MICROBIOME COMPARISON POST-INOCULATION WITH PHYTOPATHOGENIC XANTHOMONAS AND ANTIBIOTIC-PRODUCING ISOLATES

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ABSTRACT

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Xanthomonas oryzae pv oryzae (Xoo) and *Xanthomonas oryzae pv oryzicola* (*Xoc*) are notable phytopathogens that cause tremendous harm to the rice industry by causing bacterial leaf blight and leaf streak in rice, respectively. We aimed to investigate the impact of *Xoo* and *Xoc* on soil microbiomes by utilizing safe relatives inoculated into soil. We further investigated the effects of *Xanthomonas*-inhibiting, antibiotic-producing isolates inoculated into the soil microbiome in isolation and the presumed post-*Xanthomona*s inoculation.

Microcosms were designed containing garden soil, which was inoculated with designated bacteria, and samples were taken for DNA extraction every week; the V4 region of the 16s rRNA was utilized for microbiome analysis. The *Xanthomonas* did not establish in the soil, and antibiotic-producing inoculations were not found to alter the microbiome alpha or beta diversity. The addition of the antibiotic-producing isolates was also found not to significantly to alter the microbiome. However, significant differences were found in the beta diversity when antibiotic-producing isolates were introduced post-*Xanthomonas*.

The soil microbiome is known to impact the course of disease development in plants and act as a defense against the establishment of pathogens. Due to the high competition for resources within soil microbiomes, pathogen establishment can be deterred by previous soil occupants. When soil is found to resist invasion with pathogens, it can be considered disease suppressive.

KEYWORDS: Xanthomonas; Phytopathogens; Rice; Soil microbiome; Inhibition

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CHAPTER I

Introduction

Soil harbors a multitude of unique niches containing diverse microbiomes. A microbiome is the collection of microorganisms within a given environment. A single gram of soil may have 10^9–10^{10} prokaryotic cells (bacteria and archaea), 10^4–10^7 protists, ~100 m of fungal hyphae, and 10^8–10^9 viruses (Tecon & Or, 2017; Xun et al., 2021). Soil is diverse and heterogeneous, with many variables affecting it and the resultant microbiome. However, it has fundamental qualities, such as the arrangement of particles of varying sizes with pathways allowing for gas exchange and cell distribution. Water diffusion is a significant determinant of microbial life and processes, which runs in space and time. Soil's large degree of spatial and temporal heterogeneity makes for packets of niches hosting a myriad of microbial adaptations (Bakker et al., 2012; Tecon & Or, 2017).

Soil microbes drive critical biogeochemical cycles, including carbon, nitrogen, phosphorous, iron, and more. Thus, they contribute to many of the soil's ecosystem functions, making microbes and their activity crucial for the health and fertility of the soil. Plant cover, animal activity, wetness, fertilizer, pH, and salinity affect soil and shape the microbial community (Tecon & Or, 2017; Xun et al., 2021).

The rhizosphere, a zone encompassing the soil directly in contact with roots and their exudates, has an increased microbial abundance and interactions with both microbes and plants, making it one of the most unique and complex ecosystems on earth (Mendes et al., 2013). Plant-microbiome-soil interactions assist in nutrient uptake for the plant and act against abiotic and biotic stressors. The rhizosphere is of utmost importance to

agriculture across the globe. The composition and relative abundance of the rhizosphere microbiome is either plant species-specific (plant-dominated) or soil-specific (soil dominated). The microbiome assembly is governed by abiotic (soil properties and climate) or biotic (plant species and immunity) factors combined with biotic interactions (Xun et al., 2021).

Many times, the rhizosphere microorganisms' diversity can influence plant biomass with respect to composition and productivity. Often the health of the soil and plants in the soil is a direct indicator of the rhizosphere community; this is particularly true when one considers that the microbiome of the rhizosphere can directly influence the nutrient uptake of the plants (Mendes et al., 2013).

Health and Stability

In healthy ecosystems, there are nutrient cycles, energy flow, and resilience to disturbances or stress. In a healthy ecosystem, there is a sense of stability, where disturbances cause minor fluctuations that eventually restore themselves. Populations will fluctuate in an oscillatory manner before returning to relative stability. Resiliency is inversely related to the time it takes for a population to restore itself. Ecosystem health is defined as resilience combined with the ability to manage pathogen levels in biological communities. (van Bruggen et al., 2019)

Stability is often a property of microbiomes, which encounter many natural disturbances. A microbiome responds to stressors by varying the composition or dormancy of specific population sectors. Principally species diversity is considered an essential aspect of microbiome stability, with a strong positive correlation between the two (Xun et al., 2021).

The microbial diversity and their interspecific interactions play a prominent role in the plants' invasion of pathogen disease development. Often the diversity of the microbiome of the rhizosphere suppresses the pathogens. Even introduced pathogens can be temporary and limited in healthy soil, which comprises a high level of diversity. A greater microbial diversity of microbiota provides a greater chance of antagonists, resource competitors, and pathogen predators (van Bruggen et al., 2019).

Soil is either conducive (favorable) for the pathogen and its respective disease or is disease suppressive. Suppressive soil does not allow the disease to establish or persist. Two types of suppressiveness have been identified, general and specialized. General suppression is associated with competition for resources, while specialized suppression involves interference with the pathogen lifecycle at some stage. General suppressiveness is known to be transferable from soils, while specialized suppressiveness is not and is specific to the soil type from which it originates (Gómez Expósito et al., 2017; Weller et al., 2002). There is a range of mechanisms that microbes use to promote plant growth and protect plants from pathogens. Their strategies include biofertilization, root growth stimulation, rhizoremediation, abiotic stress control, and disease control. The rhizosphere microbiome typically consists of several members who can directly antagonize plant pathogens prior to and during infection. Antibiosis, trace element competition, parasitism, quorum sensing interference, and induction of systemic defense are all strategies employed by microbes to antagonize pathogens (Mendes et al., 2013; Ray et al., 2020). Even introduced human enteric pathogens can be suppressed when introduced into microbially diverse soils (Mallon et al., 2018).

Importance for Agriculture

Plants provide more than 80 percent of the world's food supply and all the food for livestock. Plant diseases can be devastating and result in food shortages and major economic losses. It is estimated that plant diseases cause an estimated 40 billion dollars in losses each year, with around 20- 40 percent of the losses resulting from pathogen infections (Roberts, n.d.; Savary et al., 2012). Global losses of staple crops yielded have been as high as 30 percent, resulting in billions of dollars lost. Stable agriculture and the resultant crops are the foundation of modern society (Rizzo et al., 2021).

Xanthomonas

Xanthomonas is a genus of phytopathogens capable of causing disease in over 400 species of plants, causing devastation worldwide to a slew of essential crops. This gram-negative bacterial genus comprises over 35 gammaproteobacteria, including many pathovars and subspecies. Commonly infected plant species are often necessary food and cash crops, such as rice, wheat, bananas, beans, and citrus, that provide a large proportion of the world's population with food (Timilsina et al., 2020).

Xanthomonas oryzae pv oryzae (*Xoo*) is a devastating pathovar causing rice leaf blight. It became prevalent in Japan in the late 1800s and spread to other rice-growing countries. Bacterial leaf blight can reduce rice yields by 50% (Yang et al., 2018). Blight symptoms are displayed on the leaves after entering hydathodes or wounds and traveling through the xylem while multiplying. For *Xoo* to effectively cause disease, it must form a biofilm. It is an important virulence factor that allows sustainment on leaves and inside plants (Syed Ab Rahman et al., 2018).

Xanthomonas oryzae pv oryzicola (*Xoc*) leads to bacterial leaf streak in rice. *Xoc* enters through stomata or wounds and then utilizes the sub-stomatal cavity for multiplication (Cao et al., 2020). *Xoo* and *Xoc* are prevalent in Asia, Africa, and Australia. *Xoo* has been reported in some USA states and other countries in the Americas. For both pathovars, the primary host is *Oryza sativa* and Poaceae weeds (Jeger et al., 2018). Traditional control methods involve inorganic copper compounds and antibiotics, which have caused substantial pollution and contributed to human health issues (Vishakha et al., 2022).

Xoo and *Xoc* are important phytopathogens that are becoming more problematic worldwide as they spread, causing economic losses to the rice industry worldwide. The bacterium can survive in soil, seeds, and crop remains and interacts with insects. While standard transmission involves contaminated seeds, there is the possibility that agricultural practices, such not cleaning equipment, can spread phytopathogens (Marin et al., 2019). *Xanthomonas* infections have continually been cropping up around the world.

Biocontrol

An emerging trend is attempting biocontrol with biological organisms. Biocontrol allows for the sustainment and growth of beneficial microorganisms while eliminating detrimental ones. In soil, microorganisms produce antibiotics or antimicrobial compounds and naturally tailor population dynamics. While concentrated antibiotics inserted into the ground negatively alter diversity, niche structure, and total microbial mass. The reasoning for using microbes with antibiotic capabilities instead of antibiotics themselves is to prevent detriment to the environment and avoid manipulating the microbiome negatively (Cycoń et al., 2019; Syed Ab Rahman et al., 2018).

In the rhizosphere, beneficial microbes work with plants to prime their induced systemic resistance against pathogens(Pieterse et al., 2014). Microbes are also beneficial because they assist in nutrient acquisition and protection from abiotic and biotic stressors.

University Past Work

Sam Houston State University has been screening Texas soil samples for antibiotic-producing bacteria with the capability to inhibit surrogate relatives of the two *Xanthomonas* pathovars *in vitro*. We have found several antibiotic-producing isolates that can act as potential biocontrol agents for the *Xanthomonas* phytopathogens. To find the agents, over 500 soil isolates were screened against the pathovars/strains of *Xanthomonas* used in this research. Out of those isolates, many were found that inhibit at least one pathovar/strain, and several inhibited more than one. Inhibition was considered to occur if, during screening, a zone of inhibition was exhibited.

For the *in vivo* portion, we will use naturally occurring soil isolates to investigate *Xanthomonas* suppression in a natural soil mixture. Our research is promising because utilizing bacteria instead of traditional control methods, such as chemicals or heavy metals, is less detrimental to the environment. The antibiotic-producing isolates obtained in past research were used in this study to examine their effects in vivo.

Hypothesis

The central hypothesis of this proposal is that the *Xanthomonas* phytopathogens can be suppressed in a natural soil mixture using naturally acquired antibiotic-producing soil isolates. Our objective is to determine the impact of inoculation of *Xanthomonas* in natural soil on the microbiome; to be followed by an investigation of how antibiotic-producing bacteria impact the phytopathogens and the soil microbiome. Our long-term

goal is to understand better how to inhibit *Xoo* and *Xoc* using natural soil-derived bacteria to eliminate disruption to the natural ecosystem while preventing the devastation of crops.

Specific Aim 1: Determine the impact of adding phytopathogenic *Xanthomonas* to the natural soil mixture. Our working hypothesis is that insertion of foreign phytopathogenic *Xanthomonas* into the natural soil will result in changes in species richness and diversity within the microbiome which will prevent the invading species from becoming dominant or utilizing too many of the limited resources. The rationale is that the natural soil microbiomes avoid invasion by shifting the composition, or evenness and diversity of the microbiome?, to create an unfavorable dynamic for the invader. Microbiome avoidance of invasion is exemplified by the failure of the pathogen *Escherichia coli* to invade bacterial communities, as they were able to shift the carbon utilization away from the pathogen's niche (Mallon et al., 2018).

This aim's objective is to characterize changes to the natural soil microbiome upon inoculation of pathogenic *Xanthomonas* to the soil mixture. The working hypothesis is that the phytopathogenic *Xanthomonas* will shift the microbiome species richness and diversity, either allowing the *Xanthomonas* to become established or preventing pathogen invasion. The null hypothesis is the phytopathogenic *Xanthomonas* will not alter the microbiome in either species richness or diversity. The rationale for hypothesis one is that the microbiomes that have resisted invasion in vitro have done so by altering the composition and the resultant niche structure. If the bacterial communities do not shift adequately, then there will likely be an exponential growth of the phytopathogenic

Xanthomonas, with the potential for them to become the dominant species present (De Roy et al., 2013; Mallon et al., 2018; Tracanna et al., n.d.).

Specific Aim 2: Determine the impact of antibiotic-producing isolates' inoculation in natural soil and soil invaded with a *Xanthomonas* phytopathogen. Our working hypothesis is that the insertion of the antibiotic-producing soil isolates will influence the microbiome diversity and influence the population levels. The null hypothesis is that the insertion of antibiotic-producing soil isolates will not influence microbiome diversity or population levels. Our hypothesis is founded on the rationale that the isolates detained from the soil could inhibit the growth of the phytopathogenic *Xanthomonas* under lab conditions (Cycoń et al., 2019).

The objective is to investigate how adding antibiotic-producing isolates from the soil will impact the natural soil microbiome post-inoculation with *Xanthomonas*. The working hypothesis is that there will be an alteration of the natural soil microbiome after adding the antibiotic-producing isolates. The bacteria will likely affect the *Xanthomonas* population through the production of antibiotics, which would effectively alter the composition of the biome. The rationale behind this is that upon interacting *in vitro*, the antibiotic-producing isolates inhibited *Xanthomonas* growth; therefore, *in vivo*, they should also influence population levels.

CHAPTER II

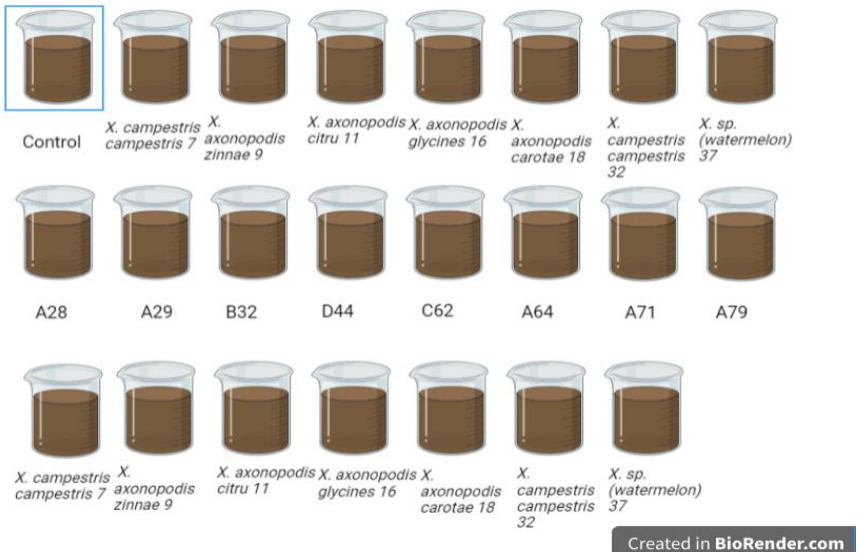
Methods

Microcosm Establishment

The soil was collected from a garden at coordinates 30° 54' 35.2" N and 95° 19' 57.5" W on 9 October 2021. The soil was then sifted and sieved through five-millimeter mesh to remove organic material and homogenize the soil mixture. Next, 300 grams of soil was weighed and placed into each of 23 autoclaved 600-milliliter beakers and loosely covered with sterile tin foil to allow for oxygen exchange (Figure 1); this created microcosms to analyze the microbiome. For this portion of the aim, eight microcosms were used, one designated as control and seven to receive a *Xanthomonas* strain. Every month, 20 mL of sterile DI water was added to the soil to maintain adequate moisture where the soil was damp but not soaking wet; this was determined using a mock microcosm.

Figure 1

The Layout of Microcosm Containers



Note. The control container is outlined in blue.

Inoculation

On day 1, microcosms (two through eight) were inoculated, receiving the following pathovars/strains: *Xanthomonas campestris campestris 7, Xanthomonas axonopodis zinnae 9, Xanthomonas axonopodis citru 11, Xanthomonas axonopodis carotae 18, Xanthomonas axonopodis glycines 16, Xanthomonas campestris campestris 32, Xanthomonas sp (watermelon) 37*. One container was designated as a control and received sterile DI water.

Additionally, antibiotic-producing bacteria were inoculated into the microcosms (nine through sixteen): *Pseudomonas USDA D44 286.3411 286.3411 (D44), Pseudomonas USDA B32 286.3393 286.3393 (B32), Pseudomonas USDA Sample A79 286.3388 286.3388 (A79), Bacillus USDA A28 1386.1892 1386.1892 (A28), Pseudomonas USDA A29 286.3410 286.3410 (A29), Pseudomonas USDA C62 286.3396 286.3396 (C62), Burkholderia USDA A64 32008.710 32008.710 (A64) and, Burkholderia USDA 32008.711(A71).*

Lastly, containers (seventeen through twenty-three) were inoculated with the following: *Xanthomonas campestris campestris 7, Xanthomonas axonopodis zinnae 9, Xanthomonas axonopodis citru 11, Xanthomonas axonopodis carotae, 18, Xanthomonas axonopodis glycines 16, Xanthomonas campestris campestris 32, Xanthomonas sp (watermelon) 37*. Then on day 22 (3 weeks post-inoculation), the antibiotic-producing strain, D44, was introduced into the soil microcosms. This will allow for examination of Xanthomonas levels post introduction of a susceptible antibiotic producer.

For inoculation, bacteria were grown on selective media; a colony was selected and mixed into sterile DI water until it was diluted to a .5 MacFarland Standard,

indicating approximately 1.5 x 108 CFU/mL. Lastly, two mL of the bacteria solution was poured into the soil microcosms, which were then mixed using a sterile spatula. The same procedure was used to inoculate with the D44 on 8 December 2021.

DNA Extraction

Starting on 16 November 2021, the total soil microbiome DNA was extracted according to the DNeasy PowerSoil Pro Kit (Qiagen) manufacturer's instructions. Samples were collected for a total of 10 weeks, every seven days, from each of the containers. The microcosms were adequately mixed then 0.25 grams of soil was selected for extraction. Following extraction, the extracted DNA samples were quantified on the NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer. Absorbance was measured at A260/280 and A260/230 to measure contamination and protein concentration.

Subsequently, samples were sent off for PCR and sequencing to Colorado State University. For the initial step, PCR, the 515F/806R primers were used. The primers are universal and garner the broadest range of bacterial DNA for the 16S SSU rRNA Variable 4 region. The amplified products were then prepared for sequencing with the addition of barcodes and adaptors. The prepared samples were then sequenced using Illumina MiSeq yielding paired-end reads.

Data Processing

All data was processed in QIIME2 2021.8. Sequenced reads were imported into QIIME2, yielding 11,580,939 demultiplexed sequences, which were then assessed for quality. Phred scores of the sequenced reads were determined, and the scores were plotted using a random sampling of 10000 out of 11,580,939 sequences without

replacement. In Divisive Amplicon Denoising Algorithm 2 (DADA2), reads were trimmed ten base pairs from the left and truncated to 234 base pairs to keep the quality scores above the 30 thresholds. The reads were then de-replicated and denoised using the default parameters (18, 19). Feature table and feature data summaries based on ASV (Amplicon Sequence Variants) were then generated with mitochondria and chloroplast removed using QIIME2 feature-table plugin. This also allowed for the separation of the containers of interest and their respective reads.

Taxonomic references were generated in QIIME2 using a rescript package, which allowed for the alignment of the representative sequences to the SILVA 138-99 SSU database (Liu et al., 2021). A rarefaction curve was created using a sampling depth of 25,894 for downstream analysis. For alpha diversity values of Peilou's evenness, Shannon's Diversity Index, Faith Phylogenetic Diversity, and the Observed Features were calculated between the control container and the Xanthomonas containers with a sampling depth of 25894. Then Kruskal-Wallis statistics were performed on the computed alpha diversity values for containers compared to the control, yielding a p-value with a value of less than 0.05 was considered to be significant. Beta diversity was compared between controls and Xanthomonas: Jaccard, Bray-Curtis, Weighted Unifrac, and Unweighted Unifrac. Those metrics allowed for a permutational multivariate analysis of variance (PERMANOVA) to be performed for each metric that utilized 999 permutations, and a value of less than 0.05 is significant.

For alpha and beta diversity calculation purposes, each container is compared to the control container; then containers are again compared by inoculation status. For Xanthomonas and antibiotic-producing containers, the comparison was between control

samples (none for inoculations) and inoculated samples (one for inoculations). For the Xanthomonas that were also inoculated with D44, the comparisons were made by container, comparing the inoculated container to the uninoculated one. Then to further analyze inoculation status, the *Xanthomonas*-only containers were included to allow for more samples for comparison. These samples were compared by zero inoculation for the control (none), one inoculation (the Xanthomonas), and two inoculations (the D44).

CHAPTER III

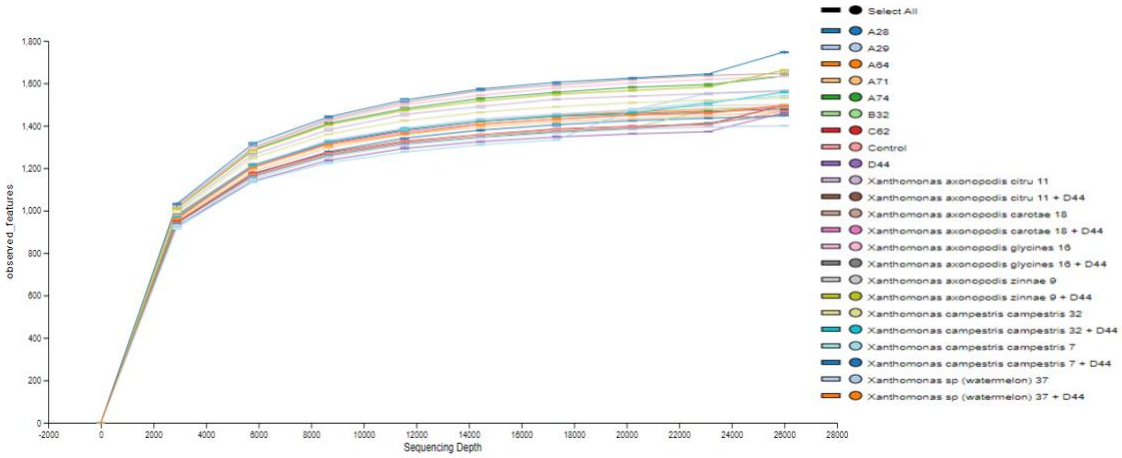
Results

Sequencing Results

After sequencing, 11580939 forward and reverse reads were obtained, with 39,239 ASV found and a total frequency of 9,413,826, with the mean frequency per sample being 40,929. Samples were rarefied to a sampling depth of 25894 ASV (Figure 2), which retained 57.21% of features in 90.43% of samples. Taxonomy was assigned to the ASV, and 77 phyla were found in the samples, with the predominant phyla as follows: Proteobacteria (21.3% to 29.6%); Firmicutes (17.3% to 36.2%); Acidobateriota (9.5% to 16%) comprising approximately over half of the sample composition. Sequences that were not able to be classified into any known group were Unassigned.

Figure 2

Alpha Rarefaction Curve of all the Containers



Note. The plateau indicates sufficient sampling depth for downstream processing.

Xanthomonas Only

Seven *Xanthomonas* pathovars/strains were inoculated into seven individual microcosms, and the microbiome was analyzed for the following ten weeks. *Xanthomonas* was found only in two inoculated containers, *X. campestris campestris 7* and *X. axonopodis carotae 18*, on week 2, with a frequency of 8 and 6, meaning the ASV associated with the *Xanthomonas* was only detected 8 and 6 times respectively in the two containers at any given time and was not found to persist in the soil. There was not any statistical difference in alpha or beta diversity when examined by container or inoculation status for all Xanthomonas containers ($p > .126$); there was one container, *X. axonopodis glycines 16*, when examined by inoculation status in Pielou's evenness that did approach significance ($p = .086$).

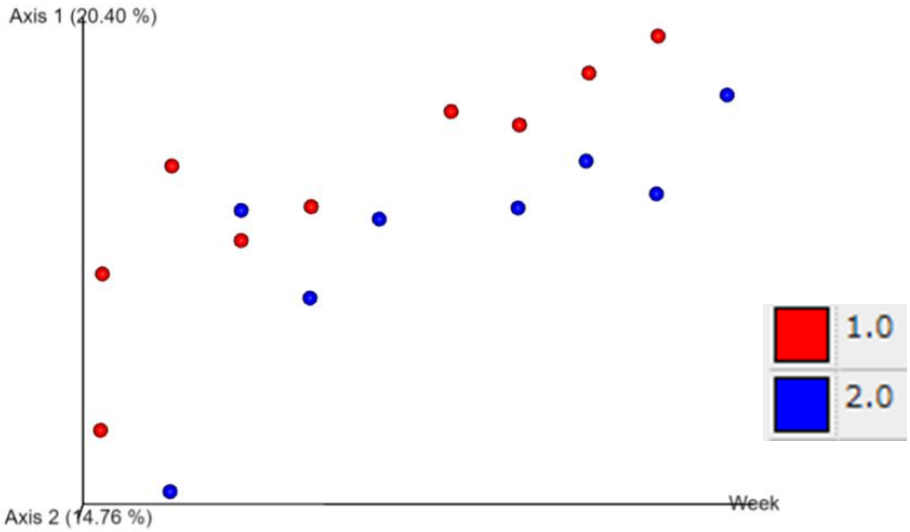
Antibiotic-Producers

There was no statistical difference in the alpha diversity when compared using all the metrics, with the lowest p-value being 0.12. Only one beta diversity metric, Weighted Unifrac, yielded significant results for strain A74 when compared by control to the inoculated container ($p=.021$) and when examined by inoculation status ($p=.026$) (Figure 3).

Weighted Unifrac, which factors in the relationship of the bacteria within the samples and weighs the sum of their branch lengths (Chang et al., 2011). The difference in community structure was, therefore, due to the difference in the familial relationship of the resident bacteria post-inoculation, as this diversity metric is sensitive to abundant taxa. All other antibiotic-producing strains inoculated into the soil did not cause significant changes in the microbiome.

Figure 3

Pcoa Of Weighted Unifrac for Antibiotic-Producing Isolate A74



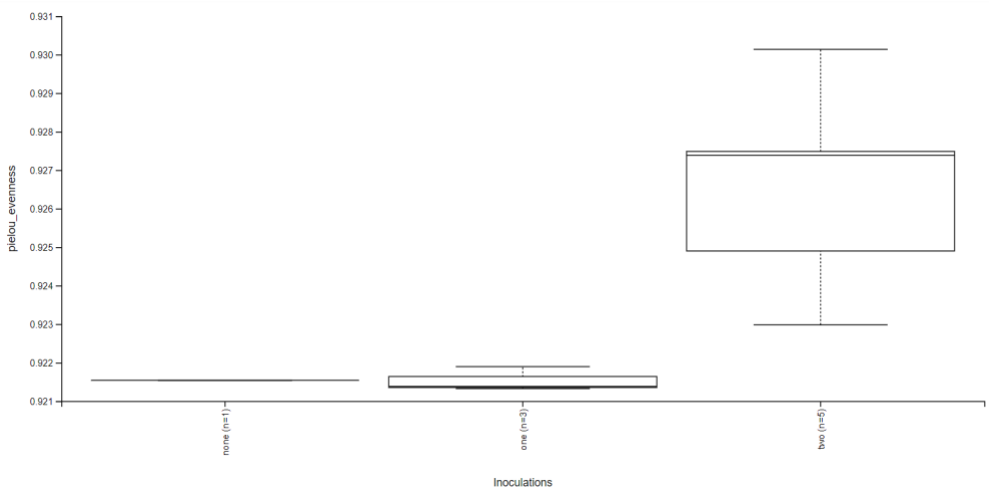
Note. Beta Diversity of control and A74 by inoculation status.

Antibiotic Producers Post-Xanthomonas

Alpha diversity was only found to be significant in *Xanthomonas* containers inoculated with D44 for one aspect in the container with *X. axonopodis carotae 18*. The one aspect showing significance was Pielou's evenness (Figure 4) between treatment one (*Xanthomonas* inoculation) and treatment two (D44) (p=.027). All other aspects of alpha diversity on the four metrics were insignificant (p > .08).

Figure 4

Boxplot Of Pielou's Evenness for X. Axonopodis Carotae 18 with D44 by Inoculation Status



Note. None is the control with zero inoculations; one inoculation is the *Xanthomonas,* while two is the D44.

Beta diversity, when examined by container, was also not significantly altered; there were multiple significant differences when examined by the inoculation status. There were no significant changes between the control (no inoculations), but there were significant differences in multiple containers between the one inoculation (*Xanthomonas*) and two inoculations (antibiotic producer D44) and again between no inoculation and two inoculations (D44) (Table 1).

The beta diversity was significantly different for several of the *Xanthomonas* and D44 treatments. For *Xanthomonas campestris campestris 7* with D44, all four beta diversity metrics yielded significant differences between inoculation one and two, along with none and two inoculations (Figures 5-8). This means the antibiotic producer D44 changed diversity when compared to multiple metrics that examined community structure

and membership. With Jaccard and Bray Curtis, the differences were non-phylogenetic, while the UniFrac metrics were both phylogenetic.

Xanthomonas axonopodis glycines 16 with D44 was only significantly different on two metrics, Bray Curtis and Weighted UniFrac, between inoculation one and two, along with none and two inoculations (Figures 9-10). Both metrics use abundance to analyze community structure, suggesting that D44 altered the abundance of the present bacteria to yield significant differences.

All four beta diversity metrics yielded significant differences between inoculation one and two, along with none and two inoculations for *Xanthomonas axonopodis carotae 18* with D44 (Figures 11-14). These metrics examined community structure and membership both phylogenetically and non-phylogenetically, meaning the community was altered by the addition of the D44. Lastly, for *Xanthomonas campestris campestris 32* with D44 (Figure 15), the only beta diversity metric that was significantly different was the weighted UniFrac, which relies on abundance and relates to the community structure on a phylogenetic measure.

Table 1

Beta Diversity by Inoculation Status for Xanthomonas with D44

Container	Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
X. campestris campestris 7 with D44	none	one	19	0.905041	0.923	0.923
Bray Curtis		two	15	1.383824	0.006	0.018
	one	two	14	1.339482	0.02	0.03
X. campestris campestris 7 with D44	none	one	19	0.950606	0.924	0.924
Jaccard		two	15	1.129592	0.02	0.06
	one	two	14	1.120195	0.04	0.06
X. campestris campestris 7 with D44	none	one	19	0.991281	0.496	0.496

(continued)

Container	Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
Unweighted Uni Frac		two	15	1.208922	0.015	0.036
	one	two	14	1.165828	0.024	0.036
X. campestris campestris 7 with D44	none	one	19	0.863382	0.743	0.743
Weighted Uni Frac		two	15	2.084739	0.008	0.012
	one	two	14	1.924842	0.006	0.012
X.axonopodis zinnae 9 with D44	none	one	20	0.914322	0.884	0.884
Bray Curtis		two	16	1.169023	0.062	0.186
	one	two	16	1.091417	0.165	0.2475
X. axonopodis zinnae 9 with D44	none	one	20	1.013227	0.317	0.317
Jaccard		two	16	1.063409	0.1	0.3
	one	two	16	1.036091	0.207	0.3105
X. axonopodis zinnae 9 with D44	none	one	20	0.969671	0.672	0.672
Unweighted Uni Frac		two	16	1.059439	0.203	0.609
	one	two	16	0.998289	0.466	0.672
X. axonopodis zinnae 9 with D44	none	one	20	0.769422	0.903	0.903
Weighted Uni Frac		two	16	1.743883	0.036	0.108
	one	two	16	1.256733	0.129	0.1935
X. axonopodis citru 11 with D44	one	two	18	1.087677	0.151	0.2325
Bray Curtis		zero	22	0.939611	0.747	0.747
	two	zero	16	1.108284	0.155	0.2325
X. axonopodis citru 11 with D44	one	two	18	1.021771	0.25	0.45
Jaccard		zero	22	0.959904	0.864	0.864
	two	zero	16	1.019025	0.3	0.45
X. axonopodis citru 11 with D44	one	two	18	1.043028	0.249	0.5415
Unweighted Uni Frac		zero	22	0.96332	0.683	0.683
	two	zero	16	1.014735	0.361	0.5415
X. axonopodis citru 11 with D44	one	two	18	1.267755	0.155	0.2325
Weighted Uni Frac		zero	22	1.051865	0.343	0.343
	two	zero	16	1.465505	0.071	0.213
X. axonopodis glycines 16 with D44	none	one	22	0.876779	0.971	0.971
Bray Curtis		two	14	1.178426	0.056	0.084
	one	two	16	1.209725	0.047	0.084
X. axonopodis glycines 16 with D44	none	one	22	0.934131	0.969	0.969
Jaccard		two	14	1.055848	0.089	0.267
	one	two	16	1.019953	0.302	0.453

(continued)

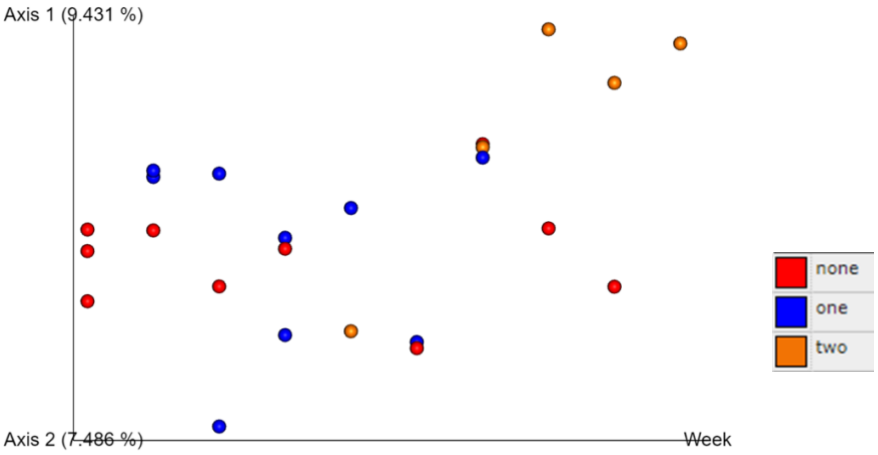
Container	Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
X. axonopodis glycines 16 with D44				0.943506	0.814	0.814
Unweighted Uni Frac		none	one	22	0.122	0.3135
	one	two	16	1.05002	0.209	0.3135
X. axonopodis glycines 16 with D44	none	one	22	0.865264	0.651	0.651
Weighted Uni Frac		two	14	1.667152	0.035	0.0525
	one	two	16	1.971182	0.017	0.051
X. axonopodis carotae 18 with D44	none	one	20	0.987681	0.519	0.519
Bray Curtis		two	14	1.439876	0.002	0.0045
	one	two	16	1.337448	0.003	0.0045
X. axonopodis carotae 18 with D44	none	one	20	0.969507	0.809	0.809
Jaccard		two	14	1.162746	0.003	0.009
	one	two	16	1.11624	0.018	0.027
X. axonopodis carotae 18 with D44	none	one	20	1.008954	0.383	0.383
Unweighted Uni Frac		two	14	1.261101	0.003	0.009
	one	two	16	1.19323	0.017	0.0255
X. Axonopodis carotae 18 with D44	none	one	20	1.053793	0.33	0.33
Weighted Uni Frac		two	14	2.662743	0.003	0.0045
	one	two	16	2.057837	0.003	0.0045
X. campestris campestris 32 with D44	none	one	22	0.898599	0.934	0.934
Bray Curtis		two	14	1.10358	0.124	0.228
	one	two	16	1.081156	0.152	0.228
X. campestris campestris 32 with D44	none	one	22	1.021515	0.266	0.328
Jaccard		two	14	1.013278	0.328	0.328
	one	two	16	1.041546	0.187	0.328
X. campestris campestris 32 with D44	none	one	22	1.03866	0.27	0.27
Unweighted Uni Frac		two	14	1.111364	0.07	0.21
	one	two	16	1.082786	0.161	0.2415
X. campestris campestris 32 with D44	none	one	22	0.736575	0.93	0.93
Weighted Uni Frac		two	14	1.590349	0.042	0.072
	one	two	16	1.512232	0.048	0.072
X. sp (watermelon) 37 With D44	none none	one	22	0.960387	0.673	0.673
Bray Curtis	one	two	14	1.098223	0.12	0.2655
	one	two	16	1.080642	0.177	0.2655
X. sp (watermelon) 37 with D44	none none	one	22	1.002968	0.442	0.442
Jaccard	one	two	14	1.003546	0.419	0.442

(continued)

Container	Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
	one	two	16	1.025841	0.214	0.442
X. Sp (watermelon) 37 with D44 Unweighted Uni Frac	none	one	22	0.937337	0.824	0.824
	none one	two	14	1.04604	0.229	0.3915
	one	two	16	1.036765	0.261	0.3915
X. sp (watermelon) 37 With D44 Weighted Uni Frac	none	one	22	0.897265	0.627	0.627
	none one	two	14	1.239845	0.157	0.2355
	one	two	16	1.26557	0.126	0.2355

Figure 5

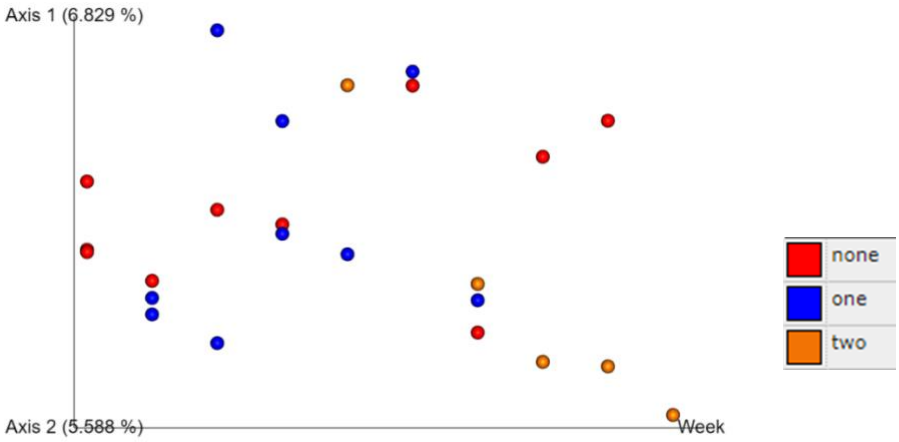
PCoA of Bray Curtis Diversity for X. Campestris Campestris 7 with D44



Note. Bray Curtis Diversity for *Xanthomonas campestris campestris 7* with D44 by inoculation status. Inoculations none compared to one yielded a p-value of .923; none compared to two inoculations yielded a p-value of .006, and one compared to two yielded a p-value of .02.

Figure 6

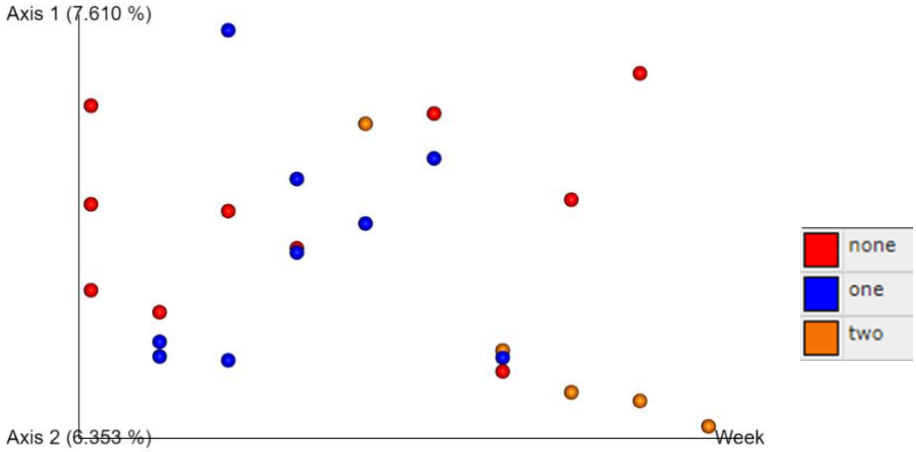
PCoA of Jaccard Diversity for X. Campestris Campestris 7 with D44



Note. Jaccard Diversity for *Xanthomonas campestris campestris 7* with D44 by inoculation status. Inoculations none compared to one yielded a p-value of .924; none compared to two inoculations yielded a p-value of .02, and one compared to two yielded a p-value of .04.

Figure 7

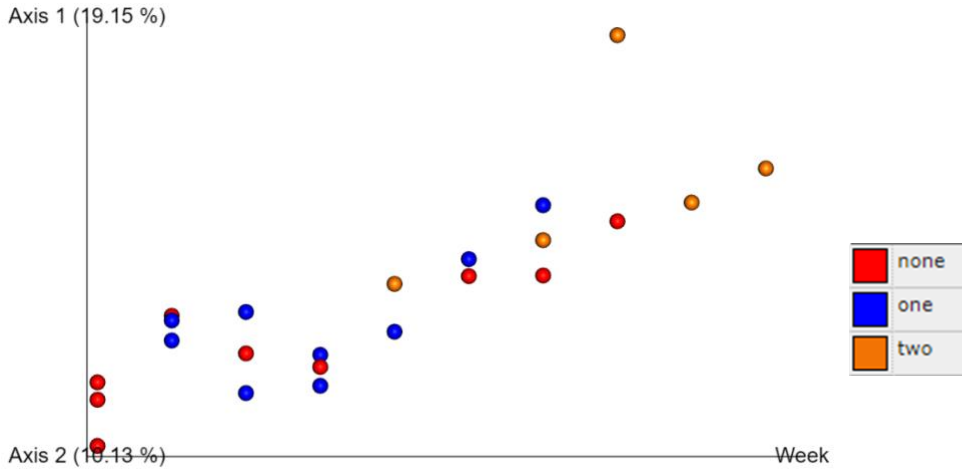
PCoA of Unweighted UniFrac Diversity for X. Campestris Campestris 7 with D44



Note. Unweighted Unifrac Diversity for *Xanthomonas campestris campestris 7* with D44 by inoculation status. Inoculations none compared to one yielded a p-value of .496; none compared to two inoculations yielded a p-value of .015, and one compared to two yielded a p-value of .024.

Figure 8

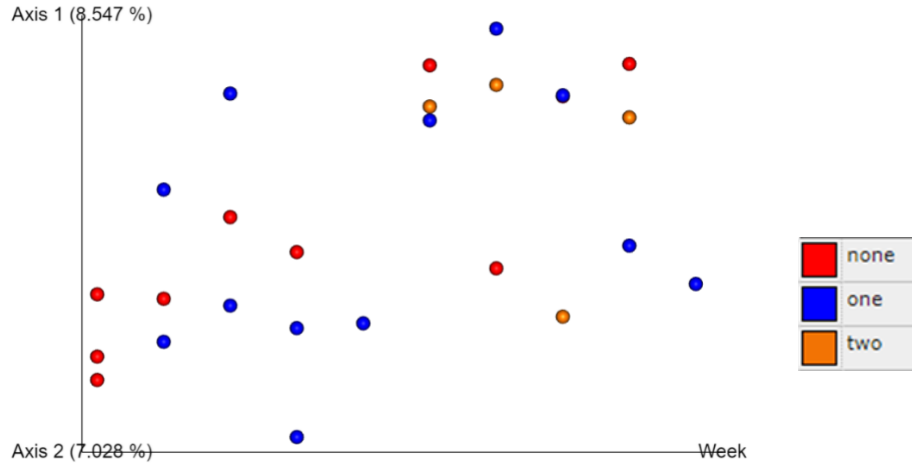
PCoA Weighted UniFrac Diversity for X. Campestris Campestris 7 with D44



Note. Weighted Unifrac Diversity for *Xanthomonas campestris campestris 7* with D44 by inoculation status. Inoculations none compared to one yielded a p-value of .743; none compared to two inoculations yielded a p-value of .008, and one compared to two yielded a p-value of .006.

Figure 9

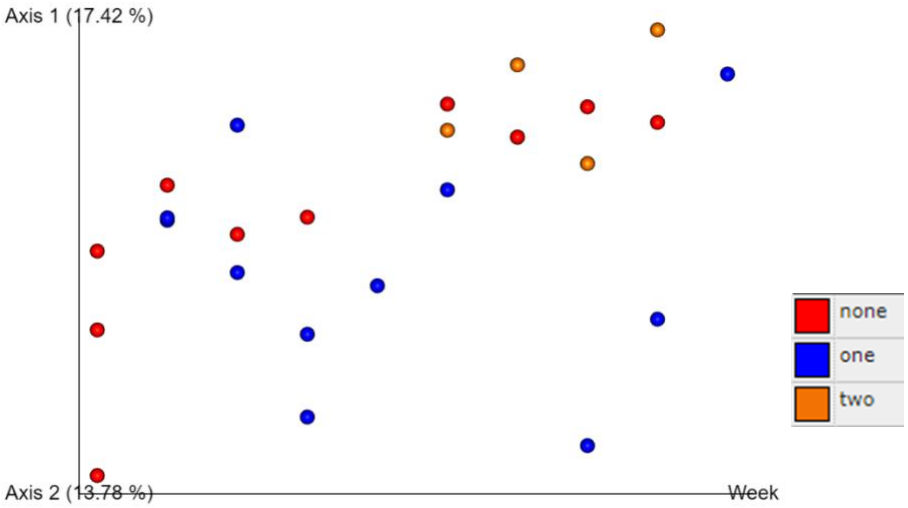
PCoA of Bray Curtis Diversity for X. Axonopodis Glycines 16 with D44



Note. Bray Curtis Diversity for *Xanthomonas axonopodis glycines 16* with D44 by inoculations. Inoculations none compared to one yielded a p-value of .971; none compared to two inoculations yielded a p-value of .056, and one compared to two yielded a p-value of .047.

Figure 10

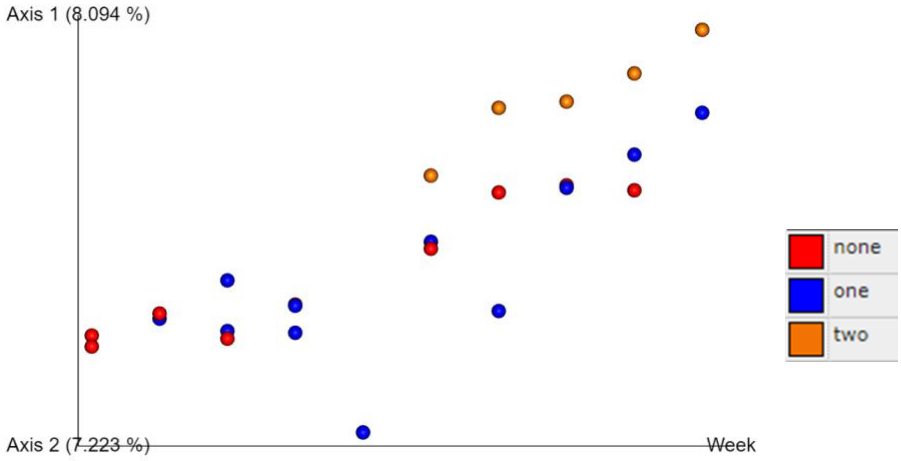
PCoA of Weighted UniFrac Diversity for X. Axonopodis Glycines 16 with D44



Note. Weighted Unifrac Diversity for *Xanthomonas axonopodis glycines 16* with D44 by inoculations. Inoculations none compared to one yielded a p-value of .651; none compared to two inoculations yielded a p-value of .035, and one compared to two yielded a p-value of .017.

Figure 11

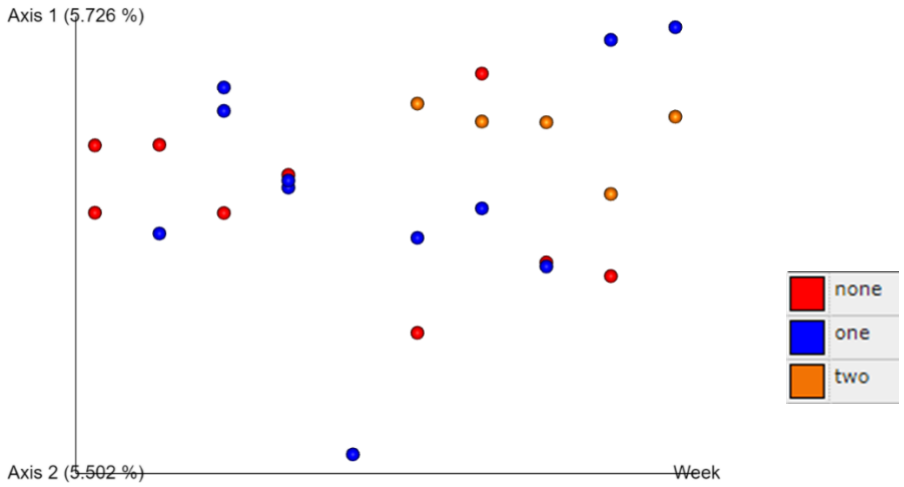
PCoA of Bray Curtis Diversity for X. Axonopodis Carotae 18 with D44



Note. Bray Curtis Diversity for *Xanthomonas axonopodis carotae 18* with D44 by inoculation status. Inoculations none compared to one yielded a p-value of .519; none compared to two inoculations yielded a p-value of .002, and one compared to two yielded a p-value of .003.

Figure 12

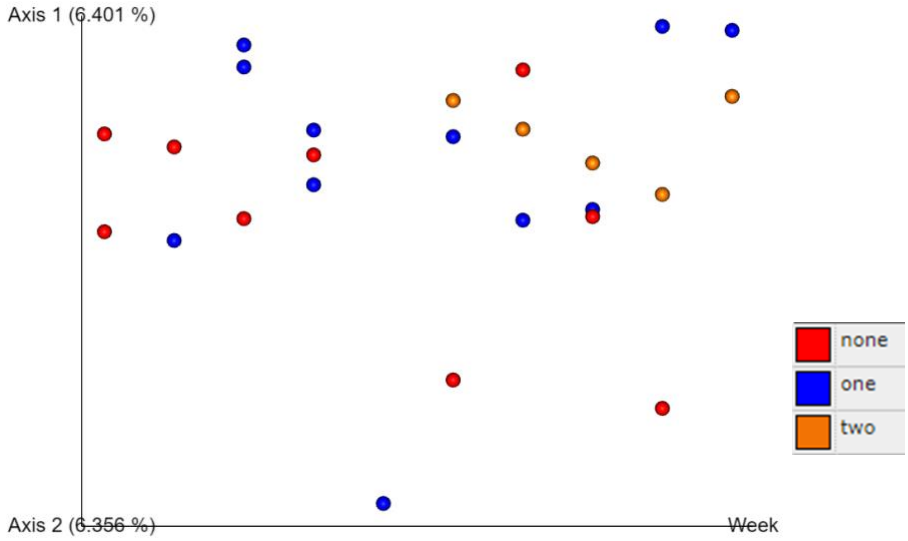
PCoA of Jaccard Diversity for X. Axonopodis Carotae 18 with D44



Note. Jaccard Diversity for *Xanthomonas axonopodis carotae 18* with D44 by inoculation status. Inoculations none compared to one yielded a p-value of .809; none compared to two inoculations yielded a p-value of .003, and one compared to two yielded a p-value of .018.

Figure 13

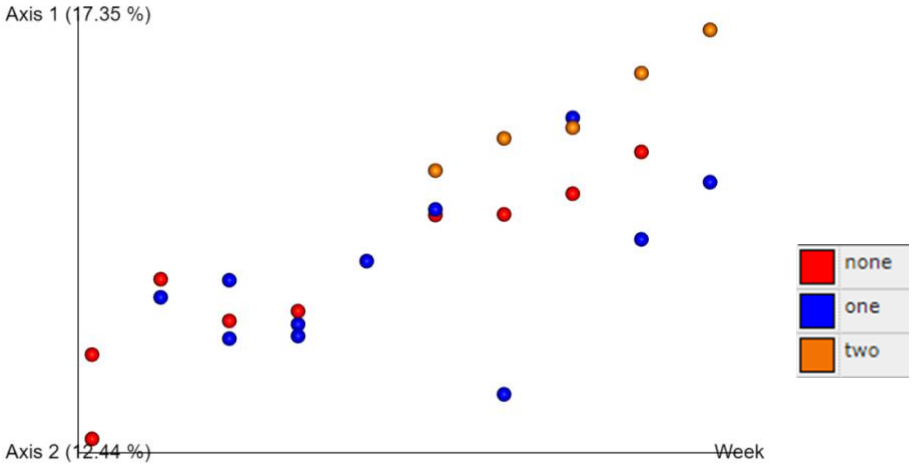
PCoA of Unweighted UniFrac Diversity for X. Axonopodis Carotae 18 with D44



Note. Unifrac Diversity for *Xanthomonas axonopodis carotae 18* with D44 by inoculation status. Inoculations none compared to one yielded a p-value of .383; none compared to two inoculations yielded a p-value of .003, and one compared to two yielded a p-value of .017.

Figure 14

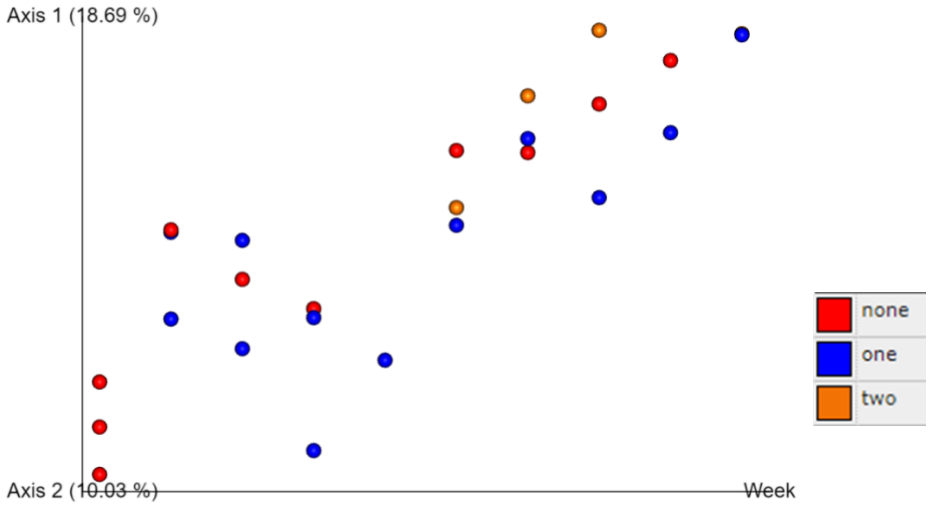
PCoA of Weighted UniFrac Diversity for X. Axonopodis Carotae 18 with D44



Note. Weighted Unifrac Diversity for *Xanthomonas axonopodis carotae 18* with D44 by inoculation status. Inoculations none compared to one yielded a p-value of .33; none compared to two inoculations yielded a p-value of .003 and one compared to two yielded a p-value of .003.

Figure 15

PCoA Weighted UniFrac Diversity for X. Campestris Campestris 32 with D44



Note. Weighted Unifrac for *Xanthomonas campestris campestris 32* with D44 by inoculation status. Inoculations none compared to one yielded a p-value of .93; none compared to two inoculations yielded a p-value of .042, and one compared to two yielded a p-value of .048.

CHAPTER IV

Discussion

Soil health plays a large role in a plant's ability to grow, produce, and resist disease. Alteration of the microbiome can play a role in a plant's ability to uptake nutrients. Traditionally microbes have frequently been used to promote growth in plants and assist in acquiring nutrients. When microbes assist in plant growth promotion, that also improves resilience against both biotic and abiotic stressors. Microbes can also be beneficial in acting as biocontrol agents by reducing or preventing the deleterious effects of phytopathogens (Ray et al., 2020).

The overall goal of this research was to determine if antibiotic producers who previously had shown inhibition to the *Xanthomonas* pathovars/strains would reduce the level of *Xanthomonas* in the soil and how they would impact microbiome composition. We also wanted to examine how the antibiotic-producing strains would impact the microbiome. The purpose was to find a way to inhibit *Xanthomonas* in soil without negatively affecting the microbiome to find an inoculum for agricultural purposes that is safe to use and will not be detrimental to the environment.

Xanthomonas In the Soil

The *Xanthomonas* were only detected in very small amounts with DNA sequencing in the containers with only *Xanthomonas*. It only showed up in trace amounts immediately after inoculation and was not further detected; this likely means it did not persist in the soil in which it was inserted. This is also corroborated by the fact that the microbiome diversity was not significantly altered when examined by container vs. control or when examined by inoculation status.

A possible reason the *Xanthomonas* may not have persisted is initial is the inoculum dose; the dose may not have been in high enough concentrations to adequately disperse the *Xanthomonas* into the soil. Another reason may be that the *Xanthomonas* pathovars/strains may not be conducive to subsisting without a host plant. There are conflicting reports on whether *Xanthomonas* is capable of persisting in the soil as the genus is highly specialized for the host plants which they infect (An et al., 2020; Cao et al., 2020).

Another reason *Xanthomonas* may not have persisted in the soil is that the soil may have contained microbes that directly competed with the *Xanthomonas*, either directly via the production of antimicrobial compounds or indirectly through competition by utilizing resources that the *Xanthomonas* may have needed (Bauer et al., n.d.). The soil itself could have been disease suppressive due to the general composition of the bacteria that it contained and the qualities of the soil itself (Gómez Expósito et al., 2017; Weller et al., 2002).

Antibiotic-Producers

The containers consisting of only the antibiotic producers were only significantly different in one container for one aspect of beta diversity. While antibiotic-producers post-*Xanthomonas* were significantly different for four of the containers when examined by inoculation status, suggesting that the antibiotic-producer D44 did alter the microbiome when examined on several metrics. The D44 alteration, however needs further examination to determine if this was in a negative or positive manner for soil health.

Conclusion

We were unable to address the initial hypothesis on how Xanthomonas changes the microbiome due to the lack of establishment of the microorganisms. Therefore, we cannot say that Xanthomonas significantly altered the diversity of the microbiome. Further, due to the lack of establishment of *Xanthomonas*, we were unable to address if population levels of the bacteria were altered due to the insertion of antibiotic producer's isolates. The microbiomes of the microcosms all were similar in composition and had the same relative frequency of phyla.

To examine if the microbial community in which the *Xanthomonas* was inserted was disease suppressive functionality of the present microbes could be assessed. This could be done by seeing which genes are up and down-regulated in response to the insertion of the foreign pathogen. There is the potential that in the experiment, the soil was already disease suppressive, and this would need to be assessed in future experimentation.

Future Directions

If the hypothesis was to be tested again, prior to beginning the experiment, serial dilutions using different concentrations of the pathovar should be performed using specialized media to detect *Xanthomonas*. This would allow for the level at which *Xanthomonas* establishes in the soil to be determined; then experimentation can be performed.

Many times, when *Xanthomonas* is used in experimentation, live plants or actual field plots are utilized (Chen et al., 2020; Hop et al., 2014; Villamizar et al., 2020). The experiment could be done using live plants that are susceptible to the *Xanthomonas*

pathovar/strain. The procedures typically inoculate plants with the pathogen, then inoculate with an inhibiting bacterium. With this procedure, you can visibly measure the lesions on plants. As *Xanthomonas* pathovars/strains are unique to their host plants, it would be crucial to correctly identify the host plants to use (An et al., 2020; Timilsina et al., 2020).

Recent research has come to light that the most effective way for bacteria to successfully integrate into the soil and effectively reduce disease is for inoculum to be a mixed culture of bacteria (Ray et al., 2020). Therefore, in further research, it would be beneficial to find a mixture of bacteria that inhibit the pathogens. This could be done with a traditional screening of the *Xanthomonas* using mixed soil isolates.

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