Differences in soil bacterial diversity after treatment with benzyl isothiocyanate, an allelopathic secondary metabolite derivative of Alliaria petiolata (garlic mustard)

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Differences in soil bacterial diversity after treatment with benzyl isothiocyanate, an allelopathic secondary metabolite derivative of *Alliaria petiolata* (garlic mustard).

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TA: Sandra Simon

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Abstract

The species, *Alliaria petiolata* (garlic mustard), has overtaken the understory of North American forests for over a century, but how the plant has become invasively successful is not well understood. There is some indication that the secretion and breakdown of its allelopathic metabolic compounds, known as glucosinolates, contribute to its success. Benzyl isothiocyanate, an end product of garlic mustard glucosinolate breakdown, has been found in previous studies to inhibit seed germination and the growth of certain mycorrhizal fungi species. Soil bacterial communities, in addition to fungi, play an important role in the surrounding abiotic and biotic environment. This study aims to better understand the effect garlic mustard and benzyl isothiocyanate might have on the surrounding bacterial communities. Soil samples were collected from two locations, one where garlic mustard was present and one where the plant was not present. The samples collected from each location were divided into a treatment group with benzyl isothiocyanate and a control group. Bacterial DNA extracted from each of the four experimental groups was amplified and sequenced at the V3-V4 16S rRNA region. Alpha and beta diversity, as well as OTU abundance were analyzed using QIIME2 software and two-way statistical analyses. Significant differences in bacterial diversity were only observed for experimental groups based on location from where the soil samples were derived. Except for one bacteria taxon, there were no significant differences in bacterial diversity or abundance observed between treatment groups. A significant difference of an interaction between treatment and location of the experimental groups was not found. More studies of the effects garlic mustard’s glucosinolate derivates have on the microbial environment need to be conducted.
Introduction

Garlic mustard, *Alliaria petiolata* of the *Brassicaceae* family, is an invasive plant species to North American forests that has been spreading across the understory for over a century. Invasive species impose a threat to the survival of surrounding native plants and microbes in the environment. Multiple studies have been performed to assess the environmental impact a non-native plant species can have on species adjacent to the location of the plant (Bartz & Kowarik, 2019; Johnson, 2002). Garlic mustard is an allelopathic plant that secretes chemicals into the soil which help the plant proliferate in its invasive environment. The degree to which the secretion of these allelopathic chemicals, over the use of other methods, allow garlic mustard to be invasively successful is not fully understood (Cipollini & Cipollini, 2016; Rodgers et al., 2008). Plants of the *Brassicaceae* family produce compounds known as glucosinolates, which are a type of sulfur and nitrogen containing secondary metabolite derived from glucose and an amino acid. Garlic mustard contains two types of glucosinolates, glucotropaeolin and sinigrin (Vaughn & Berhow, 1999). When these glucosinolates are broken down by myrosinase, the hydrolyzed products have been found to have allelopathic effects on the germination and growth of other plants (Vaughn & Berhow, 1999;).

Isothiocyanates are one of the enzymatic products produced from glucosinolate breakdown. Studies with wheat and garden cress in the presence of these compounds during initial growth have demonstrated the phytotoxic effects that garlic mustard can have on surrounding agriculture (Bialy et al., 1990; Cipollini & Cipollini, 2016; Vaughn & Berhow, 1999). When glucosinolates are secreted from the plant tissue and broken down in the soil, there is a lethal effect on some of the surrounding organisms. The effects of each chemical breakdown that leads to toxicity in the soil microbial community is not well understood. Some of the
allelopathic compounds have a longer half-life than others in addition to differences in chemical structure, which leads to a variation in the impact each chemical has on the environment and organisms living within it (Brown et al. 1991; Gimsing & Kirkegaard, 2009, Prati & Bossdorf, 2004). A strong relationship exists between plants and the surrounding microbial community. The functions of these organisms coincide and work together to create a healthy efficient environment. The introduction of new chemicals into the environment, such as the allelopathic secretions of an invasive plant, can disrupt the mutually cooperative balance between species.

The isothiocyanates derived from garlic mustard tissue secretions have not only been found to inhibit the germination of other plants, but also the growth of fungal communities. Samples where *Alliaria petiolata* or its allelopathic chemicals were present, were found to inhibit the sporulation of mycorrhizal fungi species (Cantor et al., 2011; Wolfe et al., 2008).

Mycorrhizal fungi form a symbiotic relationship with a plant’s root system to increase water and nutrient uptake. Bacterial communities share similar plant relationships with regards to decomposition, nitrogen fixation, nutrient acquisition, and other biological processes. Studies that researched the effects of glucosinolate derivatives on different plant and fungi germination commonly used allyl isothiocyanate and benzyl isothiocyanate as treatment chemicals (Cantor et al., 2011; Fahey et al., 2001; Sofrata et al., 2011; Wolfe et al., 2008). The natural soil concentrations of these isothiocyanates as a product of glucosinolate breakdown was determined by liquid chromatography and gas chromatography mass spectrometry methods (Baily et al., 1990; Brown et al., 1991; Cantor et al., 2011; Vaughn & Berhow, 1999). Although much research has been conducted to understand how garlic mustard effects other plant and fungal communities, not much research has been conducted to understand the relationship between bacterial communities and the allelopathic plant (Beevie et al., 2009; Tierens, 2001).
In order to help better understand the relationship between bacterial communities and garlic mustard, this study aims to observe how the presence of the glucosinolate derivative, benzyl isothiocyanate, affects the bacterial diversity amongst different soil samples. Samples were collected from areas where *Alliaria petiolata* basal rosettes were found to grow (GM), as well as a nearby area where the plant was not found (NGM). Bacterial diversity between locations and treatment groups are expected to be significantly different. Untreated NGM samples were expected to have greater bacterial diversity than soil samples collected from around the basal rosettes since the NGM samples were not exposed to the allelopathic chemicals secreted from garlic mustard. The difference in bacterial diversity between the untreated and treated groups for the GM location was expected to be smaller than for NGM samples since GM samples were preconditioned to allelopathic compounds from the secretions of the basal rosettes. Taxonomic microbial diversity was analyzed after sequencing at the 16S V3-V4 region of bacteria ribosomal RNA. Measuring the effects on bacterial diversity due to the presence of benzyl isothiocyanate can provide a greater understanding of how *Alliaria petiolata* works as an invasive species to outcompete the survival of surrounding understory plants.

**Methods**

**Sample Collection**

Soil samples used in this experiment were collected from the West Virginia University (WVU) Core Arboretum in an area of the floodplain between the Caperton Rail-Trail and the Monongahela River, west of marked trail loops (latitude: 39.6483, longitude: -79.9857). Two sample locations were selected within the floodplain area to maintain soil composition between location groups. One group of soil samples were collected from an area directly below and surrounding garlic mustard basal rosettes. The second location of soil samples were collected
from an area approximately 10m east of the first location where no garlic mustard basal rosettes or second-year flowering garlic mustard plants were visible. The soil samples from each of the locations were retained in the separate covered containers for transport. 12 replicates of soil collected from each location were aliquoted into separate labeled planting containers. To maintain consistency between replicates each sample was measured for volume and average weight.

**Incubation and Treatment**

The replicates from each soil location were incubated for 48 hours at 25°C without light exposure. When the soil sample replicates were removed from the incubation chamber each location group, garlic mustard (GM) and no garlic mustard (NGM), was divided into two experimental groups, so each of the 4 groups consisted of 6 replicates. The experimental groups were labeled: treatment with benzyl isothiocyanate (T) and no treatment (NT). The control groups, GM-NT and NGM-NT were given the same volume of ddH$_2$O so that the soil was saturated and ddH$_2$O seeped out from the bottom of the planting container. GM-T and NGM-T groups were treated with 500 ppm benzyl isothiocyanate vortexed in ddH$_2$O. The concentration was determined based on a natural concentration found to be inhibitory in previous research studies (Bialy et al., 1990). The replicates were randomized between experimental groups and location then placed in an incubation chamber at 25°C without light exposure for one week.

**Wet Laboratory Work and Sequencing**

The 24 total replicates were removed from the incubation chamber. Extraction of the genomic microbial DNA present in each soil sample was performed using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo, 2019). The procedure was followed according to protocol specifications for soil samples. To determine the volume of soil that should be used for
each extraction, a portion of one replicate was measured out by weight according to the Zymo Soil Microbe Miniprep Kit protocol and the rest of the replicate extraction volumes were determined by visual comparison to the first measured sample. The extracted genomic DNA from each replicate was quantified on the NanoDrop Quantification instrument; DNA concentration (ng/µL) and purity 260nm/280nm ratio values were recorded. Samples were diluted with DNA Elution Buffer so that the measured concentration was less than 75 ng/µL.

The extracted DNA samples were amplified on the thermocycler using forward and reverse primers (Table 1) at the 16S rRNA V3-V4 region following the puRETaq Polymerase Chain Reaction (PCR) protocol (illustra™, 2019). The PCR Master Mix was prepared following protocol specifications for soil samples and a reagent volume of 30 reactions. Thermocycling conditions for amplification consisted of one 5-minute initial denaturation step at 94°C; 30 amplification cycles to denature, anneal, and extend at 94°C (30 seconds), 60°C (30 seconds), and 72°C (1 minute), respectively; 5 minutes at 68°C for the final extension; and an indefinite hold period at 10°C. The success of the PCR reaction was verified with gel electrophoresis using a 1.5% agarose gel and ZipRuler Express Set 1 Ladder (Figure 1). PCR products were purified with iTruMagNA Magnetic Beads before sending the samples to the WVU Genomics Core Facility for library preparation and sequencing. Library preparation of the sample input was completed using barcode tailing addition and Axygen Bead Cleanup. A Qubit High Sensitivity DNA Kit was used for quantification of the sequencing libraries, and sequencing of each replicate was performed on the Illumina Miseq instrument using paired end 250 chemistry.

**Bioinformatics and Statistical Analysis**

The sequenced sample data and metadata were transferred to Quantitative Insights into Microbial Ecology (QIIME2) software. Following the QIIME2 Tutorial, alpha rarefaction curves
of the data were generated to determine the sequencing depth for which statistical analysis of community metrics would be performed. Alpha diversity, beta diversity, and OTU abundance were assessed using Past3 software. Alpha diversity was measured using Faith’s phylogenetic distance and a two-way ANOVA univariate test to analyze community composition variation within samples. To analyze community composition differences between samples, beta diversity, a multivariate two-way PERMANOVA test was performed. Both unweighted and weighted UniFrac distances were evaluated to analyze rare and common taxa present in the samples, respectively. Individual OTU abundance was used to generate a heat diagram of the hierarchical clustering of different bacteria taxa present amongst different samples with SAS/JMP software. Specific bacterial taxa were tested for presence/absence among experimental groups using OTU abundance and a Fisher’s Exact Test. Individual OTU abundance was evaluated using two-way ANOVA statistical analysis and Turkey’s Post Hoc test, any p-value <0.05 was considered to be statistically significant for all analyses performed.

Results

All 24 replicates matched the desired amplicon size for post-PCR amplification success verification (Figure 1). A sequencing depth of 500 was utilized for all subsequent alpha diversity, beta diversity, and OTU analyses based on the alpha rarefaction curve generated by the QIIME2 software after sequencing on the Illumina Miseq instrument (Figure 2). The analysis of alpha diversity within samples using a two-way ANOVA test in Past3 found that community composition was significantly different only for the location variable (Table 2; F = 9.533, p-value = 0.007062). Turkey’s post-hoc means test determined a significant difference between the GM-NT and NGM-NT groups in the study (Figure 4; p-value = 0.02828). Treatment and
interaction variables for alpha diversity were not found to be significantly different between any of the experimental groups.

With a two-way PERMANOVA statistical test of beta diversity using the Past3 software, significant differences were found for both weighted and unweighted UniFrac distances (Table 3). The weighted UniFrac distance PERMANOVA test found significantly different bacteria community composition for the location variable (Table 3; $F = 2.1997$, p-value = 0.0042). The location variable was found to be significantly different for the unweighted UniFrac distance of beta diversity amongst samples as well (Table 3; $F = 2.2876$, p-value = 0.0009). A PCoA plot of beta diversity for the unweighted UniFrac distance matrix (Figure 5) showed GM-NT to be closely clustered together in 3-dimensional space of the plot, while NGM samples were spaced further away from the clustered GM-NT samples. Experimental groups, other than GM-NT, were less clustered and more spread out in the 3-dimensional space of the PCoA plot (Figure 5). The GM-NT and NGM-T samples were clustered with their respective experimental groups in separate areas of the PCoA plot representing beta diversity for the weighted UniFrac distance matrix (Figure 6). The GM-T samples for both weighted and unweighted UniFrac distances were spaced far apart from each other on the PCoA plots (Figures 5 and 6).

The OTU abundance heat map clustered each of the experimental group samples that fell within the sequencing depth of 500 based on hierarchical similarity of the samples’ OTU abundance profiles (Figure 3). For the first seven clustered samples at the top of the heat map, five of the seven were GM location collected samples. Four of the NGM-NT samples are closely clustered in middle portion of the heat map. There appears to be no trend of treatment clustering, or location + treatment clustering between the four experimental groups (Figure 3).
For OTU abundance analysis, six different bacteria taxa were found to be significantly different between different experimental groups (Table 4). The bacteria classes Ellin6529 and S085, as well as, the bacteria family *Kouleothrixaceae* belong to the phylum Chloroflexi and were found to be significantly different in relative abundance means between two separate experimental groups. Bacteria class Ellin6529 was identified to be significantly different between the NGM-NT and NGM-T experimental groups (Figure 7; $p$-value = 0.03661). This bacterium taxon was the only statistical test performed for the entire research study that found a significant difference in diversity between a treatment and a control group. The bacteria class S085 (Figure 8; $p$-value = 0.004389) and family *Kouleothrixaceae* (Figure 9; $p$-value = 0.004245) were determined to be significantly different among the GM-NT and NGM-NT groups.

The bacteria family *Rhodospirillaceae* showed significant difference in abundance means between the GM-NT and NGM-NT groups (Figure 10; $p$-value = 0.02741). The GM-T and NGM-T experimental groups showed significant differences in relative abundance means for both bacteria genera *Actinoplanes* (Figure 11; $p$-value = 0.022) and *Hyphomicrobium* (Figure 12; $p$-value = 0.01129).

**Discussion**

The results of this experimental study indicate the hypothesis that a difference in bacterial diversity would be observed between untreated and treated samples with benzyl isothiocyanate from the garlic mustard absent location is not supported. The alpha diversity statistical test indicated that there was only a significant difference in bacterial community composition for samples derived from the different locations. Soil samples collected from areas where garlic mustard basal rosettes were present were found to have greater bacterial diversity than the
communities within samples collected from areas where garlic mustard was not present. The introduction and availability of different nutrients and chemicals into the environment due to garlic mustard’s presence may account for the greater diversity of bacterial communities compared to soil samples collected where garlic mustard was not present to contribute new compounds to the environment.

Analysis of beta diversity for both unweighted and weighted unifrac distances also only found significant differences in the community composition for samples that were collected from the two different locations. These statistical results indicate that the bacterial community amongst samples collected from where garlic mustard is present and not present are inherently different from each other. However, the results do not definitively prove that the presence or lack of presence of garlic mustard directly impacted the difference in bacteria community composition observed. The observed differences in composition could just be a factor of the sample collection sites originating from two separate areas of the Core Arboretum even though each was collected in relatively close proximity of each other. The structure of microbial communities is shaped by the nutrients and other organisms present in the soil (Xue et al., 2018).

Both alpha and beta diversity statistical analysis of the sequenced bacteria data found no significant differences between treatment groups of the samples tested. There was also not a significant difference observed for the interaction variable to indicate variation in community composition either within individual samples or among samples tested. These results indicate that addition of the glucosinolate derivative, benzyl isothiocyanate produced by garlic mustard, had no significant impact on the differences in bacteria community composition between samples from individual experimental groups. While these results indicate that the byproduct alone does not have an effect on the bacteria community composition, the results cannot
substantiate that glucosinolate derivatives produced by garlic mustard have no effect on bacteria community composition of the surrounding soil.

Garlic mustard produces more than one type of glucosinolate, which can be broken down and secreted into the soil (Bialy et al., 1990; Vaughn & Berhow, 1999). Treatment with a different glucosinolate derivative, other than benzyl isothiocyanate, could demonstrate a statistical difference in bacteria community composition between treated and untreated sample groups. Allyl isothiocyanate has been found to be effective in inhibiting plant germination and could result in a significant effect on the bacteria community (Bialy et al., 1990). An experimental study that is more representative of garlic mustard’s effect on bacterial communities could involve testing the combined effect of multiple glucosinolate derivatives on treated samples. Garlic mustard does secrete one glucosinolate at time into the soil, but rather a mixture of glucosinolates that are broken down into different allelopathic compounds (Bialy et al., 1990; Gimsing & Kirkegaard, 2009; Vaughn & Berhow, 1999). A combination of chemicals together are sometimes more toxic or harmful to organisms than the isolated compounds would be (Brosche & Backhaus, 2010).

Analysis of OTU relative abundance among the different experimental groups found six independent bacteria taxa that were significantly different. Three of the bacteria taxa found to be significantly different were of the same Chloroflexi phylum: Ellin6529, S085, and Kouleothrixaceae. Chloroflexi is one of 34 phyla in the bacteria domain. This bacterium is common to the soil environment at the subsurface layer where samples in our study were collected from (Oni et al., 2015; Wright et al., 2017). Chloroflexi use carbon present in the soil organic matter to survive and have been found to breakdown fresh organic matter more efficiently than other bacteria taxa (Oni et al., 2015; Meng et al., 2019). Chloroflexi Ellin6529
was the only bacteria taxa where a significant difference between treatment groups of samples collected was observed. The ability of Chloroflexi bacteria to thrive in environments where organic compounds need broken down could explain why the phylum is present in greater abundance for samples where garlic mustard and its organic components were present in the soil.

The bacteria family *Rhodospirillaceae* were found in higher abundance for samples collected from the control group where garlic mustard was not present than the control group where garlic mustard was present. This bacteria family plays are role in fixing nitrogen present in the environment so that nitrogen gas is converted to its reduced form ammonia (Madigan *et al*., 1983). Nitrogen fixation is a necessary process for molecules such as amino or nucleic acids to be synthesized by plants and other living organisms in the environment (Madigan *et al*., 1983). A study on garlic mustard secretions found out of the forty-four allelopathic compounds researched, multiple volatile compounds were non-nitrogen containing (Blazevic & Mastelic, 2007). The lack of nitrogen containing compounds secreted by garlic mustard into the soil compared to other plant types living and decomposing in the non-garlic mustard environment could help explain the decreased abundance of *Rhodospirillaceae* found in the garlic mustard control samples.

The bacteria genera *Actinoplanes* and *Hyphomicrobium* both were significantly higher in relative abundance for samples collected from where garlic mustard was growing than samples where no garlic mustard was visibly present. *Actinoplanes* is a member of the spore-forming bacteria family *Micromonosporaceae* commonly found in soil environments whose function in the surrounding environment is not well understood (Trujillo *et al*., 2014). *Hyphomicrobium* is a non-spore-forming bacterium, the presence of which has been found to be positively correlated with the degradation of different herbicide compounds toxic to plant growth (Albers *et al*., 2018;
A research study on the efficacy of specific treatments for removing garlic mustard found exposure to herbicides to be immediately effective for getting rid of garlic mustard but long-term results of the treatment were found to be ineffective (Shartell et al., 2012). The study also suggested that application of herbicides damages native plant species which unintentionally may contribute to the invasive success of garlic mustard (Shartell et al., 2012).

The ability of *Hyphomicrobium* to degrade compounds in herbicides may explain its greater abundance in the garlic mustard samples, if the area where those samples were collected in the WVU Core Arboretum had been previously treated with herbicides in attempt to control garlic mustard growth.

The presence of garlic mustard in the environment may allow for a greater abundance of certain bacteria taxa in the surrounding soil that are not as relatively abundant in soil areas where garlic mustard is not present. Garlic mustard growth could promote the abundance of these bacteria taxa to promote the plant’s own growth, rather than reducing the abundance of bacteria taxa with the secretion of its allelochemicals and inhibiting the growth of other plant types. This research study only observed the possible effects a single glucosinolate derivate could have on the bacteria community composition, with no supportive results to indicate an effect occurred. Future research should be conducted to examine how multiple compounds in combination might affect the microbial community in the soil. Prospective studies may also want to control factors such as soil composition between sample location collection groups more closely and treat samples with the allelopathic compounds in a continual fashion, rather than a single treatment. The results of this study aid in the understanding of how the invasive garlic mustard plant affects the surrounding soil environment in which it flourishes.
Figures and Tables

Figure 1. 1.5% Agarose gel results of PCR products using ZipRuler Express Set 1 Ladder. PCR amplification success was verified for all 24 replicates in the study using gel electrophoresis at 120V for 1.5 hours. The desired amplicon size was 475-500bp. P represents the positive control and N represents the negative control. Replicates 1-6 represent NGM-T, 7-12 NGM-NT, 13-18 GM-T, and 19-24 GM-NT.

Figure 2. QIIME2 output rarefaction curve of sequencing data. A sequencing depth of 500 was selected to maximize the amount of OTU observed, as well as minimize loss of replicates for each experimental group.
Figure 3. OTU abundance heatmap generated by SAS JMP software. The replicates from each of the experimental groups are clustered vertically into a hierarchy of similarity based on their OTU abundance profiles for the most common bacteria taxa. Blue boxes indicate absence/low OTU abundance, and red boxes indicate high abundance of OTU. The branches at the bottom of the heatmap represent the different identified bacteria sorted based on their taxonomic groups.

Figure 4. Alpha diversity output from two-way ANOVA test. Each bar represents the mean and standard errors for the bacteria community composition within samples from each experimental group. Turkey’s post-hoc means test determined that GM-NT and NGM-NT were significantly different in community composition (bracketed groups, * p-value < 0.05).
Figure 5. PCoA plot of beta diversity unweighted UniFrac distance matrix. The plot illustrates variation in community composition between samples for uncommon bacteria taxa. Sample points that are closer in 3-dimensional space are more similar in community composition than those points that are further apart. Four sample replicates from the GM-NT experimental group are clustered together while three samples from NGM-NT and NGM-T are clustered together in separate location of the 3-D plot.

Figure 6. PCoA plot of beta diversity weighted UniFrac distance matrix. The plot illustrates variation in community composition between samples for common bacteria taxa representing the overall community structure. Sample points that are closer in 3-dimensional space are more similar in community composition than those points that are further apart. All five the sample replicates from GM-NT evaluated at a sequencing depth of 500 are clustered together near the bottom of the plot.
Figure 7. Relative OTU abundance of Chloroflexi bacteria class: Ellin6529. Plot illustrates the OTU relative abundance diversity means and standards errors for each experimental group. Turkey’s pairwise means test determined NGM-NT and NGM-T were significantly different in OTU abundance for Ellin6529 which belongs to the phylum Chloroflexi (bracketed groups, * p-value < 0.05).

Figure 8. Relative OTU abundance of Chloroflexi bacteria class: S085. Plot illustrates the OTU relative abundance diversity means and standards errors for each experimental group. Turkey’s pairwise means test determined GM-NT and NGM-NT were significantly different in OTU abundance for S085 which belongs to the phylum Chloroflexi (bracketed groups, * p-value < 0.05).
Figure 9. Relative OTU abundance of bacteria family: *Kouleothrixaceae*. Plot illustrates the OTU relative abundance diversity means and standards errors for each experimental group. Turkey’s pairwise means test determined GM-NT and NGM-NT were significantly different in OTU abundance for *Kouleothrixaceae* which belongs to the phylum Chloroflexi (bracketed groups, *p*-value < 0.05).

Figure 10. Relative OTU abundance of bacteria family: *Rhodospirillaceae*. Plot illustrates the OTU relative abundance diversity means and standards errors for each experimental group. Turkey’s pairwise means test determined GM-NT and NGM-NT were significantly different in OTU abundance for *Rhodospirillaceae* (bracketed groups, *p*-value < 0.05).
Figure 11. Relative OTU abundance of bacteria genus: *Actinoplanes*. Plot illustrates the OTU relative abundance diversity means and standards errors for each experimental group. Turkey’s pairwise means test determined GM-T and NGM-T were significantly different in OTU abundance for *Actinoplanes* (bracketed groups, * p-value < 0.05).

Figure 12. Relative OTU abundance of bacteria genus: *Hyphomicrobium*. Plot illustrates the OTU relative abundance diversity means and standards errors for each experimental group. Turkey’s pairwise means test determined GM-T and NGM-T were significantly different in OTU abundance for *Hyphomicrobium* (bracketed groups, * p-value < 0.05).
Table 1. Forward and reverse primer names and sequences used for PCR amplification at the 16S V3-V4 rRNA bacteria region.

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<th>Primer</th>
<th>Sequence</th>
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<td>I5_320Class_</td>
<td>AATGATACGGCCGACACCAGATCTACACCNNNNNNNNTCGTCGGCAACGTCAGATGTGTATAAGAGACAGCC</td>
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<td></td>
<td>Meta_Primer</td>
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<td>16S Reverse</td>
<td>I7_320Class_</td>
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<td></td>
<td>Meta_Primer</td>
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Table 2. ANOVA results of rarefied alpha diversity. Three variables for alpha diversity were evaluated to determine the presence of variation in bacterial community composition within samples.

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<th>Variable</th>
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<th>p-value</th>
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<td>Interaction</td>
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Table 3: PERMANOVA results of rarefied beta diversity. Three variables for weighted and unweighted Beta diversity were evaluated to determine the presence of variation in bacterial community composition amongst samples.

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<td></td>
<td>Treatment</td>
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<tr>
<td></td>
<td>Interaction</td>
<td>0.72582</td>
<td>0.3141</td>
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Table 4. ANOVA results for OTU abundance of different bacteria taxa. The bacteria Ellin6529, S085, and *Kouleothrixaceae* belong to the phylum: Chloroflexi.

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<th>OTU Taxa Tested</th>
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<th>p-value</th>
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<td></td>
<td>Treatment</td>
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<td>0.02481</td>
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<td></td>
<td>Interaction</td>
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<td>Interaction</td>
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