THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

ANALYZING *IN VITRO* AND *IN VIVO* GROWTH INTERACTIONS BETWEEN *BORDETELLA* AND HOST MICROFLORA SPECIES

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ABSTRACT

The host-specificity of the animal pathogen *Bordetella bronchiseptica* and the human pathogens *Bordetella pertussis* and *B. parapertussis* have previously been noted; however, the principles underlying this host-specificity are unclear. We hypothesize that host microflora, the endogenous microbes found on a host's epithelial lining, could inhibit the growth of *Bordetella* through bacterial competition, leading to host-specificity. Using *in vivo* mouse experiments, it was observed that *B. pertussis* was more effective at colonizing mice depleted of culturable host microflora (HMF) than wild-type mice, while *B. bronchiseptica* was able to clear culturable HMF in mice three days post-inoculation. To study these bacterial interactions more specifically, murine and human HMF were isolated, identified, and competed against *Bordetella* using the *in vitro* techniques of cross-streaking, co-culture, and spot-plating. The results of these experiments indicate that *B. pertussis* and *B. parapertussis* can more effectively compete with human microflora than murine microflora. Conversely, *B. bronchiseptica* is able to inhibit the growth of several murine microflora species on agar but not in culture. These findings suggest that HMF interactions play a critical role in *Bordetella* infection. Once the interactions are understood more fully, commensal organisms could be used as therapeutics to prevent *Bordetella* colonization.

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Introduction

Nine Gram-negative organisms that infect both humans and animals comprise the bacterial genus *Bordetella*. The three classical *Bordetella*, *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* are of particular interest because of their stark differences in host pathology and host-specificity despite their high level of genetic similarity (1).

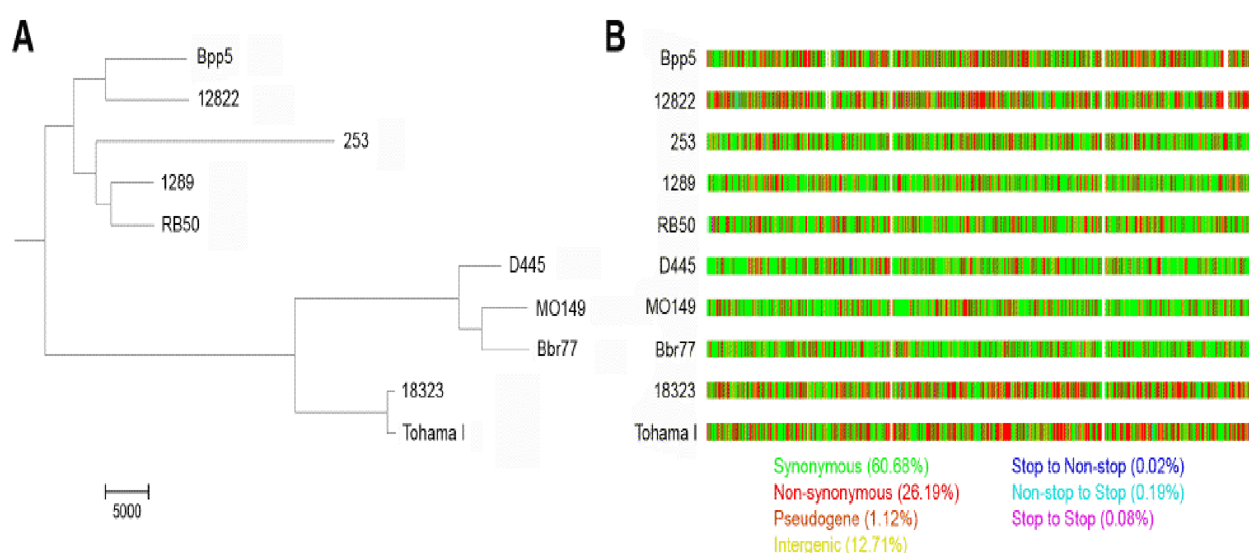


Figure 1: This diagram outlines the evolutionary branching of several species of *Bordetella* (A) as well as the similarity of the genomes of these species of *Bordetella* (B). This work was compiled by Jihye Park.

In fact, these three classical bordetellae contain an unusually high number of conserved genes, ranging from 60-86 % genetic conservation between species (Figure 1) (2). However, there are significant differences between the organisms as well. For example, even though *B. pertussis* and *B. parapertussis* are primarily human pathogens, they separately evolved from and are more closely related to the animal pathogen *B. bronchiseptica* than to each other (2). Furthermore, through evolution, the *B. bronchiseptica* progenitor lost a significant amount of DNA to eventually lead to *B. pertussis* and *B. parapertussis* (2). In *B. bronchiseptica*, the excess of genes is from prophage, and the lost genes in *B. pertussis* and *B. parapertussis* coded for small

molecule metabolism, membrane transport, and biosynthesis of surface structures (2,3). Overall, since all three pathogens are still highly prevalent, a major goal of my research is to understand the differences between the pathogens and identify novel therapeutics to prevent infection.

As mentioned above, *B. pertussis* and *B. parapertussis* are human pathogens responsible for causing whooping cough, an upper respiratory tract infection characterized by frequent spasms of violent coughing (4). The disease was rampant in the early twentieth century with about 175,000 diagnosed cases per year in the United States (5). This burden of illness spurred vaccine development, which lead to implementation of the whole-cell pertussis vaccine in the 1940's (5). The widespread use of whole-cell pertussis vaccine decreased the number of reported whooping cough cases to 2,900 per year between 1980 and 1990 (5). However, this annual incidence has sharply increased recently and reached an apex in 2004 with 25,287 reported cases, a level not reached since 1959. Moreover, though the disease is rising in frequency, many clinicians have not had much experience with whooping cough patients, so the illness might be initially misdiagnosed (6). Thus, it is believed that the actual incidence of the disease could be three times greater than the reported number (6). There are several reasons why experts believe that the number of cases of whooping cough has increased, including the development of new diagnostic tests, insufficient adolescent and adult boosting, and genetic changes within the pathogen that allow it to escape vaccine induced immunity (7). Thus, there is a need to develop a novel technique to complement vaccination for controlling whooping cough.

Different from the human pathogens, *B. bronchiseptica* is an animal pathogen known to cause kennel cough in dogs, atrophic rhinitis in pigs, and snuffles in rabbits (8). Thus, from agriculture to veterinary medicine, there is a vested interest in determining how this organism infects its host so that methods can be developed to inhibit colonization and infection. For

example, *B. bronchiseptica* infection of agricultural swine can have severe effects. *B. bronchiseptica* infection leads to disfigured or ill swine, leading to loss of sales, veterinary fees, and offspring damage (8). In a *B. bronchiseptica* study of infected swine, there were significantly more stillborn pigs, with the surviving offspring growing at a depressed growth rate (8). Furthermore, the true nature of economic losses due to *B. bronchiseptica* infection might be clouded as commercial farmers would not want to divulge such information to the public (8). Thus, with all of these agricultural problems present, there needs to be better understanding of how of *B. bronchiseptica* causes disease and how this process could be manipulated to prevent disease.

As mentioned above, uncovering novel methods for controlling or preventing bordetellae infections would be highly beneficial. Thus, my research focuses on understanding the competitive interactions between host microflora, bacteria endogenous to the epithelial lining of its host, and bordetellae as a potential mechanism to prevent pathogen colonization. I wanted to explore the possibility of using host microflora (HMF) to prevent pathogen colonization as this strategy has been shown to work experimentally. For example, nonpathogenic *Staphylococcus aureus* was implanted onto the nostrils, chest, axillae, groin, and rectal area of individuals with pathogenic *S. aureus* colonization. Twenty-three weeks after implantation, the pathogenic *S. aureus* was still missing in 73% of test subjects (9). In a separate study, *Cornyebacterium* was implanted into the nares of *S. aureus* carriers; and in 71% of test subjects, the *Cornyebacterium* eradicated the pathogen (10). In yet another study, inhibition of *Helicobacter pylori* stomach colonization was observed 8 and 49 days after *Lactobacillus acidophilus* treatment (11). Furthermore, *Veillonella*, *Neisseria*, and *Steptococcus* are known health promoting taxa and *Peptostreptococcus* is thought to be an interfering species that can inhibit upper respiratory

pathogens (12). Based on these precedents, I wanted to examine the bacterial growth interactions between HMF and Bordetellae, with the idea of gathering new information that could be used to potentially develop respiratory tract probiotics.

My research focuses on determining the level of growth competition between HMF and bordetella in vitro; however, generally understanding how bacteria can compete amongst each other is also beneficial because these mechanisms can be manipulated for therapeutics. Strategies that mediate direct competition include production of bacteriocins, acid, hydrogen peroxide, and nutrient competition (13). For example, *Staphylococcus epidermis* secretes a serine protease which prevents *S. aureus* growth and *Streptococcus salivarius* inhibits *Sreptococcus mutans* bio-film formation (14, 15). Furthermore, biosurfactants secreted by lactobacilli can prevent pathogen adherence to mucosal surfaces (16). In each of the previous examples, a species or group of bacteria inhibits pathogen colonization, independent of host function; this is the type of direct inhibition that I will be testing. Conversely, HMF also have the potential to inhibit pathogen colonization through immune-mediated responses such as Stimulated Innate Response (STIR), where HMF activate the host innate immune response in order to prevent infection (17).

To test the direct growth interactions between HMF and *Bordetella*, I used in vitro techniques such as cross-streak, co-culture, and spot-plate because forms of these experiments have been used in the past (18, 19). By changing the environment or time of incubation, each experiment has unique components which might uncover some level of bacterial inhibition. In the future, if any inhibition is observed, one can co-inoculate an inhibitory HMF with a species of bordetellae in a host to determine if *in vitro* interactions translate into *in vivo* effects. Overall, though this research is in the very early stages, it can provide valuable information that could

lead to a therapy for treating infections that are resurging in society and causing severe medical and financial stress.

Materials and Methods

Bacterial Growth

Bordetella pertussis strain 536, a streptomycin-resistant derivative of Tohama I, *B. parapertussis* strain 12822, an isolate from German clinical trials, and *B. bronchiseptica* strain RB50, an animal pathogen, were used in this study along with murine and human HMF identified in Table 1. *Bordetellae* species were cultured in Stainer-Scholte media (20) and the HMF were cultured in Luria-Burtani (LB) broth. *Bordetella* were maintained on Bordet-Gengou (Difco) agar supplemented with 10% defibrinated sheep blood (Hema Resources) and 20 µg/mL streptomycin (Sigma-Aldrich), while Blood Agar (BA) (Difco) supplemented with 10% defibrinated sheep blood was used to isolate HMF CFUs.

Murine Host Microflora Isolation and Identification

Three C57BL/6 mice (Jackson Laboratories) were co-housed for five weeks and then euthanized via CO_2. The nasal cavities of each collected and homogenized on PBS. The diluted nasal cavity samples were plated onto BA supplemented with 10% defibrinated sheep blood (Hema Resources). Each distinct bacterial species on the plate was isolated, characterized, counted and cultured. After overnight incubation at 37° C, the culture was stored as a one ml 20% glycerol solution in a -80° C freezer. For bacterial identification, the freezer stock was struck onto a BA plate and incubated at 37°C. Genomic DNA was isolated from the bacteria after vortexing in distilled water. The genomic DNA of each species was placed in a PCR reaction containing forward primer AGAGTTTGATCATGGCTCAG and reverse primer AAGGAGGTGATCCAACC which are specific for the 16S ribosomal region of prokaryotes. The PCR protocol had the following stages: 95° C for 7 minutes; then 30 cycles of 90°C for 30 seconds, 54° C for 30 seconds, and 72° C for 90 seconds; finishing with a single cycle at 75° C for 7 minutes. After PCR, samples were run on a 1% agarose gel at 150 volts for 30 minutes.

Using a UV machine (FotoPrep), the 16S RNA band of each bacterium, approximately 1,500 base pairs in length, was excised out of the gel. The nucleic acid within the gel was extracted following the protocol found in the E.Z.N.A gel extraction kit and was sent to the Penn State Genomics Core Sequencing Facility for DNA sequencing. NCBI blast was run on the sequences and entries with 95% or greater sequence identity were chosen as the bacterium's identity.

Human Host Microflora Isolation and Identification

Human sputum samples were collected at the Hershey Medical Center in Hershey, PA and transported to The Pennsylvania State University, University Park, PA on ice. Each sputum sample was diluted with PBS and plated onto six different agar plates, including Blood Agar (BA), Bordet-Gengou (BG) agar, Luria Burtani (LB) agar, Nutrient agar, MacConkeys agar, and Tryptic Soy Agar (Difco). The plates were then incubated at 37° C for twenty-four hours. Each distinct bacterial species was isolated, characterized, counted, and cultured. After overnight incubation at 37° C, the culture was stored as a one ml 20% glycerol solution in a -80° C freezer. 16S PCR amplification and gene sequencing as outlined above was also used for human HMF identification.

Chart of Bacterial Strains

Bacterial Species/Strain	Host Organism	Date Isolated	Method used for Sequencing
Bacillus clausii	Mouse	1/26/2011	16SRNA
Staph cohnii	Mouse	1/26/2011	16SRNA
Klebsiella	Mouse	1/30/2011	16SRNA
Staph. saprophiticus	Mouse	1/30/2011	16SRNA
Enterococcus	Mouse	1/26/2011	16SRNA
Rhizobium	Mouse	1/12/2011	16SRNA
Staph. species T-02	Mouse	1/26/2011	16SRNA
Micrococcus	Mouse	9/15/2010	16SRNA
Staph. xylosus	Mouse	1/26/2011	16SRNA
Enterobacter hormaechi	Mouse	9/15/2010	16SRNA
Staph. lentus	Mouse	1/26/2011	16SRNA
Staph species S16	Mouse	1/26/2011	16SRNA
Neisseria	Human	6/14/2010	16SRNA
Morococcus cerebrosus	Human	7/28/2011	16SRNA
Actinomyces naeslundi	Human	7/28/2011	16SRNA

Table 1: This table lists all of the strains of HMF used in competition with three different *Bordetellae* species. The table includes the host each bacterium was isolated from, the date of isolation, the type of sequencing used to determine its identity. This sequencing work was done by Dr. Laura Weyrich and Sarah Muse.

Co-Culture

Bacterial cultures of *B. bronchiseptica* (RB50), *B. pertussis* (BP 536), *B. parapertussis* (BPP 12822), and HMF were cultured to an optical density (OD) between 0.8-1.2, representing ≈ $8x10^8$-$1.2x^9$ CFUs/mL. The HMF cultures were diluted to a concentration of 10^4 CFUs/mL with LB broth, and the *Bordetellae* cultures were diluted to an inoculum concentration of 10^6 CFUs/mL with SS media. The HMF and *Bordetellae* inoculums were mixed to produce a 1 mL co-culture sample with the HMF concentration being 10^3 CFUs/mL and the *Bordetellae* concentration being $9x10^5$ CFUs/mL. For controls, the same concentration of HMF and *Bordetellae* were reproduced in 1 ml individually, instead of combined together, using SS media. All co-culture samples and controls were reproduced in triplicate. The co-culture samples and

controls were incubated while shaking for one hour at 37 °C. After incubation, 100 µL of each HMF control and co-culture sample were plated on BA to quantify surviving HFM. Additionally, 100 µL of each *Bordetellae* control and an additional 100 µL of each co-culture sample were serially diluted with PBS and plated on BG to quantify *Bordetellae*. The log of the number of HMF and *Bordetellae* CFU during co-culture was compared to the log of the bacterial CFU when cultured in isolation to determine the effect of bacterial interaction.

Cross-Streak

To analyze direct interactions, HMF were grown directly on top of *Bordetellae* on an agar plate. Due to differences in growth rate, *B. bronchiseptica*, *B. pertussis*, or *B. parapertussis* were struck down the middle of a BA plate and incubated at 37° C before the application of HMF. RB50 was incubated for two days while BP and BPP were incubated for four days. After the incubation period, an HMF species was struck perpendicularly across the *Bordetellae* streak in one, fluid motion. The plate was then additionally incubated overnight at 37° C. After this incubation, the cross-streak would be photographed and analyzed for inhibition. Quantitatively, the bacterial intersection was carefully examined. Any change in the size, density, or color of the bacteria was noted as inhibition. If HMF streak grew completely over *Bordetella* streak, then *Bordetella* was considered to be inhibited. If *Bordetella* streak overlapped the HMF streak, then the *Bordetella* was not considered to be inhibited.

Spot-Plating

Spot-plating was done to analyze *Bordetellae* and HMF growth interactions when both bacteria had the same incubation time. RB50 and HMF cultures were grown to an OD of ≈ 0.8-1.2. The RB50 culture was diluted to a concentration of 10^8 CFUs/mL using filter sterilized PBS, while HMF cultures were diluted using PBS to the following four concentrations: 10^8, 10^7, 10^6,

and 10^5 CFUs/mL. 10^8 CFU of *B. bronchiseptica* were spread on BA to create a lawn. After allowing the BA plate to dry, 10 ul spots of each HMF concentration were plated at the four corners of the plate onto the lawn. After the spots dried, the BA plate was incubated at 37° C for two days and then photographed for growth inhibition analysis. For most spots, individual colonies could not be counted, but the qualitative growth of spots was examined. Since each spot should have the same number of bacteria regardless of species used, the different HMF spots were compared against each other to check for inhibition.

Animal Experiments

C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and bred in a bordetella-free breeding room at The Pennsylvania State University. Newborn pups were co-housed for five weeks to allow for nasal microflora equilibration. For *B. pertussis* inoculation, the mice were lightly sedated with 5% isoflurane (Abbot Laboratories) in oxygen and inoculated by pipetting 10 µl with either 100, 500, 1000, 5000, or 10,000 CFU of bacteria onto the external nares. For *B. bronchiseptica* inoculation, the mice were lightly sedated with 5% isoflurane (Abbot Laboratories) in oxygen and inoculated by pipetting 10 µl with 100 CFU bacteria onto the external nares. To quantify bacterial numbers, nasal cavities of mice were excised after euthanizing with CO_2. Tissues were homogenized in 1 ml PBS, serially diluted, and plated on BG agar with 20 µg/mL streptomycin for bordetellae quantification and on BA for HMF quantification. BG plates with *B. bronchiseptica* and *B. pertussis* were incubated for two and five days respectively, while BA plates were incubated for two days (21). The lower limit of detection was 10 CFU. All protocols were reviewed and approved by The Pennsylvania State University Institutional Animal Care and Use Committee, and all animals were handled in accordance with institutional guidelines.

Results

<u>Murine Host Microflora (HMF) Inhibit *B. pertussis* Colonization *In vivo*</u>

Bordetella pertussis (BP 536) does not colonize murine nasal cavities as effectively as *Bordetella bronchiseptica,* and the reason for this reduced infectiousness is not well understood. Differential abilities to compete with nasal cavity flora may explain why these two evolutionarily related pathogens exhibit differences in upper respiratory tract colonization. To determine if HMF effect *B. pertussis* colonization, one group of mice was treated with enrofloxacin, a broad spectrum antibiotic, to clear a broad range of HMF, while one group was left untreated to preserve microbial diversity in the nasal cavity; all mice were subsequently inoculated with varying *B. pertussis* doses. The figure indicates that 10,000 were enough to colonize the nasal cavity three days post-inoculation, while only 100 bacteria were required for *B. pertussis* colonization in antibiotic treated mice (Figure 1). Therefore, antibiotic treatment reduced the necessary infectious dose by 99%, indicating that the presence of murine nasal cavity HMF inhibited initial *B. pertussis* colonization.

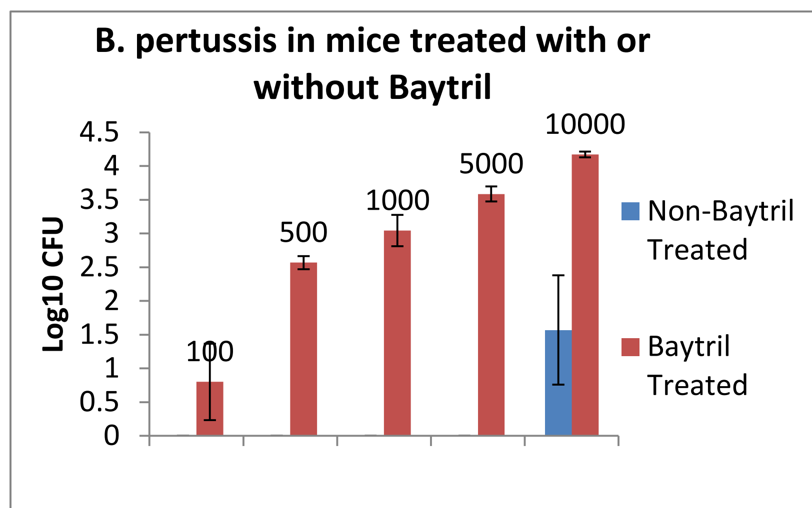
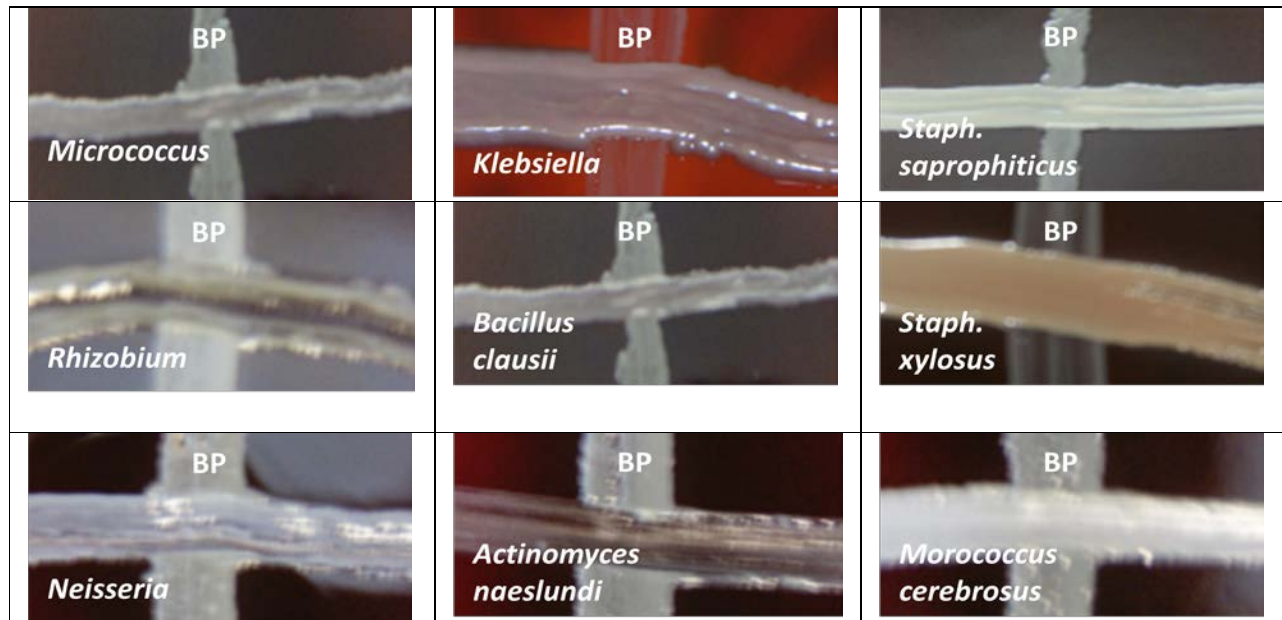


Figure 2: For each dose of inoculum, three antibiotic treated mice and three non-treated wild-type mice were used. Baytril was added to the animal's water source sixteen hours pre-inoculation. The nasal cavity of each mouse was dissected and cultured three days post-inoculation.

Murine HMF Inhibit *B. pertussis* and *B. parapertussis* Growth *In vitro* on Agar

We hypothesized that the inhibition of *B. pertussis* colonization was a result of direct bacterial competition, such as bacteriocin release or biofilm inhibition, between murine HMF species and *B. pertussis*. Thus, cross-streaking, a method in which HMF are grown adjacent to *B. pertussis,* was utilized to test whether murine HMF directly inhibited *B. pertussis* growth. Furthermore, human HMF isolated from the lower respiratory tract were also cross-struck with *B. pertussis* because *B. pertussis* is strictly a human pathogen. Since *B. parapertussis* is also a closely related human pathogen, the same nine HMF species were cross-struck with *B. parapertussis* as well to compare the interactions between the two species of *Bordetella* and HMF.



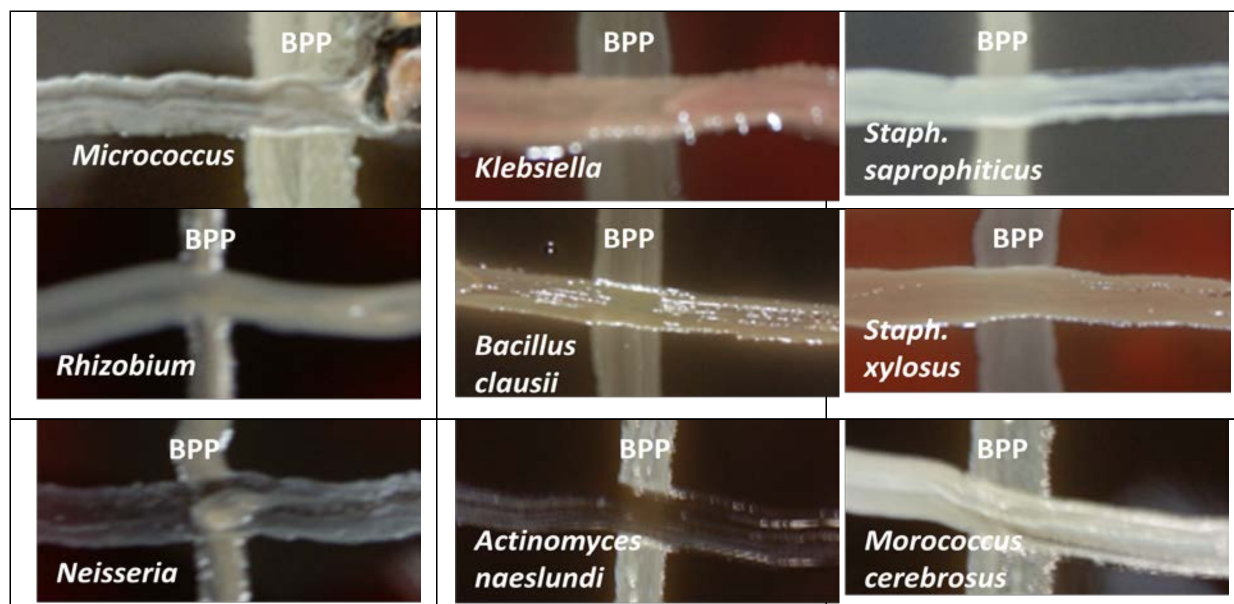


Figure 3: The middle of a BA plate was struck with either BP 536 (top) or BPP 12822 (bottom) and incubated for four days at 37°C. After incubation, a species of HMF was perpendicularly struck across the *Bordetella* streak, and the plate was incubated for an additional day at 37°C. The top two rows of each image contain mouse HMF struck against *Bordetella* while the bottom row contains human HMF struck against *Bordetella*.

In most of the murine HMF tests, excluding the *Micrococcus* test for both species of *Bordetella*, a thick streak of HMF grew completely over the individual *B. pertussis* and *B. parapertussis* streaks, indicating that *in vitro*, the mouse HMF inhibited both species of *Bordetella*. Furthermore, when examining human HMF cross-streaks, *B. pertussis* and *B. parapertussis* growth is also inhibited at the bacterial intersection. These differences in interactions suggest that *B. parapertussis* and *B. pertussis* are not effective at competing with HMF.

B. pertussis and *B. parapertussis* Inhibit Human HMF *In vitro* in Culture

Co-culture, another *in vitro* technique, was employed to observe indirect bacterial interactions, such as nutrient competition, between *B. pertussis* and *B. parapertussis* and HMF in culture. We hypothesized that altering the environment, from agar in cross-streaking to liquid broth in co-culture, would require bacteria to change their method of interactions, which could be observed as differences in bacterial competition between cross-streak and co-culture. Thus, in co-culture, two different concentrations of HMF and *Bordetella* were mixed in a 1 mL volume

and incubated for 1 hour because these conditions replicate an *in vivo* model of bacterial antagonism.

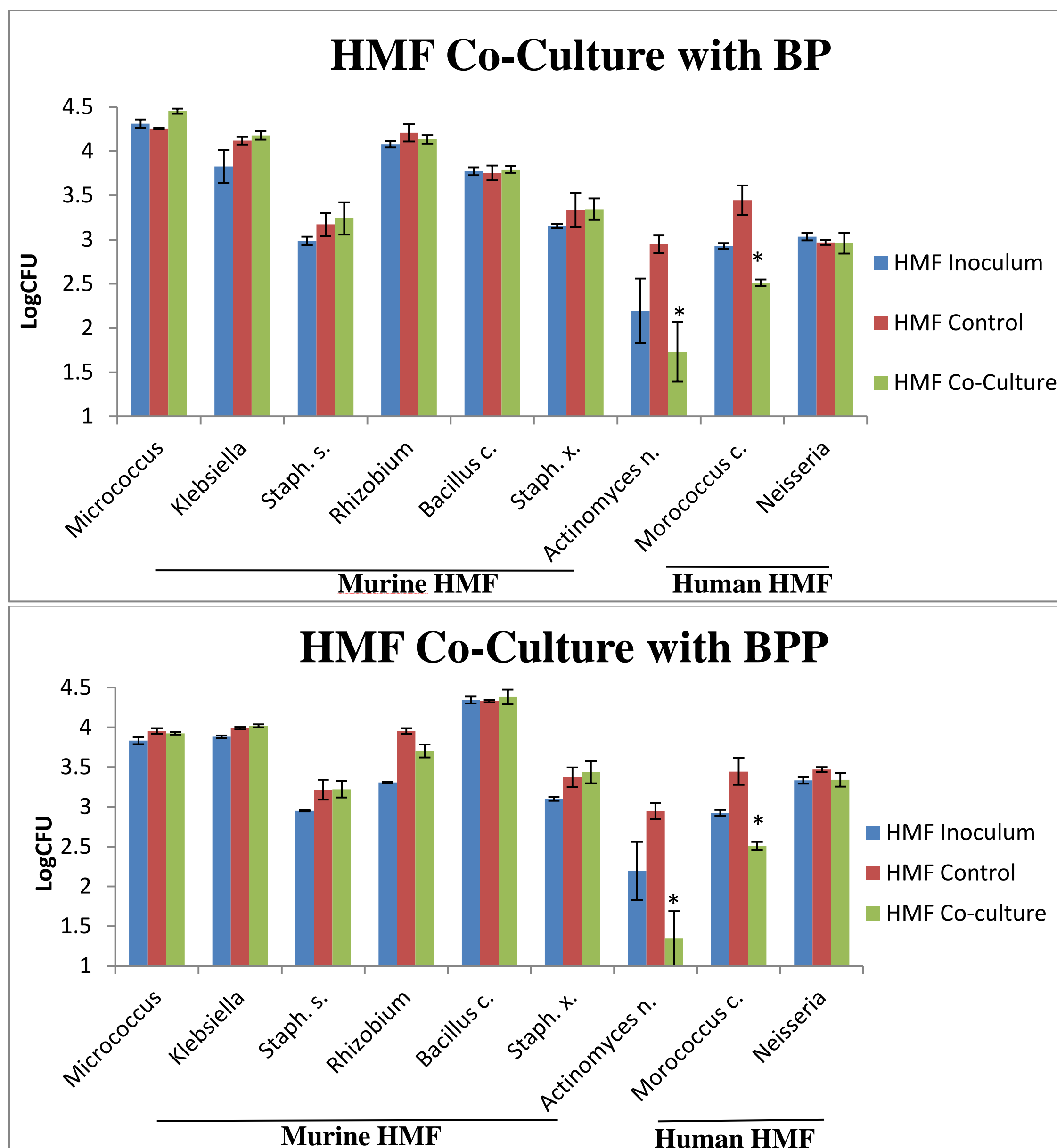


Figure 4: The following graph depicts the log10(CFU) of six murine and three human HMF after a 1 hour co-culture with *B. pertussis* (top) and *B. parapertussis* (bottom). The inoculum represents the log10CFU of the HMF at the start of the experiment. The control represents the log10CFU of the HMF after 1 hour incubation with SS media only. The co-culture represents the log10CFU of the HMF after 1 hour co-culture with *Bordetella*. For the HMF that are abbreviated, *S. saprophiticus.* is *Staphylococcus saprophiticus*; *Bacillus* is *Bacillus clausii*; *S. xylosus* is *Staphylococcus xylosus*; *Actinomyces* is *Actinomyces naeslundi*; and *Micrococcus c.* is *Micrococcus cerebrosus.* The controls and co-cultures were incubated in a shaking 37° C incubator. * Indicates statistical significance by a Student's *t* test between the co-culture and media control with a p-value ≤ 0.05.

When co-cultured with *B. pertussis* and *B. parapertussis*, most murine HMF show no significant change in growth compared to when they are cultured alone in media control, indicating that these two species of *Bordetella* do not significantly affect HMF growth (Figure 4). However, when the human HMF *Actinomyces naeslundi* and *Micrococcus cerebrosus* are co-cultured with *B. pertussis* and *B. parapertussis*, there is a statistically significant reduction in HMF CFU of approximately 90-95% between the media controls and co-cultures. This finding indicates that the human pathogens *B. pertussis* and *B. parapertussis* are more efficient at out-competing human HMF than murine HMF in culture.

Bordetella bronchiseptica Inhibits Mouse HMF *In vivo*

After analyzing HMF interactions with *B. pertussis* and *B. parapertussis*, competition between the natural animal pathogen *B. bronchiseptica* and HMF was studied to determine if a pathogen's host-specificity affected how it interacted with HMF. Because *B. pertussis* is ineffective at colonizing mice due to its inability to compete with murine HMF, we hypothesized that *B. bronchiseptica* would be more effective at out-competing murine HMF because its natural host is animals such as mice. To test our theory, we designed an *in vivo* experiment to examine how HMF colonization changed over time in a murine nasal cavity after *B. bronchiseptica* inoculation.

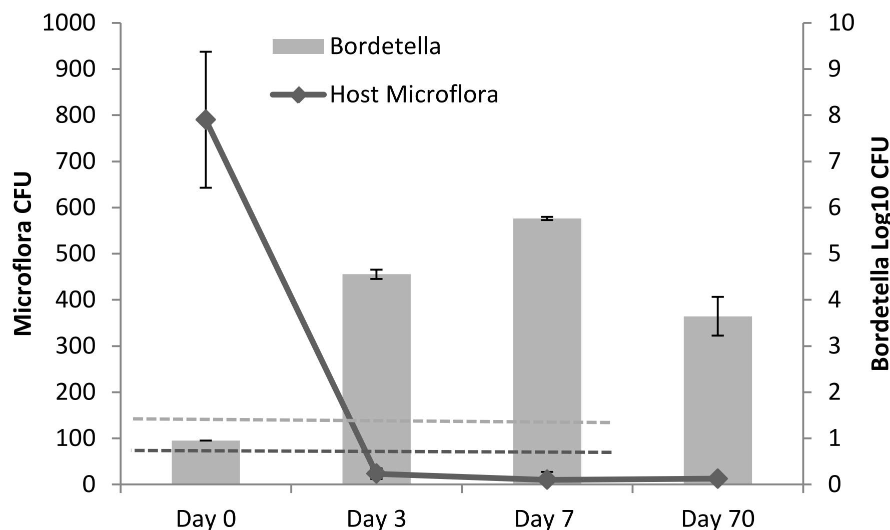


Figure 5: Twelve mice were inoculated with 100 CFUs of *B. bronchiseptica* in 10 ul. The nasal cavities were removed and cultured for *B. bronchiseptica* and HMF three, seven, and 70 days post-inoculation. The nasal cavities of three mice were also cultured directly after inoculation to serve as a control. This work was done by Dr. Laura Weyrich

In wild-type mice, the detectable colonization of *B. bronchiseptica* in a mouse's nasal cavity is paralleled with a statistically significant decrease of HMF to below detectable levels three days post-inoculation (Figure 5). Moreover, even seventy days post-inoculation, there is a detectable level of *B. bronchiseptica*, while culturable HMF colonization is absent. This observation indicates that *B. bronchiseptica* can colonize the nasal cavity at low doses and, in the process, can clear culturable HMF from the nasal cavity.

B. bronchiseptica Inhibits Mouse HMF *In vitro* on Agar

Since Figure 4 indicates that *B. bronchiseptica* colonizes a mouse while displacing culturable nasal HMF, we wanted to analyze *Bordetellae* and HMF growth interactions *in vitro*. We hypothesized that by isolating mouse HMF and performing cross-streaks, we would observe inhibition of a few species of bacteria. Thus, following a similar protocol as previous cross-

streak experiments, we struck one CFU of mouse HMF species over one CFU of *B. bronchiseptica* that had been incubated for two days.

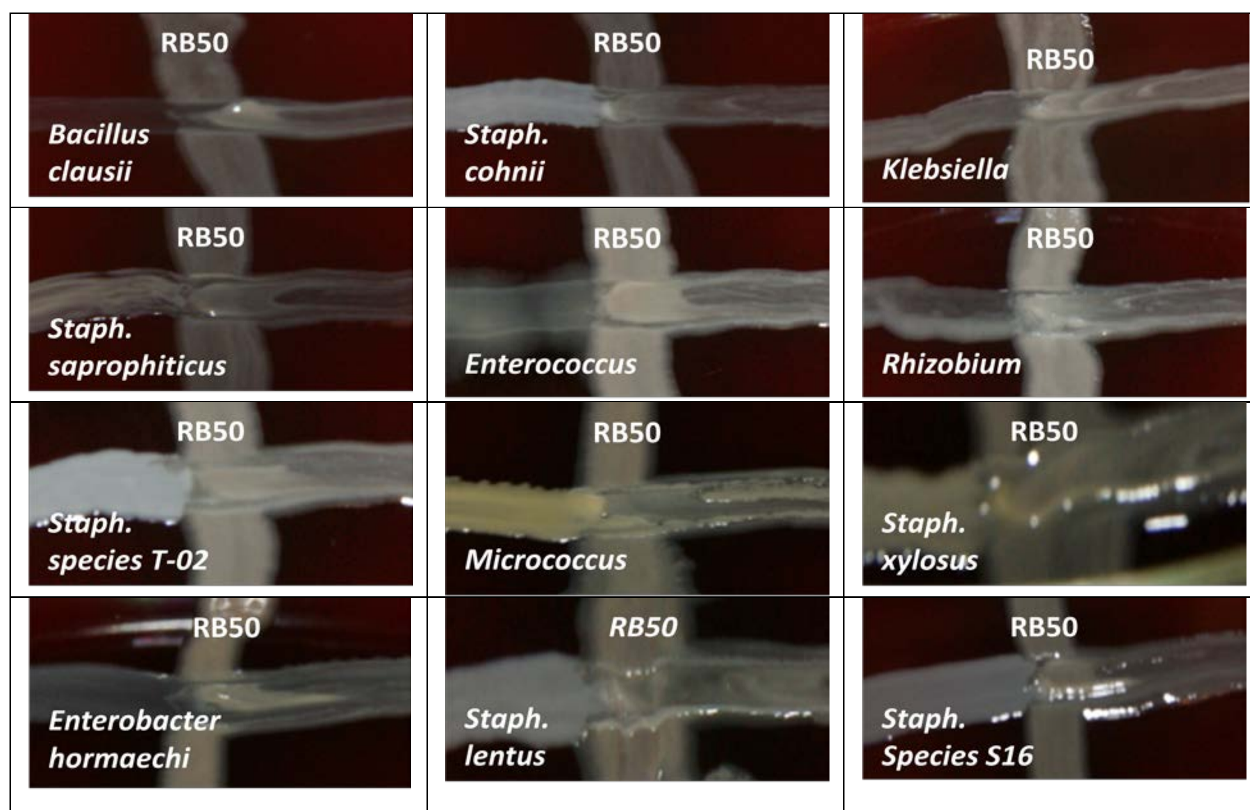
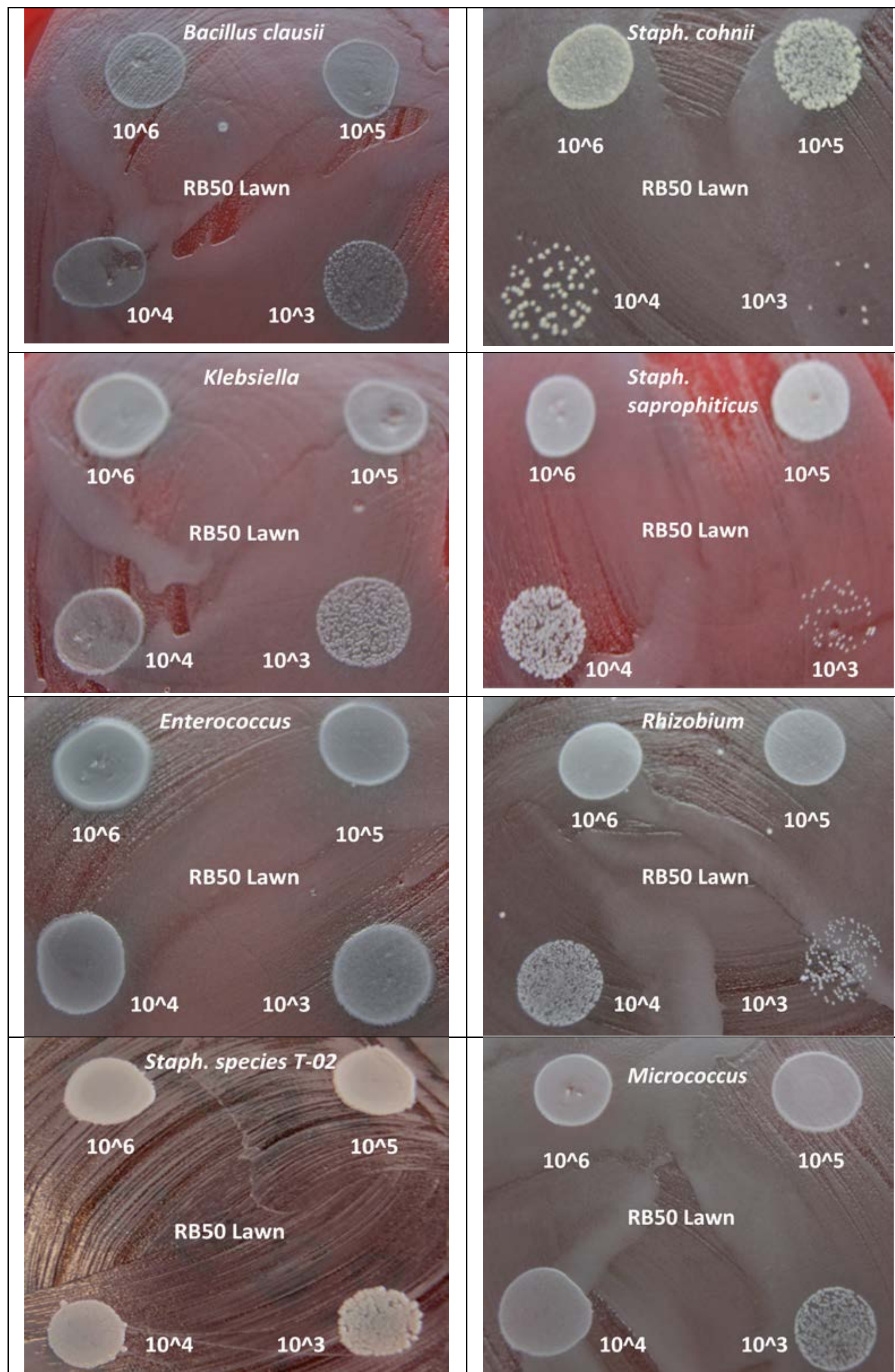


Figure 6: One CFU of *B. bronchiseptica* was struck down the middle of a BA plate which was then incubated at 37° C for two days. After incubation, one CFU of a mouse HMF species was struck perpendicularly across the *B. bronchiseptica* streak and the plate was incubated for an additional day at 37° C. The chart includes twelve mouse microflora.

There are varying degrees of growth inhibition when mouse HMF are grown in competition with *B. bronchiseptica* (Figure 6). For example, *B. bronchiseptica* significantly altered the size, shape, or color of the bacterial streaks of *Rhizobium, Staphylococcus cohnii*, and *Staphylococcus species T-02*, indicating inhibition. Alternatively, *B. bronchiseptica* did not have as clear inhibition of species such as *Klebsiella*. In comparing Figures 3 and 4, we see that there are significant differences between how murine HMF interact with the human pathogens *B. pertussis* and *B. parapertussis*, and the animal pathogen *B. bronchiseptica*, which could provide insight into each pathogen's host specificity. Overall, Figure 6 illustrates that different mouse HMF

interact very differently with *B. bronchiseptica* on an agar plate, indicating that *B. bronchiseptica* could displace some of these bacteria when it colonizes a mouse.

Since *B. bronchiseptica* inhibited HMF in cross-streaking, bacterial concentration was altered to determine how this factor impacted bacterial competition. We hypothesized that *B. bronchiseptica* would become more effective at inhibiting HMF as the concentration of HMF decreased to several times smaller than the concentration of *B. bronchiseptica*. Thus, spot-plating, a technique where four different concentrations of HMF are spotted onto a lawn of *B. bronchiseptica,* was utilized. This technique differs from co-culture because the bacteria were grown on an agar plate and not in culture, and differs from cross-streaking because the bacteria were given the same time for incubation. This technique could not be employed in the growth interaction analysis of *B. pertussis* and *B. parapertussis* because the growth rates of these human pathogens are significantly slower than those of HMF.


Bacillus clausii
10^6
10^5
RB50 Lawn
10^4
10^3
Staph. cohnii
10^6
10^5
RB50 Lawn
10^4
10^3
Klebsiella
10^6
10^5
RB50 Lawn
10^4
10^3
Staph. saprophiticus
10^6
10^5
RB50 Lawn
10^4
10^3
Enterococcus
10^6
10^5
RB50 Lawn
10^4
10^3
Rhizobium
10^6
10^5
RB50 Lawn
10^4
10^3
Staph. species T-02
10^6
10^5
RB50 Lawn
10^4
10^3
Micrococcus
10^6
10^5
RB50 Lawn
10^4
10^3

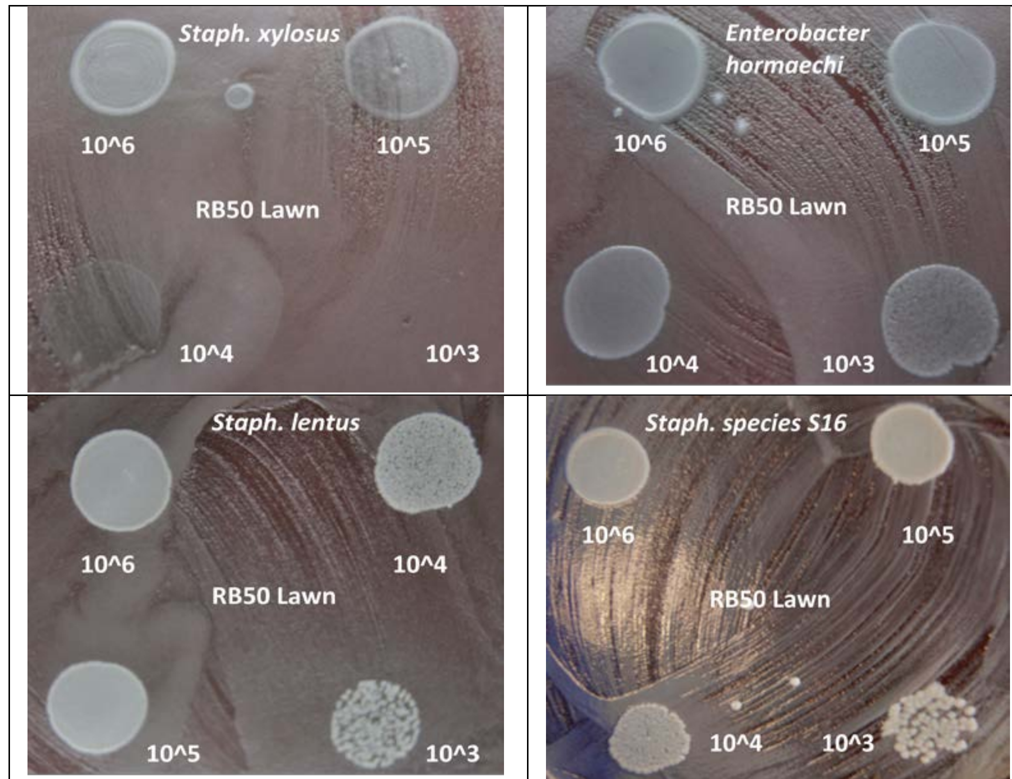


Figure 7: The spot plates were performed on BA media to allow for both HMF and RB50 growth. Approximately 10^7 CFUs of RB50 were spread on the plate, and it was left to dry for 30 minutes. After the plate dried, the four concentrations of HMF were plated in the four quadrants of the plate. After waiting 30 minutes for the spots to dry, the plates were incubated at 37°C for two days and then photographed.

Spot-plating reaffirms the observation from cross-streaking that different murine HMF species interact differently with *B. bronchiseptica* (Figure 7). *Bacillus clausii*, *Enterococcus*, *Klebsiella*, *Enterobacter hormaechi*, and *Staphylococcus lentus* grew without any visible impairment, even when the lowest concentration of HMF spots, containing 10^3 CFUs, were surrounded by a thick lawn of *B. bronchiseptica*, containing over a thousand more times more bacterial CFUs. Conversely, other bacteria such as *Staphylococcus cohnii*, *Staphylococcus saprophiticus*, and *Staphylococcus xylosus* did not grow as well. However, this lack of growth cannot be conclusively explained by bacterial competition from *B. bronchiseptica* with this data

alone. Thus, this indicates that many murine HMF are capable of growing *in vitro* in the presence of *B. bronchiseptica*.

<u>*B. bronchiseptica* Does Not Inhibit Murine HMF *In vitro* in Culture</u>

After observing varying degrees of inhibition of HMF when put in competition with *B. broncshiseptica* on agar, growth interactions were tested in culture to determine the degree of bacterial competition when the bacteria were not in continual contact. We hypothesized that *B. bronchiseptica* would kill several murine HMF species based on the *in vivo* results of Figure 4 because a cultured broth has a dynamic environment more representative of *in vivo* conditions. Thus, using the same protocol as before, we performed co-culture experiments between twelve HMF species and *B. bronchiseptica*.

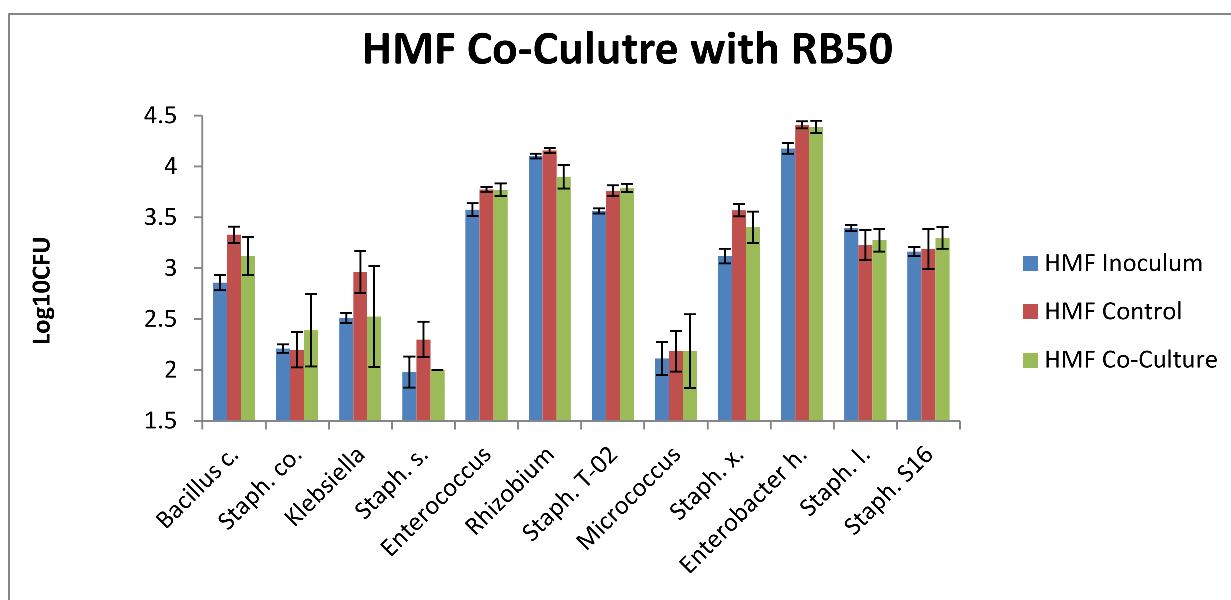


Figure 8: The following graph depicts the log10CFU of twelve murine HMF after 1 hour co-culture with *B. bronchiseptica*. The inoculum represents the log10CFU of the HMF at the start of the experiment. The control represents the log10CFU of the HMF after 1 hour incubation with SS media only. The co-culture represents the log10CFU of the HMF after 1 hour co-culture with *B. bronchiseptica*. For the HMF that are abbreviated, *Bacillus c.* is *Bacillus clausii*; *Staph. co.* is *Staphylococcus cohnii*; *Staph. s.* is *Staphylococcus saprophiticus*; *Staph T-02* is *Staphylococcus species T-02*; *Saph. x.* is *Staphylococcus xylosus*; *Enterobacter h.* is *Enterobacter hormaechi*; *Staph. l.* is *Staphylococcus lentus*; and *Staph. S16* is *Staphylococcus species S16*. The controls and co-cultures were incubated in a shaking 37° C incubator.

Even though *Rhizobium* inhibition in the presence of *B. bronchiseptica* is not statistically significant in culture, *Rhizobium* is inhibited by *B. bronchiseptica* during *in vitro* agar competition. Thus, this finding indicates that many mouse HMF are not affected by the presence of *B. bronchiseptica* in culture.

Many Culturable Murine HMF Species Do Not Alter the Growth of *B. bronchiseptica*

In addition to observing a change in HMF CFU due to co-culture, the change in *B. bronchiseptica* CFU was also examined to determine if HMF affected the animal pathogen's growth. Since there were varying degrees of inhibition seen with bacterial competition on agar (Figures 5 and 6), we hypothesized that the clearance of HMF seen in Figure 4 could have been immune –mediated rather than due to direct bacterial interactions. Thus, when HMF are put into competition against *B. bronchiseptica*, with the host removed, the HMF could have the potential to inhibit *B. bronchiseptica*. To test our hypothesis, we quantified the *B. bronchiseptica* CFU after a one hour co-culture with twelve murine HMF species.

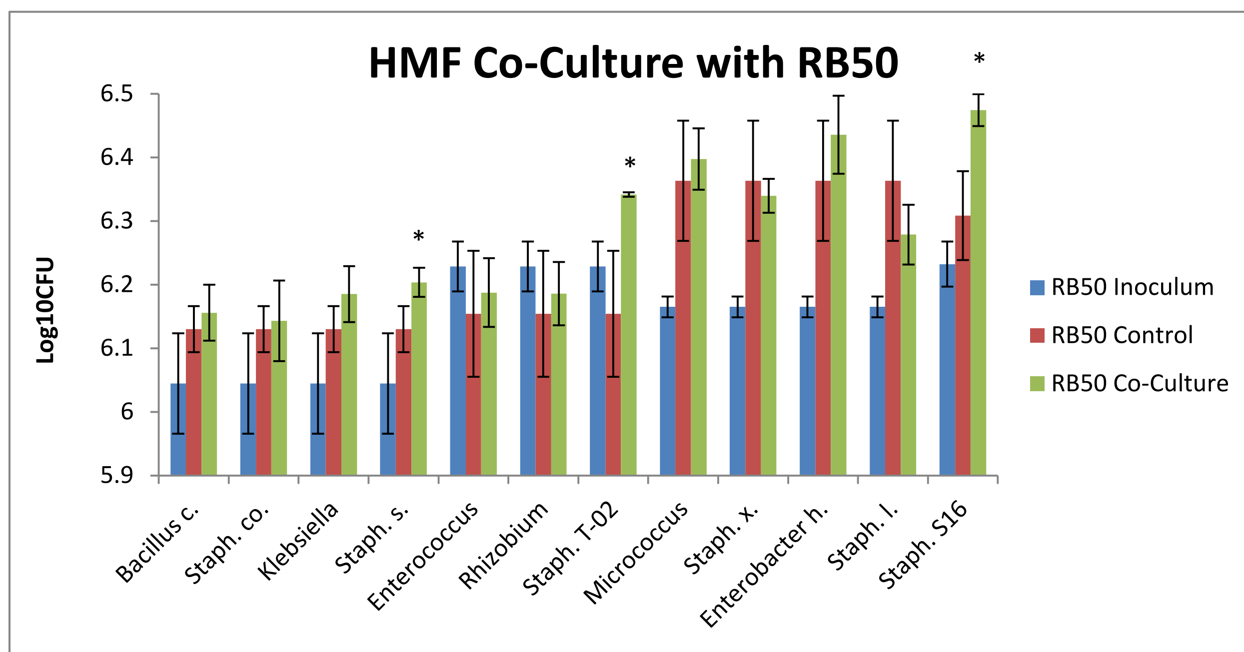


Figure 9: The following graph depicts the log10CFU of *B. bronchiseptica* after 1 hour co-culture with HMF. The inoculum represents the log10CFU of *B. bronchiseptica* at the start of the experiment. The control represents the log10CFU of *B. bronchiseptica* after 1 hour incubation with SS media only. The co-culture represents the log10CFU of *B. bronchiseptica* after 1 hour co-culture with HMF. For the HMF that are abbreviated, *Bacillus c.* is *Bacillus clausii*; *Staph. co.* is *Staphylococcus cohnii*; *Staph. s.* is *Staphylococcus saprophiticus*; *Staph T-02* is *Staphylococcus species T-02*; *Saph. x.* is *Staphylococcus xylosus*; *Enterobacter h.* is *Enterobacter hormaechi*; *Staph. l.* is *Staphylococcus lentus*; and *Staph. S16* is *Staphylococcus species S16.* The controls and co-cultures were incubated in a shaking 37° C incubator. * Indicates statistical significance by a Student's *t* test between the co-culture and media control with a p-value ≤ 0.05

When grown with most murine HMF in culture, *B. bronchiseptica* growth is not significantly altered (Figure 9). However, in the presence of *Staphylococcus saprophiticus*, *Staphylococcus species S16,* and *Staphylococcus species T-02*, there is a statistically significant increase of *B. bronchiseptica* CFU of about 20-30% in co-culture when compared to the media control. Overall, this figure indicates that while most murine HMF do not affect the growth of *B. bronchispetica,* the presence of some HMF actually facilitates *B. bronchiseptica in vitro* growth.

Discussion

Host- specificity differences between the animal pathogen *B. bronchiseptica* and the human pathogens *B. pertussis* and *B. parapertussis* had been previously noted; however, the reason for these differences are not well understood. The results presented here from *in vitro* and *in vivo* experiments indicate that HMF competition could be responsible for *Bordetella* host-specificity. Cross-streak, co-culture, and spot-plate experiments illustrate that *B. pertussis* and *B. parapertussis* cannot compete with murine microflora as effectively as with human microflora, while they illustrate that *B. bronchiseptica* inhibits the growth of murine microflora. These findings suggest that *Bordetella* have adapted to successfully compete only with bacteria common to their hosts. Medically, this could mean that researchers could take an innocuous, commensal microorganism that inhibits *Bordetella* growth and implant it to prevent *Bordetella* infection. The development of these types of probiotics could be a novel method for treating upper respiratory infections in humans and animals.

After our *in vitro* testing, we conclude that many species of murine microflora were able to inhibit *B. pertussis* and *B. parapertussis* growth in cross-streaking, while only *Klebsiella* had a similar inhibitory affect on *B. bronchiseptica* using the same experimental technique. Since cross-streaking examines direct competition because the bacteria are placed in direct contact with one another, this observation suggests that many murine microflora utilize direct bacterial mechanisms for competition. For example, it has been shown that Gram-negative bacteria such as *Citrobacter, Enterobacter,* and *Klebsiella* are able to inhibit the growth of other Gram-negative and Gram-positive organisms by secreting membrane vesicles containing peptidolgycan hydrolases that cause cell lysis (22). In addition, formic and acetic acid production of *Klebsiella*

and *Enterobacter* could cause *B. bronchiseptica* inhibition by manipulating the type three secretion system of the animal pathogen, in a method similar to the inhibition of *Shigella flexneri* which also contains a type three secretion system (23,24,25). Microflora species could also be secreting special antimicrobial molecules called bacteriocins to cause *Bordetella* inhibition. For example, *Bordetella* species could be susceptible to inhibition by the *Klebsiella* bacteriocin klebocin (26). In yet another method, since *Staphylococcus epidermis* is capable of secreting a serine protease which inhibits *Staphylococcus aureus* growth, other *Staphylococcus* microflora species could also secrete enzymes which inhibit the growth of *Bordetella* (14). Overall, HMF could use any of the methods stated above, independent of host function, to cause *Bordetella* inhibition.

Even though HMF have the ability to impede pathogen growth, *Bordetella* also have mechanisms for competing against commensal organisms, as seen by *B. pertussis* and *B. parapertussis* inhibition of *Actinomyces naeslundi* and *Neisseria* in cross-streaking and co-culturing as well as *B. bronchiseptica* inhibition of murine microflora in cross-streaking. For example, Hamada et al. and Tamura et al. have shown that oral bacteria such as *Actinomyces* rely on their fimbriae for bacterial competition and are susceptible to killing by catechins, so we can hypothesize that *B. pertussis* or *B. parapertussis* are inhibiting fimbriae activity or secreting a molecule similar to catechins (27,28). Furthermore, *Bordetella* could be preventing prokaryotic growth through contact dependent growth inhibition, in which proteins are secreted by a bacterial species in response to direct cell contact (29). This secreted protein binds to a protein receptor on the target cell, preventing bacterial growth (29). Alternatively, *Bordetella* with lysogenic phage is able to out-compete *Bordetella* that is phage sensitive; in the same manner, *Bordetella* has the potential of inhibiting HMF that are phage sensitive (30). In yet another method, *B.*

bronchiseptica could be inhibiting the growth of murine microflora through the type six secretion system (T6SS) (31). A peptide secreted by the T6SS in *Pseudomonas aeruginosa* was able to hydrolyze the peptidolgycan of prokaryotes; thus, the T6SS in *B. bronchiseptica* could be utilizing the same method for bacterial competition (32). However, *B. bronchiseptica* inhibition of murine microflora *in vitro* is not as ubiquitous as would be expected based on murine microflora clearance seen in *in vivo* with *B. bronchiseptica* infection. Since there was varying degrees of inhibition of murine microflora in cross-streaking and co-culturing, it is possible that direct bacterial competition alone cannot explain the culturable microflora clearance seen *in vivo* during *B. bronchiseptica* infection. Immune-mediated mechanisms could also be an integral factor. Overall, *Bordetella* are also equipped with mechanisms to compete with HMF in order to colonize a host.

The observation that *B. bronchiseptica* clears culturable microflora when it infects a murine nasal cavity suggests that the ability to displace HMF may be a mechanism for colonization. This implies that if an animal experienced HMF depletion, then that animal may be even more susceptible to *B. bronchiseptica.* Observations such as this could help explain why *B. bronchieptica* has great potential to cause infections in livestock, primarily swine. As early as 1950, Jukes et al. showed that giving livestock antibiotics can help accelerate animal growth (33). As a result, 79.8% of the antibiotics produced in the U.S. in 2009 were put into animal feed because farmers want to hasten livestock growth and increase their profit (34). Moreover, a survey done by the Department of Agriculture's Animal and Plant Inspection Service in 1999, 2001, and 2006 indicates that 84% of farmers that raise swine put antibiotics into their feed (34). This large scale antibiotic use could severely affect the microbial environment within a pig, increasing the likelihood of *B. bronchiseptica* infections. *B. bronchiseptica* infections in swine

already have severe economic impacts, and the widespread use of nonspecific antibiotics will likely only exacerbate the problem.

In addition to affecting *B. bronchiseptica* infection in swine, HMF interactions with the human pathogens *B. pertussis* and *B. parapertussis* could have several medical implications. For example, just as *B. pertussis* was more effective at colonizing mice after culturable HMF were removed with antibiotics, widespread antibiotic use in humans could make individuals more vulnerable to *B. pertussis* infection (35). Consequently, pathogens naturally inhibited or out-competed by resident flora could take advantage of this environmental disruption and colonize someone that would normally be protected with a diverse microbial population. Furthermore, excessive antibiotic use can disturb microbial homeostasis by leading to the development of antibiotic-resistant pathogens (35). Overall, antibiotics need to be prescribed with care because they may be as harmful as they are therapeutic. Thus, a new way to combat *B. pertussis* infections may be to explain that HMF can prevent infection and that unnecessary antibiotic use is killing this first line of pathogen defense.

Probiotics have long been used as a health measure to prevent pathogen colonization Understanding the nature of microbial interactions could allow researchers to create novel probiotics, eventually implanting bacteria into humans or animals to prevent disease. For example, HMF species identified in this study that inhibited *B. pertussis in vitro* could be co-inoculated with *B. pertussis* into a murine nasal cavity to determine if *in vitro* trends continued *in vivo*. If this experiment proved successful, then the next step would be to perform similar *in vivo* trials on humans to determine therapeutic efficacy. Currently, *Enterococcus, Bacillus,* and *Lactobacillus* are being used as probiotics in the intestinal tract; however, comparable probiotics for upper respiratory pathogens have not yet been developed (36,37). Thus, this study could

progress to provide a basic understanding of *Bordetella* competition with HMF, leading to novel upper respiratory probiotics. As a supplement, since babies acquire their HMF from the environment and their mothers, they could also be implanted with beneficial probiotics at birth to prevent the colonization by pathogens (38). Overall, using probiotics to combat pathogens could lead to a future where *Bordetella* infections, in humans and animals, could be prevented by regular intake of probiotic bacteria.

Through *in vivo* analysis of bacterial growth interactions, it has been determined that *B. pertussis* is unable to colonize mice that have a diverse microbial population because *B. pertussis* and *B. parapertussis* are inhibited by murine HMF but not human HMF. Alternatively, *B. bronchiseptica* is able to outcompete culturable HMF *in vitro* and clear them during infection in a mouse. Although the interactions between HMF and *Bordetella* are not completely understood, this work indicates that HMF interactions are important for initial *Bordetella* colonization. Thus, once these interactions are more fully described, bacterial competition may be used therapeutically to combat disease.

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