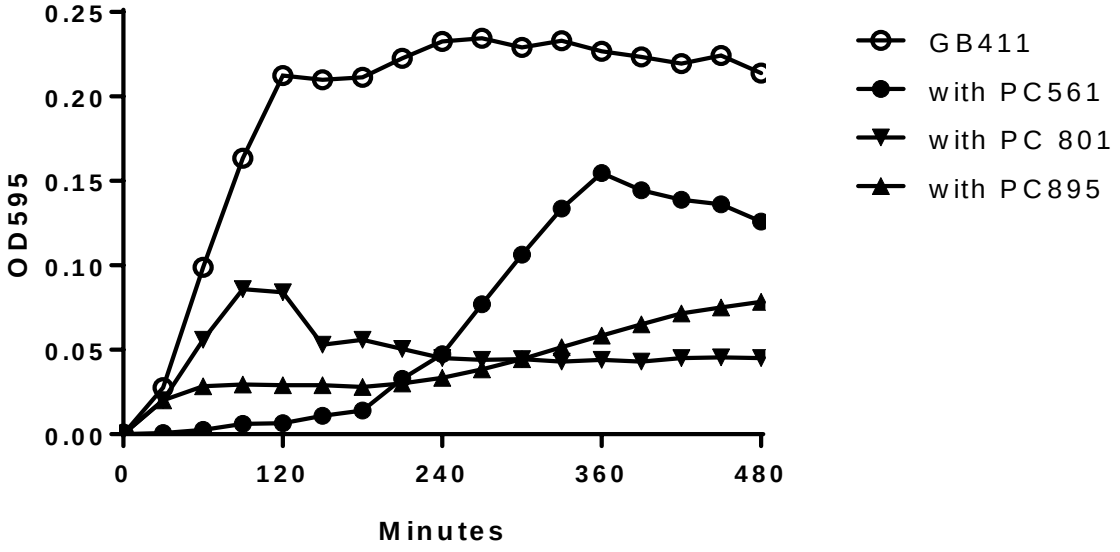


Figure 4.2. CRISPR spacer regions differ across GBS strains. GBS CRIPSR Spacers. CRISPR spacers were extracted from whole sequenced genomes and given a unique number. Red shading in the "Number of Spacers" column represents the 25% quartile of the number of spacers, while green shading represents the 75% quartile. Strains are organized by GB strain number. Spacer colors represent a unique spacer.

GB Strain	CRISPR Information	
	Number of Spacers	Spacers
GB00012	15	99 100 101 102 103 104 105 106 107 108 109 110 107 111 102
GB00020	9	120 121 122 123 124 125 116 79 97
GB00084	14	13 65 66 67 77 68 69 70 71 72 73 74 75 76
GB00097	26	97 96 95 94 119 118 93 92 88 87 86 85 117 83 82 81 80 79 117 117 116 125 90 179 180 78
GB00112	11	97 96 92 91 163 89 88 87 85 191 192
GB00279	8	30 31 54 55 56 57 58 59
GB00555	8	112 58 57 56 55 54 31 30
GB00557	11	41 13 33 34 35 36 37 38 39 28 40
GB00561	8	42 43 44 45 28 29 40 13
GB00590	4	98 29 40 13
GB00651	16	181 43 182 183 184 185 146 145 137 186 37 38 138 28 40 13
GB00653	13	1 2 3 4 5 6 7 8 9 10 11 12 13
GB00663	20	14 15 16 17 18 16 17 18 19 20 21 25 23 24 25 26 27 28 29 13
GB00037	24	97 96 95 94 162 119 118 93 92 91 163 87 88 81 80 79 117 164 169 165 166 167 168 170
GB00045	20	78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97
GB00066	15	113 114 115 116 117 79 81 82 83 92 118 119 95 96 97
GB00079	19	171 172 173 174 175 176 177 178 114 115 89 163 91 92 118 119 95 96 97
GB00374	10	30 31 54 55 60 61 62 63 32 64
GB00377	14	64 63 126 127 128 56 62 61 60 55 54 31 30 32
GB00390	31	13 40 29 28 45 138 38 37 139 161 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160
GB00411	10	155 154 153 152 145 121 141 29 40 13
GB00418	3	30 31 32
GB00438	4	30 31 54 126
GB00910	17	129 130 131 132 133 134 34 35 36 135 44 136 137 37 38 138 13
GB01007	10	13 29 28 27 26 25 24 7 6 5
GB01454	10	13 29 7 6 5 187 188 189 189 190
GB01455	11	13 40 29 46 47 48 49 53 50 51 52

Figure 4.3. The number of CRISPR spacers varies by capsule type. The number of spacers were examined across sequence (A) clinical (B) and (C-D) capsule type. Statistical differences were calculated using chi square tests.

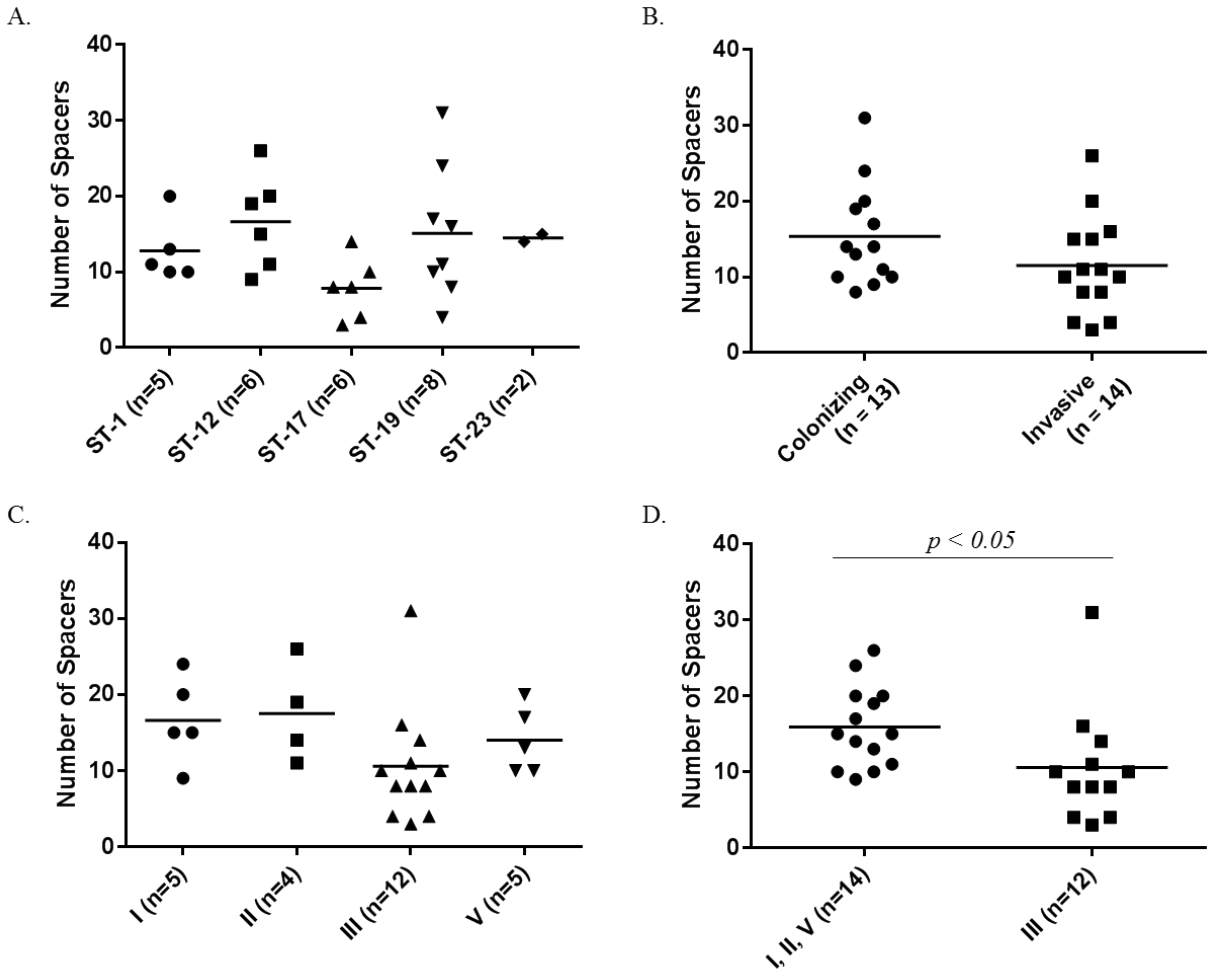


Figure 4.4. The number of CRISPR spacers and the percent GBS affect likelihood of lysis. The number of CRISPR spacers and the percent of GBS in the sample significantly impacts the ability of phage communities to inhibit growth. The 25 (Low) and 75% (High) quartile of phage communities were selected based on the percent of GBS reads in the metagenomic sample. The 25 (Low) and 75% (High) quartile of host samples were selected based on the number of spacers. Combinations that resulted in at least a 10% reduction of growth were coded as 0's. Those above the cutoff were considered resistant (1).

			Phage community Information													
			ER0960	ER1016	ER0788	ER0779	ER1008	ER0974	ER0959	ER1017	ER0886	ER0961	ER0948	ER1015	ER1010	ER1014	ERIN Sample Number
Host Information			0.90309	1.113943	1.30103	1.380211	1.491362	1.491362	1.531479	2.423246	2.591065	2.732394	2.771587	2.928396	2.98317507	3.123525	Percent GBS in sample
GBS Strain	Number of Spacers		Low							High							
GB00653	3		0	0	1	0	0	0	0	0	1	0	0	0	1	0	
GB00112	4		0	0	1	0	0	0	0	0	0	0	0	0	1	0	
GB00663	4		0	1	1	0	0	0	0	0	0	0	0	0	1	1	
GB00079	8	Low	0	0	1	0	0	0	0	0	0	0	0	0	0	1	
GB00084	8		0	0	1	0	0	0	0	0	0	0	0	0	0	0	
GB00279	8		0	1	1	0	0	1	0	1	0	1	0	1	1	1	
GB00020	9		0	0	1	1	0	1	0	0	0	0	1	1	1	0	
GB00910	17		1	1	1	0	1	1	1	1	1	1	1	1	1	1	
GB00555	19		0	0	1	0	0	0	0	0	0	0	0	0	0	0	
GB00390	20		1	1	1	1	1	1	1	1	1	1	1	1	1	1	
GB00418	20	High	0	1	1	0	0	0	0	1	0	0	0	0	1	1	
GB00411	24		0	0	0	0	0	0	0	0	0	0	0	0	0	0	
GB00045	26		0	0	1	0	0	0	0	0	0	0	0	0	0	0	
GB00590	31		0	1	1	0	1	0	0	0	1	0	0	1	1	1	

Figure 4.5. Phage titer experiments result in clearing or a confluent lawn between dilutions.

1:1 dilutions of PC801GB112 was added THB with 0.7% soft agar and overlaid on THA plates. Dilutions (A.) 1:2 and (B.) 1:3 are shown. Similar patterns were observed in BHI.

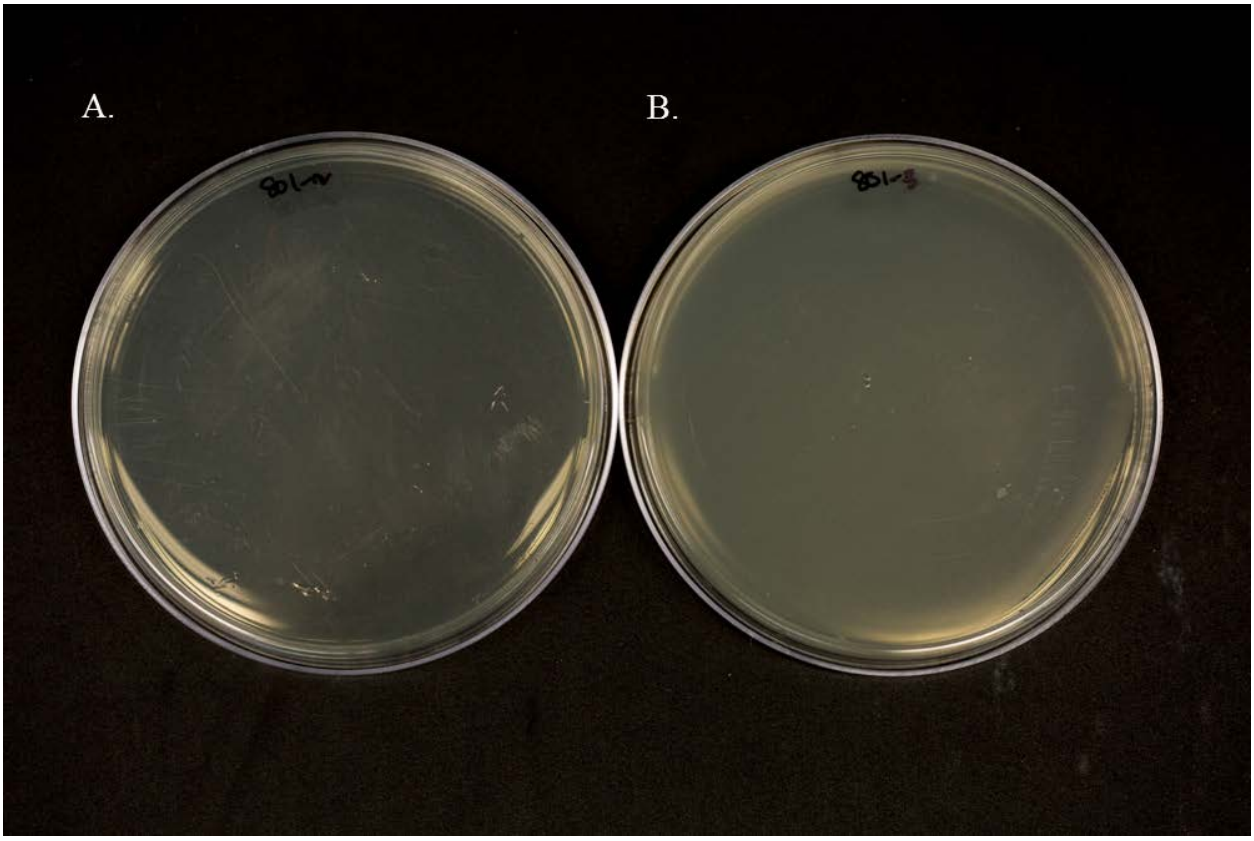


Figure 4.6. Use of BHI media resulted in clearer spot plates with clear lysogenic rings. A lawn of GB411 was created in BHI with 0.7% BHI agar overlay. Ten microliters of (A) PC801, (B) PC801GB411 and (C) a negative control of PC801 enriched without a bacterial host added.

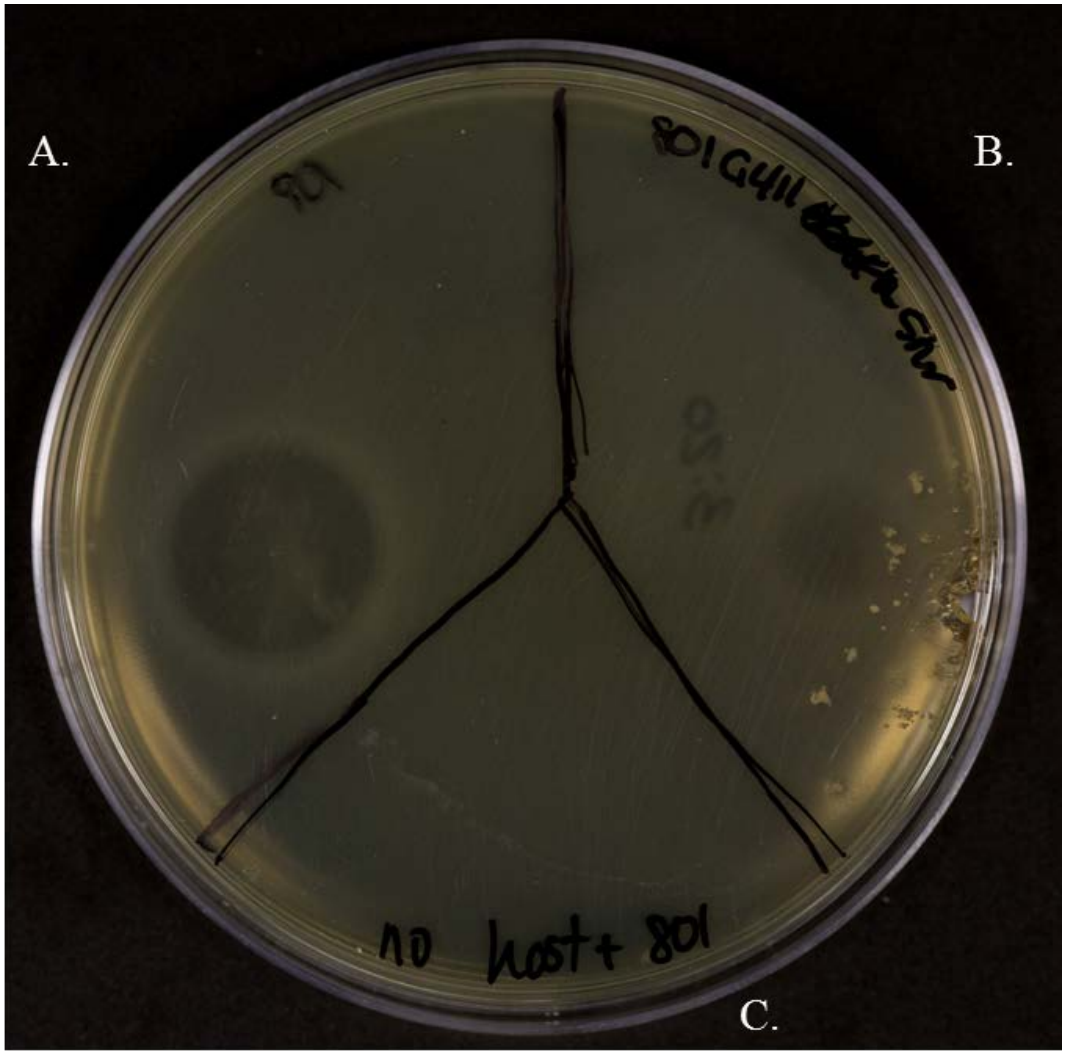


Figure 4.7. Enrichment is not sufficient to isolate phage. PC561 was added to actively growing GB112 and allowed to grow for five hours. Remaining bacteria were pelleted and the supernatant was filter sterilized. The resulting enrichment (PC561.112) was added to actively growing (A) GB411, (B) *Lactobacillus* and (C) *E. coli*. Growth was monitored for eight hours on a plate reader (OD595).

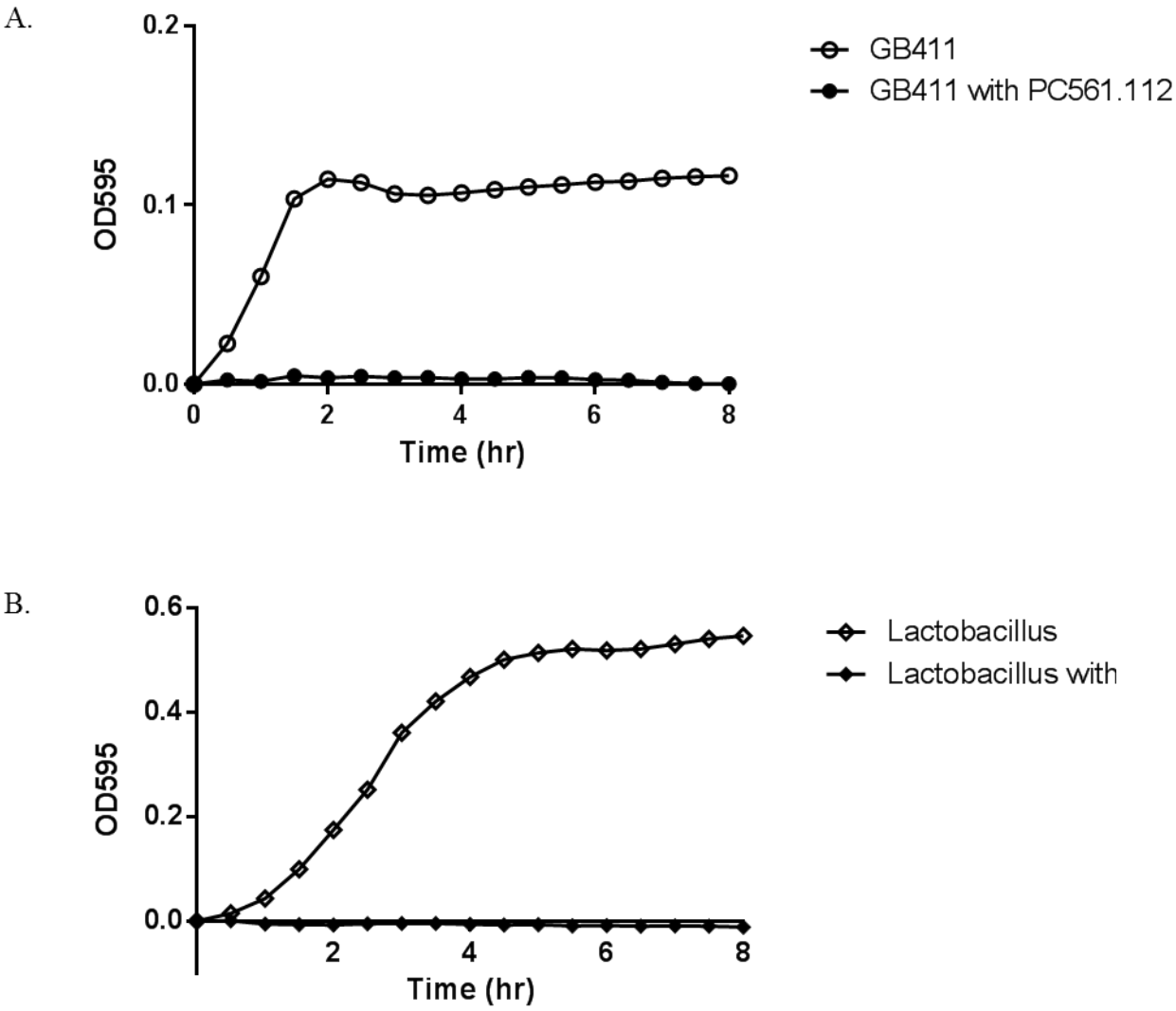


Figure 4.7 (cont'd)

C.

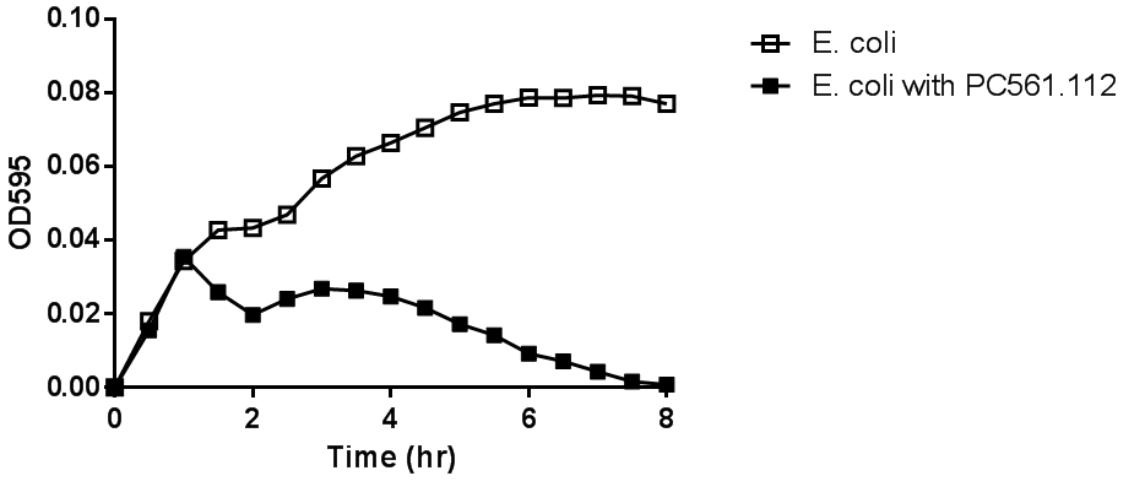
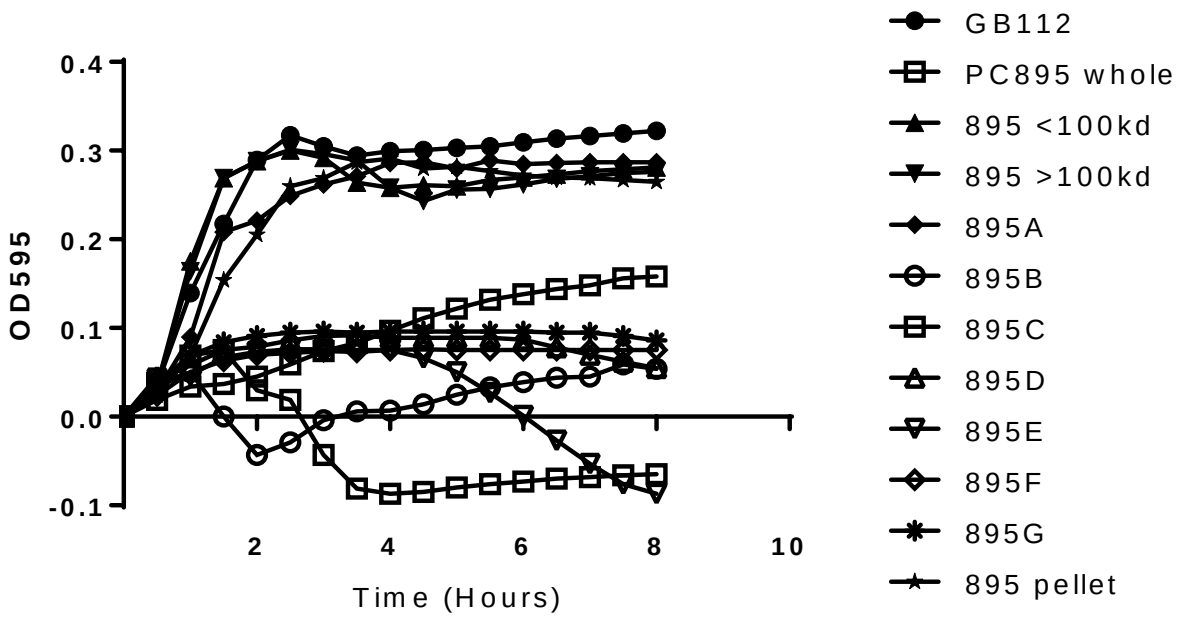


Figure 4.8. Fractions from a cesium chloride gradient maintained infectivity. An enriched sample of PC895 was fractionated by a cesium chloride gradient, resulting in 0.5ml of sample. Lowest density samples are labeled A, while higher density components would be found in the pellet and fraction G. 10% v/v of individual fractions were added to actively growing GB112. Growth was monitored for eight hours on a plate reader (OD595).



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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The microbiota, or the microbes that inhabit the human body, have been extensively studied for their role in human health but can also serve as a reservoir of opportunistic pathogens. The advent and application of metagenomic sequencing has increased our understanding of which microbes are present in the microbiome and has also begun to challenge the sterility of a variety of sites in the human body (e.g., upper reproductive tract, inner ear). Though controversial, such studies have found low loads of bacteria in the placental membranes, including *Lactobacillus*, a commonly used probiotic. While there are studies that assess the functionality of the microbiota in other body sites, no such study has been performed with a model of the placental membranes. Hence, this thesis sought to gain a better understanding of how a typically commensal bacteria, *Lactobacillus*, affects this important barrier during pregnancy. Further, we sought to examine if probiotic properties of *Lactobacillus* observed in other body sites could be extended to the placental membranes to impact an opportunistic pathogen, Group B *Streptococcus* (GBS), which invades these membranes from the vaginal tract and is a leading cause of preterm birth, stillbirth, and neonatal infections. Finally, this thesis sought to examine whether an understudied component of the microbiome, bacteriophage, could impact GBS.

Herein, we characterized four different strains of *Lactobacillus* for their ability to interact with a cell line model representing the outermost layer of the placental membranes, decidual cells. We found that while each strain could associate with the cells, *L. crispatus* associated significantly better. As this is one of the *Lactobacillus* strains most commonly found in the placental membranes, it would be interesting to further examine properties of this strain that allow it to better attach to this cell type in comparison to the other *Lactobacillus* strains. Further, we found that none of *Lactobacillus* strains appear to cause an inflammatory response or host

cell death in the decidual cell model. These finding suggests that if *Lactobacillus* was present in the placental membranes, it would not likely induce inflammation through the decidual cells. However, this model does not evaluate interactions with other cell types in the placental membranes including macrophages, which as professional phagocytes, likely play a larger role in inducing inflammation *in vivo*. For this reason, future directions require investigation of these other cell types individually and in combination. *Ex vivo* models using isolated placental membranes attached to a transwell would be an interesting model to further assess the effects of *Lactobacillus* on intact placental membranes containing multiple cell types.

Because we determined that all four *Lactobacillus* strains associate with decidual stromal cells without inducing damage, we next asked if these strains inhibit pathogens such as GBS, that are capable of invading the placental membranes. As a member of the vaginal microbiome of approximately 30% of the population, GBS has the opportunity to ascend from the vaginal tract to the uterine cavity, where it can associate with the placental membranes. Association with these membranes could result in inflammation leading to premature birth, while invasion of these membranes could result in an *in utero* infection of the fetus. We found that live *Lactobacillus* does not directly inhibit growth or impact biofilm production in two distinct GBS strains. While a certain strain of *Lactobacillus, L. gasseri* 33323, increased association of GBS with decidual stromal cells, this interaction did not result in increased invasion or cell death in the host cells. Hence, this finding suggests that *L. gasseri* may interact with the GBS on the placental membranes. It would be interesting to examine this relationship further to determine if *L. gasseri* and GBS are co-aggregating. The biofilms could also be examined to determine if the two species are forming a multi-species biofilm and how these strains are affecting each other in this environment.

As *Lactobacillus* spp. are known to secrete many inhibitory compounds, we also examined the effect of *Lactobacillus* supernatants on GBS. We found that these supernatants were able to inhibit growth and biofilm formation in GBS, though this inhibition was dependent on the strain of *Lactobacillus*. Nonetheless, these supernatants also negatively affected host cells through GBS-induced host cell death as *Lactobacillus* supernatants alone had no effect. Finally, to assess the potential use of the supernatant on other strains of GBS, we confirmed that supernatant from *L. reuteri* 6475 broadly inhibited 35 GBS strains of different sequence, capsule and clinical types. Collectively, these data suggest that both live *Lactobacillus* and its supernatant could impact GBS interactions with the placental membranes. The variability observed between strain combinations underlines the importance of studying multiple strains when examining these interactions. Future work could focus on further characterization of the supernatants. Fractionation of the supernatants will help determine the causative compound in the supernatants that is contributing the observed phenotypes. Reducing the complexity of the supernatants may also separate the different phenotypes and remove the negative effects we found on host cell death.

Lastly, though bacteriophage are abundant in the microbiome, their role in the colonization of opportunistic pathogens like GBS, which are commonly found in the microbiome, remains unknown. We hypothesized that phage communities isolated from fecal samples would contain phage capable of lysing GBS because GBS is commonly isolated using fecal swabs. We found that 6% of the 130 tested phage communities were capable of lysing a representative GBS strain. We further characterized the interactions between two complex phage communities and multiple GBS hosts (n = 38) and found that capsule, sequence and clinical types of the strains did not affect lysis patterns. However, examination of Clustered regularly

interspaced palindromic repeats (CRISPR) in 27 GBS genomes did reveal differences. These repeats serve as an adaptive immune system against invading foreign DNA including phage; therefore, we hypothesized that higher numbers of sequences would result in strains that are more resistant to lysis by phage communities. Comparing GBS strains with high and low numbers of spacers revealed that strains with fewer spacers were more likely to be lysed by a phage community. As we had observed the ability to these communities to inhibit GBS *in vitro*, we hypothesized that they would also be able to affect GBS *in vivo*, resulting in reduced levels of GBS in the corresponding bacterial metagenomic reads of each sample. While this correlation did not exist across all GBS strains tested, sensitive strains of GBS were significantly more likely to be inhibited by phage communities with a lower abundance of GBS. This finding highlights the importance of both the GBS strain and the phage community in this interaction. Collectively, these data suggest that the phage component of the intestinal microbiome could impact GBS colonization. Outside of the potential role of phage communities in GBS colonization in the rectal tract, it is also attractive to consider the use of bacteriophage to preferentially remove GBS from the microbiota. To examine this, an individual lytic phage would need to be isolated; however, we could not successfully isolate an individual phage. While examination of growth inhibition allowed us to examine a large number of interactions, it may not be optimal for isolating a lytic phage. Instead, it may be more useful to begin with identifying a strain that can form plaques.

This work contributes to several current gaps in our understanding of the microbiota's impact on health. First, the placental membranes were previously considered sterile, but we have characterized potential interactions between a cell line model and *Lactobacillus* and GBS. We have also expanded our understanding of GBS rectal colonization in humans by examining the

potential role of the bacteriophage component of the microbiome. As *Lactobacillus* and bacteriophage have been proposed as alternative therapies for GBS, this work may have downstream impacts on GBS disease prevention.