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Sherry Tsui April 10, 2023

Evolution of Antibiotic Resistance in Bumblebee Gut Symbionts

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An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

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Abstract

Evolution of Antibiotic Resistance in Bumblebee Gut Symbionts

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Exposure to antibiotics can often cause adverse effects on beneficial organisms, such as bumblebees, in cropping systems. It is vital to address this issue, as bee pollination plays a major role in the global economy. Field studies have indicated that bee-associated microbes can develop antibiotic resistance. However, it is still unclear how bumblebees acquire antibiotic-resistant genes within their gut microbiome, and how readily these mutations could arise in specific symbionts. Various environmental conditions within the gut, such as biofilm formation, could impact the ability of symbionts to gain antibiotic resistance, yet it is unclear whether biofilm formation plays an important role in facilitating this process. By using an evolution passaging assay with increasing antibiotic concentrations on static and shaking plates, we explored the potential for inducing antibiotic resistance in a strain of the bumblebee gut symbiont *Gilliamella* via *de novo* mutation. Furthermore, we explored whether biofilm formation could impact the evolution of resistance in *Gilliamella*. Previous literature has indicated frequent presence of antibiotic-resistance genes in bee symbionts *Gilliamella* and *Snodgrassella* in natural populations. Thus, I hypothesized that *Gilliamella* can gain antibiotic resistance through *de novo* mutations. Cultures grown in a planktonic environment are found to have the potential to develop antibiotic resistance at higher concentrations, thus I hypothesized that strains grown in shaking conditions can have a higher resistance to antibiotics. We found that antibiotic resistance in the bumblebee symbiont *Gilliamella* could be evolved via passaging and the state, either biofilm or planktonic, in which *Gilliamella* grew did not impact the evolution of resistance. However, we found that the state did impact the symbiont's growth rate. Further experiments will allow us to better understand and manage antibiotic resistance within the bee community. Moreover, the design of these experiments can be a tool to study how antibiotic resistance affects the functions of bee core symbionts and their impacts on bee behavior. Microbial interactions could further impact a symbiont's ability to evolve antibiotic resistance. In future studies, community-level evolution assays should be conducted to further explore symbionts' ability to evolve in a community setting.

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TERMINOLOGY

Terminology	Definition	Reference
Minimum inhibitory concentration (MIC)	The lowest antibiotic concentration that inhibits visible growth of a microbe.	(Andrews, 2001)
Tolerance	The ability of bacteria to temporarily withstand an antibiotic treatment, while lacking the ability to grow, without a change in MIC.	(Windels et al. 2019)
Resistance	The ability of a bacteria to overcome an antibiotic treatment.	(Windels et al., 2019)
	It is caused by an inherited mutation and is quantified by MIC. Strains with a higher MIC are considered more phenotypically resistant.	(Brauner et al. 2016)
Susceptible isolate	An isolate that is not resistant to any of the antibiotics tested.	(Centers for Disease Control and Prevention, 2019)
Passage/Passaging	Also known as subculturing, is the transfer of cells from a previous culture in fresh grown medium.	(Davies et al., 1999)

INTRODUCTION

The use of agricultural chemicals and antibiotics plays an essential role in suppressing bacterial crop diseases, and over the past decade, there has been an increase in the use of antibiotic applications in US orchards (National Agricultural Statistics Service, 2018). This is worrisome as there is scant knowledge about how off-target field organisms, like bees, respond to these agricultural practices. Honeybees and bumblebees are effective and widely employed for crop pollination, contributing to approximately one-third of total human dietary supplies (Khalifa et al., 2021). They also have a highly conserved and specialized gut microbiome (Raymann and Moran, 2018), or beneficial bacteria that live in their gut. This microbiome plays a critical role in bee metabolism (Zheng et al. 2016), immune function (Kwong, Mancenido, et al., 2017), and behavior (Zheng et al., 2017; Raymann and Moran, 2018).

Laboratory evidence suggests that antibiotic exposure is detrimental to bee functioning. Studies have shown that intra-colony use of antibiotics such as oxytetracycline and tetracycline can disrupt the honeybee gut microbiome leading to negative effects on worker survival (Raymann and Moran, 2018). Furthermore, long-term exposure to tetracycline in honeybees can alter the native gut microbiome and promote sensitivity to viral infections (Deng et al., 2022). Tetracycline can also delay the onset of foraging in honeybees (Ortiz-Alvarado et al., 2020). Additionally, our lab found that bumblebees' ability to associate a color with a sucrose reward declined when exposed to a high streptomycin concentration, yet the mechanism is unknown (Avila et al., 2022). In this case, it remains unclear whether the antibiotic impact on bee functioning is due to disturbances of the bee microbiome or direct toxicity.

Field studies show that the symbionts of bees might be able to evolve resistance and withstand antibiotic disruption. The animal gut microbiome is a large reservoir of antibiotic-resistance genes, and the diversity of antibiotic-resistance genes reflects the antibiotic spectrum in the environment (Sun et al., 2022). For example, Tian et al. (2012) and Ludvigsen et al. (2018) found that honeybees sampled in the US carry more oxytetracycline and streptomycin, respectively, resistance genes than their European counterparts; these antibiotics are used in agriculture in the US but not in Europe. Zhang et al. (2021) compared bumblebees collected in China and the USA and did not find markers of tetracycline resistance in Chinese bees. Presumably, this is driven by the more common use of antibiotics in US agriculture and beekeeping compared to China and other regions of the world. However, the mechanisms behind how beneficial organisms in cropping systems evolve and maintain these antibiotic-resistance genes in their gut microbiome are poorly described (Sun et al., 2022).

Environmental factors play a role in the evolution of antibiotic resistance. Factors such as concentration and period of exposure to agricultural antibiotics might play a role. Additionally, the presence of other environmental resistant bacteria could be key for gaining resistance. For example, strains of the core bee gut symbiont *Snodgrasella* have acquired resistance to streptomycin (*strA-strB*) via horizontal gene transfer from *Erwinia amylovora,* a crop pathogen that itself has developed resistance to streptomycin (Ludvigsen et al., 2018). Another study revealed that transferrable antibiotic-resistance genes are frequently found in the honeybee gut microbiome, and plasmids could be transferred between honey bee gut symbionts by conjugation (Sun et al., 2022).

There are also intrinsic factors that might impact the evolution of antibiotic resistance. For example, the ability of the symbionts to form biofilms could play a role (Vuotto et al., 2014). Bacteria form biofilm in response to environmental stress (Jefferson, 2004) and genetic analyses has indicated that different symbiont species within the bee gut are capable of biofilm formation

(Engel et al., 2012). Many studies have been interested in determining whether growth in biofilms can facilitate the evolution of antibiotic resistance. Studies have indicated that biofilm formation aids antibiotic resistance. Bowler et al.(2020) found that biofilm can not only facilitate the transfer of antibiotic resistant genes within the biofilm, they also have an innate phenotypic tolerance to antibiotics. Sharma et al.(2019) found that the biofilm matrix gives bacteria resistance from both environmental stressors and antibiotics. However, due to this protective mechanism, it is harder for cultures in biofilm to evolve to a higher antibiotic resistance due to low levels of antibiotic exposure. In a study testing the evolution of resistance to ciprofloxacin (CIP) in *Acinetobacter baumannii* populations, it was found that clones adapted to a biofilm environment were less resistant than planktonic clones with a lower MIC but are more fit without the presence of drugs when exposed to increasing sublethal levels of antibiotic concentration (Santos-Lopez et al., 2019). Similarly, in a study exploring the evolution of antibiotic resistance in biofilm and planktonic Gram-negative *Pseudomonas aeruginosa*, it was found that MICs were lower in CIP-resistant isolates from biofilm populations than planktonic cultures similarly when exposed to sublethal levels of antibiotics (Ahmed et al., 2018). These results could possibly be explained by low-level drug exposure in biofilm formation, which facilitates low-level resistance in biofilm cultures (Ahmed et al., 2018). Thus, in the context of experimental evolution, it is possible that planktonic growth can better facilitate the evolution of antibiotic resistance as compared to biofilm cultures.

The objectives of this thesis are to answer two complementary questions:

(1) Can we evolve antibiotic resistance in bumblebee symbionts through *de novo* mutations?

(2) Does biofilm formation impact the evolution of resistance through *de novo* mutations?

Research has indicated that antibiotic-resistant genes are more prevalent in honey bee-specific gut members *Gilliamella* and *Snodgrassella* (Sun et al., 2022) than in other bees symbionts. Thus, **(1) I hypothesize that *Gilliamella* will be able to gain antibiotic resistance through *de novo* mutations.** Furthermore, given that *Gilliamella* and *Snodgrassella* are capable of biofilm formation (Engel et al., 2012), and that biofilm could lead to low level resistance due to less exposure to antibiotics, **(2) I hypothesize that strains in a planktonic environment will be more likely to evolve resistance than strains within a biofilm.**

In addition to gaining insights into these fundamental questions, evolving antibiotic-resistant symbionts could serve as a tool to study if antibiotic resistance protects the functions of core bee symbionts when their hosts are exposed to antibiotics. Additionally, such a tool could make it possible to tease apart whether antibiotics impact bee behavior through disruption of the microbiome or through direct toxicity. Specifically, bees could be inoculated with antibiotic-resistant and antibiotic-susceptible symbionts, and their behavior could be measured pre and post antibiotic exposure.

We focused on streptomycin to address these questions, given its use in broadcast spraying of citrus, apple and pear orchards in the USA to control crop diseases, during periods of high bee activity (Sundin and Wang, 2018). The maximum concentration tested was 200 ppm. Higher concentrations of other antibiotics are used within honey bee hives (Raymann et al. 2017), but streptomycin is typically sprayed below 200 ppm (Avila et al., 2022).

METHODS

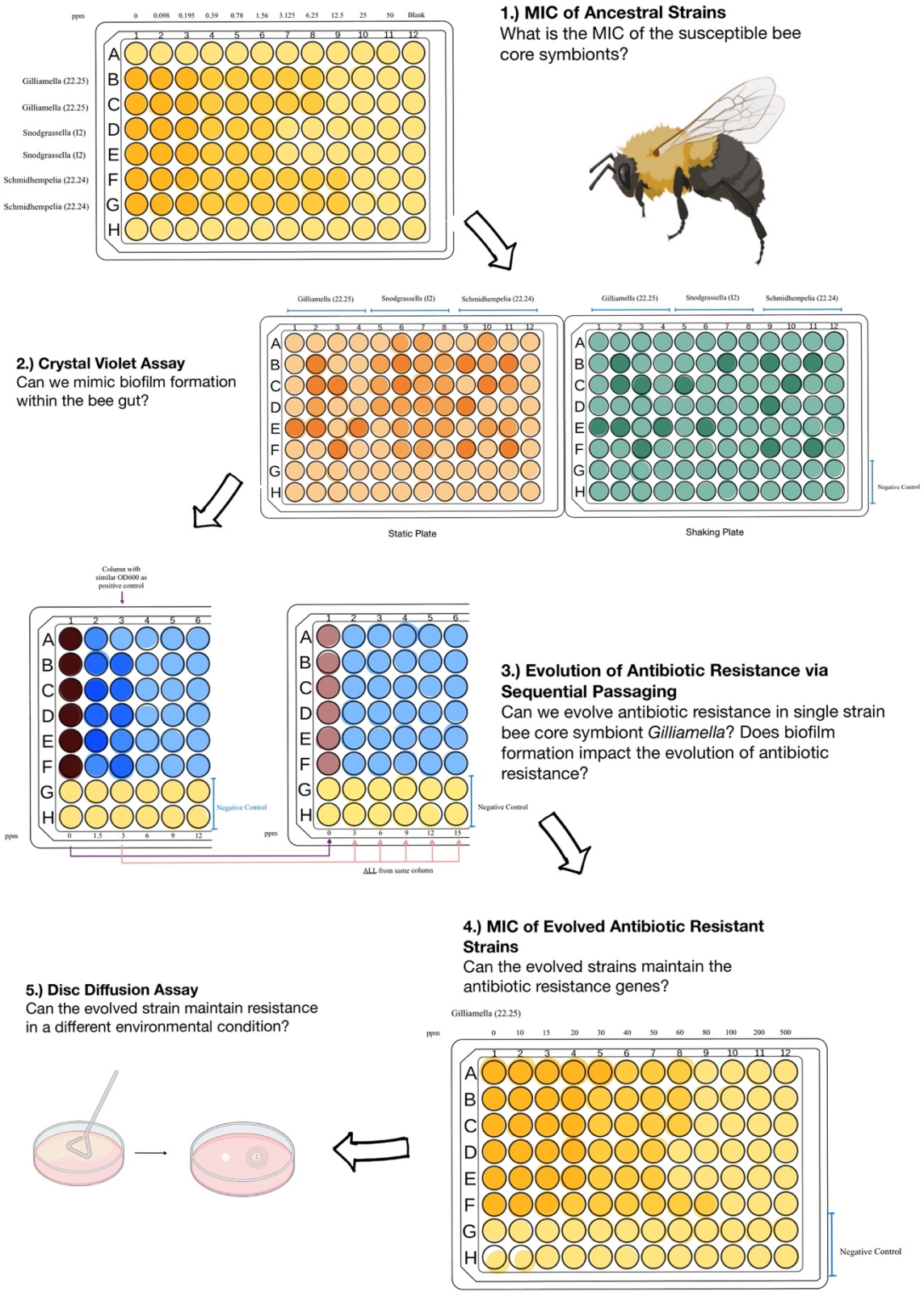

Figure 1. Experimental setup and overview. Created with BioRender.com.

Strains Description

Four major symbionts that are found in the bumblebee gut include bacteria in the genera *Snodgrassella*, *Gilliamella*, *Schmidhempelia,* and *Lactobacillus*. To carry out the experiments, I used strains of the first three. The strains were isolated from eastern bumblebees (*Bombus impatiens*). Strains were obtained from the reference collection at Ben Sadd's lab at Illinois State University (Appendix 2 and 3 for 16sRNA sequences and material transfer agreement)

Table 1. Bee core symbiont classification. Sources: (1) Ludvigsen et al., 2018; (2) Kwong and Moran, 2013; (3) Zhang et al. 2020; (4) Moran, 2015

Phylum	Family	Genus/species	Strain	Gram	Antibiotic Resistance	Biofilm Formation
Proteobacteria	Neisseriaceae	*Snodgrassella alvi*	*wkB2*	-	Streptomycin, tylosin (1), tetracycline (2)	+ (4)
Pseudomonadota	Orbaceae	*Gilliamella apicola*		-	tetracycline, polymyxin, aminocoumarin, macrolide, rifampin, multidrug (3)	+ (4)
Proteobacteria	Orbaceae	Candidatus *Schmidhemplia*		-		

1. Minimum Inhibitory Concentration (MIC) Assay for Ancestral Susceptible Strains

To determine the minimum inhibitory concentration (MIC) of ancestral, susceptible strains, I cultured core bacterial symbionts, retrieved from a cryobank (received from Sadd Lab at Illinois State University), in 1000µL Insectagro (Corning) in a 5% CO2 incubator at 35ºC up to an OD600 of 1. Fifteen µL of each strain (1 in 150 dilution from an OD of 1) was then transferred to a 96 well-plate prepared with 136 µL media and a two-fold increase in streptomycin concentrations per row (0, 0.098, 0.195, 0.39, 0.178, 1.56, 3.125, 6.25, 12.5, 25 and 50 ppm) (Figure 1). Two replicates per each strain were grown at each antibiotic concentration. The plate was placed on a shaker in a 5% CO2 incubator at 35ºC until cultures were turbid. OD600 measurements were taken daily with a plate reader (Synergy HT) to measure growth for ten days.

2. Crystal Violet Assay

We determined whether symbionts could form biofilm by comparing the biofilm of a static 96-well plate against a shaking plate. We measured biofilm formation with a crystal violet assay (O'Toole, 2011). We cultured 5 µL of each core symbiont in 100µL of Insectagro in two 96-plates. Both plates were placed in a 5% CO2 incubator at 35ºC; one plate was placed on a shaker (Thermo Scientific 4625, orbital shaking motion, 900 rpm), and the other plate was static. After 24 hours, all strains were poured out of the well, and plates were rinsed in water to remove any detached cells. Each well was filled with 125µL of crystal violet, rinsed in water and dried overnight. Each well was filled with 200µL of 95% ethanol, and an OD550 measurement was taken in a plate reader (Synergy HT) to determine biofilm formation. Pictures were also taken with a Olympus XM10 monochrome camera on an Olympus SZX16 microscope (1.25X magnification, 1X objective lens, DF (darkfield illumination), 30 ms exposure, Gain 9.3 dB)

3. Evolution of Antibiotic Resistance via Sequential Passaging

To test the hypothesis, we attempted to evolve antibiotic resistance through passaging (Davies et al., 1999). I established two different plates for passaging. One plate was shaking, which disrupts

biofilm formation, and the other plate was static, which allows biofilm formation. I revived each core symbiont on Columbia Blood Agar (CBA) amended with 5% sheep blood, and one colony was picked for each strain to culture in Insectagro up to an OD600 of 1. For the first evolution assay, 3 passages were carried out with 2-fold increments of streptomycin (0, 6, 12, 25, 59, 75 ppm). For the second evolution assay, to set up a shaking and a non-shaking plate, 190µL of Insectagro with streptomycin concentration of 3 ppm increments were added to each column until 50 ppm. Then 10µL of each ancestral strain was added to each well, and the plates were placed in a 5% CO2 incubator at 35ºC for 24 – 48 hours, depending on growth. OD600 was then measured for each well daily. When the OD600 of the 0ppm column and one of the antibiotic columns reached an OD600 of close to 1 or was visually turbid, 10 µL of culture from the wells of both columns were passaged to the next plate using the same conditions. The evolved strains were also passaged to two additional columns with increased streptomycin concentration of 3 ppm increments. This process was repeated until streptomycin reached a concentration of 50 ppm. Additionally, strains were also cultured in Insectagro with 200 ppm and 500 ppm of streptomycin.

"**Growth**" was scored with a value of 1 or 0. Wells with a higher than the mean OD600 of negative control wells with no bacteria (mean across all plates) received a score of 1. Wells with an OD600 equal to or lower than the mean OD600 of negative control wells received a score of 0.

We estimated the proportion of wells that grew for each streptomycin concentration for each passage. This was calculated by adding up the number of wells with "growth" (as defined above) and dividing by the total number of wells for that streptomycin treatment (generally six wells in total). This metric indicated the "potential for resistance to evolve," as each replicate is separately passaged although they originate from the same ancestral strain. Additionally, the OD600 was plotted against the streptomycin concentration for all those wells with growth (score of 1).

"**Evolution" of resistance** was likely if: 1) the bacteria grew (as defined previously) at a streptomycin concentration above the MIC for the susceptible ancestral strain; and 2) the bacteria growing in Insectagro with antibiotics were growing as well as the passaged susceptible control (bacteria grown at 0 ppm streptomycin in the passaging plates). In other words, the mean OD600 of the bacteria passaged in streptomycin was statistically indistinguishable from the passaged susceptible control strain. We did not compare the mean OD600 of the bacteria passaged in streptomycin with a positive control with antibiotic resistance because currently there are no *Gilliamella* strains that are found to be resistant to streptomycin.

4.1. MIC Assay for Putatively Evolved strains - Ability to Maintain Resistance

The increased sub inhibitory concentration was determined separately for each evolved strain at several of the passaged streptomycin concentrations. For *Gilliamella,* these passages were 33, 36 and 90 ppm. The passaged susceptible control and ancestral (not passaged) strains were assessed. All these passage treatments were transferred to a 96 well-plate prepared with Insectagro media and increased streptomycin concentrations (0, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, 200, and 200 ppm for 33 and 36 ppm passaged strains; 0, 10, 15, 20, 30, 40, 50, 60, 80, 100, 200, and 500 ppm for 90 ppm passaged strains). Six replicates were tested for each antibiotic concentration. Each

plate had twenty-four negative control replicates. The plate was placed in a 5% CO2 incubator at 35ºC for 24 hours or until cultures were turbid. OD600 measurements were taken with a plate reader (Synergy HT). The OD600 values were plotted for all the strains of the putatively evolved treatments against the MIC test streptomycin concentrations.

4.2. Strain Sequencing

To confirm the identity of the bacteria, the strains were diluted in PBS with a 2 in 200 dilution and 50µL were spotted on CBA plates to send for sequencing. Additionally, the evolved strains were amplified with strain-specific primers to confirm strain identity (see Appendix 1 for primers). A total of 0.6 µL of Proteinase K and 20µL of lysis buffer were used to lyse 1µL of each sample. Then 10µL platinum direct PCR universal master mix, 2µL of strain-specific forward primer, 2µL of strain-specific reverse primer, 4µL of water for each sample and 2µL of supernatant were used to amplify each sample (Invitrogenä PlatinumäSuperFiä; Appendix 1 for strain specific primers). The samples were amplified using a thermocycler (Eppendorf Mastercycler EP Gradient S). Initial denaturing was run for 5 minutes at 95 °C, followed by 35 cycles of denaturing at 95 °C for 15 seconds, annealing at 60 °C for 15 seconds, and elongation at 72 °C for 30 seconds, with a final elongation of 7 minutes at 72 °C, and a hold at 4 °C until removed. Gel electrophoresis was used to visualize the PCR products. Negative controls were run along with the samples.

Additionally, for any in-house failed PCR reaction (i.e., no bands show in gel electrophoresis of PCR reactions for *Snodgrassella* and *Schmidhempelia*), three wells were randomly selected, and three 50 µL aliquots per well were spotted in individual CBA plates. Plates were incubated following the same settings as described previously and sent for PCR (using primers 27F & 1492R) and Sanger sequencing to Genewiz (Azenta, South Plainfield, NJ). Resulting sequences were identified using BLAST Nucleotide and Genome Query (National Library of Medicine, n.d.).

5. Disc Diffusion Assay - Ability to Maintain Resistance in a Different Environmental Condition

To determine whether the strains maintained antibiotic-resistance, a disc diffusion assay was used. From the previously cultured ancestral strain, the previously passaged 0 ppm strain, and the putatively evolved strains (33, 66, and 50 ppm), an aliquot of each culture was diluted up to 100 µL in Phosphate Buffered Saline (PBS). The passaged susceptible control dilutions were 1:100 µL, 5:100 µL and 10:100 µL, with three replicates each, for a total of nine plates. The ancestral dilutions were 5:100 µL and 10:100 µL, with three replicates each for a total of six plates. A 5:10 µL dilution was used for the 33-ppm and 36 ppm evolved strains, with three replicates each, for a total of six plates. A 10:100 µL dilution was used for the 50-ppm passaged strain, for a total of three plates. Then 50µL of each dilution was spread and dried on a CBA plate. A blank disc (negative control disk) and a 10-µg streptomycin disc were placed on the agar plate and the plates were incubated in a 5% CO2 incubator at 35ºC until growth was observed. Pictures of each plate were taken with Olympus XM10 monochrome camera on an Olympus SZX16 microscope to observe the formation of a zone of inhibition. The number of plates with inhibition

zones around the antibiotic disc was tallied, a proportion was estimated, 95% binomial confidence intervals were calculated for each proportion, and the data was plotted. The diameter around the disk was not measured.

Statistical Analyses

Data were plotted and analyzed using R (v.4.2.0). If the continuous variables did not follow a normal distribution, they were either analyzed with a non-parametric statistical test or log/square root transformed before statistical analysis.

To test for statistical differences in biofilm formation in the static versus shaking plates, crystal violet OD600 data was analyzed with a (non-parametric) Wilcoxon paired test, using the 'car' package.

A binomial regression was implemented with the 'stats' package to model to model the effect of streptomycin treatment, biofilm formation, and passage number on the proportion of wells with growth ("potential for resistance to evolve") for all passages. Next, an analysis of deviance was used to assess the statistical significance of streptomycin treatment, biofilm formation, and passage number on the proportion of wells with growth (sequential comparison of models adding each of the tested variables). Given heteroscedasticity in the model residuals, the data was also analyzed with a beta regression implemented in the 'betareg' package. For the beta regression, proportions equal to 1 had to be converted to 0.999 and results are presented in the Appendix (Table A2).

A linear model was implemented in the 'stats' package to model the effect of streptomycin treatment, biofilm formation, and passage number on the OD600 values of samples from passaged strains that exhibited growth (as defined earlier). The OD600 values were log-transformed to achieve normally distributed model residuals. An ANOVA was estimated with the 'car' package Dunnet linear contrasts were carried out between the passaged susceptible control against all the passaged streptomycin concentrations. A lack of statistically significant differences between the growth (mean OD600) of the passaged susceptible control vs. the passaged streptomycin concentrations indicated evolution of resistance (as defined earlier). Only the last measurement for passages seven to nine were analyzed, as those represented the highest passage concentrations. Instead of a comparison between strains growing at a MIC of 25 ppm against all the strains passaged in streptomycin concentrations, we chose to do a comparison between the passaged susceptible control against all the passaged streptomycin concentrations because we wanted to account for the strains' adaptation to the media. The strains in the first MIC assay from the susceptible ancestral strains at 25 ppm had a low OD600 value and they were not passaged as frequently as during the sequential passaging assay, so we are not considering the impact of the adaptation to Insectagro with each sequential subculture. Furthermore, we did not compare the growth of strains in the presence of antibiotics to the OD600 value of the strains growing at 25 ppm during the sequential passaging at 3 ppm increments because there is a variation in the number of passaging as we were only able to putatively evolve strains to 25 ppm by the 6th passage.

A final MIC assay was used to assess the ability of two “evolved” strains (33 and 36 ppm) to maintain their resistance at various streptomycin concentrations. An ANOVA was used to assess the statistical effect of streptomycin concentration and treatments (the two evolved strains, a failed-to-evolve strain at 90 ppm, a passaged susceptible control, and an ancestral strain) on mean growth (OD600). To determine differences in the mean growth between the passaged susceptible control and ancestral strains versus the passaged strains (33, 36, and 90 ppm), We carried out pairwise Tukey linear contrast with Bonferroni corrections in the ‘multicomp’ package.

RESULTS

The MIC assay for susceptible ancestral strains includes data from all three bee core symbionts. However, Sanger sequencing of the strains indicated contamination issues during the passage of *Snodgrassella* and *Schmidhempelia*. Therefore, only results from *Gilliamella* are presented for the remaining assays.

1. MIC Assay for Susceptible Ancestral Strains

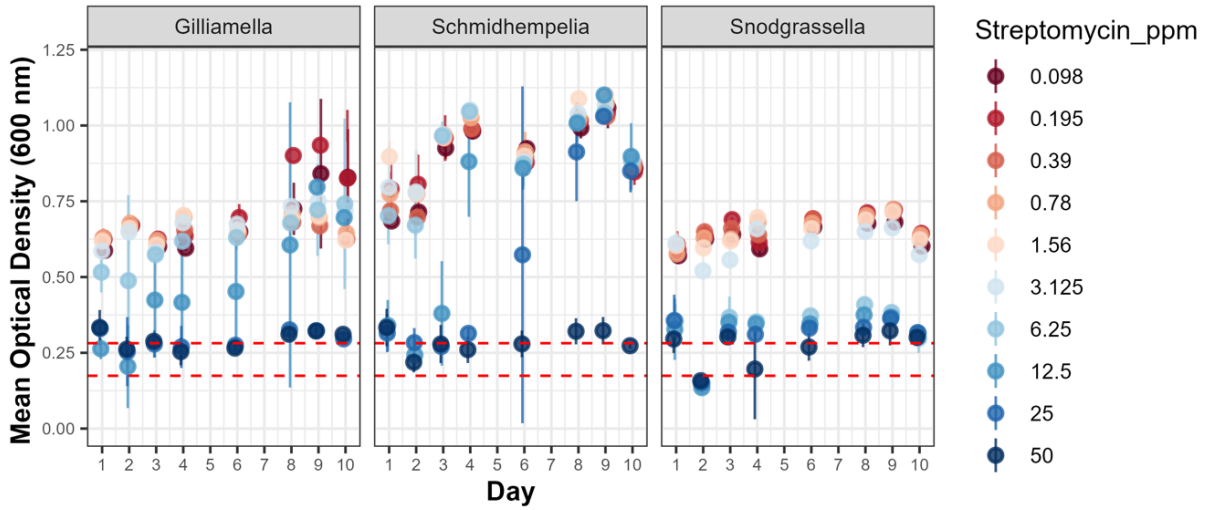

Figure 2. MIC assay for the bee gut symbiont ancestral strains susceptible to streptomycin. All three bacteria genera were tested in a single MIC plate. There is no reading for time "0" after the inoculum was added to the wells. Red dotted lines indicate 95% CI for the OD600 values of the negative control (n = 30).

Antibiotic-resistant genes have previously been found in bee core symbionts in the field, however, the exact MIC of streptomycin for many core symbionts has yet to be determined. The mean OD600 of each core symbiont was measured over ten days, and the MIC range was determined. In general, a clear difference, or breakpoint, in the mean OD600 for each strain is observed as the strain is cultured in media with an antibiotic concentration that falls within the MIC range (Figure 2).

The MIC value of *Gilliamella* lies near 25 ppm. Specifically, at streptomycin concentrations of 12.5 ppm and below, the OD600 of strains measured are all above 0.50 by the end of ten days. Low OD600 values near 0.25 were measured in strains grown at streptomycin concentrations of 25 ppm and above, indicating little to no growth over this period.

The MIC value of *Schmidhempelia* lies within the range of 25 ppm to 50 ppm. At streptomycin concentrations of 25 ppm and below, the OD600 of strains measured are all above 0.75 by the end of ten days, indicating a substantial amount of growth. Low OD600 values of around 0.25 and 0.725 were measured in strains grown in streptomycin concentrations of 25 ppm and above, indicating little to no growth over the ten days.

The MIC value of *Snodgrassella* lies within the range of 3.125 ppm to 6.25 ppm. Over ten days, the OD600 value of cultures growing in streptomycin concentrations of 3.125 ppm and below are above 0.50. The OD600 values of cultures growing in streptomycin concentrations of 6.25 ppm and above were relatively stable at around 0.25 and 0.725 and no visible growth was observed.

2. Crystal Violet Assay

Our results indicate that there is a significantly greater amount of biofilm formation when *Gilliamella* strains are cultured in a static plate (Figure 3).

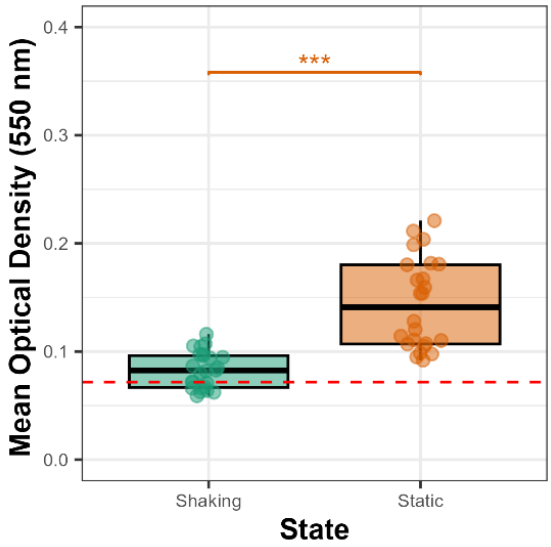

Figure 3. Biofilm Assay for *Gilliamella*. Bacteria were grown on shaking and static plates for 24 hours. A crystal violet assay was performed, and plates were read at OD550. The red line indicates the average OD550 of the negative control (the media Insectagro with no strain added)

OD550 was significantly higher in the static plate as compared to the shaking plate (Wilcoxon paired test, W=33.5, p-value < 0.0001)

Qualitatively, by comparing selected wells from the shaking and static plates under the microscope, more biofilm formation is observed in the static plate (Figure 4).

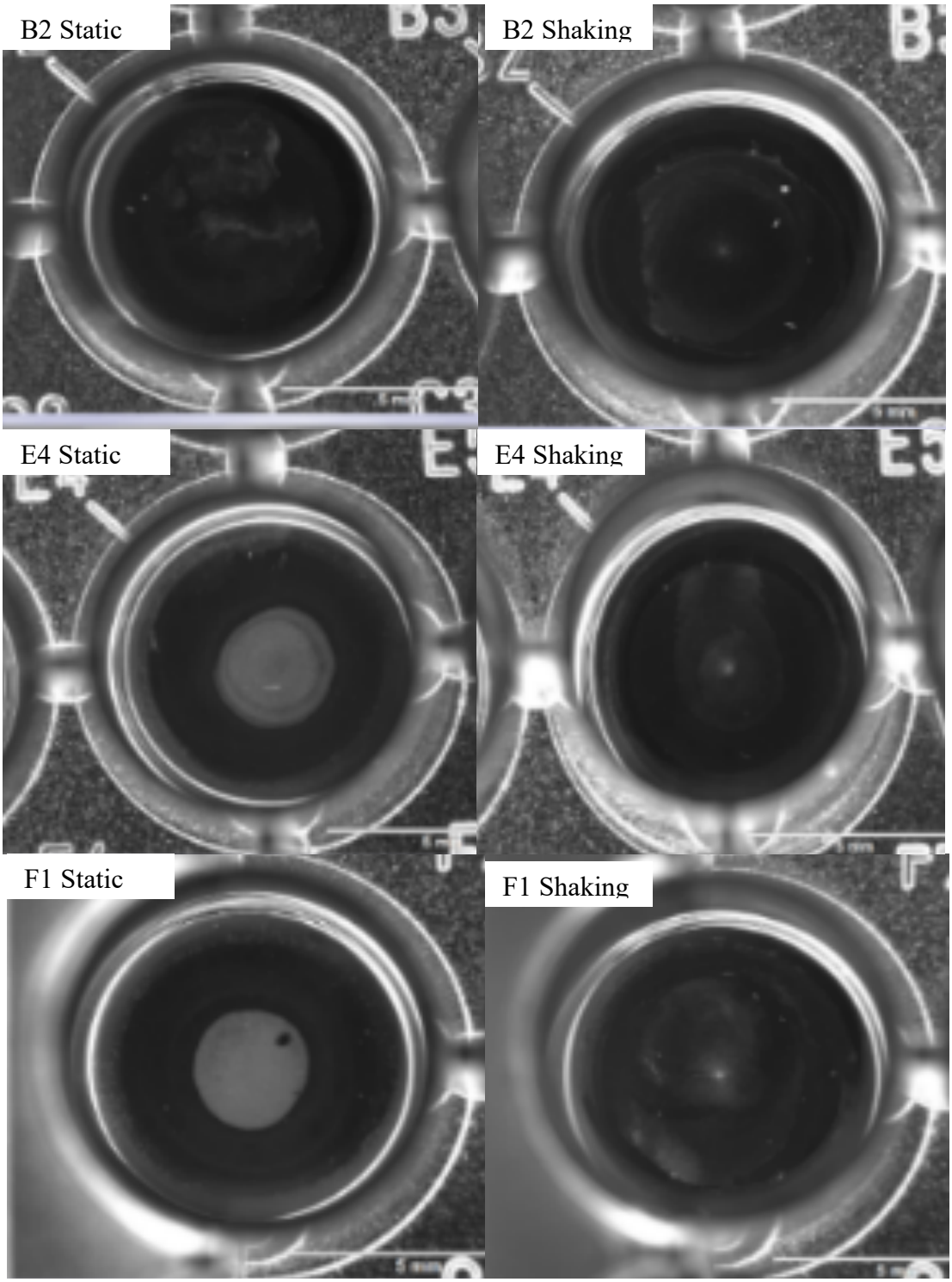

Figure 4. Biofilm assay visualization. Pictures of wells after crystal violet staining with Olympus XM10 monochrome camera on an Olympus SZX16 microscope. *Gilliamella* was cultured in all wells shown with Insectagro (Corning).

3.1. Evolution of Antibiotic Resistance via Sequential Passaging: Two-Fold Increments

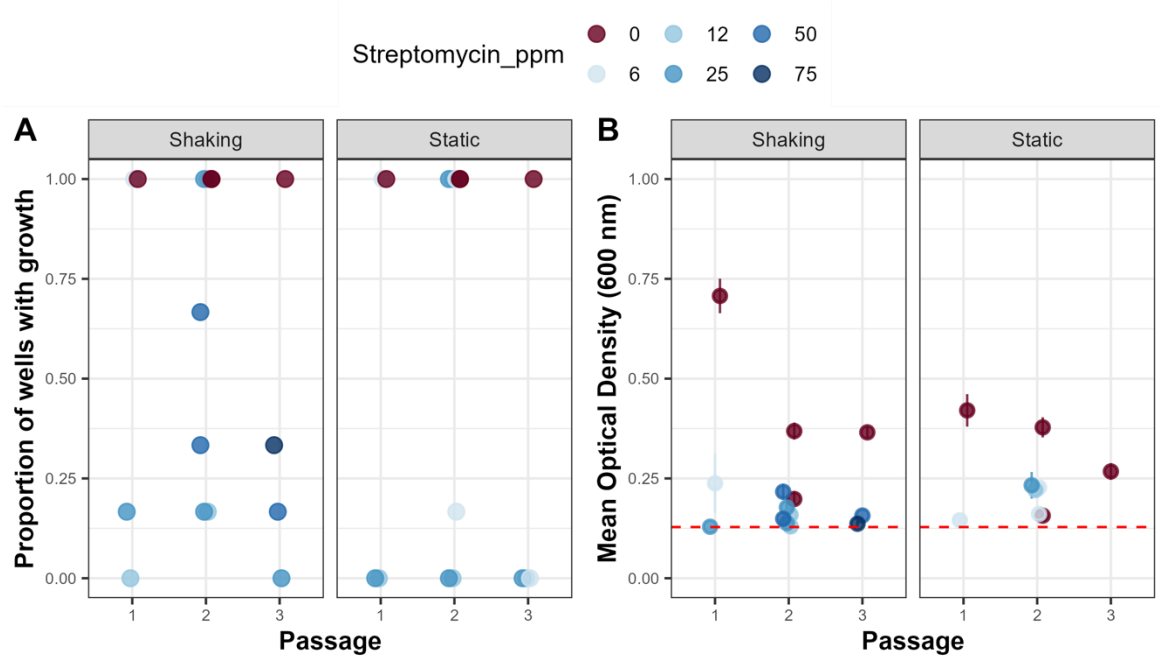

Figure 5. The proportion of *Gilliamella* samples with growth, and growth of those samples, during the first passaging experiment. Strains were passaged in two-fold streptomycin increments. Labelled facets show data segregated by shaking and static plates. Each streptomycin concentration has six replicates. Passage 2 was read twice on two consecutive days (days 5 and 6) due to insufficient growth from the reading on the first day. **A)** Proportion of wells with growth (OD600 nm > than OD600 for negative control samples) for the strains passaged from 0 to 75 ppm streptomycin through three passages. **B)** Mean OD for the wells with growth. The red line indicates the average OD600 of the negative control (the media Insectagro with no strain added).

In Figure 5A, only strains grown in 0 ppm (passaged susceptible control) had growth in all wells during each passage for both the shaking and the static plate. For the strains grown in presence of streptomycin, the proportion of wells that had growth decreased with each consecutive streptomycin passage in both the shaking and the static plates. By the third passage, only the passaged susceptible control had growth in all six replicates.

In Figure 5B, there is a decline in the mean OD600 read for all strains across each passage. Although the mean OD600 for the passaged susceptible control was the highest for each passage, it also showed a decline over time. The mean OD600 of strains grown in antibiotics was at or barely higher than the negative control by the third passage, in both the shaking and static plates.

3.2. Evolution of Antibiotic Resistance via Sequential Passaging: 3 ppm Increments

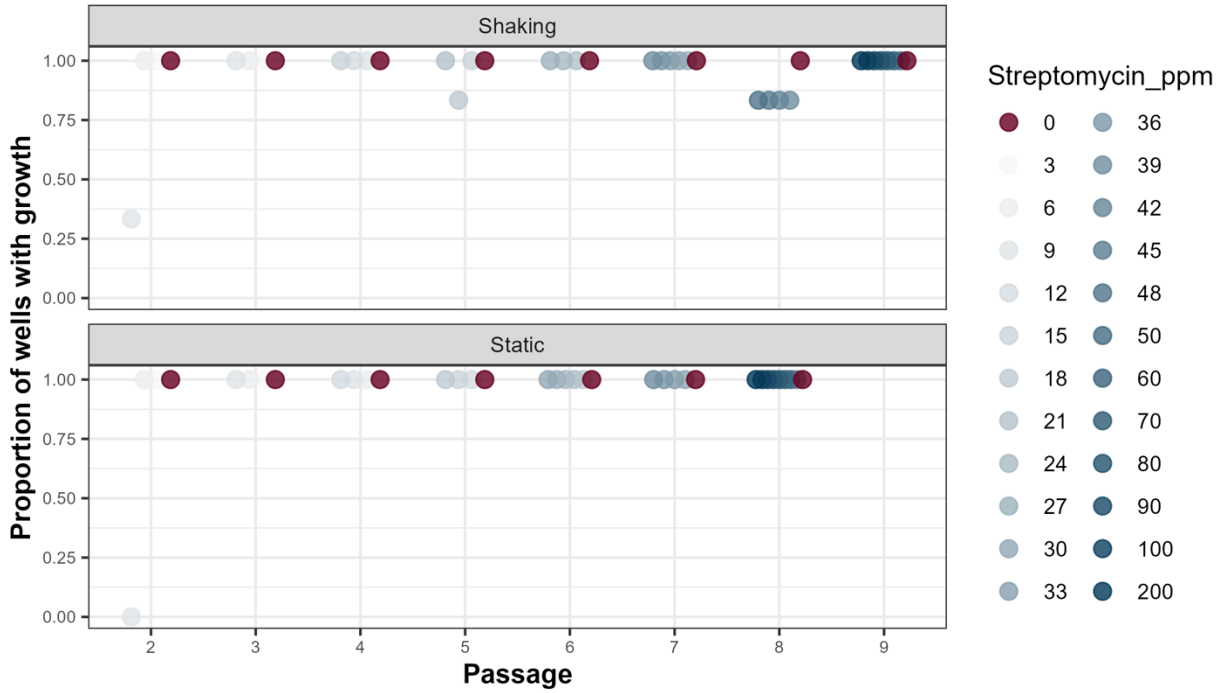

Figure 6. Proportion of *Gilliamella* samples with growth during second passage experiment. Proportion of wells with growth (OD600 > negative control samples OD600) for the strains passaged from 0 to 200 ppm streptomycin through (nine passages for shaking treatment, eight for static treatment). Labelled facets show data segregated by shaking and static plates. Strains were passaged in 3 ppm streptomycin up to 39 ppm (static plate) or 48 ppm (shaking plate), in increments of 10 ppm afterwards up to 100 ppm and finally with a 100 ppm increment to 200 ppm. There are six replicates for each concentration at each passage per plate. There are three readings from three consecutive days for passage nine of the shaking plate and passage eight of the static plate, as there was not sufficient growth observed in the first two days.

Compared to when the strains were passaged at two-fold increments, a greater proportion of strains on each plate in 3 ppm antibiotic increments survived and continued growing after each passage. Growth was observed in all wells by the last passaging in both the shaking and the static plate (Figure 6, Figure A2).

Table 2. Analysis of deviance for the binomial models of the proportion of wells with growth during the sequential passage. Data for all passages, ranging from 0 to 200 ppm of streptomycin.

Variables	**Chi-Square**	**DF**	***P***
State	0.0049	1	0.9437
Streptomycin treatment	0.0180	1	0.8931
Passage	0.2655	1	0.6064
State x Streptomycin	0.0038	1	0.9502

There was no impact of passage treatment (static vs shaking), antibiotic treatment, nor passage on the proportion of wells with growth during sequential passaging in the second passaging experiment (Table 2; Table A4 for binomial regression model).

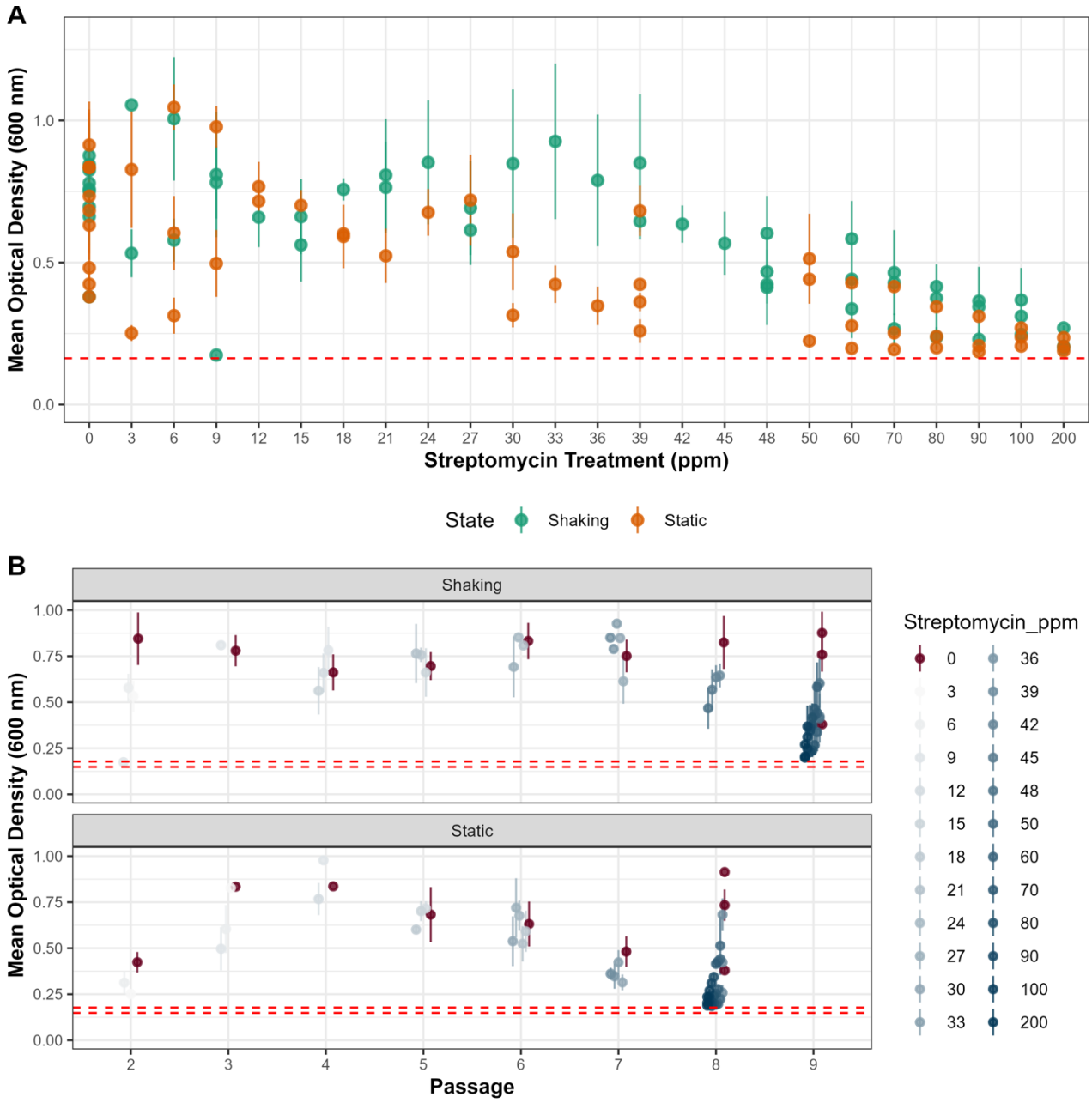

Figure 7. *Gilliamella* samples with growth during second passaging experiment. Both panels represent the same dataset. Negative control (red dotted line, n= 216 replicates) consisted of liquid media (Insectagro) with no bacteria added. Strains were passaged in 3 ppm streptomycin initially and higher concentrations once they had been passaged up to 39 ppm (static plate) or 48 ppm (shaking plate). There are six replicates for each concentration at each passage per plate. There are three readings from three consecutive days for passage nine of the shaking plate and

passage eight of the static plate, as there was not sufficient growth observed in the first two days. **A)** Mean Optical Density (OD at 600 nm) at different antibiotic concentrations. Green and orange circles indicate readings from shaking and static plates, correspondingly. From 39 to 48 ppm, no readings were done for the static plate as antibiotic treatments were increased in greater increments. **B)** Mean Optical Density (OD at 600 nm) at each passage. Readings of samples at low streptomycin concentrations were removed for each consecutive passaging because the goal is to evolve strains to be resistant at higher streptomycin concentrations.

There was a decrease in the mean optical density of *Gilliamella* for both the shaking and static plate with an increase in streptomycin concentration. Additionally, a higher mean OD600 was observed in the shaking plate as compared to the static plate. For both shaking and static plates, minimal growth was observed at a streptomycin concentration of 100 ppm and 200 ppm. In the shaking plates, strains growing up to 60 ppm and in the static plates, strains growing up to 50 ppm had a mean OD600 of above 0.5. These observed streptomycin concentrations are higher than the previously identified *Gilliamella* MIC of 12.5 ppm to 22.5 ppm (Figure 7A).
The strains grown in 0 ppm streptomycin on the static plate consistently had an OD600 reading above 0.625 for all passages except for passage 1 which had a mean OD600 reading of 0.375 (Figure 7B).

The strains grown in 0 ppm streptomycin on the static plate reached an OD600 of above 0.5 by the third passage, however, the mean OD600 started to decline in the following passages. A mean OD600 of strains grown in 0 ppm streptomycin did reach above a mean OD600 of 0.5 in two of the readings in passage 9. This could be an indicator that the growth of strains in the static plate is slower than that of the shaking plate (Figure 7B).

Table 3. ANOVA for the OD600 growth of strains in passage seven, eight & nine, for a total of n=168 wells, ranging from 27 to 200 ppm of streptomycin.

Variables	**F-value**	**DF**	***P***
State (Shaking or Static)	55.1635	1	**<0.000001**
Streptomycin treatment	145.1263	1	**<0.000001**
Passage (7,8 or 9)	0.2900	1	0.5909
State x Streptomycin	1.1665	1	0.2817

There was an effect of the state of the plate (shaking vs static) and streptomycin concentration on the observed growth of the strains. There was no effect observed of passage when comparing sequential passages 7, 8, and 9 (Table 3; A5 Table 2 for linear regression model).

Table 4. Linear contrasts for mean OD600 growth of evolved strains. Comparison of the log mean OD values for passaged susceptible control samples against the mean OD for all other streptomycin treatments. The null hypothesis is that the OD means of antibiotic passaged strains are not different than those of control passaged strains. A Dunnett test was applied.

Contrast	**Estimate**	**Standard Error**	**t-value**	***P(>|t|)***
27 ppm – 0 ppm	-0.24072	0.16853	-1.428	**0.81582**
30 ppm – 0 ppm	-0.20931	0.13150	-1.592	**0.69936**

33 ppm – 0 ppm	-0.02302	0.13150	-0.175	**1.00000**
36 ppm – 0 ppm	-0.20439	0.13150	-1.554	**0.72763**
39 ppm – 0 ppm	-0.06068	0.10842	-0.560	**0.99994**
48 ppm – 0 ppm	-0.58313	0.16957	-3.439	0.00722
50 ppm – 0 ppm	-0.25318	0.16377	-1.546	0.73381
60 ppm – 0 ppm	-0.71102	0.13150	-5.407	< 0.001
70 ppm – 0 ppm	-0.89381	0.13150	-6.797	< 0.001
80 ppm – 0 ppm	-1.02159	0.13150	-7.769	< 0.001
90 ppm – 0 ppm	-1.16544	0.13150	-8.863	< 0.001
100 ppm – 0 ppm	-1.03534	0.13150	-7.873	< 0.001
200 ppm – 0 ppm	-1.38625	0.13150	-10.542	< 0.001

Growth of strains at streptomycin concentration of up to 39 ppm was similar to the control passaged strains. This is also true for strains grown at 50 ppm. However, growth of the rest of the strains at other antibiotic concentrations was different than that of the control passaged strains. (Table 4).

4. MIC Assay of Putatively Evolved Strains

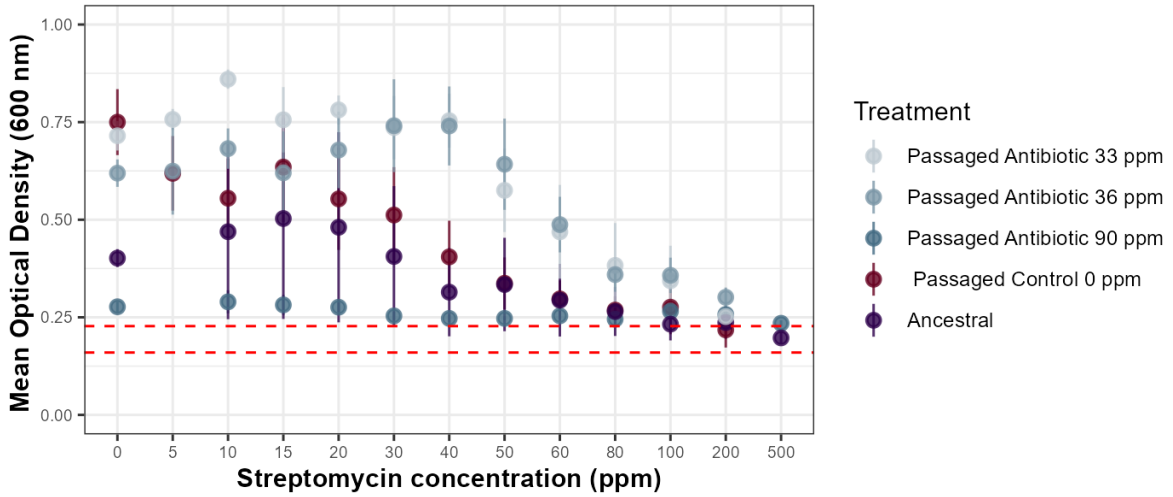

Figure 8. Final MIC of passaged strains from the biofilm plate that showed increased MIC (putative evolved strains for 33ppm and 36ppm). Each passaged strain had its own MIC plate (n=6 wells per streptomycin concentration, n=24 negative control wells). The "Ancestral" strain was not passaged, and the 0 and 90ppm strains did not exhibit any indication of evolution of antibiotic resistance, and thus also serve as controls. Red dotted lines indicate 95% CI for the OD600 values of the negative control. Circles indicate means and vertical lines 95% CI. Notice the break between 200 and 500 ppm. Only ancestral treatment and passaged antibiotic 90 ppm were tested at 500 ppm.

The putatively evolved strains passaged to 33 ppm and to 36 ppm exhibited increased antibiotic resistance, relative to the control and ancestral strains, when they were grown again in streptomycin, as their OD600 was greater than the control 0 ppm strains, and ancestral strains.

This increased OD600 was present up to 100 ppm streptomycin concentration, indicating that the evolved strains have developed a new MIC. At the 200 ppm streptomycin concentration, all strains had little to no growth (Figure 8). The strain passaged to 90 ppm did not grow well in all antibiotic concentrations due to a low OD600 in the initial inoculum for the MIC assay (see Figure 8). Both the original passaged treatment and the streptomycin treatment in the MIC had an impact on the OD600 value observed (Table 5).

Table 5. ANOVA for the OD600 growth of the final MIC for ancestral strain, control passaged strain, antibiotic-passaged strains with presumed evolution of resistance (passaged to 33 ppm, 36 ppm of streptomycin), antibiotic-passaged strain with lack of evolution of resistance (passaged to 90 ppm of streptomycin). The OD600 values were square root transformed to improve normality.

Variables	**F-value**	**DF**	***P***
Treatment	74.525	4	<0.00001
Streptomycin treatment	187.804	1	<0.00001

Table 6. Linear contrasts for mean OD600 growth of antibiotic passaged strains (across all antibiotic concentrations). Comparison of the mean OD values for passaged susceptible control samples against the mean OD for all other streptomycin treatments. The alternative hypothesis is that the OD600 means of antibiotic passaged strains are higher than those of the control strain & ancestral strain. Tukey's pairwise test was applied with Bonferroni correction. OD600 values were square root transformed.

Contrast	**Estimate**	**Standard Error**	**t-value**	***P(>|t|)***
Control – 33 ppm	0.11433	0.01658	6.895	<0.00001
Control – 36 ppm	0.09037	0.01658	5.450	<0.00001
Control – 90 ppm	-0.13515	0.01708	-7.914	1.00000
Ancestral – 33 ppm	-0.18370	0.01708	-10.757	1.00000
Ancestral – 36 ppm	-0.15974	0.01708	-9.354	1.00000
Ancestral– 90 ppm	0.06578	0.01732	3.798	0.000867
Control – Ancestral	-0.06937	0.01708	-4.062	1.00000

The OD600 of strains passaged at 33 ppm and 36 ppm was significantly higher than those of the control strain and ancestral strain. There was no difference in growth between the control and ancestral strain ($p > 0.05$), nor the control and a strain passaged at 90 ppm ($p > 0.05$) (Table 6).

5. Disc Diffusion Assay- Ability to maintain resistance in different environmental conditions

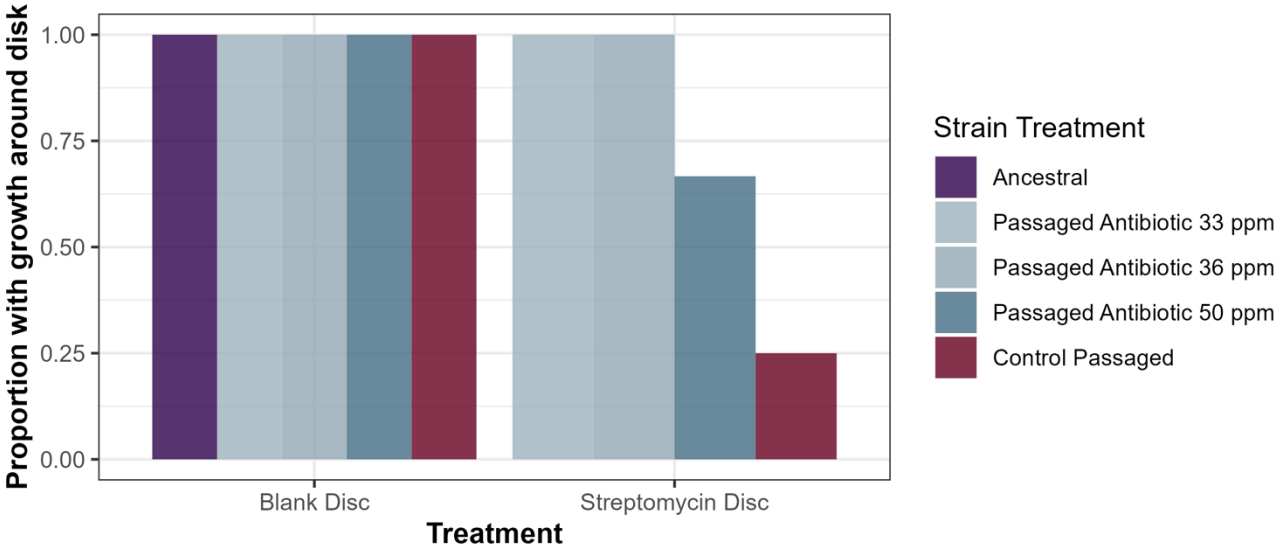

Figure 9. Disc diffusion assay of passaged strains from biofilm plate that showed increased MIC (putative evolved *Gilliamella* strains). The proportion of plates that exhibited growth around a blank disk and a streptomycin disk (10 ug). Control and streptomycin disks were placed within each plate replicate. Brackets show the estimated binomial 95% confidence intervals. The passage control strain had eight replicate plates (one plate did not grow). No bar is shown for ancestral as none of the three replicates had growth around the disk (three other replicates did not grow). All other treatments had three plate replicates only.

All replicates for strains from all treatments grew around the blank disc with no streptomycin. All replicates from strains that putatively evolved in 33 and 36 ppm grew around the streptomycin disc, which indicates antibiotic resistance. Two out of three replicates of strains passaged to 50 ppm did not grow around the streptomycin disc. Two out of nine replicates of the control passaged strains grew around the streptomycin disc and both of these replicates are from a 10:100 µL dilution while the rest of the replicates are from a 1:100 µL and 5:100µL dilution (Figure 9).

DISCUSSION

Experimental evolution of resistance to streptomycin in bee symbionts is possible, yet it happens at a slow pace. We found that: a) the MICs of the ancestral bumblebee core symbionts *Gilliamella*, *Snodgrassella* and *Schmidhempelia* are all below 50 ppm, and specifically *Gilliamella* has an MIC below 25 ppm streptomycin (Figure 2); b) the MIC for *Gilliamella* could be increased to beyond 100 ppm and susceptible ancestral strains evolved antibiotic resistance to 39 ppm through sequential passaging, suggesting that we can select single strain core bee symbionts *in vitro;* c) this resistance is stably maintained in selected cultures, as these strains can grow better than passaged susceptible controls and ancestral strains at higher antibiotic concentrations; and d) lastly, we have discovered that biofilm formation does not impact the selection of resistance in directed experimental evolution, but that it can affect the growth rate of selected strains.

Initial MIC determination

The MIC of the three symbionts tested is below 50 ppm streptomycin (Figure 2). While previous studies have identified antibiotic resistance genes in isolates of *Gilliamella*, their MIC to streptomycin has not been tested (Ludvigsen et al., 2018; Zhang et al. 2020). In our study, ancestral *Gilliamella* strains isolated from bumblebee guts had a MIC near 25 ppm streptomycin. The MICs from susceptible and resistant *Snodgrassella* strains isolated from honey bee guts are 0.75 and 12 ppm of streptomycin, respectively (Ludvigsen et al., 2018). The MIC we measured for the ancestral *Snodgrassella* strain isolated from bumblebee guts (6.25 ppm of streptomycin) is below the previously reported 12 ppm. We did not find any published literature regarding the presence of antibiotic resistant genes and MICs for *Schmidhempelia,* but we determined that its MIC was higher than for the other two bee gut symbionts (within the range of 25 ppm to 50 ppm). Overall, we contribute to the literature by reporting for the first time an MIC for streptomycin in *Gilliamella* and *Schmidhempelia* strains.

The ancestral strains probably experienced a prolonged lag phase (period prior to exponential growth in bacteria), during the initial MIC assay. Given the slow growth of the symbionts, we measured OD600 in the MIC plate over a ten-day period. We discovered that *Gilliamella* and *Schmidhempelia* at 12.5 and 25 ppm, respectively, arrested growth in the presence of streptomycin then increased in growth after three to four days, as indicated by an increase in OD600 (Figure 2). Studies have indicated that the lag phase can last from several hours up to several days and some cells within the same population can take much longer than others despite being genetically identical (Vermeersch et al. 2019). In addition, the nutrients available may also extend lag phase (Jacob and Monod, 1961). Thus, this increase in lag phase could be explained by the bacteria adapting to Insectagro, in addition to the added selective pressure from antibiotics. For example, lack of simple carbohydrates could have delayed *Gilliamella's* growth, as it needs these as energy source (Kwong, Engel, et al., 2014). This potential mechanism is supported by the fact that passaged susceptible control strains seemed to adapt to the Insectagro media and grew to higher OD600 than all the other passaged and ancestral strains in the passaging experiments (but their MIC were still around 25 ppm).

Evolution of Resistance

Gilliamella was able to putatively evolve resistance, regardless of biofilm condition. First, passaged *Gilliamella's* MIC increased to a range between 100 to 200 ppm and the ancestral susceptible strain is now resistant to 39 ppm of streptomycin. In this experiment, we tried evolving the ancestral susceptible strains with two-fold ppm and three ppm increments. In the

two-fold increment evolution assay, the bacterial strains did not survive through each passaging, thus the protocol needed to be adjusted. There was an overall decrease in OD600 after each subsequent passaging in the passaged susceptible control (0 ppm), which indicated that some other factor, besides antibiotic presence, was impacting microbial survival. Thus, we decided to increase the inoculation size from 2μL to 10μL per passaging to increase possible bacterial growth. For the time of incubation, we decided to passage bacterial strains with an OD600 close to the passaged susceptible control to ensure that there was sufficient bacterial growth for passaging. Lastly, we altered the antibiotic concentration with smaller increments, which could potentially help the strains to adjust to a gradual increase in antibiotic concentration. With these adjustments, we were able to evolve to an streptomycin resistance of 39 ppm, shown by the fact that the strain passaged at this concentration grew as well as the passaged susceptible control without antibiotic ($p<0.999$). A 25 ppm MIC is considered a low MIC, and a 39 ppm sub inhibitory concentration is considered an intermediate level of antibiotic resistance (low≤16ppm, intermediate: 32-64 ppm, high ≤64 ppm) as defined by Tudó et al. (2010). Thus, the observed increase in antibiotic resistance could be considered intermediate. We did observe strains growing at streptomycin concentrations up to 100 ppm, however 39 ppm is the minimum antibiotic concentration in which the growth rate was similar to the passaged susceptible control. It could be possible that the increased MIC lies within the range of 100 to 200 ppm. Second, we confirmed that *Gilliamella* forms biofilms (Moran, 2015), and that its biofilm can be disrupted via shaking. Previous studies have indicated that planktonic cultures are more likely to facilitate the evolution of antibiotic resistance in single strains of other bacteria species (Santos- Lopez, 2019; Ahmed et al., 2018). However, our results indicate that the state in which the strains were grown did not have an impact on the evolution of antibiotic resistance (Table 3). Contrary to past literature indicating that biofilm formation could lead to a higher growth rate than planktonic cultures of *Pseudomonas sp* strains (Bester et al., 2005), we observed that strains grown in a static plate had a lower OD600 values relative to shaking plates at the same antibiotic concentrations.

We do not know the exact mechanism for the putative evolution of resistance. Streptomycin is found to directly interact with four nucleotides of 16S rRNA and the ribosomal protein S12 ribosomal subunit to disrupt protein translation, resulting in truncated peptides (Raymann et al., 2017; Sundin and Wang, 2018). There are several mechanisms that streptomycin resistance determinants employ, such as encoding enzymes that confer resistance through the inactivation of the streptomycin molecule through either phosphorylation or adenylation (Shaw K J et al., 1993) and through spontaneous mutations (Sundin and Wang, 2018). The most common spontaneous mutations occur in the *rrs* and *rpsL* genes, which lead to alteration in the streptomycin binding site in the ribosome (Ozaki et al., 1969). Examples of genes that confer streptomycin resistance via horizontal gene transfer include, *strA- strB, apb6-1a, apb6-1b, apb6-1c, ant (3"), ant (6)* and *aadA* and its relevant alleles (Sundin and Wang, 2018). Among these, the *strA- strB* gene pair and *aadA* are two of the most widely distributed streptomycin resistance determinants (Sundin and Wang, 2018). However, horizontal gene transfer would not be the source of antibiotic resistance in our experiment as there are no streptomycin resistant donors for the bee core symbionts. The permeability of the bacterial outer membrane can also play a role in antibiotic resistance, specifically drug resistance could involve the modifications of lipid and protein compositions of the outer membrane(Delcour, 2009). Antibiotics can travel through the outer membrane through either a lipid mediated pathway or through general diffusion porins depending on whether if the antibiotic is hydrophobic or

hydrophilic (Delcour, 2009). For hydrophilic antibiotics like streptomycin, antibiotics, addition and modifications of general diffusion porins can increase and decrease antibiotic permeability respectively (Waters & Tadi, 2023). Antibiotic resistance could also be acquired through chromosomal alterations from an unknown mechanism (Björkman et al., 1999). Genome sequencing of ancestral, passaged susceptible control and evolved strains would be needed to better understand the reason behind the increase in *Gilliamella* resistance in our experiments.

The putatively evolved *Gilliamella* was able to maintain antibiotic resistance, without evident fitness costs *in vitro.* Here we use OD600 as a proxy for growth and fitness. By checking the growth of strains passaged at 33 ppm and 36 ppm, we found that the evolved strains were not only able to grow at higher OD600 than the passaged susceptible control strains, but they could also grow at higher antibiotic concentrations surpassing the MIC of susceptible ancestral strains. Strains passaged up to 33 and 36 ppm grew better than the control strain at concentrations higher than 25 ppm (Figure 8). From the putatively evolved strains MIC assay, we observed that they are able to grow in streptomycin concentrations of up to 100 ppm (Figure 8). The evolution and acquisition (e.g., horizontal gene transfer) of antibiotic resistance could directly alter the fitness of microbes. Currently, the fitness costs of harboring resistance genes are still poorly understood (Rajer and Sandegren, 2022). However, research has indicated that, generally, mutations that confer resistance are costly, though some have little or no cost (Melnyk et al., 2015). This might be the case, at least for honey bees. For example, Raymann et al., (2017) found that there is an increase in the accumulation of resistance genes in the gut microbiome after long-term antibiotic exposure and that these genes continue to persist in low frequencies even in honeybee hives with no recent antibiotic treatment (Raymann et al., 2017). Additionally, Ludvigsen et al (2018) found streptomycin resistant genes (*strA-strB)* in bees from Arizona, an area where streptomycin is not used in crops, indicating that the gut symbionts in these colonies had maintained acquired genes in the absence of direct selective pressure. Given its persistence under no selective pressure of antibiotic resistance, it could be possible that there are compensatory mutations that restore fitness, or there is a rare occurrence of a cost-free mutation (Andersson and Hughes, 2011). This could possibly be explained by the "plasmid paradox" (Haavisto, 2023). It could be possible that plasmids persist in the microbiome even if it does not directly benefit the bacteria because the antibiotic resistant plasmids may carry an unknown fitness other than antibiotic resistance, or the plasmid may be acting as selfish DNA that is only concerned for its own persistence (Haavisto, 2023). In line with previous literature (Andersson and Hughes, 2011; Haavisto, 2023), the fitness of the evolved antibiotic resistant *Gilliamella* strains was even better than the ancestral strain, as the OD600 measured was higher without the presence of antibiotics (Figure 8). However, the OD600 was slightly lower than strains passaged at 0 ppm possibly due to these strains increased ability to adapt to Insectagro (Figure 8).

Resistance seems to persist in different environmental conditions. The strains evolved in biofilm liquid culture, yet they exhibited resistance to streptomycin when placed in solid media. Specifically, the evolved *Gilliamella* survived the exposure to streptomycin discs (10 ug) placed on CBA plates. We were also able to confirm that the streptomycin passaged strains were resistant relative to the passaged susceptible control and ancestral strains in this solid media. Aside from differences in solid state, these two media could have varied nutrient content. However, we cannot make inferences regarding nutritional differences because the exact composition of Insectagro is a “trade secret”. CBA (ThermoFisher) has a limited source of sugars, as only cornstarch is found in the formula. The observed slow growth rate of *Gilliamella* in both media (48 hrs needed for visible colonies in agar and generally low OD600 values in

liquid culture) could be explained by a limited source of simple sugars, which in turn could also mediate tolerance to antibiotics. Even if their formulations are not ideal for *Gilliamella* growth, Insectagro and CBA are commonly used to culture bee symbionts (Leonard, 2017; Kwong and Moran, 2013). Therefore, we were able to confirm that susceptibility and increased resistance to antibiotics could be tested in either.

Shortcomings

All three bumblebee core symbionts *Gilliamella, Snodgrassella,* and *Schmidhempelia* were passaged in these experiments, however only *Gilliamella* was used for final MIC assays, as the other two core symbionts were contaminated. These contamination issues could be indicative of the difficulty of culturing bee core symbionts out of a community context. Previous studies have indicated that biofilm formation within the gut is known to have a protective function against pathogens (Steele et al., 2021). Although biofilm formation was detected in the static plates, the amount of biofilm formed *in vitro* could still be different than the amount of biofilm formation within the bee gut microbiome, resulting in the ability of other microbes to proliferate. Within a community setting, the interactions between different microbes could also have collective protective behaviors (Bottery et al., 2021). This could explain why the two single strain bee core symbionts were easily contaminated.

We were also not able to use a positive control with streptomycin resistance to compare the growth of strains passaged in antibiotics. This is because there are currently no true positive controls present as no *Gilliamella* strains are found to have resistance to streptomycin.

Future Directions

Emerging evidence suggests that interspecies interaction within a microbiome can alter bacteria responses to antibiotics (Bottery et al., 2021), and collective protective behaviors such, as collective resistance, collective tolerance and exposure protection could either increase the MIC of bacteria or facilitate the survival of bacteria exposed to antibiotics (Vega and Gore, 2014; Meredith et al., 2015). The evolution of antibiotic resistance was tested on single strain bumblebee core symbionts; however, community level evolution assays should be conducted in the future. Additionally, studies have found that *Gilliamella* typically grows on top of *Snodgrassella* within the bee gut, and the two symbionts interact, with *Gilliamella* providing food sources to *Snodgrassella* for energy metabolism in the Krebs cycle (Kwong, Engel, et al., 2014). Experiments that mix different strains could enrich our knowledge of how microbial interactions could impact the development of antibiotic resistance and fitness within the bee gut microbiome. Each unique bee core symbiont could be tagged with fluorophore to distinguish each species when cultured in a consortium. Previous literature has indicated that active bacterial starvation responses could mediate antibiotic tolerance in biofilms and nutrient limited bacteria (Fung et al., 2010; Nguyen et al., 2011). Therefore, it would be important to explore whether different nutritional states could impact *Gilliamella's* evolution of resistance. Finally, *in vivo* experiments would be necessary to understand whether the evolved strains are able to establish inside the bee gut and be passed on to other naïve hosts, without detrimental consequences to microbe and host fitness.

Big Picture

From the results of this experiment, we have found that long-term exposure to antibiotics and incremental exposure to increasing antibiotic concentrations are needed for ancestral susceptible symbionts to evolve resistance. It took sixteen days for susceptible *Gilliamella* to

evolve resistance up to a sub inhibitory concentration of 39 ppm of streptomycin. We have also found that bee symbionts are only able to evolve at small antibiotic increments as a sudden increase in antibiotic concentration may directly kill the bee core symbionts during passaging. Given that antibiotic resistance genes have been found in the field where antibiotics have been used in agriculture, the presence of antibiotics could provide selective pressure for the bee symbionts to evolve antibiotic resistance (Zhang et al. 2020). However, if the bee symbionts did evolve resistance in the field through *de novo* mutations, as indicated by our results, they would need to be exposed to antibiotics for a long period of time at sublethal levels. Perhaps the bee symbionts may rely on other means to acquire resistance, such as horizontal gene transfer (Ludvigsen et al., 2018).

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APPENDIX

Table A1. Strain primers

Strain Primer	Primers Sequences (5’ - 3’)
Snod-F	CTTAGAGATAGGAGAGTGCCTT
Snod-R	AACTTAATGATGGCAACTAATGACAA
GbombiF1	CGTGAATCAGAATGTCACG
GbombiR1	CCGTGGTAAACGCCCTCCCGAAG
GbombiF2	GACCTCGCGAGAGCAAG
GbombiR2	AAACGCCCTCCCGAAG
SchmidF1	CGGAACCCACAGCTCAAG
SchmidR1	AAAACTGGTCGTCTGGAGT
SchmidF2	CCCCTCGCGGTTAAGCTATC
SchmidR2	GAGAAGCGAGCCAGCGAT

Table A2. Strain Sequences for Ancestral Strains

Genus Original	Strain/Tube Contents	BLAST Genus	Species	% match	Nucleotide sequence
Snodgrassella	I2	Neisseriaceae /Snodgrassella	alvi	99.19%/99.07%	CGNNNNAGCGACCN CNNNGCGGTTAGTCT ACCCAATTNNTGGTA TCCCCCACTCCCATG GTGTGACGG GCGGTGTGTACAAGA CCCGGGAACGTATTC ACCGCAGTATGCTGA CCTGCGATTACTAGC GATTCCGACTTCATG CACTC GAGTTGCAGAGTGCA ATCCGGACTACGACC GGCTTTCTGAGATTG GCTTCACCTCGCGGC TTCGCTACCCTCTGT ACCGA CCATTGTATGACGTG TGAAGCCCTGGTCAT AAGGGCCATGAGGA CTTGACGTCATCCCC ACCTTCCTCCGGTTT GTCACC GGCAGTCTCATTAGA GTGCCCAACTTAATG ATGGCAACTAATGAC AAGGGTTGCGCTCGT TGCGGGACTTAACCC AACAT CTCACGACACGAGCT GACGACAGCCATGCA GCACCTGTGTTACGG CTCCCGAAGGCACTC TCCTATCTCTAAGAG ATTCC GTACATGTCAAGACC AGGTAAGGTTCTTCG CGTTGCATCGAATTA ATCCACATCATCCAC CGCTTGTGCGGGTCC CCGTC AATTCCTTTGAGTTTT AATCTTGCGACCGTA

					CTCCCCAGGCGGACA ATTTCACGCGTTAGC TTCGCTACTAAAACA TCTA GTGTCCCAACAGCTA ATTGTCATCGTTTAG GGCGTGGACTACCAG GGTATCTAATCCTGT TTGCTACCCACGCTT TCGAG CATGAACGTCAGTGT TATCCCAGGAGGCTG CCTTCGCCATCGGTA TTCCTCCACATCTCT ACGCATTTCACTGCT ACACG TGGAATTCTACCTCC CTCTGACACACTCTA GTTAACCAGTTTCAA ATGCACGTCCCAAGT TGAGCTCGGGGATTT CACAT
Gilliamella apicola	22.25 #2	Gilliamella	bombi / apicola	0.9976 %	GNAACGCCCTCCCGA GGTTAAGCTATCTAC TTCTGGTGCAACCCA CTCCCATGGTGTGAC GGGCG GTGTGTACAAGGCCC GGGAACGTATTCACC GTGACATTCTGATTC ACGATTACTAGCGAT TCCGACTTCATGGAG TCGAG TTGCAGACTCCAATC CGGACTTAGACGTAC TTTGTGAGGTCCGCT TACTCTCGCGAGGTC GCCTCCCTTTGTATA CGCCA TTGTAGCACGTGTGT AGCCCTGGTCGTAAG GGCCATGATGACTTG ACGTCGTCCCCACCT TCCTCCGCTTTATCA ACGGC AGTCTCCTTTGAGTT CCCGACCTAATCGAT GGCAACAAAGGATA AGGGTTGCGCTCGTT GCGGGACTTAACCCA ACATTT CACAACACGAGCTGA CGACAGCCATGCAGC ACCTGTCTCACAGTT CCCGAAGGCACTCTC GTATCTCTACAAGAT TCTGT GGATGTCAAGACCAG GTAAGGTTCTTCGCG TTGCATCGAATTAAA CCACATGCTCCACCG CTTGTGCGGGCCCCC GTCAA TTCATTTGAGTTTTAA CCTTGCGGCCGTACT CCCCAGGCGGTCGAT TTATCGCGTTAGCTT CGGAGCCCATCACTC TAGG CAACAAACTCCAAAT CGACATCGTTTACAG CGTGGACTACCAGGG TATCTAATCCTGTTTG CTCCCCACGCTTTCG CATC TCAGCGTCAGTATCT GTCCAGAAGGCCGCC TTCGCCACCGGTATT

					CCTCCACATCTCTAC GCATTTCACCGCTAC ACGTG GAATTCTACCTTCCT CTACAATACTCTAGT TAACCAGTTTTAAGT GCAATTCCCTAGGTT GAG
Schmidhempelia	22.24	Candidatus Schmidhempelia	bombi	0.9940 %	GCCCCCCTCGCGGTT AAGCTATCTACTTCT GGTGCAACCCACTCC CATGGTGTGACGGG CGGTGTGTACAAGGC CCGGGAACGTATTCA CCGTAGCATTCTGAT CTACGATTACTAGCG ATTCCGACTTCATGG AGTCG AGTTGCAGACTCCAA TCCGGACTTAGACGT ACTTTATGAGGTCCG CTCACCATCGCTGGC TCGCTTCTCTTTGTAT ACGC CATTGTAGCACGTGT GTAGCCCTGGTCGTA AGGGCCATGATGACT TGACGTCGTCCCTGC CTTCCTCCACTTTATC AATG GCAGTCTCCTTTGAG TTCCCGACCTGACCG CTGGCAACAAAGGAT AAGGGTTGCGCTCGT TGCGGGACTTAACCC AACAT TTCACAACACGAGCT GACGACAGCCATGCA GCACCTGTCTCACAG TTCCCGAAGGCACTC TCGCATCTCTGCAAG ATTCT GTGGATGTCAAGACC AGGTAAGGTTCTTCG CGTTGCATCGAATTA AACCACATGCTCCAC CGCTTGTGCGGGCCC CCGTC AATTCATTTGAGTTTT AACCTTGCGGCCGTA CTCCCCAGGCGGTCG ATTTAACGCGTTAGC TCCGGAACCCACAGC TCAA GACCGCAAATTCCAA ATCGACATCGTTTAC AGCGTGGACTACCAG GGTATCTAATCCTGT TTGCTCCCCACGCTT TCGCA TCTCAGCGTCAGTAT CTGTCCAGAAGGCCG CCTTCGCCACCGGTA TTCCTCCACATCTCT ACGCATTTCACCGCT ACACG TGNAATTCTACCTTN NNTCTACAATACTCC AGACGACCAGTTTTA AATGC

A3. Material Transfer Agreement

Number: A18-0133 - Fully Executed - Material Transfer Agreement
Principal Investigator: Sadd, Benjamin
Department: Biological Science
ISU Sponsor: National Institute of Health
ISU Project Number: A18-0133
ISU Project Name: Host and microbe factors influencing composition and functioning of an accessible gut microbiota model
Secondary Agreement Party: Gerardo, Nicole M. - Emory University
Emory Sponsor: National Institute of Food and Agriculture
Emory Project Number: NIFA # 13372589
Emory Project Name: Assessing the impact of field-relevant agricultural antibiotics on bee microbiome and host behavior
Agreement Dates: 02/01/2022 – 02/01/2023
Agreement Type: Material Transfer Agreement

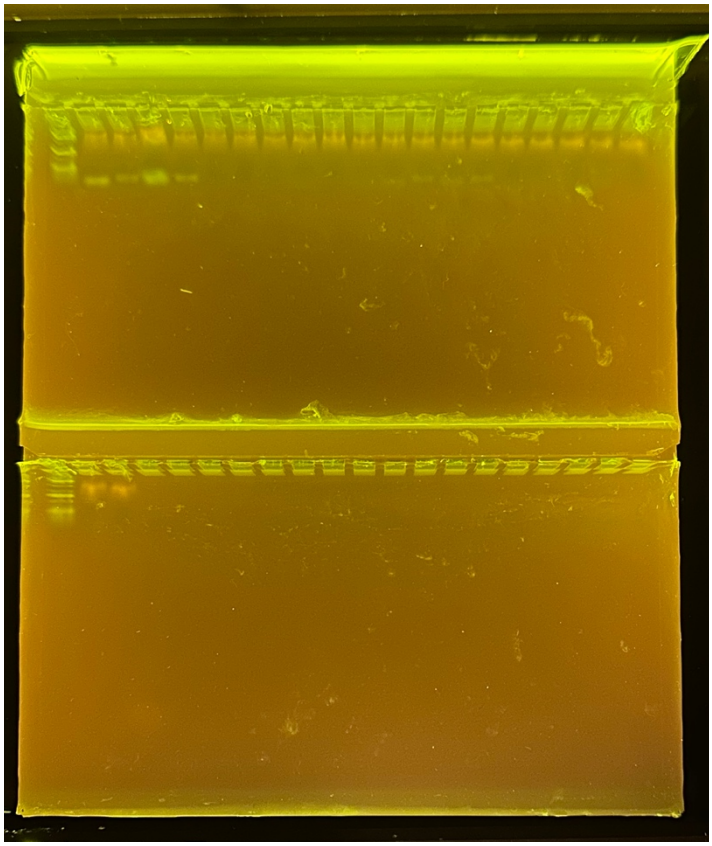

Figure A1. Gel visualization of passaged *Gilliamella* strain. Strain passaged to 18 ppm (from 5th passage of biofilm plate as indicated in columns 1-4 on gel) and 27 ppm (from 6th passage of shaking plate as indicated in columns 11-14 on gel). *Gilliamella* strains were amplified with strain specific primers (Table A1)

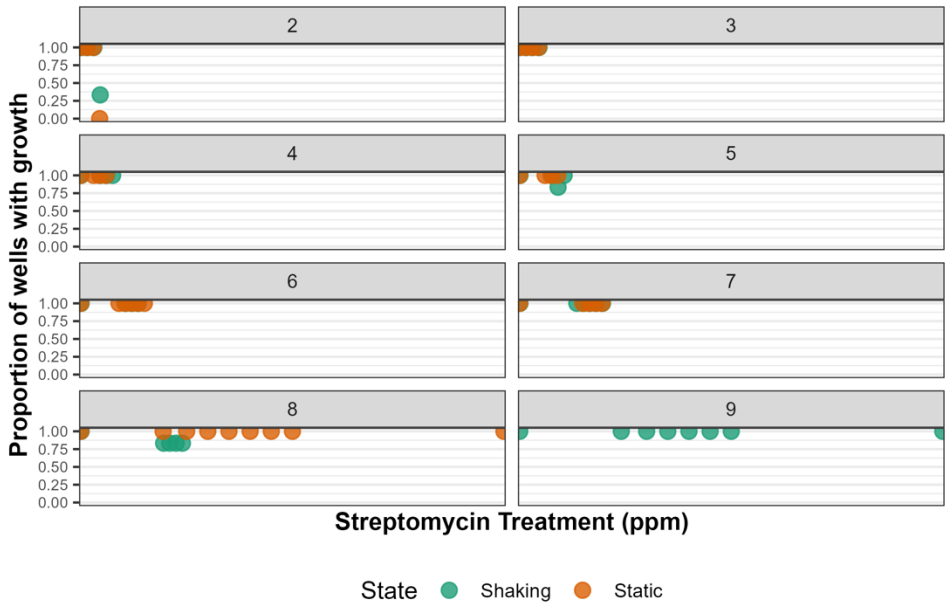

Figure A2. Proportion of *Gilliamella* samples with growth during second passage experiment. Proportion of wells with growth (OD600 > negative control samples OD600) for the strains passaged from 0 to 200 ppm streptomycin through (nine passages for shaking treatment, eight for static treatment). Labelled facets show data segregated passage. Strains were passaged in 3 ppm streptomycin up to 39 ppm (static plate) or 48 ppm (shaking plate), and in increments of 10 ppm afterwards up to 100 ppm. There are six replicates for each concentration at each passage per plate. There are three readings from three consecutive days for passage nine of the shaking plate and passage eight of the static plate, as there was not sufficient growth observed in the first two days.

Table A3. Analysis of deviance for the proportion of wells with growth during the sequential passaging estimated with a beta-regression. Data for all passages, ranging from 0 to 200 ppm of streptomycin.

Variables	**Chi-Square**	**DF**	***P***
State	0.0189	1	0.8906
Streptomycin treatment	0.1666	1	0.6831
Passage	1.4418	1	0.2298
State x Streptomycin	0.1084	1	0.7419

Table A4. Linear Model for the proportion of wells with growth during the sequential passaging at 3 ppm increments. Data for all passages, ranging from 0 to 200 ppm of streptomycin.

Variables	**log Odds**	**Standard Error**	**z-value**	***P***
Intercept	-0.127898	0.2044250	-0.626	0.532
State (Static)	0.010706	0.1517216	0.071	0.944
Streptomycin	-0.000249	0.0018526	-0.134	0.893
Passage	0.016168	0.0313833	0.515	0.606

State (Static) x Streptomycin	0.000142	0.0022727	0.062	0.950

Table A5. Linear Model for the OD600 growth of evolved strains. Data for passage 7, 8 & 9, for a total of n=168 wells, ranging from 27 to 200 ppm of streptomycin

Variables	**log (OD600)**	**Standard Error**	**t-value**	***P***
Intercept	-0.0738149	0.2651713	-0.278	0.781
State (Static)	-0.4975713	0.0936073	-5.316	**<0.00001**
Streptomycin	-0.0077087	0.0009461	-8.148	**<0.00001**
Passage	-0.0079389	0.0197668	-0.402	0.688
State (Static) x Streptomycin	0.0013023	0.0012058	1.080	0.282
R2/R2 adjusted	0.53/0.542			

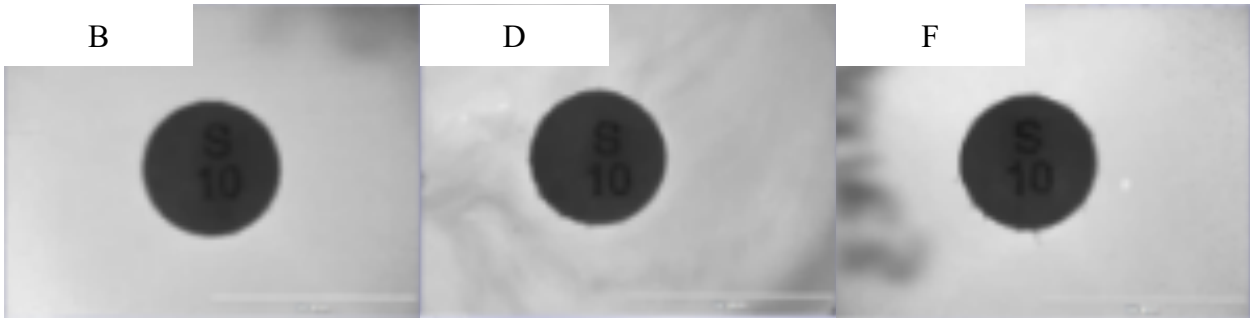

Figure A3. *Gilliamella* passaged in 0 ppm with a 1 in 100 dilution with 10 mg streptomycin disc.

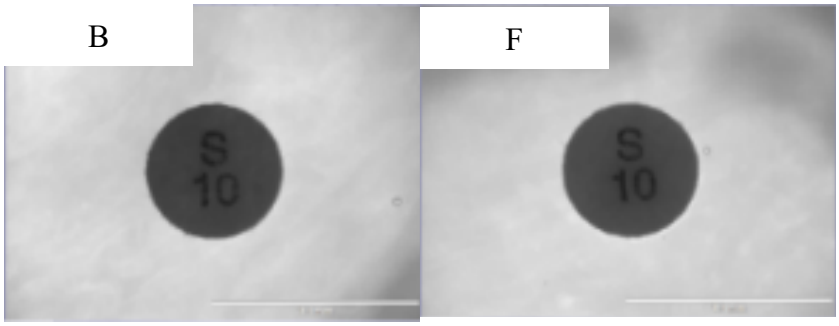

Figure A4. *Gilliamella* passaged in 0 ppm with a 10 in 100 dilution with 10 mg streptomycin disc.

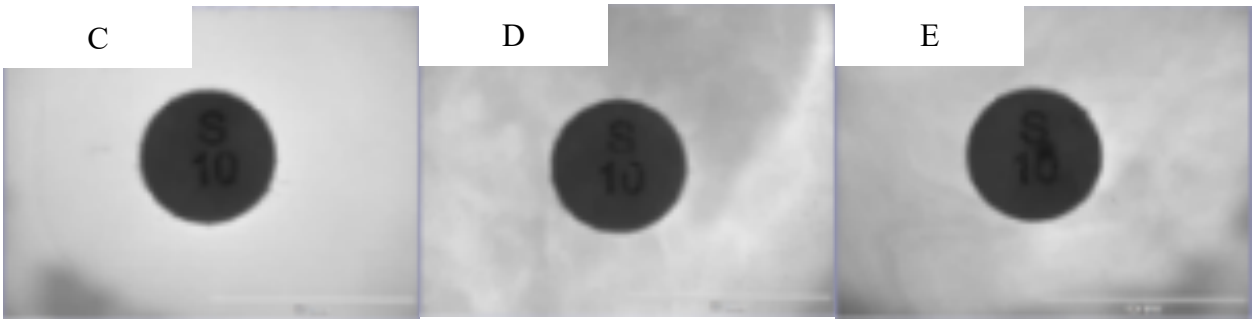

Figure A5. *Gilliamella* passaged in 50 ppm with a 10 in 100 dilution with 10 mg streptomycin disc

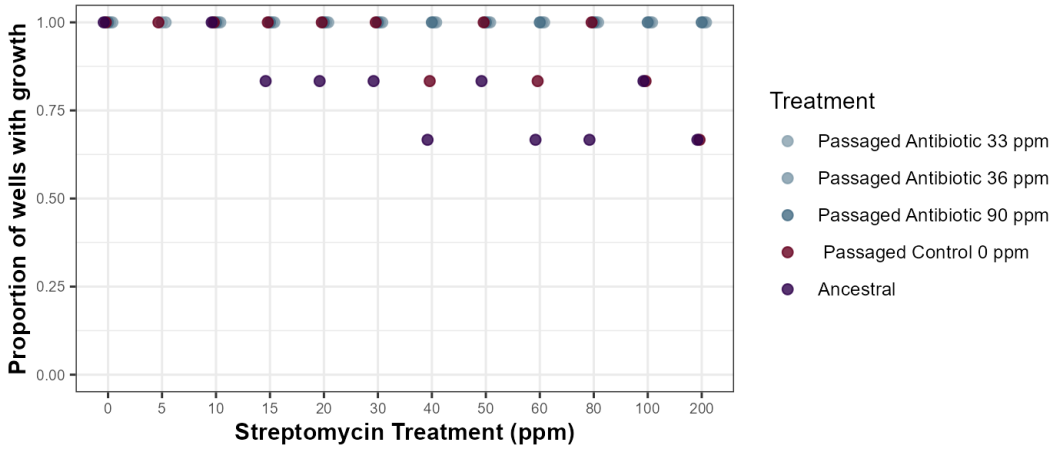

Figure A6. Proportion of wells with growth for the MIC of passaged strains.