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Investigating soil microbiome changes during *Panax quinquefolius* cultivation in new gardens

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Abstract

Ginseng (*Panax quinquefolius*) is susceptible to ginseng replant disease (GRD), resulting in root rot and decreased yield when ginseng is grown in soils previously used for ginseng crops. Although fungal and oomycete pathogens are implicated, GRD is a complex disease and the changes in the soil when initially cultivating ginseng that lead to GRD remain unclear. This thesis reported changes in the soil microbiome from three new gardens in Norfolk County, Ontario over three years starting from seeding with ginseng. Over this period of cultivation, metabarcoding of the V4 region of the 16S ribosomal RNA gene clearly showed a decrease in alpha diversity of the microbiome and a shift in microbial community composition. Families of microbes that are known to perform nitrogen fixation, ammonia oxidation, and toxin degradation increased in relative abundance. Future investigations should confirm whether any of the changes in microbial taxa during ginseng cultivation contribute to GRD.

Keywords

Summary for Lay Audience

Ginseng is grown for its roots, which have been used in traditional Chinese and Korean medicine for thousands of years. However, ginseng cultivation is prone to ginseng replant disease (GRD) which results in poor growth of ginseng and lower yields when planted in soils previously used for ginseng crops. This is a major concern for ginseng growers. Pathogens in the fungi and oomycetes are known to contribute to GRD. In addition, ginseng roots can produce ginsenosides which can accumulate over time and cause changes to beneficial or pathogenic bacteria in the soil and contribute to GRD. However, the specific changes in the soil microbiome when ginseng is first grown that end up causing GRD are unclear.

This study aimed to investigate how microorganisms in new ginseng garden soils changed over a period of three years. Between October 2018 and November 2021, soil samples were collected every season from three new gardens in Norfolk County, Ontario. They were compared to see how their microbial communities changed as ginseng grew. The study found that microorganisms known to perform nitrogen fixation, ammonia oxidation, and toxin degradation increased after three years.

In summary, the diversity of the soil microbiome where ginseng was grown had evidently changed. These changes in community composition could be directly due to the growth of ginseng or be affected by the farmers' cultivation practices, such as fertilization, and should be further examined to determine whether the bacterial changes are related to the development of GRD, and to develop specific tests to diagnose the soil.

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List of Abbreviations

16SV4   Hypervariable region 4 of the 16S ribosomal small subunit gene

ALDEx2   Analysis of differential abundance taking sample variation into account

ANCOM-BC Analysis of composition of microbes, with bias correction

ASV   Amplicon sequence variant

DGGE   Denaturing gradient gel electrophoresis

GRD   Ginseng replant disease

IMP   *Ilyonectria mors-panacis*

PCDD   Polychlorinated dibenzodioxins

PCoA   Principal coordinates analysis

PCR   Polymerase chain reaction

QIIME2   Quantitative insights in microbial ecology version 2

RSD   Reductive soil disinfection

Chapter 1

1   Introduction

1.1   Ginseng and its global history

Ginseng, a perennial herbaceous plant that belongs to the genus *Panax* in the family
Araliaceae, is cultivated due to its commercially and medicinally valuable roots. Thirteen
species of ginseng have been described: *P. ginseng*, *P. japonicus*, *P. major*, *P.
notoginseng*, *P. omeiensis*, *P. pseudoginseng*, *P. quinquefolius*, *P. sinensis*, *P.
stipuleanatus*, *P. trifolius*, *P. wangianus*, *P. zingiberensis*, and *P. vietnamensis* (Yun et
al., 2001).

Ginseng has a long history as a medicinally valuable plant. Ginseng root has been used
for centuries in Chinese and Korean traditional medicines, and the exportation of ginseng
from China to various parts of Eastern Asia has been documented from as early as the
14th century (Pan et al., 2014). Modern studies have reported that ginseng has a range of
pharmacological effects such as on the cardiovascular and central nervous systems, in
treating diabetes, and anti-tumour properties (Jin et al., 2010; Li et al., 2010; Yuan &
Dey, 2001). These effects are thought to be attributed to a group of chemical compounds
produced by ginseng called ginsenosides, which can be found in all parts of the plant,
including the roots, leaves, stems, flower buds, and berries (Qi et al., 2011; Nicol et al.,
2002). Ginsenosides are saponins, a group of plant secondary metabolites found naturally
in many plant species and are used to protect against pathogens (Goodwin & Best 2023).

Currently, ginseng is commercially distributed to 35 countries and two of them, South
Korea, and China, are both major exporters and importers (Baeg & So, 2013). The three
main species that are cultivated and available commercially are Chinese ginseng (*P.

notoginseng) in China, Korean ginseng (*P. ginseng*) in South Korea, and American ginseng (*P. quinquefolius*), which is the species cultivated in North America. The exportation of American ginseng from Canada to countries including China, Singapore, the United States, Taiwan, Vietnam, Australia, Netherlands, Italy, Germany, Malaysia, and the United Kingdom as well as regions like Hong Kong, has an average annual market value of approximately CA$90.5 million (Statistics Canada, 2021). In addition, the third most exported product from Canada to Hong Kong is ginseng, demonstrating the high demand and value of this product (Oddleifson et al., 2020).

In North America, wild *P. quinquefolius* is an understory plant in deciduous and mixed forests in the regions of northeastern United States and the Canadian provinces of Ontario and Quebec (Proctor & Bailey, 1987). Forests that are dominated by sugar maples (*Acer saccharum*) are suitable for its growth (Charron & Gagnon, 1991). American ginseng was collected and exported for medicinal purposes to Asian countries from the time of its discovery by French explorers and Jesuit missionaries in Quebec in the 1700s. Ginseng roots were exported by Chinese merchants due to its remunerative prices, which was comparable to fur trading at the time (Schorger, 1969). The demand for ginseng was so high that it became rare in Quebec forests (Carlson, 1986), which resulted in a shift towards agricultural production. In North America as a whole, wild ginseng populations began to decrease dramatically in the 1900s due to overexploitation and harvesting (Punja, 2011) and as a result *P. quiquefolius* is now listed as endangered by the Canadian Species at Risk Act (SARA) (Environment Canada, 2015) and the Ontario Endangered Species Act. The latter act forbids one to buy, harvest, possess, plant, sell, or trade ginseng roots that are harvested from the wild without permission.

North American ginseng was first cultivated in New York by George Stanton in the 1870's and Abraham Whisman from Virginia (Pearsons, 1994). Commercial cultivation

of North American ginseng started occurring in the late 19th century in the states of Wisconsin, Michigan, North Carolina, Ohio, and Tennessee in the United States, while this occurred mostly in Ontario and British Columbia in Canada (Punja, 2011; Proctor & Bailey, 1987). *Panax quinquefolius* has since become one of the most economically important species from the *Panax* genus (Carpenter & Cottam, 1982).

1.2 Cultivation of North American ginseng

Ginseng can only be cultivated either under artificial shades or forest conditions that mimic the natural habitat of hardwood forests (Proctor, 1996). There are three major cultivation methods for ginseng, which are field cultivated, wood grown, and wild stimulated. For field cultivation of ginseng, ginseng is grown in agricultural lands under artificial shade structures that emulate the wild conditions in the forests. Wood grown ginseng varieties are planted in the forest with standard agricultural practices including soil preparation, fertilisation, and pest-control. Finally, wild stimulated ginseng is grown by planting seeds in the forest without any standard agricultural practices.

In Ontario, the production of wood grown and wild stimulated ginseng is illegal under the Ontario Endangered Species Act; therefore, ginseng that is commercially grown in Ontario are 100% field cultivated (Samur, 2020). North American ginseng is normally cultivated for four years or until its roots reach a harvestable size to sell. When cultivating ginseng, there are many factors that are considered to obtain the highest yield. Soils used for ginseng cultivation need to have excellent drainage with high organic matter and preferably with a pH level of at least 6.5 as acidic conditions often results in lower yield (Behdarvandi, 2020; Samur, 2020). However, the climate and soil conditions in Ontario, and particularly Norfolk County, are suitable for growing ginseng despite a low organic matter. In addition, seed stratification is needed for 18 to 20 months before planting into raised soil beds prior to germination. Stratification is the process of storing

seeds at cold temperatures to simulate winter conditions which seeds must experience before overcoming seed dormancy in order to germinate.

Stratified seeds available commercially are almost guaranteed to have been contaminated by spores from known fungal pathogens such as those from the genera *Alternaria*, *Rhizoctonia*, *Fusarium*, and *Phytophthora*. Therefore, it is often recommended to surface sterilise seeds with a bleach solution prior to planting them in the soil bed. A layer of mulch is then applied onto the soil bed, such as shredded hardwood bark, aged leaves, or sawdust to retain soil moisture and for winter protection. Once planted, ginseng plants are grown for up to four years, and then the roots are dug up for harvest, dried, and stored in a dry, cold location prior to shipping to international markets.

1.3 Replant Disease

Although ginseng can be cultivated on a large scale, ginseng growers are facing a problem in growing a high-quality crop consistently due to a condition called ginseng replant disease (GRD). Replant disease is a phenomenon whereby plants of the same species are unable to be cultivated in the same soil after the initial plants are removed. Replant disease occurs in many perennial horticultural crops, including apples, cherries, pears, roses, plums, as well as ginseng (Mazzola & Manici, 2012; Mai & Abawi, 1978; Yim et al., 2020; Traquair, 1984; Li, 1995). The main symptoms are poor growth and development, as well as a decrease in yield and quality (He et al., 2022). Replant disease for fruit crops is caused by factors such as allelopathy, autotoxicity, and an imbalance of both soil physical-biochemical traits (e.g., soil texture, pH, organic matter content, and aeration or water saturation) (Hanschen & Winkelmann, 2020) as well as pathogenic and non-pathogenic fungi and bacteria (He et al., 2020).

One of the most extensively studied horticultural crops that suffers from replant disease is apple. Apple replant disease (ARD) has been a serious problem for orchard farmers. It causes the delay of an apple tree's fruit production for up to 50% of its lifetime (Van Schoor et al., 2009). For a period of over ten years, ARD can reduce the value of the crop by up to 40,000 dollars per acre in gross return (Mazzola, 1998). Apple trees that suffer from ARD can be severely stunted in height, and have shortened internodes, rosette leaves (i.e., small leaves with poor nutrition that are denser than normal conditions), smaller root systems, decayed or discoloured roots, and most importantly a reduction of productivity (Mazzola, 1998). In apple orchards that are suffering from ARD, young trees will have such poor growth that it can eventually lead to the death of these trees.

Numerous abiotic factors have been suggested to cause ARD, including low or high soil pH, phytotoxins from fumigants, an imbalance of soil nutrition, metal contamination, poor soil structure (with limited large or medium sized pores) and drainage, as well as cold or drought stress (Mai & Abawi, 1981; Traquair, 1984; Willett et al., 1994). Although such abiotic factors are suggested to be the cause of replant disease for apples, other fruit trees have been able to grow normally in the same orchard (Mazzola, 1988). In addition, the use of soil pasteurisation (Hoestra, 1968; Jaffee et al., 1982a) and fumigation (Mai & Abawi, 1981; Slykhuis et al., 1985) results in a dramatic increase of growth which suggests that the cause is a biotic factor instead (Mazzola, 1998).

Soilborne organisms such as plant parasitic nematodes from the genus *Pratylenchus* have been reported to play a major role in ARD in eastern United States (Jafee et al., 1982b; Mai & Abawi, 1981) as well as in British Columbia (Utkhede et al., 1992a). Bacterial pathogens such as actinomycetes and fluorescent pseudomonads (Mazzola, 1998) have also been shown to be a potential cause of ARD. Furthermore, a number of fungi and oomycetes have been reported as being associated with ARD, including *Pythium* spp.

(Jaffee et al., 1982a; Sewell, 1981), *Cylindrocarpon* spp. (Braun, 1991; Braun, 1995; Sewell, 1981), *Phytophthora* spp. (Utkhede et al., 1992b), *Armillaria mellea* (Mazzola, 1998), *Peniophora sacrata* (Taylor & Wallace, 1970), *Trichoderma hamatum*, *Torulomyces lagena*, and *Mortierella* sp. (Utkhede et al., 1992a).

Similarly, replant disease also occurs in *Panax* species and is often termed ginseng replant disease (GRD) (Li, 1995), but the causes for GRD are not well known. Based on the current literature, the main cause of GRD-associated root rot involves the fungal pathogen, *Ilyonectria mors-panacis* (IMP), which is formerly identified as *Cylindrocarpon destructans*. However, other abiotic and biotic factors likely also contribute to GRD overall (Westerveld & Shi, 2021). Many researchers have suggested abiotic and biotic factors that include the imbalance of soil physical-biochemical traits, changes in the soil microbiome (e.g., increase or decrease of beneficial and pathogenetic microbes) (Dong et al., 2018a), and autotoxicity from ginsenosides released from the plant itself (Yang et al., 2015; Westerveld & Shi, 2021). GRD can occur in subsequent ginseng plants that are grown 8 to 15 years after the first crop is planted (Westerveld & Shi, 2021), causing a lack of suitable lands for ginseng cultivation to be a serious issue for farmers.

1.4   Ginseng Replant Disease (GRD)

1.4.1   Pathogenic organisms associated with GRD

The ginseng industry has placed considerable effort into investigating and identifying potential pathogens, which are suggested to be a main cause of GRD. Shortly after ginseng fields were set up by growers in the 1880s, they started to notice that the ends of the roots begin to disappear after attempting to grow ginseng on the same land. The issue was first attributed to the prevalence of disease-causing organisms that would accumulate

during the first cultivation of the plant, subsequently resulting in a failure to grow in the same site. A series of experiments found that fungi within the *Ramularia* genus were associated with symptoms of rust and rotting for ginseng plants (Hildebrand, 1935). This genus has been reclassified and these fungal pathogens are now identified as *Ilyonectria mors-panacis* (IMP) or *Ilyonectria robusta* (Cabral et al, 2012). The pathogens were first identified by Zinssmeister (1918), and first associated with GRD found in Asian ginseng (*P. ginseng*) (Westerveld & Shi, 2021). The association of GRD with North American ginseng was not documented until 1994 (Li, 1994). IMP was firstly isolated by Reeleder et al. (2002), as *C. destructans* from replanted gardens in Norfolk and Brant counties in southwestern Ontario and was later confirmed and renamed by Cabral et al. (2012). IMP is known to affect *Panax* species, and this fungal species is the primary pathogen associated with GRD, but this species needs to be more accurately identified globally (Westerveld & Shi, 2021).

Fusarium species have also been shown to be associated with GRD, which can lead to both root rot and rusty root, and subsequently cause a decrease in quality and production (Lee et al., 2011; Wang et al., 2019; Miao et al., 2006). In a preliminary study conducted by Punja et al. (2006), *Fusarium* species were observed to be one of the most frequently isolated organisms in ginseng tissues with rusty root. *Fusarium equiseti* and *Fusarium solani* have both been shown to have the ability to cause root decay on ginseng seedlings (Punja, 1997). A study conducted by Punja et al. (2007) has revealed that *Fusarium* species (e.g., *Fusarium equiseti*) are found in straw mulch that are used during ginseng cultivation, which caused root discolouration symptoms that resemble those of rusty root seen in earlier studies (Hildebrand, 1935). Seed borne *Fusarium* species were also found to be able to infect ginseng seedlings as well as two-year-old plants, resulting in root rot (Guan et al., 2020).

In addition to fungi, Oomycota (a phylum of fungus-like Chromista, commonly referred to as oomycetes), include soil-borne pathogens of ginseng from the genera *Pythium* and *Phytophthora* (Kernaghan et al., 2008; Bae et al., 2004). *Pythium irregulare* is commonly isolated from diseased seedlings in ginseng gardens in southwestern Ontario (Reeleder & Brammall, 1994), which symptoms including root rot, damping off to seedlings, and reddish-brown roots. *Phytophthora cactorum* was isolated in commercial ginseng gardens, and the recovery of *P. cactorum* from ginseng seedlings suggested that the reoccurrence of *P. cactorum* occurred through seed transmission (Hill & Hausbeck, 2008).

Although fungal and oomycete pathogens are typically associated with GRD, there are observations that suggest that there may be other factors that contribute to GRD. Firstly, the GRD can remain in replanted gardens for a very long time, ranging from 50 to 80 years after the first crop is harvested, which is atypical for fungal pathogens (Westerveld & Shi, 2021). Fumigation practices for ginseng planting fields have been applied, but GRD remained and appeared to spread evenly across a field which is also not usual for a fungal pathogen (Westerveld & Shi, 2021).

1.5 Mitigation of replant disease

Several strategies have been developed to counter replant diseases. Crop rotation, fumigation, reductive soil disinfestation, and solarization, are common strategies that have been applied to perennial horticultural crops such as apples, and ginseng. Crop rotation could help reduce replant disease for ginseng farmers. A study conducted by Jiao et al. (2019) assessed the effects of maize rotation by examining nutrients and microbial communities in soil used in *P. quinquefolius* cultivated soil in Beijing, China. It was found that biomass of ginseng roots planted in soils used previously in ginseng cultivation followed by 3 years of maize rotation increased, and root disease from these

soil samples also decreased. However, a study conducted by Li et al. (2021) in Weihai in China showed contrasting results. It was revealed that *P. quinquefolius* grown in pots with soil previously used in ginseng cultivation that were subjected to 10 year crop rotation had a lower survival rate relative to control soils. This suggests that crop rotation may not be effective even after long periods as GRD conditions may still remain in the soil. Furthermore, because of industrial standardized set ups for specific crops like apples, crop rotation is strongly limited as other crops cannot be efficiently harvested. For instance, in modern orchard infrastructures, there are fences, hail nets, wells, pipes, and complicated irrigation systems in order to protect the orchard trees (Winkelmann et al., 2019). For ginseng, structures to provide shade are necessary, and need to be taken down for other crops. The costs of setting up the shade structures could be offset if ginseng could be grown in the same site after the first crop (Westerveld & Shi, 2021). Ultimately, the main problem for GRD is that it can remain in soil for a long time even though crop rotation might alleviate this disease.

Fumigation is a method for chemically sterilizing soil, which was developed in the 1960s primarily using methyl bromide or chloropicrin. Methyl bromide is used because it is effective against a wide spectrum of plant pathogens and pests, which include fungi, bacteria, nematodes, insects, mites, and rodents, and also weeds (Duniway, 2002). However, it has been classified as a class I stratospheric ozone-depleting agent since 1993 in Canada, and many alternatives (e.g., chloropicrin, and 1,3-dichloropropene) have slowly replaced it (Martin, 2003). In modern agricultural practices, chloropicrin is mixed with other chemicals and applied to the replanted soil for crops like apples and prunes (Browne et al., 2018; Nyoni et al., 2019). Similarly, fumigants are also applied to ginseng replanted sites, but the symptoms are only alleviated and not particularly effective (Westerveld & Shi, 2021).

Reductive soil disinfestation (RSD) is also a possibility for reducing the severity of replant diseases. This is often done by adding easily decomposable organic matter into the soil followed by saturating the soil with water. The soil is then covered with a plastic film and exposed to high temperatures for a period ranging from three to four weeks. During this time, the high decomposition rates lead to the soil becoming anaerobic (Momma et al., 2013; Butler et al., 2014). As a result, pathogens, and other soil organisms, are killed by the anaerobic conditions. However, replant disease control has been reported to be inconsistent (Hewavitharana & Mazzola, 2016). RSD has also been applied to Asian ginseng, and a study conducted by Li et al. (2019) showed that the population of *Fusarium oxysporum* and the relative abundance of *Fusarium* were significantly reduced when RSD was followed by crop rotation. Furthermore, using crop rotation following RSD promoted the restoration of microbial activity as well as ginsenoside degradation, and ultimately alleviated replant failure when compared with applying RSD treatment alone (Li et al., 2019).

Finally, solarization is a non-chemical disinfestation method that is used by farmers in which heat is used to reduce various pathogens (e.g., bacteria, fungi, nematodes), weeds, and insects. This is often done during the hottest part of the year and requires the use of a plastic tarp. It has been shown that this method is effective when coupled with the use of methyl bromide, e.g., *Verticillium* wilt infecting globe artichokes was controlled for three continuous cropping seasons (Tjamos et al., 1988). In addition, solarization applied along with methyl bromide to raised soil beds used for strawberry cultivation significantly reduced soil-borne disease (e.g., *Rhizoctonia* spp. and *Phytophthora cactorum*) (Benlioğlu et al., 2005). Solarization alone has also been tested in raised soil beds from a 12-year-old ginseng replant garden in Norfolk Country (Rabas, 2021). Results showed that a minimum treatment of four weeks of solarization before seeding can partially

alleviate the severity of GRD caused by IMP on ginseng roots at least during the first year of cultivation.

Unfortunately, the current existing mitigation strategies do not eliminate GRD effectively and permanently, so there remains a need to investigate the underlying cause. Most methods used are aimed at removing potential pathogens, which include fumigation and reductive soil disinfestation, to alleviate the symptoms of GRD. However, the effects caused by GRD are not fully eliminated by these treatments, which suggests that other soil microbes and other factors are involved.

1.5.1   The impact of ginseng on soil microbiomes

The soil microbiome collected from ginseng gardens has been investigated in recent years, and changes have been observed in regards to many different aspects, in addition to GRD. The rhizosphere microbiome in ginseng has been shown to change while the plant grows (reviewed by Goodwin, 2022). A study conducted by He et al. (2022) examined how the type of soil used (farmland and forest) and cultivation age (4, 5, and 6 years) affected the rhizosphere microbiome of *P. ginseng* in nine farms from the Jilin Province of China.

With the use of high-throughput sequencing of the 16S rRNA gene, the relative abundance and the diversity of microbes in the rhizosphere was shown to differ. Acidobacteria, Proteobacteria, Actinobacteria and Chloroflexi were the predominant bacterial phyla from the nine farm sites. The diversity of bacterial communities was lower from forest soil relative to farmland soil. The cultivation age was also shown to have an effect on the microbial composition, in which bacteria and fungi including *Acidothermus*, *Bryobacter*, *Chaetomium*, *Cryptococcus*, and *Sphingomonas* were negatively correlated with cultivation age (He et al., 2022). Nguyen et al. (2016) also

examined changes in the bacterial microbiome from soil samples at different depths (0–10 cm, 10–20 cm, and 20–30 cm) obtained after 2, 4, and 6 years after the first and second round of *P. ginseng* cultivation in Korea. Bacterial diversity decreased over time, and it was significantly different between 2 and 6-year-old soil samples. The authors suggests that this observation supports the hypothesis that bacterial diversity decreases over longer cultivation time, which could be explained by the fact that older roots secrete lower amounts of organic matter (Li et al., 2012; Li et al., 2014). The temporal dynamics of microbial communities in the rhizosphere, collected across the life cycle of Asian ginseng (*P. notoginseng*) in Yunnan Province, China, have also been investigated and have shown that the bacterial communities were shaped by the plant's developmental stages (Wei et al., 2022). The authors in this study proposed that *P. notoginseng* may select a specific set of microbes throughout its life cycle, in which the microbial community changes are caused by the changes in plant trait expression and functional requirements across different growth stages.

The rhizosphere microbiome of healthy ginseng roots and diseased roots have also been compared. Wu et al. (2015) used high-throughput sequencing of the V4 region from the 16S rRNA to examine soil from the rhizosphere in 19 different *P. notoginseng* plantations ranging from cultivation age of 2 to 4 years old in the Yunnan province in China. The microbial communities from the rhizosphere and plant roots were of lower diversity in diseased plants compared to healthy ones. Based on their results, the authors suggested that microbial diversity can serve as an indicator for disease outbreaks for ginseng. Similarly, Jiang et al. (2019) assessed microbial communities collected from rhizosphere soils of healthy *P. quinquefolius* roots and root rot diseased roots across four years from Shaanxi province in China. Overall, the dominant bacteria phyla were reported as Proteobacteria, Actinobacteria, Chloroflexi, Acidobacteria, Gemmatimonadetes and Nitrospirae in all samples, but the communities were dissimilar

between soils collected from healthy and diseased ginseng roots. Bacteria including *Pedobacter*, *Pseudomonas*, *Sphingopyxis*, and *Dactylosporangium* were more abundant in the rhizosphere soils of ginseng with root rot. The authors stated that *Pseudomonas* are more abundant in rhizosphere soils of root rot ginseng because many bacteria from this genus produce anti-fungal compounds (e.g., 2,4-diacetylphloroglucinol and/or hydrogen cyanide). These chemicals are found to be antagonistic to *Thielaviopsis basicola*, which is a fungal pathogen that causes black root rot to tobacco (Stutz et al., 1986; Ramette et al., 2003).

Microbiome studies have also looked at the change of soil microbiomes under continuous cropping of ginseng soils. Dong et al. (2017) examined the differences between soils used for used for maize cultivation and North American ginseng cultivation in farms located in the Huairou district of Beijing in China. The bacterial diversity (i.e., Chao1, Phylogenetic diversity, Shannon diversity index) in soil samples used for ginseng cultivation was reported to have significantly declined relative to fungal diversity when compared to soil samples used for maize cultivation. The authors suggested that as ginseng is a perennial plant, root exudates can accumulate in the rhizosphere, which may act as substrates for specific groups of bacteria. For instance, Proteobacteria and Bacteroidetes have shown to increase in their relative abundance in soil plots that are rich in nitrogen (Fierer et al., 2012). In another study conducted by Zhang et al. (2020a), the microbiome was observed to change in bulk soil samples obtained in gardens used for continuous cropping of *P. quinquefolius* in the Shaanxi province in China. A number of bacteria with biodegradation functions including *Methylibium*, *Sphingomonas*, *Variovorax*, and *Rubrivivax*, were found to decrease in abundance in soils used in ginseng cultivation relative to controls. For instance, *Rubrivivax gelatinosus* can degrade pollutants found in effluents produced from fish farms in fish industry effluent (de Lima et al., 2011). The

authors suggested that the decrease of these specific bacteria with biodegradation abilities may subsequently lead to the accumulation of toxic compounds in soil over time.

Most microbiome studies mentioned earlier have examined the rhizosphere of *P. ginseng*, *P. notoginseng*, *P. quinquefolius* and were predominantly done in Asia. Furthermore, the majority of ginseng microbiome studies examined how soil microbiomes collected from healthy and diseased roots differed, as well as how continuous cropping affects the composition of the microbiome. However, ginseng cultivation practices in Asia are different relative to North America in multiple ways. Therefore, less is known about ginseng diseases and ginseng soil microbiomes in North America.

Plantings are established through direct seeding in soil for cultivation in North America (Beyfuss, 2017), which are different from cultivation practices in China. In China, seeds can be planted either by direct seeding or first planted in pots then transplanted to seedbeds, and seeds can also be planted in spring or autumn, or planting in forests versus shaded gardens (Zhao & Xu, 2021). In addition, transplanting will often result in fewer but larger roots (Proctor et al., 1988). Secondly, the types of materials used in mulching are also different in both countries. In North American cultivation practice, mulching involves the use of small grain straws or fall leaves (Proctor et al., 1988), and it is maintained throughout the life of the crop. Fallen leaves of *Metasequoia glyptostroboides* are used instead for mulching in Asia, and the amount of covered *Metasequoia* branches regulates how much sun is allowed to permeate and is variable based on the growth stages of the plant (Guo et al., 2010). The cultivation practice for *P. notoginseng* in China and *P. ginseng* in Korea is to select specific strains that are adapted to different climatic conditions and be resistant to soil-borne pathogens while maintaining high-quality and high-yield roots (Zhang et al., 2020b). Finally, seasonal changes in soil conditions in North America and China will be different. These differences will mean that observations

of ginseng soil microbiomes from Asia may not reflect changes that occur in North American soil.

1.5.2 The effect of allelopathy and ginsenosides towards the microbiome

Allelopathy is a process in which plants release chemicals (e.g., acetic acid, cinnamic acid) in order to compete through molecular interactions for resources or affect the growth of other organisms including individuals of the same species. These chemicals are referred to as allelochemicals if they contribute to allelopathy. Ginsenosides are considered as allelochemicals and are released as autotoxins through root exudations from the plant to alleviate inter-plant competition (Samur, 2020; Yang et al., 2015; Yousef & Bernards, 2006). They also attract beneficial microbes such as *Bacillus amyloliquefaciens* subsp. *plantarum*, as well as harmful microbes such as *Pythium irregulare* and *Ilyonectria mors-panacis* towards the ginseng roots (Nicol et al., 2003; Ma et al., 2013). Ginsenosides have also been shown to enrich taxa of pathogenic fungi including *Fusarium* and *Alternaria* in Asian ginseng (*P. notoginseng*) (Li et al., 2020).

Ginseng also produces other allelochemicals. Dong et al. (2018b) reported that there was an acidification of ginseng garden soil as well as an accumulation of the autotoxic diisobutyl phthalate (DiBP), which was negatively correlated with the abundance of *Arthrobacter*, *Burkholderia*, *Rhodanobacter*, and *Sphingobacterium*. The authors also showed that the inoculation of ginseng seeds with *Sphingobacterium* sp. PG-1 helped with the growth of ginseng relative to control samples, suggesting that this particular bacterium has beneficial functions and could be selected to improve the growth of ginseng seeds and alleviate GRD in continuously cropped fields. This study provides insights into how the manipulation of soil microbial communities could be an effective

strategy for alleviating the problems caused by GRD and increase the productivity of the plant.

These previous studies indicate that ginsenosides can influence the composition of the soil microbiome which may contribute to the process of allelopathy. Ginsenosides have been shown to accumulate over the first two years of ginseng cultivation (Kaberi, 2021) and may be an important factor contributing to changes in the soil microbiome and susceptibility to GRD and other diseases in subsequent cultivation of ginseng plants.

1.6   Research objectives

This study is part of a larger project to track the origin of development of GRD in southern Ontario soils as a ginseng crop is grown from seed to harvest in ginseng-naïve soils. There are no currently known North American studies that have examined changes in the microbiome in ginseng-naïve soils due to the growth of *P. quinquefolius* from direct seeding to cultivation, and this project will characterize how soil microbiomes shift across time. This thesis specifically focuses on the bacteria and archaea over the first 3 years of ginseng cultivation addressing two main research objectives: (1) to characterize changes in the bacterial and archaeal microbiome with the use of DNA metabarcoding of the V4 region of the 16S rRNA gene, and (2) to identify bacterial and archaeal taxa that respond similarly as ginseng is cultivated to potentially identify indicators of or contributors to GRD.

DNA metabarcoding is a method in which environmental samples are analyzed by sequencing short genetic marker regions to identify members of the community and their relative abundances (Rousk et al., 2010). In this project, the V4 region from the 16S rRNA gene, which is universally common to bacteria and archaea, will be amplified and sequenced (Thompson et al., 2017; Yeh et al., 2021). Changes in the soil microbiome will

be examined through multivariate analyses to determine whether there are taxa or groups of taxa that increase or decrease in abundance as ginseng is grown, which may indicate that they play a role in setting up the conditions for ginseng replant disease.

Chapter 2

2 Materials and Methods

2.1 Farm site and Experimental Design

Three farm sites located in Norfolk County, Ontario were used for this thesis (Figure 2.1). These farm sites were newly planted ginseng gardens in which the soil had not been previously used for ginseng cultivation. The sites were prepared for ginseng cultivation by creating raised soil beds and erecting solar shades in October (Figure 2.2) and ginseng seeds were planted in October 2018.

Garden soil samples were collected throughout the cultivation of ginseng from the raised soil beds in which ginseng was planted; these are referred to as the experimental samples. Control samples were soils collected from outside of the gardens. At each site, experimental plots were subject to standard farming practices, e.g., application of fertilizers and pesticides. Control plots were not shaded, and not purposefully treated and maintained.

Experimental and control soil samples were collected immediately after seeding in the Fall of 2018, then seasonally, four times a year (spring, early summer, late summer, fall), for three consecutive years. For each sampling date, five replicate samples were collected within the ginseng beds from each garden (experimental), and outside of the beds (control). For each replicate sample, three 30 cm soil cores were collected with a LaMotte 1055 soil sampler (2.5 cm diameter, 30.5 cm long) (Figure 2.3A), which was sterilized between replicates using 70% ethanol. The three soil cores from each replicate were combined together. Control samples were collected with a trowel (Figure 2.3B) in three different spots and combined together for each of five replicates. Both experimental and

control samples were stored in plastic sealable bags and temporarily stored in an ice box while in the field. All soil samples were stored at -20°C until further processing.
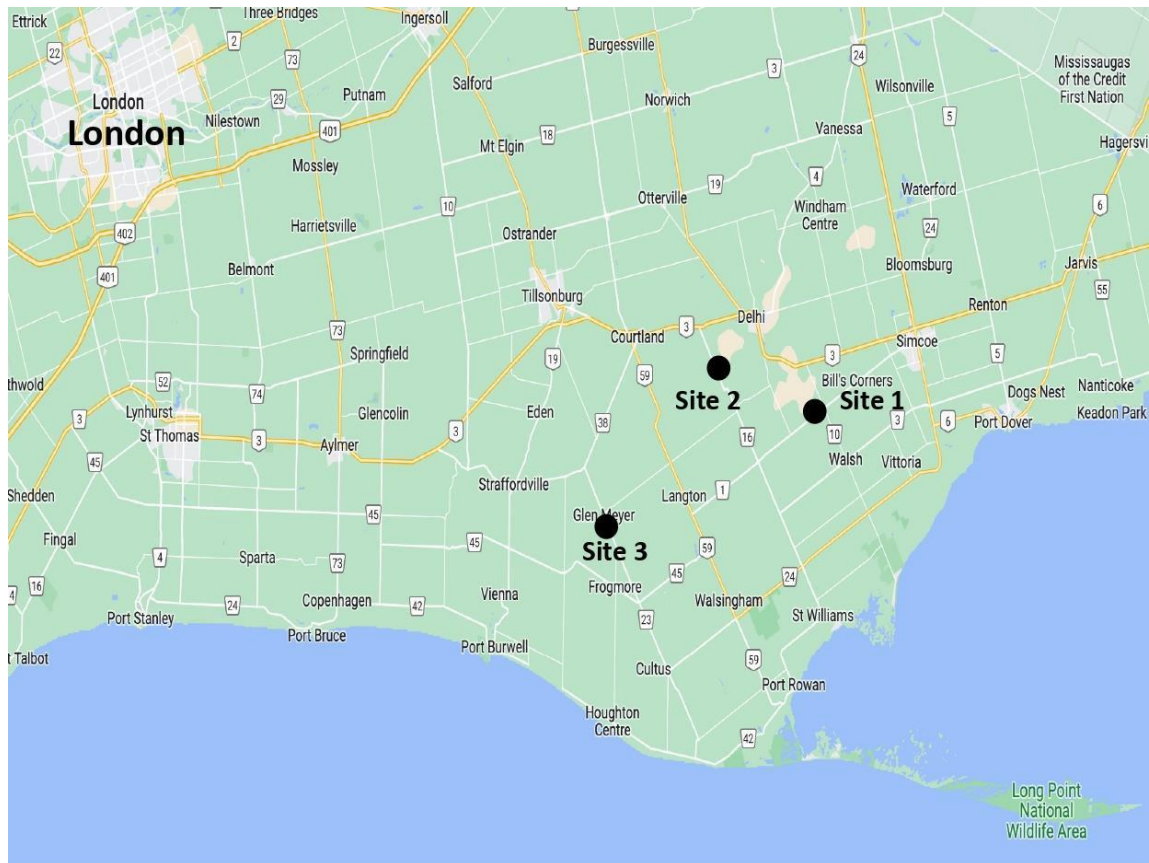
Figure 2.1: Approximate locations of each farm site investigated in Norfolk County, Ontario.

Figure 2.2: Raised soil beds from Site 1 with the solar shade structures provided for ginseng cultivation.
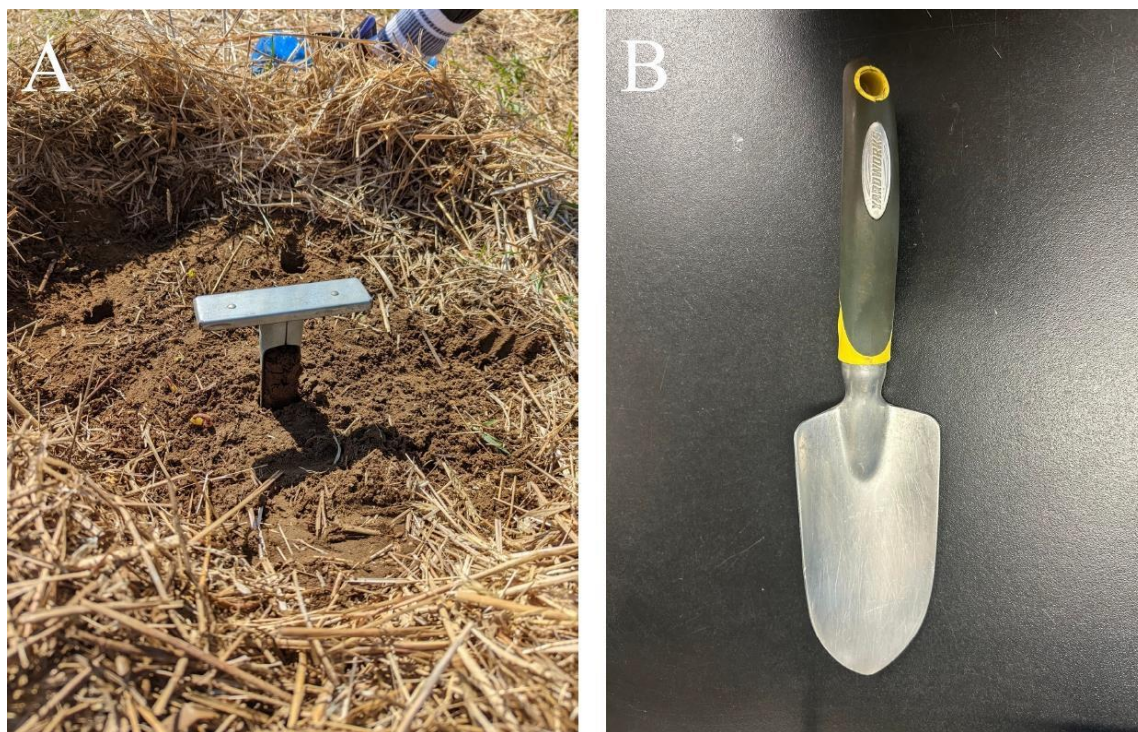
Figure 2.3: Sample soil collection tools. A) Taking a soil core with a LaMotte 1055 soil sampler. Soil samples where ginseng was grown were collected seasonally with a LaMotte 1055 soil sampler, stored in plastic sealable bags, and temporarily placed in an ice box while in the field. For each replicate, three cores were collected then combined together for all sites. B) Control samples outside of the ginseng gardens were collected seasonally with a small trowel.

2.2 Soil sieving

Prior to DNA extraction, soil samples were removed from the -20°C freezer and thawed overnight at 4°C before sifting. Soil was placed into a sieve with 0.2 mm mesh to remove any debris (e.g., sticks and stones). Sieved soil was placed in a new plastic bag, and stored at -20°C.

2.3 Molecular methods

2.3.1 DNA extraction

A modified DNA extraction protocol based on Kamble and Singh (2020) was used to extract DNA from 1 g of soil. Soil samples were placed in a 5 mL falcon tube along with 2 g of 1 mm zirconium beads (Fisher Scientific) and 3.2 mL lysozyme solution (0.15 M Tris- HCl, 0.1 M EDTA, 30 mg/mL lysozyme (Bio Basic)), which was then placed into a bead beater (Bullet Blender 5E Pro) at speed 16 for 15 minutes, followed by an incubation at 37°C for 30 minutes. The samples were centrifuged at 6000 x g for 10 minutes, and the supernatant was transferred into a 15 mL screw cap tube. One-tenth volume of 3 M sodium acetate and 0.6 volume of 100% isopropanol were added to precipitate DNA at room temperature for 5 minutes, then centrifuged at 6000 x g for 20 minutes to yield a light yellowish brown pellet. The supernatant was discarded and 5 mL of cold 70% ethanol was added to wash the DNA pellet. After centrifugation at 16,000 x g for 10 minutes, the supernatant was discarded and the pellet air dried. The DNA pellet was processed and cleaned with ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Germany) as per the manufacturer's instructions with minor modifications. Briefly, the DNA pellet was first resuspended with 400 µL of Zymobiomics Lysing Buffer, then transferred into a Zymo-Spin III-F filter and centrifuged at 8000 x g for 1 minute. ZymoBIOIMCS DNA Binding buffer (1200 µL) was then added to the filtrate and

mixed. The mixture was transferred to a Zymo-Spin IICR column and was centrifuged at 10,000 x g for 1 minute. The flow-through from the column was discarded, 400 µL of ZymoBIOMICS Wash Buffer 1 was added to the IICR column and centrifuged at 10,000 x g for one minute. The flow-through was from the column was discarded, 700 µL of ZymoBIOMICS DNA Wash Buffer 2 was added to the IICR column and centrifuged at 10,000 x g for one minute. The flow-through from the column was discarded, 200 µL of ZymoBIOMICS DNA Wash Buffer 2 was added and centrifuged at 10,000 x g for one minute. The IICR column was then transferred to a sterilized 1.5 mL microcentrifuge tube. To elute the DNA from the column, TE buffer (10 mM Tris pH 8, 0.1 mM EDTA) was first heated to 65°C in order to help with the elution process, then 50 µL added to the column. The column was centrifuged at 10,000 x g for one minute to collect the eluted DNA. DNA concentrations, A260/A230, and A260/A280 ratios were determined with the NanoDrop One Spectrophotometer (Thermo Scientific, Burlington ON).

2.3.2   Two Step PCR of the V4 region of the 16S ribosomal RNA gene for metabarcoding

16S rRNA V4 amplicons were prepared for Illumina Miseq sequencing using a dual-indexing protocol with two separate PCR reactions (2-step PCR) similar to that described by Gohl et al. (2016). In the first PCR reaction, the V4 region of the 16S rRNA gene was targeted with 515F and 926R primers (Table 1, Figure 2.5A, Parada et al., 2016; Yeh et al., 2021). In the first PCR reaction, each reaction contained 6.25 µL of AccuStart II PCR ToughMix, 0.625 µL each of 10 µM 515F and 926R primers, 3.75 µL of water, 0.25 µL of 50X loading dye, and 1 µL of soil DNA for a total reaction volume of 12.5 µL. The PCR was also repeated using 1:10 diluted soil DNA. The PCR conditions for the first PCR was: the initial polymerase activation and DNA denaturation step took place at 94°C for two minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, then a final extension step

at 72°C for 5 minutes. The final hold temperature was 4°C before products were stored in a 4°C fridge.

In the second PCR, unique 8 bp sequences (index) were added to distinguish each sample as well as the remainder of the Illumina adaptor (Figure 2.5B, Table 1), producing dual indexed amplicons that were compatible with an Illumina Miseq instrument (Figure 2.5C). In the second PCR, each reaction contained 10 µL of Phusion® Hot Start Flex 2X Master Mix (New England Biolabs), 3.2 µL (5 µM) of each forward and reverse index primers (unique for each sample), 1 µL of DNA from the first PCR, and water for a total volume of 20 µL. The cycling conditions for the second PCR were: the initial activation and DNA denaturation step at 95°C for 5 minutes, followed by 10 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for one minute, then a final extension step at 72°C for 5 minutes. The final holding temperature was at 10°C before they were stored at 4°C.
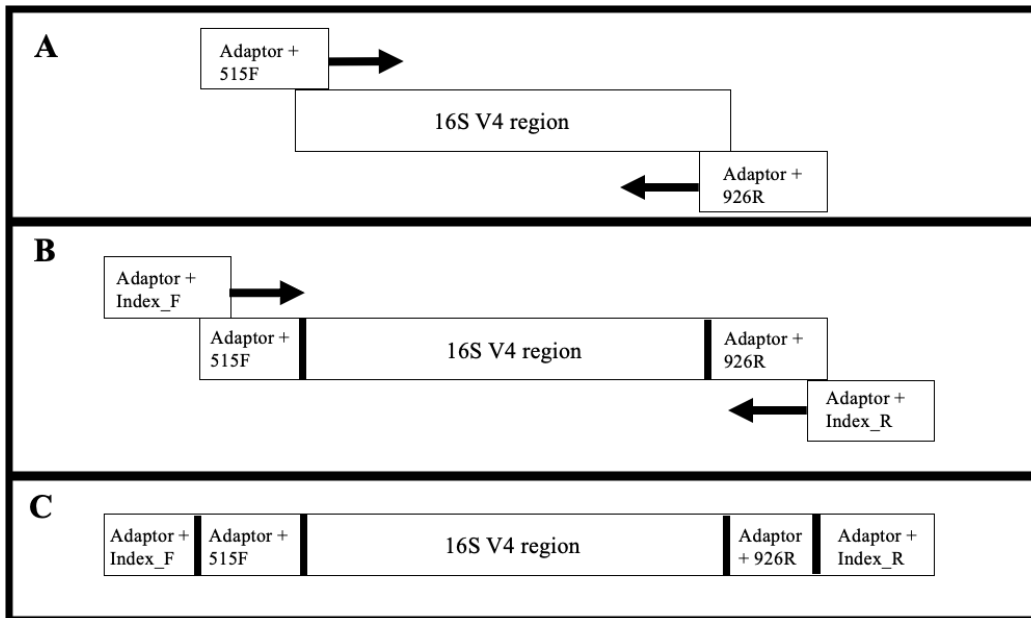
Figure 2.4: Two step PCR protocol for producing the 16S V4 dual indexed sequence amplicons. A) Primers used in the first round of PCR is marker specific. The forward and reverse primers (515F and 926R) target that 16S V4 region and also contain Illumina-compatible adaptor sequences. B) The second round of PCR amplifies the products produced from the first round of amplification, which are sample specific indices with adaptor sequences on both ends of the amplicon product. C) The final PCR product. The size of this PCR product was confirmed with gel electrophoresis before sending for Illumina Miseq sequencing.

Table 1: Primers used to universally amplify the 16S V4 rRNA region with MiSeq sequencing adaptors from bacteria and archaea. Bolded sequence indicates oligonucleotides used to target the 16S V4 region. Italicized sequence indicates complementary sequences for priming the second step PCR. Underlined sequence indicates index sequences.

Primer Name	Sequence
16SV4_515F_NexPad	*TCGTCGGCAGCGTC*AGATGTGTATAAGAGACAG**GTGY CAGCMGCCGCGGTAA**
16SV4_926R_NexPad	*GTCTCGTGGGCTCGG*AGATGTGTATAAGAGACAG**CCG YCAATTYMTTTRAGTTT**
Adaptor + Index_F	AATGATACGGCGACCACCGAGATCTACAC<u>N NNNNNN</u> N*TCGTCGGCAGCGTC*
Adaptor + Index_R	CAAGCAGAAGACGGCATACGAGAT<u>NNNN NNNN</u>*GTCT CGTGGGCTCGG*

2.3.3   Product confirmation and Sequencing

Agarose gel electrophoresis was used to confirm the size of the PCR products from the first round of PCR. Similarly, gel electrophoresis was run for the final PCR products to ensure that they were consistent with a predicted size of around 450 bp. For each sample, equal volumes of the 2nd PCR reactions from the 1X and 1:10 diluted soil DNA were combined, and the DNA concentrations were quantified by London Regional Genomics using a Qubit fluorometer. Samples were pooled by normalizing the DNA concentration for each sample and the pooled DNA was then sequenced with an Illumina Miseq instrument to acquire 2 X 300 bp sequencing reads. phiX (20%) was added to the pooled DNA in order to provide a diversity of sequences needed by the Illumina method to discriminate bases.

2.4   Bioinformatics and statistical analysis

2.4.1   Sequencing processing

Sequence analysis programs available from QIIME2 (version 2022.2) (Bolyen et al., 2019) were used to process and taxonomically classify the sequences reads. Sequence reads from all three sites were first combined prior to any processing. Sequences were trimmed to 292 and 192 bp for forward and reverse reads, respectively, resulting in an overlap of 107 bases between the forward and reverse reads. DADA2 was used to trim primer sequences and bases with a quality score lower than 20, remove chimeric sequences, and correct sequencing errors, resulting in a table of unique amplicon sequence variants (ASVs) and their abundances in each sample (Callahan et al., 2016). Samples with a low abundance of sequence reads (<300) were removed.

2.4.2   Taxonomic assignment

ASVs were classified taxonomically using a scikit-learn naive Bayes machine-learning classifier trained on the SILVA database for the V4 region of the 16S rRNA gene (Quast et al., 2012). ASV counts from each sample were transformed into relative abundances and grouped by phylum to examine community composition, which was then visualized by bar plots created from ggplot2 (Wickham, 2016).

2.4.3   Community Analysis

Statistical and phylogenetic analyses as well as graphs were created with various R packages including phyloseq (McMurdie & Holmes, 2013), vegan (Dixon, 2003), and ggplot2 (Wickham, 2016) installed in R version 4.2.2 (R Core Team 2020). It is important to note that when evaluating the results by year, the Fall 2018 samples were grouped with the 2019 samples (Table 2).

Table 2: Soil samples collected from Fall 2018 to Fall 2021 in a three-year time period. Fall 2018 samples are grouped into the year one category for analyses.

Collection year	Year	Samples
2018	1	Fall 2018
2019	1	Spring 2019, Summer A 2019, Summer B 2019, Fall 2019
2020	2	Spring 2020, Summer A 2020, Summer B 2020, Fall 2020
2021	3	Spring 2021, Summer A 2021, Summer B 2021, Fall 2021

Alpha diversity for each soil sample across the three sites was measured using the Shannon diversity index in phyloseq (McMurdie & Holmes, 2013) and Faith's phylogenetic diversity in Picante (Kembel et al., 2010). Statistically significant differences between control and experimental samples and across three years was evaluated with a linear fixed effects model and a post hoc test (Tukey's range test) from nlme and lsmeans packages, respectively (Pinheiro & Bates, 2000; Lenth, 2018).

To examine differences in community composition (beta diversity), the data was first rarefied to an even sampling depth and the unweighted UniFrac metric (Lozupone et al., 2011) was calculated to measure the dissimilarity between samples. Principal coordinate analysis (PCoA) was used to determine whether the community composition from experimental samples from three sites shifted over time, or whether control and experimental samples differed by site and time. For comparisons between control and experimental samples, sites were first analyzed separately using PCoA. This was then followed by comparing control and experimental samples. Permutational multivariate analysis of variance (PERMANOVA) tests were conducted to test whether UniFrac distances between site, time, interaction between site and year, interaction between site and sample type groups were significantly different. After examining the PCoA of all samples, year 1 control and experimental samples were similar but differences between community composition became evident starting in year 2. In addition, samples from each site did not overlap, so each site demonstrated distinct changes. In order to examine differences between control and experimental samples, analyses were conducted with only year 2 (2020) and 3 (2021) samples, and each site was analyzed individually. For these analyses, PERMANOVA were conducted based on sample type.

From the PCoAs, species loading data were used to assess which ASVs provided the highest contributions to the variation in the experimental samples across time. Firstly, all

species loadings were plotted as arrows on a PCoA biplot, which was then followed by selecting arrows in a specific range of angles that best explained the spread of the samples over time. The top 10% of ASVs represented by these arrows contributing the most to the spread of the samples over time were considered further. Similarly, the comparison of control and experimental samples collected at year 2 (2020) and 3 (2021) was also assessed by using species loading data. The species loading arrows were plotted on the PCoA, and arrows in a specific range were selected that best explained the spread of the data between control and experimental samples. The top 10% of ASVs from these arrows were selected for further consideration. From these lists of ASVs, the number of ASVs for a taxonomic family were counted and bubble plots were made to examine the composition of taxa that primarily contributed to the differences in experimental samples over time, or between control and experimental samples.

Analysis of compositions microbiomes with bias correction 2 (ANCOM-BC2) (Lin & Peddada, 2020) and ANOVA-like differential expression tool for compositional data (ALDEx2) (Fernandes et al., 2014) were performed to identify taxa that were significantly different in abundance in control and experimental samples, and in experimental samples across time. The experimental data were analysed by ANCOM-BC2 by grouping the samples by year (Table 2) as seasonal variations in community composition were not a dominant trend in the PCoA. To examine differences between control and experimental samples, each site was analyzed separately as the community changes were not consistent for all sites based on the PCoA. Control and experimental samples in year 2 were compared relative to each other, and this was repeated for year 3. For ALDEx2, the experimental data from all three sites were compared pairwise between year 1 and year 2 and between year 2 and year 3. Control and experimental samples were also compared pairwise for each year of samples, but for this analysis, sites were analyzed separately. Wilcoxon signed-rank test and Welch's t-test were used to test

whether specific ASVs in the microbial community were significantly different in abundance.

Chapter 3

3 Results

3.1 Sequencing results

After Illumina sequencing, an average of 15871, 22729, and 23636 reads per sample in site 1, 2, and 3, respectively, were produced from the V4 region from the 16S rRNA gene (16S V4) region using bacterial primers pairs (515F and 926R) (Table 3). Samples from site 3 contained the highest number of merged sequences and of ASVs compared to the other two sites. Even though the number of ASVs was distinctly lower from samples in site 1, rarefaction curves indicate that ASV diversity was sufficiently sampled from the soil samples (Appendix A, Figure A1 – A3).

Table 3: Average and total number of Illumina Miseq reads from all soil samples per site taken from ginseng garden and control soils after quality filtering steps using DADA2.

	Average Site 1 results	Average Site 2 results	Average Site 3 results	Total number of sequences analyzed
Input sequences	15,871	22,729	23,636	62,236
Filtered sequences	6403	14396	15084	35,883
Denoised sequences	2858	8201	10380	21,439
Merged sequences	594	1661	3575	5830
Non-chimeric sequences	532	1243	1918	3693

3.2 Overview of the diversity of soil communities in ginseng garden soils

The mean relative abundance of bacterial and archaeal ASVs grouped at the phylum level for control and experimental soil samples from sites 1, 2, and 3 are plotted by sampling date from fall 2018 to fall 2021 (Figures 3.1). The phyla identified from control and experimental samples and across all three sites were generally similar. Common phyla found in all samples from all three sites include Acidobacteriota, Actinobacteriota, Bacteroidota, Chloroflexi, Crenarchaeota, Firmicutes, Gemmatimonadota, Plantcomycetota, and Proteobacteria. In general, ASVs from the Crenarchaeota and Proteobacteria increased in relative abundance over the three year time frame, while those from the Firmicutes decreased in relative abundance for all samples from the three sites. ASVs from Acidobacteriota increased over time, and those from Actinobacteriota decreased over time for all samples except for control samples from site 2. ASVs from the Cyanobacteria were only identified in control samples. Furthermore, experimental and control soil samples from site 2 tend to have a similar microbiome profile, but a notable difference was that the relative abundance of Verrucomicrobia increased in year 2 and year 3 for the controls (Figure 3.1 C-D). Finally, trends observed from site 3 were similar overall to site 2 samples, compared to site 1 samples which had a higher relative abundance of Firmicutes particularly in the experimental samples (Figure 3.1). Phyla that were lower in relative abundance and not consistently greater than 1% in relative abundance, such as GAL 15, Zixibacteria, RCP2-54, and Abditibacteria, might also be observed in other samples but would be included in the taxa that had <1% relative abundance.
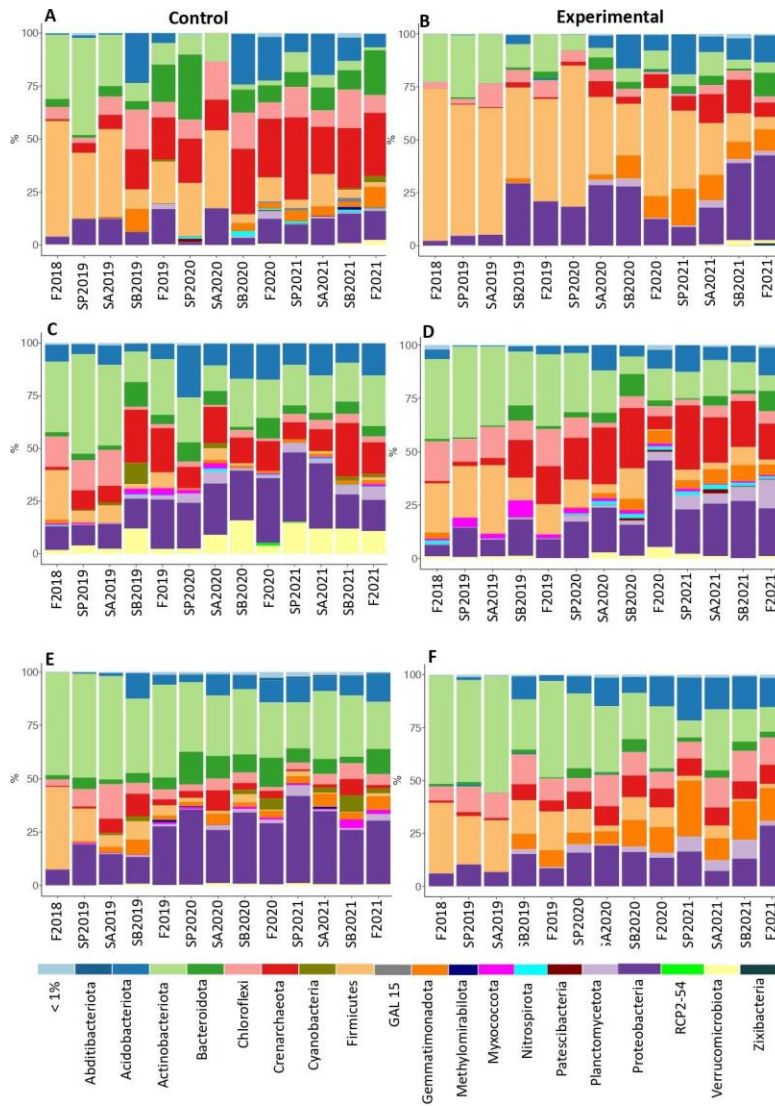
Figure 3.1: Relative abundance barplot of taxa grouped at the phylum taxonomic rank across time. A) Site 1 control samples. B) Site 1 experimental samples. C) Site 2 control samples. D) Site 2 experimental samples. E) Site 3 control samples. F) Site 3 experimental samples. Rarer taxa with a mean abundance of less than one percent are grouped together as the < 1% group and not shown specifically.

Alpha diversity of the soil microbiomes was assessed by calculating the Shannon diversity index and Faith's phylogenetic diversity. Shannon diversity index has a similar distribution for control samples over time but decreased in the experimental samples (Figure 3.2A). A linear mixed effect model confirmed that there was no significant change in Shannon diversity indices measured in control samples through time suggesting that species diversity remained consistent. However, the Shannon diversity indices for experimental samples grouped by year were significantly different (year 1 and year 2, $p < 0.0001$ and year 1 and year 3 ($p < 0.0001$)). In contrast, using the Faith's phylogenetic diversity, the distribution of the diversity indices for control and experimental samples was similar over time (Figure 3.2B). A linear mixed effect model was not significant for both sample types grouped by year. Microbes from specific taxonomic groups or evolutionary lineages did not dominate more frequently in these sample groups.
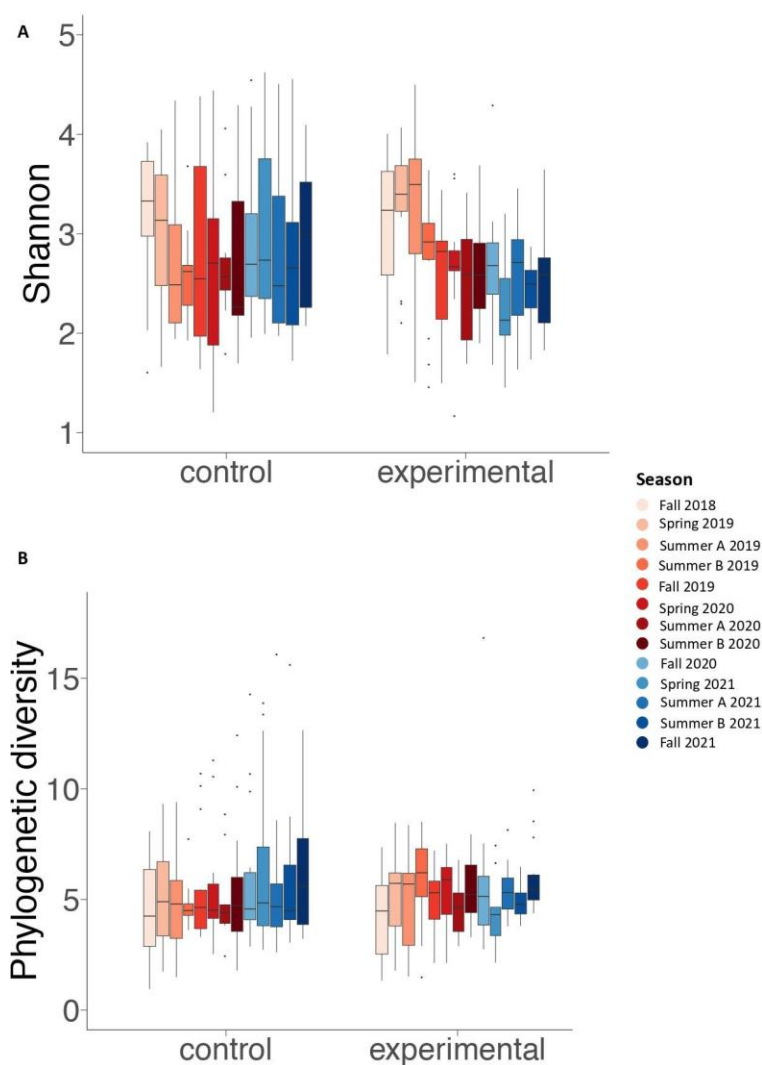
Figure 3.2: Boxplot for alpha diversity values for soil microbiomes in control and experimental samples across time for sites 1, 2, and 3. A) Shannon diversity index. B) Faith's phylogenetic diversity. The midline indicates the median and the upper and lower ends of the box represent the upper and lower quartile, respectively. The extent of the whisker lines represents the maximum and minimum values. Black dots represent outliers.

Principal coordinates analyses (PCoA) of unweighted UniFrac distances were used to assess whether samples from three sites differed significantly in composition, and whether samples grown with or without ginseng from the three sites were significantly different across time. A PCoA of all samples (Figure 3.3A), indicates that both control and experimental samples overlapped more so for sites 2 and 3 than for site 1 samples suggesting that the composition of communities from site 1 was significantly different from sites 2 and 3. The same PCoA plot, but with the samples coloured by time clearly shows consistent changes in community composition over time for all samples (Figure 3.3B). Both control and experimental samples shifted across the first principal component axis from Fall 2018 to 2021 (year 3) indicating that time was a primary factor correlating with changes in the microbiomes. These observations were confirmed by a PERMANOVA, which was significant when considering sample type only, year only, and their interaction ($p<0.001$), while a beta dispersion analysis was not significant, ($p=0.705$)), indicating that the dispersion of samples was the same for each group. Control and experimental samples are not clearly different when all samples were analyzed together, but there are distinct differences when sites were analyzed separately (see section 3.4).
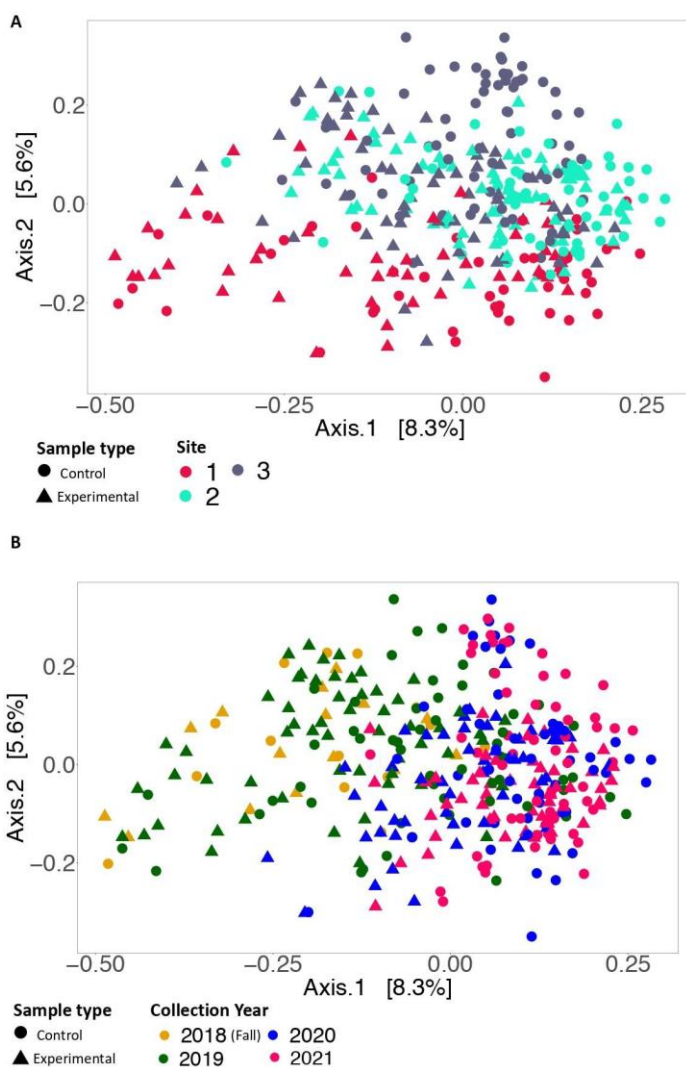
Figure 3.3: Principal coordinate analysis using unweighted Unifrac for measuring the dissimilarity in the composition of amplicon sequence variants (ASVs) from all control and experimental soil samples. A) Samples coloured by site. B) Samples coloured by sampling year. Each year consisted of four sampling points (Spring, Summer A, Summer B, and Fall), except for 2018 when soil was sampled only in the fall when seeds were first planted.

3.3 Microbiome changes in garden soil over time

To specifically examine changes in the soil microbiomes in the experimental samples (i.e., ginseng garden soils), a PCoA with only experimental samples for all three sites was conducted (Figure 3.4). This analysis confirms a consistent shift in community composition across time in samples where ginseng was grown. A PERMANOVA for the experimental samples only considering pairwise comparisons of all time points was also significant ($p<0.001$, beta dispersion of samples grouped by time point was not significant ($p=0.558$)).
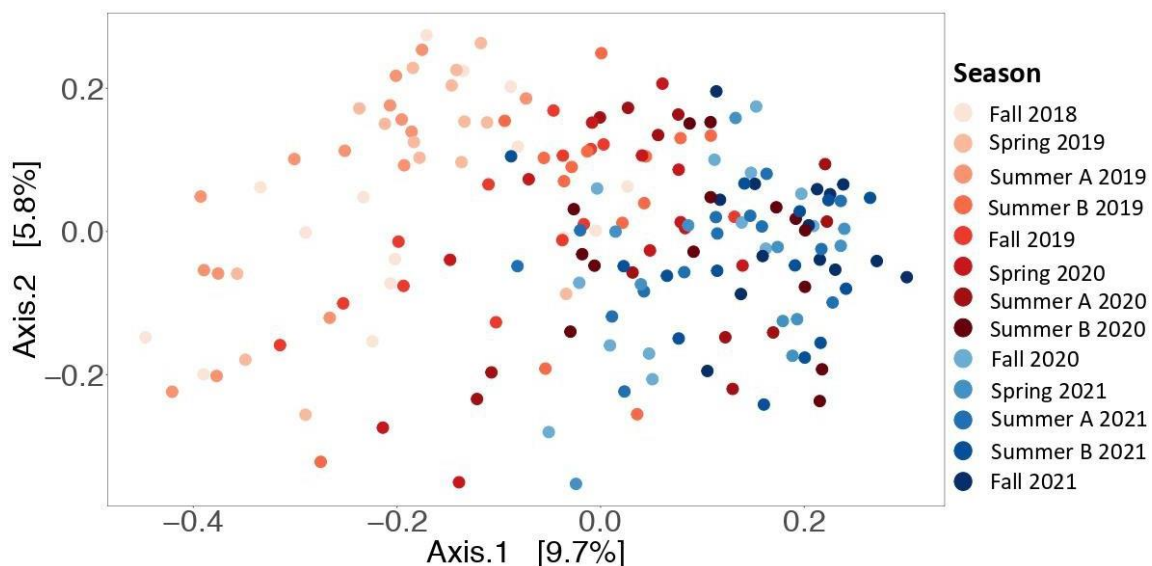
Figure 3.4: Principal coordinate analysis using unweighted Unifrac for measuring the dissimilarity in the composition of amplicon sequence variants (ASVs) from experimental soil samples only for site 1, site 2, and site 3.

To identify taxa contributing to the shift in microbiome composition over time in the experimental samples, species loadings were analyzed, indicating the contribution of each ASV to the ordination of samples in the PCoA. The species loadings for each ASV were

filtered by selecting the top 10% of ASVs contributing to the shift in the microbiome composition over time (i.e., across axis 1 in the PCoA). The taxonomic composition of these ASVs is shown as a bubble plot indicating the number of ASVs in a taxonomic family that contributed to the differences in composition over time (Figure 3.5 A-C). Several ASVs increased in relative abundance from families belonging to the Crenarchaeota and Proteobacteria (Figure 3.5A), and taxonomy barplots also showed an increased relative abundance of reads from these phyla (Figure 3.1). The bacterial families that increased in these phyla are known to be associated with particular metabolic functions, biochemical conversions, or are relevant to bioremediation functions. Several families contain microbes involved in the nitrogen cycle: Beijerinckiaceae (Proteobacteria) is a family known to have nitrogen fixing bacteria, both Nitrososphaeraceae (Crenarchaeota) and Nitrosopumilaceae (Crenarchaeota) are families with ammonia oxidizing archaea, and Nitrospiraceae (Nitrospirota) contain nitrate oxidizing bacteria. Xanthobacteraceae (Proteobacteria) was also found to have increased in experimental samples over time, and bacteria belonging to this family are known to degrade toxic compounds such as chlorinated alkanes, alkenes, polyaromatic compounds, thiophenes (Oren, 2014). ASVs that decreased in samples over time (Figure 3.5B) include ASVs from the Aeromonadaceae (Proteobacteria), and bacteria from this family include known pathogens found in fish. However, there are no known applications towards agricultural uses or in the food industry. ASVs also decreased from families including Alicyclobacillaceae, Bacillaceae, Clostridiaceae, Hungateiclostridiaceae, and Lachnospiraceae, which belong to the phylum Firmicutes. Clostridiaceae, Hungateiclostridiaceae, and Lachnospiraceae are grouped under the class, Clostridia.

Families including C0119, Gitt-GS-136, Roseiflexaceae, and SHA-26, all belong to the phylum Chloroflexi. Although bacteria belonging to the Roseiflexaceae have been identified in extreme environments (e.g., hot springs), no known functions have been

identified. Finally, there are also families with ASVs from the top 10 % that increased and others that decreased over time. For instance, the family, Sphingomonadaceae (Proteobacteria) comprised ASVs that increased and decreased (Figure 3.5C).
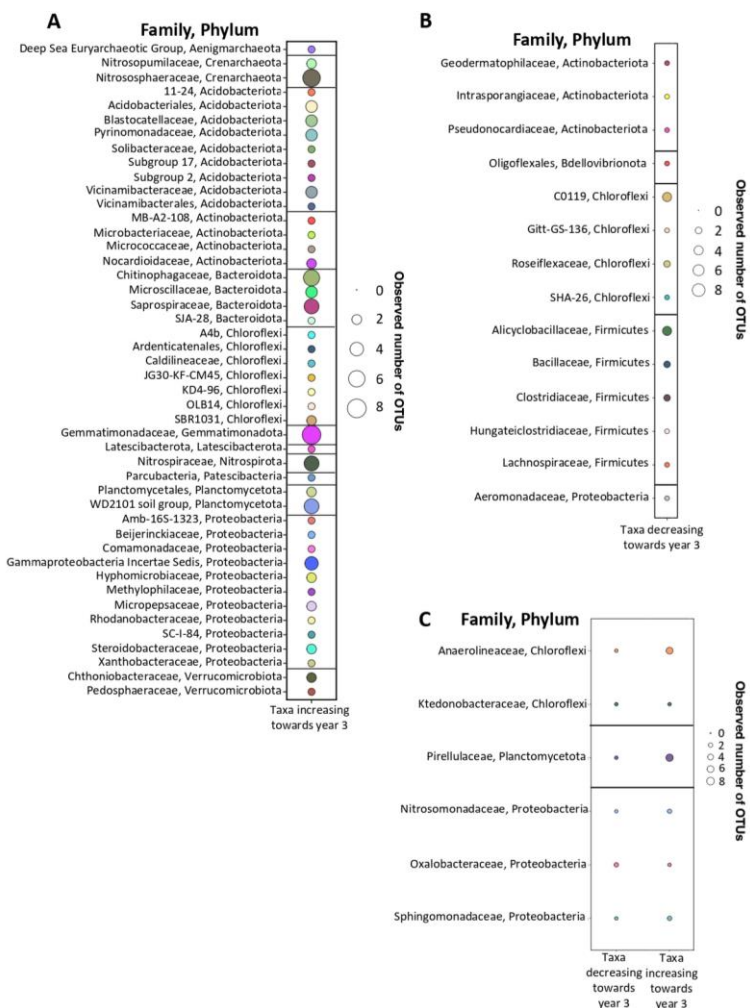
Figure 3.5: Bubble plot indicating the number of ASVs per taxonomic family from the top 10% of the ASVs with the highest contribution to the spread of the data across axis 1 (correlating with changes over time) in the PCoA analysis for experimental samples. The number of ASVs observed for each family corresponds with the size of the bubbles. A) Bacterial and archaeal families that increased over time. B) Bacterial families that decreased over time. C) Bacterial families that increased and decreased over time.

3.3.1 Differential abundance analyses of samples from ginseng garden soils over time

Differential abundance analyses using analysis of compositions of microbiomes with bias correction 2 (ANCOM-BC2) and ANOVA-like differential expression tool for high throughput sequencing data (ALDEx2) were used as alternative methods to determine whether there are specific taxa that changed significantly over time. ANCOM-BC2 was run with only experimental samples from three sites over time at the ASV level and by grouping ASVs into their taxonomic families to compare with the results from the species loadings analysis. Overall, the number of taxa that were found to differ significantly was low (Figure 3.6). Oddly, there were no taxa that were significantly different in abundance when comparing year 3 and year 1 samples in the ANCOM-BC2 analysis, although these sample groups were clearly different in composition based on the PcoA (Figure 3.4).

When grouping ASVs into families, taxa with an increase in relative abundance between year 2 and 3 included Gemmatimonadaceae (Gemmatimonadota), Microscillaceae (Bacteroidota), Nitrosophaeraceae (Crenarchaeota), Xanthobacteraceae (Proteobacteria), and Vicinamibacteraceae (Acidobacteriota) (Figure 3.6), and ASVs from these families also were shown to increase over time in the species loadings analysis. In contrast, when examining the results of the change in relative abundance between years 1 and 2, the same taxa decreased from year 1 to 2, suggesting that changes in abundance over time were not linear or consistent trends. At the ASV level, only one ASV is identified to change significantly in abundance from year 2 to 3, an unidentified species from Bacillaceae (Firmicutes) which decreased. This Bacillaceae (Firmicutes) ASV and 3 Micrococcaceae (Actinobacteriota) ASVs increased from year 1 to 2 and did not change significantly from year 2 to 3.

ALDEx2 confirmed the lack of taxa that were differentially abundant in the ANCOM-BC2 analysis. Using ALDEx2, no significant taxa were identified in comparing year 1 versus year 2, and year 1 versus year 3 experimental samples from all three sites.

|	Year 2 vs Year 1	Year 3 vs Year 1	Year 3 vs Year 2
(Proteobacteria) Xanthobacteraceae	−1.21	0	1.72
(Acidobacteriota) Vicinamibacteraceae	−0.71	0	1.26
(Planctomycetota) uncultured_13	−0.45	0	0
(Actinobacteriota) Solirubrobacteraceae	0.72	0	0
(Chloroflexi) Roseiflexaceae	1.07	0	−1.46
(Actinobacteriota) Pseudonocardiaceae	0.53	0	0
(Firmicutes) Planococcaceae	0.89	0	−1.31
(Planctomycetota) Pirellulaceae	−0.86	0	0.89
(Actinobacteriota) Nocardioidaceae	0.72	0	−1.02
(Nitrospirota) Nitrospiraceae	−0.52	0	0
(Crenarchaeota) Nitrososphaeraceae	−0.84	0	1.41
(Bacteroidota) Microscillaceae	−0.6	0	1.07
(Actinobacteriota) Micrococcaceae	0.95	0	−1.05
(Chloroflexi) JG30−KF−CM45	0.67	0	−1.05
(Actinobacteriota) Geodermatophilaceae	1.07	0	−1.51
(Gemmatimonadota) Gemmatimonadaceae	−1.72	0	2.16
(Firmicutes) Clostridiaceae	1.3	0	−1.74
(Firmicutes) Bacillaceae	1.3	0	−1.28
(Chloroflexi) Anaerolineaceae	−0.71	0	1
(Firmicutes) Alicyclobacillaceae	1.75	0	−2.14

Figure 3.6: ANCOM-BC2 analysis for experimental samples from sites 1, 2, and 3 together comparing groups pairwise for samples from years 1, 2, and 3. ASVs were grouped at the family level. Red and blue colours indicate an increase or decrease in relative abundance for a particular family, respectively.

3.4 Compositional differences in experimental samples compared to control.

The microbial composition of control and experimental samples from all sites analyzed together could not be clearly distinguished in the PCoA, indicating that there was not a consistent difference in composition when considering all sites (points for these sample groups overlapped) (Figure 3.3). However, when the data was separated into individual sites, there was a clear separation between control and experimental samples collected in 2020 (year 2) and 2021 (year 3) but not from fall 2018 and 2019 (year 1) samples (Appendix A, Figure A4). Therefore, a PCoA from year 2 and 3 experimental and control samples was performed to focus on the differences that developed between control and experimental samples in the latter years (Figure 3.7). The top 10% of ASVs contributing to the difference in microbiome composition between control and experimental samples (i.e., across axis 1 in the PCoA) for each site was considered and shown as bubble plots (Figure 3.8, Appendix A, Figure A6). Overall, there were more ASVs that were observed to have a higher relative abundance in site 3 control samples relative to all the other groups. Taxa where more ASVs were lower in experimental samples are from bacterial families including 67-14 (Actinobacteriota), Solirubrobacteraceae (Actinobacteriota), Bdellovibrionaceae (Bdellovibrionota), Chroococcidopsaceae (Cyanobacteria), Methyloligellaceae (Proteobacteria), Nitrosomonadaceae (Proteobacteria), Xanthobacteraceae (Proteobacteria), Opitutaceae (Verrucomicrobiota), and Pedosphaeraceae (Verrucomicrobiota) (Figure 3.8). Taxa where more ASVs were higher in experimental samples were from bacterial families including Micrococcaceae (Actinobacteriota), KD4-96 (Chloroflexi), Ktedonobacteraceae (Chloroflexi), Alicyclobacillaceae (Firmicutes), Clostridiaceae (Firmicutes), and Planococcaceae (Firmicutes). In general, the taxa for which ASVs were higher in relative abundance in

either control or experimental samples were consistently identified across one or more sites. For instance, Nitrosomonadaceae (Proteobacteria) was found to increase across controls across all sites (Figure 3.8). In addition, there were many families with ASVs identified to have increased and decreased in experimental samples which were also shown in bubble plots (Appendix A, Figure A6).
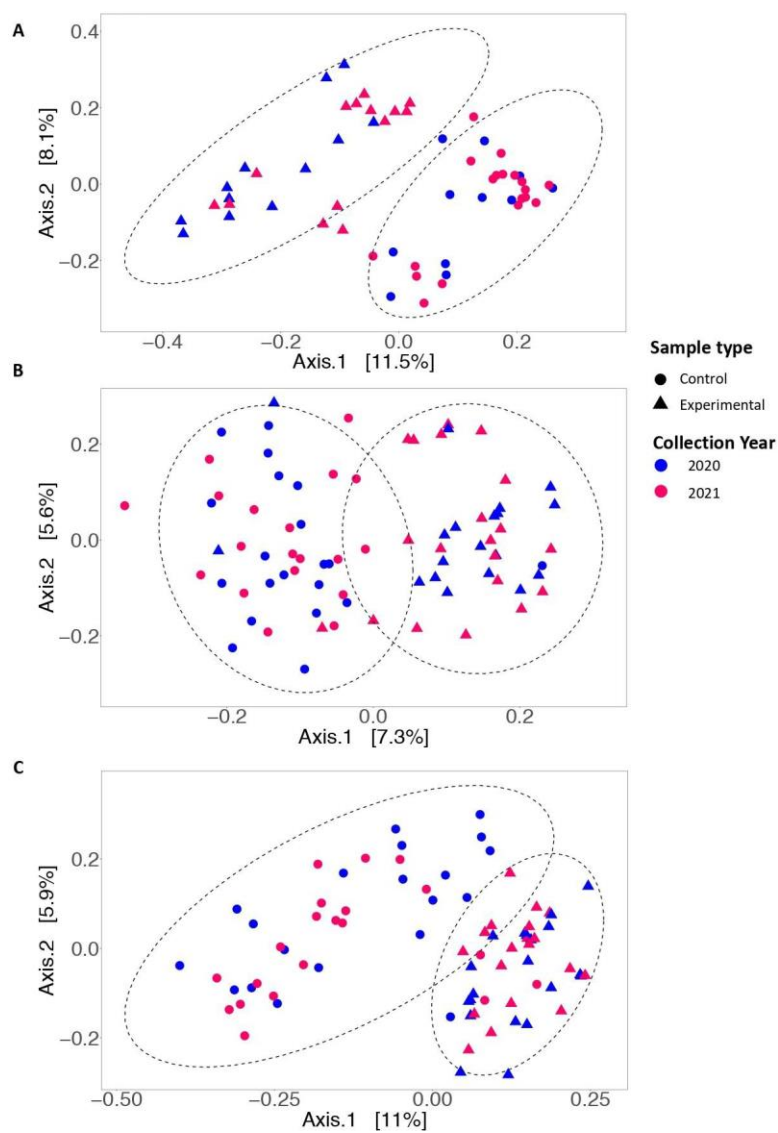
Figure 3.7: Principal coordinate analysis using unweighted Unifrac for measuring the dissimilarity in the composition of amplicon sequence variants (ASVs) from control and experimental soil samples collected in 2020 and 2021. A) Site 1 samples. Site 2 samples. C) Site 3 samples.
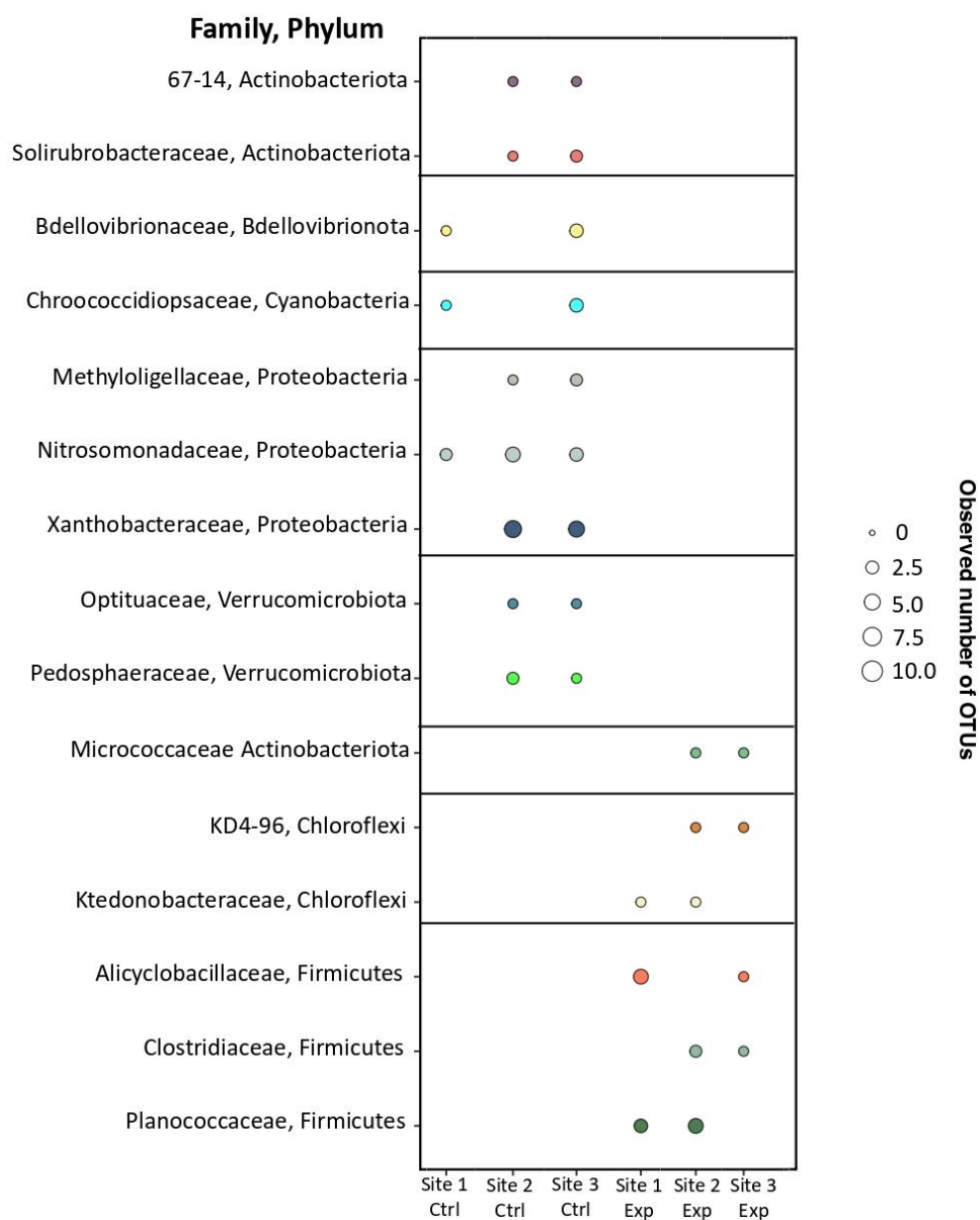
Figure 3.8: Bubble plot indicating bacterial families with ASVs that either increased only in experimental samples relative to control samples (Exp) or decreased only (i.e., increased in control samples) (Ctrl) from 2020 and 2021 samples for each site.

Chapter 4

4   Discussion

The purpose of this study was to investigate how the soil microbiome in first-time commercial American ginseng (*P. quinquefolius*) gardens shifts from seed planting through time. These changes in the soil microbiome may be linked to ginseng replant disease (GRD), a condition in which subsequent ginseng crops planted in previous ginseng gardens have high incidence of root rot and low yields. This study aimed to identify bacterial species or groups associated with ginseng cultivation that could potentially contribute to GRD or be used to diagnose soils that would cause GRD. The potential functional roles of bacteria that have changed in their relative abundance as ginseng is grown were also examined.

4.1   General overview of shifts in the soil microbiome in ginseng gardens

After 3 years of cultivation, the predominant phyla observed in this study from the bulk soil collected over three years were shown from the taxonomic composition bar plots (Figure 3.1). Experimental and control soil samples from site 2 and 3 were more similar, dominated by Actinobacteriota and Proteobacteria. In comparison, the major taxa observed in previous studies that examined the rhizosphere microbiomes of *P. notoginseng*, *P. ginseng*, and *P. quinquefolius*, were also dominated by Proteobacteria, and then followed by either Acidobacteria or Actinobacteria (reviewed by Goodwin 2022). There was a higher proportion of Actinobacteriota and Firmicutes in the early years (i.e., Fall 2018, Spring 2019, and Summer A 2019) in site 1 (Figure 3.1A-B), which could be due to sample processing in which these soil samples were erroneously placed in

a heated oven to dry. This may explain why samples from site 2 and 3 were more similar in composition relative to site 1 (Figure 3.3A).

The diversity decreased and the microbial community of the soil shifted in composition over time, with a distinct separation of first year soils from year 2 and 3 soils (Figure 3.3B). In this study, the Shannon diversity index decreased significantly in ginseng garden soils, which is an expected result as ginseng roots can have an influence on the soil microbiome over time through plant, soil, and microbe interactions. This was also observed in a study conducted by Tong et al. (2021), in which the rhizosphere microbiome of *P. ginseng* in two types of soil (farmland and forest soil) was found to have a decrease in alpha diversity regardless of the soil type. Another study by Chen et al. (2022) examined and compared the structure of the microbial community in rhizospheric soil of *P. quinquefolius*, and there was a decrease in the diversity of bacteria in the rhizospheric soils as *P. quinquefolius* grew over time. The authors showed that the abundances of bacteria from the Proteobacteria declined, but Proteobacteria increased or remained consistent in this study. Overall, the alpha diversity of ginseng garden soil was expected to change in comparison to control soil due to the change of plants in the soil, but ginseng may also have caused particular changes to the microbial communities over time perhaps due to its secondary metabolites (e.g., ginsenosides). However, Faith's phylogenetic diversity did not change for both control and experimental samples over time. The similarities of this index between the control and experimental samples, and over time, indicates that the microbial communities covered a similar degree of phylogenetic diversity. This diversity metric is not commonly calculated and has not been measured in most ginseng microbiome studies available from the literature. Future studies may want to consider this metric when analysing the diversity of ginseng grown soil.

Species loadings analysis at the ASV level identified several ASVs that contributed to the shift in microbiome over time and between control and experimental samples, but most of these were not confirmed by ANCOM-BC2 or ALDEx2. This may be due to a lack of statistical power in the data due to a low number of merged reads. Further analysis should be considered for ANCOM-BC2 analysis by using only the forward reads as the number of reads per sample was found to be significantly higher relative to the merged reads. In addition, there was also an inconsistency between the results from ALDEx2 and the species loadings results. Based on the plots created by ALDEx2, there were no significant taxa identified despite the clear separation from year 1 and 3 experimental samples from the PCoA plot (Figure 3.4). An ALDEx2 analysis of just the forward reads may also be needed to further explore these results. Nevertheless, some interesting changes occurred that might be indicative of microbes that contribute to GRD.

4.2 Potential functions of microbes increasing in ginseng garden soils

The microbial community definitely changed during the growth of ginseng and these changes might be potentially responsible for GRD but must be examined further. The changes observed in this study could be due directly to ginseng growth, or could be caused by ginseng cultivation practices. Some of the ASVs that increased in experimental samples over time are from families that are known to perform particular biochemical transformations. Whether or not these contribute to GRD should be tested.

Based on the bacterial families identified to increase in experimental samples, a lot of them contribute to nitrogen cycling. Many bacteria from the Beijerinckiaceae are known for their nitrogen fixing ability and they are commonly found in acidic soils. It can be completely normal to find this particular family in ginseng garden soil as it has been found that soils used in ginseng cultivation lead to acidity (You et al., 2015). Both

Nitrosopumilaceae and Nitrososphaeraceae are archaeal families, with species that can oxidize ammonia (Clark et al., 2021; Ren & Wang, 2022). Ammonia is used as a fertilizer to increase crop yield, and is commonly added to ginseng soil in gardens prior to seeding and during cultivation. Their relative abundance could have increased over time as fertilizers with ammonia were added. It is possible that the presence of these archaeal families are beneficial to ginseng by helping to convert nutrients, which are absorbed by the roots of ginseng over time. Lastly, the bacterial family Nitrospiraceae comprises species that are capable of converting nitrite to nitrate, which is the second step of nitrification in the nitrogen cycle (Off et al., 2010). Bacteria from the Nitrospiraceae family are important as nitrite is toxic for organisms including humans, fauna, and flora (Philips et al., 2002), and they may have an important role in the health of ginseng as they can prevent nitrite to accumulate over time.

As ginseng plants produce many different types of secondary metabolites including ginsenosides, these are likely to influence the soil microbiome over time. Several bacterial families from Proteobacteria were found to increase in experimental samples, and studies have indicated that certain genera are known to have the ability to degrade toxic chemicals. It is therefore a hypothesis to consider is that the increase in their relative abundance could be due to the presence of ginseng or root exudates that remained from a previous cultivation cycle.

In considering specific genera that increased from the phylum Proteobacteria, *Sphingomonas* and *Burkholderia* are known to degrade toxic chemicals such as polychlorinated dibenzodioxins (PCDD) and reduce soilborne plant pathogens (e.g., *Alternaria*) by prohibiting spore germination (Holmes et al., 1998). Furthermore, eight strains of *Burkholderia-Caballeronia-Paraburkholderia* (BCP) group and a *Burkholderia* isolate B36 had the ability to not only degrade autotoxic ginsenosides (Rb1, Rg1, and

Rd), but also antagonize *Ilyonectria mors-panacis*, which is a known soil-borne pathogen of ginseng and linked to ginseng replant disease (Luo et al., 2021). The genus, *SC-I-84* (*Burkholderia*) was found to increase in experimental samples collected in 2020 (year 2) and 2021 (year 3) in this study. It is possible that the increase in relative abundance of this specific genus from *Burkholderia* might help to promote the growth of ginseng roots, as the study by Dong et al. (2023) showed that its presence had a positive correlation with the biomass and fruit yield of cherry tomatoes. However, further research will be needed to confirm whether a similar effect can be observed in ginseng.

Methylibium (Proteobacteria) and *Sphingomonas* (Proteobacteria) were also found in ginseng soil microbiomes (Zhang et al., 2020a). In this study, species in the family Methylophilaceae (Proteobacteria) increased in soil samples over time (Figure 3.5A), and *Methylibium* belongs to this family. Similarly, the bacterial family Sphingomonadaceae, which includes *Sphingomonas*, was also identified as increasing in relative abundance over time in ginseng garden soils. However, sequence variants from this family also decreased over time (Figure 3.5C), therefore, bacteria from this family have varying responses in the ginseng garden samples, and their responses cannot be straightforwardly or simply interpreted.

Both *Methylibium* and *Sphingomonas* also possess the ability to biodegrade toxic compounds. For instance, the *Methylibium* genus has been shown to have the ability to biodegrade toxic compounds such as methanol, formaldehyde, methylated amines, and dichloromethane (Doronina et al., 2014). Cultures of *Methylobacillus* sp. have the ability to eliminate methanol produced from industrial sewage (Trotsenko et al., 2005). Similarly, specific species like *Sphingomonas wittichii* RW1[T] were used for the bioremediation of polychlorinated dibenzodioxins (PCDD) in contaminated incinerator fly ash (Nam et al., 2005). As ginsenosides and ginseng root exudates can remain in soils

during the lifetime of the plant and likely for a long time after they are harvested due to small unharvested root remnants, it is possible that both will accumulate during continuous cultivation of this plant. In addition, ginsenosides are autotoxic to ginseng (Yang et al., 2015); however the presence of *Methylibium* and *Sphingomonas* may reduce the levels of these toxic compounds and thereby increase the growth of ginseng or alleviate the conditions of GRD. For instance, *Sphingomonas kyungheensis*, which has ginsenoside converting ability, was isolated from soil of a ginseng field in South Korea (Son et al., 2013). However, further tests would be needed to see whether its ability can alleviate the autotoxic conditions caused by ginsenosidses.

Variovorax, and *Rubrivivax* have also been found in ginseng garden soils and have potential biodegrading abilities (Zhang et al., 2020a). Species from *Variovorax* can degrade specific toxic chemicals like polycyclic aromatic hydrocarbons, chlorinated hydrocarbons, and methyl tertiary butyl ether. In addition, they can degrade specific herbicides such as isoproturon, linuron, and atrazine. These genera, however, were not found in this study to have a significant contribution to changes in the microbiome of ginseng garden soils over time.

Xanthobacteraceae was also identified to have an increased in relative abundance in ginseng garden soils over time. This family contains species from genera such as *Xanthobacter*, *Ancylobacter*, and *Labrys* that have the ability to degrade toxic compounds including chlorinated alkanes, alkenes, polyaromatic compounds, and thiophene, which may be found in polluted environments (Oren, 2014). Nonetheless, these particular genera were not identified from the top 10% of ASVs (Figure 3.5A) as majority of these Xanthobacteraceae ASVs could not be identified past the family level.

4.3 Shifts in composition generally similar to ginseng soil microbiome studies from Asia.

The type of *Panax* species, cultivation methods (e.g., direct seeding and transplantation), the type of soil used for cultivation (e.g., farmland and forest soil), the type of soil analyzed (e.g., bulk soil and rhizosphere), as well as the type of material used in cultivation practices, have been examined in previous microbiome studies to see how they can contribute to GRD. However, the majority of these studies are conducted in Asia, where the type of soil, nutrient content, and cultivation practices may be different and can significantly affect how the microbiome community changes over time during ginseng cultivation.

However, major shifts in composition, at the phylum level are generally similar between this study of North American ginseng gardens and studies of ginseng gardens in Asia. In my study, soil samples were collected from three new ginseng gardens and compared with control soil samples which were located outside of the gardens. The alpha diversity particularly the Shannon diversity index was found to have decreased over time. Similar findings from the study conducted by Dong et al. (2017) also showed that the bacterial diversity (i.e., Chao1, phylogenetic diversity, and Shannon diversity index) decreased in soils used for ginseng cultivation, but the control samples compared by Dong et al. (2017) were soil used in maize cultivation instead. However, there were discrepancies between my study and this study, Dong et al. (2017) showed that the relative abundances of Bacillales (Firmicutes) decreased when compared to rhizosphere soil used in maize cultivation, but Bacillales (Firmicutes) was found to have increased in experimental samples in site 3. Furthermore, the relative abundance of Nitrospirales (*Nitrospira*) decreased in their study but specific ASVs were shown to increase in this study from experimental samples collected over time.

This study showed that there was a decrease in alpha diversity in soil samples grown with ginseng over time. The study by Xiao et al. (2016) which evaluated the effects of age and type of cultivation on the rhizosphere soil in *P. ginseng* from the Jilin province in China also showed similar findings. The authors stated that the alpha diversity in the bacterial community was higher in lower cultivation ages of direct seeding when compared to higher cultivation ages. In addition, Proteobacteria was found to be one of the predominant bacterial phyla detected in their study, which was also found in this study for samples from the new gardens. However, there are differences when comparing the two studies. Results obtained in this study specifically examined how the microbiome shifted over time in gardens that had no known history of ginseng cultivation by directly planting ginseng seeds in the soil, but the study by Xiao et al. (2016) demonstrated that transplantation method allowed an increase in microbial diversity to alleviate the problems caused by continuous cultivation of ginseng.

The mean relative abundance of Firmicutes for experimental samples in this study decreased over time (Figure 3.1), in which bacterial families in Firmicutes including Alicyclobacillaceae, Bacillaceae, Clostridiaceaeare, Hungateiclostridiaceae, and Lachnospiraceae were observed to decrease in experimental samples over time. Similar results were also shown in the study by Dong et al. (2018a), which the bacterial class Clostridia (Firmicutes) decreased in the relative abundance in soils collected from 3- and 4-year-old plants at the fruiting stage. However, the authors examined the rhizosphere microbial communities of different stages (2-, 3-, and 4-year-old) of *P. ginseng*, whereas the microbiome was tracked from seed to 3-year-old of *P. quinquefolius* in this study.

The types of soil used in continuous cultivation of ginseng have also been investigated to see how they may affect the rhizosphere microbiome of *P. ginseng*. Although only soil samples from new ginseng farms used in ginseng cultivation were examined, there were

similar findings relative to the study done by Tong et al. (2021). The rhizosphere microbiome of *P. ginseng* in two types of soils (farmland and forest soil) used for continuous ginseng cultivation in Jilin province in China was found to be dominated by Acidobacteria, Actinobacteria, Chloroflexi, and Proteobacteria (Tong et al., 2021). The authors compared how the bacterial community would be affected by ginseng cultivation on both types of soils. The relative abundance of Acidobacteria decreased in ginseng farmlands while Bacteroidetes increased (Tong et al., 2021). Acidobacteria was shown to increase in ginseng garden soil collected across the three sites in this study; whilst the relative abundance of Bacteroidetes remained constant. Similarly, when bacterial communities were compared between forest soil used for ginseng cultivation and ones that were not, Actinobacteria, Bacteroidetes, and Cyanobacteria were found to increase relative to non-ginseng cultivated forest soil (Tong et al., 2021). In this study, where farmland soil was analyzed, Cyanobacteria was only observed in control samples (Figure 3.1). However, Cyanobacteria might be categorized in the <1% label for the experimental samples (Figure 3.1).

Most previous studies have examined the rhizosphere of ginseng soils, but in this study bulk soils were examined instead. This is because ginseng roots are pulled after they reach a marketable size, and new seeds are grown again. Therefore, it was decided to examine the bulk soil of these ginseng gardens in order to determine what potential bacterial taxa may be responsible for GRD for this study. The microbial community may also be affected differently from the rhizosphere microbiome relative to the bulk soil microbiome. The rhizosphere soil is considered as soil that is associated with the roots of a plant, whereas bulk soil is soil outside of the rhizosphere and not penetrated by the roots. In this study, bulk soil of both experimental and control samples was collected and compared instead of the rhizosphere soil samples. The comparison between these two kinds of soils obtained from growing ginseng plants of 1 to 5-year-olds with two different

planting methods (i.e., planting in farm and understory planting) was compared in *P. notoginseng* (Kui et al., 2021). The authors suggested that in the case of *P. notoginseng*, rhizospheric soil and bulk soil samples may be more or less homogenous. However, the authors did find that the alpha and beta diversity of the rhizosphere microbiome were significantly different, suggesting that the type of planting will shape distinctive unique rhizosphere microbiome profiles. Nevertheless, it is not certain whether the rhizosphere microbiome would be different as the rhizosphere was not collected in this study.

Finally, the repetitive use of pesticides and the type of mulch applied during ginseng cultivation can also affect the soil microbiome. For example, the type of mulch used in ginseng cultivation was shown to affect the rhizosphere microbiome in *P. ginseng*, which the abundance of Bacteroidetes decreased compared to other bacteria when conifer leaf litter mulch was used instead of broad leaf litter mulch (Sun et al., 2017). In addition, the type and amount of fertilizer used may also influence the soil microbiome. A higher amount of nitrogen due to the application of urea has increased root exudates which affected the abundance of soil bacteria in crops like maize (Hao et al., 2020). This may also be possible for the ginseng microbiome with the repetitive use of nitrogen fertilizer in continuous cultivation practices (Zhu et al., 2016). In this study, control samples were not treated with any fertilizers, while experimental samples were treated with standard agricultural practices used in ginseng cultivation. Ultimately, these changes observed in the microbial community over time may not be solely due to the presence of ginseng itself as they could have been increased by agricultural practices. In addition, both control and experimental soils would have experienced the same weather which can also contribute to these changes.

4.4 Limitations of the current study

The shift in the microbiome is thought to be influenced by ginseng root exposure over time, but it is not known how this exposure contributes to microbiome changes. Previous studies have shown that chemical variables (e.g., amount of nutrients and pH) can affect how the microbial community changes and how ginseng or ginseng cultivation affects these variables should be investigated in the future. In addition, methods to measure ginsenosides have been developed (Kaberi, 2021), so these can also be evaluated for their effects on the soil microbiome in future.

For the majority of ASVs that contributed to the microbiome shifts, known characteristic functions could not be identified. For instance, many of the ASVs that belong to Chloroflexi have unknown functions even though they were shown to decrease in relative abundance in experimental samples over time. In order to provide an ecological function for specific microbes, metatranscriptomics or metagenomics might be an option to better understand the functions for specific species in bacterial families. These methods could identify potential biodegradation or bioremediation abilities that increased as ginseng was grown. After DNA sequences extracted from soil samples are sequenced and cloned, these short reads can be assembled to predict individual genomes or parts of genomes that originated from the environmental samples. Ultimately, species diversity and the potential functions from the microbial community may be determined. The functions of microorganisms that increased with ginseng growth can also be examined by matching ASVs generated from 16S rRNA gene sequencing with bioinformatic packages such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PiCRUST) (Langille et al., 2013).

Culturing these microorganisms could also help to determine whether they also possess the ability to degrade ginsenosides or contribute to GRD conditions. However, although

many bacteria increased in relative abundance when ginseng was grown, their increase may not solely be due to the growth of ginseng, or the increasing presence of ginsenosides or other ginseng exudates. For example, the increase of bacterial and archaeal families including Beijerinckiaceae, Nitrosopumilaceae, Nitrososphaeraceae, and Nitrospiraceae, that are known to oxidise ammonia and nitrite, may be linked to the addition of fertilizers that are often used as standard practice during ginseng cultivation. There are inconsistencies in current microbiome studies on GRD in Asia based on the factors that are examined, as well as a lack of microbiome studies in North America. It is therefore challenging to compare results in this study to previous microbiome studies and may suggest that a diagnostic tool may only be developed locally for a specific region for ginseng growers.

4.5   Summary and future directions

This thesis examined the soil microbiome from seeding with *Panax quinquefolius* to the end of the third year of growth in three new gardens with no known history of ginseng cultivation in Norfolk County, Ontario. The diversity of the microbiome decreased and a shift in microbial community composition was clearly evident over time. The relative abundance of specific microbial families changed over time, and many are associated with biodegradative abilities. However, it must be further investigated whether the observed increases or decreases in abundance directly contribute to GRD conditions. The results from this study revealed that the soil microbiome is affected by ginseng cultivation, either directly due to ginseng growth or ginseng-farming practices. In addition, as there were inconsistencies from results obtained from species loadings, ANCOM-BC2, and ALDEx2, the change in abundances for taxa from specific groups of family may need further examination with the use of only forward reads instead of merged reads to have more sequences to analyze.

Soil samples from the same gardens in this study should also be collected by planting a subsequent generation of ginseng for four years to test whether GRD has developed. The changes that occur in the soil microbiome may then be confirmed to contribute to GRD. In addition, as soil samples collected in this study were only from seeding to the third year of the cultivation cycle, it will be necessary to obtain and perform metabarcoding on soil samples in the fourth year for comparison as ginseng roots are normally grown for four years prior to harvest. The growth and maturity of the ginseng roots may further affect the microbial community, which would be crucial to understand how this contributes to the susceptibility to GRD. The experimental design used in this thesis should be repeated by collecting soil samples from three new ginseng gardens from a new collection in order to provide a comparison with the current results. Finally, the changes in the microbiome should be observed in other perspectives such as network analysis (Layeghifard et al., 2017). Bacteria are represented by nodes and their interactions are represented in edges. By investigating the microbe interactions within the microbiome at a specific time point as ginseng grows through time, the presence of key hubs (i.e., bacterium with more than one edge with other ones) may be determined and selected for further investigation. These key hubs may potentially be important taxa within the community that are associated or affected by GRD. If so, it could be possible to increase or reduce the abundance of them in order to alleviate or potentially eliminate GRD.

It is crucial for future studies to know which of these changes are ginseng-specific and whether these changes contribute to GRD. By identifying the potential changes in the soil microbiome throughout the ginseng cultivation process, this project will contribute to developing a potential diagnostic tool for ginseng farmers in Ontario where the history of the soil is not known. As the cultivation of ginseng roots normally take up to four years of growth prior to reaching a marketable stage, farmers can also take preventive measures

(e.g., increase bacteria beneficial to growth of ginseng) before planting to reduce losing a large number of roots during cultivation.

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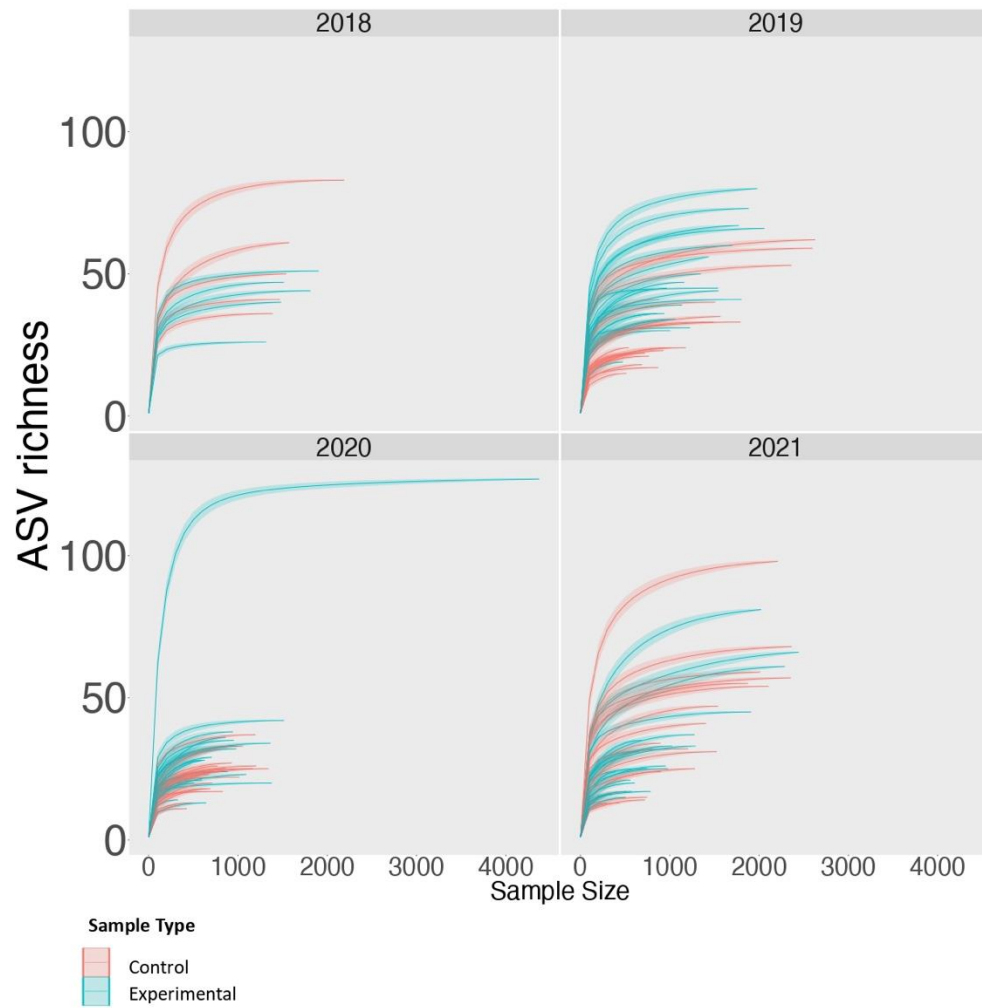
Appendix

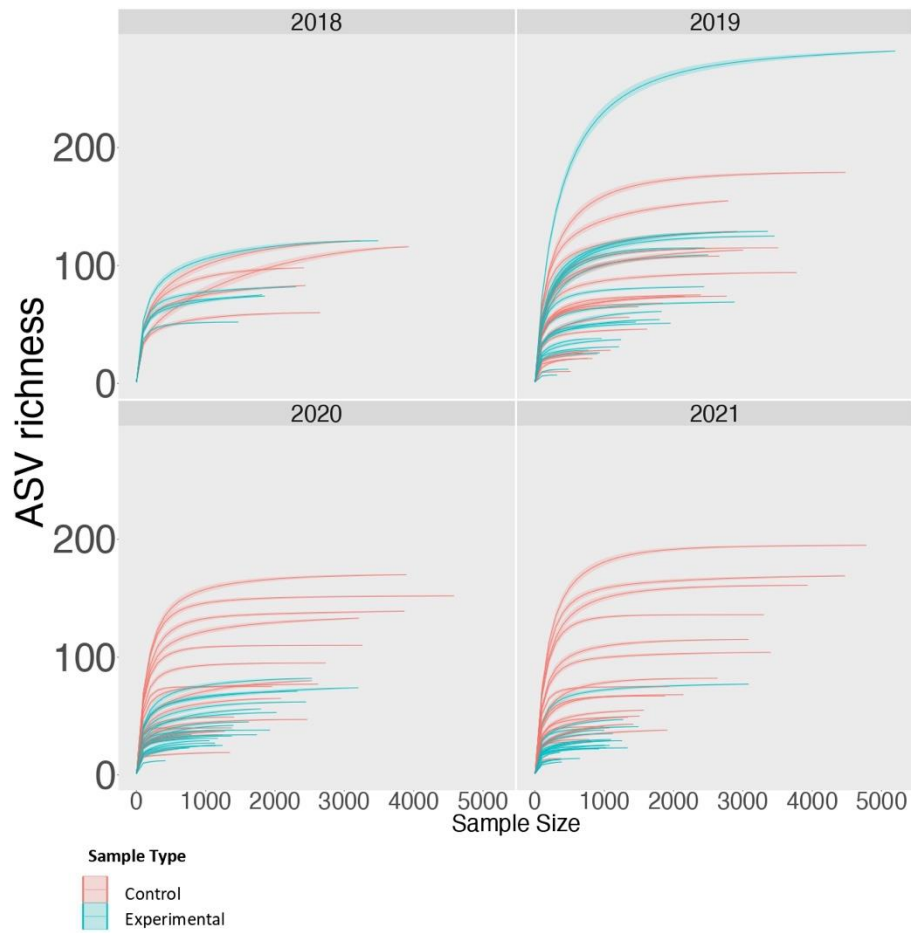Appendix A: Chapter 3 supplementary material
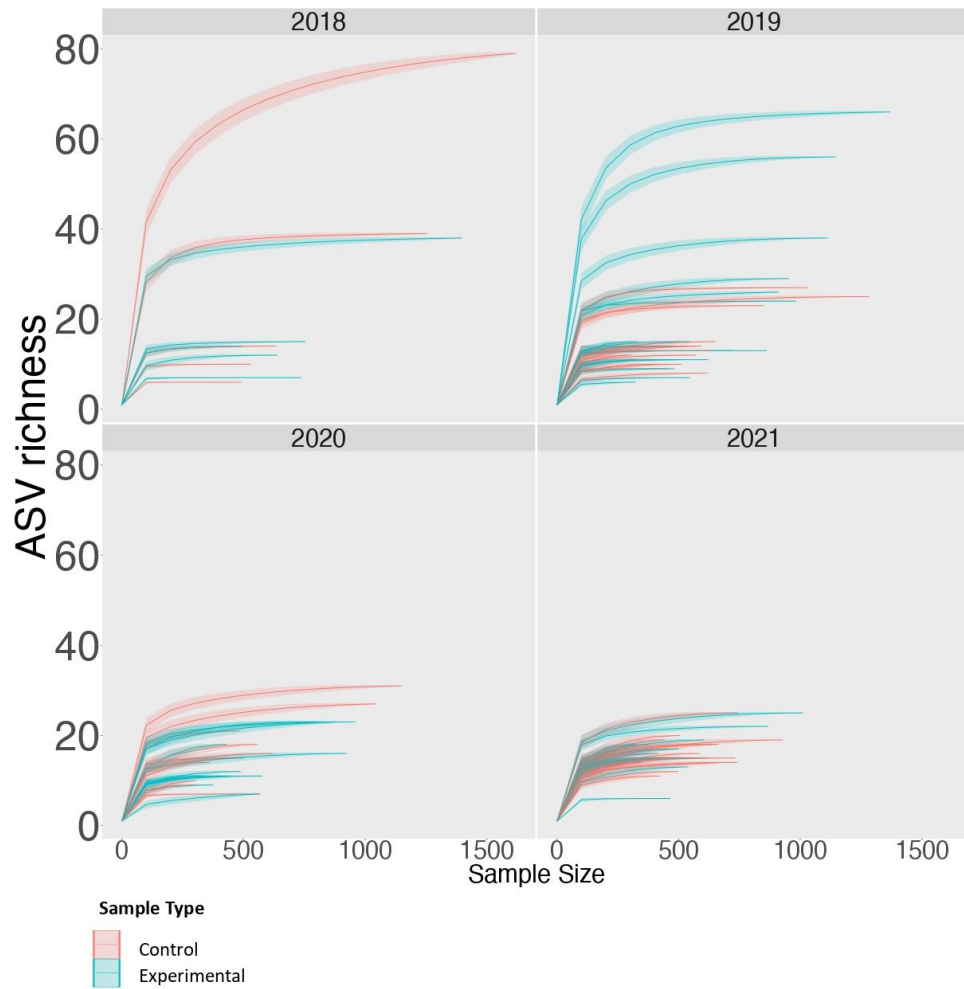
Figure A1: Rarefaction curves of bacterial amplified sequence variants (ASVs) sequenced from site 1 samples across year.
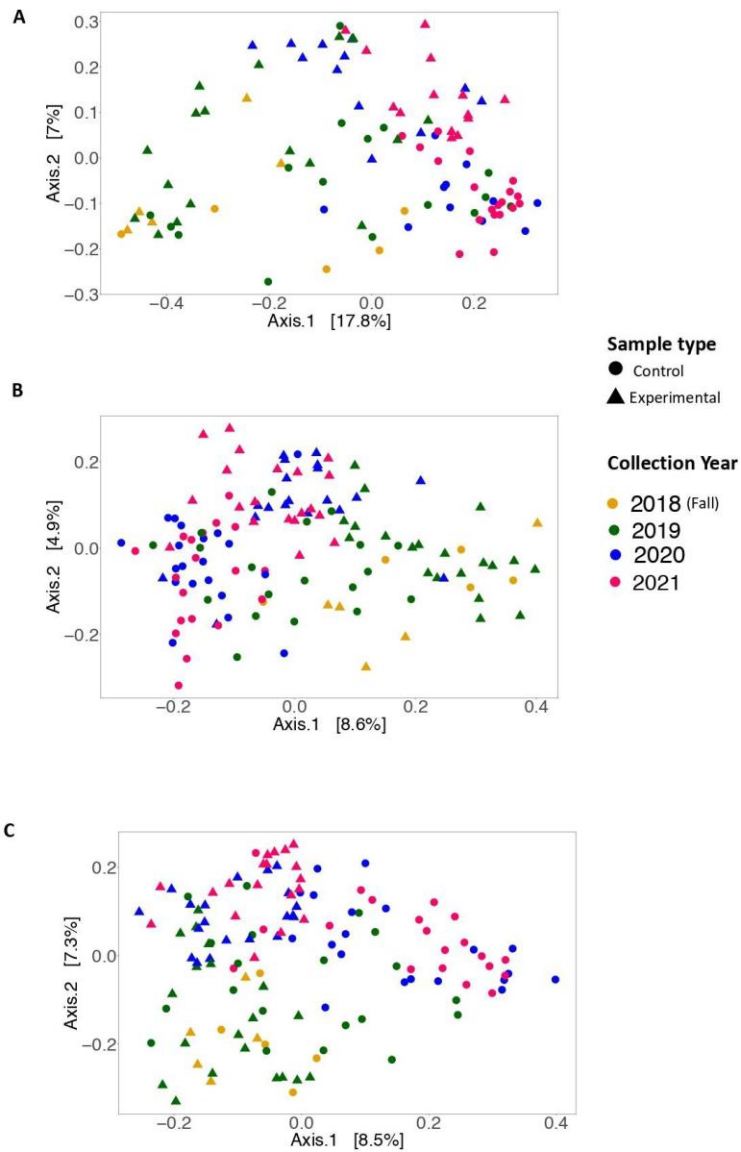
Figure A2: Rarefaction curves of bacterial amplified sequence variants (ASVs) sequenced from site 2 samples across year.

Figure A3: Rarefaction curves of bacterial amplified sequence variants (ASVs) sequenced from site 3 samples across year.

Figure A4: Principal coordinate analysis using unweighted Unifrac for measuring the dissimilarity in the composition of amplicon sequence variants (ASVs) from control and experimental soil samples across collection year for A) site 1 B) site 2 and C) site 3.
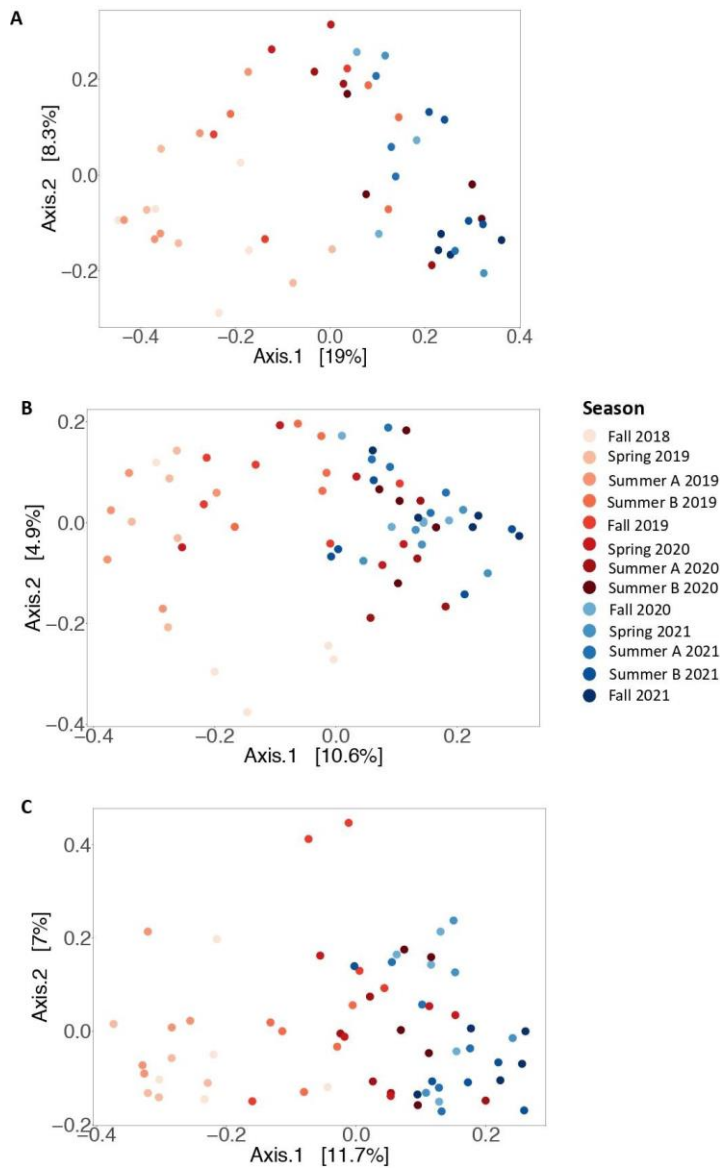
Figure A5: Principal coordinate analysis using unweighted Unifrac for measuring the dissimilarity in the composition of amplicon sequence variants (ASVs) from experimental soil samples only across collection year for A) site 1 B) site 2 and C) site 3.

A Family, Phylum

B Family, Phylum
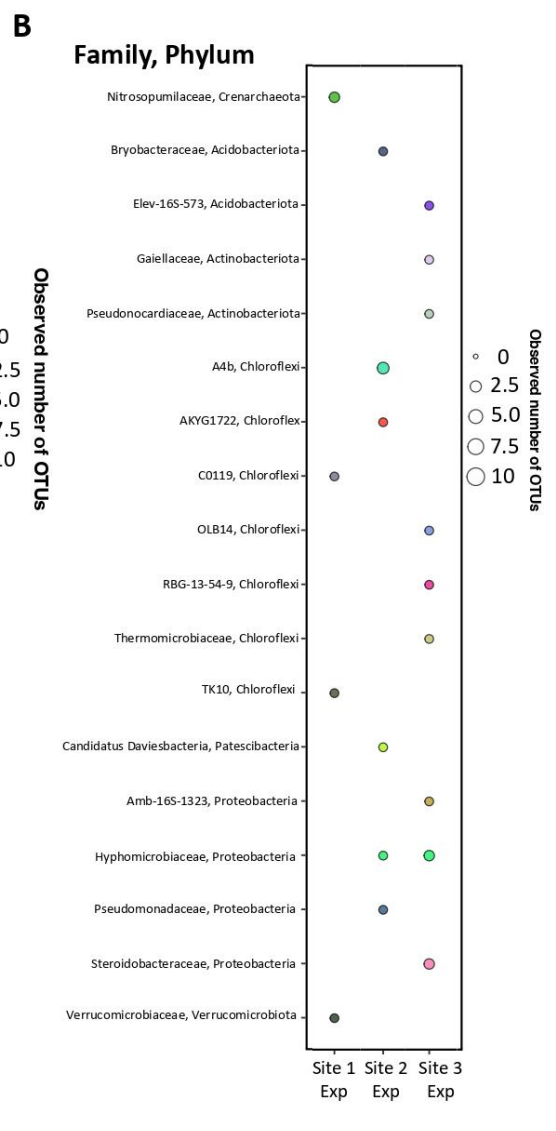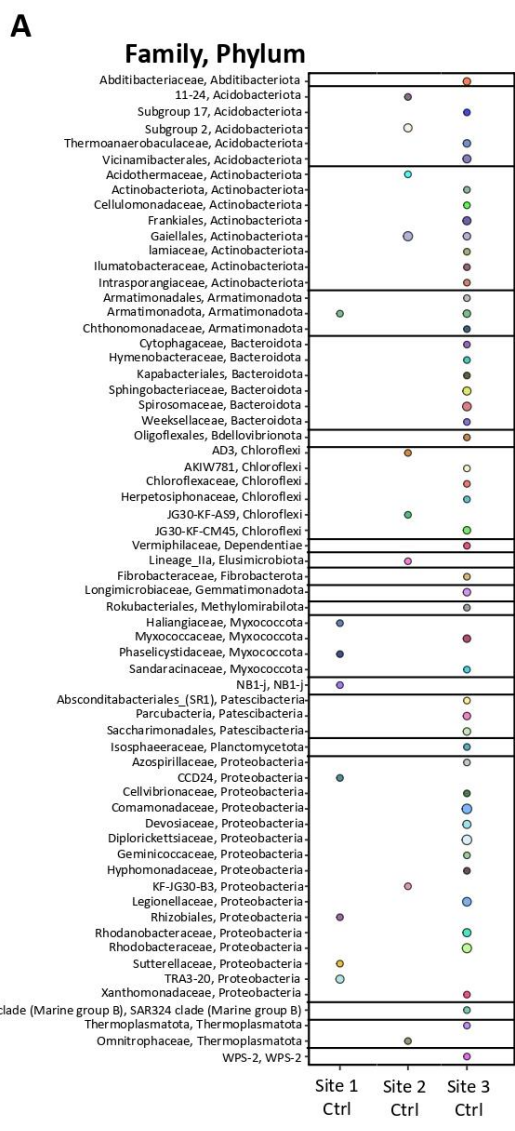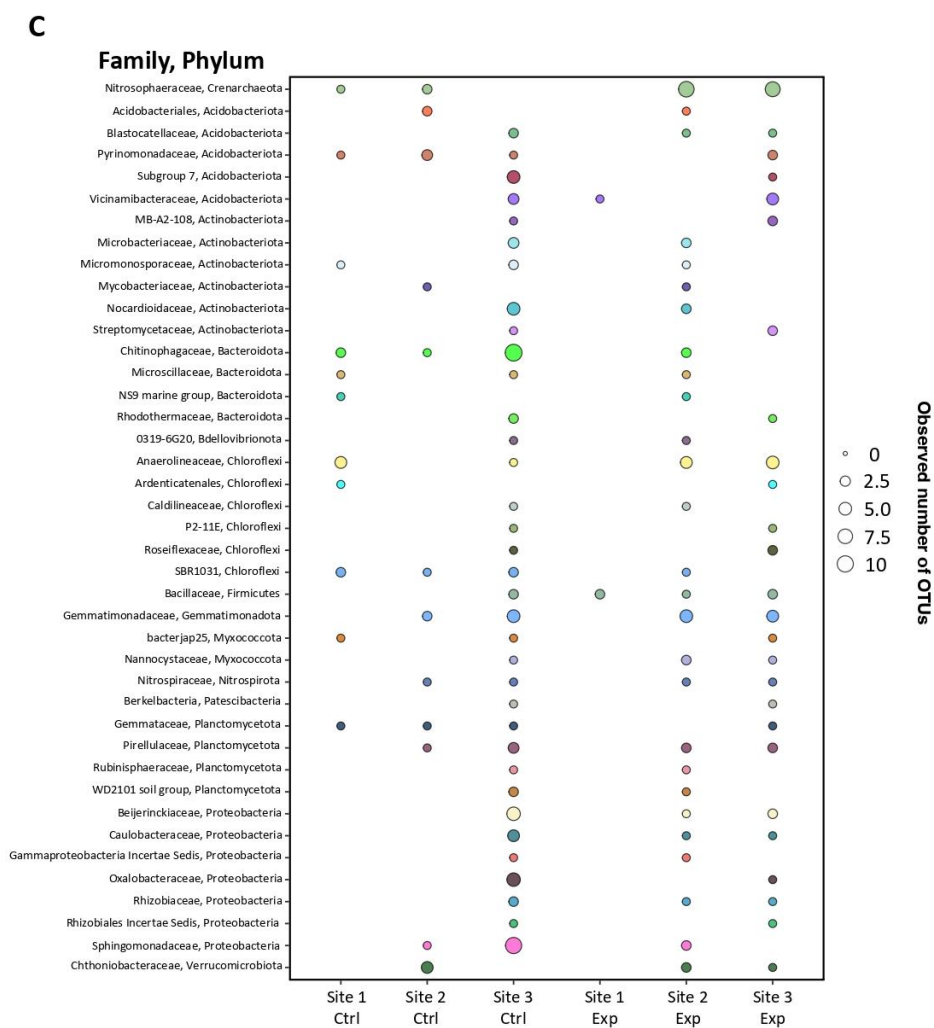
Figure A6: Bubble plot indicating the number of ASVs grouped by taxonomic family for the top 10% of ASVs with the highest contribution to the difference between control and experimental samples in 2020 and 2021 for each site. A) Bacterial and archaea families that decreased in abundance in experimental samples only. B) Bacterial and archaea families that increased in experimental samples only. C) Bacterial and archaea families that increased or decreased in experimental and control.

Curriculum Vitae

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