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The impact of *Lactobacillus* on group B streptococcal interactions with cells of the extraplacental membranes

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Abstract

Group B *Streptococcus* (GBS) causes adverse pregnancy outcomes and neonatal disease. The recommended preventative measure is intrapartum antibiotic prophylaxis, which impacts early onset neonatal disease but fails to protect against chorioamnionitis, preterm labor, stillbirth, or late-onset disease. Novel prevention methods are therefore needed. Use of probiotics including *Lactobacillus* spp., has been suggested given that they are dominant members of the lower reproductive tract microbiome. Although *Lactobacillus* was shown to reduce recto-vaginal colonization of GBS, no studies have examined how *Lactobacillus* impacts GBS in the extraplacental membranes. As *Lactobacillus* has been detected in the placental membranes, we sought to characterize GBS-*Lactobacillus* interactions *in vitro* using a colonizing and invasive GBS strain. While live *Lactobacillus* did not affect growth or biofilms in GBS, co-culture with *L. gasseri* contributed to an over 200-fold increase in GBS association with decidualized human endometrial stromal cells for both GBS strains ($p < 0.005$). Increased association did not result in increased invasion ($p > 0.05$) or host cell death, though some GBS and *Lactobacillus* combinations contributed to a significant reduction in host cell death ($p < 0.05$). Since *Lactobacillus* secretes many inhibitory compounds, the effect of *Lactobacillus* supernatants on GBS was also examined. The supernatants inhibited GBS growth, biofilm formation and invasion of host cells, though strain dependent effects were observed. Notably, supernatant from *L. reuteri* 6475 broadly inhibited growth in 36 distinct GBS strains and inhibited GBS growth to an average of 46.6% of each GBS strain alone. Together, these data show that specific *Lactobacillus* strains and their secreted

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products have varying effects on GBS and its interaction with cells of the extraplacental membranes that could impact pathogenesis. Understanding these interactions could help guide new treatment options aimed at reducing GBS-associated maternal complications and disease.

Keywords

Lactobacillus; group B Streptococcus; pregnancy; placental membranes; biofilm; probiotic

1. INTRODUCTION

Group B *Streptococcus* (*Streptococcus agalactiae*, GBS) is a global human health threat, contributing to neonatal infections and deaths as well as adverse pregnancy outcomes such as chorioamnionitis (infection of the extraplacental membranes), premature birth, preterm premature rupture of membranes (PPROM), and stillbirth [1]. GBS remains the leading cause of neonatal pneumonia, sepsis and meningitis in the United States [2]. In 2018, the Centers for Disease Control and Prevention reported the incidence of early- and late-onset GBS disease to be 0.25 and 0.28 per 1,000 live births, respectively [3]. GBS contributes to premature birth and stillbirth by infecting the fetus following its ascension from the vagina [4], while early-onset neonatal disease can occur due to aspiration of GBS during childbirth following ascension [1]. Although maternal vaginal GBS colonization is typically asymptomatic, GBS chorioamnionitis has been linked to inflammation and subsequent fetal infections [5,6].

Current consensus guidelines in the United States recommend that mothers testing positive for GBS between 36 and 37 weeks gestation receive intrapartum antibiotic prophylaxis (IAP) [2]. This practice has reduced the rate of early-onset neonatal sepsis occurring within the first week of life but has not affected late onset disease [7] or other adverse pregnancy outcomes. While effective at protecting against early-onset infection, several hundred women need to be treated with IAP for each case of early-onset sepsis that is averted [8]. Additionally, antibiotic treatment is known to affect both the maternal vaginal microbiome as well as the neonatal gut microbiome, which are both important for neonatal development [9–11]. The multiple shortcomings with IAP [12] combined with growing concerns about antibiotic resistance have resulted in increased interest in alternative therapies for the prevention of GBS-associated neonatal and pregnancy complications disease. As maternal GBS colonization is the primary risk factor for neonatal infection [1], novel therapies should aim to eradicate colonization or reduce the bacterial density.

Lactobacillus, a dominant member of the vaginal and cervical microbiota [13], has been examined for use as a probiotic against a variety of bacteria including GBS. For example, human trials using orally-administered *Lactobacillus* alone or in combination with other probiotic species, have shown variable, but promising success in reducing recto-vaginal colonization in pregnant mothers [14–17]. Reasons for variability include differences in the length of intervention, strain characteristics and dosage [16], though less is known about how *Lactobacillus* affects GBS and ascending infections. Some studies, however, have found that *Lactobacillus* can reduce the risk of premature birth triggered by inflammation [18–20].

To our knowledge, no prior studies have been conducted to determine how *Lactobacillus* affects GBS ascension of the vaginal tract or GBS virulence phenotypes such as attachment to and invasion of placental cells.

Since *Lactobacillus* has been identified in the human extraplacental membranes using metagenomic techniques [21], we aimed to determine whether different *Lactobacillus* spp. can alter 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 impact on host cell death. These *in vitro* studies reveal potential mechanisms used by *Lactobacillus* to affect colonization and premature birth, allowing for better understanding of its use as a probiotic as well as its limitations.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

GBS strains were selected based on multilocus sequence type (ST) designation, capsular serotype, and source. *Lactobacillus* and GBS interactions were performed using two serotype III GBS strains belonging to ST-17, which has been most associated with severe disease in neonates [22,23]. One strain, GB00112 (GB112), was isolated using a vaginal-rectal swab from a colonized mother after childbirth [24] and the second, GB00411 (GB411), from the blood of a septic newborn [25]. GBS strains were cultured in Todd-Hewitt broth (THB) or half-concentrated THB with agar (THA) at 37°C with 5% CO₂.

Lactobacillus strains were selected to represent species that have been found in both the vaginal tract and extraplacental membranes via metagenomics [21] as well as the gastrointestinal tract. These species included *L. reuteri*, *L. gasseri*, and *L. crispatus* (Table 1). *L. reuteri* 6475 was isolated from breast milk, *L. gasseri* 33323 from the vagina [26], and *L. crispatus* 19390 from stool. *L. reuteri* 17938 is the daughter strain of *L. reuteri* ATCC 55730, which was isolated from breast milk and was previously shown to harbor potentially transferable resistance traits for tetracycline and lincomycin [27]. This daughter strain, however, no longer carries this plasmid. All strains were cultured in de Man, Rogosa and Sharpe (MRS, Difco 288130) broth or agar at 37°C with 5% CO₂. Plating of lactobacilli and GBS co-cultures, however, was performed on Tryptic Soy Broth (TSB) supplemented with 5% Sheep Blood (Northeast Lab Services) at 37°C and 5% CO₂. *Lactobacillus* spp. colonies were given 48 hours to grow.

In addition to live *Lactobacillus* cultures, supernatants were also isolated to evaluate the impact of *Lactobacillus*-associated secreted factors on GBS. Each *Lactobacillus* strain was loop-inoculated into 10 mL of MRS broth with or without 5 mM glycerol in a 15 mL 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 µm filter. Supernatants were used immediately. The effect of the *L. reuteri* supernatants on GBS growth was evaluated using a diverse set of 36 GBS strains (Table S1), while supernatant pH and hydrogen peroxide concentrations were measured in biological triplicate. The pH levels were measured using a pH reader (SevenEast pH, Mettler-Toledo GmbH). Hydrogen peroxide concentrations (µM) were determined using 50µl of supernatant in a Fluorometric

Hydrogen Peroxide Assay Kit as described by the manufacturer (Sigma Aldrich; Lot#187037).

2.2. Cell Culture

Telomerase-immortalized human endometrial stromal cells (T-HESC; ATCC CRL-4003) [28] were cultured in DMEM/Nutrient Mixture Ham's F-12 with L-glutamine (Sigma) supplemented with 1% ITS+ universal cell culture supplement premix (BD Biosciences), 1.5 g/liter sodium bicarbonate, 2% penicillin / streptomycin, and 10% charcoal-treated fetal bovine serum (HyClone), which is referred to as HESC medium herein. For all cell experiments, the T-HESC line was decidualized (dT-HESCs) by incubating with 0.5 mM 8-Bromo-cyclic AMP (cAMP) (Sigma) for three to six days as described [29]. These cells are also referred to as decidual cells. Assays were only performed when cells reached a 100% confluent monolayer so that only decidual cell surfaces were available for bacterial interactions.

2.3. Bacterial growth curves

To examine the effect of live *Lactobacillus* and its supernatants on GBS, growth curves were generated by serial plating or using a plate reader (Beckman Coulter, Inc). For the *Lactobacillus* and GBS co-culture experiments, overnight cultures 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, 100 μ L of 0.1 OD 600 culture in HESC infection media was added to a 96-well plate with 25 μ L of supernatant or additional infection media. Time points were collected every 15 minutes for eight hours by plate reader (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.

2.4. Biofilm assays

Biofilm production was quantified as described in our prior study [30]. Briefly, overnight cultures of GBS and *Lactobacillus* were diluted to an equivalent OD₆₀₀ of 0.1 and resuspended in Tryptic Soy Broth supplemented with 1% dextrose (TSBd); 50 μ l of each culture was added to a 96-well plate for co-culture biofilms. Mono-culture wells contained 50 μ l of culture and 50 μ l of media. *Lactobacillus* supernatant was added to GBS in monoculture 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% CO₂. After incubation, wells were washed twice and 100 μ l of crystal violet was added. After a ten-minute incubation, crystal violet was removed, and the wells were washed four times with 150 μ l of PBS. Remaining crystal violet was solubilized with 100% ethanol; 50 μ l was taken from each well, and absorbance at OD₅₉₅ 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.

2.5. Association with and invasion of decidual cells

Monolayers of dT-HESCs 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) cells per host cell (MOI = 10) for both monoculture and co-culture wells to assure the same number of GBS cells were available to affect the decidual cells.

Co-culture wells had an additional MOI of 10 of each *Lactobacillus* strain. For the experiments with *Lactobacillus* supernatants, 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°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 [31]. 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 the Triton-X treatment and enumeration steps described above. The percent of associated cells was 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.

2.6. 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 4 µM ethidium homodimer 1 (Molecular Probes) suspended in PBS as described previously [32]. 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.

3. RESULTS

3.1. Impact of live *Lactobacillus* strains on growth of GBS

To evaluate growth of each GBS strain in the T-HESC media used for each experiment, growth curves were performed with GBS in monoculture and co-culture with *Lactobacillus*. GBS growth was measured for the two GBS strains, GB00112 (GB112) and GB00411 (GB411), grown alone and in co-culture with the four *Lactobacillus* strains (Figure S1A). To

determine if there was a difference in growth, the AUC was calculated and compared across strains (Figure S1B). Co-culturing GBS with any of the four *Lactobacillus* strains, however, did not significantly affect the growth of GBS ($p > 0.05$). Because colonies of *Lactobacillus* were not detectable in co-culture after the first time point due to the higher concentration of GBS, we were unable to calculate *Lactobacillus* growth in co-culture.

3.2. Effect of live *Lactobacillus* on GBS biofilm formation

As biofilms are thought to be important for colonization and persistence [33], 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 monoculture and to each GBS strain in co-culture with all four *Lactobacillus* strains. The colonizing GB112 strain formed a biofilm of 0.86 in monoculture, while co-culturing with all four *Lactobacillus* strains resulted in an increase in biofilm production in this strain (Figure S2A). Specifically, co-culturing GB112 with *L. reuteri* 6475, *L. gasseri* 33323, *L. reuteri* 17938, and *L. crispatus* 19390 increased biofilm production to absorbance values of 1.36, 1.37, 1.49, and 0.97, respectively. This increase in biofilm production, however, was not statistically significant ($p > 0.05$). While the invasive GB411 strain formed a stronger biofilm than GB112 with an absorbance value of 1.22, the difference between the two strains was not significant ($p > 0.05$). Similarly, no difference was observed in GB411 biofilm production following co-culture with *L. reuteri* 6475, *L. gasseri* 33323, *L. reuteri* 17938, and *L. crispatus* 19390, which had absorbance values of 1.44, 1.50, 1.80 and 1.25, respectively ($p > 0.05$) (Figure S2B). No differences were observed between GB112 and GB411 and co-cultured with each *Lactobacillus* strain.

3.3. *Lactobacillus* variably affects GBS association with decidual cells

As we have demonstrated previously, dT-HESCs serve as a model of GBS attachment to and invasion of the extraplacental membranes [30–32]. We have also previously established that *Lactobacillus* is capable of associating with this cell line [34]. Hence, the goal of this experiment was to determine whether live *Lactobacillus* affects the ability of GBS to interact with this important fetal barrier. While no difference was observed in the level of association with dT-HESCs between the colonizing and invasive GBS strains of GBS ($p > 0.05$), differences were observed in response to co-culture with *Lactobacillus*. Co-culture with *L. gasseri*, for example, contributed to a significant 224-fold increase in association of the colonizing GB00112 strain compared to the monoculture ($p < 0.005$; Figure 1A). A similar effect was observed for the invasive GB00411 strain as association to dT-HESCs increased 233-fold in co-culture with *L. gasseri* relative to the invasive strain alone ($p < 0.05$; Figure 1B). Association to dT-HESCs following co-culture with *L. gasseri* 33323 was significantly greater for both GBS strains when compared to all other *Lactobacillus* and GBS combinations. No significant differences in association levels were observed between the GBS strains in monoculture compared to co-culture with both *L. reuteri* strains and *L. crispatus* ($p > 0.05$). Both GBS strains also demonstrated similar responses to the addition of *Lactobacillus*, with no observed statistical differences between each co-culture condition.

GBS invasion of the dT-HESCs was also examined. No significant difference in percent invasion was observed between the colonizing and invasive GBS strains in monoculture ($p >$

0.05). The percentages, however, were slightly higher for the colonizing GB112 strain. Although the addition of each *Lactobacillus* strain did not affect the ability of either GBS strain to invade the dT-HESCs ($p > 0.05$; Figures 1C and 1D), calculating the percent of associated cells that invaded resulted in 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 *L. reuteri* 6475, *L. gasseri*, *L. reuteri* 17938 and *L. crispatus*, respectively. A similar reduction was observed with the invasive strain of GBS, with 3.68% of GBS invading in monoculture 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, however, we cannot determine if these differences were statistically significant as they cannot be paired in a way to allow for biological replicates.

3.4. Variation in the impact of *Lactobacillus* strains on GBS-induced host cell death

GBS is known to lyse host cells and this hemolysis could influence the ability of GBS to cross barriers like the extraplacental membranes [5]. To determine if *Lactobacillus* can prevent GBS-mediated cell lysis, we performed host cell permeability assays. Our prior study demonstrated that the same live *Lactobacillus* strains do not induce dT-HESC death [34]; therefore, the impact of GBS co-cultured with each *Lactobacillus* strain was evaluated. When evaluated in monoculture, both the colonizing (Figure 2A) and invasive (Figure 2B) GBS strains significantly damaged the host cells. The colonizing GB112 strain resulted in 71.16% ($p < 0.00005$) cell death in the four-hour period compared to the mock infection (24.85%), while the invasive GB411 strain caused 70.78% cell death ($p < 0.00005$). No significant difference in cell damage was observed between the colonizing and invasive strains ($p > 0.05$). When GBS was co-cultured with *Lactobacillus*, however, certain strain combinations contributed to a significant reduction in cell death. These effects were dependent on the GBS strain. For the colonizing GB112 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 2A). Conversely, in the invasive GB411 strain, only *L. reuteri* 6475 significantly reduced host cell death from 70.78% to 53.25% ($p < 0.05$; Figure 2B)

3.5. *Lactobacillus* supernatants inhibit GBS growth

Because *Lactobacillus* was unable to grow in the HESC infection media, we hypothesized that it may not be producing the secondary metabolites that could inhibit GBS. To evaluate 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 AUC. Unlike the live *Lactobacillus* co-culture experiments, the supernatants from *L. reuteri* 6475, *L. gasseri* 33323 and *L. reuteri* 17938 reduced the AUC of the colonizing GB112 strain from 1.44 to 0.46 ($p < 0.00005$), 0.25 ($p < 0.00005$) and 0.53 ($p < 0.00005$), respectively (Figure 3A). 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 3B). 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.

Because *L. reuteri* has been shown to use glycerol to synthesize reuterin, a compound that has broad spectrum antimicrobial activities [35], we also assessed growth effects on GBS when glycerol was added. Intriguingly, the addition of glycerol increased the inhibitory effect of *L. reuteri* 17938 ($p < 0.05$). For the colonizing strain, GB112, the addition of glycerol further reduced the AUC to 0.15 following co-culture with *L. reuteri* 17938 ($p < 0.05$; Figure 3A), while it further reduced the AUC of the invasive GB411 strain to 0.29 ($p < 0.005$; Figure 3B). The addition of glycerol did not significantly reduce the AUC for either GBS strain upon co-culture with *L. reuteri* 6475, *L. gasseri* 33323, or *L. crispatus* 19390 ($p > 0.05$).

3.6. *Lactobacillus* supernatants prevent GBS biofilm formation

Since we found that supernatants affected GBS growth, we hypothesized that 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. Significant differences in GBS biofilm formation were observed for all supernatants compared to the control except for the invasive GB411 strain co-cultured with *L. reuteri* 6475 supernatant (Figure 4). Supernatants of *L. reuteri* 6475, *L. gasseri*, *L. reuteri* 17938, and *L. crispatus* 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 ($p > 0.05$).

3.7. Impact of *Lactobacillus* supernatants on GBS interactions with dT-HESCs

To further assess the effects of *Lactobacillus* supernatants, we characterized how the addition of each supernatant would affect GBS association with and invasion of dT-HESCs. As this experimental design accounts for growth within the time span of the experiment by presenting the data as a percent of the CFU at the end of the incubation period, differences are independent of the growth changes observed. Because lactobacilli are lactic acid producing bacteria, we were concerned that the supernatants could damage the host cells, which are sensitive to pH changes. However, we observed no significant effect on host cell permeability for all supernatants except for those recovered from *L. gasseri* ($p < 0.05$; Figure S3). Supernatants from *L. gasseri* significantly increased host cell permeability from 27.27% to 59.84% in three hours ($p < 0.05$); therefore, results using this supernatant in combination with dT-HESCs must be interpreted carefully as changes may be due to supernatant-induced host cell permeability.

Generally, the addition of all *Lactobacillus* supernatants reduced association of GBS with dT-HESCs except for *L. reuteri*. 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 association of the colonizing GB112 strain to 35.85% ($p > 0.05$), 29.74% ($p < 0.05$) and 4.41% ($p < 0.005$), respectively (Figure 5A). The addition of glycerol during *Lactobacillus* growth also resulted in reduced association for the colonizing GB112 strain,

but this decrease was not statistically significant. By contrast, supernatants from *L. reuteri* 6475, *L. gasseri* 33323, *L. reuteri* 17938, and *L. crispatus* 19390 grown with glycerol 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 GB411 strain was also reduced by *Lactobacillus* supernatants (Figure 5B). Supernatants from *L. reuteri* 6475, *L. gasseri* 33323, *L. reuteri* 17938, and *L. crispatus* 19390 grown in the absence of glycerol reduced association of the invasive GB411 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. No significant differences were observed between the colonizing and invasive strains based on interactions with a given supernatant except for *L. reuteri* 6475 grown without glycerol. This strain contributed to increased association of the colonizing GB112 strain but reduced association of the invasive GB411 strain ($p < 0.00005$).

Because invasion of the extraplacental membranes is an important step in *in utero* infection for GBS, we also sought to assess the effect of these *Lactobacillus* 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. Controlling for variation among biological replicates, however, did not account for all the variation observed in these experiments. Indeed, the level of invasion observed for the colonizing GB112 strain was particularly variable for all supernatants except *L. crispatus*, (Figure 5C). Given this high degree of variability, no significant differences were observed for invasion by GB112 following incubation with each *Lactobacillus* supernatant ($p > 0.05$). For the invasive GB411 strain, the most variable invasion percentages were observed with the *L. reuteri* 17938 supernatant. Only the addition of *L. crispatus* supernatant contributed to a significant increase in GB411 invasion of dT-HESCs (Figure 5D).

3.8. Impact of *Lactobacillus* supernatants on host cell death differs by GBS strain

As maintenance of the extraplacental membranes is important for a successful pregnancy, we also examined if the *Lactobacillus* supernatants altered the amount of host cell damage induced by GBS. 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 GB112 strain of GBS caused 60.5% cell death alone, but with the addition of supernatants from *L. reuteri* 6475, *L. gasseri*, *L. reuteri* 17938, and *L. crispatus*, cell death increased to 74.8%, 77.0%, 75.1% and 81.0% (Figure 6). These increases were not statistically significant ($p > 0.05$). By contrast, the invasive GB411 strain had increased host cell death in the presence of *Lactobacillus* supernatants from all four strains. 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*, *L. reuteri* 17938, and *L. crispatus*, respectively. No significant differences

were observed for host cell death with the colonizing strain versus the invasive strain using the same supernatant.

3.9. Supernatant from *Lactobacillus reuteri* 6475 broadly inhibits GBS strains

To determine if growth inhibition of GBS was specific to the two strains, GB411 and GB112, a larger sampling of 36 strains representing diverse sequence types (STs), serotypes and sources (Table S1) was examined. Only the impact of *L. reuteri* 6475 supernatant was evaluated. Among all 36 strains tested, GBS growth was broadly inhibited, averaging 46.6% of each GBS strain alone and ranging from 39.2% to 54.7% (Figure S4). No significant differences, however, were observed across strains based on the level of growth inhibition or after stratifying by strain characteristics, namely ST, capsule type and source.

3.10. Supernatants have variable H₂O₂ and pH levels

To better understand the effects of *Lactobacillus* supernatants, we examined two properties of the supernatants that have been previously shown to affect other species: pH and H₂O₂ concentration. The pH was similarly acidic for all supernatants, but *L. gasseri* was significantly lower than all other strains at 3.73 (Figure 7A; $p < 0.0001$). pH of *L. reuteri* 6475, *L. reuteri* 17938 and *L. crispatus* supernatants were 4.20, 4.21 and 4.34, respectively. There were also significant differences between *L. crispatus* and both of the *L. reuteri* strains ($p < 0.001$, ANOVA). Hydrogen peroxide concentrations were also different across the *Lactobacillus* strains examined. *L. crispatus* supernatants, for instance, had a significantly greater concentration of hydrogen peroxide (0.32 μM) compared to all other strains (Figure 7B; $p < 0.0001$, ANOVA). *L. reuteri* 6475 had a greater concentration (0.11 μM) than *L. reuteri* 17938 (0.06 units per ul, $p < 0.001$, ANOVA) and *L. gasseri* (0.02 μM), but the difference was not significant for *L. gasseri*. Similarly, no significant difference was observed between *L. gasseri* and the *L. reuteri* 17938 strain ($p > 0.05$ ANOVA).

4. DISCUSSION

Efforts to reduce the burden of perinatal GBS infections through antibiotic treatment have not been fully successful [2,12]. While reducing rates of early-onset neonatal sepsis, there has been no effect on late-onset disease [7] or other GBS-related adverse pregnancy outcomes. Further, the use of antibiotics is not without potential negative effects, including those on maternal and neonatal microbiomes [9–11], increased risk of *E. coli*-associated disease, and antibiotic resistance [36,37]. For this reason, alternative therapies, including probiotics, have been suggested. Herein, we sought to characterize the effects of different *Lactobacillus* strains and their secreted factors on GBS and its interactions with cells (dT-HESCs) that represent those found in the extraplacental membranes. To determine if GBS-lactobacilli interactions vary across GBS strains, we first compared interactions among the invasive GB411 and colonizing GB112 strains, which both belong to the ST-17 lineage that has been shown to disproportionately affect neonates [22,23].

Surprisingly, we found that live *Lactobacillus* did not affect growth or biofilm production for either GBS strain. These results, however, are likely due to the inability of *Lactobacillus* to grow in both the infection media and the biofilm media, and hence, these findings may not

accurately represent the impact on GBS. Future work should therefore evaluate these interactions with different media, particularly given the inhibitory effects observed when some *Lactobacillus* supernatants were examined. It would also be interesting, for example, to determine the relative composition of *Lactobacillus* and GBS within each biofilm as previous work showed inhibition of biofilm formation in *Pseudomonas fluorescens* and *Bacillus cereus* by *Lactobacillus* spp. [38]. Given the phenotypic variation observed among the four *Lactobacillus* strains evaluated herein, it is likely that distinct *Lactobacillus* strains respond differently to different pathogens and environmental signals. Indeed, the prior study of bacterial biofilms utilized a strain of *Lactobacillus plantarum* [38], which not evaluated in our study.

Nonetheless, it is notable that certain *Lactobacillus* strains could alter GBS interactions with dT-HESCs. While we have shown that each *Lactobacillus* strain was capable of attaching to dT-HESCs [34], most of the strains evaluated did not increase the ability of GBS to associate with host cells. The exception was *L. gasseri* as co-culture significantly increased association of both the colonizing and invasive GBS strains, suggesting that these species interact in a way that promotes GBS attachment. Prior studies have shown that this strain of *Lactobacillus* can aggregate with other pathogens [39] and GBS [40], which could enhance colonization of both species if the aggregate is secured to the host cells. It is important to note that increased association may not necessarily be a negative outcome as association did not lead to an increase in invasion or death of dT-HESCs. Indeed, these findings are in line with those from a prior study that showed co-aggregation of GBS and *Lactobacillus* spp. was inhibitory on GBS viability [40]. Future studies involving the use of advanced microscopy tools could enhance understanding of co-aggregation, particularly to dT-HESCs and other cells *in vitro*.

Because of the concerns regarding poor growth of *Lactobacillus* in the media used for the experiments, we were unable to evaluate the impact of specific secreted factors that may be produced by each *Lactobacillus* strain. The role of factors such as bacteriocins, which have been isolated from *L. acidophilus* [41] and other lactic acid bacteria [42], should be evaluated in future studies. Instead, we evaluated the effect of *Lactobacillus* supernatants representing a compilation of secreted factors, on GBS growth and biofilm production. Since biofilms are a congregation of cells surrounded by a polysaccharide matrix [33], we hypothesize that the observed reduction in biofilm production may be due to the limited growth of GBS. Indeed, previous work has demonstrated the importance of environmental conditions for GBS biofilm production [43], suggesting this may be the primary reason for reduced levels. It is interesting to note, however, that though supernatant from *L. crispatus* did not significantly inhibit growth, it did significantly inhibit biofilm formation ($p < 0.005$). It is therefore possible that another mechanism of biofilm reduction exists in some strains. Nonetheless, this growth deficiency may explain the effects on biofilm production but it does not explain the effects on GBS association with dT-HESCs, which was also reduced by *L. crispatus* and other *Lactobacillus* supernatants. Because the experimental design of the association assays accounts for growth by testing the CFUs at the end of the incubation period, these reductions are due to another factor. Further characterization of the supernatants is critical to identify those factors that have contributed to reductions in GBS association levels. Indeed, we hypothesized that reuterin, a compound with broad

spectrum antimicrobial activities produced by *L. reuteri* [35], may be one of these factors. Because *L. reuteri* uses glycerol to synthesize reuterin, we evaluated whether adding glycerol would result in increased GBS inhibition, particularly for the two *L. reuteri* strains. An increase in inhibition was only observed by *L. reuteri* 17938, suggesting that it may be capable of producing reuterin unlike the other *L. reuteri* strain.

The different *Lactobacillus* supernatants also variably impacted GBS invasion. Although the host cell death assay time points are three hours later than those for the invasion assays, these results could suggest that the host cells are becoming more permeable within the time frame of the invasion assay. If this cell death is increased or decreased during the first two hours of the experiment, then it may explain the high variability between biological replicates; the assay requires the host cell to be non-permeable to protect the invaded GBS from antibiotic treatment. It is also notable that host cell death was increased with the combination of GBS plus the supernatants since the supernatants alone did not affect host cell death with the exception of *L. gasseri*. This finding suggests that GBS can alter its interactions with host cells in response to the supernatant, which was more pronounced in the invasive GBS strain compared to the colonizing strain. This variation further suggests that there may be differences in how these two strains respond to microbial cues, which is consistent with prior studies. For example, key stressors like antibiotics and reactive oxygen species, have been shown to alter GBS interactions with host cells including increasing macrophage uptake [44]. Additional experiments are needed to determine how GBS alters its transcription or protein profile to better understand these changes. An increase in GBS virulence in response to *Lactobacillus* supernatants is of particular importance because the extraplacental membranes are critical for maintaining a health pregnancy.

The examination of pH level and hydrogen peroxide concentrations in each *Lactobacillus* supernatant identified more interesting differences between the *Lactobacillus* strains. The lower pH of the *L. gasseri* supernatant may have contributed to its effect on dT-HESC viability; however, we did not see a significant difference in the relative reduction in GBS growth between the strains that could be explained solely by this difference in pH. This result differs from those findings from a prior study that showed a pH-dependent effect of the supernatants [40]. Specifically, those supernatants with a pH below 4.0 could completely kill GBS, while those with levels between 4.0 and 4.5 simply reduced growth. Despite observing a *L. gasseri* supernatant pH level of 3.73 in our study, we did not observe GBS killing, but rather, a reduction in GBS was detected. This difference could also be explained by differences in GBS strains. Similarly, differences in hydrogen peroxide concentrations were observed in the *Lactobacillus* supernatants. Hydrogen peroxide is a reactive oxygen species that can lead to peroxidation of membrane lipids and increased membrane permeability of bacteria such as *Salmonella enterica*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus faecalis* [45]. Although the *L. crispatus* supernatant had the highest concentration of hydrogen peroxide, we did not observe a corresponding difference in GBS growth inhibition, biofilm production, or association and invasion of dT-HESCs. Indeed, *L. crispatus* did not reduce GBS growth and was comparable to the other *Lactobacillus* strains for inhibition of GBS biofilms, association and invasion. Because GBS is resistant to hydrogen peroxide [46,47], these data suggest that hydrogen peroxide concentration is not likely contributing to the GBS inhibition observed.

Since we observed effects on growth and association with dT-HESCs, future work should focus on examining the different components of the supernatants to determine which components are responsible for each phenotype. 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, from negative effects including increased GBS-induced host cell death. Identification of growth inhibitory compounds would be of particular interest due to its broad impact on GBS growth across a diverse set of strains.

CONCLUSIONS

While current literature examines the effect of *Lactobacillus* on vaginal-rectal colonization, we sought to determine if distinct lactobacilli could also impact GBS phenotypes that are important for ascending infections, including growth, biofilm production, association with and invasion of decidual cells, and host cell death. Because the decidua is the outermost cell layer of the extraplacental membranes, it represents a potential early contact point for bacteria, including GBS, which ascend from the cervicovaginal region. Hence, it is biologically plausible that lactobacilli and GBS could be physically close to each other in the lower reproductive tract and near the decidua of the extraplacental membranes. While we show that live *Lactobacillus* had a minimal impact on GBS, some *Lactobacillus* supernatants variably affected growth, biofilm production, association with and death of dT-HESCs. Some of these effects, namely increased association and induction of host cell death, are concerning when considering the importance of the extraplacental membranes in maintaining a healthy pregnancy. Therefore, these findings further demonstrate the importance of a thorough examination of the effects of any potential alternative therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Several *Lactobacillus* spp. had variable effects on group B *Streptococcus* (GBS) association with decidualized human endometrial stromal cells but no effect on cell invasion or cell death
- Different *Lactobacillus* supernatants containing secreted inhibitory compounds were capable of inhibiting GBS growth, biofilm formation and host cell invasion
- Supernatant from one *L. reuteri* strain demonstrated a broad level of growth inhibition in 36 distinct GBS strains of varying serotypes and multilocus sequence types
- Collectively, these data indicate that specific *Lactobacillus* strains and their secreted products have varying effects on GBS that could impact pathogenesis or protection from invasive disease

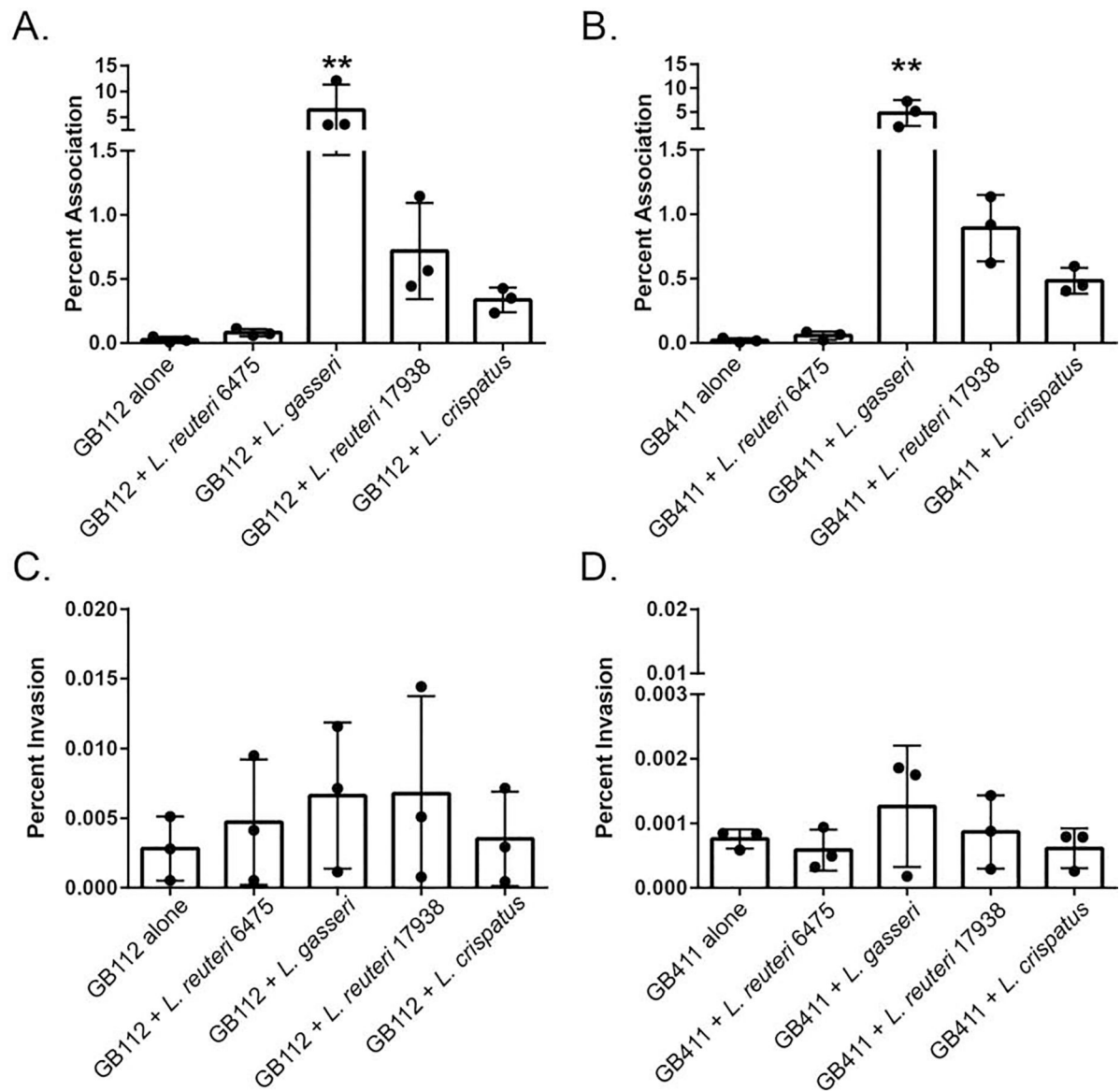


Figure 1. Live *Lactobacillus* variably impacts GBS association with decidual cells. dT-HESCs were infected with a colonizing (GB112) or invasive GBS strain (GB411) at a MOI of 10 for two hours with or without an equivalent amount of each *Lactobacillus* strain. The percent association for **A)** GB112 and **B)** GB411 as well as the percent invasion for **C)** GB112 and **D)** GB411 were calculated relative to the total number of bacteria in the well. Experiments were completed in biological triplicates of technical triplicates. Error bars represent standard deviation between biological trials. Significance was determined by an unpaired ANOVA. $p < 0.005$ **

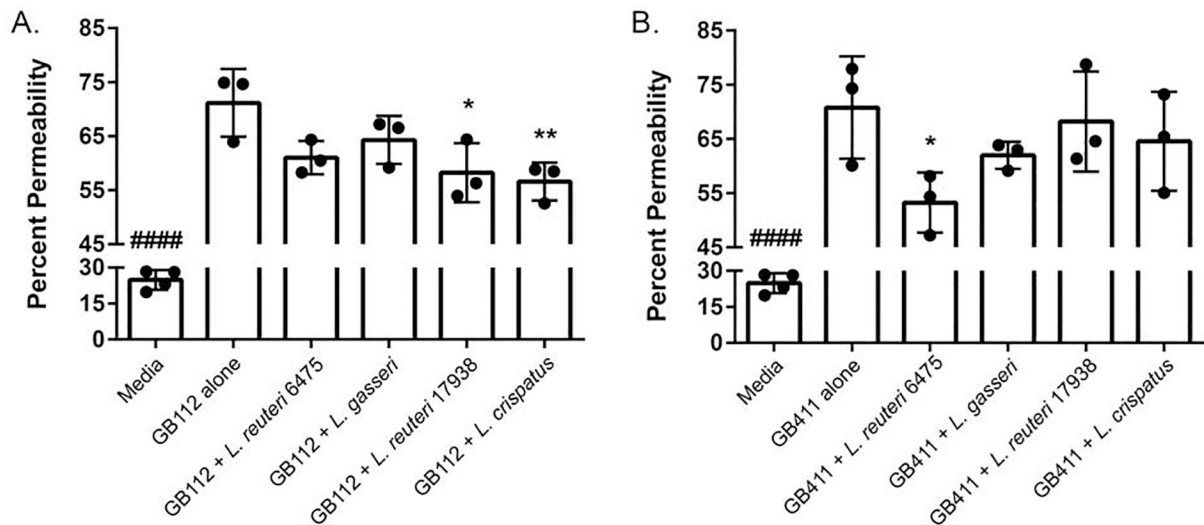


Figure 2. Co-culturing GBS with *Lactobacillus* variably affects host cell death.

dT-HESCs were infected with the **A)** colonizing GBS strain, GB112, and **B)** invasive GBS strain, GB411, at a MOI of 10 with or without the equivalent amount of *Lactobacillus*.

Following a four-hour incubation, cell permeability was detected using an ethidium homodimer, and percent permeability, or percent of cells that were permeable, was calculated by lysing the remaining cells 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 monoculture and co-culture. $P < 0.05$ *; $p < 0.005$ **; $p < 0.00005$ ##### All other conditions were significantly higher than the media control.

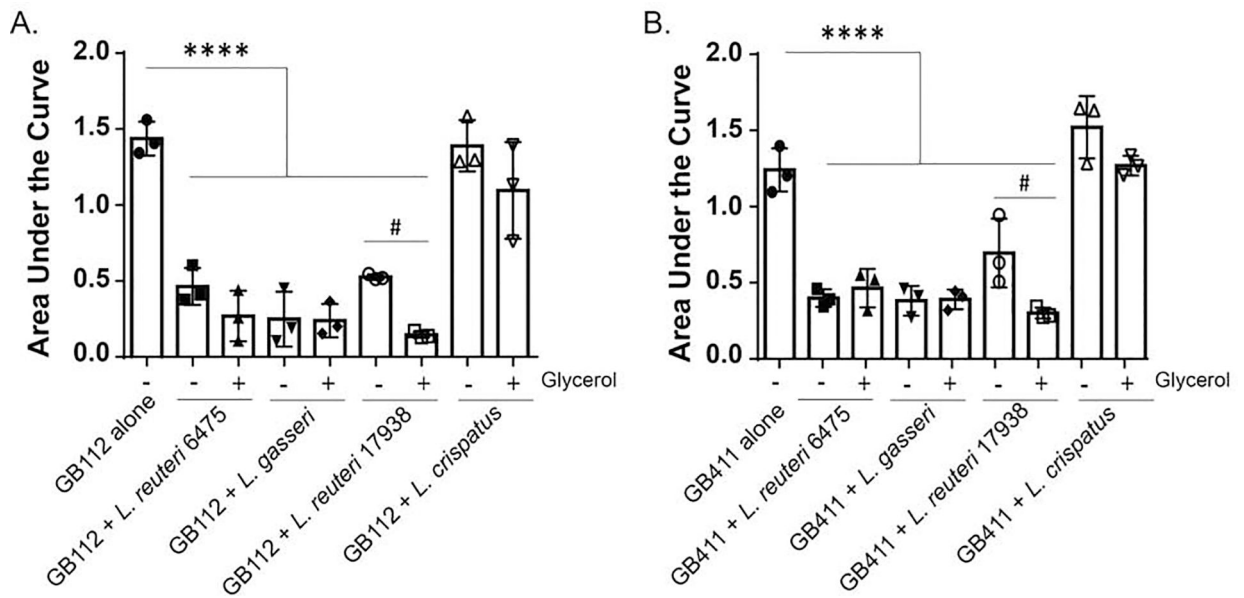


Figure 3. Effect of *Lactobacillus* supernatants on GBS growth with and without glycerol. Growth of the **A)** colonizing GB112 strain and **B)** invasive GB411 strain was monitored for eight hours at OD₅₉₅ when grown alone or with *Lactobacillus* supernatants (25% v/v) in the presence (+) and absence (-) of glycerol. The Area Under the Curve (AUC) is presented. Error bars represent the standard deviation between three biological trials. # An unpaired ANOVA was used to test differences between each supernatant condition and GBS alone. p < 0.00005 **** A Student's t-test was used to evaluate differences based on the addition of glycerol. p < 0.05

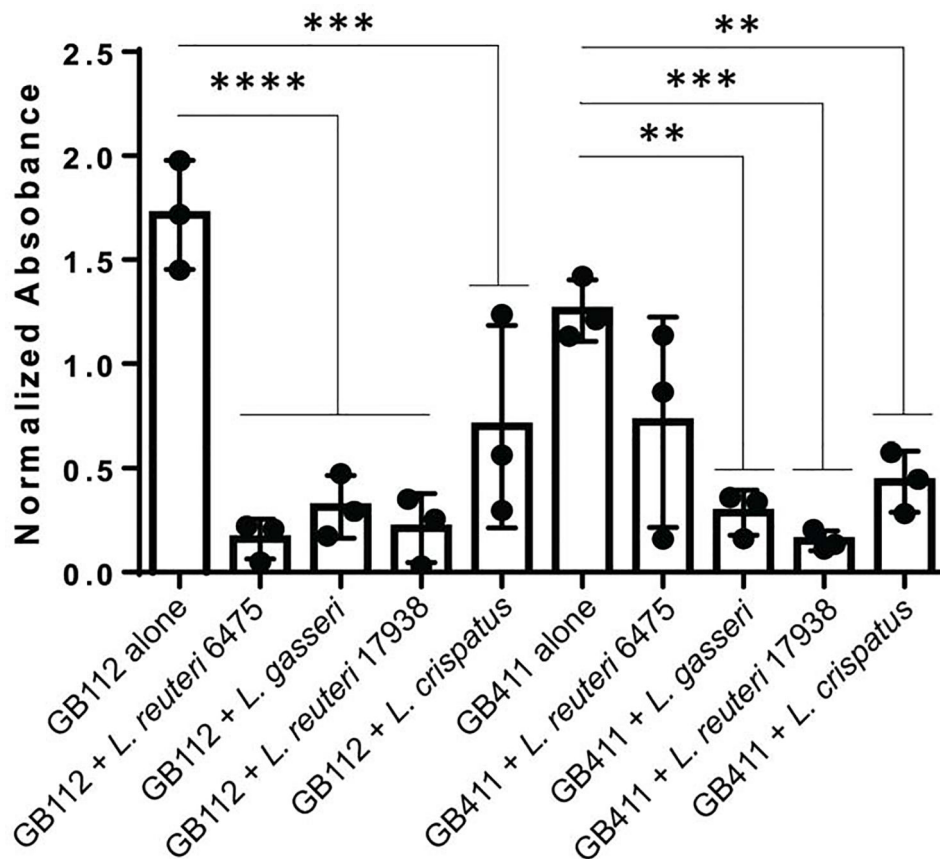


Figure 4. *Lactobacillus* supernatants reduce GBS biofilms.

GBS was grown for 48 hours with or without 10% v/v *Lactobacillus* supernatants to measure biofilm production. Normalized absorbance was calculated by taking OD₅₉₅, subtracting the media control and multiplying by four. Error bars represent the 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 ****

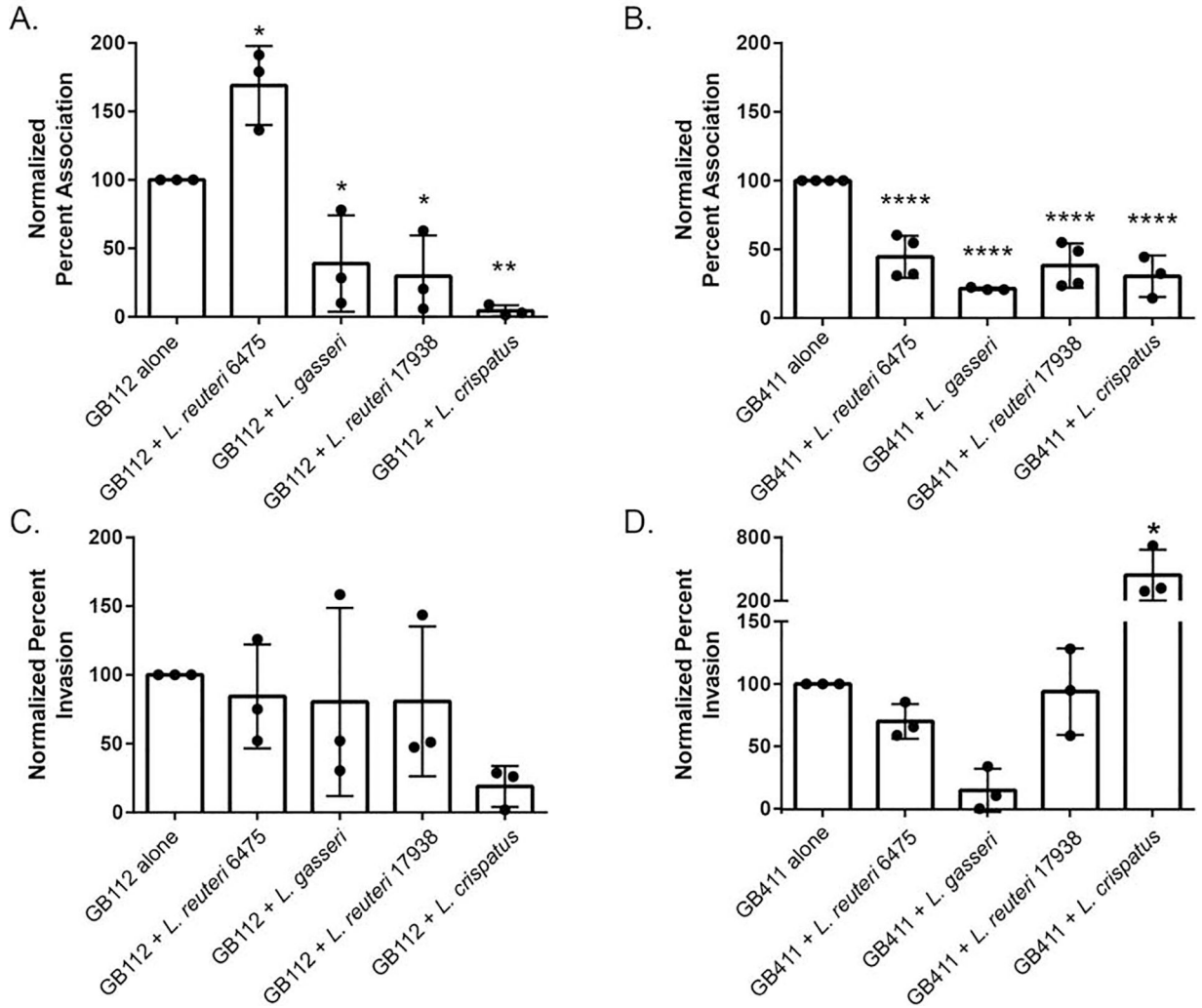


Figure 5. Effect of *Lactobacillus* supernatant on GBS association with and invasion of dT-HESCs. The colonizing GB112 and invasive GB411 strains were added to dT-HESCs at a MOI of 10 for two hours with 10% v/v of supernatant from each *Lactobacillus* strain; GBS without *Lactobacillus* supernatant was included for comparison. The percent of associated GBS was calculated for **A)** GB112 and **B)** GB411 as was the percent of invaded bacteria for **C)** GB112 and **D)** GB411. Percentages were calculated relative to the total number of bacteria in the well and were examined as a percentage of each GBS strain alone to account for variation between trials. Experiments were completed in at least biological triplicates of technical triplicates. Error bars represent standard deviation between biological trials. Significance between GBS alone and with supernatant was determined by an unpaired ANOVA. $p < 0.05$ *

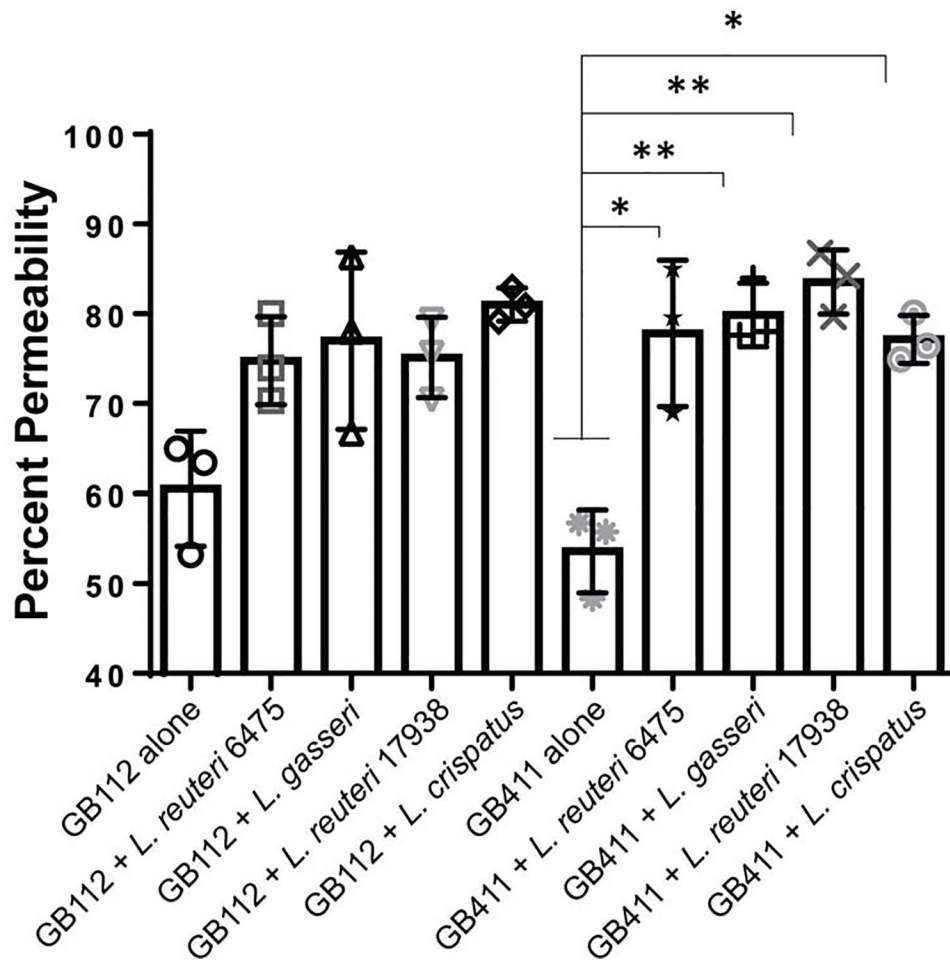


Figure 6. *Lactobacillus* supernatants increase GBS-induced host cell death.

dT-HESCs were infected with GB112 and GB411 (MOI = 10) with 10% v/v *Lactobacillus* supernatant 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 are three biological replicates with error bars to represent the standard deviation. An unpaired ANOVA tested for differences between GBS alone and with supernatant. $p < 0.05$ *; $p < 0.005$ **

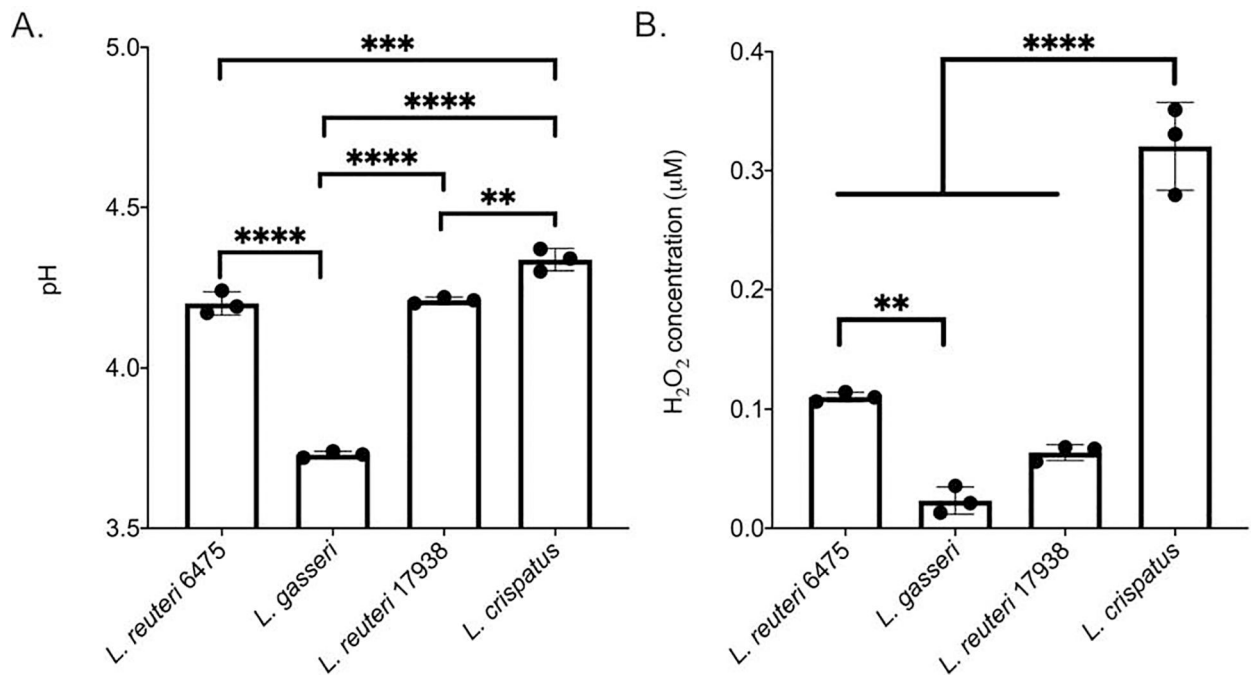


Figure 7. pH levels and hydrogen peroxide concentration vary among *Lactobacillus* supernatants.

Lactobacillus supernatants were collected from overnight cultures of each strain and evaluated for **A**) pH and **B**) hydrogen peroxide (μM) levels. Significance was measured using a one-way ANOVA after confirmation of a normal distribution by the Shapiro-Wilk test. ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$

Table 1.Source of four *Lactobacillus* strains examined in this study.

<i>Lactobacillus</i> spp.	Strain number / ATCC number	Source	Reference
<i>Lactobacillus reuteri</i>	MM4-1A / ATCC PTA-6475	Breast milk	
<i>Lactobacillus reuteri</i>	Unknown / ATCC DSM 17938	Breast milk	[27]
<i>Lactobacillus crispatus</i>	Unknown	Stool	
<i>Lactobacillus gasseri</i>	DSM 20243 [63 AM] / ATCC 33323	Vagina	[26]

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