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Effects of and Influences on Microbial Populations of Missouri Maize Fields

Madsen Paul Sullivan

A thesis submitted to the faculty of  
Brigham Young University  
in partial fulfillment of the requirements for the degree of

Master of Science

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## ABSTRACT

### Effects of and Influences on Microbial Populations of Missouri Maize Fields

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Master of Science

The role of individual soil microorganisms changes over the course of a plant's life - microorganisms that have no discernable role at one developmental stage may affect the plant later in its growth. Traditional analysis of the soil microbiome, which has focused principally on the relative abundances (RA) of individual organisms, may be incomplete, as underlying differences in population size cannot be addressed. We conducted a metagenomic analysis of soil microorganisms from various maize (*Zea mays* L.) fields at two depths, accompanied by crop yield components, to provide insight into influences of edaphic microbes on maize productivity under commercial maize production systems in Missouri. This study assesses the influence of fungi and bacteria, not only in terms of RA, but also in their estimated absolute abundances (EAA), derived by combining the results of Illumina HiSeq sequencing data and phospholipid fatty acid abundance data. Significant interactions were identified between maize yield components and soil microbes at critical developmental states. Most interactions between fungi and yield components were negative, with notable exceptions. Bacterial interactions were more complex, with most interactions during early ear development identified as positive, and most interactions during tasseling identified as negative. In addition to the effects that microbial populations have on yield, plant populations reciprocally changed the microbial community. Plant developmental state was the greatest predictor of bacteria, with the microbial communities present during the active growing season being most similar to each other, whereas the preplant microbiome and post-reproductive microbiome being most similar to each other. Fungal communities were primarily dependent on location.

Keywords: soil microbiome, DNA sequencing, yield components, relative abundance, estimated absolute abundance, differential abundance

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To Owie, my baby bug, I love you, what else can I say? You and Mama are the reason I am so happy and excited to come home every day. You are my buddy boy, and I am so happy you are in my life.

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## CHAPTER 1

### Environmental and Plant Effects on Microbial Populations of Missouri Soil in Maize Production

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## ABSTRACT

The effects of the soil microbiome on plant health is becoming increasingly recognized in agriculture. Analyses in microbial ecology frequently focus on the role and influence of the environment and abiotic variables on microbial populations. Less typically studied however, is the effect that the host plant community has on determining the community composition. Of the few studies that do discuss plant effects on the microbiome, most tend to focus on bacterial populations, rarely addressing fungi as well. In this study, we performed metagenomic soil analysis of fungal and bacterial populations from Missouri maize (*Zea mays L.*) fields to determine the effects of abiotic variables, such as soil depth and location soil, as well as the effects of the plant host community. Bacterial community composition was shown to be primarily influenced by the plant hosts, specifically the developmental state of the maize. Additionally, differentially abundant bacteria were shown to cluster primarily by maize developmental state. Fungal populations were primarily dependent on their location soil, although depth and maize developmental state contributed to fungal population composition. Additionally, differentially abundant fungi were shown to cluster primarily by location soil.

## INTRODUCTION

Many studies have detailed the effects that soil characteristics have on microbial population structure. Of these, some of the most influential have been shown to be soil classification (Bossio, Scow, et al., 1998; Garbeva, Van Veen, et al., 2004), texture (England, Lee, et al., 1993), organic matter content (Doran, 1980; Fontaine, Mariotti, et al., 2003; Haines and Uren, 1990), and pH (Anderson and Domsch, 1993; Rousk, Bååth, et al., 2010). Unfortunately, many of these are qualities that are impractical to improve in soils, such as type or texture, or they may take a long time to improve, such as organic matter. However, they are useful in ensuring the preservation of healthy microbial populations in currently arable land.

In addition to abiotic variables, the host plant community greatly determines which microorganisms are selected through the release of microbe specific exudates. Some hosts exhibit a high degree of microbial symbiont specificity, such as the rhizobia-legume interaction (Downie, 2010), while others are far more versatile, such as the colonization of roots by various forms of arbuscular mycorrhizal fungi. As Berg and Smalla highlight, there is diversity even within different hybrids and cultivars of the same crop (Berg and Smalla, 2009). Additionally, Chaparro et al. studied the effects that *Arabidopsis* populations had on their microbial communities, finding that many bacterial phyla were selected by the plant at various stage of development, indicating the plant's ability to control its microbiome (Chaparro, Badri, et al., 2014). Finally, Pfeiffer et al. investigated the effects of various maize cultivars on the soil microbiome in which they were grown, finding that the cultivars brought along unique taxa, changing the bacterial composition in ways specific to each cultivar (Peiffer, Spor, et al., 2013). This presents clear evidence that not only are there microbial influences on plants, but there

are complementary and reciprocal influences on microbes by their plant hosts, resulting in a two-sided conversation.

Maize (*Zea mays L.*) is the most widely produced crop in the United States, with 14.6 billion bushels grown on 83 million acres in 2017 (USDA, 2018). Understanding the microbiome of maize soils is an essential component of agriculture, by improving crop yield and performance, preventing damage to the environment (Michalak, Anderson, et al., 2013), and maintaining arable land (Crecchio, Mimmo, et al., 2018). Microbial groups have been shown to contribute significantly in various nutrient cycles (Bardgett, Freeman, et al., 2008; Gyaneshwar, Kumar, et al., 2002), which becomes especially important when working with maize fields, given the significant portion of land used. Many of these fields have high levels of nutrient fertilizers leaving the soil and moving into water systems, due to the relatively low levels of nitrogen-recovery efficiency (Cassman, Dobermann, et al., 2002). As Cassman et al. show, approximately only 37% of applied nitrogen fertilizer is added into maize biomass, crop residues, or incorporated into soil nitrogen stores. The remaining 63% is lost to the environment, including watersheds, which becomes significant when considering the land used. Understanding the microbial community of maize is prerequisite for future studies to analyze how maize soil microbes may allow for better use of nutrients.

While a few studies have detailed the bacterial communities within maize fields (Bakker, Chaparro, et al., 2015; Pfeiffer, Mitter, et al., 2016), there has not been a comprehensive study addressing both fungal and bacterial communities within maize soils. Understanding these maize associated microbes is necessary, given the role of fungi in plant productivity (Van der Heijden, Klironomos, et al., 1998), suppression of disease (Butt, Jackson, et al., 2001),

pathogenicity (Dean, Van Kan, et al., 2012), nutrient cycling (Hayatsu, Tago, et al., 2008), and their interactions with other microbes (Wargo and Hogan, 2006). Understanding the behaviors of both fungal and bacterial communities will be necessary to comprehensively identify how microbes may be affecting the plants, soil, and other microorganisms they live alongside.

Soil microbial communities are increasingly recognized for their contribution to plant health and productivity, especially regarding crops. Some microbial groups improve plant health by controlling pathogenic microbes (Wanner, Kirk, et al., 2014), while others improve plant tolerance for abiotic stressors (Busby, Soman, et al., 2017). However, without general community analysis first, it can be very difficult to identify how the established soil microbiome affects plant health and productivity.

The purpose of this study is to analyze the fungal and bacterial microbiome to identify significant differences between various locations, depths, and developmental states of maize. This will allow future research to consider these differences as they may relate to plant productivity or nutrient use efficiency. Therefore, our objectives were: 1) identify the role of location soil, soil depth, and maize developmental state in differentially abundant and general microbial populations; 2) identify differences in general microbial communities and specific microbial groups; and 3) establish general patterns of plant-microbe interactions.

## MATERIALS AND METHODS

### *Sampling Design*

This research came from a subset of three field sites that were a part of a 49-site research project over 8 US states and three growing seasons focused on performance and improvement of nitrogen fertilizer recommendation tools (Kitchen, Shanahan, et al., 2017). Soil cores were collected in 2016 from three farm fields in Missouri as described by Kitchen et al. Each farm had considerably different soil classifications, and were named according to their classifications – claypan, alluvial, and loess. The claypan soil was a Mexico silt loam (fine, smectitic, mesic vertic epialf), the alluvial soil was a Peers silty clay loam (fine-silty, mixed, superactive, calcareous, thermic lithic torriorthents), and the loess soil was a Higginsville silt loam (fine-silty, mixed, superactive, mesic aquic argiudolls) (Table 1-1). Each farm was planted with the same cultivar (P1197AM), and were tilled, with the previous year's crop being soybean. Each farm was divided into eight replicates, with four of the replicates receiving 200 lbs of ammonium nitrate fertilizer before planting, and the other four remaining unfertilized. Each replicate was split into two depths, 0-5 cm and 5-15 cm, with a sample taken at each depth, resulting in two samples per replicate. Samples were collected by taking multiple cores with a hand-probe from each replicate and combined to represent the sampled area. Cores were taken both between and within rows, such that samples contained variable mixes of rhizosphere and bulk soil. This was done six times over a period of approximately 4 months, with the first occurring shortly before planting and the last occurring between tasseling (VT) and blistering (R2), depending on the location (Table 1-2). Immediately following sample collection, soil samples were stored at the University of Missouri in a -20 C freezer, and upon

collection of all time periods, samples were shipped on ice to Brigham Young University, where they were stored in a -20 C freezer until further processing.

### *DNA Extraction*

Soil biota DNA was extracted using the MoBio PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following standard kit protocols with slight modifications as recommended by Lindahl (Lindahl, Nilsson, et al., 2013). Samples were homogenized with a Vortex-Genie 2 Mixer (Scientific Industries, Bohemia, NY, USA) at a setting of 10 for 15 minutes. Templates were quantified with an ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and then concentrated to 30 ng/μl.

### *Sequencing*

Two amplicon libraries were generated to assess the different populations of fungi and bacteria. Methods for sequencing are described by Smart (Smart, 2018). To assess fungal populations, we amplified the nuclear internal transcribed spacer 1 (ITS1) in a two-step PCR approach. Initial PCR amplification utilized an oligo containing the forward primer ITS1F\_KYO1 (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-NNNXXX-CTHGGTCATTTAGAGGAASTAA-3') with 3-mer, 4-mer, 5-mer or 6-mer Ns and the forward Illumina sequencing primer fused to the 5' end. The reverse oligo consisted of primer ITS2\_KYO2 (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-NNNXXX-TTYRCTRCGTTCTTCATC-3'), 3-mer, 4-mer, 5-mer or 6-mer Ns, and the reverse Illumina sequencing primer (Toju, Tanabe, et al., 2012). PCR amplification was performed with Accuprime Pfx SuperMix with the following



parameters: initial denaturation at 94°C for 2 min, 35 cycles at 98°C for 10 sec, 50°C for 30 sec, 68°C for 50 sec, with a final extension of 68°C for 5 min and a final holding temperature of 4°C. A final PCR was utilized to ligate Illumina adapters and barcodes. A second PCR process utilized forward fusion Illumina primer consisting of the P5 Illumina adaptor, an 8-mer barcode, and the 5' end of the sequencing adaptor (5'-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-TCGTCGGCAGCGTC-3'). The reverse fusion Illumina primer consisted of the P7 Illumina adaptor, an 8-mer barcode, and the 5' end of the sequence adapter (5'-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXX-GTCTCGTGGGCTCGG). The PCR parameters were as follows: initial denaturation at 94°C for 2 min, 8 cycles at 98°C for 10 sec, 50°C for 30 sec, 68°C for 50 sec, with a final extension of 68°C for 5 min and a final holding temperature of 4°C.

In bacteria, the V4 hypervariable region of the 16S rRNA gene was targeted using primers 16Sf (5'-GTGCCAGCMGCCGCGGTAA-3') and 16Sr (5'-GGACTACHVGGGTWTCTAAT-3'). Both primers, 16Sf and 16Sr, contained a series of repeating 8-bp barcodes which, in combination, facilitated a dual-indexed Illumina sequencing approach (Caporaso, Lauber, et al., 2012; Kozich, Westcott, et al., 2013). A forward or reverse Illumina primer, linker region and primer pad were also included on both 16Sf and 16Sr. Invitrogen™ AccuPrime™ Pfx SuperMix was likewise used for the generation of 16S amplicons. Thermocycler conditions were performed by running the following protocol: Initial denaturation was 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 60 seconds, and elongation at 72°C for 90 seconds. A final elongation step was set for 72°C for 10 minutes and all samples were held at 4°C.

Following amplification of either library, normalization of amplicons occurred using SequelPrep™ Normalization Plate (96) Kit (Invitrogen, Carlsbad, CA, USA). All multiplexed samples will be pooled and have their concentration quantitated on a Qubit™ 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). Following quantitation, all samples were submitted to Brigham Young University where they underwent further quality control. At Brigham Young University, all samples were tested for size distribution, size confirmation and PCR artefacts using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Library quantification using Kapa PCR was utilized prior to sequencing (Kapa Biosystems, Wilmington, MA, USA). Paired-end sequencing (2x250) was carried out on the Illumina HiSeq 2500 platform (2x250) (Illumina Biotechnology, San Diego, CA, USA).

#### *Soil Chemical and Physical Property Analyses*

Soil analyses were performed by the University of Missouri Soil Health Assessment Center. Soil properties of interest and method used included texture (pipette), cation exchange capacity (ammonium acetate), total organic C (dry combustion), organic matter (dry combustion), pH (saturated paste), and bulk density (core). For more detail, refer to Kitchen et al. (Kitchen, Shanahan, et al., 2017).

#### *Microbial Data Analysis*

Illumina sequence reads were demultiplexed according to Illumina protocol at the Brigham Young University sequencing center. Demultiplexed paired-end sequence reads were imported to QIIME 2 (v. 2.2018.6), available at <https://qiime2.org/> (Caporaso, Kuczynski, et al.,

2010). Within QIIME 2, sequences underwent quality filtering, chimera removal, and were used to create a feature table. This was done using the QIIME 2 plugin DADA2 (Callahan, McMurdie, et al., 2016). Taxonomy was assigned to bacteria using the SILVA 18S 128 QIIME release (Quast, Pruesse, et al., 2012). Taxonomy was assigned to fungi using the UNITE ITS database (Kõljalg, Larsson, et al., 2005).

Alpha and beta diversity were calculated within QIIME 2, and distance matrices were produced. Fungal and Bacterial OTU tables and taxonomic groupings, along with other similar files, were exported and analyzed by R (Team, 2018), through RStudio (Team, 2016). Exported files were then combined into a single object using the Phyloseq package (McMurdie and Holmes, 2013). Distance matrices were analyzed by ADONIS from the R package vegan (Oksanen, Blanchet, et al., 2018), identifying influential factors. Additionally, differential abundances of bacterial and fungal taxa were tested as a function of depth, developmental state, location, and combinations of these factors, as well as other factors, using QIIME 2's native support of ANCOM.

## RESULTS

### *Dominant Bacterial Phyla are Proteobacteria, Acidobacteria, and Bacteroidetes*

Bacterial 16S reads obtained from the QIIME2 workflow totaled 20,812,906. Each sample was rarefied to a depth of 15,000 reads, as this number maintained the highest number of samples while maximizing reads, and alpha-rarefaction curves plateaued or were beginning to plateau around this value; samples less than 15,000 reads were removed (Figure 1-9). Overall, this retained 223 of the 287 samples. The top 3 bacterial phyla were Proteobacteria (24.28%), Acidobacteria (22.88%), and Bacteroidetes (11.88%) (Figure 1-1). The other 7 phyla shown comprised 35.87%. Other phyla not shown comprised 5.09% of overall RA.

### *Sordariomycetes are the Dominant Fungal Class*

Fungal ITS reads obtained from the QIIME2 workflow totaled 12,518,568. Each sample was rarefied to a depth of 2700 reads, as this number maintained the highest number of samples while maximizing reads, and alpha-rarefaction curves plateaued or were beginning to plateau around this value; samples comprised of less than 2,700 reads were removed (Figure 1-10). Overall, this retained 234 of the 290 samples. Fungal classes Sordariomycetes (49.83%), Mortierellomycetes (14.57%), and Dothideomycetes (11.73%) were the most abundant (Figure 1-2). The remaining six fungal classes had a total overall RA of (22.11%). Other classes not shown comprised 1.75% of overall RA.

### *Growing Season Bacteria are Highly Similar, Although Differences Do Exist*

Bacterial PCoA utilized the weighted bacterial UniFrac matrix to plot sample dissimilarity. Samples from developmental periods 1-4 were highly similar to each other, regardless of the location or depth from which they were sampled. Notably, these time periods are all part of the active yet still early growing season for maize. Samples from developmental periods 0 and 5 were also highly similar to each other, although they were quite different from 1-4. They also clustered together regardless of their location and depth. Location and depth had a very minor effect on other clustering patterns, although they were only apparent when viewing weaker principal components and were therefore not included. (Figure 1-3).

Samples within developmental periods 1-4 also utilized the weighted bacterial unifrac to plot sample dissimilarity for bacterial PCoA. Samples differed primarily by the location from which they were taken, rather than depth or developmental state. (Figure 1-4)

### *Fungal Communities are Location Dependent*

Fungal PCoA utilized the unweighted fungal jaccard matrix to plot sample dissimilarity. Samples were most similar to each other based on location. They also clustered together regardless of the depth or maize phenology. (Figure 1-5). As was done with bacterial samples, fungal samples were analyzed both by all maize phenologies, as well as by the active growing season alone. Again, the unweighted fungal jaccard matrix was used to plot sample dissimilarity for fungal PCoA. As was seen with bacterial samples during the growing season, fungal samples differed primarily by the location from which they were taken, rather than depth or developmental state. (Figure 1-6)

### *All Variables Affect Bacterial Community Composition*

The three variables location, depth, and phenology contributed significantly ( $P < 0.01$ ) to differential population abundances among the bacterial phyla, when considering samples from all time periods. There were also significant ( $P < 0.01$ ) interactions between location and phenology, as well as depth and phenology, that contributed to differential population abundances. Nitrogen was not found to significantly contribute to differential population abundances. (Table 1-3)

However, when considering the active growing season only (phenologies 1-4), all four variables contribute significantly ( $P < 0.01$ ) to differential population abundance. However, the only significant interaction in the active growing season is phenology and location. (Table 1-4)

Differentially abundant bacterial families at each combination of location soil and maize developmental state tended to cluster together more based on developmental timepoint rather than location. Earlier points in development were highly similar across samples, although the later points in development had some variation. Notably, the families *Gemmatimonadaceae*, *Tepidisphaeraceae*, *Xanthobacteraceae*, and *Flavobacteriaceae* tend to cooccur and are predominantly found in all location fields after ear initiation begins, being especially abundant in the period between ear initiation and tasseling. Additionally, the families *Comamonadaceae*, *Cytophagaceae*, *Desulfurellaceae*, *Nitrosomonadaceae*, and *Planctomycetaceae* are primarily abundant during the period following tasseling. Finally, the families *Gaiellaceae*, *Bacillaceae*, *Rhodospirillaceae*, and *Nitrospiraceae* are preferentially abundant during early ear initiation at all farms. (Figure 1-7)

### *Fungal Community Composition Affected by Location and Depth*

All fungal variables and variable interactions that are significant ( $P < 0.01$ ) are listed in Table 1-5. When considering all sampling periods, all variables found to be significant for bacteria were also found to be significant for fungi as well. However, in contrast with bacteria, when considering only the active growing season, nitrogen does not affect the fungal populations. The growing season fungal samples also differ from the bacteria in their significant interactions. Phenology by depth was identified as significant, as was location by depth. (Table 1-6)

Differentially abundant fungal species tended to cluster together primarily based on location, rather than developmental timepoint. However, within each location, samples were distributed somewhat on developmental period. Notably, *Cercophora samala*, *Paraphoma chrysanthemicola*, and *Minimedusa polyspora* are preferentially abundant only in the claypan soil, while *Mortierella samyensis* is abundant not only in the claypan soil, but also the loess. The alluvial soil had relatively high abundances of *Sordaria fimicola* and *Acremonium persicinum*, while loess uniquely had high abundances of *Trichoderma spirale* and *T. piluliferum*. (Figure 1-8)

## DISCUSSION

The variable “location” represents numerous variables. In effect, it is a composite of different soil parent material, precipitation, wind, and temperature. It is by far the least precise variable. What is particularly interesting about this, is that while it does have a strong effect on some microbial populations, it doesn’t determine the microbial communities as much as would be expected. While the fungal samples were most affected by location, within each location they are very similar to each other based on the developmental timepoint. Additionally, the bacterial samples were most affected by developmental period when considering all sampling periods, with location contributing primarily to sample similarity during the active growing season.

Fungal taxonomic assignment had excellent OTU identification, with only approximately 15% of OTUs being unassigned or unidentified at a class level or higher taxonomic level. Recently, Smart used a combination of the fungal UNITE ITS database and an oomycete specific database (Sapkota and Nicolaisen, 2015) to improve taxonomic assignment of fungal ITS OTUs (Smart, 2018). This combined use of fungal and oomycete database allowed many initially misidentified basidiomycetes to be correctly assigned as oomycetes, drastically improving overall taxonomic assignment. However, when we attempted to test this same technique on our fungal ITS data, not only was oomycete assignment very poor, but many OTUs that had previously been assigned a fungal taxonomy with high confidence were unable to be assigned to any specific group, with overall unassigned or unidentified OTUs now comprising approximately 40% at a class level or higher taxonomic level. While combining databases



seemed to work for Smart, in our data this was not found to be the case, and fungi were assigned taxonomy using the UNITE ITS database alone.

General bacterial populations were found to be highly dependent on developmental timepoint of the maize. Because of planting date, soil, and local weather variation, the maize crop from some fields developed faster than others. Because of this, developmental timepoint and sampling periods were not necessarily the same. Therefore, while highly related to each other, the sampling period and developmental timepoint at sampling were able to be used as distinct variables. This is important because in addition to analyzing sample dissimilarity in PCoA through the lens of developmental timepoint, we also analyzed it through the lens of sampling period. Understandably, the samples clustered quite heavily by sampling period, however, they were not as clearly differentiated as developmental period. This leads us to believe that it is indeed the developmental state of the plant that causes community wide changes in bacteria, rather than simply the time of year.

General fungal sample composition was found to be dependent on location, depth, and developmental timepoint. However, of these variables, by far the most influential was location, which is not especially surprising, given that other studies have found similar results (Smart, 2018). However, the most interesting difference between fungi and bacteria is the role of nitrogen in each population. When bacterial populations were analyzed as a whole, nitrogen was not identified as a significant factor. Although, when limiting the samples to just the active growing season, nitrogen became a heavy influencer of the bacterial populations. The fungal populations also are not influenced by nitrogen during the whole sampling timeline, or when only considering the active growing season. This provides strong indication that fungal

populations are not nitrogen limited, whereas bacterial populations are. When nitrogen is applied to corn fields, bacterial populations change, although fungal populations are unaffected.

Regarding differential abundance of fungi and bacteria, the two populations grouped quite different from each other. Differentially abundant fungi grouped together first by location, then by developmental timepoint, while bacterial families grouped independent of location, and were more similar based on developmental timepoint. This is a strong indication that these specific bacterial taxa are driven more by their plant hosts over the course of the growing season, whereas the fungal taxa are more limited by which fungal spores and other survival structures are in the soil at the beginning of the growing season, and their populations change over time according to those initial limitations. It is especially reassuring that our data shows that bacteria grouped by developmental state of the plant, given that recent research agrees with this find. Pfeiffer et al. recently studied the rhizosphere microbiome of *Solanum tuberosum* under variable conditions (Pfeiffer, Mitter, et al., 2016). Specifically, the study considered various factors and environmental conditions, including variable soil characteristics, climatic conditions, elevation, agronomic practices, and developmental state. Through analysis on both taxonomic composition and core microbiome, Pfeiffer identified plant developmental state to be the greatest predictor of microbial composition. While Pfeiffer studied potato, we would presume that the same type of result would occur regardless of host plant.

The effects of location and developmental timepoint were not necessarily characteristic of the overall microbial communities. As bacterial PCoA showed, there is a strong effect by developmental timepoint on the bacterial populations, which does agree with the differentially

abundant bacterial families. However, as mentioned earlier, the fungal populations were somewhat more dissimilar from each other, although location was found to be highly influential, followed by depth and developmental timepoint. Therefore, not only were certain fungal species heavily affected by location, the community  $\beta$  diversity was as well, contributing to the evidence that location affects fungal populations far more than bacterial. This is not the first study to find this, as Smart identified similar patterns in fungal populations from Idaho (Smart, 2018). While Smart identified specific grouping patterns in fungal populations when comparing Idaho and Minnesota, within each state there were no statistical differences, as found in our study. While general trends at a class taxonomic level or higher are quite similar between samples, analysis at a species or OTU level reveals complete dissimilarity between samples.

However, there is a possibility that fungal community structure is more similar between samples when considering the functional role of individual microbes, rather than taxonomic or phylogenetic assignment. This study did not intend to identify or analyze the functional role of specific microbial taxa, although tools exist for this, such as FunGuild and PICRUSt, and others (Aßhauer, Wemheuer, et al., 2015; Langille, Zaneveld, et al., 2013; Manter, Korsa, et al., 2016; Nguyen, Song, et al., 2016). Other studies that have analyzed the microbiome, focusing on functional profiles rather than taxonomic, have found their samples to be more similar to each other than taxonomic analysis (Maherali and Klironomos, 2007; Sarathchandra, Ghani, et al., 2001). Future studies would likely benefit in investigating the functional role of soil microorganisms regarding diversity estimates and community profiling.

One of the limitations to this study is the inability to distinguish bulk soil from rhizosphere, since samples were taken from within the row of maize. Some samples may have contained more roots, and therefore more rhizosphere, while others contained more bulk soil. Fortunately, this limitation did not appear to affect our results and data analysis. This may be due to the high density of maize roots, especially during later stages of development (Tardieu, 1988), making each sample relatively similar in bulk soil/rhizosphere composition.

## CONCLUSION

While there are many biotic influences on microbial populations of Missouri maize field soils, not all contribute equally. Bacterial populations are heavily influenced by the developmental state of the maize, such that the communities at various points during the growing season are highly similar to each other, and bacterial communities outside of the growing season are highly similar to each other, but not the active growing season. Within the growing season, bacterial populations are location dependent, but also affected by nitrogen and depth. Total fungal populations are primarily influenced by location, although depth and maize developmental state both play roles. Additionally, individual fungal taxa are differentially abundant by location, and individual bacterial taxa are differentially abundant by maize developmental timepoint.

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## CHAPTER 2

### Effects of Microbial Populations on Maize Productivity

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#### ABSTRACT

The role of individual soil microorganisms changes over the course of a plant's life - microorganisms that have no discernable role at one developmental stage may affect the plant later in its growth. Traditional analysis of the soil microbiome, which has focused principally on the relative abundances (RA) of individual organisms, may be incomplete, as underlying differences in population size cannot be addressed. We conducted a metagenomic analysis of soil microorganisms from various maize (*Zea mays L.*) fields under commercial maize production systems in Missouri at two depths, accompanied by crop yield components, to provide insight into influences of edaphic microbes on maize productivity. This study assesses the influence of fungi and bacteria, not only in terms of RA, but also in their estimated absolute abundances (EAA), derived by combining the results of Illumina HiSeq sequencing data and PLFA abundance data. Significant interactions were identified between maize yield components and soil microbes at early ear development (V6-V8) and tasseling (VT), as these developmental periods have been shown to be highly influential on maize yield and quality. Most interactions between fungi and yield components were negative, with notable exceptions. The fungal pathogen – *Fusarium solani* – was negatively correlated with yield at all locations and depths, while the known biocontrol – *Clonostachys rosea* – was positively correlated with yield at both depths of two locations. Bacterial interactions were more complex, with most interactions

during early ear development identified as positive, and most interactions during tasseling identified as negative. However, there were exceptions, such as *Rubrobacteriaceae* which was negatively correlated with yield at all locations, both depths, and both developmental timepoints.

## INTRODUCTION

Soil microorganisms have significant roles in plant life cycles, from acting as regulators of growth (Hamidi, Chaokan, et al., 2009) to managers of nutrients (Adesemoye, Torbert, et al., 2009). These roles change over the course of the life of the plant - some microorganisms that have no discernable role at one developmental stage may affect the plant later in its growth. A soil microorganism that encourages vegetative growth may be beneficial during preflowering stages but could potentially have a negative effect on other characteristics not directly related to vegetative growth, such as delaying reproductive development (Adesemoye, Torbert, et al., 2009). Understanding the role of the microbiome at various points of plant development is useful, as it allows land managers to promote the growth of beneficial microbes and suppress harmful microbes at critical developmental stages.

Although the entirety of a crop's life directly or indirectly affects yield, stages of reproductive growth and development disproportionately contribute to yield (Cakir, 2004). In maize, two of these important stages are ear initiation and tasseling. While tasseling has a well-defined developmental point in traditional staging methods (VT), ear initiation does not. Typical ear initiation occurs around V6 (Stevens, Stevens, et al., 1986), and is characterized by the division of kernel rows, which ultimately determines the potential size of the ear (Abendroth,

Elmore, et al., 2011). While the number of potential kernels per ear is highly dependent on ear initiation, the number of harvestable kernels is especially dependent on the time period around VT, as this period is highly correlated with pollen shed and ultimately pollination success (Abendroth, Elmore, et al., 2011). Together, ear initiation and tasseling are key determinants of overall grain production.

Given the importance of these periods of reproductive development, they have been studied and researched extensively, especially focusing on external factors that improve or reduce grain yield or quality. It has been established that stressors, such as drought (Cakir, 2004) and heat (Wilhelm, Mullen, et al., 1999), around these critical developmental periods have detrimental effects on yield, however there have not been any studies that consider the positive and negative effects of the commonly occurring maize soil microorganisms at these time periods on yield or yield components. Plants and microbes have coevolved, with some microorganisms developing intricate relationships with their plant hosts that mutualistically benefit both organisms (Nihorimbere, Ongena, et al., 2011). Host plants are able to communicate with microbes through the release of root exudates, which recruit specific microbes, depending on the types, combinations, and concentrations of released root exudates (Bais, Park, et al., 2004; Walker, Bais, et al., 2003). It should be noted that while all plants prioritize establishing these relationships, maize has been shown to release up to 52% of all carbon transferred to the roots as a means of communicating with nearby microbes (Whipps, 1985). These recruited microorganisms are capable of conferring many beneficial properties to their hosts, including increased nutrient uptake (Adesemoye and Kloepper, 2009), promotion of vegetative and reproductive growth (yield) (Xie, Zhang, et al., 2009), and tolerance of numerous

abiotic stressors. These complex relationships between microorganisms and plants allow for significant increases in plant productivity and health.

While a few studies have assessed the interactions between microbial populations in maize soils and their plant hosts, there have been limitations. One study focusing on the effects of plant growth promoting rhizobacteria (PGPR) found that PGPRs did affect maize phenology in numerous beneficial ways, such as the acceleration of tasseling and silk emergence, as well as increases in yield in some hybrids (Hamidi, Chaokan, et al., 2009). However, nearly all PGPRs are introduced microbial strains which are not otherwise found in established maize soils and may not be viable in all agricultural soils (Bashan, Holguin, et al., 2004; Burr, Schroth, et al., 1978; Kloepper, 1992; Lifshitz, Kloepper, et al., 1986). Li et al. analyzed the effects of nitrogen and phosphorous fertilization on bacterial communities over the course of a maize growing season, finding changes in microbial biomass only in early stages of development (Li, Liang, et al., 2008). Although biomass remained relatively the same, bacterial counts and diversity were shown to change in response to different fertilization treatments and environmental factors. However, the study utilized colony forming unit (CFU) enumeration for density analysis, which is unable to address community wide changes in microbial density due to the limitation that only approximately 1% of soil bacteria are culturable, meaning that the vast majority of bacteria are ignored entirely (Handelsman, 2004; Hugenholtz, Goebel, et al., 1998; Schmeisser, Steele, et al., 2007; Ward, Weller, et al., 1990). While these studies have certainly assessed aspects of the role of soil microbes in agricultural soils and their environment, they do not attempt to explore the interactions between the established soil microorganisms and the plants with which they grow.

Although many studies focus on the effects of PGPRs and other introduced soil microbes, they still provide valuable insight into the potential benefits that established microorganisms could confer on their plant hosts. One of the most common plant properties gained through these microbial associations is an increase in vigor and overall growth. Xie et al. showed that after an *Arabidopsis* population was exposed to volatile emissions of the beneficial soil bacterium *Bacillus subtilis* for 3 weeks, not only was the plant fresh weight double the weight of unexposed controls, but the dry plant weight was also double the weight of the unexposed controls (Xie, Zhang, et al., 2009). Additionally, exposed plants had more rosette leaves than the unexposed controls and approximately 50% more siliques. While increases in plant biomass are useful for plants whose vegetative tissue is the primary food product, most cultivated plants are not grown for their vegetative tissue, rather for their reproductive structures. The increase in *Arabidopsis* silique count demonstrates the capacity of soil microorganisms to increase reproductive growth, and therefore yield. In order for microbes to be particularly useful, they will need to be capable of increasing yield, as this is generally the most useful trait that a microorganism can confer on an agronomically important host, under otherwise adequate growth conditions.

Additionally, soil microorganisms have been shown to improve the nutrient acquisition of their plant hosts. Adesemoye et al. demonstrated this in a greenhouse study using tomato plants inoculated with both bacterial and fungal plant symbionts (Adesemoye, Torbert, et al., 2009). In this study, inoculated plants were supplemented with only 75% of the recommended fertilizer rate, yet were just as effective in nutrient acquisition and fruit production as the uninoculated plants with the full fertilizer rate, while uninoculated controls had significantly



reduced nutrient uptake at the 75% fertilizer rate. In addition to this research, Cavagnaro et al. established differences in tomato nutrition in a field study (Cavagnaro, Jackson, et al., 2006). Rather than focusing on both bacterial and fungal inoculants, arbuscular mycorrhizal fungi (AMF) were exclusively assessed, specifically on their effects on plant nutrition. While both inoculated and uninoculated plants had similar aboveground biomass, fruits from inoculated plants contained 41% more phosphorous and 24% zinc than their uninoculated counterparts. Shoots and vegetative tissue of inoculated plants also had higher concentrations of important nutrients, including nitrogen, phosphorous, and zinc. Improved nutrient acquisition can reduce the amount of fertilizer required for crop production, as well as improve the quality of the crop.

As previously mentioned, many studies have detailed the effects that soil characteristics have on microbial population structure. Of these, some of the most influential have been shown to be type (Bossio, Scow, et al., 1998; Garbeva, Van Veen, et al., 2004), texture (England, Lee, et al., 1993), organic matter content (Doran, 1980; Fontaine, Mariotti, et al., 2003; Haines and Uren, 1990), and pH (Anderson and Domsch, 1993; Rousk, Bååth, et al., 2010). Unfortunately, many of these are qualities that are impractical to improve in soils, such as type or texture, or they may take a long time to improve, such as organic matter. However, they are useful in ensuring the preservation of healthy microbial populations in currently arable land.

In addition to the abiotic variables, the host plant community greatly determines which microorganisms are selected through the release of microbe specific exudates. Some hosts exhibit a high degree of microbial symbiont specificity, such as the rhizobia-legume interaction (Downie, 2010), while others are far more versatile, such as the colonization of roots by various forms of AMF. As Berg and Smalla highlight, there is diversity even within different hybrids and

cultivars of the same crop (Berg and Smalla, 2009). This presents clear evidence that not only are there microbial influences on plants, but there are complementary and reciprocal influences on microbes by their plant hosts, resulting in a two-sided conversation.

Given the role of microbes and their influence on plants, it is imperative to accurately measure and identify microbes and their differences between samples. However, as previously mentioned, culture-based techniques are inadequate and imprecise sources for determining which microbes are present in samples. High-throughput metagenomics is able to overcome this limitation by using variable regions of DNA to not only identify all microbes present, but also the relative abundance (RA), which is the percent of the sample that is made up by any given organism (Schmeisser, Steele, et al., 2007). RA is very useful and is the standard method of metagenomic microbial analysis, however it may have limitations. Because RA is all based on percentages of an unknown total, it cannot address actual abundances in a sample (Zhou, He, et al., 2015). Samples with identical RAs may have drastically different biomasses or cell counts. RA provides standardized and reliable information about microbial populations; however, it does not intend to address changes in biomass, and is therefore unable to analyze changes in actual microorganism abundances.

Analysis of actual abundances would allow for additional relationships to be discovered in microbial samples. Recently, Zhang et al. demonstrated how to generate absolute abundance data by combining RA with culture-independent methods of quantification (Zhang, Qu, et al., 2017). One such measurement was phospholipid fatty acids (PLFA), which was combined with RA to produce estimates of absolute abundance (EAA). These estimates were comparable with other methods of determining absolute biomass, including quantitative real-time PCR, microbial

biomass carbon, and adenosine tri-phosphate. This study demonstrated that absolute quantities are essential for a more comprehensive analysis and understanding of microbial communities.

The primary purpose of this study is to define significant interactions between the maize yield components and soil microbes at ear initiation and tasseling, under commercial maize production systems in Missouri, and secondarily, determine whether estimated absolute abundance revealed considerably more about microbial interactions than relative abundance alone. Therefore, our objectives are: 1) identify specific organisms and taxonomic groups that are highly correlated with yield and yield components, given maize phenology and site variation, and 2) compare RA and EAA, identify differences between the two, and determine the usefulness of EAA in this study.

## MATERIALS AND METHODS

### *Sampling Design*

This research came from a subset of three field sites that were a part of a 49-site research project over 8 US states and three growing seasons focused on performance and improvement of nitrogen fertilizer recommendation tools (Kitchen, Shanahan, et al., 2017). Soil cores were collected in 2016 from three farm fields in Missouri as described by Kitchen et al. Each farm was divided into eight replicates, with four of the replicates receiving 200 lbs of ammonium nitrate fertilizer before planting, and the other four remaining unfertilized. Each replicate was split into two depths, 0-5 cm and 5-15 cm, with a sample taken at each depth, resulting in two samples per replicate. Samples were collected by taking multiple cores with a hand-probe from each replicate and combined to represent the sampled area. Cores were taken both between and within rows, such that samples contained variable mixes of rhizosphere and bulk soil. This was done six times over a period of approximately 4 months, with the first occurring shortly before planting and the last occurring between tasseling (VT) and blistering (R2), depending on the location. Immediately following sample collection, soil samples were stored at the University of Missouri in a -20 C freezer, and upon collection of all time periods, samples were shipped on ice to Brigham Young University, where they were stored in a -20 C freezer until further processing.

### *DNA Extraction*

Soil biota DNA was extracted using the MoBio PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following standard kit protocols with slight modifications as

recommended by Lindahl (Lindahl, Nilsson, et al., 2013). Samples were homogenized with a Vortex-Genie 2 Mixer (Scientific Industries, Bohemia, NY, USA) at a setting of 10 for 15 minutes. Templates were quantified with an ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and then concentrated to 30 ng/μl.

### *Sequencing*

Two amplicon libraries were generated to assess the different populations of fungi and bacteria. Methods for fungal ITS1 sequencing are described by Smart. Briefly, the ITS1 region was amplified using a two step-PCR approach, consisting of an initial amplification of metagenomic ITS1 DNA using the ITS1F\_KYO1 and ITS2\_KYO2 primers (Toju, Tanabe, et al., 2012), as well as attachment of Illumina adapters, followed by a second PCR to ligate the Illumina adapters of the first amplification with barcodes for demultiplexing (Smart, 2018). Methods for bacterial V4 sequencing are also described by Smart. Briefly, the V4 region of the 16S was amplified using the 16Sf and 16Sr primers in a single amplification, in which the primers contained a series of repeating 8-bp barcodes which facilitated a dual-indexed sequencing approach (Caporaso, Lauber, et al., 2012; Kozich, Westcott, et al., 2013; Smart, 2018). Samples were submitted to Brigham Young University where they underwent further quality control. Paired-end sequencing (2x250) was carried out on the Illumina HiSeq 2500 platform (2x250) (Illumina Biotechnology, San Diego, CA, USA) at Brigham Young University.

### *Soil Analysis and Weather Data Collection*

Soil analyses were performed by the University of Missouri Soil Health Assessment Center. Soil properties of interest and method used included texture (pipette), cation exchange capacity (ammonium acetate), total organic C (dry combustion), organic matter (dry combustion), pH (saturated paste), and bulk density (core). Other parameters and methods used included grain N (dry combustion), tissue N (dry combustion), and N uptake (calculations based on biomass and N concentrations). Weather data for the growing season was collected with HOBO (model U30) weather stations (Onset Corporation, Bourne, MA) located at each site. Raw and summarized data were uploaded to a DuPont Pioneer cloud server, as DuPont Pioneer had assumed a central leadership role. These data were then quality checked against interpolated temperature data from Multi-Radar/Multi-Sensor (MRMS) rainfall data. Any outliers and/or missing values were identified and replaced by the interpolated temperature or MRMS rainfall estimates. For more detail, refer to Kitchen et al. (Kitchen, Shanahan, et al., 2017).

### *Phospholipid Fatty Acid Analysis*

A total of 93 PLFA were detected and identified. As recommended by Frostegård, absolute biomass was calculated by combining the biomass of multiple PLFA types together (Frostegård and Bååth, 1996), using the MIDI Sherlock system for PLFA biomarkers (MIDI, Newark, DE, USA). Total bacterial biomass incorporated the following groups (Table 2-1): Gram-negative, Gram-positive, and actinobacteria. Total fungal biomass incorporated the following groups: AM fungi and fungi. Bacterial cell counts were then calculated by using a standard

conversion factor of  $1.40 \times 10^{-8}$  nmol bacterial PLFA cell<sup>-1</sup>. Fungal cell counts were not determined and remained as individual PLFA counts.

### *Data Analysis*

Illumina sequence reads were demultiplexed according to Illumina protocol at the Brigham Young University sequencing center. Demultiplexed paired-end sequence reads were imported to QIIME 2 (v. 2.2018.6), available at <https://qiime2.org/> (Caporaso, Kuczynski, et al., 2010). Within QIIME 2, sequences underwent quality filtering, chimera removal, and were used to create a feature table. This was done using the QIIME 2 plugin DADA2 (Callahan, McMurdie, et al., 2016). Taxonomy was assigned to bacteria using the SILVA 18S 128 QIIME release (Quast, Pruesse, et al., 2012). Taxonomy was assigned to fungi using the UNITE ITS database (Kõljalg, Larsson, et al., 2005).

Fungal and Bacterial OTU tables and taxonomic groupings were produced in QIIME2, along with other similar files, then were exported and analyzed by R (Team, 2018), through RStudio (Team, 2016). Exported files were then combined into a single object using the Phyloseq package (McMurdie and Holmes, 2013). Analysis for the bacterial EAA was the same as above with three additional steps. First, OTU tables were corrected for 16S copy number at the family level via the Greengenes database (McDonald, Price, et al., 2012) and the PICRUSt analysis (Langille, Zaneveld, et al., 2013). Second, PLFA data was converted into cell counts using standard conversion factors (Frostegård and Bååth, 1996). Finally, bacterial cell counts were applied to RAs as described by Zhang et al (Zhang, Qu, et al., 2017). Fungal EAA generation was similar to bacterial EAA generation, although with fewer steps. Copy number correction

was unnecessary, and since there are no standard conversion factors to convert fungal PLFA data into cell counts, PLFA biomass alone was used (Frosteegård, Tunlid, et al., 2011). Therefore, fungal EAA was generated by combining biomass and RA alone.



## RESULTS

### *Upper Soil Depths have Greater Bacterial Biomass than Lower Soil Depths*

Biomass was combined with the copy number corrected 16S RAs, producing estimated absolute abundances (EAAs) for bacteria. Whereas RAs appeared to be similar across samples, EAAs appeared to be very different. Notably, the upper depth of 0-5 cm consistently had larger EAAs for all bacteria when compared to the lower depth of 5-15 cm. Additionally, the loess soil had considerably lower EAAs when compared with the claypan and alluvial soils. This was true regardless of the higher taxonomic groups (phylum, class, order) studied (Figure 2-1)

### *Upper Soil Depths have Greater Fungal Biomass than Lower Soil Depths*

Fungal biomass was combined with the ITS RAs, producing fungal EAAs. As with the bacterial RAs and EAAs, fungal EAAs appeared to vary from their RAs. The upper depth of 0-5 cm had larger EAAs for all fungi when compared to the lower depth of 5-15 cm; the alluvial soil varied most, with the average upper depth containing 300% more PLFA (8262 nmol/g) than the average lower depth (2052 nmol/g). Next was the claypan soil, with the average upper depth containing approximately 100 % more PLFA (7243 nmol/g) than the average lower depth (3267 nmol/g). Finally, the loess soil was the most similar between depths, with the average upper depth containing approximately 50% more PLFA (5336 nmol/g) than the average lower depth (3405 nmol/g). While the loess soil also had larger EAAs in the upper depth compared with the lower, it was less extreme (5336 nmol/g) than either the claypan (7243 nmol/g) or alluvial (8262 nmol/g). (Figure 2-2)

### *Bacterial Correlations with Yield Depend on Developmental Period*

Correlation analysis of bacterial relative abundances revealed that there are more positive associations during the EI period, shown in blue, such as the families *Phycisphaeraceae*, *Paenibacillaceae*, and *Acidimicrobiaceae* (Figure 2-3). However, there were mostly negative associations during the VT period, shown in red, such as the families *Phaselicytidaceae*, *Parachlamydiaceae*, and *Rhodobiaceae*. Additionally, the families that have an effect during both time periods tended to have the same correlation during both. Notably, while a bacterial family may be correlated with a yield component at all three locations, never was a family correlated with a yield component at all three locations at the same depth. For example, *Thermoactinomycetaceae* was identified as having a negative correlation during early ear development at the claypan soil in the upper depth of soil, and the alluvial and loess in the lower depth of soil. (Figure 2-3)

Even more than the RA, there tend to be more positive correlations at the early period of reproductive development, and negative effects at the later point of development. Again, the direction of the correlations did not change between developmental periods or depths, although there were fewer instances in which a family was correlated at both time periods. However, fewer families were identified than the RA correlation, and there were multiple cases in which a family was correlated with a yield component at all three locations and the same depth. One such family, *Rubrobacteriaceae* was negatively correlated at all three locations during both periods of development. Additionally, *Cyclobacteriaceae* and *Blastocatellaceae* were negatively correlated at all three locations during tasseling. (Figure 2-4)

### *Fungal Correlations with Yield are Mostly Negative*

Fungal correlations are mostly negative, also shown in red, with notable exceptions (Figure 2-5). First, *Latorua caligans* was identified as being significantly correlated at all three locations in the upper depth of soil. However, during ear initiation it was negatively correlated with yield, but during tasseling it was positively correlated. The other exception was *Clonostachys rosea*, a known biocontrol of nematodes and fungi (Yu and Sutton, 1997; Zhang, Yang, et al., 2008), which was positively correlated with yield at both the loess and alluvial soils. Of the other fungal correlations, two of the notable species were *Fusarium solani* and a *Cladosporium* species, both of which are known maize diseases. *Fusarium solani* was negatively correlated with yield at all three locations during tasseling, while *Cladosporium* was negatively correlated only in the loess soil during ear development. (Figure 2-5)

As with RA, most correlations between fungi and yield components were negative. Again, *Clonostachys rosea* was an exception, although it was only positively correlated during the period of ear development. *Latorua caligans* was also different from other groups by being initially negatively correlated, then positively correlated with yield, although not in the claypan soil. Another exception to the negative correlations is an unidentified *Mortierella* species, which is positively correlated with yield, in the upper depth of soil. (Figure 2-6)

## DISCUSSION

Traditional bacterial analysis has focused on percentages of the 16S rRNA gene to determine patterns and community structure. However, there may be limitations to analyzing

samples this way. First, since the abundance results are given simply as a percentage or proportion of the total sample (relative abundance), the actual size of the community is unknown. Two samples with drastically different biomasses may have highly similar relative abundances, which traditional analysis would view as being very similar, if not indistinguishable from each other. Second, since bacteria contain multiple copies of the 16S rRNA gene region, community analysis of 16S rRNA copies has the limitation that it is not necessarily an analysis of the microbes, but rather an analysis of the copies of the 16S rRNA gene region. By no means do we intend to say that 16S rRNA analysis is not a good method of studying microbial communities, quite the contrary – not only is it the standard method of assessing microbial communities, with nearly all studies on metagenomics using relative abundances from either the 16S rRNA gene, or one of the eukaryotic DNA regions, making all these types of studies comparable to each other, but the analysis performed on these microbial communities have been shown to contain useful, practical, and applicable information, ranging from insights in the human gut microbiome (Gill, Pop, et al., 2006; Zoetendal, Akkermans, et al., 1998) to improved techniques in soil management (Carbonetto, Rascovan, et al., 2014; Fierer, Lauber, et al., 2012) to a more complete understanding of changes in microbial populations during algal blooms (Williams, Wilkins, et al., 2013; Yang, Li, et al., 2015). Overall, relative abundance is a necessary component of metagenomic analysis, and we do not intend to suggest that it should or can be replaced using estimated absolute abundances. Rather, we hypothesize that estimated absolute abundances reveal additional insights into the microbial ecosystem, and our findings, along with other studies (Props, Kerckhof, et al., 2017; Zhang, Qu, et al., 2017), support this hypothesis.

Some may have a concern with the 16S copy number correction, such as Louca et al. recently discussed (Louca, Doebeli, et al., 2018). However, of the various methods of 16S correction, PICRUSt not only has more citations than the other methods of 16S copy number corrections by orders of magnitude (Angly, Dennis, et al., 2014; Bowman and Ducklow, 2015; Langille, Zaneveld, et al., 2013), but is recognized as the standard method of correction within the scientific community. Using this method of correction is by far the most reliable, not only because of its accuracy of the 16S copy numbers, but also because of the widespread use of PICRUSt in microbial literature.

Bacterial cell counts were generated by using a standard conversion factor of  $1.40 \times 10^{-8}$  nmol bacterial PLFA cell<sup>-1</sup> (Frostegård and Bååth, 1996), after having corrected for bacterial 16S copy number. This was not done however, for fungal biomass, given that while PLFA can be used as a good indicator of fungal biomass, there is no standard conversion factor for fungal PLFA per cell (Frostegård, Tunlid, et al., 2011). This is because fungal PLFA content is inversely related to the hyphal diameter of fungi, meaning larger hyphae have a lower density of PLFA than smaller hyphae, making cell count estimates imprecise (Frostegård and Bååth, 1996; Klamer and Bååth, 2004).

An important note to make with the following analysis is the understanding that these results are correlations, and unless stated otherwise, we do not intend to say that a specific microbe or taxonomic group causes a certain response, simply that these are observations of interest and provide direction for current analysis as well as future studies. However, while these results are simply correlations, these microbial relationships are still useful, as they provide a reasonable prediction of what overall yield and yield components may be at the end

of season. Using this data, other researchers can test maize soils for these microbial groups to see how their populations may be at a given developmental timepoint, and how it may relate with yield. Additionally, many of the identified microbial groups have literature supporting their interactions with maize and other plants. Between identifying strong interactions and supporting literature, we feel confident in designating some of the microbial groups as not only being correlated with yield or yield components, but as actually causing the noted change.

Regarding the correlation analysis of bacterial relative abundance, despite never being correlated at all locations at the same depth, the microbial groups consistently have the same positive or negative correlation. The one exception to this was the family *Haliangiaceae*, which was identified as having a positive correlation during EI at Ghebhart, then a negative correlation during VT at Troth. We believe this is an artifact of the correlation analysis, and while these relationships truly exist between *Haliangiaceae* and yield components, there is no underlying change in biology or ecological role of the family between the two developmental states.

The estimated absolute abundance data was considerably different and more separated by developmental timepoint and location than the relative abundance correlation analysis. Having taken abundance and 16S copy number into account for the correlation, it is not surprising that fewer families are identified as being significant for the bacteria. Adding more parameters that must be met should reduce the total identified. It is interesting however, that families' correlations were more consistent across locations, and that fewer families were significantly correlated at both developmental time periods when compared to RA.

While the correlations between yield components and specific families are important to explore and understand, it is far more interesting that EI tends to have more positive correlations and VT tends to have more negative correlations. We believe this reveals insight into how the relationship between microbes and maize changes as a function of plant development. These results indicate that bacteria are more likely to form a positive relationship with plants early on in reproductive development, which is supported by plant physiology studies demonstrating that plants actively release high amounts of root exudates during this period (Bais, Weir, et al., 2006).

While bacteria tended to have positive correlations during early ear development and negative correlations during tasseling, fungal species predominantly had negative correlations with yield components. While some of these relationships may be pathogenic in nature, we cannot confidently say that the decrease in yield is due to these organisms. Given that many of the listed species are facultative saprophytes (Ainsworth, 2008; Setälä and McLean, 2004), they may be simply decomposing maize that has already died for other reasons. If a plant were to die, saprophytes would begin decomposing it and therefore increasing in abundance, creating a negative correlation. Therefore, we cannot say with confidence that the negative correlations between these fungal species and yield are causal.

However, for fungal relative abundance, there were a few notable exceptions. Not only was the known biocontrol, *Clonostachys rosea*, (Zhang, Yang, et al., 2008) correlated with increased yield, but the other location soils in which it was correlated, loess and alluvial, were categorized as being more productive than the claypan soil. With this information, along with the known function of this fungal species, we believe that *Clonostachys rosea* causes higher

yield by suppressing pathogenic fungi and nematodes. Unfortunately, the other positively correlated species, *Latorua caligans*, lacks literature about its function and ecological role. Known to cause rot in maize, *Fusarium solani* (Morales-Rodríguez, de Yañz-Morales, et al., 2007) was negatively correlated with yield at all three locations, although only during tasseling. Along with *Clonostachys rosea*, we believe that *Fusarium solani* is one of the organisms that is not only correlated with yield, but is responsible for changes in yield. Given the pathogenic role of the fungus, along with the correlation analysis, we are confident that *Fusarium solani* decreases yield by harming maize during tasseling. Additionally, *Cladosporium*, which causes a form of ear rot, was identified as being correlated with yield decreases. However, since we were only able to identify the organism to a genus level, we do not feel confident that this species is responsible for the negative correlation.

Unlike the relationship between bacterial RA and EAA, fungal EAA correlations were not nearly as differentiated by developmental timepoint when compared with fungal RA. A notable genus, *Trichoderma*, was identified as having a negative correlation with yield. *Trichoderma* species are commonly used as biocontrols, which would have a positive correlation with yield. However, the species was identified as *piluliferum*, which has received very little attention regarding its potential as a biocontrol (Rekha, Patil, et al., 2012; Thakur and Harsh, 2014). Therefore, we believe the negative correlation of this species to be due to its ability to behave as a saprophyte.

Overall, there are many bacterial families and fungal species that we have identified as being highly correlated with yield. We recommend these microbial groups be further investigated for the yield changes they may potentially cause, as it relates to maize grain



productivity. We specifically recommend that microbial taxa with multiple correlations be prioritized, including the bacterial families *Shingomonadaceae*, *Sphaerobacteraceae*, *Rubrobacteriaceae*, *Cyclobacteriaceae*, and *Blastocatellaceae*, as well as the fungal genera and species *Latorua caligans*, *Mortierella*, *Minimedusa polyspora*, *Preussia terricola*, *Metarhizium marquandii*, and *Trichoderma piluliferum*.

Estimated absolute abundances did contribute additional findings to this study, although they were limited. When analyzing general community structure and composition, relative abundance and biomass individually revealed basically the same results as we found with the estimated absolute abundances. The main difference is whether those findings can be displayed in a single image, or if they need to be in two separate images. However, regarding bacterial correlation analysis, estimated absolute abundances were quite helpful – not only did they identify fewer correlated bacterial families, effectively trimming the results, but they also identified additional correlations of previously identified families, at previously uncorrelated locations and depths, strengthening the existing correlations.

If we only intended to perform analysis on community structure and composition, relative abundance would likely have been sufficient for this study. We would still recommend performing measurements of biomass, whether through PLFA or another measurement tool, as this metric did improve our overall understanding of how microbial groups are differentially abundant in the soil. However, for understanding correlations, combining biomass and relative abundance was very helpful for bacterial correlation analysis, and we recommend future studies consider using estimated absolute abundances, to both improve quality of results, as

well as contribute to the limited number of studies regarding the absolute quantification of microbial taxa.

## CONCLUSION

Of the causations that exist in these maize fields, certain bacterial families are promoting growth at the beginning of the ear development, while entirely different bacterial families are directly or indirectly harming the plant during the tasseling developmental stage. Additionally, of the fungi that influence maize, most of these interactions are negative. However, there are exceptions to these fungi; some fungal species appear to control maize diseases and pests.

Estimated absolute abundances contributed additional information to this study, although it was limited. Performing analysis of relative abundance and determining community biomass independently was as useful as combining them. Estimated absolute abundances revealed insights primarily regarding the bacterial correlation analysis. We recommend that future studies on microbial community analysis include them, as they may contribute additional findings.

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TABLES

Table 1-1. Soil Characteristics and Properties

Year	Location	Horizon	Depth Start (cm)	Depth End (cm)	Clay (%)	Silt (%)	Sand (%)	Cation-Exchange Capacity	Total Organic Carbon (%)	Organic Matter (%)	Nitrogen (%)	pH	Bulk Density
2016	Claypan	Ap	0	22	18.6	75.7	5.7	15.26	0.89	2	0.108	6.76	1.33
2016	Claypan	Btg1	22	41	41.7	55.3	3	27.64	0.62	1.96	0.077	5.59	1.38
2016	Claypan	Btg2	41	65	43.1	54.6	2.3	30.11	0.46	1.57	0.063	5.42	1.49
2016	Claypan	Btg3	65	89	33.45	63.45	3.1	26.05	0.21	1	0.039	5.77	1.58
2016	Claypan	Btg4	89	113	31.1	66.5	2.4	24.1	0.17	0.86	0.036	6.09	1.56
2016	Loess	Ap	0	17	21.8	72.9	5.4	17.16	1.42	2.7	0.146	6.58	1.47
2016	Loess	A2	17	33	27.1	68.6	4.3	19.6	0.79	2.02	0.091	6.41	1.53
2016	Loess	Bt1	33	52	29.5	66.4	4.1	21.1	0.58	1.72	0.075	6.58	1.48
2016	Loess	B2	52	79	27.8	68.4	3.8	21.35	0.4	1.34	0.06	6.72	1.44
2016	Loess	BC	79	118	25.9	70.3	3.8	20.87	0.23	1.04	0.045	6.73	1.46
2016	Alluvial	Ap	0	22	37.2	52.4	10.4	30.26	1.26	2.42	0.129	7.59	1.42
2016	Alluvial	A	22	42	42.9	49.7	7.4	32.49	0.92	2	0.095	7.67	1.51
2016	Alluvial	Bt	42	59	34.3	58.9	6.8	27.23	0.8	1.72	0.079	7.78	1.29
2016	Alluvial	Bq1	59	80	18.2	56.5	25.4	16.15	0.53	1.1	0.049	7.89	1.34
2016	Alluvial	Bq2	80	110	16.5	30	53.6	13.55	0.38	0.84	0.04	7.89	1.41



Table 1-2. Sampling Period Dates and Phenology

<b>Location</b>	<b>Sampling Period</b>	<b>Date</b>	<b>Phenology</b>
Loess	1	5/5/16	Pre-Ear
Alluvial	1	5/5/16	Pre-Ear
Claypan	1	5/6/16	Pre-Ear
Loess	2	5/19/16	Ear Initiation
Alluvial	2	5/20/16	Ear Initiation
Claypan	2	5/20/16	Pre-Ear
Loess	3	6/7/16	Between
Alluvial	3	6/6/16	Between
Claypan	3	6/7/16	Ear Initiation
Loess	4	6/24/16	Tasseling
Alluvial	4	6/23/16	Tasseling
Claypan	4	6/24/16	Between
Loess	5	7/8/16	Post-Tasseling
Alluvial	5	7/7/16	Post-Tasseling
Claypan	5	7/8/16	Tasseling

Table 1-3. Bacterial ADONIS results, all developmental points.

a: Pre-plant, Pre-ear, Ear Initiation, Between, VT, Post-VT; b: 0lbs, 200lbs; c: 0-5 cm and 5-15 cm; d: Claypan, Loess, and Alluvial

Number of permutations: 999	Df	SumOfSqs	MeanSqs	F.Model	R <sup>2</sup>	Pr(>F)	
Phenology <sup>a</sup>	5	1.9312	0.38624	21.1288	0.26	0.001	***
Nitrogen <sup>b</sup>	1	0.0505	0.05053	2.7641	0.0068	0.014	*
Depth <sup>c</sup>	1	0.4353	0.43527	23.8112	0.0586	0.001	***
Location <sup>d</sup>	2	0.3787	0.18935	10.3581	0.05098	0.001	***
Phenology:Nitrogen	5	0.1139	0.02278	1.2463	0.01534	0.145	
Phenology:Depth	5	0.238	0.04759	2.6034	0.03204	0.001	***
Nitrogen:Depth	1	0.02	0.02005	1.0966	0.0027	0.343	
Phenology:Location	9	0.8911	0.09901	5.4163	0.11997	0.001	***
Nitrogen:Location	2	0.0295	0.01475	0.8068	0.00397	0.65	
Depth:Location	2	0.039	0.0195	1.0669	0.00525	0.332	
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1							

Table 1-4. Bacterial ADONIS results, growing season.

a: Pre-ear, Ear Initiation, Between, VT; b: 0lbs, 200lbs; c: 0-5 cm and 5-15 cm; d: Claypan, Loess, and Alluvial

Number of permutations: 999	Df	SumOfSqs	MeanSqs	F.Model	R <sup>2</sup>	Pr(>F)	
Phenology <sup>a</sup>	3	0.5452	0.18172	10.1377	0.11497	0.001	***
Nitrogen <sup>b</sup>	1	0.063	0.06304	3.5168	0.01329	0.001	***
Depth <sup>c</sup>	1	0.4873	0.48727	27.1838	0.10277	0.001	***
Location <sup>d</sup>	2	0.3341	0.16706	9.3201	0.07047	0.001	***
Phenology:Nitrogen	3	0.0905	0.03018	1.6837	0.0191	0.027	*
Phenology:Depth	3	0.0856	0.02852	1.591	0.01804	0.033	*
Nitrogen:Depth	1	0.0253	0.02528	1.4102	0.00533	0.18	
Phenology:Location	6	0.4209	0.07015	3.9137	0.08877	0.001	***
Nitrogen:Location	2	0.0339	0.01694	0.9448	0.00714	0.513	
Depth:Location	2	0.0438	0.02189	1.2209	0.00923	0.218	
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1							

Table 1-5. Fungal ADONIS results, all developmental periods.

a: Pre-plant, Pre-ear, Ear Initiation, Between, VT, Post-VT; b: 0lbs, 200lbs; c: 0-5 cm and 5-15 cm; d: Claypan, Loess, and Alluvial

Number of permutations: 999	Df	SumOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Phenology <sup>a</sup>	5	4.681	0.9361	6.3551	0.09733	0.001	***
Nitrogen <sup>b</sup>	1	0.175	0.1746	1.1851	0.00363	0.203	
Depth <sup>c</sup>	1	1.05	1.0504	7.1308	0.02184	0.001	***
Location <sup>d</sup>	2	7.23	3.6151	24.542	0.15034	0.001	***
Phenology:Nitrogen	5	0.524	0.1048	0.7116	0.0109	0.992	
Phenology:Depth	5	1.328	0.2655	1.8025	0.02761	0.001	***
Nitrogen:Depth	1	0.105	0.1055	0.7162	0.00219	0.851	
Phenology:Location	9	3.395	0.3773	2.5612	0.0706	0.001	***
Nitrogen:Location	2	0.249	0.1245	0.8452	0.00518	0.764	
Depth:Location	2	0.534	0.2671	1.813	0.01111	0.002	**
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1							

Table 1-6. Fungal ADONIS results, growing season.

a: Pre-ear, Ear Initiation, Between, VT; b: 0lbs, 200lbs; c: 0-5 cm and 5-15 cm; d: Claypan, Loess, and Alluvial

Number of permutations: 999	Df	SumOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Phenology <sup>a</sup>	3	2.426	0.80858	4.9906	0.06729	0.001	***
Nitrogen <sup>b</sup>	1	0.186	0.18597	1.1478	0.00516	0.265	
Depth <sup>c</sup>	1	1.012	1.01151	6.2431	0.02806	0.001	***
Location <sup>d</sup>	2	5.078	2.53883	15.6698	0.14084	0.001	***
Phenology:Nitrogen	3	0.376	0.12532	0.7735	0.01043	0.928	
Phenology:Depth	3	0.7	0.23348	1.4411	0.01943	0.005	**
Nitrogen:Depth	1	0.149	0.14889	0.9189	0.00413	0.58	
Phenology:Location	6	2.104	0.35061	2.164	0.05835	0.001	***
Nitrogen:Location	2	0.334	0.16694	1.0304	0.00926	0.411	
Depth:Location	2	0.5	0.25008	1.5435	0.01387	0.019	*
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1							

Table 2-1. Microbial group assignments used by the MIDI Sherlock system for PLFA biomarkers

Microbial Group	Markers			
AM Fungi	16:1 w5c			
Fungi	18:2 w6c			
Gram Negative	10:0 2OH	10:0 3OH	12:1 w8c	12:1 w5c
	13:1 w5c	13:1 w4c	13:1 w3c	12:0 2OH
	14:1 w9c	14:1 w8C	14:1 w7c	14:1 w5c
	15:1 w9c	15:1 w8c	15:1 w7c	15:1 w6c
	15:1 w5c	14:0 2OH	16:1 w9c	16:1 w7c
	16:1 w6c	16:1 w4c	16:1 w3c	17:1 w9c
	17:1 w7c	17:1 w6c	17:0 cyclo w7c	17:1 w5c
	17:1 w4c	17:1 w3c	16:0 2OH	17:1 w8c
	18:1 w8c	18:1 w7c	18:1 w6c	18:1 w5c
	18:1 w3c	19:1 w9c	19:1 w8c	19:1 w7c
	19:1 w6c	19:0 cyclo w9c	19:0 cyclo w7c	19:0 cyclo w6c
	20:1 w9c	20:1 w8c	20:1 w6c	20:1 w4c
	21:1 w9c	20:0 cyclo w6c	21:1 w9c	21:1 w8c
	21:1 w6c	21:1 w5c	21:1 w4c	21:1 w3c
	22:1 w9c	22:1 w8c	22:1 w6c	22:1 w5c
	22:1 w3c	22:0 cyclo w6c	24:1 w9c	24:1 w7c
Gram Positive	11:0 iso	11:0 anteiso	12:0 iso	12:0 anteiso
	13:0 iso	13:0 anteiso	14:1 iso w7c	14:0 iso
	14:0 anteiso	15:1 iso w9c	15:1 iso w6c	15:1 anteiso w9c
	15:0 iso	15:0 anteiso	16:0 iso	16:0 anteiso
	17:1 iso w9c	17:0 iso	17:0 anteiso	18:0 iso
	19:0 iso	19:0 anteiso	20:0 iso	22:0 iso
Actinobacteria	16:0 10-methyl	17:1 w7c 10-methyl	17:0 10-methyl	20:0 10-methyl
	18:1 w7c 10-methyl	18:0 10-methyl	19:1 w7c 10-methyl	

## FIGURES

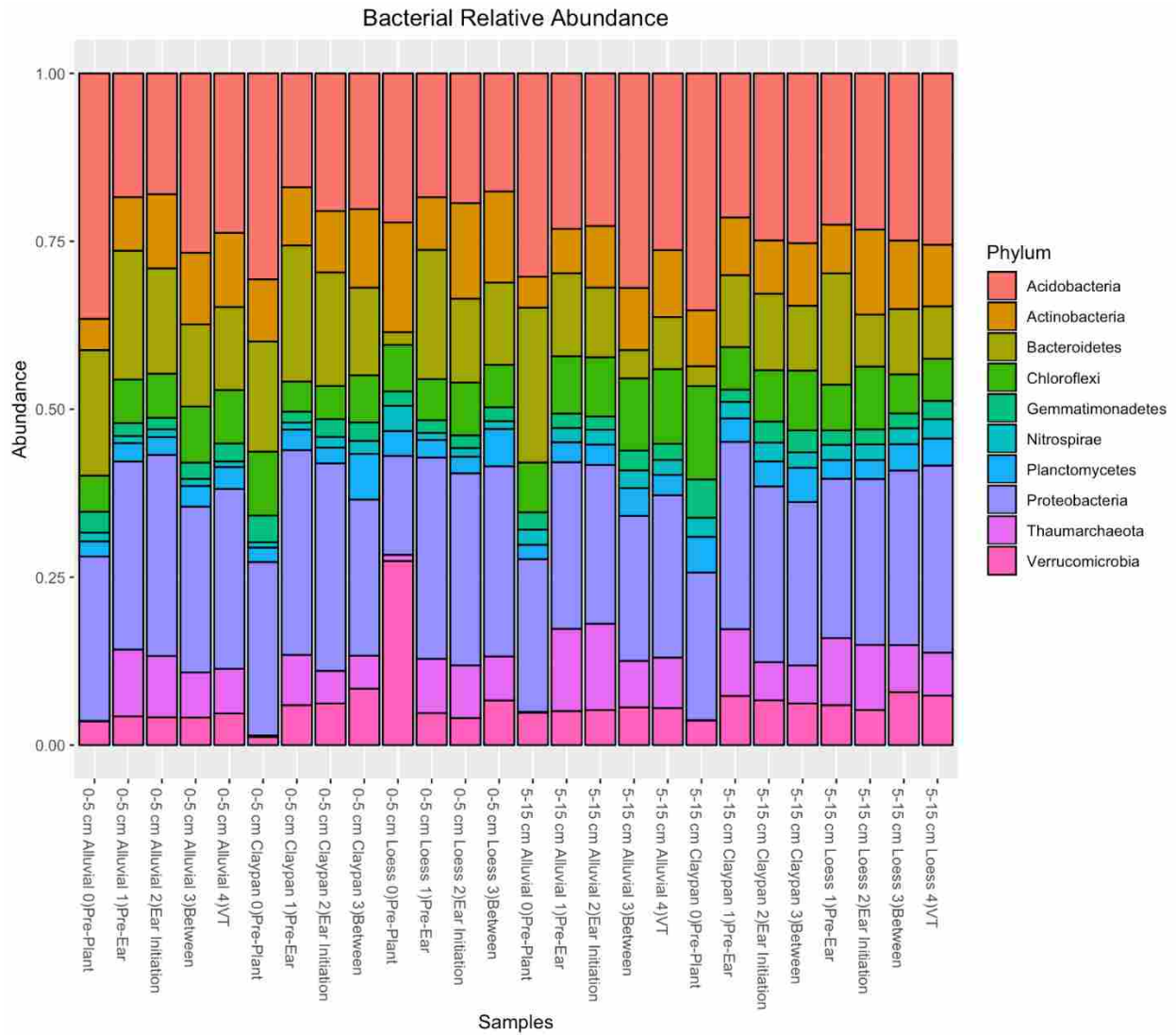


Figure 1-1. Relative Abundances of top 10 most abundant bacterial phyla across sample replicates.

Phylum level barplot displaying relative bacterial populations across all timepoints at each field location. Phylum level was selected as it allowed most taxa to be shown. All samples were rarefied to 15,000 reads and combined by location, depth, and developmental timepoint they were sampled. The top ten most abundant phyla were selected.

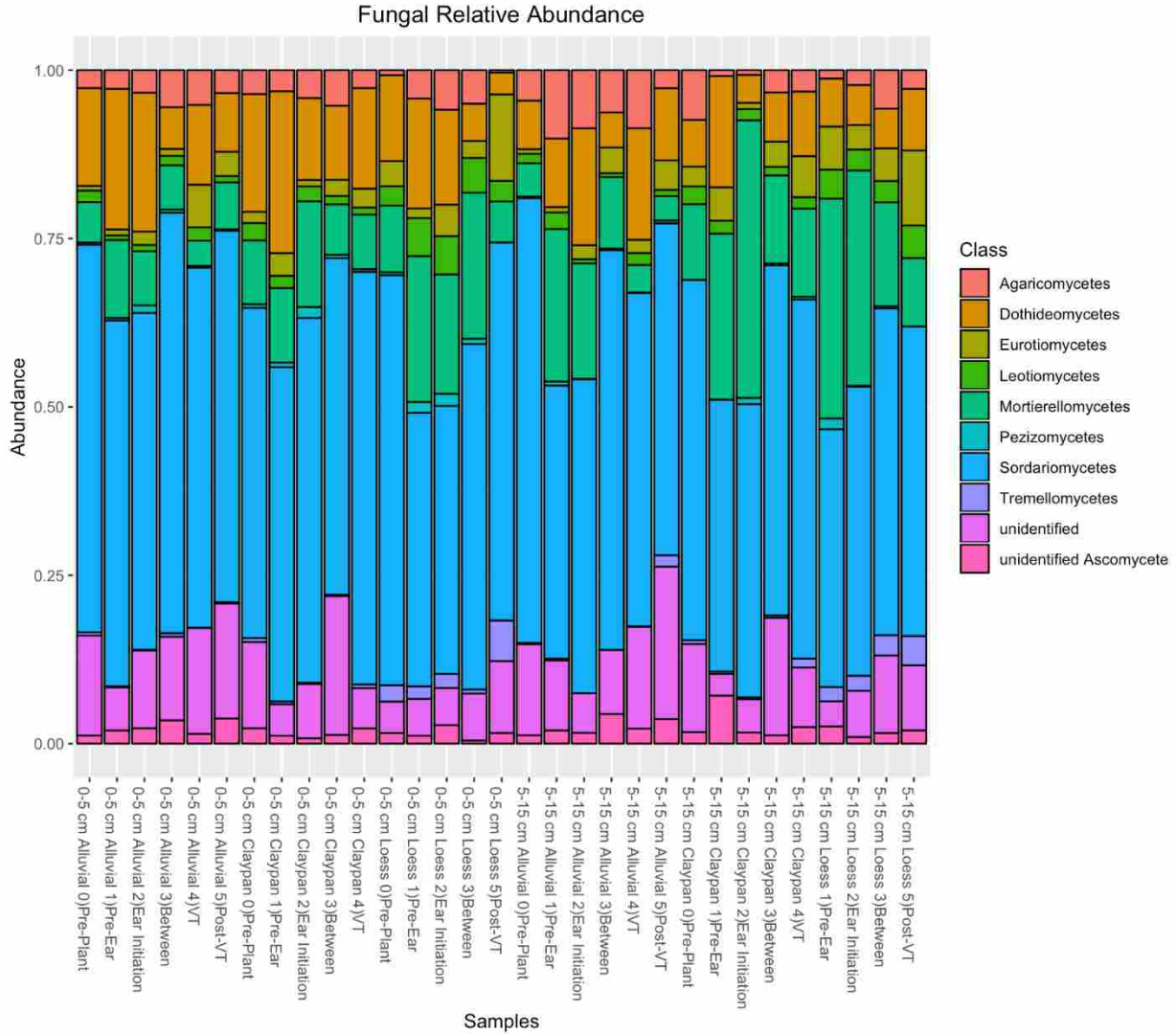


Figure 1-2. Relative Abundances of top 10 most abundant fungal classes across sample replicates.

Class level barplot displaying relative bacterial populations across all timepoints at each field location. Class level was selected as it allowed most taxa to be shown. All samples were rarefied to 15,000 reads and combined by location, depth, and developmental timepoint they were sampled. The top ten most abundant phyla were selected.



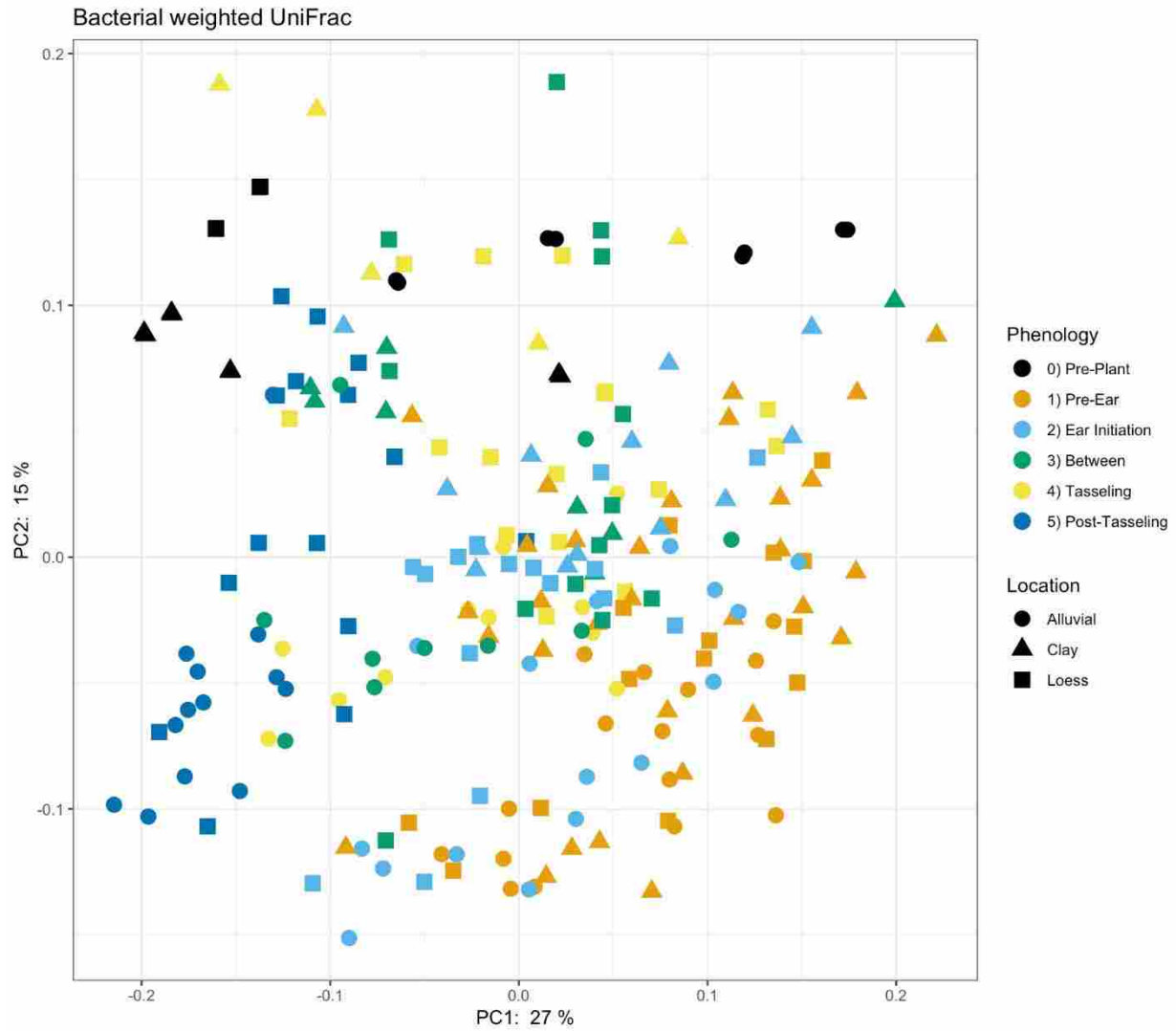


Figure 1-3. Bacterial weighted UniFrac PCoA results of all samples

PCoA plot representing the differences in populations between all samples, colored by developmental state of maize.

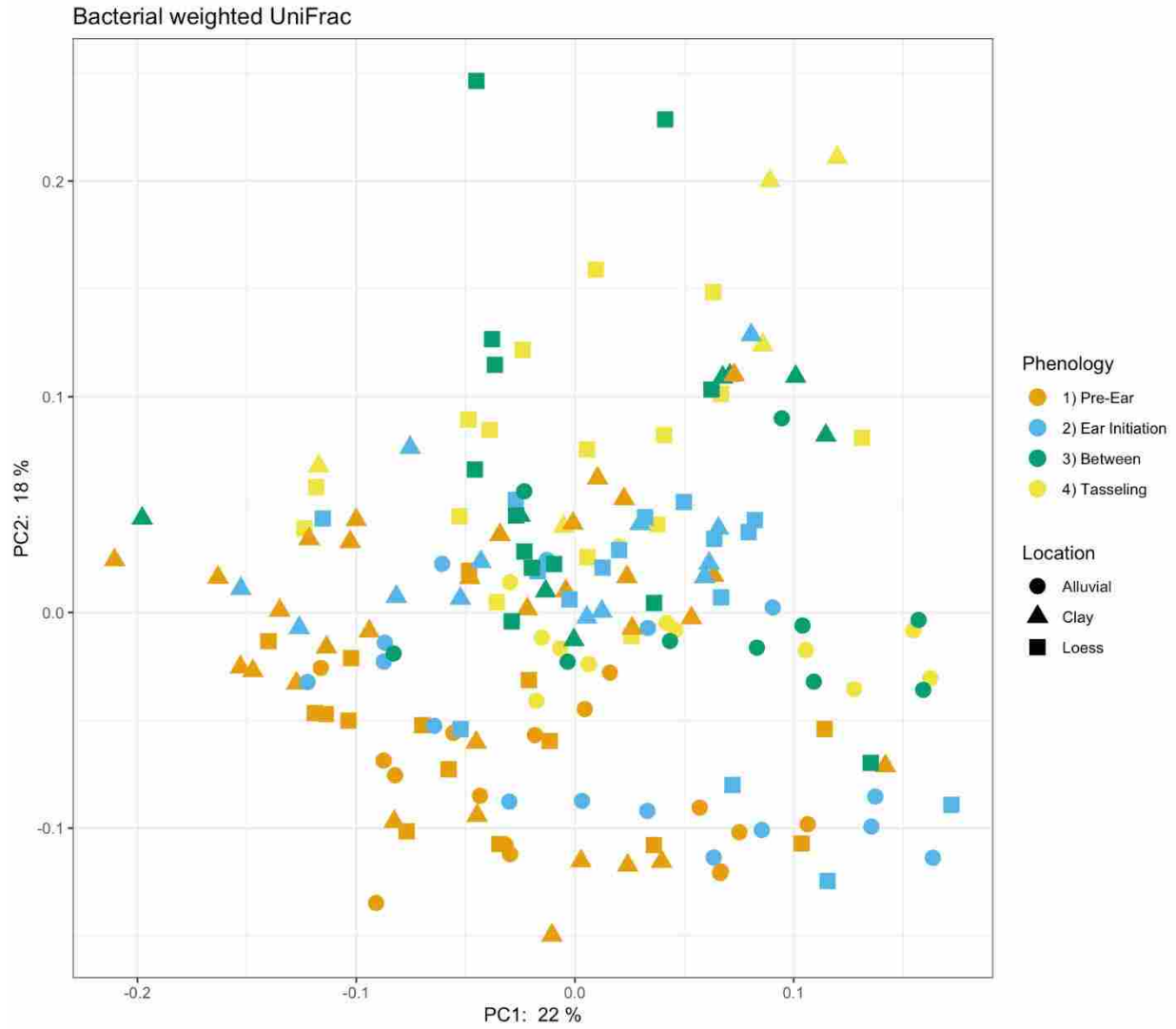


Figure 1-4. Bacterial weighted UniFrac PCoA results of active growing season

PCoA plot representing the differences in populations between samples taken during the active growing season, colored by developmental state of maize.

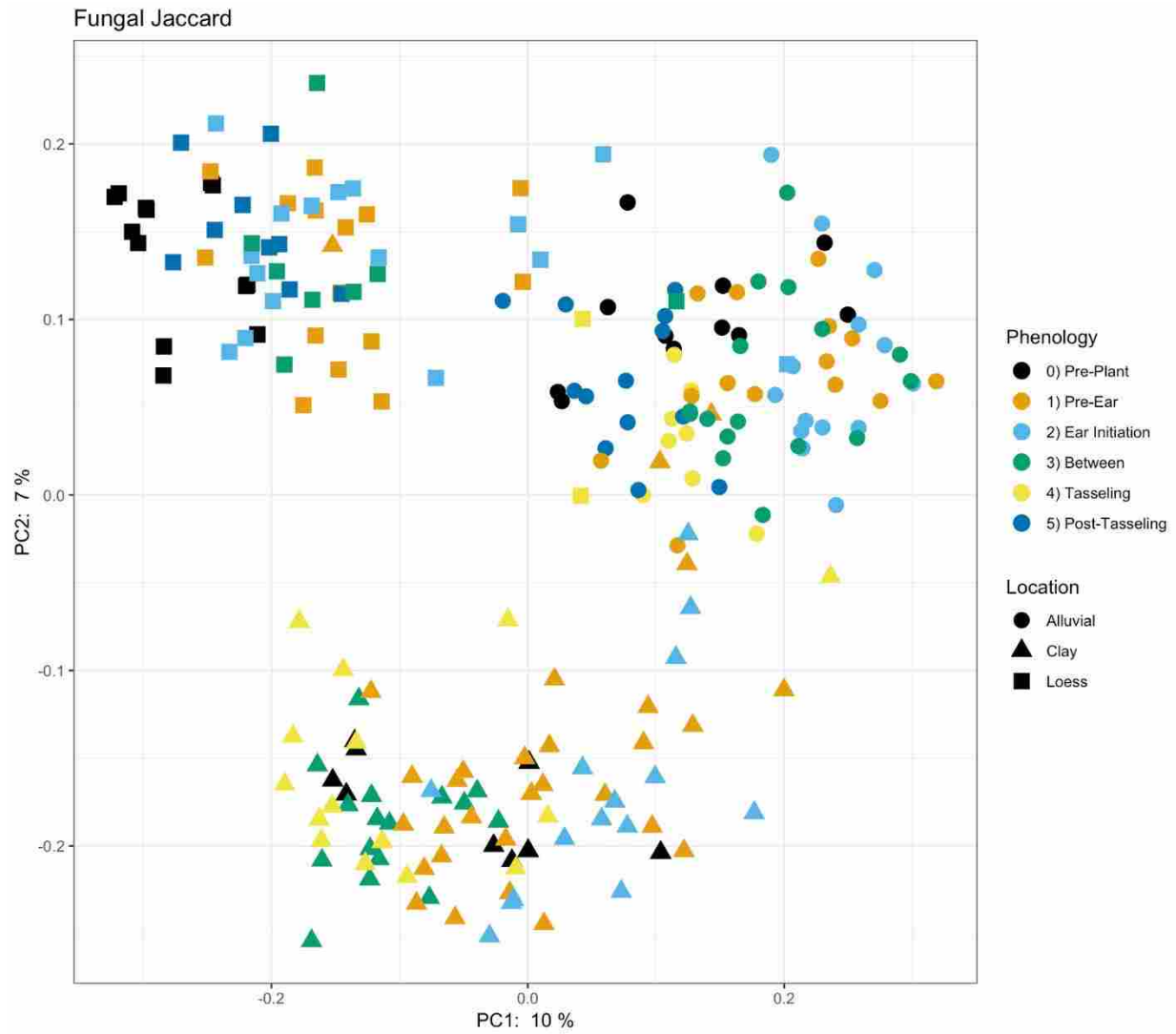


Figure 1-5. Fungal unweighted Jaccard PCoA results of all samples

PCoA plot representing the differences in populations between all samples, colored by developmental state of maize.

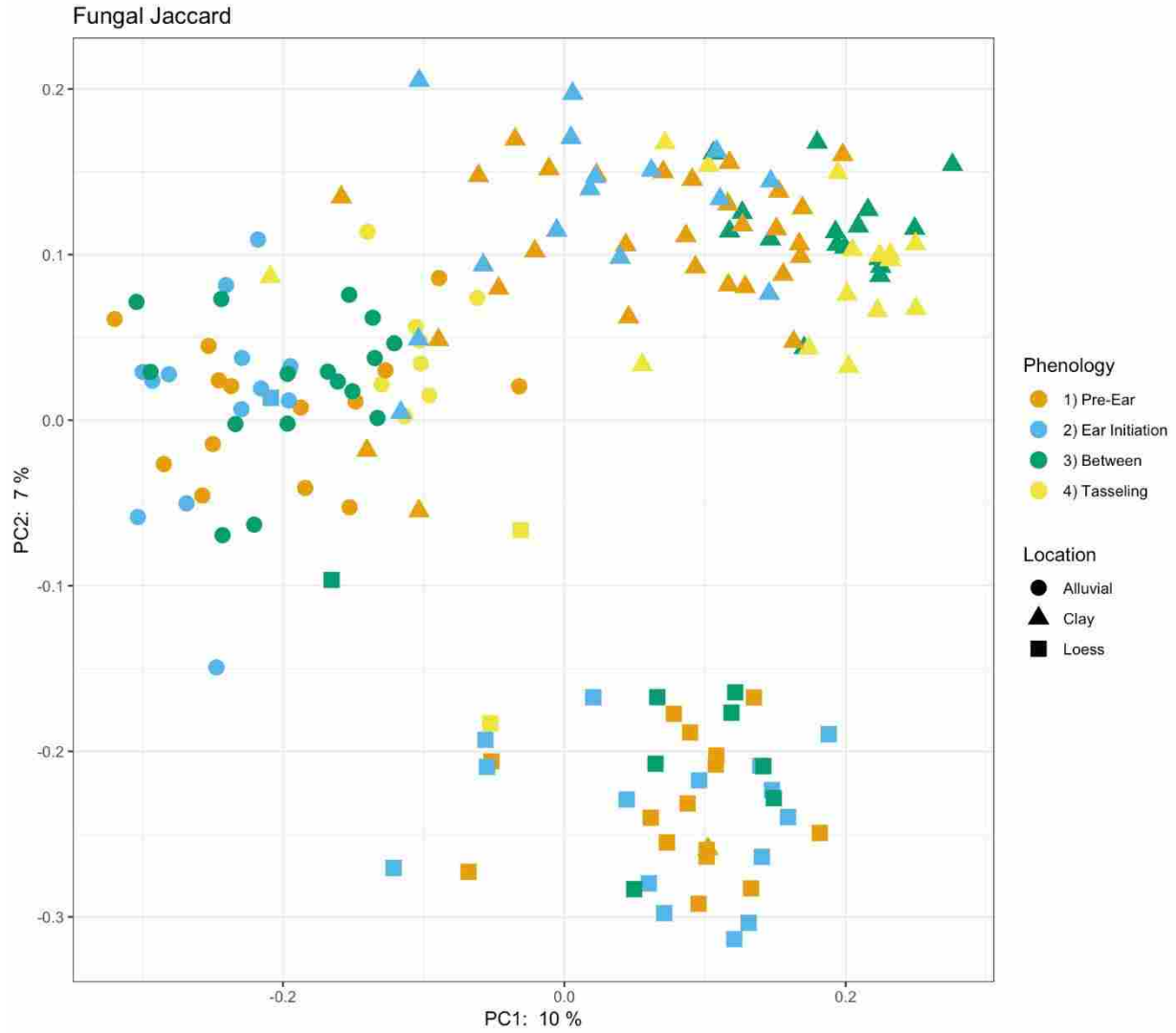


Figure 1-6. Fungal unweighted Jaccard PCoA results of active growing season

PCoA plot representing the differences in populations between samples taken during the active growing season, colored by developmental state of maize.

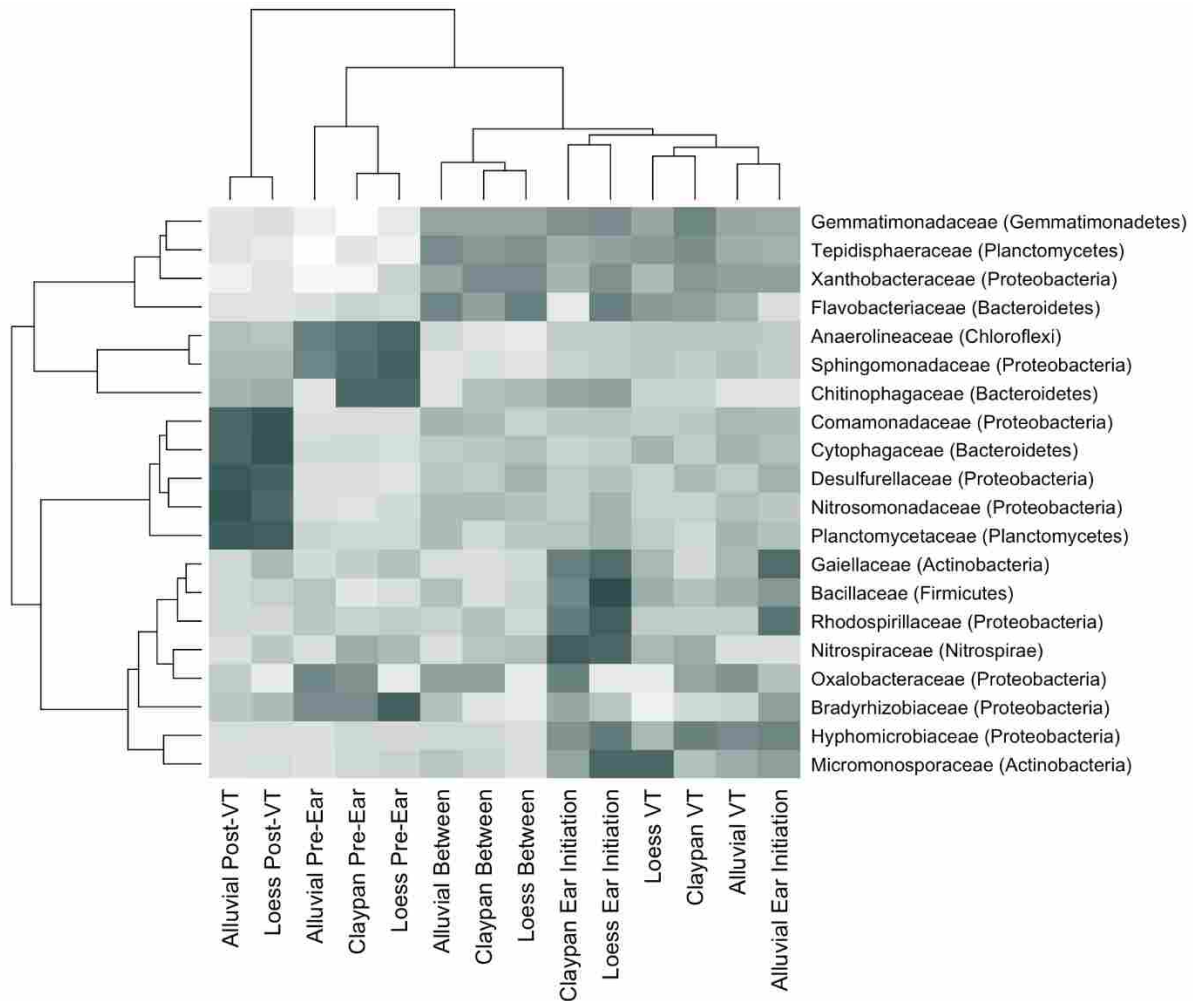


Figure 1-7. Bacterial ANCOM results – Family Level.

Heatmap illustrating soil abundance of taxa characterized by 16S rRNA gene amplification sequencing. Occurrence of *Comamonadaceae*, *Cytophagaceae*, *Desulfurellaceae*, *Nitrosomonadaceae*, and *Planctomycetaceae* are significantly different between the Post-VT developmental period and all other developmental states. Additionally, *Oxalobacteraceae*, *Bradyrhizobiaceae*, *Anaerolineaceae*, *Sphingomonadaceae*, and *Chitinophagaceae* are differentially abundant, occurring primarily during the growing season prior to ear development. Ear development had especially high occurrences of *Gaiellaceae*, *Bacillaceae*, *Rhodospirillaceae*, and *Nitrospiraceae*.

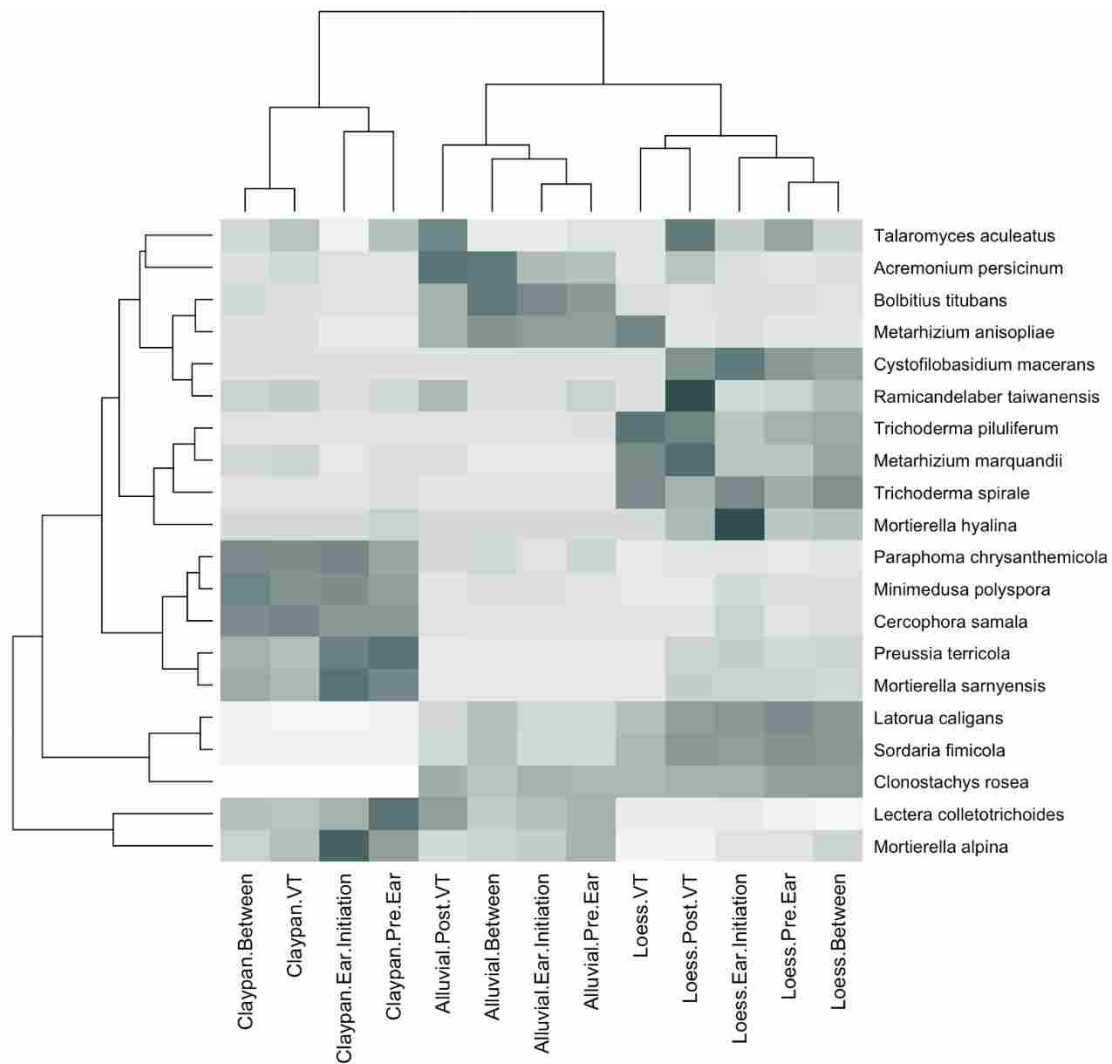


Figure 1-8. Fungal ANCOM results – Species Level

Heatmap illustrating soil abundance of taxa characterized by ITS amplification sequencing. Occurrence of *Cercophora samala*, *Paraphoma chrysanthemicola*, *Minimedusa polyspora*, and *Mortierella sarnyensis* were predominantly found in the claypan soils. Additionally, *Lactoria caligans*, *Bolbitius tibutans*, *Preussia terricola*, and *Metarhizium anisopliae* are differentially abundant, occurring primarily in the alluvial soils. The loess soil had especially high occurrences of *Trichoderma spirale*, *Trichoderma piluferum*, and *Clonostachys rosea*, although they were not necessarily unique to the loess soils.

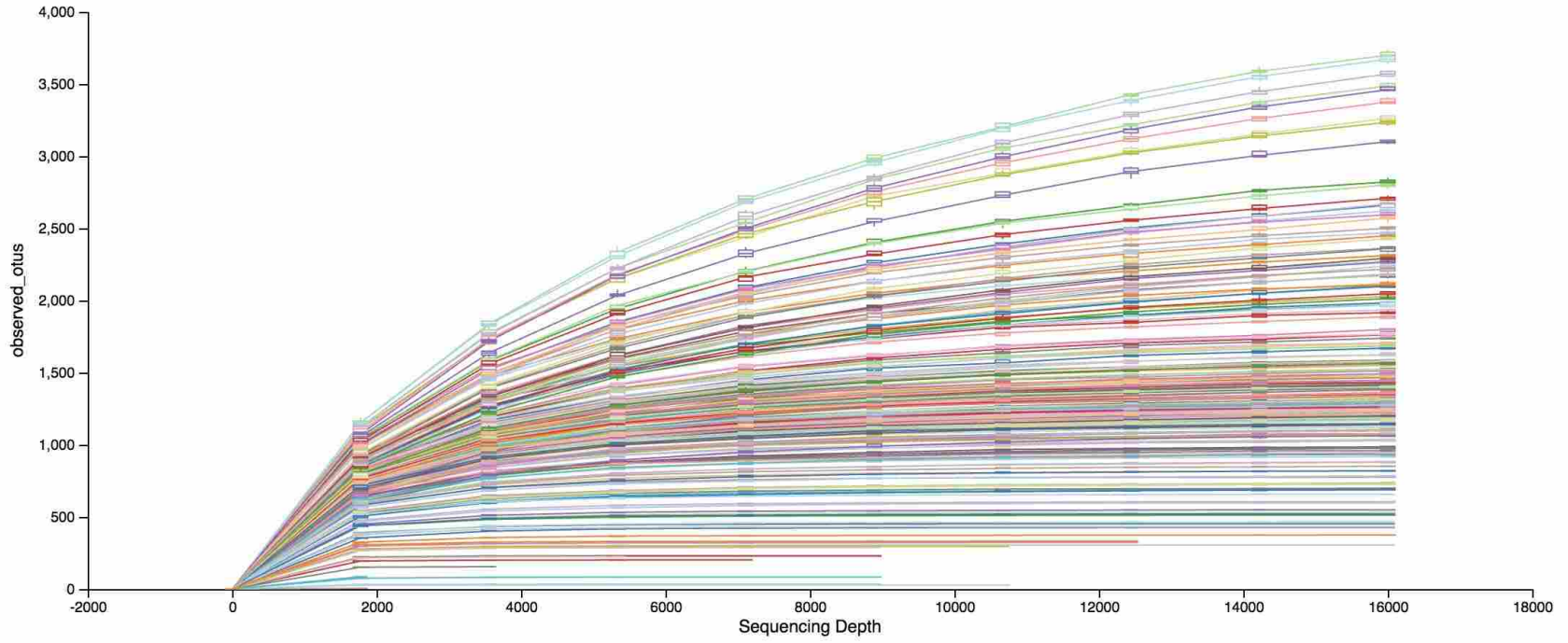


Figure 1-9. Alpha Rarefaction of Bacterial Samples

Alpha rarefaction to 16,000. While there are a few outliers, most samples have plateaued or begin to plateau around 15,000 reads.

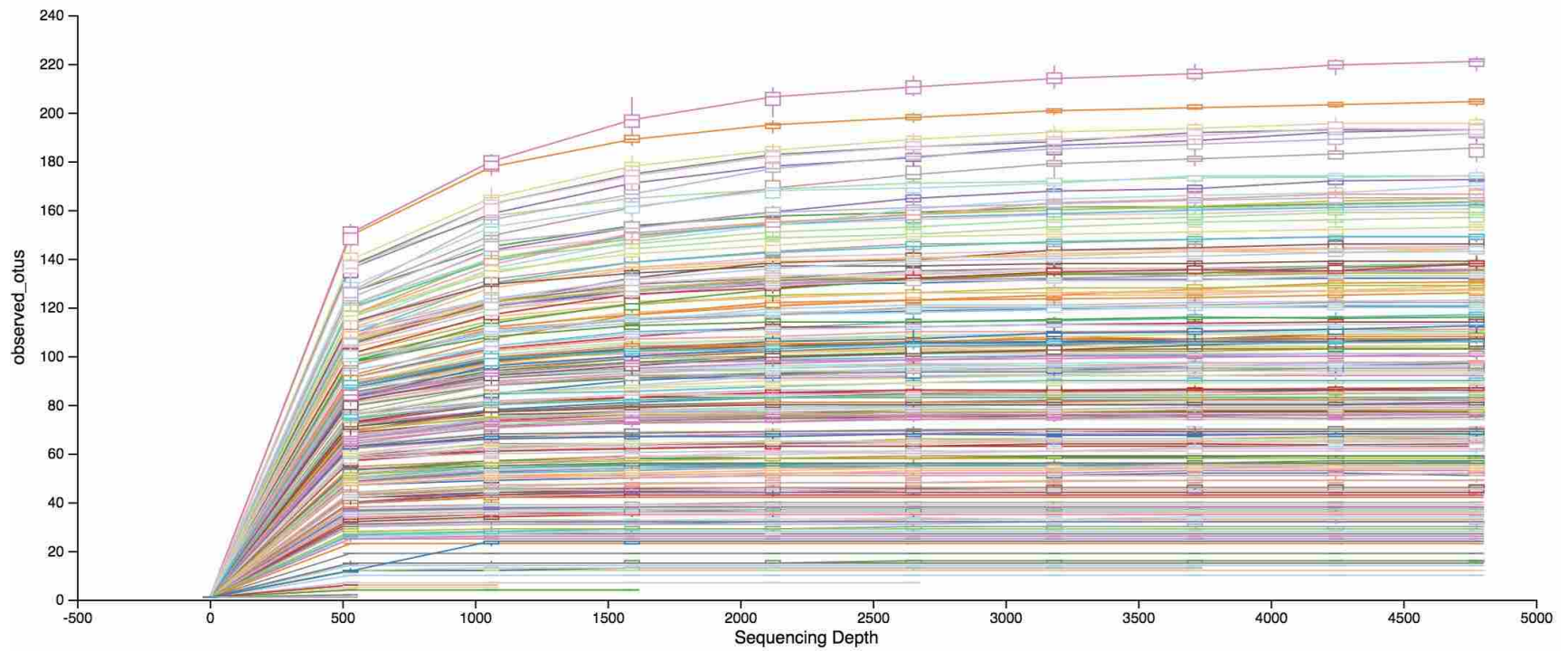


Figure 1-10. Alpha Rarefaction of Fungal Samples

Alpha rarefaction to 5,000. Most samples have plateaued or begin to plateau around 2,700 reads.



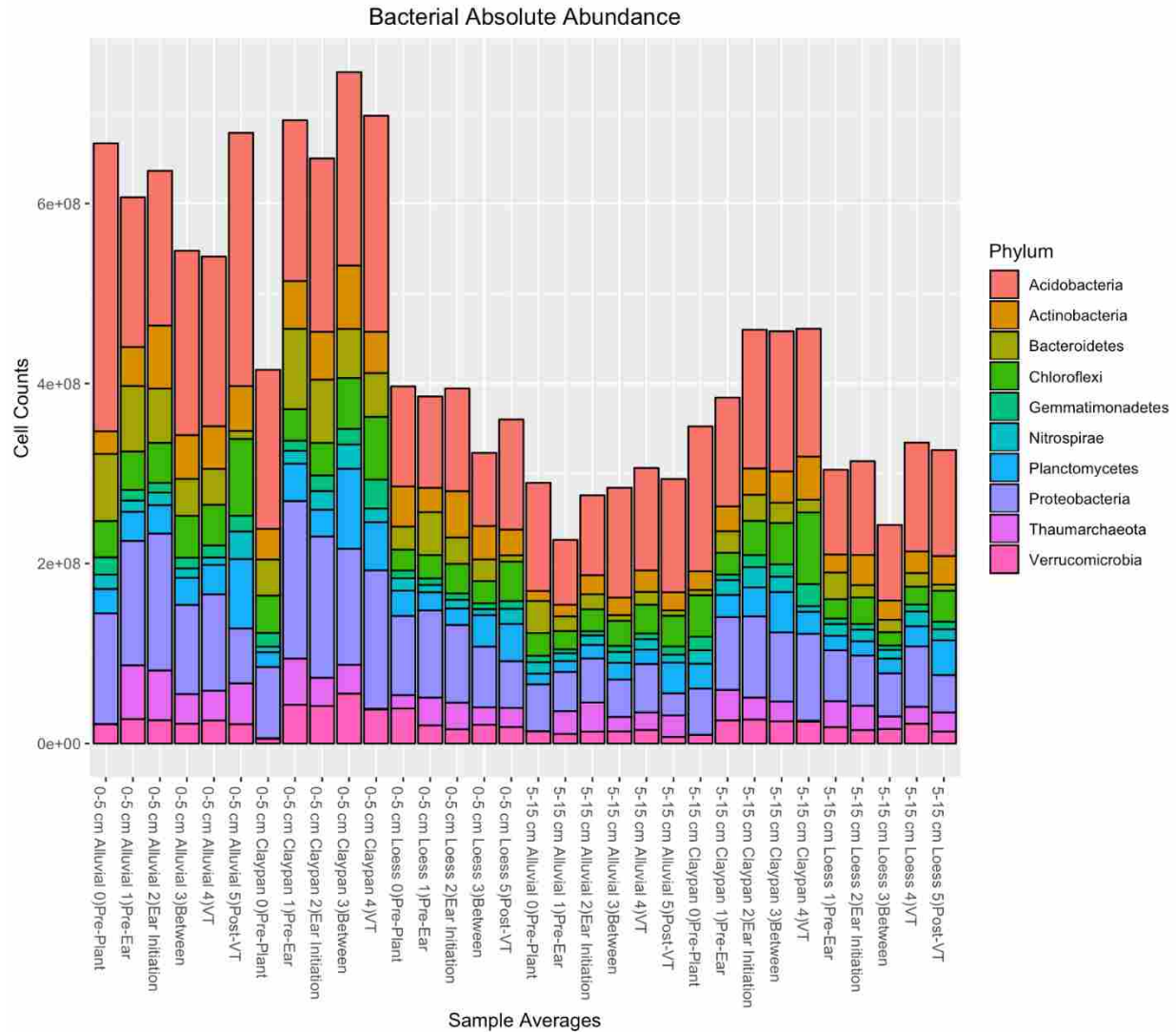


Figure 2-1. Estimated Absolute Abundances of top 10 most abundant bacterial phyla across sample replicates.

Phylum level barplot displaying absolute bacterial populations across all timepoints at each field location. Phylum level was selected as it allowed most taxa to be shown. All samples were rarefied to 15,000 reads and combined by location, depth, and developmental timepoint they were sampled, after which the combined samples were corrected for copy number and multiplied by their absolute abundances and converted into cell counts. The top ten most abundant phyla were selected.

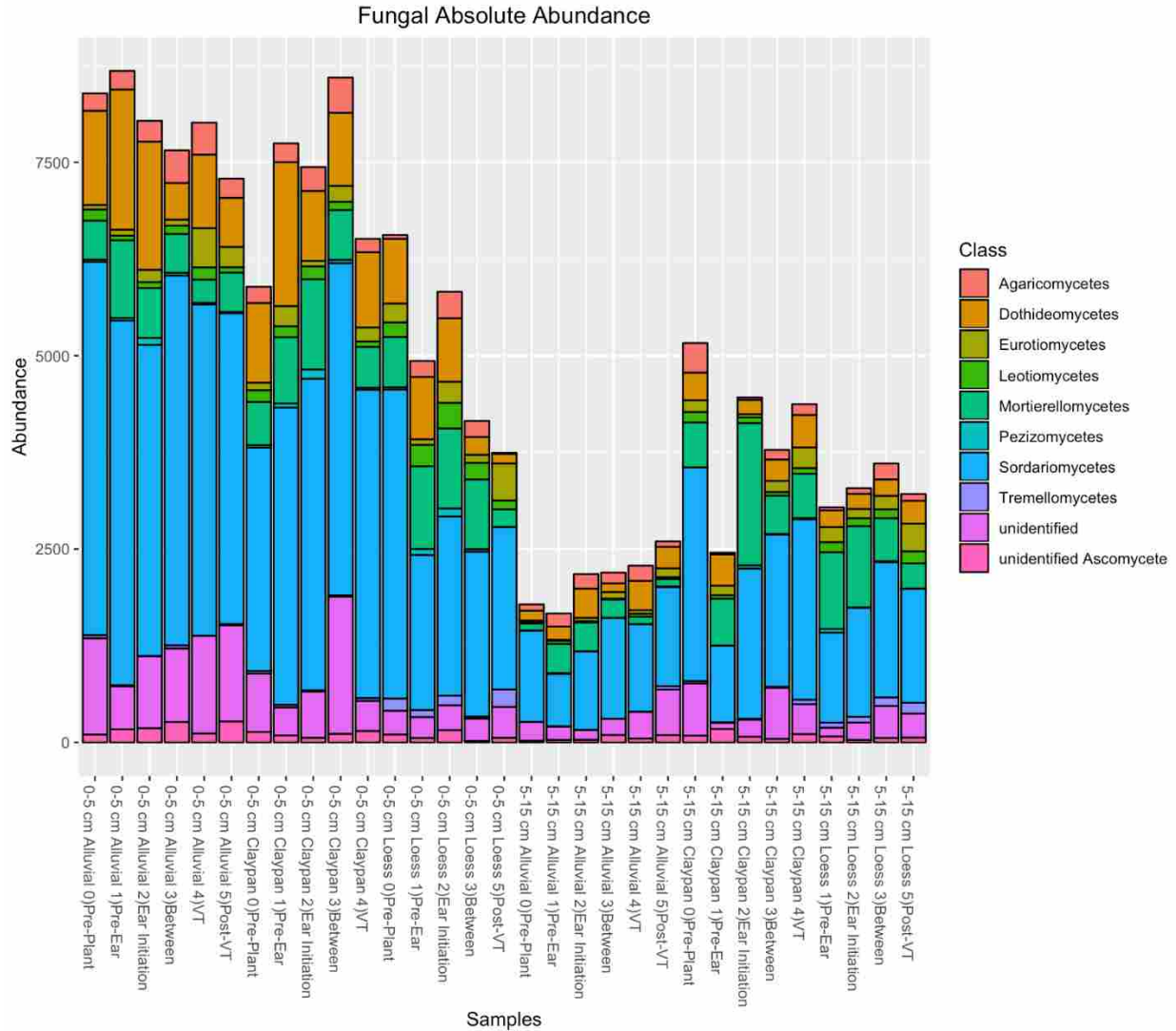


Figure 2-2. Estimated Absolute Abundances of top 10 most abundant fungal classes across sample replicates.

Class level barplot displaying absolute fungal populations across all timepoints at each field location. Class level was selected as it allowed most taxa to be shown. All samples were rarefied to 15,000 reads and combined by location, depth, and developmental timepoint they were sampled, after which the combined samples were multiplied by their absolute abundances and converted into cell counts. The top ten most abundant classes were selected.

Bacteria RA	Ear Development		Tasseling	
	0-5 cm	5-15 cm	0-5 cm	5-15 cm
Phycisphaeraceae	C,L	C,L		A
Paenibacillaceae	A,C	A,C		
Legionellaceae	C	A,C	A	
Ruminococcaceae	C	C,L		L
Nocardiaceae	A,L	C		
Acidimicrobiaceae	A,C			
Sphaerobacteraceae	C	A,L		
Caldilineaceae	C,L	C		
Thermoactinomycetaceae	C	A,L		
Thermomonosporaceae		C,L	A	L
Sphingomonadaceae	C,L		A,L	
Erythrobacteraceae	C	C	A,L	
Nakamurellaceae	L	C	L	L
Planctomycetaceae	L	C	A,L	
Pseudonocardiaceae	L	C		L
Sphingobacteriaceae	C,L			L
Peptostreptococcaceae	A	A		L
Comamonadaceae	L	L	L	
Geobacteraceae	G	C	L	
Haliangiaceae	L	L		A
Xanthobacteraceae	L	C	L	
Parachlamydiaceae		C		A,L
Rhodobiaceae		C	A,L	
Patulibacteraceae		C	L	L
Cyclobacteriaceae	C		A,L	
Nitrospiraceae	A		A	L
Chthoniobacteraceae	C		A,L	
Herpetosiphonaceae		C	L	L
Phaselicystidaceae			A,L	A

Figure 2-3. Bacterial Correlation Analysis– Relative Abundance.

Family level bacterial relative abundance correlation analysis results at early ear development (EI) and tasseling (VT), between bacteria and yield components. Family level was selected as lower taxonomic levels had progressively lower confidences in their assignment. Blue shading represents a positive correlation between a yield component and a bacterial family. Red shading represents a negative correlation between a yield component and a bacterial family. C: Claypan, L: Loess, A: Alluvial

Bacteria EAA Families	Ear Development		Tasseling	
	0-5 cm	5-15 cm	0-5 cm	5-15 cm
Alcaligenaceae	C	A		
Bacillaceae	C,L			
Thermoactinomycetaceae	C	L,A		
Sphingomonadaceae	C,L			
Paenibacillaceae	C	C		
Oligoflexaceae	C	C,L		
Nakamurellaceae	L	C		
Sphaerobacteraceae	C	L,A		
Rhodocyclaceae	C	L	C	L
Thermomonosporaceae		C,L	A	L
Planctomycetaceae	L	C	L,A	
Rubrobacteriaceae		C,L,A		C,L,A
Oxalobacteraceae	L	C		
Phycisphaeraceae	L	L		
Comamonadaceae	L	L		
Veillonellaceae		C,A		
Mycobacteriaceae	A,C			
Cyclobacteriaceae			C,L,A	
Methylophilaceae			L,A	
Legionellaceae			L,A	
Blastocatellaceae			C,L,A	
Caulobacteraceae			L	L
Hyphomonadaceae			L	C
Erythrobacteraceae			L,A	
Frankiaceae			A	C
Solirubrobacteraceae			L,A	
Verrucomicrobiaceae			L,A	
Saprospiraceae			L,A	

Figure 2-4. Bacterial Correlation Analysis– Estimated Absolute Abundance.

Family level bacterial estimated absolute abundance correlation analysis results at early ear development (EI) and tasseling (VT), between bacteria and yield components. Family level was selected as lower taxonomic levels had progressively lower confidences in their assignment. Blue shading represents a positive correlation between a yield component and a bacterial family. Red shading represents a negative correlation between a yield component and a bacterial family. C: Claypan, L: Loess, A: Alluvial

Fungi RA	Ear Development		Tasseling	
	0-5 cm	5-15 cm	0-5 cm	5-15 cm
Latorua caligans	C,L,A		C,L,A	
Clonostachys rosea	L,A	L,A	L,A	L,A
Penicillium sp	L	C,L		
Preussia terricola	C		C	
Trichoderma piluliferum	L	L		
Cladosporium sp	L	L		
Plectosphaerella cucumerina	C	A		
Microdochium sp	C	C		
Fusarium solani			C,L,A	C,L,A
Fusarium sp			C	C
Metarhizium marquandii			C	C
Podospora sp			C	C

Figure 2-5. Fungal Correlation Analysis– Relative Abundance.

Family level fungal relative abundance correlation analysis results at early ear development (EI) and tasseling (VT), between bacteria and yield components. Species level was selected as taxonomic assignment had sufficiently high confidence. Blue shading represents a positive correlation between a yield component and a bacterial family. Red shading represents a negative correlation between a yield component and a bacterial family. C: Claypan, L: Loess, A: Alluvial

Fungi EAA	Ear Development		Tasseling	
Families	0-5 cm	5-15 cm	0-5 cm	5-15 cm
<i>Preussia terricola</i>	C	C	C	C
<i>Colletotrichum</i> sp	C	C	C	C
<i>Clonostachys rosea</i>	L,A	L,A		
<i>Penicillium</i> sp	L	L		
<i>Trichoderma piluliferum</i>	L	L		
<i>Cladosporium</i> sp	L	L		
<i>Paraphoma chrysanthemicola</i>	C		C	
<i>Latorua caligans</i>	L,A		L,A	
<i>Mortierella</i> sp	L,A		L	
<i>Metarhizium anisopliae</i>	C		C	
<i>Minimedusa polyspora</i>	C,L,A			
<i>Fusarium</i> sp			C	C
<i>Podospora</i> sp			C	C
<i>Mortierella sarnyensis</i>		C		C
<i>Metarhizium marquandii</i>			C	C

Figure 2-6. Bacterial Correlation Analysis– Estimated Absolute Abundance.

Family level fungal estimated absolute abundance correlation analysis results at early ear development (EI) and tasseling (VT), between bacteria and yield components. Species level was selected as taxonomic assignment had sufficiently high confidence. Blue shading represents a positive correlation between a yield component and a bacterial family. Red shading represents a negative correlation between a yield component and a bacterial family. C: Claypan, L: Loess, A: Alluvial