Analysis of the Stability of Microbial Consortia Grown on Pectin

by

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Introduction

Bacteria are social organisms that interact within and between other species while simultaneously responding to external stimuli from their surrounding environment (11). Bacterial communities are among the most diverse of ecological communities in that they vary drastically in species composition, niches occupied, and influence on different environments. Understanding the development and implications of these bacterial communities is of great importance, because they not only influence our personal health, but also our surrounding environment. Determining exactly how these communities form is a complex issue that is not completely understood.

Bacteria within a community are not homogenously distributed; rather they are patterned based on their interactions with other neighboring cells. The abiotic environment also determines the patterning of bacterial growth based on the bacteria's metabolic and physiological needs (11). It is also thought that a bacteria's growth rate is shaped by natural selection in response to resource conditions over the course of the bacteria's ancestral history. This is because no bacteria encounters all environments and since carrying non-essential, or unused genes, is a burden to a cell due to extra metabolic costs, bacterial genomes become adapted for growth within particular environments based on natural selection (11). Another phenomenon that occurs within microbial communities is horizontal gene transfer (HGT), which in simple terms is the transfer of genetic information between different species. One possible benefit of horizontal gene transfer is its potential to enhance the functional diversity of microbial communities to improve their performance in changing a specific environment (3). By sharing genetic information across different species of bacteria a more cohesive environment could be

formed. Figure 1 shows the ways in which horizontal gene transfer can occur within microbial communities.

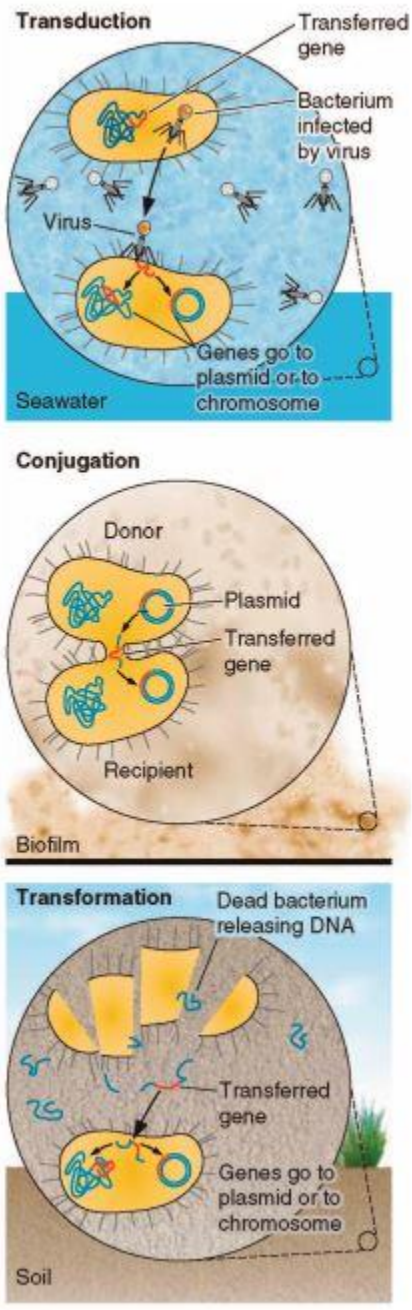

Figure 1. These are the three major mechanisms in which horizontal gene transfer can occur among microbes in a given environment. An environment where each process has been observed and documented is shown and is thought to affect microbial community fitness (3).

Another important consideration when assessing bacterial growth is that bacteria do not typically occur in axenic culture, therefore metabolic needs have been influenced by the metabolic functions of neighboring bacteria of the same and different species (11). Bacteria are very synergistic organisms, that is different species of bacteria may produce different enzymes that work together to help degrade a nutrient source or perhaps the byproduct of one bacterium is beneficial to another. This "cross feeding" that has been observed in bacteria and other organisms is termed syntrophy. However, bacteria may also compete against each other for resources. For example, when bacteria are grown in multispecies communities they often employ "high risk, high reward" strategies based on their specific metabolic adaptations to outcompete their neighbors (11). Therefore it is thought that bacteria that are better able to exploit a particular environment will perhaps produce more progeny. In other cases when multiple species of bacteria with similar metabolic adaptations inhabit the same environment, they may inhibit or kill competitors (11). That is, certain bacteria will release diffusible antagonists such as toxins or antibiotics to create an inhospitable zone for the competing bacteria (11).

The environment that will be created for this experiment to assess bacterial communities is a pectin environment. Pectin is a component of plant cell walls and functions in cell adhesion and cell wall hydration (Figure 2) (13). Pectin makes up anywhere from a quarter to a half of the dry mass of a typical plant cell wall from non-woody plant tissue (6).Pectin is commonly extracted from plant material such as citrus peel, apple pomace, mango waste, and sugar beet waste (9).

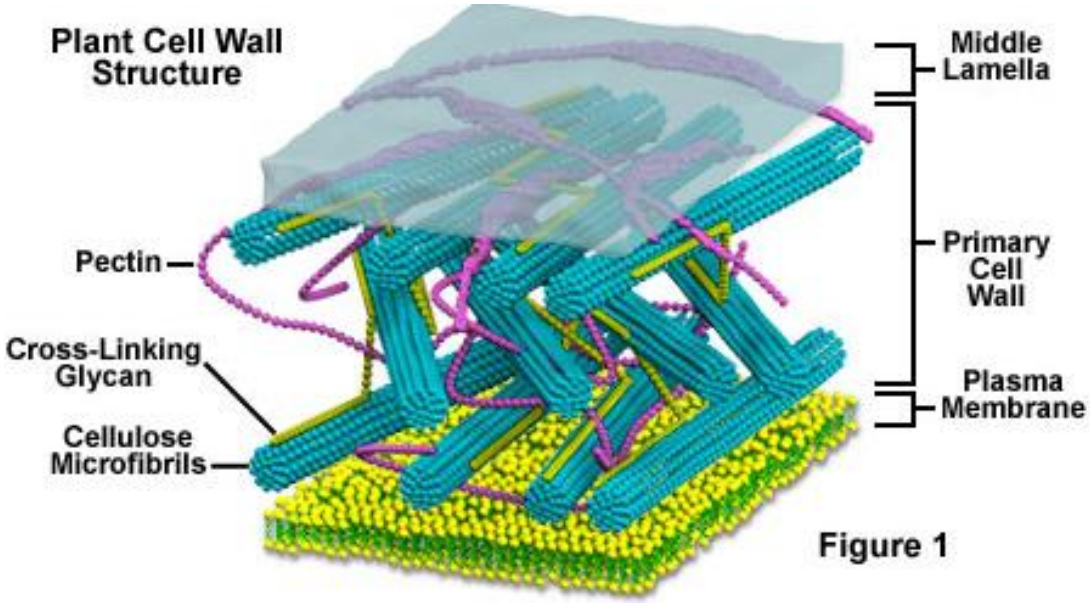

Figure 2.Plant cell wall structure along with the location of pectin within the cell wall.

Pectins are polysaccharides that are composed of four subclasses: homogalacturonan (HG), rhamnogalacturonan (RG-I), RG-II, and xylogalacturonan (XGA) (12). The backbones of HG, RG-II, and XGA consist of α-(1-4) linked galacturonic acid (GalA) residues that can be methylesterified at the C6 carboxyl group and/or acetylated at the O2 or O3. It is these galacturonic acid monomers that can be fermented into alcohols to create biofuels. The backbone of RG-1 is composed of alternating rhamnose and GalA residues. RG-II contains complex side chains that consist of at least 12 different types of sugars, whereas RG-I contains structurally diverse side chains which contains mostly arabinose and galactose as well as some other sugars. XGA is essentially the same as HG, with the exception of an added β-1,3-xylosyl side groups (13).

Soil samples were collected and placed into a pectin environment so that common soil bacteria could be assessed. Aside from assessing the total bacteria in a sample,

Firmicutes and *Actinobacteria* were assessed because they are both common bacteria found in soil. *Firmicutes* are mainly Gram-positive bacteria and can be either cocci or rod-shaped (5). Some *Firmicutes* are important pathogens (5). *Actinobacteria* are also a group of Gram-positive bacteria and play key roles in decomposition (1). All *Actinobacteria* are aerobic and a select few are pathogens (1).

The bacterial composition of each sample was assessed using a method called real-time PCR also known as quantitative PCR (qPCR). Real-time PCR refers to the fact that measurements can be made during amplification rather than at the end of PCR (12). Real-time PCR reveals not only the types of bacteria that are present, but also their relative abundances. A dye called SYBR Green will be used to detect the bacterial DNA in a sample. SYBR Green dye only binds to double-stranded DNA and when bound it exhibits fluorescence, as seen in Figure 3. When the DNA is denatured the dye no longer binds, but when the DNA is amplified such that now there is twice the DNA as there was previously, SYBR Green binds again and an increase in fluorescence can be detected. This occurs again and again until the threshold value is met, which is the point when the fluorescence is stronger than the background noise.

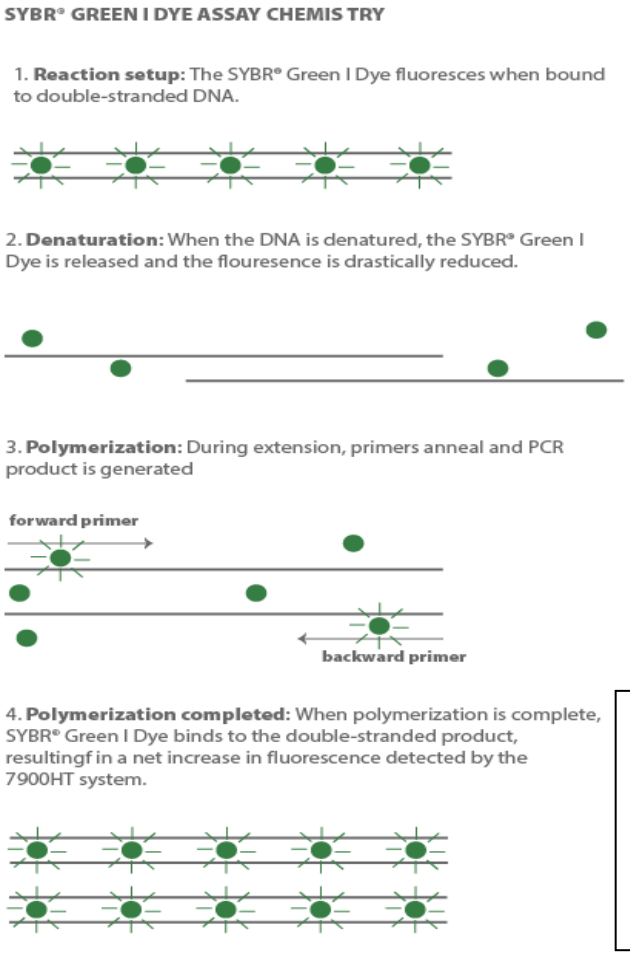

Figure 3. The process SYBR Green dye goes through to provide fluorescence for the detection of bacterial DNA in qPCR.

When using qPCR, primers must be specific to the particular types of bacteria that are to be amplified. There are also primers designed to amplify all bacteria, so that the total abundance of a particular sample can be determined. For this experiment primers will be used to amplify all bacteria, *Firmicutes*, and *Actinobacteria*. The primers were designed based on the 16S rDNA sequence, which is a section of prokaryotic DNA found in all bacteria and archaea (2). The 16S sequence is found in the small subunit (30S) of the ribosome and it binds complementary to the Shine-Dalgarno sequence, which is a consensus sequence only found in bacteria (7). This binding plays an important role in translation for prokaryotes. The 16S sequence is targeted because it is highly conserved

among prokaryotes and is also relatively short at 1.5kb, making it faster and cheaper to sequence than many other unique bacterial genes (2). The sequences of the primers used to amplify "all bacteria" were; forward primer-5'ACT CCT ACG GGA GGC AGC AG 3' and reverse primer-5'ATT ACC GCG GCT GCT GG 3'. The *Actinobacteria* primer sequences were; forward primer-5' CGC GGC CTA TCA GCT TGT TG 3' and reverse primer- 5'ATT ACC GCG GCT GCT GG 3'. The *Firmicute* primer sequences were; forward primer-GCA GTA GGG AAT CTT CCG 3' and reverse primer- 5'ATT ACC GCG GCT GCT GG 3'. All primer sequences used in this experiment were developed by Fierer et al (4).

Quantitative PCR is also a helpful tool in identifying changes in bacterial communities. Part of this experiment will focus on the stability of microbial communities once they have reached equilibrium. To assess the stability of microbial communities, each culture will be perturbed by a bacterium called *Pectobacterium chrysanthemi*, which is very well adapted to a pectin environment. *P. chrysanthemi* belongs to the family *Enterobacteriaceae. P. chrysanthemi* is a plant pathogen and since pectin is one of the main components in plant cell walls, *P. chrysanthemi* has evolved several enzymes that can efficiently degrade the molecule. Three such pectinases have been identified in this bacterium thus far: two pectin methylesterases, a polygalacturonase, and eight pectate lyases (8).

Using qPCR to assess the composition of the bacterial communities, this experiment attempts to answer two questions. First, what factors are largely responsible for determining the composition of a bacterial community and second, once a bacterial community is established how resilient is it to perturbation? There are three possible

factors controlling the establishment of bacterial communities; the environment, initial inoculum, or it could be completely chaotic. If the environment is responsible for the development of the bacterial communities, then all of the samples should look the same. For example, species found in all cultures should eventually come to similar abundances in each culture. If the initial inoculum is largely responsible for the development of bacterial communities, then each culture made from the same soil sample should look more similar to each other than cultures made from different soil samples. Lastly, if the development of bacterial communities is a chaotic process, then there should be no trends and each bacterial community should come to a different composition. It is my hypothesis that the environment is largely responsible for controlling the development of bacterial communities and once bacterial communities are formed they, should be very resilient to any type of perturbation.

Materials/Methods

Collection of Soil Samples

The soil samples were labeled A, B, and C throughout the experiment. Soil sample A was collected from a compost pile. Soil sample B was collected from underneath rotting wood. Soil sample C was collected from the bottom of a pool of stagnant water in trial, 1 but in trial 2 soil was collected from a field.

Pectin Broth

Two liters of pectin broth were made by adding 12g of Na_2HPO_4, 6g of KH_2PO_4, 1g of NaCl, and 2g of NH_4Cl in a 2 liter flask with 1 liter of water. The mixture was stirred until all salts were dissolved. In a separate 2 liter flask, 1 liter of water was heated up and stirred with a stir bar while slowly adding 20g of pectin from citrus peel. Once everything was dissolved, both flasks were put in the autoclave. Once the flasks cooled, the mixtures were added together and 1ml of 1M $MgSO_4$ and 10ml of 0.01M $CaCl_2$ were added. The mixture was stirred until everything was evenly dispersed.

Nystatin

The nystatin was brought to a concentration of 100U/ml by adding 16.86mg of nystatin to 10ml of 95% ethanol. The stock concentration was 5932U/mg. The nystatin was supplied by the Sigma company.

Creating the Environment

There were a total of 9 flasks: 3 A samples, 3 B samples, and 3 C samples. Each flask contained 20ml of pectin broth, 200ul of nystatin (to kill any fungi), and 0.1g of soil from the appropriate sample. These flasks were then placed on a shaker table and gently spun. Once the cultures matured (at about one week), they were transferred to another

sterile 9 flasks. Everything was the same except soil was not added; instead, 100ul of the old culture served as the inoculum.

Taking Absorbance's Using Spectrophotometer

To determine when the cultures reached maturity, absorbance's were taken once a day to see when they plateaued. Absorbance's were taken by transferring 1ml of culture into a cuvette and read at 600nm. The blank was pectin broth.

Creating Pellet and Soup

When each generation matured, a pellet and the "soup" were taken from each sample before the flasks were cleaned out. To create the pellet and soup, 1ml of each culture was pipetted into a 1.5ml microcentrifuge tubes. The nine tubes were centrifuged at 14,000rpm for 10 minutes. The soup was then pipetted off from each sample and put into another tube. Both the soup and the pellet were then put in the freezer.

DNA Extraction

Using the pellets collected from previous generations DNA was extracted following the Gram-positive procedure for the NORGEN BIOTEK Bacterial Genomic DNA Isolation Kit.

Adding Perturbing Bacteria

After ample time was given for the cultures to potentially reach equilibrium the perturbing bacteria *Pectobacterium chrysanthemi* was added to each of the cultures. The concentration of bacteria added was 1 million bacteria per ml. A series of 10 fold dilutions were made and the colonies were then counted for each plate. The 10^{-6} plate was used because it contained 134 colonies which was between 30 and 300 colonies. The number of bacteria per milliliter were determined by multiply 134 x 10 (because only

.1ml was added) x10^6. From here this number was divided by 1000 to get the number of bacteria per microliter, then the number of bacteria needed over the number of bacteria per microliter gave the amount of microliters of culture that needed to be added to each sample. In this case it was $\frac{19\ million}{1.34\ million}$= 14.18ul of broth to be added (19 million because 1ml was taken out of each sample for absorbance's).

qPCR Using SYBR Green

To use qPCR to assess bacterial composition a master mix was made using .25ul of forward and reverse primer (primers were at a concentration of 25nM), 7ul of molecular grade water, and 12.5 ul of SYBR Green per reaction. Then in a 96 well block, 20ul of master mix was added to each well along with 5ul of DNA. The DNA was diluted in molecular grade water 10 fold and 100 fold. Doublets of each reaction were made. The negative control was molecular grade water. Using the Quant Studio 3 Real-Time PCR system made by Thermo Fischer Scientific, the following cycling conditions were carried out.

Hold stage: Step 1. 50°C for 02:00, Step 2. 95°C for 10:00

PCR stage: Step 1. 95°C for 01:00, Step 2. 60°C for *Actinobacteria* and *Firmicutes* but 53°C for all bacteria, run for 00:30, Step 3. 72°C for 01:00.

Melt Curve stage: Step 1. 95°C for 00:15, Step 2. 60°C for 01:00, Step 3. 95°C for 00:01

Converting Ct values to Relative Abundances.

The lowest Ct value served as 1, which was assumed to be the highest abundance the bacteria could be found at in the pectin environment. Then all other Ct values had the lowest Ct value subtracted from it. Then .5 was raised to the value found by subtracting the Ct values. This gave all Ct values an abundance relative to the highest abundance.

Graphing Relative Abundances

Using Microsoft Excel, a line graph was used to show the relative abundances of each bacteria type in each sample over the generations it was cultured. The cultures from the same soil sample were coded with the same color line in the graph.

Results

The qPCR graph depicted in Figure 4 shows the amplification of both *Actinobacteria* and *Firmicutes*. The horizontal lines running across the graph depict the threshold value for the samples. When the DNA for each bacteria has been amplified enough to cross the threshold value this gives the Ct value, which is the intersection between the two lines. The Ct value is the number of cycles a particular sample had to go through to reach the threshold value. As seen in Figure 4, *Actinobacteria* reaches the threshold value at a much lower Ct value than does *Firmicutes*. Each difference in the Ct value is a twofold event such that there is twice as much bacteria in a sample that has a Ct value one less.

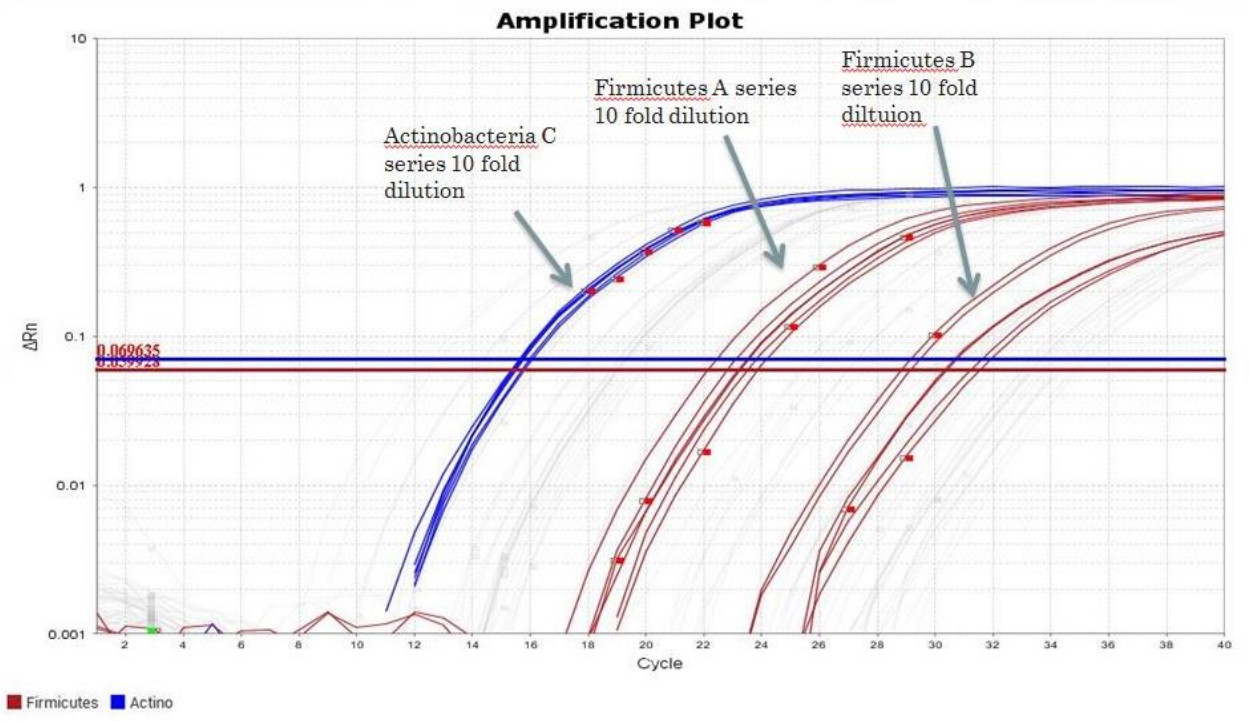

Figure 4.qPCR graph. From left to right: C series *Actinobacteria* 10 fold dilution, A series *Firmicutes* 10 fold dilution, and B series *Firmicutes* 10 fold dilution, all from generation 19.

Figure 5 shows DNA on a 1% agarose gel. This is proof that the DNA was successfully extracted from the pellets collected from each generation. This DNA was then used in the qPCR to assess the composition of each sample for each generation.

Figure 5. Extracted DNA ran on a 1% agarose gel. Lanes 1 through 3 shows DNA successfully extracted from generation 4.

Figure 6 shows the relative abundances for all bacteria for all nine samples across generations 3,7,11,16,17,18, and 19. The lines are color coded such that the red lines refer to the A samples, the blue lines refer to the B samples, and the yellow lines refer to the C samples. The line running vertical across the samples represents the time at which the perturbing bacteria, *Pectobacterium chrysanthemi*, was added. In Figure 6 it can be seen that there is a lot of variability between cultures of the same series, no real trends seem to be present. In Figure 7, primers specific for *Firmicutes* were used to amplify only *Firmicute* DNA. Again, all nine samples were run for each of the generations mentioned previously and then graphed. The *Firmicutes* Graph shows a lot less variability between

cultures of the same series. In Figure 8, *Actinobacteria* primers were used such that only *Actinobacteria* DNA was amplified. Again there are some similarities that can be seen among cultures of the same series.

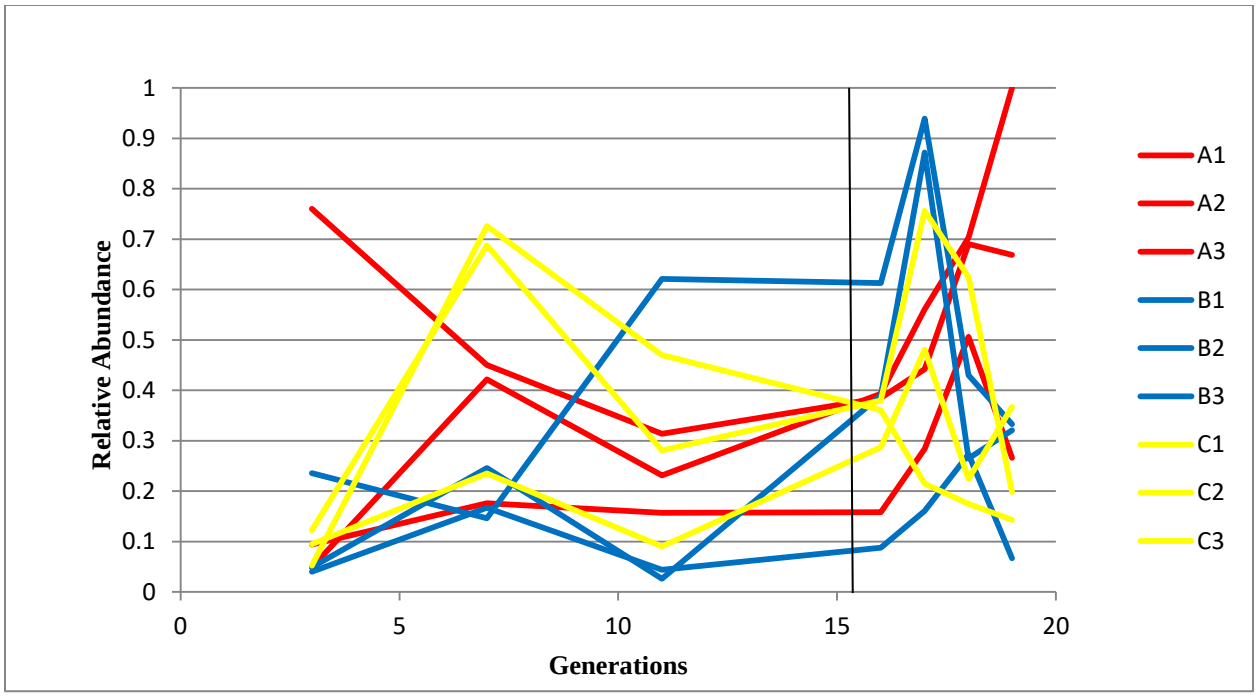

Figure 6. All bacteria abundances for all 9 samples relative to the highest all bacteria abundance across generations 3,7,11,16,17,18, and 19. The vertical line represents when *P. chrysanthemi* was added.

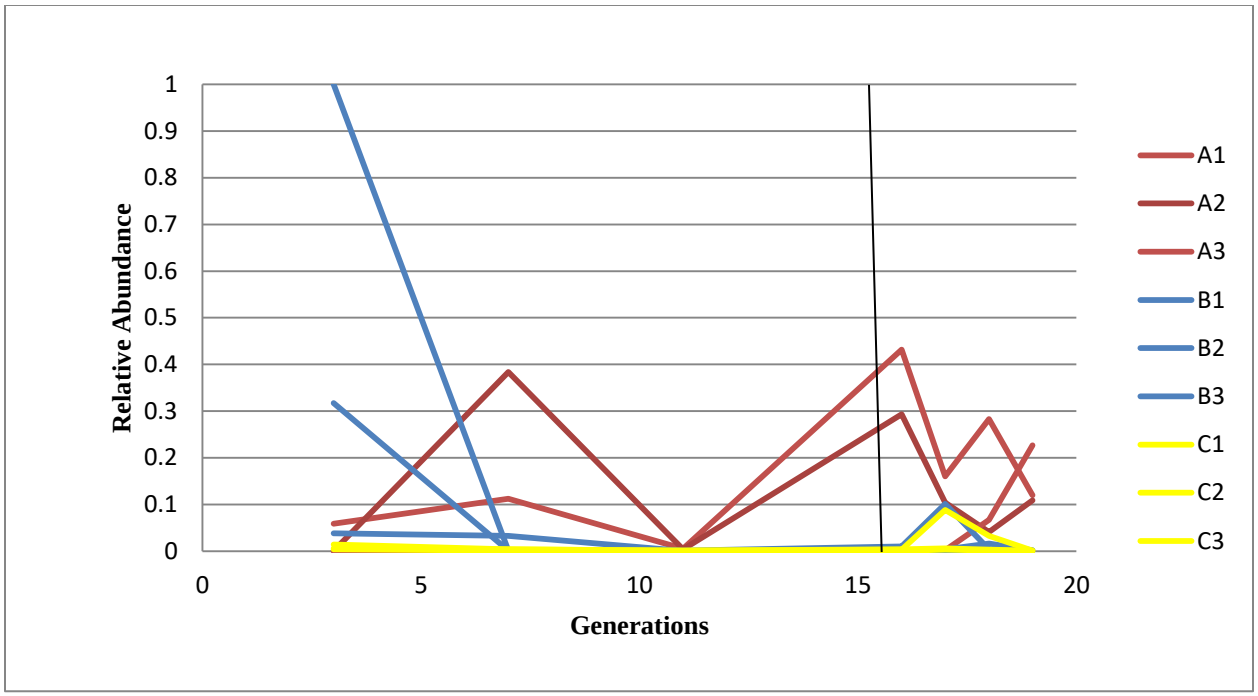

Figure 7. *Firmicutes* abundances for all 9 samples relative to the highest *Firmicutes* abundance across generations 3,7,11,16,17,18, and 19. The vertical line represents when *P. chrysanthemi* was added.

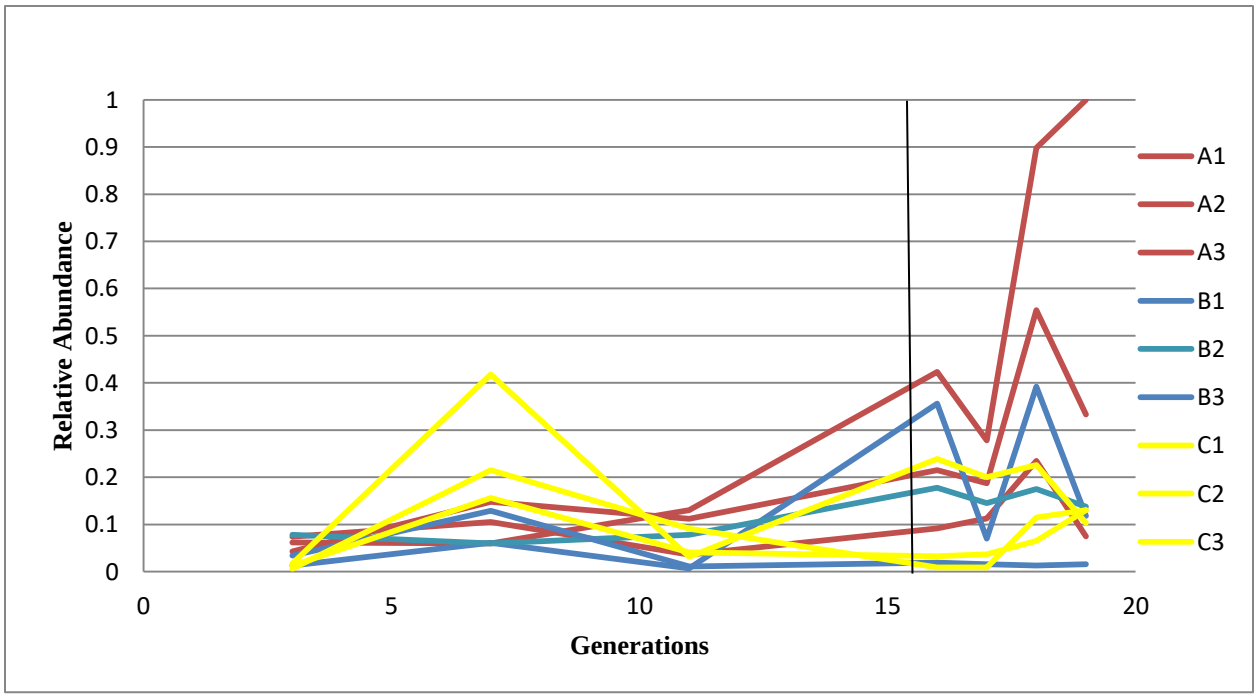

Figure 8. *Actinobacteria* abundances for all 9 samples relative to the highest *Actinobacteria* abundance across generations 3,7,11,16,17,18, and 19. The vertical line represents when *P. chrysanthemi* was added.

In Figure 9, 10, and 11, DNA extracted from trial 2 was used to create the graphs. Soil samples A and B were the same as in the first trial, but soil sample C was taken from a field as opposed from under water. In trial 2, there was only a total of six generations, whereas in trial 1 there was a total of 19 generations. Again the line running vertical through the nine samples represents the point at which *Pectobacterium chrysanthemi* was added. Each of the graphs shows the relative abundance of bacteria in relation to a specific generation. In Figure 9 there are more distinctive trends for the all bacteria than in trial 1. Cultures in the C series seem to have the most bacteria while cultures in the A series seem to have the least. In Figure 10 there are very similar trends among the *Firmicutes* as compared to the results of Figure 7 in trial 1. In Figure 11 it can be seen that cultures in the A series seem to have the most *Actinobacteria* while cultures in the C series seem to have the least *Actinobacteria.*

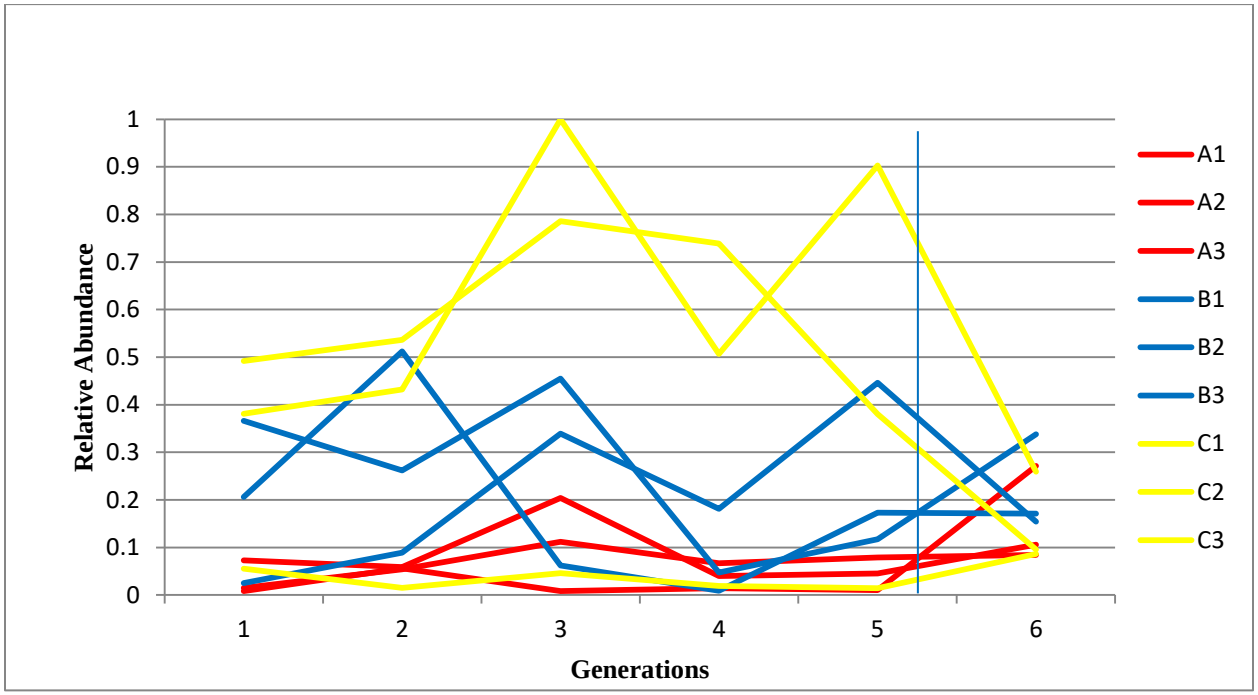

Figure 9. Trial 2 all bacteria abundances for all 9 samples relative to the highest all bacteria abundance in trial 2. The vertical line represents when *P. chrysanthemi* was added.

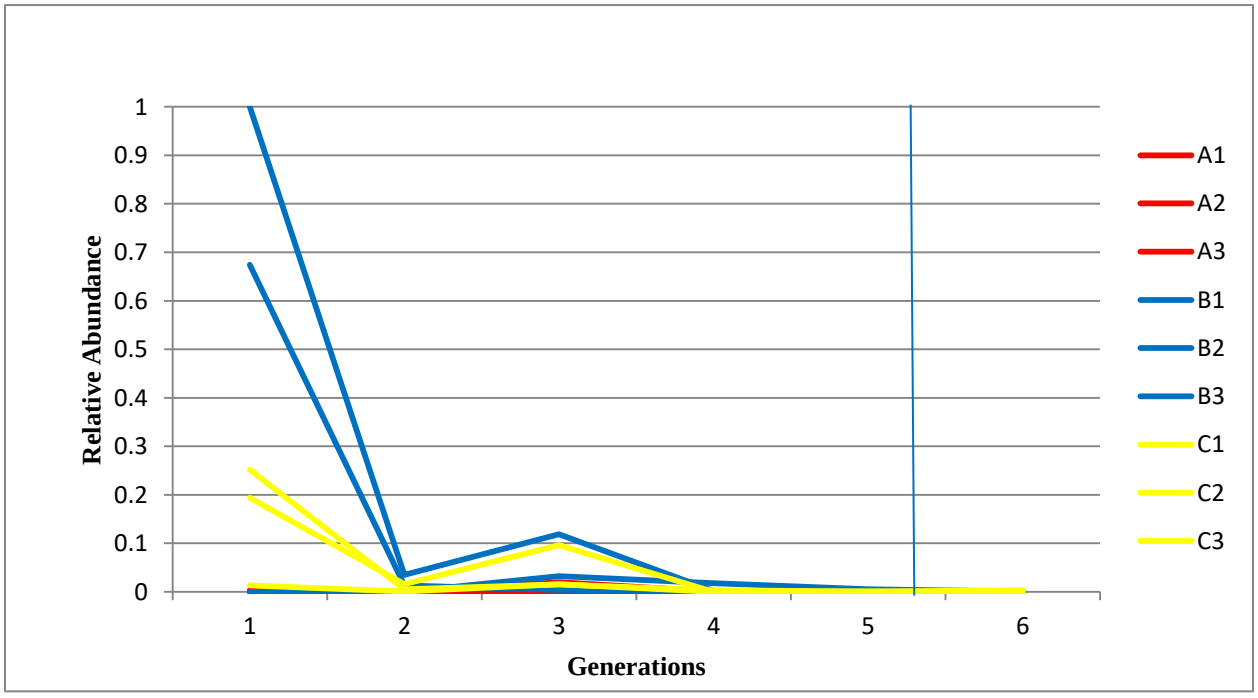

Figure 10. Trial 2 *Firmicute* abundances for all 9 samples relative to the highest *Firmicute* abundance for trial 2. The vertical line represents when *P. chrysanthemi* was added.

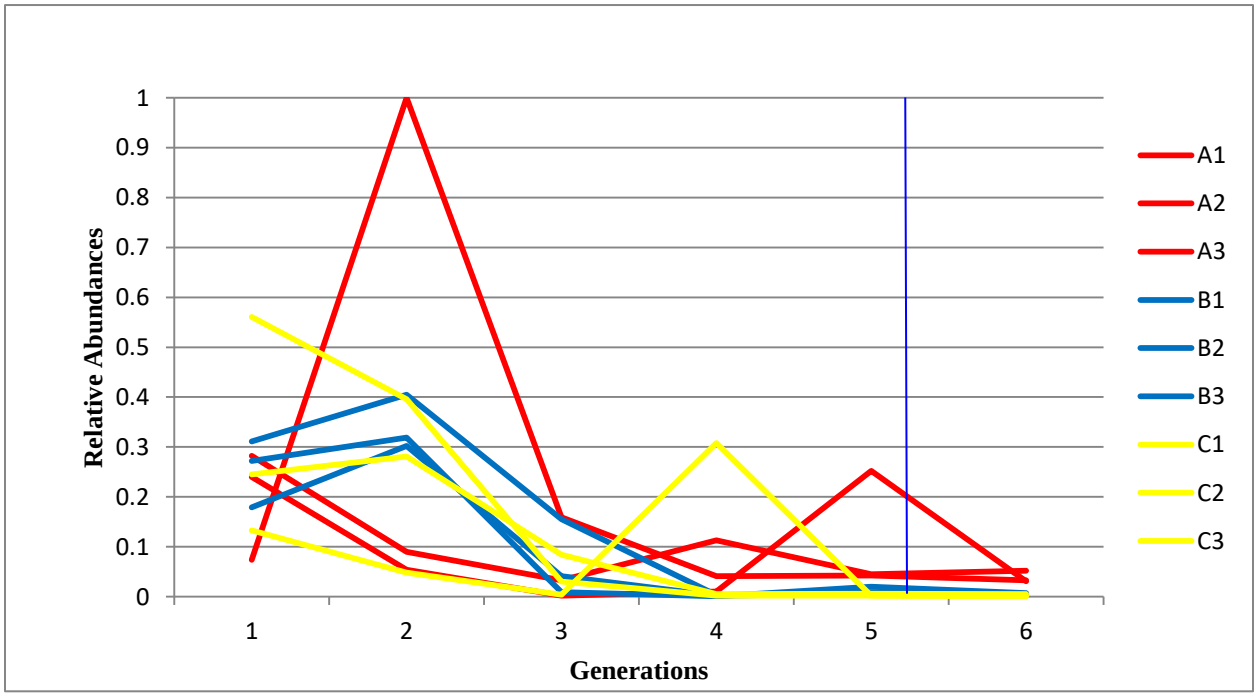

Figure 11. Trial 2 *Actinobacteria* abundances for all 9 samples relative to the highest *Actinobacteria* abundance for trial 2. The vertical line represents when *P. chrysanthemi* was added.

Discussion

It was my hypothesis that the environment would be largely responsible for controlling the development of bacterial communities and once the bacterial communities were formed, they should be very resilient to any type of perturbation. In Figure 5 it can be seen that DNA was successfully extracted. It was then used to run qPCR reactions as seen in Figure 4. In Figure 4 when comparing the 10 fold diluted *Firmicutes* to the 10 fold diluted *Actinobacteria* it is apparent that the *Actinobacteria* is present at much higher abundances than *Firmicutes*. This was the case for all samples and all generations for both trials 1 and 2. Since all of the line graphs that were made (Figures 6-11) were relative to the specific bacteria being graphed, we can only visually see the differences in that particular bacteria versus other samples. The graphs do not show how *Actinobacteria* was at much higher abundances than *Firmicutes*. However, after calculating the difference between the lowest Ct values for both *Firmicutes* and *Actinobacteria*, it was determined that the most *Firmicutes* found in a sample was only 0.003% of the most *Actinobacteria* found in a sample. Since all samples in both trials were consistent in that *Actinobacteria* was present at much higher abundances than *Firmicutes*, we can conclude that the environment does in fact play a role in the development of bacterial communities. What these data are showing is that the environment is preferentially selecting for *Actinobacteria* over *Firmicutes*, most likely due to *Actinobacteria* being better adapted for a pectin environment.

In Figures 7, 8, 10, and 11 it is apparent that the trends in the A, B, and C cultures are more closely related to themselves than they are to other cultures from the different soil samples. This is good evidence that the initial inoculum is also playing a crucial role

in the development of bacterial communities. Because all of the cultures from the same soil sample show close trends, this indicates that when the initial inoculum is consistent in regards to abundance of specific strains of bacteria, development of bacterial communities follows a similar trend. In contrast, when the initial inoculum varies in composition, different bacterial composition arises in the different bacterial communities. Additional evidence that supports the idea that initial inoculum plays a role in the development of bacterial communities is seen in Figures 7 and 10. In both trials soil sample B was taken from the same location, under rotting wood. In each case it can be seen that the *Firmicutes* start off at high concentrations in the B samples and then quickly plummets. In this case, the results were not only reproducible, but nearly identical. The initial inoculum was the same and the same results occurred in both cases. This is also evidence that the environment plays a role in the development of bacterial communities as this is an extreme example of how the environment is not selecting for *Firmicutes*.

In Figure 6, the pattern amongst the samples seems to be a bit chaotic, in that there are no real trends that can be seen. However, we might expect this because these soil samples were taken from different environments, therefore the total abundance of bacteria may be similar, but the particular strains that largely make up the total bacteria most likely vary. So, essentially cultures from different soil samples will most likely respond differently to the pectin environment, but the total abundance of bacteria across all cultures from all soil samples should be relatively similar. There is evidence from the Human Microbiome Project that supports this idea (10). In this study they found that species of bacteria varied markedly across most individuals at a given anatomical site. However, the metabolic functions of these bacteria found at particular anatomical sites

was in fact quite similar. To relate this to my study, perhaps the carrying capacity of this environment is fairly fixed, that is this particular pectin environment design can only support a certain number of bacteria. However, the composition of the bacteria that make up the total bacterial community can vary drastically as long as particular duties are being carried out. Since different species of bacteria can perform similar functions, the types of bacteria found in the initial inoculum can vary quite significantly and thus have a profound effect on the final composition of the bacterial community.

In Figure 9 which depicts all bacteria for trial 2, we can see there are considerably different results from trial 1. In trial 2, the all bacteria graph shows distinct differences between cultures inoculated with the different soil samples. C Cultures show relatively high abundances of bacteria whereas A cultures show relatively low abundances of bacteria. This contradicts what was seen in trial 1. What might explain this is that C cultures already possessed a high abundance of bacteria that were well suited for the pectin environment whereas A cultures did not. Since this was only a 6 week trial, perhaps the A cultures did not have enough time to fully develop so that the bacteria that were better suited for a pectin environment could reach peak abundances.

Furthermore, it can be concluded that a steady equilibrium for both trial 1 and 2 was never obtained. Because of this, the effects that *P. chrysanthemi* may have had on the cultures from each trial cannot be determined. There is no way of determining whether any of the changes seen in the cultures was due to the addition of *P. chrysanthemi* or the bacteria themselves still trying to find their niche. Another alternative explanation is that instead of the cultures never coming to a steady equilibrium perhaps they came to a cyclic equilibrium. In a cyclic equilibrium there would be constant fluctuations in the

abundances of the various bacterial species present in the cultures. Statistical analysis could have been performed on the data in the graphs to attempt to detect any patterns. However, we were unable to come up with a way of analyzing this data for the detection of patterns among the various cultures.

In conclusion, the results from the experiment seem to indicate that both the environment and the initial inoculum play significant roles in the development of bacterial communities in a pectin environment. Additional experiments should be conducted on this subject to see if the results can be duplicated. Furthermore, this experiment should be expanded by assessing the types of enzymes released into the pectin broth. If the different enzymes that the varying bacteria in the community release can be analyzed, identified, and then assessed for efficiency, this may be a way to create an enzymatic cocktail that efficiently degrades pectin. This would have huge implications to the production of biofuels. If communities of bacteria could efficiently degrade pectin and ultimately turn it into ethanol, this would be a way to create biofuels from the byproducts of farming. Another area that this research applies to is the effects of probiotics on the human microbiome. What these results suggest is that if the proper environment is not maintained in the human gut, then essentially taking probiotics may have no effect. One way to try and establish a favorable environment for healthy gut bacteria is to eat a diet high in raw vegetables and stay away from sugars and fats. It has been found that healthy gut bacteria need the polysaccharides from sources like raw vegetables for nutrients. Conversely harmful bacteria in the gut thrive on sugars and fats. If a diet high in sugars and fats is being consumed, it will favor harmful gut bacteria which may out compete the healthy bacteria. However, if a proper diet is being consumed

than this will better enable healthy gut bacteria to flourish and eliminate the more harmful gut bacteria.

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