DISSERTATION

BACTERIOMES OF PEACH ORCHARD SOIL AND COVER CROPS

Submitted by

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ABSTRACT

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Replant syndrome (RS) of fruit and nut trees causes reduced tree vigor and crop productivity in orchard systems due to repeated plantings of closely related tree species. Although RS etiology has not been clearly defined, the causal agents are thought to be a complex of soil microorganisms combined with abiotic factors and susceptible tree genetics. Different soil disinfection techniques alleviate RS symptoms by reducing the loads of the deleterious microbiome; however, the positive effect on crop growth is temporary. Here, the current understanding of RS in orchards from a soil microbiome perspective is reviewed. The resolution to RS will require experts to outline explicit descriptions for its symptoms, determine its etiology, identify the primary phytopathogens, and fully explore sustainable treatments which alleviate RS. Two sustainable treatments of RS were selected to explore at a deeper level, soil disinfection and increasing crop diversity to observe what technique could help establish a healthy soil bacteriome. In a greenhouse study, soil disinfection via autoclave was then followed by cover cropping. It was found that soil disinfection increases plant biomass as compared to the control for only the first crop cycle while non-autoclaved soils with a history of cover cropping alleviated RS in RS-susceptible 'Lovell' peach seedlings. Although soil disinfection via autoclave was found to distinctly alter the peach soil bacteriome for the full duration of the study, this sustainable practice mimicking solarization failed to provide relief from RS for peach seedlings. Instead of long-term benefits, differential abundance comparisons displayed a loss of potentially beneficial bacteria due to soil disinfection. *Paenibacillus castaneae* and *Bellilinea caldifistulae* were beneficial bacterial species which uniquely colonized peach rhizosphere of

non-autoclaved soils with a cover crop history. As a promising sustainable technique, a greater understanding of how inter-/intra-specific competition of cover crops can influence the bulk soil bacteriome was pursued. Alfalfa, brassica, and fescue were grown in 7 different plant combinations (1. alfalfa, 2. brassica, 3. fescue, 4. alfalfa-brassica, 5. alfalfa-fescue, 6. brassica-fescue, 7. alfalfa-brassica-fescue) across 3 density concentrations (low: 1–3 plants, medium: 24 plants, and high: 48 plants) for a greenhouse microcosm experiment. It was found that even in highly competitive space beneficial bacteria were enriched, however, there was an apparent trade-off where different plant combinations enriched distinct beneficial bacteria. As an example, even if a free-living nitrogen fixing bacteria such as an Azospirillum spp. was enriched in the bulk soil of alfalfa and brassica monocultures, it was not enriched in the bulk soil of an alfalfa-brassica plant mixture. Instead *Pseudarthrobacter phenanthrenivorans*, a phytohormone producer, was enriched in alfalfa-brassica plant mixtures. When zooming into the rhizosphere compartment of these microcosms, it was found that regardless of plant neighbor identity or density, a few rhizobacteria were highly correlated with a specific plant species. Meanwhile, certain plant species specific rhizobacteria were enriched only if specific conditions such as plant neighbor identity or density were met. Overall, our research found that growing genetically distinct plants prior to the re-establishment of a peach orchard could alleviate RS symptoms. Furthermore, that cover crops can enrich for different microbes when grown together as opposed to when grown separately. Lastly, although plants recruit a particular set of bacteria, this recruitment can shift depending on plant neighbor identity or density. Further study of cover crops may identify how they can alleviate RS in orchards worldwide.

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CHAPTER 1 REVIEWING THE CURRENT UNDERSTANDING OF REPLANT SYNDROME IN ORCHARDS FROM A SOIL MICROBIOME PERSPECTIVE[1]

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Synopsis

Replant syndrome (RS) of fruit and nut trees causes reduced tree vigor and crop productivity in orchard systems due to repeated plantings of closely related tree species. Although RS etiology has not been clearly defined, the causal agents are thought to be a complex of soil microorganisms combined with abiotic factors and susceptible tree genetics. Different soil disinfection techniques alleviate RS symptoms by reducing the loads of the deleterious microbiome; however, the positive effect on crop growth is temporary. The goals of this introduction are: (1) to conceptualize the establishment of the syndrome from a microbiome perspective and (2) to propose sustainable solutions to develop a beneficial microbiome to inhibit the onset of RS. The second chapter is a greenhouse study which looks how RS is alleviated by soil disinfection via autoclave and/or a crop rotation which tested 4 genetically different crops. The third chapter further examines how the bulk soil bacteriome is influenced by plant diversity and density. Lastly, the fourth chapter explores how rhizobacteria colonization is influenced by plant identity and density.

Introduction

The agronomic challenges that arise during the re-establishment of a closely related tree fruit/nut species are collectively known as replant syndrome (RS). RS symptoms can be observed in the first replant generation (second orchard generation) and persist for several years or even

decades (Simon et al., 2020, Mahnkopp et al., 2018). RS has been vaguely characterized by reduced tree growth, lifespan, fruit yield, and fruit quality attributes such as soluble sugar level and sugar–acid ratio (He et al., 2018; Mahnkopp et al., 2018; Yang et al., 2012; Rumberger et al., 2007). More descriptive characterizations have included reduced branching, shortened internodes, deformed leaves, root necrosis/discoloration, and reduced root growth (Liu et al., 2014; Yang et al., 2012; Rumberger et al., 2007; Mazzola, 1998). However, even these more descriptive symptoms are not diagnostic (Jafee et al., 1982) and often trees grown in replant soils need to be compared to trees grown in previously non-orchard soils in order to fully grasp the detrimental consequences of replant syndrome. Furthermore, most reports of RS longevity in the soil are merely anecdotal, since controlled research paired with accurate and detailed, multidecade cropping histories has been practically impossible to obtain (Westerveld & Shi, 2021; Zhao et al., 2016).

In addition to ambiguous symptom descriptions, there is no consensus on terminology as synonyms for RS include soil sickness, soil fatigue, replant problem, replant disease (Hanschen et al., 2020), soil exhaustion, replant disorder (Mazzola et al., 2012), and specific replant disease (Caruso et al., 1989). Here, the term replant syndrome is used since the condition's onset is driven by repeated monocropping (Ma et al., 2022; Wu et al., 2018). Additionally, while a "disease" has distinguishing symptoms typically derived from a single known cause (i.e., a specific pathogen), a "syndrome" refers to a group of signs, phenomena, or symptoms that occur together (Agon-Levin et al., 2012) with an uncertain underlying primary cause.

There is no unanimous agreement on the etiology of RS, which has largely remained an enigma for over 300 years (Mazzola, 1998; Sewell, 1981). The mechanisms by which consecutive monocultures give rise to a decline in crop productivity are still subject to debate.

While RS has been reported in many crops, its negative impacts have been more notable among fruit/nut trees in the Rosaceae family such as almond (Browne et al., 2013), apple (Mazzola & Manci, 2012), cherry (Mai & Abawi, 1978), pear (Mai & Abawi, 1978), and peach (Hanschen et al., 2020; Yang et al., 2012). Citrus species (Rutaceae family) are greatly affected by RS (Chen et al., 2015; Thakur et al., 2018). Multiple factors such as autotoxin production leading to accumulation, an imbalance of soil nutrients, and an imbalance of the microbial community structure have been credited with exacerbating RS (Chen et al., 2015). While abiotic factors such as autotoxins and nutrient imbalance decrease soil fertility (Spath et al., 2015), they are not necessarily the direct cause of RS, but rather, may increase the survival and competitiveness of phytopathogens (Winkelmann et al., 2019). A critique of the idea that autotoxicity relates to RS is that fallow periods of up to three years fail to suppress RS and improve tree growth (Li et al., 2020). While chemicals causing autotoxicity are unlikely to be stable enough to persist for years, they may result in longer term shifts in the soil microbiome (Winkelmann et al., 2019). Thus, instead of one specific phytopathogen, the primary cause of RS is suspected to consist of a complex of soil phytopathogens which have been shown to be enriched by autotoxins (Zhao et al., 2016; Mazzola & Mullinix et al, 2005). For example, *Panax notoginseng* was found to produce autotoxic ginsenosides which enriched potential phytopathogens (*Alternaria, Cylindrocarpon*, *Fusarium*, *Gibberella*, and *Phoma*); meanwhile, relative abundances of beneficial taxa (*Acremonium*, *Mucor*, and *Ochroconis*) decreased (Mazzola & Mullinix, 2005). As the microbiome shifts, plant-growth-promoting microbes could become outnumbered by phytopathogens.

Microbiome shifts as the possible underlying cause of RS is further supported by studies showing that plants grown in autoclaved RS soil experience a remarkable increase in growth

relative to plants grown in untreated replant soils (Newberger et al., 2023; Hanschen et al., 2020; Li et al., 2019). Similarly, fruit tree biomass has been shown to increase in RS soils treated with chemical fumigation, resulting in reduced microbial biomass carbon with no apparent effect on other soil properties (basal respiration, ergosterol content, pH, electrical conductivity, and most nutrient and metal contents) (Spath et al., 2015). Finally, when apple trees exhibiting RS symptoms were transplanted from RS soils into healthy soils the RS symptoms reversed (Winkelmann et al., 2019).

This reversibility is particularly interesting given the identification/involvement of several potential soilborne plant pathogens in RS. For example, phytopathogens frequently associated with RS are oomycetes *Pythium* and *Phytophthora*, bacterial taxa from actinomycetes and genera of *Bacillus* and *Pseudomonas*, and the root lesion nematode (Spath et al., 2015; Yang et al., 2012). Fungal suspects are *Cylindrocarpon*, *Rhizoctonia*, *Fusarium* sp., *Alternaria* sp., *Myrothecium verrucaria*, and *Mycelia sterilia* with many of these taxa being frequently isolated from the rhizosphere (soil surrounding plant roots) (Winkelmann et al., 2019; Liu et al., 2014). However, microbe–microbe and microbe–plant interactions are complex, and site-to-site variation has yielded contradicting results. For instance, *Phytopythium vexans* was found to be a pathogen in one site but acted as a biological control at a different location (Liu et al., 2014). Virulence differences of *P. vexans* strains compounded with different abiotic or biotic soil factors could explain these discrepancies (Liu et al., 2014).

In summary, the diversity and abundance of phytopathogens cause RS, with abiotic factors and autotoxicity instigated by the previous monocrop acting as positive feedback mechanisms for phytopathogen recruitment and development. RS etiology appears to depend not

only on the presence of phytopathogens but also on the overall balance of the soil microbial community.

Developing a Soil Microbiome Model to Understand Replant Syndrome

Tree growth in an orchard's second generation (replanting) is not as vigorous as its first generation. Often, young, transplanted trees die in sites exhibiting severe RS (Mazzola, 1998). Additionally, peach seedlings display reduced height and trunk width compared to a control group grown in fumigated soil in as little as 10 weeks (Liu et al., 2014). Some studies have found that shoot growth was reduced by 66.9–71% with shoot masses staying consistently low after multiple replanting generations (Mahnkopp et al., 2018). Less severe cases have noted that trees can overcome an initial delay in growth, eventually reaching the size and annual yields of those grown in healthy soils (Foy et al., 1996; Arneson & Mai, 1976). Nonetheless, recovery is time-consuming, taking valuable years and resources, which ultimately reduces the profitability of the orchard (Rumberger et al., 2007). In these less severe sites, the fruiting of trees can be delayed 2 to 3 years and still never attain comparable yields to those of the first cycle of planting (Mazzola, 1998). Even in instances where RS causes a reduction in fruit yield or a shortened production life without ending in plant death, the resulting reduction in profits has been estimated at 10–20% (Liu et al., 2014).

RS can persist in fallowed soil for several years or even decades following the removal of the first established orchard (Li et al., 2020; Mazzola & Manici, 2012). It is believed that RS symptoms can be observed even if the roots of previous plants were in an area for only a few months. When young saplings are transplanted, the young root systems interact with populations of phytopathogens from the plant matter residue of the previous trees. There are examples of

literature indicating younger plants are more susceptible to diseases compared to their adult counterparts (Li et al., 2019). As such, peach saplings are known to struggle in RS soil.

Traditionally, it is believed that RS primarily affects the next cropping cycle if the consecutive plant species are closely related (i.e., peaches following peaches). Specific RS, like specific apple replant disease, is a buildup of non-generalist pathogens tailored for the genotype of the host tree with host plant residues playing a key role (Jaffee et al., 1982). This would support the possibility that with a decrease in the number of tree hosts, there would be a decrease in the specific replant microbes. Nonetheless, even with the removal of tree hosts, specific phytopathogens can be sequestered in plant residues until complete decomposition. A non-competing concept is that the pathogen build-ups are often composed of ubiquitous generalists (Forge et al., 2016). For example, a build-up of phytopathogenic nematodes has been found to be partially responsible for the nonspecific replant symptoms (Jaffee et al., 1982). Once the orchard is newly planted, the RS microbiome will exponentially colonize these recently introduced tree hosts.

Orchard management practices use natural tree physiology to dictate the processes to which the peach tree should direct its energy (Chalmers et al., 1981). Traditional horticultural practices in orchards do not focus on encouraging the tree host to expend its energy in recruiting beneficial microbes for the sake of immune defense. However, recent studies have investigated sustainable techniques like intercropping and how different cover crops influence soil microbial communities in apple orchards (Li et al., 2022). Plants have been found to use between 5–25% of all photosynthetic net fixation of $CO2$ for root exudation of carboxylates (Neumann & Römheld, 2000) which are critical for attracting plant-growth-promoting rhizobacteria to the plant (Hassan et al., 2019). Consequently, there is a possibility that the RS microbiome develops instead of a

beneficial microbiome tailored to the peach plant, negatively impacting the peach orchard even within the first generation. The disease may not be readily apparent, and the damage observed may be misidentified as part of the aging process. It is generally agreed that the process of RS is initiated by repeated monoculture, and here it is highlighted that RS begins to establish, even in the first generation, if the fruit trees are relatively asymptomatic (Mazzola, 1999). Incidentally, significant shifts in the soil microbial community have been detected between non-cultivated, first-year, and second-year apple trees of the first planting (Mazzola, 1999). In support of our hypothesis, when second-generation apple trees were planted in steamed disinfected soils where first-generation apple trees had been grown for only three years, the increase in growth was equal to that achieved in non-cultivated soils (Mazzola, 1999). In short, the precursor phytopathogenic replant microbes existed in the soil before the orchard was established and, with time, the environment began to evolve virulent traits that were increasingly effective, building an inhospitable environment for the next planting of fruit trees.

Agricultural practices such as pruning initiate a stress response, which stimulates growth to replace the lost biomass (Suchocka et al., 2021). As a result, exposed tissue can become infected (Zhang et al., 2018). It is known that common pathogens, like the *Cytospora leucostoma*, have great difficulty colonizing trees except through open wounds induced by injuries such as drought injury, winter injury, or pruning (Alfieri et al., 1973). These wounds allow repeated recolonization/co-colonization of multiple strains of phytopathogens which should increase their virulence as observed in other pathogens (Chao et al., 2015). Here, it is posited that the replant microbiome virulence levels build up gradually over time, and microbial populations approach higher levels as the first-generation plants are maturing for the first cycle of growth. The chronological age of a plant has been correlated with increased pathogen resistance (Li et al.,

2020). Although immune signaling can increase from early developmental stages to reproductive stages, the fitness of a plant's immune system decreases during the reproductive stage as a possible function of host senescence (Li et al., 2020). These findings need to be correlated for fruit trees with a longer lifespan.

Here, the proposed model (Figure 1) is based on broad patterns in an attempt to link tree development through time and the RS microbiome build-up to monitor the development of RS. The purpose of this model is to represent a hypothetical replant situation. Peach, *Prunus persica*, was selected as the example. The first generation of an orchard is defined as an area where peaches have not been grown previously. The timeline starts with the trees planted from seedlings or transplanted saplings (Figure 1a). First-generation orchards do not exhibit replant symptoms (Atucha & Litus, 2015), since neither allelochemicals nor the replant microbiome are present in the soil in detrimental concentrations (Lü & Wu, 2018; Liu et al., 2014). Typically, peach trees take 1–3 years to be established in the soil and have the potential to provide a commercial crop during the second year (Li et al., 2022). As the tree roots are established, the tree canopy is trimmed and trained to bear larger branches that can hold a heavy load of fruit (Teskev, 2012). This is a large energy expenditure since the more trimming, the more vigorous epicormic growth occurs (Dejong et al., 2012). Peak fruit set starts at 4 years of age for what is considered a mature tree (Paço et al., 2006; Sofo et al, 2005), and peaks at eight years with yields being around 50–150 pounds of fruit per year (Warmund, 2009). After year 8, the fruit set decreases, with year 12 possibly having minimal fruit sets. In orchards, dwarfing rootstocks are used to reduce vegetative vigor by controlling root growth, which in turn can divert sugars to fruit production, especially in young trees (Li et al., 2022). Peach dwarfing rootstocks typically live about 10–15 years in an intensive orchard setting (Warmund, 2009).

Microbial communities in the bulk soil are extremely diverse, with estimates of 10 billion bacteria classified under thousands of different species in just 1 g of soil (Badri & Vivanco, 2009). Plants secrete root exudates to culture beneficial microbes in the rhizosphere that are tailored to the plant's needs (Vives-Peris et al., 2020). However, in addition to symbionts, phytopathogens can also be attracted to this chemical communication (Figure 1b) (Steinkellner et al., 2007). Thus, the precursor microbes that make up the RS microbiome are most likely already present in the bulk soil and can proliferate as the composition of the bulk soil shifts (Yang et al., 2021).

Breaking the Cycle of Replanting Syndrome

Several solutions have been proposed to combat the problem of RS, each with varying degrees of success. These solutions may be viewed in two ways: (1) a single application of a pre-plant soil disinfection strategy and (2) a continuously implemented biological strategy that increases either plant or microbial diversity. Soil disinfection methods include chemical fumigation, solarization, anaerobic soil disinfestation, autoclaving, soil amendments (*Brassica napus* seed meal, biochar), or even soil replacement in severe cases. Biological strategies are polyculture (cover crops or intercropping), rootstocks, or plant-growth-promoting inoculations and use concepts drawn from the intermediate disturbance hypothesis (IDH). These strategies are designed to avoid the shift towards an RS microbiome.

Pre-plant soil disinfection strategies typically yield more consistent successes, even if temporary, while a biological strategy that increases diversity often varies in success. Sterilization is defined as a process that effectively eradicates all viable microorganisms (including bacterial spores) from a surface or product (Silindir et al., 2009). Since sterilization of bulk soil is incredibly challenging, the term "soil disinfection" is used here in place of "soil

sterilization" to convey a process that reduces the microbial load of a surface McDonnell & Burke, 2011). Although other soil microbe eradication techniques such as microwaving and gamma radiation exist, methods such as soil replacement, chemical fumigation, and solarization are the most common practices implemented in orchards for soil disinfection. Pre-plant fumigation has the remarkable ability to reduce RS; however, its benefits are temporary, and it is primarily a pre-plant method. Although chloropicrin has shown effectiveness in reducing RS that was not nematode related, the fumigant was deemed "unpleasant to handle" (Ross et al., 1983). Preliminary field and greenhouse trials testing Vorlex have shown promising results and could be an alternative fumigant to chloropicrin for ameliorating RS soils (Ross et al., 1983). Currently, chloropicrin is heavily restricted, and Vorlex's registration has been cancelled since 1992. Methyl bromide is a chemical fumigant that was used for RS until 2005 but has since been phased out by U.S. and European governments, since it was found to deplete the ozone layer (Eayre et al., 2000). Other chemical fumigants, such as Methyl iodide, have been shown to be as effective against RS as methyl bromide (Eayre et al., 2000). Although methyl iodide does not deplete the ozone layer and was approved by the Environmental Protection Agency in 2008, by 2011 the Pesticide Action Network of North America characterized the fumigant as a neurotoxin and carcinogen (Guthman & Brown, 2017). This led Arysta LifeScience to withdraw methyl iodide from the United States and other markets (Guthman & Brown, 2017). Chemical fumigants are becoming more restricted since they are considered non-sustainable methods for soil remediation (Hestmark et al., 2019). Solarization, the technique of trapping the sun's radiation in the soil using tarps, has reduced soil fungal phytopathogens such as *Fusaruim* spp., *Verticillium* spp., and *Ilyonectria morspanacis* (responsible for RS in ginseng) (Westerveld et al., 2023). Anaerobic soil disinfestation builds upon solarization through the addition of carbon substrates and water to the

soil, which in-creases soil temperature and slows down gas exchange (Browne et al., 2018). Anaerobic soil disinfestation has demonstrated potential for reducing soil microbial loads (fungi, oomycetes, bacteria, and nematodes) in different soil types and is comparable to soil fumigation (Browne et al., 2018). Anaerobic soil disinfestation has been shown to increase trunk cross-sectional area in almond trees by 148–214% compared to controls (Browne et al., 2018). Autoclaving the soil as a pre-planting method has increased peach tree biomass (Mazzola & Mullinix, 2005). Gamma radiation appears to be the most effective method for soil sterilization (Stroetmann et al., 1994), but this method is impractical at an orchard scale. In an attempt to reduce RS-related microbes, the complete removal of the RS soils and replacement with healthy/non-pathogenic soil has been in practice (Kelderer et al., 2012). Nonetheless, an inoculation of merely 1% of RS soil is sufficient for the associated microbes to re-establish and reduce tree growth (Bent et al., 2009). Soil amendments with *Brassica napus* seed meal were effective starting in the third year of application (Li et al., 2020). Additionally, soil amendments of pinewood biochar (10–20% (v/v)) led to an increase in total peach biomass compared to the untreated control (Atucha & Litus, 2015). Although effective in reducing RS, these pre-plant soil disinfection strategies are a temporary solution, which provide some relief from RS symptoms.

In terms of the microbiome, how RS develops could follow the intermediate disturbance hypothesis (IDH), which posits that local species diversity is optimized when environmental disturbances are not drastic in terms of magnitude and occur at a regular interval (Santillan & Wuertz, 2022). Although both “magnitude” and “regular interval” are ambiguous (Sheil & Burslem, 2003), the management practices of an orchard—such as irrigation, fertilizer, and pesticides—might provide an ideal environment for pathogens and microbial competitors to enhance their virulence and colonization of the rhizosphere. Since microbes can quickly undergo

multiple generations, they can evolve in a relatively short time span. If placed in an ideal setting, bacteria can, therefore, evolve resistance to antibacterial within 10 days (Dößelmann et al., 2017). The bulk orchard soil of an orchard experiences much less disturbance than annual crops, so 12 years should be sufficient time for the convergent evolution of several microbes to develop virulent functionalities towards their host.

Biological strategies (cover crops and rootstocks) used to remedy RS are continuously implemented, with success being site-dependent unlike pre-plant soil disinfection strategies (Yim et al., 2017; Roberts et al., 2005; Gu & Mazzola, 2003). These strategies aim to increase diversity in the field by using genetically distinct rootstocks and cover crops, which in turn can increase microbial diversity (Li et al., 2020). Sustainable practices such as increasing plant diversity through polyculture, crop rotation, intercropping, and cover crops have been shown to improve soil health unlike monoculture. Cover crops can improve soil health by increasing nitrogen levels (legumes) or increasing antimicrobial glucosinolates (Brassica). Furthermore, the planting of multiple genetically distinct species from the previous crop in polyculture can dilute the build-up of autotoxic compounds by contributing a mix of different plant residues (Hooks et al., 2010). Although one year of using wheat as a cover crop gave rise to enhanced vegetative growth and apple tree yield, it was not as effective as methyl bromide (Li et al., 2020). A cover crop of wheat showed promising results, but to further reduce RS there needs to be an antimicrobial aspect as well. Incorporation of cover crops which are resistant to generalists phytopathogens, such as nematode-resistant cowpea Vigna unguiculata (L.), have been shown to increase tomato yields more than the growth and incorporation of susceptible cowpea or non-incorporation of cowpea (Wang et al., 2002). Other promising cover crops that may be used to

manage generalist phytopathogens such as plant-parasitic nematodes are *Crotalaria* spp. and *Tagetes* spp. (Abd, 2018; Wani et al., 2012; Wang et al., 2002).

The development of genetic tools such as rootstocks have shown potential. Peach rootstocks with resistance to root-knot nematodes have been developed (Cesarano et al., 2017; Schneider et al., 2003). Furthermore, peach rootstocks—such as Evrica, PAC 9801-02, ROOTPAC® 40, and Tetra—appear to be tolerant to replant soils (Jiménez et al., 2011). Additionally, the drawbacks of monoculture can be mitigated by using rootstocks that are genetically different from their scions which could be used to promote plant diversity while maintaining the same fruit crop type in the orchard (Warschefsky et al., 2016). However, RS-resistant rootstocks need to be able to tolerate regional abiotic conditions such as climate, soil type, pH, salinity, etc. (Wang et al., 2019).

Beneficial microbe inoculums with antimicrobial properties have been developed to enhance crop productivity (Kloepper et al., 1989), but these are still in development for RS. Generalized conclusions have surmised that more than 60% of the strains isolated from healthy soils corresponded to *Pseudomonas* sp. (Benizri et al., 2005). More specifically, Pseudomonas putida has been found to isolate suppressed replant-contributing phytopathogens—such as the growth of *Rhizoctoni*a and *Pythium* spp. in vitro—and could control Rhizoctonia root rot for apple trees (Mazzola & Manici, 2012). Arbuscular mycorrhiza fungi (AMF) form symbiosis with the roots of approximately 80% of studied land plants (Gao et al., 2020). Arbuscular mycorrhiza has been tested by using inoculations of *Acauloapora scrobiculata* in replant soils, resulting in significantly increased shoot biomass and root phosphorus, potassium, calcium, copper, zinc, iron, and boron concentrations (Lǚ et al., 2019).

The effect of soil disinfection is effective but temporary and requires a complimentary technique. Figure 2 conceptualizes how the phytopathogen load of peach orchard (orange) soils gradually increase once a tree of the same genotype is re-planted. However, it is possible that, even in untreated soils, the phytopathogen concentrations could plateau. The microbial composition of the rhizosphere, in terms of both bacterial and fungal communities, has been found to be highly variable and to change over seasons and years (Rumberger et al., 2007), which could indicate that the players that cause RS shift even within the same site.

The rhizosphere may not be space-limited, but rather nutrient-limited, since direct observations of roots have shown the majority of the root surface is open space and remains uncolonized (Weller et al., 1988). This would mean that the rhizosphere has a carrying capacity, and the total abundance of rhizosphere microorganisms may be consistent with changes occurring in the composition of the rhizosphere (Weller et al., 1988). As soil disinfection lowers the phytopathogen population in replant soils of a peach orchard (blue), there is temporary relief from RS. This population could eventually recover, and disinfected replant soils may require continuous measures to increase microbial diversity, such as those highlighted previously.

RS is a multifaceted issue, thus requiring a multifaceted solution. For example, combinations of cover cropping and *Brassica napus* seed meal soil amendment improved the initial peach growth equivalent to a fumigation treatment using 1,3-dichloropropene-chloropicrin (Li et al., 2020). However, using an autoclave as the pre-plant soil disinfection method prior to having the cover crops established, the soil was not amended in a way that was conducive to inducing a biomass increase in the following peach tree planting as compared to the non-autoclaved with no cover crop controls. Additionally, in the same study, not all cover crops induced peach growth equally (Newberger et al., 2023). Soil disinfection can be challenging to

incorporate in multifaceted approaches, since this strategy can decrease not only phytopathogens but beneficial bacteria like nitrogen-fixing Rhizobium (D'Addabbo et al, 2010). A common goal of soil disinfection is to reduce all microbial life, which can be accomplished by heating moist soil to 63 °C for 30 min as it is known to eliminate most pathogenic fungi, bacteria, and viruses (Baker & Chandler, 1957). However, solarization practices which induced soil temperatures that did not exceed 41 °C at depths of 30–46 cm still greatly reduced soil population densities of fungal phytopathogens such as *Verticillium dahlia* Kleb., *Pythium ultimum* Trow., *Rhizcotonia solani* Kuehn, and *Thielaviopsis basicola* (Pullman et al., 1981). Similar solarization studies also found that lethal temperatures for thermal sensitive phytopathogens have been reported to be less than 41 °C (ED90 of Verticillium dahlia after 14 h at 37 °C, 50–100% mortality of *Rosellinia necatrix* Berl. ex Prill after 4 h at 38 °C, mycelium mortality of *Phytophthora cinnamomi* after 1–2 h at 38–40 °C, *Macrophomina phaseolina* and *Pythium aphanidermatum* (strongly declined after 24 h at 40 °C) (D'Addabbo et al., 2010). *Rhizobium* spp. have an upper temperature limit range of 37–47 °C with some strains still capable of nodulation at 45 °C (Patel et al., 2020). Although solarization heat treatment has been shown to decrease soil abundances of *Rhizobium* spp., these bacteria quickly recovered after the establishment of a legume crop (D'Addabbo et al., 2010). Therefore, a multifaceted solution including soil disinfection and retaining beneficial microbes may benefit if soil temperatures do not go above 41 °C. Regardless, strategies which mitigate RS are not always as effective when they are combined unless all the factors are considered.

Conclusions

Arriving at a solution to RS will require experts to reach a consensus on RS-related terminology, develop explicit descriptions for its symptoms, define its etiology, and identify its primary

phytopathogens. Consistent terminology would facilitate compiling the literature. Explicit symptom descriptions may aid in detangling compound issues like depleted soil nutrients and autotoxicity, each of which can lead to reduced overall plant biomass in monocultures. The buildup of RS-causing microbes needs to be reduced. The soil disinfection method outlined should also allow the survival of beneficial microbes in the soil instead of aiming for the total elimination of the soil's microbial load. Once the microbial load of RS soils is reduced, then multiple continuous biological methods should be used to keep RS under control. Such methods include RS-resistant rootstocks, poly-cropping, and inoculations of beneficial microbes. Continuous efforts to use these biological methods to increase plant/microbe diversity is critical, since RS-causing microbes will continuously attempt to build up in the soil throughout this time as well. The goals of this introduction were: (1) to conceptualize the establishment of the syndrome from a microbiome perspective and (2) to propose sustainable solutions to develop a beneficial microbiome to inhibit the onset of RS. The second chapter is a greenhouse study which looks how RS is alleviated by soil disinfection via autoclave and/or a crop rotation which tested 4 genetically different crops. The third chapter further examines how the bulk soil bacteriome is influenced by plant diversity and density. Lastly, the fourth chapter explores how rhizobacteria colonization is influenced by plant identity and density.

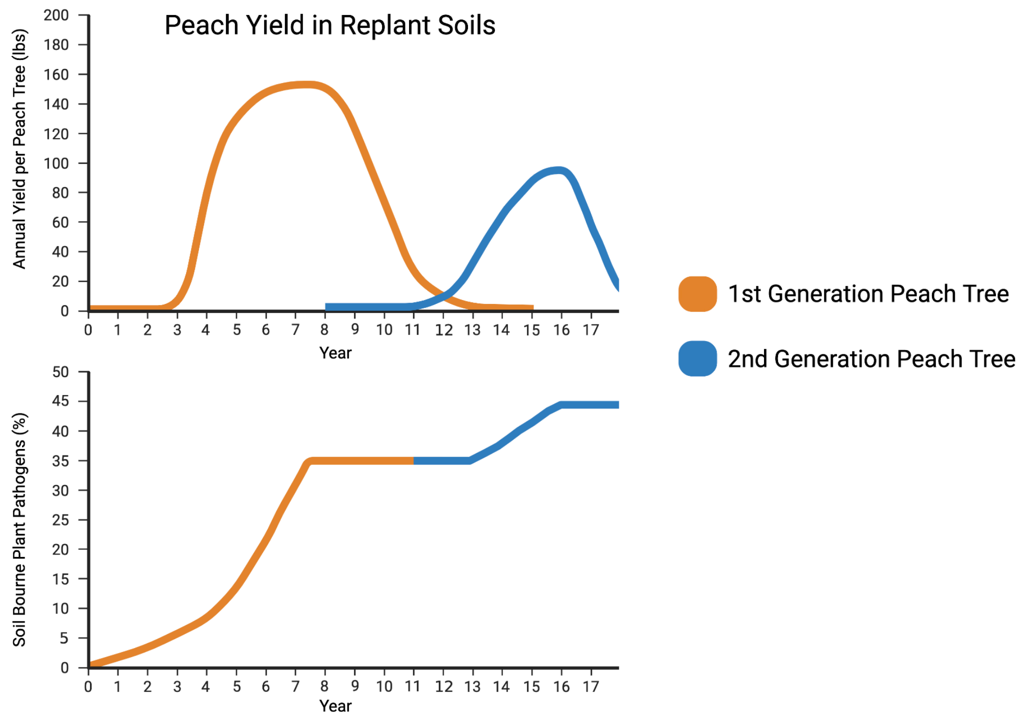

Figure 1. Proposed concept of the development of a replant syndrome microbiome. (a) First-generation peach trees show expected and typical growth. In the first replanting or second generation, the symptoms of the replant microbiome are observed by the impact it has on the developing fruit tree's crop yield. (b) A replant microbiome is established within the first generation of a monocropping orchard. Replant symptoms are immediately evident on newly planted saplings since the pathogens in the soil microbiome have been established previously. Phytopathogens that make up the replant microbiome can potentially specialize to be specific to the orchard geno-type, and the pathogenic microbial load is at its peak biomass when crop production is at its high-est.

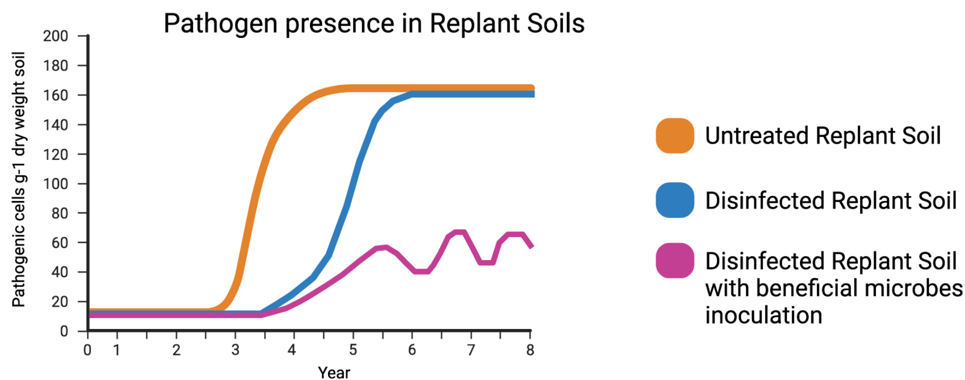

Figure 2. Proposed concept of the relative pathogenic gene expression or pathogenic cells g-1 dry weight soil. Phytopathogen load in untreated replant soils of a peach orchard (orange) gradually increases once the host peach tree is planted with possibly plateauing. Phytopathogen load of disinfected replant soils of a peach orchard (blue) show temporary RS relief. Disinfected

replant soil with repeated inoculations of beneficial microbes (violet) would fail to remove all phytopathogens but could control RS microbiome populations.

REFERENCES

Simon, M.; Lehndorff, E.; Wrede, A.; Amelung, W. In-field heterogeneity of apple replant disease: Relations to abiotic soil properties. *Sci. Hortic.* **2020**, *259*, 108809.
Mahnkopp, F.; Simon, M.; Lehndorff, E.; Pätzold, S.; Wrede, A.; Winkelmann, T. Induction and diagnosis of apple replant disease ARD): A matter of heterogeneous soil properties? *Sci. Hortic.* **2018**, *241*, 167–177.

He, J.-D.; Shu, B.; Wu, Q.-S. Mycorrhizosphere of fruit crops: Nature and properties. In *Fruit Crops*; Elsevier: Alpharetta, GA, USA, **2020**; pp. 325–338.

Rumberger, A.; Merwin, I.A.; Thies, J.E. Microbial community development in the rhizosphere of apple trees at a replant disease site. *Soil Biol. Biochem.* **2007**, *39*, 1645–1654.

Yang, J.-I.; Ruegger, P.M.; McKenry, M.V.; Becker, J.O.; Borneman, J. Correlations between root-associated microorganisms and peach replant disease symptoms in a California soil. *PLoS ONE* **2012**, *7*, e46420.
Liu, E.-T.; Wang, G.-S.; Li, Y.-Y.; Shen, X.; Chen, X.-S.; Song, F.-H.; Wu, S.-J.; Chen, Q.; Mao, Z.-Q. Replanting affects the tree growth and fruit quality of Gala apple. *J. Integr. Agric.* **2014**, *13*, 1699–1706.

Mazzola, M. Elucidation of the microbial complex having a causal role in the development of apple replant disease in Washington. *Phytopathology* **1998**, *88*, 930–938.

Jaffee, B.; Abawi, G.; Mai, W. Role of soil microflora and Pratylenchus penetrans in an apple replant disease. *Phytopathology* **1982**, *72*, 247–251.

Westerveld, S.M.; Shi, F. The history, etiology, and management of ginseng replant disease: A Canadian perspective in review. *Can. J. Plant Sci.* **2021**, *101*, 886–901.

Zhao, Y.-P.; Lin, S.; Chu, L.; Gao, J.; Azeem, S.; Lin, W. Insight into structure dynamics of soil microbiota mediated by the richness of replanted Pseudostellaria heterophylla. *Sci. Rep.* **2016**, *6*, 26175.

Hanschen, F.S.; Winkelmann, T. Biofumigation for Fighting Replant Disease—A Review. *Agronomy* **2020**, *10*, 425.

Mazzola, M.; Manici, L.M. Apple replant disease: Role of microbial ecology in cause and control. *Annu. Rev. Phytopathol.* **2012**, *50*, 45–65.

Caruso, F.L.; Neubauer, B.F.; Begin, M.D. A histological study of apple roots affected by replant disease. *Can. J. Bot.* **1989**, *67*, 742–749.

Ma, W.; Liao, X.; Wang, C.; Zhang, Y. Effects of four cropping patterns of lilium brownii on rhizosphere microbiome structure and replant disease. *Plants* **2022**, *11*, 824.

Wu, L.; Chen, J.; Khan, M.U.; Wang, J.; Wu, H.; Xiao, Z.; Zhang, Z.; Lin, W. Rhizosphere fungal community dynamics associated with Rehmannia glutinosa replant disease in a consecutive monoculture regime. *Phytopathology* **2018**, *108*, 1493–1500.

Agmon-Levin, N.; Mosca, M.; Petri, M.; Shoenfeld, Y. Systemic lupus erythematosus one disease or many? *Autoimmun. Rev.* **2012**, *11*, 593–595.

Sewell, G. Effects of Pythium species on the growth of apple and their possible causal role in apple replant disease. *Ann. Appl. Biol.* **1981**, *97*, 31–42.

Browne, G.; Lampinen, B.; Holtz, B.; Doll, D.; Upadhyaya, S.; Schmidt, L.; Bhat, R.; Udompetaikul, V.; Coates, R.; Hanson, B. Managing the almond and stone fruit replant disease complex with less soil fumigant. *Calif. Agric.* **2013**, *67*, 128–138.

Mai, W.; Abawi, G. Determining the cause and extent of apple, cherry, and pear replant diseases under controlled conditions. *Phytopathology* **1978**, *68*, 1540–1544.

Chen, T.; Lin, S.; Wu, L.; Lin, W.; Sampietro, D.A. Soil sickness: Current status and future perspectives. *Allelopath. J.* **2015**, *36*, 167–196.

Thakur, K.K.; Sharma, D.P. Pot-culture studies on soil and leaf nutrients status of peach in response to different soil management techniques for preventing replant disease. *J. Pharm. Innov.* **2018**, *7*, 1046–1053.

Spath, M.; Insam, H.; Peintner, U.; Kelderer, M.; Kuhnert, R.; Franke-Whittle, I.H. Linking soil biotic and abiotic factors to apple replant disease: A greenhouse approach. *J. Phytopathol.* **2015**, *163*, 287–299.

Winkelmann, T.; Smalla, K.; Amelung, W.; Baab, G.; Grunewaldt-Stöcker, G.; Kanfra, X.; Meyhöfer, R.; Reim, S.; Schmitz, M.; Vetterlein, D. Apple replant disease: Causes and mitigation strategies. *Curr. Issues Mol. Biol.* **2019**, *30*, 89–106.

Li, Y.; Dai, S.; Wang, B.; Jiang, Y.; Ma, Y.; Pan, L.; Wu, K.; Huang, X.; Zhang, J.; Cai, Z. Autotoxic ginsenoside disrupts soil fungal microbiomes by stimulating potentially pathogenic microbes. *Appl. Environ. Microbiol.* **2020**, *86*, e00130–e00120.

Mazzola, M.; Mullinix, K. Comparative field efficacy of management strategies containing Brassica napus seed meal or green manure for the control of apple replant disease. *Plant Dis.* **2005**, *89*, 1207–1213.

Newberger, D.R.; Minas, I.S.; Manter, D.K.; Vivanco, J.M. A Microbiological Approach to Alleviate Soil Replant Syndrome in Peaches. *Microorganisms* **2023**, *11*, 1448.

Li, K.; DiLegge, M.J.; Minas, I.S.; Hamm, A.; Manter, D.; Vivanco, J.M. Soil sterilization leads to re-colonization of a healthier rhizosphere microbiome. *Rhizosphere* **2019**, *12*, 100176.

Arneson, P.; Mai, W. Root diseases of fruit trees in New York State. VII. Costs and returns of preplant soil fumigation in a replanted apple orchard. *Plant Dis. Rep.* **1976**, *60*, 1054–1057.

Foy, C.L.; Drake, C.R.; Pirkey, C.L. Impact of herbicides applied annually for twenty-three years in a deciduous orchard. *Weed Technol.* **1996**, *10*, 587–591.

Forge, T.; Neilsen, G.; Neilsen, D. Organically acceptable practices to improve replant success of temperate tree-fruit crops. *Sci. Hortic.* **2016**, *200*, 205–214.

Chalmers, D.; Mitchell, P.; Van Heek, L. Control of Peach Tree Growth and Productivity by Regulated Water Supply, Tree Density, and Summer Pruning[1]. *J. Am. Soc. Hortic. Sci.* **1981**, *106*, 307–312.

Li, X.; Chu, Y.; Jia, Y.; Yue, H.; Han, Z.; Wang, Y. Changes to bacterial communities and soil metabolites in an apple orchard as a legacy effect of different intercropping plants and soil management practices. *Front. Microbiol.* **2022**, *13*, 956840.

Neumann, G.; Römheld, V. The release of root exudates as affected by the plant's physiological status. In *The Rhizosphere: Biochemistry and Organic Substances at The Soil-Plant Interface*; Marcel Dekker Inc.: , New York, NY, USA, **2000**; pp. 41–93.

Hassan, M.K.; McInroy, J.A.; Kloepper, J.W. The interactions of rhizodeposits with plant growth-promoting rhizobacteria in the rhizosphere: A review. *Agriculture* **2019**, *9*, 142.

Mazzola, M. Transformation of soil microbial community structure and Rhizoctonia-suppressive potential in response to apple roots. *Phytopathology* **1999**, *89*, 920–927.

Suchocka, M.; Swoczyna, T.; Kosno-Jończy, J.; Kalaji, H.M. Impact of heavy pruning on development and photosynthesis of Tilia cordata Mill. trees. *PLoS ONE* **2021**, *16*, e0256465.

Zhang, L.; Koc, A.; Wang, X.; Jiang, Y. A review of pruning fruit trees. *IOP Conf. Ser. Earth Environ. Sci.* **2018**, *153*, 062029.

Alfieri, S., Jr.; Seymour, C.; French, W. Cytospora Canker of Peach in Florida. In Proceedings of the Florida State Horticultural Society, Miami, FL, USA, 6–8 November **1973**.

Chao, Y.; Marks, L.R.; Pettigrew, M.M.; Hakansson, A.P. Streptococcus pneumoniae biofilm formation and dispersion during colonization and disease. *Front. Cell. Infect. Microbiol.* **2015**, *4*, 194.

Li, P.; Lu, Y.-J.; Chen, H.; Day, B. The lifecycle of the plant immune system. *Crit. Rev. Plant Sci.* **2020**, *39*, 72–100.

Atucha, A.; Litus, G. Effect of biochar amendments on peach replant disease. *HortScience* **2015**, *50*, 863–868.

Lü, L.-H.; Wu, Q.-S. Mitigation of replant disease by mycorrhization in horticultural plants: A review. *Folia Hortic.* **2018**, *30*, 269–282.

Teskey, B.J. *Tree Fruit Production*; Springer Science & Business Media: Berlin, Germany, 2012.

Dejong, T.M.; Negron, C.M.; Favreau, R.; Day, K.; Costes, E.; Lopez, G. Using concepts of shoot growth and architecture to understand and predict responses of peach trees to pruning. In Proceedings of the 7th International Peach Symposium. *Acta Hortic.* **2012**, *962*, 225–232.

Paço, T.A.; Ferreira, M.I.; Conceição, N. Peach orchard evapotranspiration in a sandy soil: Comparison between eddy covariance measurements and estimates by the FAO 56 approach. *Agric. Water Manag.* **2006**, *85*, 305–313.

Sofo, A.; Nuzzo, V.; Palese, A.M.; Xiloyannis, C.; Celano, G.; Zukowskyj, P.; Dichio, B. Net CO_2 storage in Mediterranean olive and peach orchards. *Sci. Hortic.* **2005**, *107*, 17–24.

Warmund, M. *Fruit Production*; University of Missouri Extension: Columbia, MO, USA, **2009**, pp. 1–20.

Badri, D.; Vivanco, J. Regulation and function of root exudates Plant. *Cell Environ.* **2009**, *32*, 666–681.

Vives-Peris, V.; de Ollas, C.; Gómez-Cadenas, A.; Pérez-Clemente, R.M. Root exudates: From plant to rhizosphere and beyond. *Plant Cell Rep.* **2020**, *39*, 3–17.

Steinkellner, S.; Lendzemo, V.; Langer, I.; Schweiger, P.; Khaosaad, T.; Toussaint, J.-P.; Vierheilig, H. Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic plant-fungus interactions. *Molecules* **2007**, *12*, 1290–1306.

Yang, F.; Chen, Q.; Zhang, Q.; Long, C.; Jia, W.; Cheng, X. Keystone species affect the relationship between soil microbial diversity and ecosystem function under land use change in subtropical China. *Funct. Ecol.* **2021**, *35*, 1159–1170.

Silindir, M.; Özer, A.Y. Sterilization methods and the comparison of E-beam sterilization with gamma radiation sterilization. *Fabad J. Pharm. Sci.* **2009**, *34*, 43–53.

McDonnell, G.; Burke, P. Disinfection: Is it time to reconsider Spaulding? *J. Hosp. Infect.* **2011**, *78*, 163–170.

Ross, R.G.; Delbridge, R.; Kimpinski, J.; McRae, K. Control of apple replant disease in Nova Scotia by soil fumigation with Vorlex and chloropicrin. *Can. J. Plant Pathol.* **1983**, *5*, 177–180.

Eayre, C.; Sims, J.; Ohr, H.; Mackey, B. Evaluation of methyl iodide for control of peach replant disorder. *Plant Dis.* **2000**, *84*, 1177–1179.

Guthman, J.; Brown, S. How Midas Lost Its Golden Touch: Neoliberalism and Activist Strategy in the Demise of Methyl Iodide in California. In *The New Food Activism: Opposition, Cooperation, and Collective Action*, Alkon, A.H., Guthman, J., Eds.; University of California Press: Oakland, CA, USA, **2017**; pp. 80–106.

Hestmark, K.; Fernández-Bayo, J.; Harrold, D.; Randall, T.; Achmon, Y.; Stapleton, J.; Simmons, C.; VanderGheynst, J. Compost induces the accumulation of biopesticidal organic acids during soil biosolarization. *Resour. Conserv. Recycl.* **2019**, *143*, 27–35.

Westerveld, S.M.; Riddle, R.N.; Shi, F. Efficacy of fumigants and biofumigants for the control of replant disease of American ginseng (Panax quinquefolius). *Can. J. Plant Pathol.* **2023**, *45*, 405–419.

Browne, G.; Ott, N.; Poret-Peterson, A.; Gouran, H.; Lampinen, B. Efficacy of anaerobic soil disinfestation for control of Prunus replant disease. *Plant Dis.* **2018**, *102*, 209–219.

Stroetmann, I.; Kämpfer, P.; Dott, W. The efficiency of sterilization methods for different soils. *Zentralbl. Hyg. Umweltmed.* **1994**, *195*, 111–120.

Kelderer, M.; Manici, L.M.; Caputo, F.; Thalheimer, M. Planting in the 'inter-row'to overcome replant disease in apple orchards: A study on the effectiveness of the practice based on microbial indicators. *Plant Soil* **2012**, *357*, 381–393.

Bent, E.; Loffredo, A.; Yang, J.-I.; McKenry, M.V.; Becker, J.O.; Borneman, J. Investigations into peach seedling stunting caused by a replant soil. *FEMS Microbiol. Ecol.* **2009**, *68*, 192–200.

Santillan, E.; Wuertz, S. Microbiome assembly predictably shapes diversity across a range of disturbance frequencies in experimental microcosms. *npj Biofilms Microbiomes* **2022**, *8*, 41.

Sheil, D.; Burslem, D.F. Disturbing hypotheses in tropical forests. *Trends Ecol. Evol.* **2003**, *18*, 18–26.

Dößelmann, B.; Willmann, M.; Steglich, M.; Bunk, B.; Nübel, U.; Peter, S.; Neher, R.A. Rapid and consistent evolution of colistin resistance in extensively drug-resistant Pseudomonas aeruginosa during morbidostat culture. *Antimicro. Agents Chemother.* **2017**, *61*, e00043-17. http://doi.10.1128/aac. 00043–00017.

Yim, B.; Nitt, H.; Wrede, A.; Jacquiod, S.; Sørensen, S.J.; Winkelmann, T.; Smalla, K. Effects of soil pre-treatment with Basamid® granules, Brassica juncea, Raphanus sativus, and Tagetes patula on bacterial and fungal communities at two apple replant disease sites. *Front. Microbiol.* **2017**, *8*, 1604.

Gu, Y.-H.; Mazzola, M. Modification of fluorescent pseudomonad community and control of apple replant disease induced in a wheat cultivar-specific manner. *Appl. Soil Ecol.* **2003**, *24*, 57–72.

Roberts, P.; Matthews, W., Jr.; Ehlers, J. Root-knot nematode resistant cowpea cover crops in tomato production systems. *Agron. J.* **2005**, *97*, 1626–1635.

Hooks, C.R.; Wang, K.-H.; Ploeg, A.; McSorley, R. Using marigold (*Tagetes* spp.) as a cover crop to protect crops from plant-parasitic nematodes. *Appl. Soil Ecol.* **2010**, *46*, 307–320.

Wang, K.-H.; Sipes, B.; Schmitt, D. Crotalaria as a cover crop for nematode management: A review. *Nematropica* **2002**, *32*, 35–58.

Abd Alhady, M.R.A. In vitro propagation for peach rootstock (Nemaguard). *Egypt. J. Desert Res.* **2018**, *68*, 1–13.

Wani, I.A.; Ahanger, R.A.; Bhat, H.A.; Lone, A.A.; Bhat, T.A.; Malik, I.A.; Hassan, G. Rootstocks of almond. *J. Plant Dev. Sci. Vol.* **2012**, *4*, 137–150.

Schneider, S.M.; Rosskopf, E.N.; Leesch, J.G.; Chellemi, D.O.; Bull, C.T.; Mazzola, M. United States Department of Agriculture—Agricultural Research Service research on alternatives to methyl bromide: Pre-plant and post-harvest. *Pest Manag. Sci. Former. Pestic. Sci.* **2003**, *59*, 814–826.

Cesarano, G.; Zotti, M.; Antignani, V.; Marra, R.; Scala, F.; Bonanomi, G. Soil sickness and negative plant-soil feedback: A reappraisal of hypotheses. *J. Plant Pathol.* **2017**, *99*, 545–570.

Jiménez, S.; Pinochet, J.; Romero, J.; Gogorcena, Y.; Moreno, M.Á.; Espada, J.L. Performance of peach and plum based rootstocks of different vigour on a late peach cultivar in replant and calcareous conditions. *Sci. Hortic.* **2011**, *129*, 58–63.

Warschefsky, E.J.; Klein, L.L.; Frank, M.H.; Chitwood, D.H.; Londo, J.P.; von Wettberg, E.J.; Miller, A.J. Rootstocks: Diversity, domestication, and impacts on shoot phenotypes. *Trends Plant Sci.* **2016**, *21*, 418–437.

Wang, Y.; Li, W.; Xu, X.; Qiu, C.; Wu, T.; Wei, Q.; Ma, F.; Han, Z. Progress of apple rootstock breeding and its use. *Hortic. Plant J.* **2019**, *5*, 183–191.

Kloepper, J.W.; Lifshitz, R.; Zablotowicz, R.M. Free-living bacterial inocula for enhancing crop productivity. *Trends Biotechnol.* **1989**, *7*, 39–44.

Benizri, E.; Piutti, S.; Verger, S.; Pages, L.; Vercambre, G.; Poessel, J.; Michelot, P. Replant diseases: Bacterial community structure and diversity in peach rhizosphere as determined by metabolic and genetic fingerprinting. *Soil Biol. Biochem.* **2005**, *37*, 1738–1746.

Gao, W.-Q.; Lü, L.-H.; Srivastava, A.; Wu, Q.-S.; Kuča, K. Effects of mycorrhizae on physiological responses and relevant gene expression of peach affected by replant disease. *Agronomy* **2020**, *10*, 186.

Lǚ, L.-H.; Zou, Y.-N.; Wu, Q.-S. Mycorrhizas mitigate soil replant disease of peach through regulating root exudates, soil microbial population, and soil aggregate stability. *Commun. Soil Sci. Plant Anal.* **2019**, *50*, 909–921.

Weller, D.M. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Ann. Rev. Phytopathol.* **1988**, *26*, 379–407.

D'Addabbo, T.; Miccolis, V.; Basile, M.; Candido, V. Soil solarization and sustainable agriculture. In *Sociology, Organic Farming, Climate Change and Soil Science*; **2010**; Springer Dordrecht: Dordrecht, Netherlands, pp. 217–274.

Baker, K.F.; Chandler, P.A. UC system for producing healthy container-grown plants. *AIBS Bull.* **1957**, *8*, 46.

Pullman, G.; DeVay, J.; Garber, R. Soil solarization and thermal death: A logarithmic relationship between time and temperature for four soilborne plant pathogens. *Phytopathology* **1981**, *71*, 959–964.

Patel, A.K.; Banjare, U.; Kumari, A.; Singh, R.K.; Pandey, K.D. Temperature tolerant Rhizobium leguminosorum bv. viciae strains with plant growth promotion traits. *J. Pure Appl. Microbiol.* **2020**, *14*, 2603–2609.

CHAPTER 2 A MICROBIOLOGICAL APPROACH TO ALLEVIATE SOIL REPLANT SYNDROME IN PEACHES[2]

[2]This research was originally published in Microorganisms Journal by Newberger, D. R., Minas, I. S., Manter, D. K., & Vivanco, J. M. (2023). A Microbiological Approach to Alleviate Soil Replant Syndrome in Peaches. *Microorganisms*, *11*(6), 1448.

Synopsis

Replant syndrome (RS) is a global problem characterized by reduced growth, production life, and yields of tree fruit/nut orchards. RS etiology is unclear, but repeated monoculture plantings are thought to develop a pathogenic soil microbiome. This study aimed to evaluate a biological approach that could reduce RS in peach (*Prunus persica*) orchards by developing a healthy soil bacteriome. Soil disinfection via autoclave followed by cover cropping and cover crop incorporation was found to distinctly alter the peach soil bacteriome but did not affect the RS etiology of RS-susceptible 'Lovell' peach seedlings. In contrast, non-autoclaved soil followed by cover cropping and incorporation altered the soil bacteriome to a lesser degree than autoclaving but induced significant peach growth. Non-autoclaved and autoclaved soil bacteriomes were compared to highlight bacterial taxa promoted by soil disinfection prior to growing peaches. Differential abundance shows a loss of potentially beneficial bacteria due to soil disinfection. The treatment with the highest peach biomass was non-autoclaved soil with a cover crop history of alfalfa, corn, and tomato. Beneficial bacterial species that were cultivated exclusively in the peach rhizosphere of non-autoclaved soils with a cover crop history were *Paenibacillus castaneae* and *Bellilinea caldifistulae*. In summary, the non-autoclaved soils show continuous enhancement of beneficial bacteria at each cropping phase, culminating in an enriched rhizosphere which may help alleviate RS in peaches.

Introduction

Replant syndrome (RS), commonly referred to as replant disease, is a global soil-related challenge induced in trees newly planted upon old orchard sites where repeated monoculture leads to stunted tree growth and reduced yields (Manganaris et al., 2022; Minas at al., 2018; Thakur & Sharma, 2018; Zhu et al., 2016). RS etiology is not fully understood, but reduced orchard productivity due to RS is caused by a microbial complex of phytopathogens/competitors (Mazzola & Manici, 2012). Replant symptoms are nonspecific, affect multiple genera of fruit trees, and often correlate with pathogenic generalists such as root-lesion nematodes and *Fusarium* spp. (Manici et al., 2017; Merwin & Stiles, 1989; Traquair, 1984). Biotic factors such as microorganisms contribute to RS, which is supported by studies where *Prunus persica* (peach tree) biomass is higher in autoclaved soils than in non-autoclaved soils (Li et al., 2019). Nonetheless, abiotic factors such as decreased soil fertility, poor soil structure, and nonoptimal pH can exacerbate RS (Hanschen & Winkelmann, 2020; Mazzola & Manici, 2012). The consensus that the previous plant of a similar genotype is responsible for initiating RS has supported the notion of intraspecific allelopathy, known as autotoxicity, is a contributing factor (Hanschen & Winkelmann, 2020; Wang et al., 2017). However, RS can persist in soils for several years, and it is unknown whether these chemicals are stable for years (Li et al., 2019). Recent understanding of RS and autotoxicity suggests these chemicals are rapidly degraded by rhizosphere and soil microbes but may induce a microbial composition shift in the soil from beneficials to pathogenic or nutrient competitors (Hanschen & Winkelmann, 2020).

The soil microbiome is highly connected, and disturbances can affect bacteriome composition and functionality (Smith et al., 2016). Cover crops, tillage, solarization, and fumigation can change microbial communities. Previous management of RS involved chemical fumigation of orchard soils before planting seedlings (Zhu et al., 2016; Kanaan et al., 2018). Soil

fumigation induced a growth response for trees in RS soils which lasted one year, but RS symptoms reappeared within two growing seasons (Wang & Mazzola, 2019). Environmental regulations restrict the use of fumigants that restrict their use in orchards (Zhu et al., 2016). Thus, sustainable soil practices are needed to alleviate RS.

Cover cropping is a sustainable soil strategy where crops are planted to regenerate soil health rather than to be harvested for economic value. Cover cropping can conserve soil, decrease water runoff, and enhance soil organic matter content (Altieri et al., 2015). Since cover crops affect the chemical and physical properties of the soil, they also modify the biological properties of the rhizosphere, i.e., the narrow region of soil where root–microbial associations occur (Abán et al., 2021). Root–microbial associations within the rhizosphere can potentially improve soil fertility and degrade toxic chemicals (Hrynkiewicz et al., 2012). Furthermore, beneficial associations in the rhizosphere can influence pathogen populations (Peralta et al., 2018).

In the current study, alfalfa, fescue, corn, and tomato were tested as cover crops for the purpose of reducing RS symptoms in peach. Additionally, autoclaving was used to determine if the benefits of soil disinfection could complement those of cover crops. Previous studies focused on the identification of reoccurring phytopathogenic instigators of RS, such as fungi, oomycetes, and nematodes. The scope of this study was to emphasize sustainable agricultural techniques that promoted peach health and to identify potential plant growth promoting rhizobacteria for future inoculation studies. These findings show some drawbacks of soil disinfection with cover cropping as a favorable soil regenerative strategy. Furthermore, correlations between microbial taxa and RS alleviation in peaches were identified.

Materials and Methods

Soil Sampling Site and Disinfection

RS soil for the experiment was acquired from a peach orchard research block, which was established in 2007 at the Colorado State University's experimental orchard at the Western Colorado Research Center in Orchard Mesa, CO. This peach orchard was established using *Prunus persica* (peach) 'Cresthaven' scions with grafted peach 'Lovell' rootstocks. The soils from this area have been described as Billings silty clay loam (calcareous, mesic Typic Torrifluvents). RS soils were transported to Colorado State University's Horticultural Center.

In the Horticultural Center, soils were passed through a metal sieve (2 cm wide) and homogenized. Samples of the replant soil were collected before and after autoclaving and stored at −80 °C to be used as controls for soil bacteriome analysis. Soil was placed in autoclave bags and then in a STERIS brand steam autoclave set on the 40 min liquid cycle at 121 °C, which was run three times. In between cycles, bags carrying the soil were shaken to redistribute the soil before being returned to the autoclave for a second and third time.

Then, 4 L black plastic pots (n = 100) were lined with Vigoro weed control fabric medium duty, placed on Vigoro 15.24 cm plastic plant saucers, and filled with c. 2.1 kg of either untreated RS soil (n = 50) or autoclaved RS soil (n = 50).

Seed Sterilization and Density for Cover Crops

Four crops were selected for this study: natural sweet F1 OG hybrid bicolor Sh2 corn (*Zea mays*), hybrid cherry tomato SUN gold F1 (*Solanum lycopersicum*), ranger alfalfa (*Medicago sativa*), and a fine fescue species mixture of chewing fescue (*Festuca rubra* ssp. *Commutate*), hard fescue (*Festuca longifolia*), and creeping red fescue (*Festuca rubra*). Alfalfa and fescue were selected since these cover crops are known to successfully establish in Colorado soils (Braun et al., 2020; Ervin & Koski, 1998). Tomato and corn have been shown to induce

microbial shifts in RS soils under autoclaved conditions, which could potentially create a beneficial microbiome for the incoming peach crop cycle (Li et al., 2019). All cover crops were considered genetically distant from peaches. Seed densities were calculated by using recommended crop seed count or weight per square meter and adjusted by the 0.0222 m^2 surface area of a 4 L pot. Corn and tomato treatments had one plant per pot (Ara et al., 2007; Ilker, 2011). For fescue, the recommended use of 50 lbs of seed per acre for high elevation soil in the western United States was used to calculate 0.54 g of fescue seeds per pot (Laycock, 1982). For alfalfa, the recommended use of 75 seeds of alfalfa per square foot was used to calculate 0.038 g of alfalfa seeds per pot (Rankin, 2008). For seed sterilization, 15 mL falcon tubes with seeds were filled with 3% NaOCl and vortexed at max speed (setting 10: 600–2700 RPM) for one minute. NaOCl was removed and seeds were then rinsed with autoclaved distilled water and vortexed at max speed for one minute, with this rinse step being repeated 5 times. Seeds were immediately planted into the soil. Each crop treatment had 10 replicates for autoclaved and non-autoclaved soils. Pots with only autoclaved and non-autoclaved soils served as a no-plant control and were watered to water-holding capacity daily.

Establishing the Cover Crops in a Greenhouse

The experiment was factorial with two factors: soil disinfection (2 levels: autoclave and non-autoclaved) and cover crops (5 levels: corn, tomato, alfalfa, fescue, and a no-plant control). Pots (n = 100) were set in a completely randomized design (5 × 20) using an online random block design generator (https://www.randomizer.org accessed on 26 February 2021) with one treatment per row. Pots were watered at water-holding capacity (c. 200 mL) for six days per week for 12 weeks. After 12 weeks, bulk soil samples were collected with a hand-sized soil probe either in the center of the pot or 2 cm from the base of the plant at a depth of 7 cm. Above

ground crop biomass was cut into <2 cm pieces using scissors that were washed in 3% NaOCl followed by heat sterilization using a Bacti-Cinerator III™ from Monoject (St. Louis, MO, USA) in between samples. Above ground fresh biomass of cover crops was recorded, immediately incorporated into the soil within the first 3 cm of the same pot in which the crops had been grown, and left to decompose. After two weeks, bulk soil samples were collected.

Continued Greenhouse Experiment with Peaches

'Lovell' (*Prunus persica*) rootstock cultivar was grown from seeds in liners using pro-mix potting media in a greenhouse for 28 days. This RS-susceptible 'Lovell' was selected since this rootstock cultivar was grown in the orchard where the RS soil was collected. These four-week-old peach seedlings were transplanted into the pots that previously had cover crops and no-cover-crop controls. Peach seedlings were watered daily with c. 150 mL of tap water. Weeds and cover crops were continuously removed, and no fertilizer was added. Peaches grew for 22 weeks. For microbial analysis, bulk soil and rhizosphere soil was collected. Using a soil probe, bulk soil samples were collected from the top 7 cm of soil within 2 cm of the base of the tree trunk and immediately stored at −20 °C. Rhizosphere was defined as the soil adhering to the roots after the removal of bulk soil and gently shaking the root system. Rhizosphere soil was taken from light colored roots, placed into 15 mL falcon tubes, and immediately stored at −20 °C. Remaining soil on root systems were removed with tap water. Biomass was separated as either above- or below-ground and its weight was recorded. Fresh biomass samples were oven-dried at 90 °C for 72 h and were weighed for above- and below-ground dry biomass. Greenhouse experiments ran between 20 June and 1 August, humidity set point was 70%, cool set point was 24–26.5 °C, heat set point was 18–21 °C, relative humidity ranged from 21–80% (average = 55.5%), and actual temperature ranged from 18.9–38.3 °C (average = 25.4 °C).

Soil Analysis

Soil analysis (total nutrient digest and Haney H_2O extract) was performed by WARD Laboratories, Inc. (Kearney, NE, USA) (Yost et al., 2018) on three bulk soil samples per treatment. Total nutrient digest analysis quantified the total values of elements in a soil (C, N, P, K, Ca, S, Mg, B, Zn, Mn, Fe, Cu, Mo). The Haney test uses different extracts from traditional soil test labs, and the extract analysis quantifies nutrients within the soil that are available to soil microorganisms by measuring soil respiration, water-soluble organic carbon, and nitrogen. Soil analyses of total nutrient digest and Haney H_2O extract by soil treatment of autoclave, cover crop, and control treatments can be found in Table 1.

DNA Extraction

Total genomic DNA (gDNA) was extracted from 0.25 g of bulk and peach rhizosphere soil in a QIAcube instrument (Qiagen, Germantown, MD, USA) using PowerSoil® DNA kits by Qiagen. All DNA extractions were performed according to Qiagen's instructions with a final elution volume of 100 µL. DNA concentration was quantified using a Qubit with broad range assay solutions. Of the ten replicates used for biomass, a subset of five replicates were used for bacterial DNA microbial analysis. Bulk soil samples were taken after cover crops were grown for 12 weeks, after cover crops had been incorporated for two weeks, and after peach trees had been growing for 22 weeks. The controls used were pre-extracted Zymo gDNA (Zymo Research Corporation, Irvine, CA, USA) (n = 4), HPLC water (n = 3), stock soil (n = 4), non-autoclaved soil (n = 5), and autoclaved soil (n = 4). In total, 220 samples were extracted.

Oxford Nanopore Library Preparation, Sequencing, and Bioinformatics Pipeline

Based on Qubit concentrations (ng/µL), extracted DNA was diluted 10× with HPLC water to lower DNA concentrations and minimize potential PCR inhibitors. Mastermix consisted of 10 µL Phusion HSII master mix, 7.2 µL H_2O, 0.4 µL forward primer, and 0.4 µL reverse primer for a total of 18 µL Mastermix per 2 µL sample. Bacterial primers used were Bact_27F-Mn (5′-TTTCTGTTGGTGCTGATATTGCAGRGTTYGATYMTGGCTCAG-3′) and Bact_1492R-Mn (5′-ACTTGCCTGTCGCTCTATCTTC TACCTTGTTACGACTT-3′). Polymerase chain reaction (PCR) settings were 98 °C for 30 s, 98 °C for 15 s, 50 °C for 15 s, and 72 °C for 60 s for 25 cycles, and 72 °C for 5 min. After PCR, equal volumes of DNA and beads were mixed. A 96-pronged magnetic stand moved beads with adhering DNA into two 30 s rinses of 70% EtOH. DNA was eluted in a 96-well plate with 40 µL PCR grade water and beads were removed using a magnetic stand. DNA was quantified using a Qubit with high sensitivity assay solutions. The second PCR settings were 98 °C for 30 s, 98 °C for 15 s, 62 °C for 15 s, and 72 °C for 60 s for 25 cycles, and 72 °C for 5 min.

After a second PCR, DNA and barcodes were pooled in AMPure bead solution in a 96-well plate. Wells with suspended DNA and barcodes were pooled into a clean Lo-Bind tube. MinION sequencer was loaded with a flow cell (R9.4.1) and was prepared for DNA loading. To prepare the flow cell, air (c. 20 µL) was removed using a pipette. The flow cell was then primed with flush buffer, and pooled DNA was loaded into the sampling port. MinKNOW software (v23.04.5) was used to sequence the pooled library for 48 h. Raw data were downloaded and converted into fastq file format using Guppy_basecaller (v6.0.1). Barcodes were sorted by de-multiplex using Guppy_barcoder using barcode kit EXP-PBC096, trimmed, and reads were then filtered by quality and length (Filtlong minimum length: 1000 and mean quality: 70) (Cutadapt: -m 1000 -M 2000). Chimeras were identified and removed by Vsearch. Bacterial taxa were

identified using EMU NCBI Reference Database. EMU error correction removed identified bacterial taxa based on alignment and abundance profiles. Bacterial taxa with <one per 10,000 reads were removed. Sequencing data came from three separate sequence runs, which were pooled for data analysis.

Statistical Analysis

All statistical analyses were performed in RStudio Version 1.4.1103. A non-parametric test, the Kruskal–Wallis rank sum test, was used to analyze fresh cover crop biomass and peach dry biomass by soil treatment (autoclaved vs. non-autoclaved). Pairwise comparisons using the Wilcoxon rank sum exact test was used to infer differences between plant biomass and soil treatment. For regressions analyzing soil nutrients from the end of the peach experiment, peach dry biomass was used. The Lagrange multiplier test was used for the regressions fit with broom and tidyverse packages in RStudio. The vegan package was used to test for significant differences between treatments with perMANOVA and visualized with a constrained principal coordinate analysis (PCoA). Bray–Cutis was used to determine distance for PCoAs. Homogeneity of multivariate dispersions was measured using betadisper from the vegan package. Differential abundance analysis was based on bacterial species counts that were transformed using a log2 fold-change and the Benjamini and Hochberg statistical method using the false discovery rate function (FDR) and 0.05 as the accepted threshold for the adjusted *p*-value.

Results

Effect of Soil Disinfection on Cover Crop Biomass

The Kruskal–Wallis test for cover crop above-ground biomass shows the soil treatment (autoclaved vs. non-autoclaved) is significant, $\chi^2 = 16.398$ (df = 1, p-value < 0.001). All cover

crops grown in autoclaved soils have a higher biomass than cover crops grown in non-autoclaved soils. Biomass of corn ($p < 0.001$), fescue ($p < 0.001$), and tomato ($p < 0.001$) are significantly different between their respective autoclaved and non-autoclaved treatments. However, alfalfa crop biomass is not significantly different ($p = 0.459$) between the autoclaved and non-autoclaved soil treatments (Figure 1). Alfalfa's biomass in non-autoclaved soils shows a 34.6% reduction compared to alfalfa grown in autoclaved soils. Corn grown in autoclaved soils has the highest biomass out of all autoclaved and non-autoclaved cover crop treatments. In this study, tomato plants grown in untreated soils (RS soils) have a biomass reduction of 85.8% compared to tomato plants grown in autoclaved soils. This supports the trend that soil disinfection improves plant health.

Effect of Cover Crop and Biomass Incorporation on the Soil Microbiome

The bulk soil bacteriome where cover crops had been growing for 12 weeks were analyzed. The perMANOVA test shows that crop type ($p = 0.001$), autoclaved and non-autoclaved ($p = 0.001$), and the interaction ($p = 0.03$) between the two factors are significant and the CAP axes explain a total of 37.9% of the variance for all samples (Figure 2A). Separation between autoclaved and non-autoclaved soils is clear along axis 1 and explains 29.9% of the variance. For cover crops, autoclaved soils (average distance to median: 0.400) have a lower dispersion than non-autoclaved soils (average distance to median: 0.411) (Figure 2A). Within the autoclaved soil treatment, cover crop bulk soil microbiomes overlap while cover crop treatment has a greater role in shaping the microbiome in non-autoclaved soils. Corn grown in autoclaved soils has the highest biomass after 12 weeks of growth, but its microbiome does not show clear separation from the other cover crops. In the bulk soil of the cover crops, no-cover-crop controls overlap with the crop treatments in either of their respective soil treatments.

Microbes from the autoclaved soil treatment of cover crop bulk soils were of interest due to the increase in biomass in all cover crops. Although the positive effects of autoclaving on cover crop growth are primarily due to the removal of or reduction in potentially negative microorganisms, this study aimed to identify beneficial bacteria instead of highlighting deleterious bacteria that has been previously studied. Thus, differential abundance between non-autoclaved and autoclaved cover crop bulk soils highlights bacteria whose abundances are significantly different (Tables 2 and 3). There are 14 bacterial taxa whose abundance is driven by autoclaving, since all cover crops and no-cover-crop controls share these microbes. No common bacteria are found to be promoted within just the four crop treatments (indicated by the orange circle in Figure 2B). Autoclaved treatments with the highest unique bacterial taxa are no crop (n = 48) and fescue (n = 33), followed by tomato (n = 13), corn (n = 7), and alfalfa (n = 3). In addition, non-autoclaved cover crop bulk soil treatments show 26 bacterial taxa whose abundances are higher than in autoclaved soils and are shared within all cover crop treatments (Figure 10).

Bulk soils after the cover crop had been incorporated and decomposed for two weeks continued to show separation between autoclaved and non-autoclaved microbiomes (Figure 3). The perMANOVA test shows that cover crop history ($p = 0.001$) and autoclaved soil treatment ($p = 0.001$) are significant and explain a total of 35.7% of the variance. The interaction between cover crop history and autoclaved soil treatment is not significant ($p = 0.105$) (Figure 3). Similar as in crop history, the microbiome corresponding to bulk soil after cover crop incorporation shows a tighter cluster in autoclaved soils (Figure 3). Interestingly, the incorporation of alfalfa biomass in non-autoclaved soils shows an independent cluster compared to other cover crop treatments. Bacterial drivers (identified by the differential abundance) of the incorporated cover

crop bulk soil microbiome that are found in all crops and no-cover-crop controls continue to have increased abundance in their respective autoclaved soil treatment (Figure 3). In autoclaved cover-crop-incorporated soils, the bacterial species *Tumebacillus soli*, *Cytobacillus oceanisediminis*, and *Mesobacillus subterraneus* are found to be bacterial drivers of the microbiome (Figure 3) and these are the same microbes found in autoclaved cover crop soils (Table 2). In non-autoclaved soil incorporated with cover crops, the bacterial species *Vicinamibacter silvestris*, *Skermanella stibiiresistens*, *Bacillus megaterium*, *Nostoc* sp. HK-01, and *Nostoc* sp. PCC 7107 are primarily found (Figure 3), which are also present in non-autoclaved cover crop soils (Table 3).

Effect of Soil Disinfection and Cover Crop Incorporation on Peach Growth

The Kruskal–Wallis test for peach dry total biomass shows that the soil treatment effect (autoclaved vs. non-autoclaved) is significant ($\chi^2 = 35.298$, df = 1, p-value < 0.001). Biomass is higher for peach trees grown in soil that has not been disinfected via steam autoclave, and it is observed for most soil cover crop treatments, with alfalfa being the exception ($p = 0.095$) (Figure 4A). Pairwise comparisons using corn ($p = 0.002$), fescue ($p < 0.001$), and tomato ($p < 0.001$) cover crops show a significant difference in biomass within autoclaved and non-autoclaved soil treatment pairs (Figure 4A). Between the two no crop controls that later had peaches growing, there was no significant difference in biomass within autoclaved and non-autoclaved soil treatments ($p = 0.363$) (Figure 4B). Additionally, autoclaved soils with a cover crop history of fescue ($p < 0.001$) and corn ($p = 0.002$) perform worse than autoclaved soils with a history of no cover crops (Figure 4B). Within autoclaved soil treatments, peaches grown in alfalfa and tomato have a higher biomass than peach trees in soils that previously had corn and fescue. In all, peach

trees grown in non-autoclaved soils have the highest biomass compared to peaches grown in autoclaved soils.

Nitrogen and Nutrient Analysis

Nutrient analyses were performed to investigate if nutrient cycling could help explain the differences between autoclaved and non-autoclaved soil treatments. Dry peach biomass was used in regression plots with several different soil nutrient parameters. Soil nutrient parameters that are not significant predictors of peach biomass are total organic carbon, organic nitrogen, organic C/N ratio, total phosphorus (H3A), available phosphorus, potassium (H3A), and available potassium. The only positive correlation between dry peach biomass is with available organic nitrogen ($R^2 = 0.144$, p-value = 0.038) (Figure 5). Available nitrogen is statistically different by crop treatment with alfalfa and tomato having higher available nitrogen than fescue and no cover crop treatments (Figure 11). Overall, alfalfa and tomato treatments have the highest available nitrogen and are not statistically different compared to corn treatments. Fescue and no crop treatments have lower available nitrogen (Figure 5). The only negative correlation between dry peach biomass found to be significant is with ammonium (Figure 12).

Effect of Soil Disinfection, Cover Crop Incorporation, and Peach Growth on the Bulk and Rhizosphere Soil Bacteriome

Shannon index of controls and all treatments separated by soil and cover crop history shows a trend of non-autoclaved soils having a greater alpha diversity than autoclaved soils (Figure 8). For beta diversity, the autoclave soil treatment is the driver of cluster separation. Non-autoclaved and autoclaved soil bacteriomes remain separated for the entire study (cover crop bulk soil, cover crop incorporation bulk soil, peach bulk soil, and peach rhizosphere).

The bacteriome corresponding to bulk soil of peach grown under autoclaved (average distance to median: 0.402) and non-autoclaved (average distance to median: 0.387) conditions show that dispersion is greater in disinfested soils. The perMANOVA test shows that cover crop history (p = 0.001) and autoclaved soil treatment (p = 0.001) is significant and the CAP axes explain a total of 32.5% of the variance. The interaction between cover crop history and autoclaved soil treatment is not significant (p = 0.369) (Figure 6A). This result indicates how the autoclaved bacteriome continues to be prone to change, and the separation along axis 2 supports clustering by cover crop history (Figure 6A). Under both soil treatments, it is observed that previous cover crop histories create bacterial associations. Peaches grown in non-autoclaved soils with a cover history of alfalfa create a unique bacteriome and have the highest shift from the non-autoclaved centroid than other non-disinfested treatments (average distance to median for: alfalfa, 0.356; fescue, 0.343; tomato, 0.340; no cover crop, 0.327; corn, 0.279).

Peach seedlings with the highest biomass correspond to non-autoclaved soil treatments. Therefore, microbes from non-autoclaved peach bulk soils are of the most interest. Within all non-autoclaved treatments (cover crop history and the no-cover-crop history control) for the peach crop cycle, there are seven bacterial species (*Bacillus megaterium*, *Brevitalea aridisoli*, *Brevitalea deliciosa*, *Gaiella occulta*, *Nitrospira japonica*, *Skermanella rosea*, *Skermanella stibiiresistens*) whose abundance increases compared to autoclaved peach bulk soils (Table 4). Soils with cover crop histories have no additional bacterial species in common that do not increase in the no cover crop treatment (indicated by the orange circle; (Figure 6B). Non-autoclaved treatments with the highest unique bacterial species that increase compared to autoclaved soil correspond to no crop and fescue.

In contrast to the peach bulk soil, the rhizosphere soil corresponding to non-autoclaved treatment shows a tighter cluster than the rhizosphere soil of autoclaved soils (Figure 7A). Peaches grown in autoclaved soils (average distance to median: 0.319) loosely cluster based on previous cover crop and show overlap. Within non-autoclaved soils (average distance to median: 0.36), peaches that previously had a cover crop of alfalfa or fescue samples have the greatest shift away from the no crop control. The constrained PCoA shows that cover crop history (p = 0.001) and autoclaved soil treatment (p = 0.001) are significant and explain a total of 50.6% of the variance. The interaction between crop history and autoclaved soil treatment is not significant (p = 0.267) (Figure 7A). Similar to the peach bulk soil, the soil history of alfalfa grown in non-autoclaved soils develops a distinct bacteriome.

From the differential abundance of non-autoclaved peach bulk soil, out of the seven microbes found in all treatments, six of these bacterial species (*Bacillus megaterium*, *Brevitalea aridisoli*, *Brevitalea delicios*, *Gaiella occulta*, *Nitrospira japonica*, *Skermanella rosea*) are found again in all non-autoclaved peach rhizosphere soil treatments, with *Skermanella stibiiresistens* being the exception (Tables 4 and 5). Differential abundance of non-autoclaved peach rhizosphere soil per crop shows that there are 11 shared bacterial species that increase in abundance by soil treatment, regardless of cover crop (Figure 7B). Soils with cover crop histories have two additional bacteria species, *Paenibacillus castaneae* and *Bellilinea caldifistulae,* in common (Figure 7B). Bacterial species that are in higher abundancies in non-autoclaved peach rhizosphere soils with a cover crop treatment and not found in the RS symptomatic non-autoclaved soils without a crop control are *Bacillus cereus* (alfalfa, fescue, and corn), *Paenibacillus xylanilyticus* (fescue, corn, and tomato), *Baekduia soli* (corn and tomato), *Terrimonas suqianensis* (corn and tomato), *Desulfobulbus propionicus* (fescue and

corn), *Paenisporosarcina indica* (alfalfa and corn), *Desulfopila inferna* (alfalfa and fescue), and *Desulfotalea psychrophile* (alfalfa and fescue) (Table 1). Non-autoclaved no-crop treatments, notably, have the highest unique bacterial taxa (n = 53). Of the cover crops, corn (n = 10) and fescue (n = 10) have the highest counts of unique bacterial taxa in higher abundances, with tomato (n = 8) and alfalfa (n = 5) having the least (Figure 7B).

Discussion

Cover Crop Biomass and Bulk Soil Bacteriome

Biomass for all cover crop treatments is significantly higher in autoclaved soils. Pathogen accumulation in soils has been observed in continuous monocultures of a wide range of crops (Wang et al., 2017). Steam autoclave as a soil disinfection method has been shown to increase crop biomass by having decreased bacterial populations, particularly those that could have competed for nutrients and are pathogenic to the plant (DiLegge et al., 2022; Li et al., 2019; Alshaal et al., 2014; Tanaka et al., 2003). Although the mycobiome was not assessed in this study, conceptually, disinfestation would also reduce deleterious or pathogenic fungi. This coincides with the premise that non-native plants can acquire higher yields due to the low amount of specialized soil microbial pathogens and nutrient competitors (Badri & Vivanco, 2009). Tomato's biomass reduction in non-autoclaved soils suggests that they are less tolerant to RS-associated microbes than alfalfa, whose biomass reduction is less severe. Plant fitness is dependent on the strong associations with soil microbiota, and the biomass results show a strong biological component in RS soils.

Microbes associated with peach RS negatively affect cover crop biomass of genetically distant crops. However, cover crops do not appear to establish their own distinct beneficial bacteriome, as all cover crop and no crop control bacteriomes overlap (Figure 2A). Beneficial

bacterial taxa are identified in autoclaved cover crop soils, most of which fall into the categories of antimicrobial, toxic metal bioremediation or uptake, and nitrogen-related activities (Table 2). Microbes in autoclaved cover crop bulk soils with antimicrobial properties are *Janthinobacterium* sp. strain *Marseille* (antifungal), *Pseudomonas koreensis* (antifungal), *Paenibacillus typhae* (antifungal), *Cytobacillus oceanisediminis* (antibiotic), *Fictibacillus phosphorivorans* (nematicidal), *Fictibacillus arsenicus* (nematicidal), and *Verrucomicrobium spinosum* (nematicidal) (Baltaci, 2022; Yaday et al., 2022; Egamberdieva et al., 2020; Zheng et al., 2017; Haack et al., 2016; Rafikova et al., 2016; Sait et al., 2011). Microbes in autoclaved cover crop bulk soils with toxic metal bioremediation or uptake properties are *Thermincola potens*, *Arthrobacter* sp. PGP41, *Brevundimonas diminuta*, and *Ensifer adhaerens* (Rathi & Yogalakshmi, 2021; Singh et al., 2016; Xu et al., 2018; Oyes et al., 2017; Byrne-Bailey, 2010). Microbes in autoclaved cover crop bulk soils with nitrogen-related activities are *Bacillus dakarensis*, *Nostoc punctiforme*, and *Pseudomonas koreensis* [**33**,**42**,**43**]. (Yin et al., 2022; Álvarez et al., 2020; Rafikova et al., 2016). Autoclaving the soil temporarily reduces RS symptoms and maintains beneficial microbes.

Higher Peach Biomass in Non-Autoclaved Soil and Its Bacteriome

Biomass is higher in autoclaved soils for the cover crop portion of the study; however, the peach seedlings show the highest biomass results when grown in soils that have never been autoclaved. Soils of the autoclaved treatment were only autoclaved once, which was immediately before the plantings of the cover crops. Cover crops were incorporated into the same soil in which they had been grown, and peach seedlings were then planted. Non-autoclaved treatments with a cover crop history of alfalfa, corn, and tomato show a higher peach seedling biomass than

the non-autoclaved treatments with no history of a cover crop (Figure 4). Autoclaved treatments with a cover crop history do not outperform autoclaved treatments with no-cover-crop history in terms of peach seedling biomass. The benefit of autoclaving the soil is lost after the cover crop cycle. In all, peach biomass is cover crop treatment-dependent, and the benefit of soil disinfection increasing crop biomass is short term.

Autoclaving soils show slight changes in nutrient accumulation with some changes in plant macronutrients being inconsistent (Monohon et al., 2021; Li et al., 2019; Mahmood et al., 2014). In this study, trends such as a slight increase in nitrate in autoclaved soils and in ammonium in non-autoclaved soils by crop treatment are observed. Available nitrogen is positively correlated with peach biomass. Additionally, available nitrogen is significantly higher in tomato and alfalfa compared to fescue and no cover crop treatments (Figure 10). Field studies show that increasing crop biomass enhances weed suppression, decreases nitrate leaching (improved C/N ratio) and above-ground biomass N. However, a study found an increase in crop biomass negatively impacted inorganic N availability and the following cash crop's (corn) yield was decreased [46]. Similarly, in this study, a decrease in ammonium is correlated with an increase in peach seedling biomass. Overall, nutrients in the soil do not explain the decrease/increase in peach biomass between the autoclaved and non-autoclaved soils.

In the present study, while autoclaving the soil increases cover crop biomass, these same autoclaved soils do not increase peach biomass as the following crop cycle. This could be due to the non-bacterial RS pathogens recuperating to previous microbial compositions pre-disinfection. The benefits of soil disinfection are temporary, even if the impact of the overall bacteriome persists. For instance, methyl bromide treatments produce yield increases for crop cycles following the disinfection of the soil, but strategies are needed to augment the duration of these

benefits (Trout, 2004; Gamliel, 1997). This would indicate that populations of pathogenic and nutrient-competing microbes recover over time to induce replant symptoms anew. In non-autoclaved peach soils with a cover crop history, which show the highest peach biomass, the beneficial bacterial taxa identified have either antimicrobial, iron-reducing, or nitrogen-related capabilities. Bacterial species with antimicrobial capabilities are *Fimbriiglobus ruber* (antifungal), *Peribacillus simplex* (antifungal), *Bacillus altitudinis* (antifungal), *Stigmatella aurantiaca* (antifungal/antibiotic), *Bacillus halotolerans* (antifungal), *Bacillus megaterium* (antibacterial), *Bacillus cereus* (antifungal), *Bacillus pumilus* (antibiotic), *Paenibacillus castaneae* (nematicidal), and *Bacillus pumilus* (nematicidal) (Joshi, 2021; Goswami & Deka, 2019; Slama et al., 2019; Cetintas et al., 2018; Ravin et al., 2018; Schwartz, 2013; Vos et al., 2011; Höfle & Kunze, 2008; Vary et al., 2007; Ding et al., 2005; Leifert, 1995). Bacterial taxa with iron-reducing capabilities are *Desulfuromonas michiganensis*, *Desulfuromonas soudanensis*, *Pelobacter carbinolicus*, *Geobacter* sp. M2, *Aciditerrimonas ferrireducens*, *Geobacter bemidjiensis*, *Pedomicrobium Americanum*, *Geobacter uraniireducens*, *Geobacter psychrophilus*, *Pseudomonas sagittaria*, *Paenibacillus guangzhouensis*, and *Pseudarthrobacter* sp. NIBRBAC000502772 (Park et al., 2020; Badalamenti et al., 2016; Liang et al., 2016; Emtiazi & Hedayatkhah, 2015; Li et al., 2014; Aklujkar et al., 2013; Lin et al., 2013; Lovley et al., 1995). Bacterial taxa with nitrogen-related capabilities (nitrogen fixer, nitrogen reducer) are *Gaiella occulta*, *Nitrospira japonica*, *Clostridium magnum*, *Candidatus Saccharibacteria*, *Geobacter* sp., *Microvirga ossetica*, *Azospira restricta*, *Nitrosospira multiformis*, *Paenibacillus massiliensis*, *Paenibacillus xylanilyticus*, *Microvirga zambiensis*, *Aromatoleum aromaticum*, and *Bacillus megaterium* (Fujitani et al., 2020; Xiujie et al., 2019; Dahal & Kim, 2017; Safronova et al., 2017; Bahulikar

et al., 2014; Albuquerque et al., 2011; Vos et al., 2011; Norton et al., 2008; Ortíz-Castro et al., 2008; Trautwein et al., 2008; Bae et al., 2007; Ding et al., 2005; Rivas et al., 2005; Bomar et al., 1991). For the non-autoclaved cover crop treatment, alfalfa's rhizosphere bacteriome is the treatment that overlaps with the no crop control the least (Figure 7A), and the lack of bacteria species in higher abundancies that are common among multiple cover crops of three or more are few, indicating that there are multiple bacteriomes that can alleviate RS with relatively minimal shifts.

To highlight the loss of key beneficial bacterial species that do not recover their abundancies post-autoclaving, *Bacillus megaterium* is used as an example (Figure 13). This well-documented plant-growth-promoting rhizobacteria has been known to use phytohormones (auxins, gibberellins, and cytokinins), penicillin amidase for biocontrol, and could contain the nifH gene for nitrogen-fixing capabilities (Slama et al., 2019; Vos et al., 2011; Vary et al., 2007; Ding et al., 2005). Although bacterial abundancies are affected long term in autoclaved soils, RS-causing microbes are re-established while beneficial bacteria, as identified in non-autoclaved soils, fail to recover.

Reduced Peach Biomass Treatments Show Beneficial Bacteria Instead of a Myriad of Phytopathogens

RS symptoms are observed in all peach seedlings, but these symptoms are exacerbated in peach seedlings grown in soils with a history of autoclaving. It is expected that peaches that have the lowest biomass have bacteriomes that overlap with that of the original RS soils, however, this is not the case. Autoclaved soils with a history of fescue and corn are the treatments with the lowest biomass, and their bacteriomes do not overlap with the initial non-autoclaved bulk soils (Figure 9). These distinct bacteriomes that show RS symptoms indicate that the microbial

composition of RS soils is not dominated by an identical community of pathogenic and nutrient-competing microbial taxa, but that the abundancies of only a few taxa are required to cause RS symptoms, such as a decrease in biomass. Li et al. (2019) showed that even a slight shift away from the RS bacteriome of peaches reduced RS symptoms, indicating that a large shift away from the replant soil bacteriome was not required to reduce RS symptoms.

Although the control (non-autoclaved soil with no history of cover crops) has the lowest peach biomass out of the non-autoclaved treatments, the control shows the highest count of unique bacterial taxa (n = 53), many of which have previously been associated with beneficial traits. Antimicrobial bacteria are *Bacillus halotolerans* (antifungal) *Bacillus pumilus* (nematicidal), and *Paenibacillus castaneae* (nematicidal) (Slama et al., 2019; Cetintas et al., 2018). Also, *Microvirga zambiensis* and *Aromatoleum aromaticum* have previously been associated with the nitrogen cycle (Xiujie et al., 2019; Dahal & Kim, 2017). Additionally, *Azotobacter chroococcum* and *Bacillus halotolerans* have been known to aid in plant nutrition (Jiménez-Gómez et al., 2020). Multifunction microbes such as *Bacillus pumilus,* which is capable of gibberellins and antibiotics production, and *Peribacillus simplex* (previously *Bacillus simplex*), which has been shown to synthesize auxin and has anti-fungal activity, are also found in non-autoclaved soils with a no-cover-crop history (Sabir, 2013; Schwartz et al., 2013; Gutiérrez-Mañero et al., 2001; Leifert et al., 1995). Although these bacteria have previously been shown to be beneficial, further studies are needed to prove their direct influence on RS.

Conclusions: A Healthy Peach Bacteriome Progression

A robust population of beneficial bacteria are needed to remedy RS soils. Non-autoclaved soil cultivated with alfalfa, corn, and tomato as cover crops developed the best conditions for

peaches to withstand RS in this study. This further supports the idea that certain cover crops may be deployed to reduce RS, specifically for peaches. *Paenibacillus castaneae* and *Bellilinea caldifistulae,* which were cultivated exclusively in the rhizosphere of non-autoclaved soils by peaches for only cover crop histories, may be beneficial and further study could shed light on their role as general colonizers that can possibly reduce RS. Non-autoclaved bulk soils and peach rhizospheres also have an increased abundance of *Bacillus megaterium*, *Gaiella occulta*, and *Nitrospira japonica*. However, these bacterial taxa are also present in the non-autoclaved and no-cover-crop control, which did not outperform the non-autoclaved cover crop treatments (alfalfa, corn, and tomato) in terms of biomass. Nonetheless, further research should be conducted to determine the role of these bacteria in alleviating RS, as these bacteria could be specifically recruited by peaches since abundances are present in the peach rhizosphere in all non-autoclaved treatments. Future studies should use mock community inoculations to investigate the robustness of these bacterial species, since bacteriomes function as a consortia and may require one another to reduce RS.

In contrast, soil disinfection instigates the loss of bacterial species with populations unable to recover within the time frame of this study. This gives an insight into the possible consequences of effective soil disinfection techniques. The Shannon index shows how the newly autoclaved RS bulk soil control has a drastically reduced alpha diversity compared the initial untreated RS bulk soil control. However, the Shannon index supports the fact that many bacterial populations are able to recover by the time the cover crops have grown (Figure 8). This is in line with previous studies that saw benefits of reducing microbial load using soil disinfection techniques and immediately planted peach trees (Li et al., 2019; Tanner et al., 2006). Here, it is proposed that moderate soil disinfection should be used to avoid removing beneficial microbes

by using temperatures that are high enough to be lethal to poor soil competitors such as phytopathogens, but low enough for beneficial bacteria to recolonize. The present study shows that cover crops can help ameliorate RS symptoms, but not all cover crops provide equal benefit, with soil disinfection benefits being temporary.

Table 1. Soil analysis of autoclaved and non-autoclaved soils by treatment. Each treatment was a subset and consisted of n=3 replicates. Mean and standard deviation were calculated using Microsoft Excel.

Treatment	Peach Dry Total Biomass (g)	1:1 Soil pH	Organic Matter %	H2O Total N ppm	Organic N ppm	H3A Nitrate ppm	Ammonium ppm	H3A Inorganic Nitrogen ppm	H3A Total Phosphorus	Inorganic Phosphorus
Alfalfa NonAutoclaved (Mean ± STD)	4.02 ± 1.24	8.13 ± 0.25	1.8 ± 0.1	15.1 ± 2.23	9.77 ± 0.45	4.23 ± 1.63	0.7 ± 0.1	4.93 ± 1.71	4.67 ± 0.58	2.8 ± 0.26
Fescue NonAutoclaved (Mean ± STD)	2.83 ± 0.33	8.07 ± 0.12	1.77 ± 0.06	11.9 ± 2	7.93 ± 1.45	3.2 ± 0.66	0.8 ± 0.1	4 ± 0.56	6 ± 1	3.87 ± 0.75
Corn NonAutoclaved (Mean ± STD)	2.87 ± 0.58	8.1 ± 0.1	1.63 ± 0.06	12.93 ± 1.52	9.13 ± 1.64	3.17 ± 0.64	0.7 ± 0.1	3.83 ± 0.61	8 ± 2.65	5.23 ± 1.6
Tomato NonAutoclaved (Mean ± STD)	4.16 ± 0.28	8.1 ± 0.1	1.63 ± 0.06	13.8 ± 1.68	10.57 ± 1.05	2.8 ± 0.53	0.63 ± 0.06	3.43 ± 0.59	9.67 ± 0.58	6.37 ± 0.59
None NonAutoclaved (Mean ± STD)	2.37 ± 0.99	8.23 ± 0.06	1.5 ± 0	13.03 ± 0.25	8.83 ± 1.03	3.4 ± 0.46	0.37 ± 0.31	3.8 ± 0.53	10.33 ± 1.15	7.2 ± 1.21
Alfalfa Autoclaved (Mean ± STD)	1.71 ± 1.16	8.07 ± 0.06	1.7 ± 0.1	14.87 ± 1.42	7.4 ± 0.82	6.07 ± 1.68	1 ± 0.2	7.03 ± 1.72	6.33 ± 1.15	4.07 ± 0.84
Fescue Autoclaved (Mean ± STD)	0.72 ± 0.12	8.07 ± 0.06	1.6 ± 0.1	9.77 ± 1.59	6.97 ± 1.27	2.2 ± 1.91	1.17 ± 0.4	3.33 ± 1.81	4.33 ± 0.58	2.8 ± 0.44
Corn Autoclaved (Mean ± STD)	1.16 ± 0.59	8.1 ± 0.1	1.63 ± 0.12	10.5 ± 0.82	8.33 ± 0.96	1.63 ± 0.25	0.97 ± 0.47	2.6 ± 0.46	9 ± 4.58	6.43 ± 3.35
Tomato Autoclaved (Mean ± STD)	2.41 ± 0.28	8.13 ± 0.06	1.73 ± 0.12	14.7 ± 2.76	12.07 ± 0.76	2.27 ± 1.59	0.8 ± 0.1	3.07 ± 1.5	9 ± 1.73	6.17 ± 1.46
None Autoclaved (Mean ± STD)	2.75 ± 1.08	8.17 ± 0.06	1.57 ± 0.06	10.8 ± 0.95	8.87 ± 1.83	1.43 ± 0.15	0.87 ± 0.25	2.3 ± 0.1	11.67 ± 0.58	8.47 ± 0.35

Treatment	H3A Organic Phosphorus ppm	H3A ICAP Potassium ppm	Organic N:Inorganic N	Available N ppm	Available P ppm	Available K ppm	Total N Concentration %	Total P Concentration %	Total K Concentration %	CO2-C ppm
Alfalfa NonAutoclaved (Mean ± STD)	1.93 ± 0.38	90 ± 4	2.13 ± 0.67	22.03 ± 2.63	10.9 ± 1.35	4.71	0.09 ± 0.02	0.06 ± 0	0.22 ± 0.01	36.6 ± 11.70
Fescue NonAutoclaved (Mean ± STD)	2 ± 0.2	89 ± 4.58	2 ± 0.36	17.93 ± 2.32	13.43 ± 2.15	5.34	0.08 ± 0.01	0.06 ± 0	0.23 ± 0.01	26.67 ± 2.55
Corn NonAutoclaved (Mean ± STD)	2.77 ± 0.81	91 ± 5.57	2.43 ± 0.67	19.43 ± 2.34	18.2 ± 5.07	7.09	0.09 ± 0.01	0.06 ± 0	0.24 ± 0.01	26.2 ± 6.24
Tomato NonAutoclaved (Mean ± STD)	3 ± 0.3	83.67 ± 4.62	3.17 ± 0.55	21.03 ± 1.93	20.7 ± 1.8	5.29	0.08 ± 0	0.06 ± 0	0.23 ± 0.01	24.63 ± 0.67
None NonAutoclaved (Mean ± STD)	2.87 ± 0.21	89.67 ± 4.93	2.37 ± 0.57	17.6 ± 1.54	21.03 ± 2.47	107.17 ±	0.08 ± 0.02	0.06 ± 0	0.23 ± 0.01	18.17 ± 1.98
Alfalfa Autoclaved (Mean ± STD)	2.03 ± 0.55	74 ± 4.36	1.07 ± 0.25	21.7 ± 3.3	14 ± 3.3	88.9 ± 4.69	0.08 ± 0.01	0.06 ± 0.01	0.22 ± 0.02	26 ± 7.65
Fescue Autoclaved (Mean ± STD)	1.53 ± 0.4	81.67 ± 1.15	2.57 ± 1.43	14.2 ± 2.49	9.47 ± 2.53	97.8 ± 1.91	0.08 ± 0.01	0.06 ± 0	0.22 ± 0.01	21.13 ± 8.96
Corn Autoclaved (Mean ± STD)	2.6 ± 0.96	87.67 ± 9.61	3.3 ± 0.46	15.23 ± 2.85	18.97 ± 8.5	11.94	0.11 ± 0.05	0.05 ± 0.02	0.15 ± 0.1	20.17 ± 3.45
Tomato Autoclaved (Mean ± STD)	2.83 ± 0.35	82 ± 12.49	4.5 ± 1.59	22.7 ± 3.08	20.47 ± 4.05	98.43 ±	0.08 ± 0.01	0.06 ± 0	0.25 ± 0.02	37.8 ± 10.90
None Autoclaved (Mean ± STD)	3.23 ± 0.15	81.67 ± 2.08	3.87 ± 0.97	16.23 ± 2.14	25.43 ± 1.4	97.57 ±	0.07 ± 0	0.06 ± 0	0.23 ± 0.01	25.4 ± 7.73

Table 2. Differential abundance of bacterial taxa with increased abundance in autoclaved cover crop bulk soil as compared to non-autoclaved cover crop bulk soil. P-adjusted values which are <0.001 are represented as 0.000. Log2 Fold change was based on log2FC=Log2(non-autoclaved)-Log2(autoclaved) using the DESeq2 package in RStudio.

Bacterial Taxa	Log2FC	Padjust	Log2 Fold	Padjust	Log2	Padjust	Log2	Padjust	Log2	Padjust	Count
Adhaeribacter aquaticus	-8.315	0.000	-7.528	0.003	-7.560	0.016	-6.844	0.003	-7.791	0.001	5
Ammoniphilus resinae	-4.088	0.021	-4.977	0.003	-4.898	0.005	-3.487	0.006	-3.780	0.008	5
Cytobacillus firmus	-7.664	0.002	-7.795	0.000	-6.919	0.003	-8.147	0.000	-8.027	0.000	5
Cytobacillus oceanisediminis	-5.144	0.000	-5.811	0.000	-4.246	0.000	-4.406	0.000	-5.055	0.000	5
Fictibacillus arsenicus	-4.760	0.033	-5.943	0.000	-4.564	0.010	-3.500	0.002	-5.014	0.000	5
Fictibacillus phosphorivorans	-5.646	0.005	-7.064	0.000	-5.501	0.003	-4.544	0.000	-5.356	0.000	5
Mesobacillus subterraneus	-5.006	0.000	-6.017	0.000	-2.923	0.014	-3.325	0.000	-3.793	0.000	5
Planomicrobium chinense	-7.670	0.007	-9.049	0.000	-22.529	0.000	-6.451	0.012	-8.814	0.000	5
Planomicrobium glaciei	-9.974	0.000	-12.347	0.000	-7.860	0.000	-9.158	0.000	-9.662	0.000	5
Pontibacter brevis	-8.026	0.000	-8.628	0.000	-6.958	0.015	-8.983	0.000	-6.350	0.017	5
Pontibacter chitinilyticus	-7.224	0.005	-8.455	0.001	-7.023	0.014	-8.278	0.000	-6.436	0.016	5
Pontibacter korlensis	-9.310	0.000	-10.047	0.000	-8.007	0.006	-9.299	0.000	-6.923	0.001	5

Pontibacter populi	-12.511	0.000	-11.646	0.000	-10.162	0.000	-11.068	0.000	-8.624	0.000	5
Tumebacillus soli	-4.962	0.023	-6.566	0.000	-6.988	0.000	-4.569	0.000	-5.400	0.000	5
Ammoniphilus oxalaticus	-7.422	0.022	-7.543	0.001			-4.028	0.050	-5.451	0.015	4
Bacillus onubensis	-6.307	0.032	-7.211	0.000			-7.280	0.000	-7.107	0.000	4
Telluribacter humicola	-7.308	0.025	-9.784	0.000			-8.772	0.000	-8.715	0.000	4
Leptolyngbya sp. NIES-3755	-23.926	0.000	-8.488	0.001	-7.594	0.004			-5.708	0.031	4
Asprobacter aquaticus			-6.984	0.022	-6.182	0.016	-6.704	0.003	-5.028	0.011	4
Bacillus dafuensis			-5.182	0.002	-5.320	0.005	-4.363	0.006	-4.753	0.002	4
Bacillus humi			-6.042	0.000	-5.140	0.017	-4.208	0.004	-4.489	0.017	4
Bacillus infantis			-4.786	0.004	-4.110	0.032	-2.915	0.021	-3.826	0.007	4
Chungangia koreensis			-7.501	0.001	-5.877	0.041	-7.746	0.000	-6.260	0.004	4
Exiguobacterium sibiricum			-9.845	0.000	-24.061	0.000	-23.836	0.000	-7.022	0.001	4
Flavihumibacter cheonanensis			-8.992	0.000	-7.505	0.001	-11.570	0.000	-11.534	0.000	4
Flavihumibacter stibioxidans			-7.354	0.003	-6.305	0.007	-9.672	0.000	-9.517	0.000	4
Massilia armeniaca			-5.858	0.033	-6.385	0.043	-6.462	0.011	-6.363	0.007	4
Flavihumibacter petaseus	-6.839	0.036					-7.313	0.001	-7.193	0.000	3
Reyranella massiliensis	-6.467	0.028	-5.668	0.022					-5.351	0.006	3
Planococcus plakortidis	-6.806	0.020	-6.149	0.007					-5.602	0.014	3
Pontibacter rhizosphera	-7.883	0.006	-7.985	0.000			-6.821	0.007			3
Adhaeribacter aerolatus			-8.003	0.000			-6.731	0.001	-7.320	0.002	3
Alkalihalobacillus halodurans			-5.527	0.015	-6.537	0.011			-3.692	0.042	3
Bacillus methanolicus			-4.580	0.008			-2.744	0.044	-3.014	0.041	3
Cytobacillus gottheilii			-4.512	0.010			-3.065	0.013	-3.351	0.029	3
Daejeonella rubra			-7.355	0.003			-5.927	0.019	-7.643	0.000	3
Domibacillus tundrae			-4.498	0.046			-4.627	0.047	-5.228	0.029	3
Exiguobacterium mexicanum			-23.542	0.000			-9.426	0.000	-25.972	0.000	3
Exiguobacterium sp. MH3			-10.485	0.000	-24.982	0.000			-7.650	0.001	3
Exiguobacterium sp. U13-1			-9.765	0.000	-23.867	0.000			-7.249	0.002	3
Exiguobacterium sp. ZWU0009			-8.400	0.000	-7.092	0.024			-5.879	0.026	3

Flavihumibacter profundi			-6.740	0.014			-9.264	0.000	-8.827	0.000	3
Flavisolibacter sp. 17J28-1			-8.994	0.000			-5.476	0.000	-7.911	0.000	3
Noviherbaspirillum aurantiacum			-7.210	0.008	-7.304	0.011	-8.069	0.000			3
Paenibacillus yunnanensis			-6.498	0.003			-6.324	0.012	-6.639	0.005	3
Pseudomonas stutzeri			-5.964	0.003	-5.525	0.045			-9.264	0.000	3
Pseudorhodoferax soli			-6.626	0.016	-6.187	0.030	-5.754	0.040			3
Shinella sp. HZN7			-6.256	0.022			-6.381	0.021	-6.853	0.001	3
Tumebacillus algifaecis			-6.513	0.002	-7.540	0.000	-5.087	0.009			3
Tumebacillus flagellatus			-4.563	0.022	-6.563	0.000	-4.004	0.028			3
Tumebacillus ginsengisoli			-3.864	0.017	-3.962	0.000	-2.767	0.015			3
Georhizobium profundi					-6.919	0.009	-6.644	0.018	-7.101	0.001	3
Tumebacillus lipolyticus					-7.459	0.010	-7.543	0.001	-5.362	0.042	3
Adhaeribacter swui	-6.452	0.030							-5.953	0.025	2
Hyphomonas neptunium	-23.038	0.000							-7.363	0.000	2
Methylovorus glucosotrophus	-6.633	0.026							-5.982	0.025	2
Symbiobacterium thermophilum	-6.972	0.007							-6.631	0.005	2
Lysobacter oligotrophicus	-9.119	0.001			-8.316	0.004					2
Leptolyngbya boryana	-6.116	0.034			-4.935	0.029					2
Acaryochloris marina	-7.613	0.020	-6.653	0.007							2
Achromobacter spanius			-6.925	0.005					-9.262	0.000	2
Actinotalea ferrariae			-6.525	0.033					-5.570	0.037	2
Bacillus mediterraneensis			-5.241	0.005					-4.312	0.014	2
Devosia ginsengisoli			-6.073	0.014					-6.819	0.001	2
Devosia soli			-6.310	0.005					-6.547	0.013	2
Dyadobacter fermentans			-6.979	0.012					-5.779	0.029	2
Dyadobacter jiangsuensis			-7.245	0.001					-6.609	0.005	2
Exiguobacterium acetylicum			-10.469	0.000					-7.661	0.001	2
Flavisolibacter galbus			-7.452	0.001			-5.949	0.033			2

Flavisolibacter ginsengiterrae			-5.694	0.000					-2.720	0.040	2
Larkinella harenae			-5.476	0.046			-6.580	0.001			2
Noviherbaspirillum soli			-7.908	0.000			-3.964	0.030			2
Novosphingobium aromaticivorans			-6.621	0.029			-6.943	0.012			2
Planococcus rifietoensis			-4.827	0.036					-4.136	0.037	2
Pontibacter ruber			-5.513	0.046			-5.915	0.020			2
Shinella zoogloeoides			-6.923	0.005					-6.398	0.003	2
Azospirillum sp. TSH58			-6.822	0.006	-7.515	0.004					2
Oxalophagus oxalicus			-5.434	0.008	-5.078	0.019					2
Mesorhizobium sp. Pch-S					-7.132	0.006	-5.128	0.041			2
Nostoc sphaeroides					-22.872	0.000	-6.550	0.006			2
Pseudomonas sp. UW4					-6.214	0.048			-7.022	0.008	2
Aneurinibacillus migulanus							-6.362	0.022	-5.913	0.026	2
Aneurinibacillus soli							-6.325	0.012	-5.962	0.025	2
Aneurinibacillus tyrosinisolvens							-5.732	0.040	-6.318	0.017	2
Flavihumibacter sediminis							-6.069	0.030	-6.599	0.005	2
Hydrogenophaga atypica							-6.193	0.014	-5.752	0.029	2
Hydrogenophaga sp. PBC							-7.087	0.002	-10.846	0.000	2
Larkinella rosea							-6.818	0.003	-6.085	0.024	2
Methylobacillus flagellatus							-6.451	0.020	-6.817	0.004	2
Quasibacillus thermotolerans							-4.204	0.011	-3.187	0.050	2
Roseomonas aestuarii							-6.522	0.019	-6.137	0.020	2
Sinorhizobium sp. RAC02							-5.762	0.040	-6.318	0.003	2
Agaricicola taiwanensis	-22.968	0.000									1
Fischerella sp. NIES-3754	-23.771	0.000									1
Thermincola potens	-23.587	0.000									1
Alkalihalobacillus pseudofirmus			-7.491	0.000							1
Arenimonas daechungensis			-6.514	0.018							1

Arthrobacter sp. PGP41	-4.610	0.045		1
Azospirillum sp. TSA2s	-9.475	0.000		1
Bacillus dakarensis	-5.444	0.029		1
Bacillus mannanilyticus	-6.007	0.017		1
Exiguobacterium antarcticum	-6.865	0.005		1
Flaviaesturariibacter luteus	-7.523	0.006		1
Flavisolibacter ginsengisoli	-8.113	0.000		1
Flavisolibacter ginsenosidimutans	-3.263	0.036		1
Kocuria rosea	-6.861	0.025		1
Lysobacter brunescens	-6.789	0.019		1
Lysobacter solanacearum	-21.908	0.000		1
Magnetospirillum bellicus	-6.539	0.033		1
Neobacillus notoginsengisoli	-5.407	0.049		1
Niastella hibisci	-6.978	0.011		1
Nocardioides sp. JS614	-6.909	0.022		1
Paenarthrobacter nicotinovorans	-6.533	0.033		1
Paenibacillus chitinolyticus	-4.521	0.046		1
Paenibacillus ihbetae	-6.691	0.006		1
Paenibacillus typhae	-5.215	0.044		1
Pantoea agglomerans	-21.945	0.000		1
Parasegetibacter luojiensis	-12.965	0.000		1
Planctopirus ephydatiae	-6.880	0.040		1
Planococcus kocurii	-5.967	0.049		1
Planococcus sp. MB-3u-03	-6.475	0.009		1
Pontibacter amylolyticus	-9.390	0.000		1
Pontibacter diazotrophicus	-6.586	0.017		1
Pontibacter virosus	-22.928	0.000		1
Pseudarthrobacter chlorophenolicus	-6.266	0.041		1

Pseudohongiella spirulinae	-5.612	0.041							1
Sphingomonas koreensis	-6.409	0.010							1
Verrucomicrobia bacterium IMCC26134	-6.846	0.006							1
Arthrobacter sp. Rue61a			-6.727	0.024					1
Arthrobacter sp. YN			-7.152	0.013					1
Brevundimonas diminuta			-6.190	0.030					1
Paenibacillus sp. FSL H7-0357			-6.457	0.043					1
Pelosinus fermentans			-6.431	0.043					1
Rhodococcus sp. NJ-530			-6.708	0.020					1
Verrucomicrobium spinosum			-7.915	0.003					1
Brevundimonas sp. MF30-B					-6.493	0.010			1
Chthoniobacter flavus					-2.447	0.032			1
Cnuella takakiae					-4.681	0.050			1
Gemmobacter megaterium					-6.589	0.017			1
Haematobacter massiliensis					-5.542	0.020			1
Janthinobacterium sp. Marseille					-5.865	0.036			1
Nostoc punctiforme					-6.139	0.010			1
Oligoflexus tunisiensis					-4.482	0.050			1
Phenylobacterium muchangponense					-5.534	0.049			1
Rhodobacter capsulatus					-5.991	0.021			1
Rhodobacter sphaeroides					-6.161	0.014			1
Rufibacter sp. DG31D					-5.656	0.045			1
Tabrizicola piscis					-4.264	0.031			1
Achromobacter insolitus							-6.961	0.003	1
Achromobacter xylosoxidans							-6.479	0.006	1
Acidovorax defluvii							-7.067	0.007	1
Acidovorax facilis							-7.654	0.003	1
Acidovorax sp. KKS102							-6.453	0.014	1

Acidovorax sp. RAC01	-6.945	0.008	1
Agrobacterium tumefaciens	-6.047	0.037	1
Algoriphagus jejuensis	-7.189	0.003	1
Algoriphagus terrigena	-6.885	0.018	1
Aminobacter aminovorans	-7.084	0.001	1
Bacillus tianmuensis	-5.467	0.024	1
Bacillus timonensis	-5.392	0.027	1
Brevundimonas sp. M20	-5.773	0.016	1
Cellvibrio sp. KY-YJ-3	-7.647	0.004	1
Cellvibrio sp. PSBB023	-6.612	0.013	1
Cohnella candidum	-3.785	0.030	1
Devosia geojensis	-5.405	0.025	1
Devosia riboflavina	-6.162	0.033	1
Devosia sp. A16	-3.361	0.025	1
Devosia sp. I507	-5.425	0.040	1
Ensifer adhaerens	-5.309	0.018	1
Exiguobacterium aurantiacum	-8.480	0.001	1
Exiguobacterium sp. AT1b	-6.012	0.039	1
Fermentibacillus polygoni	-5.326	0.043	1
Herbaspirillum seropedicae	-6.920	0.003	1
Hydrogenophaga sp. RAC07	-23.204	0.000	1
Janthinobacterium sp. 17J80-10	-5.966	0.039	1
Lacunisphaera limnophila	-5.744	0.031	1
Legionella massiliensis	-4.140	0.015	1
Legionella saoudiensis	-5.610	0.033	1
Luteolibacter flavescens	-4.399	0.029	1
Massilia oculi	-6.008	0.025	1
Massilia sp. WG5	-6.326	0.017	1
Methyloversatilis discipulorum	-6.392	0.044	1
Microbacterium sp. ABRD_28	-22.901	0.000	1

Nibricoccus aquaticus	-3.681	0.042	1
Paenibacillus koleovorans	-5.331	0.027	1
Pararcticibacter amylolyticus	-6.651	0.001	1
Phenylobacterium haematophilum	-5.979	0.013	1
Prosthecobacter dejongeii	-6.033	0.011	1
Prosthecobacter fusiformis	-7.013	0.003	1
Pseudomonas alcaliphila	-6.584	0.038	1
Pseudomonas koreensis	-6.104	0.012	1
Pseudomonas putida	-8.428	0.000	1
Pseudoxanthomonas mexicana	-8.210	0.000	1
Shinella yambaruensis	-5.918	0.014	1
Siccirubricoccus deserti	-6.011	0.013	1
Synechococcus elongatus	-22.702	0.000	1

Table 3. Differential abundance of bacterial taxa with increased abundance in non-autoclaved cover crop bulk soil as compared to autoclaved cover crop bulk soil. P-adjusted values which are <0.001 are represented as 0.000. Log2 Fold change was based on log2FC=Log2(non-autoclaved)-Log2(autoclaved) using the DESeq2 package in RStudio.

Bacterial Taxa	Log 2	Padjust	Log 2	Padjust	Log 2	Padjust	Log 2	Padjust	Log 2	Padjust	Count
[*Brevibacterium*] *frigoritolerans*	4.22	0.01	4.11	0.03	3.97	0.02	5.56	0.00	3.99	0.00	5
Aciditerrimonas ferrireducens	6.95	0.00	5.58	0.00	9.35	0.00	5.88	0.00	6.69	0.00	5
Bacillus megaterium	5.04	0.00	4.22	0.00	6.10	0.00	6.33	0.00	4.41	0.00	5
Baekduia soli	7.91	0.00	7.23	0.00	7.76	0.00	8.35	0.00	8.74	0.00	5
Brevitalea aridisoli	8.64	0.00	8.12	0.00	8.02	0.00	8.75	0.00	9.01	0.00	5
Brevitalea deliciosa	10.43	0.00	9.73	0.00	9.85	0.00	10.57	0.00	10.75	0.00	5
Gaiella occulta	9.89	0.00	9.35	0.00	9.43	0.00	10.15	0.00	10.33	0.00	5
Methyloceanibacter caenitepidi	7.96	0.00	7.78	0.00	7.77	0.00	8.02	0.00	8.34	0.00	5
Nitrospira japonica	9.12	0.00	8.32	0.00	8.58	0.00	9.20	0.00	9.82	0.00	5
Nostoc sp. HK-01	10.57	0.00	15.00	0.00	13.10	0.00	7.93	0.00	22.17	0.00	5
Nostoc sp. PCC 7107	24.47	0.00	14.16	0.00	12.07	0.00	6.93	0.01	22.68	0.00	5
Paenibacillus castaneae	8.19	0.00	5.31	0.02	7.58	0.00	8.26	0.00	5.76	0.00	5
Planctomycetes bacterium Pla175	8.05	0.00	4.64	0.02	7.87	0.00	8.56	0.00	8.60	0.00	5
Povalibacter uvarum	8.90	0.00	6.29	0.00	4.49	0.01	8.93	0.00	4.71	0.00	5
Rhodoplanes sp. Z2-YC6860	8.28	0.00	3.61	0.03	3.97	0.02	4.30	0.00	4.30	0.00	5
Skermanella mucosa	7.36	0.00	6.42	0.02	6.61	0.01	7.25	0.00	7.62	0.00	5
Skermanella rosea	6.63	0.00	8.86	0.00	9.30	0.00	9.38	0.00	9.37	0.00	5
Skermanella stibiiresistens	10.48	0.00	10.01	0.00	10.29	0.00	10.43	0.00	10.55	0.00	5
Solirubrobacter ginsenosidimutans	7.22	0.00	6.19	0.02	6.63	0.00	7.16	0.00	7.44	0.00	5
Solirubrobacter soli	8.10	0.00	5.25	0.02	7.53	0.00	8.49	0.00	9.00	0.00	5
Sphingomonas daechungensis	7.65	0.00	6.51	0.02	7.26	0.00	7.55	0.00	8.22	0.00	5

Sphingomonas sediminicola	6.40	0.02	6.06	0.04	6.53	0.01	6.71	0.00	7.23	0.00	5
Sphingomonas sp. AE3	7.06	0.00	6.17	0.02	7.12	0.00	6.34	0.00	6.51	0.01	5
Steroidobacter denitrificans	5.33	0.00	4.92	0.00	4.11	0.01	5.94	0.00	3.96	0.00	5
Vicinamibacter silvestris	4.26	0.00	3.42	0.03	3.64	0.00	4.12	0.00	4.03	0.00	5
Woeseia oceani	7.08	0.00	6.72	0.01	6.91	0.00	7.62	0.00	7.74	0.00	5

Table 4. Differential abundance of bacterial taxa with increased abundance in non-autoclaved peach bulk soil as compared to autoclaved peach bulk soil. P-adjusted values which are <0.001 are represented as 0.000. Log2 Fold change was based on log2FC=Log2(non-autoclaved)-Log2(autoclaved) using the DESeq2 package in RStudio.

Bacterial Taxa	Log2FC	Padjust	Log2 Fold	Padjust	Log2	Padjust	Log2	Padjust	Log2	Padjust	Count
Bacillus megaterium	*5.377*	0.037	4.575	0.041	5.405	0.011	6.885	0.009	4.374	0.009	5
Brevitalea aridisoli	*7.797*	0.015	8.441	0.001	8.816	0.000	8.672	0.001	8.161	0.000	5
Brevitalea deliciosa	*9.905*	0.000	10.134	0.000	7.045	0.007	10.251	0.000	9.821	0.000	5
Gaiella occulta	*8.641*	0.001	9.749	0.000	10.088	0.000	9.882	0.000	6.480	0.005	5
Nitrospira japonica	*8.427*	0.007	8.479	0.001	9.250	0.000	9.047	0.000	8.857	0.000	5
Skermanella rosea	*9.464*	0.000	8.958	0.000	5.603	0.038	9.068	0.000	9.120	0.000	5
Skermanella stibiiresistens	*10.49*	0.000	10.010	0.000	10.585	0.000	10.348	0.000	10.235	0.000	5
Sphingomonas daechungensis	*7.679*	0.018	7.445	0.015	7.839	0.010			7.812	0.005	4
Pelobacter acetylenicus	*7.459*	0.018	8.291	0.007	7.821	0.010			7.256	0.021	4
Methyloceanibacter caenitepidi			8.223	0.007	8.453	0.001	8.131	0.002	8.261	0.000	4
Paenibacillus castaneae			7.562	0.012	8.276	0.007	8.116	0.008	8.010	0.004	4
Woeseia oceani			7.565	0.012	7.961	0.009	7.652	0.003	6.834	0.029	4
[*Brevibacterium*] *frigoritolerans*	*8.020*	0.003					5.903	0.023	4.694	0.027	3
Peribacillus simplex	*7.506*	0.018	8.187	0.007			8.242	0.008			3
Longilinea arvoryzae	*5.946*	0.031	5.366	0.047					7.466	0.020	3

[*Desulfobacterium*] *catecholicum*	*8.843*	0.001	9.651	0.000	9.146	0.003					3
Gemmata sp. SH-PL17			8.450	0.001			8.296	0.001	8.002	0.004	3
Geoalkalibacter subterraneus			7.292	0.016			6.912	0.026	6.426	0.039	3
Arenimicrobium luteum					7.477	0.014	7.120	0.022	6.451	0.039	3
Skermanella aerolata	*8.538*	0.001					7.623	0.004			2
Solirubrobacter soli	*7.638*	0.018					8.358	0.001			2
Skermanella mucosa	*7.637*	0.006					7.077	0.023			2
Stigmatella aurantiaca	*6.994*	0.035			7.263	0.018					2
Thermomarinilinea lacunifontana			7.382	0.038					6.474	0.039	2
Desulforhopalus singaporensis			7.276	0.018	6.867	0.030					2
Bellilinea caldifistulae			6.986	0.049	7.484	0.014					2
Acidimicrobium ferrooxidans					7.431	0.015			7.036	0.011	2
Aridibacter nitratireducens					9.460	0.007			7.764	0.005	2
Burkholderiales bacterium GJ-E10					7.065	0.021			6.443	0.039	2
Planctomycetes bacterium Pla175					5.722	0.029			4.844	0.039	2
Gemmata obscuriglobus					7.254	0.018	6.675	0.034			2
Actinobacteria bacterium IMCC26256							7.019	0.023	6.368	0.040	2
Anaeromyxobacter sp. K							7.535	0.014	6.672	0.035	2
Bacillus safensis							7.281	0.019	6.223	0.045	2
Bacillus thuringiensis	*7.914*	0.014									1
Clostridium magnum	*9.325*	0.004									1
Haliscomenobacter hydrossis	*8.035*	0.035									1

Clostridium saccharoperbutylacetonicum	5.523	0.049							1
Desulfuromonas acetoxidans	6.659	0.038							1
Desulfuromonas michiganensis	8.286	0.007							1
Paenibacillus sp. JDR-2	7.741	0.010							1
Pelobacter carbinolicus	7.705	0.011							1
Pelobacter sp. SFB93	7.819	0.010							1
Planctomycetes bacterium Pan216	6.494	0.042							1
Planctomycetes bacterium Pan265	6.608	0.038							1
Pseudomonas sagittaria	7.299	0.017							1
Ruminiclostridium hungatei	7.375	0.045							1
Aridibacter famidurans			7.444	0.038					1
Bacillus cereus			7.079	0.045					1
Candidatus Saccharibacteria bacterium YM_S32_TM7_50_20			7.338	0.038					1
Desulfuromonas soudanensis			7.440	0.036					1
Desulfuromonas sp. DDH964			8.219	0.007					1
Kofleria flava			6.552	0.043					1
Stanieria cyanosphaera			7.400	0.019					1
Dongia mobilis					7.133	0.022			1
Geobacter sp. M21					7.110	0.023			1
Longimicrobium terrae					6.698	0.033			1
Microvirga ossetica					8.043	0.002			1
Aciditerrimonas ferrireducens							4.680	0.032	1
Arthrobacter sp. QXT-31							8.136	0.003	1

Fimbriiglobus ruber									7.274	0.010	1
Geobacter bemidjiensis									6.654	0.035	1
Geobacter uraniireducens									6.968	0.027	1
Lysinibacillus sp. SGAir0095									6.367	0.040	1
Paenibacillus xylanilyticus									6.356	0.040	1
Pedomicrobium americanum									6.935	0.028	1
Rubrobacter radiotolerans									6.587	0.038	1
Solirubrobacter ginsenosidimutans									7.058	0.011	1

Table 5. Differential abundance of bacterial taxa with increased abundance in non-autoclaved peach rhizosphere soil as compared to autoclaved peach rhizosphere soil. P-adjusted values which are <0.001 are represented as 0.000. Log2 Fold change was based on log2FC=Log2(non-autoclaved)-Log2(autoclaved) using the DESeq2 package in RStudio.

Bacterial Taxa	Log2	Padjust	Log2	Padjust	Log2	Padjust	Log2	Padjust	Log2	Padjust	Count
Arenimicrobium luteum	7.566	0.007	7.800	0.006	7.606	0.001	8.036	0.001	7.588	0.000	5
Bacillus megaterium	5.579	0.003	6.169	0.006	3.762	0.036	5.663	0.001	4.340	0.001	5
Brevitalea aridisoli	9.138	0.000	8.884	0.001	8.627	0.000	9.228	0.000	8.715	0.000	5
Brevitalea deliciosa	10.917	0.000	11.033	0.000	10.587	0.000	10.873	0.000	10.454	0.000	5
Gaiella occulta	9.538	0.000	10.204	0.000	9.710	0.000	10.022	0.000	10.060	0.000	5
Longilinea arvoryzae	5.411	0.029	9.298	0.000	5.299	0.019	8.844	0.000	8.428	0.000	5
Methyloceanibacter caenitepidi	8.097	0.003	8.686	0.000	8.287	0.000	7.710	0.006	8.659	0.000	5
Nitrospira japonica	9.207	0.000	9.193	0.000	5.482	0.035	9.283	0.000	6.201	0.000	5
Peribacillus simplex	5.740	0.015	6.093	0.010	8.624	0.000	4.813	0.011	3.964	0.016	5
Skermanella rosea	8.776	0.000	9.159	0.000	8.647	0.000	9.340	0.000	8.846	0.000	5
Solirubrobacter soli	7.646	0.006	5.113	0.040	8.231	0.000	8.398	0.000	8.355	0.000	5
Desulfuromonas michiganensis	7.551	0.007			7.121	0.009	7.820	0.002	8.397	0.001	4

Gemmata sp. SH-PL17	6.721	0.046			7.548	0.006	8.678	0.000	8.239	0.000	4
Candidatus Saccharibacteria bacterium oral taxon 955	7.170	0.036	7.156	0.012			6.209	0.037	5.370	0.047	4
Skermanella stibiiresistens	9.914	0.000	10.526	0.000			10.408	0.000	10.231	0.000	4
[Desulfobacterium] catecholicum	9.639	0.000	4.690	0.045	4.159	0.047			8.265	0.001	4
Paenibacillus castaneae	8.491	0.000	8.964	0.000	8.299	0.000	8.970	0.000			4
Bellilinea caldifistulae	7.332	0.009	8.021	0.001	7.019	0.009	7.558	0.000			4
Acidimicrobium ferrooxidans			6.673	0.023	6.117	0.045	6.662	0.021	5.982	0.021	4
Aciditerrimonas ferrireducens			6.228	0.009	6.057	0.008	5.801	0.007	9.624	0.000	4
Actinobacteria bacterium IMCC26256			6.658	0.023	6.126	0.045	7.333	0.004	6.696	0.002	4
Geoalkalibacter subterraneus			8.253	0.000	7.795	0.001	7.606	0.002	8.095	0.000	4
Skermanella aerolata			7.352	0.010	6.816	0.012	7.207	0.004	6.931	0.001	4
Solirubrobacter ginsenosidimutans			7.139	0.012	6.712	0.013	7.208	0.004	7.145	0.000	4
Woeseia oceani			7.274	0.011	6.504	0.035	7.285	0.003	7.593	0.000	4
Pelobacter sp. SFB93	7.415	0.008			7.179	0.009			7.957	0.000	3
Pelobacter carbinolicus	7.110	0.012			6.958	0.010			7.849	0.000	3
Pedomicrobium americanum	6.666	0.022			7.666	0.001			7.536	0.000	3
Thermomarinilinea lacunifontana	7.431	0.027	8.235	0.000					6.619	0.002	3
Candidatus Accumulibacter phosphatis	7.233	0.027	7.786	0.007					7.515	0.001	3
Ornatilinea apprima	5.255	0.039	5.446	0.029					4.104	0.030	3
Bacillus cereus	7.044	0.013	7.490	0.009	7.451	0.007					3
Planifilum fulgidum			6.699	0.023	6.666	0.016			5.701	0.030	3
Skermanella mucosa			7.050	0.013	6.802	0.013			6.960	0.001	3
Bacillus safensis			7.383	0.010	7.453	0.007			6.994	0.001	3
Candidatus Koribacter versatilis			6.752	0.046	6.145	0.044			6.821	0.002	3
Paenibacillus xylanilyticus			7.231	0.011	7.217	0.008	6.860	0.016			3

Candidatus Saccharibacteria bacterium YM_S32_TM7_50_20	7.689	0.007			6.566	0.045	7.042	0.001	3
Desulfomonile tiedjei	6.693	0.048	6.566	0.016			6.928	0.001	3
Paenibacillus prosopidis	7.316	0.010	6.526	0.016			6.890	0.002	3
Paenisporosarcina antarctica	7.176	0.012	7.397	0.001			6.809	0.002	3
[*Brevibacterium*] *frigoritolerans*			4.169	0.020	6.356	0.002	3.712	0.008	3
Candidatus Promineofilum breve			6.318	0.038	7.496	0.002	5.967	0.023	3
Dechlorobacter hydrogenophilus			7.181	0.020	6.961	0.031	6.242	0.006	3
Gemmata massiliana			6.589	0.016	6.560	0.024	5.601	0.035	3
Holophaga foetida			7.465	0.007	6.972	0.016	5.810	0.027	3
Planctomycetes bacterium Pla175			4.420	0.031	4.749	0.029	4.001	0.030	3
Baekduia soli			8.134	0.000	8.545	0.000			2
Terrimonas suqianensis			6.439	0.036	6.733	0.019			2
Pelobacter acetylenicus			8.083	0.000			8.664	0.000	2
Alkalitalea saponilacus			8.066	0.010			6.913	0.016	2
Desulfobulbus propionicus	8.276	0.001	7.582	0.015					2
Arthrobacter sp. KBS0702			7.173	0.009			7.024	0.001	2
Paenibacillus sp. DCT19			7.166	0.009			6.744	0.002	2
Deferrisoma camini			6.829	0.004			5.835	0.048	2
Lutispora thermophila			6.723	0.031			6.513	0.025	2
Rhodoplanes tepidicaeni			6.632	0.016			5.860	0.025	2
Rubrivivax gelatinosus			6.594	0.035			6.117	0.018	2
Pseudolabrys sp. FHR47			6.572	0.035			6.982	0.001	2
Rubrobacter radiotolerans			6.543	0.017			6.662	0.002	2
Bacillus velezensis			6.475	0.036			6.113	0.018	2
Terrimonas pekingensis			6.403	0.037			5.986	0.008	2
Sphingomonas daechungensis			6.329	0.039			6.610	0.003	2
Desulfuromonas acetoxidans			6.312	0.038			6.068	0.019	2

Pseudolabrys taiwanensis					6.282	0.040			6.535	0.009	2
Labilithrix luteola					6.250	0.040			6.408	0.004	2
Terribacillus goriensis					6.124	0.045			5.480	0.040	2
Microvirga sp. 17 mud 1-3					6.110	0.045			5.333	0.049	2
Aquihabitans daechungensis					6.006	0.050			6.566	0.003	2
Desulfuromonas soudanensis					4.998	0.029			9.113	0.000	2
Paenisporosarcina indica	6.897	0.039			7.424	0.007					2
Desulfopila inferna	7.846	0.005	7.310	0.004							2
Desulfotalea psychrophila	7.192	0.029	7.028	0.006							2
Adhaeribacter terreus			6.746	0.048					5.382	0.046	2
Paenisporosarcina macmurdoensis			6.269	0.038					5.632	0.033	2
Vicinamibacter silvestris			3.808	0.019					3.153	0.002	2
Anaeromyxobacter sp. K			8.246	0.000					8.067	0.000	2
Bacillus funiculus							3.191	0.041	2.252	0.035	2
Bacillus pseudomycoides							6.722	0.020	5.968	0.023	2
Conexibacter woesei							7.168	0.004	7.342	0.000	2
Fimbriiglobus ruber							6.701	0.021	6.088	0.007	2
Microlunatus phosphovorus							7.100	0.015	6.444	0.003	2
Paenibacillus xylanexedens							6.706	0.019	6.679	0.002	2
Planctomycetes bacterium ETA_A8							3.836	0.025	3.287	0.049	2
Tepidiforma bonchosmolovskayae							7.501	0.003	3.908	0.032	2
Geobacter psychrophilus	8.344	0.000									1
Haliscomenobacter hydrossis	7.172	0.012									1
Methylobacter luteus	8.624	0.011									1
Methylobacter marinus	7.470	0.031									1
Pseudomonas sagittaria	7.244	0.011									1
Azospira restricta			7.171	0.012							1

Desulfocapsa sulfexigens	6.206	0.045							1
Desulfovirga adipica	6.538	0.035							1
Gallionella capsiferriformans	9.127	0.002							1
Hydrogenophaga taeniospiralis	7.191	0.038							1
Legionella clemsonensis	7.094	0.013							1
Lysinibacillus sp. SGAir0095	6.908	0.040							1
Nitrosospira multiformis	7.991	0.005							1
Oxobacter pfennigii	7.505	0.024							1
Syntrophus aciditrophicus	7.656	0.009							1
Alkalihalobacillus decolorationis			6.090	0.048					1
Azoarcus sp. CIB			6.510	0.037					1
Bacillus altitudinis			6.428	0.037					1
Bacillus thuringiensis			8.054	0.000					1
Maribellus luteus			7.283	0.036					1
Microvirga ossetica			7.375	0.007					1
Microvirga subterranea			6.340	0.038					1
Paenibacillus massiliensis			6.614	0.016					1
Paraflavitalea soli			6.155	0.044					1
Stigmatella aurantiaca			7.122	0.009					1
Gemmata obscuriglobus					6.502	0.026			1
Levilinea saccharolytica					6.940	0.006			1
Paenibacillus guangzhouensis					6.585	0.026			1
Paenibacillus sp. JDR-2					3.746	0.030			1
Paraclostridium bifermentans					7.620	0.009			1
Planctomycetes bacterium ETA_A1					6.286	0.033			1
Pseudarthrobacter sp. NIBRBAC000502772					7.261	0.013			1
Pseudarthrobacter sulfonivorans					7.665	0.008			1

[*Polyangium*] *brachysporum*	6.717	0.002	1
Acidothermus cellulolyticus	6.774	0.002	1
Anaeromyxobacter sp. Fw109-5	7.535	0.003	1
Archangium gephyra	5.978	0.023	1
Aromatoleum aromaticum	6.356	0.013	1
Arthrobacter sp. QXT-31	7.086	0.001	1
Azotobacter chroococcum	7.308	0.001	1
Bacillus halotolerans	5.565	0.019	1
Bacillus pumilus	6.187	0.016	1
Blastococcus saxobsidens	5.560	0.037	1
Burkholderiales bacterium GJ-E10	6.259	0.005	1
Chondromyces crocatus	5.769	0.029	1
Dechloromonas hortensis	3.734	0.046	1
Dechloromonas sp. HYN0024	6.306	0.004	1
Dechlorosoma suillum	5.906	0.024	1
Desulforhopalus singaporensis	6.352	0.015	1
Desulfuromonas sp. DDH964	9.162	0.000	1
Domibacillus indicus	6.440	0.003	1
Geobacter daltonii	7.333	0.001	1
Geobacter metallireducens	6.928	0.002	1
Geobacter pickeringii	8.089	0.000	1
Geobacter sp. FeAm09	6.313	0.006	1
Geobacter sp. M21	6.877	0.002	1
Geobacter uraniireducens	8.426	0.000	1
Haliangium ochraceum	6.006	0.008	1
Hydrogenispora ethanolica	5.357	0.004	1
Hyphomicrobium sulfonivorans	7.134	0.000	1

Ilumatobacter fluminis	7.194	0.000	1
Limnoglobus roseus	5.868	0.011	1
Luteitalea pratensis	2.089	0.044	1
Microlunatus ginsengisoli	5.727	0.030	1
Microvirga brassicacearum	5.338	0.049	1
Microvirga zambiensis	5.334	0.049	1
Minicystis rosea	5.780	0.028	1
Nitrosospira briensis	5.724	0.030	1
Nordella oligomobilis	3.343	0.020	1
Paenibacillus aceris	5.784	0.028	1
Paenibacillus sp. 37	6.578	0.003	1
Pelobacter propionicus	7.329	0.001	1
Planctomycetales bacterium	4.047	0.029	1
Planctomycetes bacterium Pla85_3_4	4.060	0.026	1
Polyangium fumosum	6.007	0.021	1
Rhodoplanes azumiensis	6.466	0.003	1
Rubrobacter xylanophilus	5.545	0.037	1
Shimazuella kribbensis	6.453	0.003	1
Sinorhizobium meliloti	5.752	0.030	1
Sorangium cellulosum	6.727	0.002	1
Sphingomonas lutea	5.731	0.030	1
Stenotrophobacter roseus	5.892	0.024	1
Stenotrophobacter terrae	7.380	0.001	1
Thermanaerothrix daxensis	6.528	0.003	1
Thermoanaerobaculum aquaticum	5.599	0.035	1
Zhizhongheella caldifontis	5.463	0.042	1

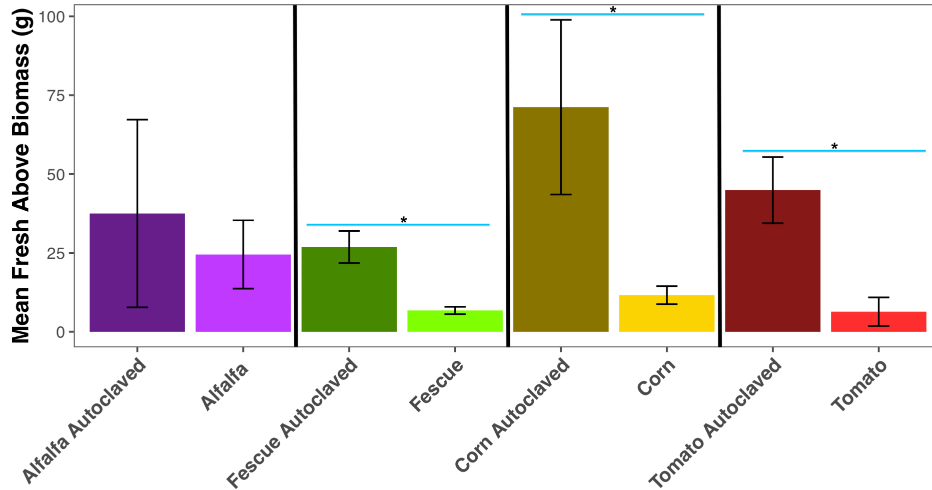

Figure 1. Above-ground fresh biomass of cover crops. Pairwise comparisons using Wilcoxon rank sum exact test to infer significance for each cover crop by autoclaved and non-autoclaved soil treatment. Error bars use the equation ymin/ymax = mean ± standard deviation. Significance ($p < 0.01$) for pairwise comparisons were denoted with *.

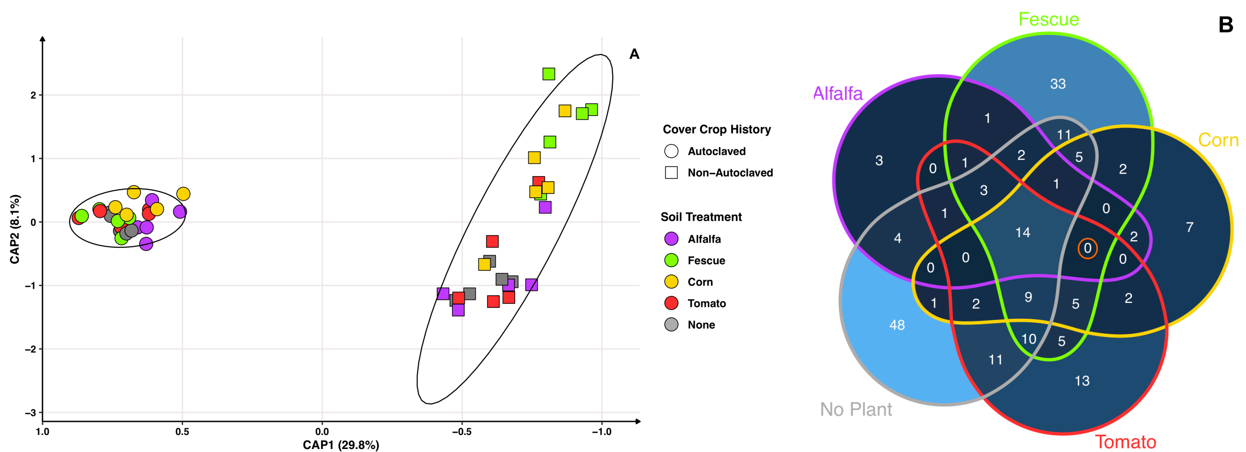

Figure 2. Cover crop bulk soil bacteriomes. (**A**) Constrained principal coordinate analysis (PCoA) using Bray–Curtis distance for cover crop bulk soil bacteriomes. Circle shape represents autoclaved and squares non-autoclaved. Colors indicate cover crop treatment: alfalfa (purple), fescue (green), corn (yellow), tomato (red), and no crop (gray). (**B**) Differential abundance Venn diagram shows a count of bacteria species in higher abundancies in autoclaved compared to non-autoclaved cover crop bulk soils separated by crop. Orange circle indicates common bacteria found to be promoted within all cover crop treatments. Color gradient displays low bacterial taxa species counts in dark blue and higher counts in light blue.

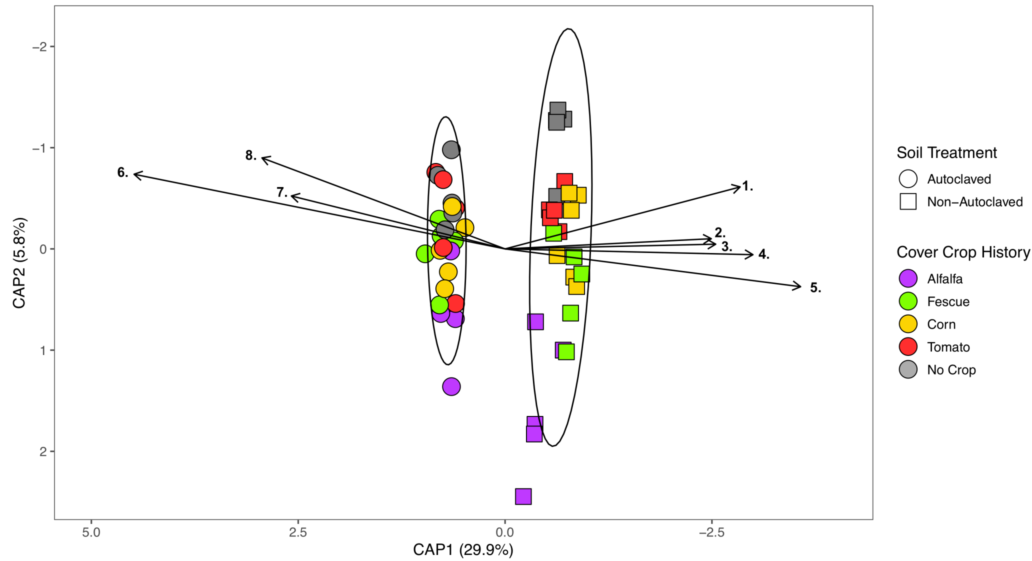

Figure 3. Biplot of a constrained principal coordinate analysis (PCoA) using Bray–Curtis distance for cover-crop-incorporated bulk soil bacteriome. Circles represent autoclaved and squares non-autoclaved. Colors indicate cover crop treatment: alfalfa (purple), fescue (green), corn (yellow), tomato (red), and no crop (gray). Bacterial taxa: 1. *Vicinamibacter silvestris*, 2. *Skermanella stibiiresistens,* 3. *Bacillus megaterium*, 4. *Nostoc* sp. PCC7107, 5. *Nostoc* sp. Hk-01, 6. *Cytobacillus oceanisediminis*, 7. *Mesobacillus subterraneus*, 8. *Tumebacillus soli*.

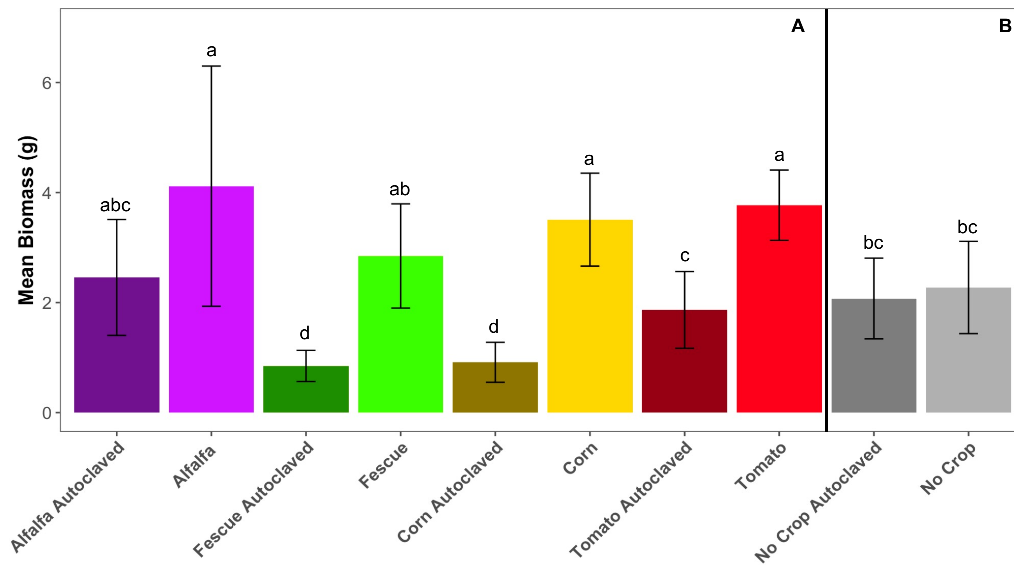

Figure 4. Peach biomass. (**A**) Total dry peach biomass for peach trees with a cover crop history. Wilcoxon rank sum exact test was used to infer significance for each cover crop and autoclaved soil treatment. Dry peach biomass was recorded. Dry biomass was used since it accounted for the fluctuating water concentrations within plant tissues. Error bars use the equation ymin/ymax = mean ± standard deviation. (**B**) Total dry peach biomass for peach trees in autoclaved and non-autoclaved no-cover-crop controls. Both autoclaved and non-autoclaved soils were initially set

up at the start of the cover crop experiment, meaning these soils experienced fallowness for 14 weeks before having peach seedlings planted. Error bars use the equation ymin/ymax = mean ± standard deviation. Different superscript letters denote significant difference ($p < 0.01$) compared with different cover crop histories and soil disinfection treatments by Wilcoxon rank sum exact test.

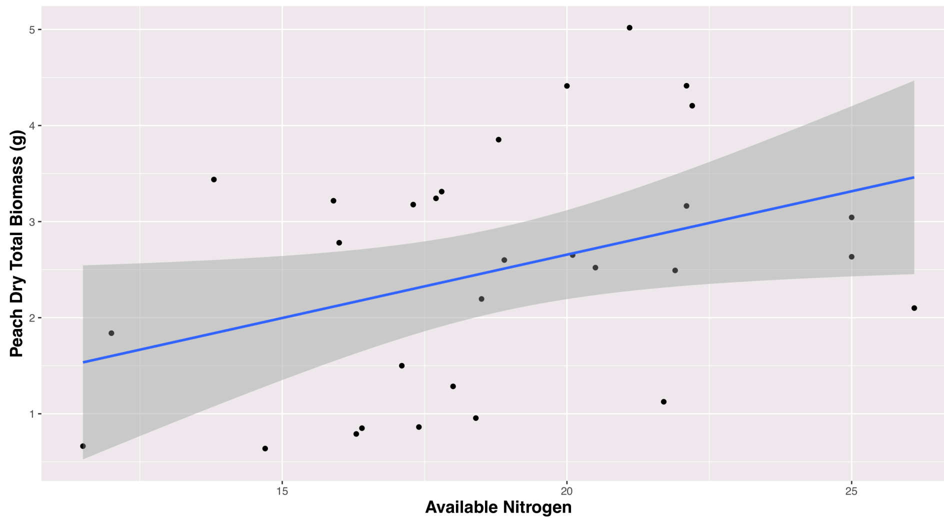

Figure 5. Available organic nitrogen in correlation with total dry peach biomass.

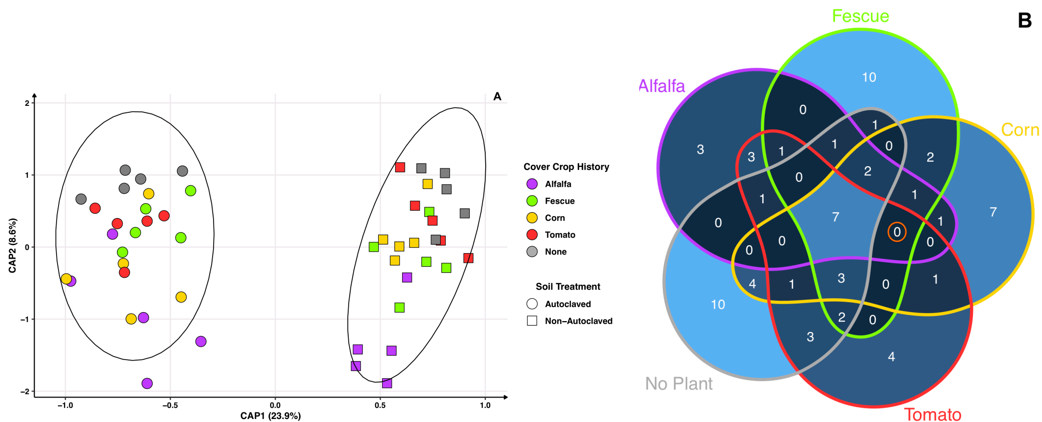

Figure 6. Peach bulk soil bacteriomes. (**A**) Constrained principal coordinate analysis (PCoA) using Bray–Curtis distance for peach bulk soil bacteriomes. Circles represent autoclaved and squares non-autoclaved. Colors indicate cover crop treatment: alfalfa (purple), fescue (green), corn (yellow), tomato (red), and no crop (gray). (**B**) Differential abundance Venn diagram shows a count of bacteria species in higher abundancies in non-autoclaved and autoclaved peach bulk soils separated by crop. Orange circle indicates common bacteria found to be promoted within all cover crop treatments. Color gradient displays low bacterial species counts in dark blue and higher counts in light blue.

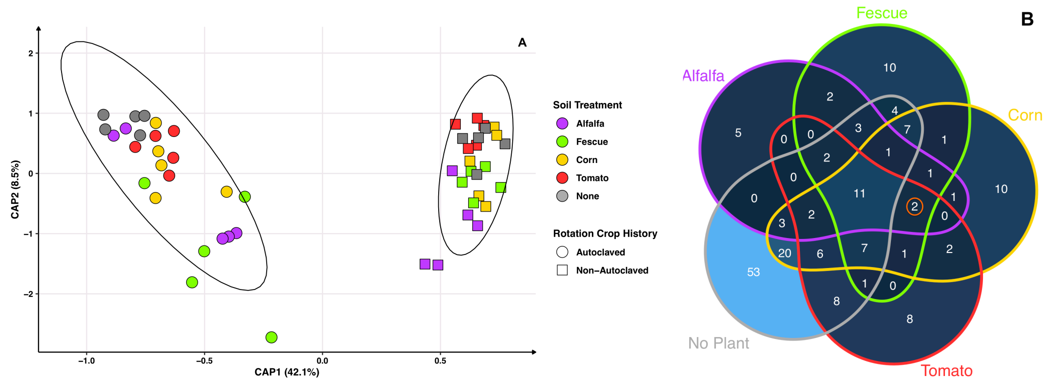

Figure 7. Peach rhizosphere bacteriomes. (**A**) Constrained principal coordinate analysis (PCoA) using Bray–Curtis distance for peach rhizosphere bacteriomes. Circles represent autoclaved and squares non-autoclaved. Colors indicate cover crop treatment: alfalfa (purple), fescue (green), corn (yellow), tomato (red), and no crop (gray). (**B**) Differential abundance Venn diagram shows a count of bacteria species in higher abundancies in non-autoclaved and autoclaved peach rhizosphere soils separated by crop. Orange circle indicates common bacteria found to be promoted within all cover crop treatments. Color gradient displays low bacterial taxa species counts in dark blue and higher counts in light blue.

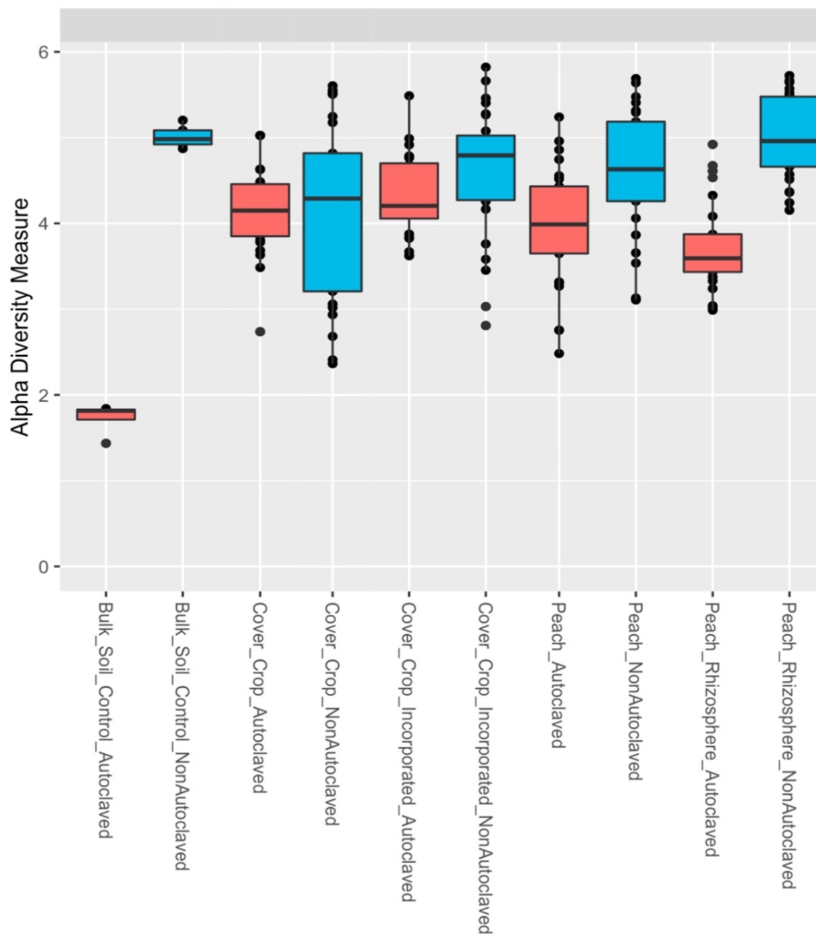

Figure 8. Shannon index of controls and all treatments separated by autoclaved (red), non-autoclaved (blue), and cover crop history.

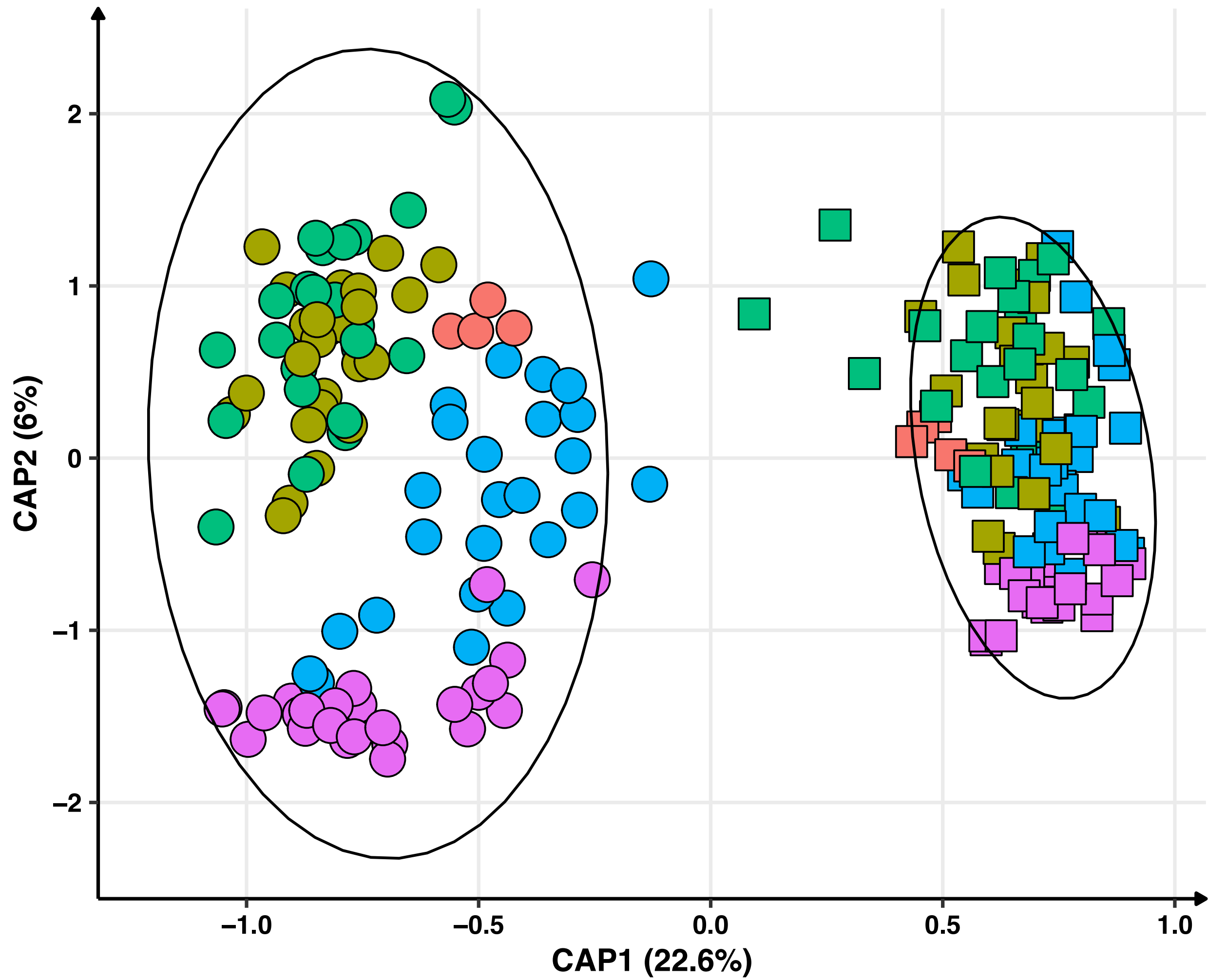

Figure 9. Principal Coordinate Analysis (PCoA) of bacterial DNA using Bray-Curtis distance for bacterial microbiomes of all samples sorted by crop cycle. Shape denotes soil treatment with circles representing autoclaved and squares non-autoclaved. Color indicated crop cycle with initial bulk soil control (before any crops) in salmon, cover crop bulk soil in olive green, incorporated cover crop bulk soil in leaf green, peach bulk soil in blue, and peach rhizosphere in purple.

Figure 10. Differential abundance Venn diagram of non-autoclaved cover crop bulk soils as compared to autoclaved cover crop bulk soils separated by crop. Orange circle indicates common bacteria found to be promoted within the 4 cover crops (alfalfa, fescue, corn, and tomato) treatments. A blue color gradient displays low bacterial taxa species counts in dark blue, with higher bacterial taxa species counts in lighter blue.

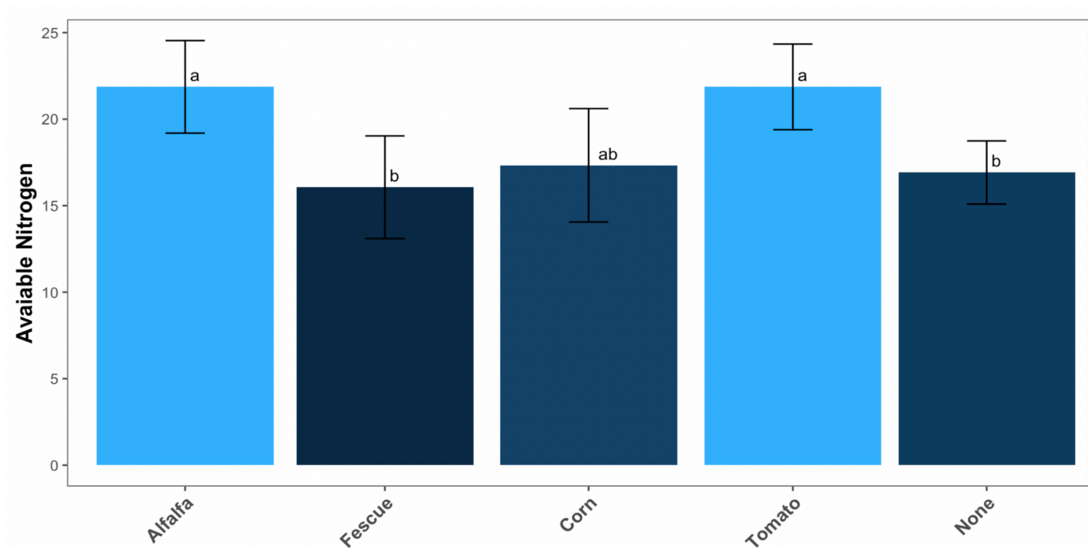

Figure 11. Available organic nitrogen in correlation with total dry peach biomass. Of the samples with the highest available nitrogen, the top eight soil samples previously had either alfalfa or tomato from both soil treatment types (autoclaved / non-autoclaved).

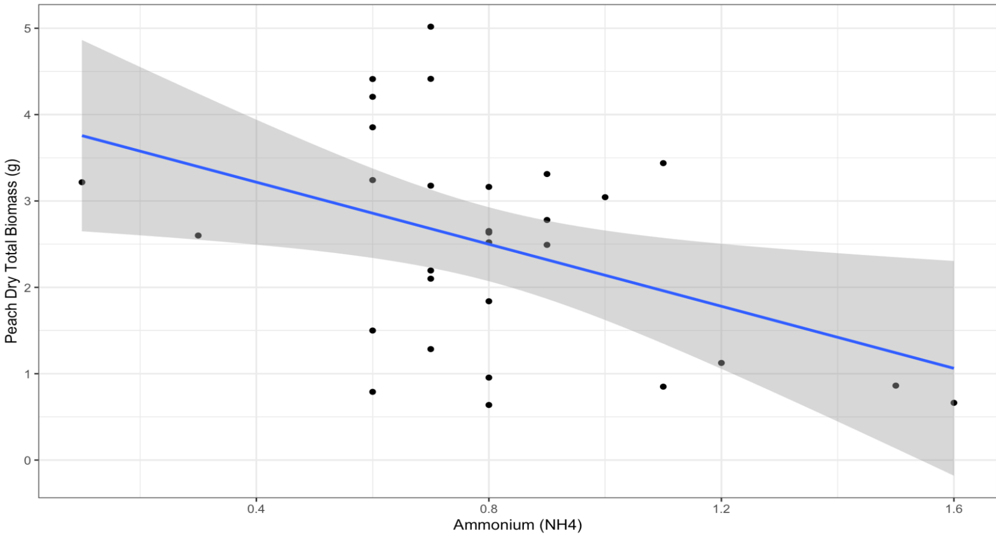

Figure 12. Ammonium (NH4) in correlation with total dry peach biomass. soil extractant (H3A-1) Correlation was positive (R^2= 0.1852, p-Value= 0.01761). Of the samples with the highest ammonium, previously disinfected soils showed consistently higher ammonium values.

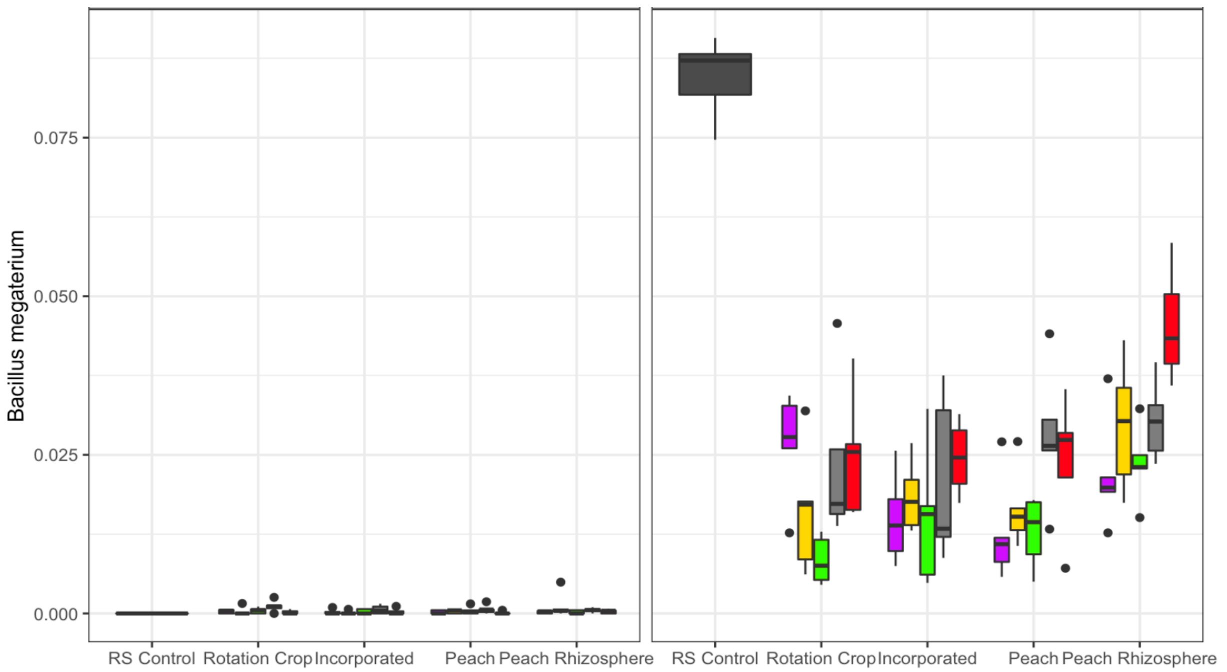

Figure 13. *Bacillus megaterium* relative abundance by crop phase. Crop history was denoted by color with alfalfa as purple, fescue as green, corn as yellow, tomato as red, no crop as light grey, and the initial bulk soil control as dark grey. (A)Autoclaved soils. (B) Non-autoclaved soils.

REFERENCES

Manganaris GA, Minas I, Cirilli M, Torres R, Bassi D, Costa G. Peach for the future: A specialty crop revisited. Scientia Horticulturae. 2022;305:111390.

Minas IS, Tanou G, Molassiotis A. Environmental and orchard bases of peach fruit quality. Scientia Horticulturae. 2018;235:307-22.

Thakur KK, Sharma DP. Effect of different soil agro-techniques on leaf nutrient status of peach grown in a replant sick soil. Journal of Pharmacognosy and Phytochemistry. 2018;7(2):3139-44.

Zhu Y, Shin S, Mazzola M. Genotype responses of two apple rootstocks to infection by *Pythium ultimum* causing apple replant disease. Canadian Journal of Plant Pathology. 2016;38(4):483-91.

Mazzola M, Manici LM. Apple replant disease: role of microbial ecology in cause and control. Annual Review of Phytopathology. 2012;50:45-65.

Manici L, Caputo F, Saccà M. Secondary metabolites released into the rhizosphere by *Fusarium oxysporum* and *Fusarium* spp. as underestimated component of nonspecific replant disease. Plant and Soil. 2017;415(1):85-98.

Merwin IA, Stiles WC. Root-lesion nematodes, potassium deficiency, and prior cover crops as factors in apple replant disease. Journal of the American Society for Horticultural Science. 1989;114(5):724-8.

Traquair JA. Etiology and control of orchard replant problems: a review. Canadian Journal of Plant Pathology. 1984;6(1):54-62.

Li K, DiLegge MJ, Minas IS, Hamm A, Manter D, Vivanco JM. Soil sterilization leads to re-colonization of a healthier rhizosphere microbiome. Rhizosphere. 2019;12:100176.

Hanschen FS, Winkelmann T. Biofumigation for fighting replant disease-A Review. Agronomy. 2020;10(3):425.

Wang GZ, Li HG, Christie P, Zhang FS, Zhang JL, Bever JD. Plant-soil feedback contributes to intercropping overyielding by reducing the negative effect of take-all on wheat and compensating the growth of faba bean. Plant and Soil. 2017;415(1):1-12.

Smith CR, Blair PL, Boyd C, Cody B, Hazel A, Hedrick A, et al. Microbial community responses to soil tillage and crop rotation in a corn/soybean agroecosystem. Ecology and Evolution. 2016;6(22):8075-84.

Kanaan H, Frenk S, Raviv M, Medina S, Minz D. Long and short term effects of solarization on soil microbiome and agricultural production. Applied Soil Ecology. 2018;124:54-61.

Wang L, Mazzola M. Field evaluation of reduced rate Brassicaceae seed meal amendment and rootstock genotype on the microbiome and control of apple replant disease. Phytopathology. 2019;109(8):1378-91.

Altieri MA, Nicholls CI, Henao A, Lana MA. Agroecology and the design of climate change-resilient farming systems. Agronomy for Sustainable Development. 2015;35(3):869-90.

Abán CL, Brandan CP, Verdenelli R, Huidobro J, Meriles JM, Gil SV. Changes in microbial and physicochemical properties under cover crop inclusion in a degraded common bean monoculture system. European Journal of Soil Biology. 2021;107:103365.

Hrynkiewicz K, Baum C. The potential of rhizosphere microorganisms to promote the plant growth in disturbed soils. Environmental protection strategies for sustainable development: Springer; 2012. p. 35-64.

Peralta AL, Sun Y, McDaniel MD, Lennon JT. Crop rotational diversity increases disease suppressive capacity of soil microbiomes. Ecosphere. 2018;9(5):e02235.

Ervin EH & Koski AJ. Drought avoidance aspects and crop coefficients of Kentucky bluegrass and tall fescue turfs in the semiarid west. Crop Science. 1998; 38(3): 788-795.

Braun RC, Patton AJ, Watkins E, Koch PL, Anderson NP, Bonos SA, et al. Fine fescues: A review of the species, their improvement, production, establishment, and management. Crop Science. 2020;60(3):1142-87.

Ara N, Bashar M, Begum S, Kakon S. Effect of spacing and stem pruning on the growth and yield of tomato. International Journal of Sustainable Crop Production. 2007;2(3):35-9.

Ilker E. Correlation and path coefficient analyses in sweet corn. Turkish Journal of Field Crops. 2011;16(2):105-7.

Laycock WA. Seeding and fertilizing to improve high-elevation rangelands: US Department of Agriculture, Forest Service, Intermountain Forest and Range Station (USA). 120. 1982.

Rankin M. Determining the Optimum Alfalfa Seeding Rate. University of Wisconsin Board of Regents; 2008.

Yost MA, Veum KS, Kitchen NR, Sawyer JE, Camberato JJ, Carter P, et al. Evaluation of the Haney Soil Health Tool for corn nitrogen recommendations across eight Midwest states. Journal of Soil and Water Conservation. 2018;73(5):587-92.

Alshaal T, Domokos-Szabolcsy É, Márton L, Czako M, Katai J, Balogh P, et al. Restoring soil ecosystems and biomass production of *Arundo donax* L. under microbial communities-depleted soil. Bioenergy Research. 2014;7(1):268-78.

DiLegge MJ, Manter DK, Vivanco JM. Soil microbiome disruption reveals specific and general plant-bacterial relationships in three agroecosystem soils. PLoS ONE. 2022;17(11):e0277529.

Tanaka S, Kobayashi T, Iwasaki K, Yamane S, Maeda K, Sakurai K. Properties and metabolic diversity of microbial communities in soils treated with steam sterilization compared with methyl bromide and chloropicrin fumigations. Soil Science and Plant Nutrition. 2003;49(4):603-10.

Badri DV, Vivanco JM. Regulation and function of root exudates. Plant, Cell & Environment. 2009;32(6):666-81.

Baltaci MO. Enhancement of cellulase production by co-culture of *Streptomyces ambofaciens* OZ2 and *Cytobacillus oceanisediminis* OZ5 isolated from rumen samples. Biocatalysis and Biotransformation. 2022;40(2):144-52.

Egamberdieva D, Shurigin V, Alaylar B, Wirth S, Bellingrath-Kimura SD. Bacterial endophytes from horseradish (*Armoracia rusticana* G. Gaertn., B. Mey. & Scherb.) with antimicrobial efficacy against pathogens. Plant, Soil and Environment. 2020;66(7):309-16.

Haack FS, Poehlein A, Kröger C, Voigt CA, Piepenbring M, Bode HB, et al. Molecular keys to the *Janthinobacterium* and *Duganella* spp. interaction with the plant pathogen *Fusarium graminearum*. Frontiers in Microbiology. 2016;7:1668.

Rafikova G, Korshunova TY, Minnebaev L, Chetverikov S, Loginov O. A new bacterial strain, *Pseudomonas koreensis* IB-4, as a promising agent for plant pathogen biological control. Microbiology. 2016;85(3):333-41.

Sait M, Kamneva OK, Fay DS, Kirienko NV, Polek J, Shirasu-Hiza MM, et al. Genomic and experimental evidence suggests that *Verrucomicrobium spinosum* interacts with eukaryotes. Frontiers in Microbiology. 2011;2:211.

Yadav M, Kumar T, Kanakan A, Maurya R, Pandey R, Chauhan NS. Isolation and characterization of human intestinal bacteria *Cytobacillus oceanisediminis* NB2 for probiotic potential. Frontiers in Microbiology. 2022;13.

Zheng Z, Zheng J, Peng D, Sun M. Complete genome sequence of *Fictibacillus arsenicus* G25-54, a strain with toxicity to nematodes. Journal of Biotechnology. 2017;241:98-100.

Byrne-Bailey KG, Wrighton KC, Melnyk RA, Agbo P, Hazen TC, Coates JD. Complete genome sequence of the electricity-producing "*Thermincola potens*" strain JR. Journal of Bacteriology. 2010;192(15):4078-9.

Oves M, Khan MS, Qari HA. *Ensifer adhaerens* for heavy metal bioaccumulation, biosorption, and phosphate solubilization under metal stress condition. Journal of the Taiwan Institute of Chemical Engineers. 2017;80:540-52.

Rathi M, Yogalakshmi K. *Brevundimonas diminuta* MYS6 associated *Helianthus annuus* L. for enhanced copper phytoremediation. Chemosphere. 2021;263:128195.

Singh N, Marwa N, Mishra J, Verma PC, Rathaur S, Singh N. *Brevundimonas diminuta* mediated alleviation of arsenic toxicity and plant growth promotion in *Oryza sativa* L. Ecotoxicology and Environmental Safety. 2016;125:25-34.

Xu X, Xu M, Zhao Q, Xia Y, Chen C, Shen Z. Complete genome sequence of Cd (II)-resistant *Arthrobacter* sp. PGP41, a plant growth-promoting bacterium with potential in microbe-assisted phytoremediation. Current Microbiology. 2018;75(9):1231-9.

Álvarez C, Navarro JA, Molina-Heredia FP, Mariscal V. Endophytic colonization of rice (*Oryza sativa* L.) by the symbiotic strain *Nostoc punctiforme* PCC 73102. Molecular Plant-Microbe Interactions. 2020;33(8):1040-5.

Yin S, Zhang W, Tong T, Yu C, Chang X, Chen K, et al. Feedstock-dependent abundance of functional genes related to nitrogen transformation controlled nitrogen loss in composting. Bioresource Technology. 2022;361:127678.

Mahmood T, Mehnaz S, Fleischmann F, Ali R, Hashmi Z, Iqbal Z. Soil sterilization effects on root growth and formation of rhizosheaths in wheat seedlings. Pedobiologia. 2014;57(3):123-30.

Monohon SJ, Manter DK, Vivanco JM. Conditioned soils reveal plant-selected microbial communities that impact plant drought response. Scientific reports. 2021;11(1):1-11.

Finney DM, White CM, Kaye JP. Biomass production and carbon/nitrogen ratio influence ecosystem services from cover crop mixtures. Agronomy Journal. 2016;108(1):39-52.

Gamliel A, Grinstein A, Peretz Y, Klein L, Nachmias A, Tsror L, et al. Reduced dosage of methyl bromide for controlling Verticillium wilt of potato in experimental and commercial plots. Plant Disease. 1997;81(5):469-74.

Trout T, Klonsky K, De Moura R, editors. Economics of methyl bromide alternatives for orchard replant in California. Methyl Bromide Alternatives and Emissions Research Conference Proceedings; 2004; 5: 1-9.

Cetintas R, Kusek M, Fateh SA. Effect of some plant growth-promoting rhizobacteria strains on root-knot nematode, Meloidogyne incognita, on tomatoes. Egyptian Journal of Biological Pest Control. 2018;28(1):1-5.

Ding Y, Wang J, Liu Y, Chen S. Isolation and identification of nitrogen-fixing bacilli from plant rhizospheres in Beijing region. Journal of Applied Microbiology. 2005;99(5):1271-81.

Goswami M, Deka S. Biosurfactant production by a rhizosphere bacteria *Bacillus altitudinis* MS16 and its promising emulsification and antifungal activity. Colloids and Surfaces B: Biointerfaces. 2019;178:285-96.

Höfle G, Kunze B. Biosynthesis of aurachins A− L in *Stigmatella aurantiaca*: a feeding study. Journal of Natural Products. 2008;71(11):1843-9.

Joshi A, Thite S, Karodi P, Joseph N, Lodha T. *Alkalihalobacterium elongatum* gen. nov. sp. nov.: An antibiotic-producing bacterium isolated from lonar lake and reclassification of the genus *Alkalihalobacillus* into seven novel genera. Frontiers in Microbiology. 2021;12.

Leifert C, Li H, Chidburee S, Hampson S, Workman S, Sigee D, et al. Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. Journal of Applied Bacteriology. 1995;78(2):97-108.

Ravin NV, Rakitin AL, Ivanova AA, Beletsky AV, Kulichevskaya IS, Mardanov AV, et al. Genome analysis of *Fimbriiglobus ruber* SP5T, a planctomycete with confirmed chitinolytic capability. Applied and Environmental Microbiology. 2018;84(7):e02645-17.

Schwartz AR, Ortiz I, Maymon M, Herbold CW, Fujishige NA, Vijanderan JA, et al. *Bacillus simplex*—a little known PGPB with anti-fungal activity—alters pea legume root architecture and

nodule morphology when coinoculated with *Rhizobium leguminosarum* bv. viciae. Agronomy. 2013;3(4):595-620.

Slama HB, Cherif-Silini H, Chenari Bouket A, Qader M, Silini A, Yahiaoui B, et al. Screening for *Fusarium* antagonistic bacteria from contrasting niches designated the endophyte *Bacillus halotolerans* as plant warden against *Fusarium*. Frontiers in Microbiology. 2019;9:3236.

Vary PS, Biedendieck R, Fuerch T, Meinhardt F, Rohde M, Deckwer W-D, et al. *Bacillus megaterium*—from simple soil bacterium to industrial protein production host. Applied Microbiology and Biotechnology. 2007;76(5):957-67.

Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, et al. Bergey's manual of systematic bacteriology: Volume 3: The Firmicutes: Springer Science & Business Media; 2011.

Aklujkar M, Coppi MV, Leang C, Kim BC, Chavan M, Perpetua L, et al. Proteins involved in electron transfer to Fe (III) and Mn (IV) oxides *by Geobacter sulfurreducens* and *Geobacter uraniireducens*. Microbiology. 2013;159(Pt_3):515-35.

Badalamenti JP, Summers ZM, Chan CH, Gralnick JA, Bond DR. Isolation and genomic characterization of '*Desulfuromonas soudanensis* WTL', a metal-and electrode-respiring bacterium from anoxic deep subsurface brine. Frontiers in Microbiology. 2016;7:913.

Emtiazi G, Hedayatkhah A. Comparative modelling of 3D-structure of *Geobacter* sp. M21 (a metal reducing bacteria) Mn-Fe superoxide dismutase and its binding properties with bisphenol-A, aminotriazole and ethylene-diurea. Iranian Journal of Environmental Technology. 2015;1(1):19-30.

Li J, Lu Q, Liu T, Zhou S, Yang G, Zhao Y. *Paenibacillus guangzhouensis* sp. nov., an Fe (III)- and humus-reducing bacterium from a forest soil. International journal of Systematic and Evolutionary Microbiology. 2014;64(Pt_11):3891-6.

Liang J, Bai Y, Hu C, Qu J. Cooperative Mn (II) oxidation between two bacterial strains in an aquatic environment. Water Research. 2016;89:252-60.

Lin S-Y, Hameed A, Liu Y-C, Hsu Y-H, Lai W-A, Chen W-M, et al. *Pseudomonas sagittaria* sp. nov., a siderophore-producing bacterium isolated from oil-contaminated soil. International Journal of Systematic and Evolutionary Microbiology. 2013;63(Pt_7):2410-7.

Lovley DR, Phillips E, Lonergan DJ, Widman PK. Fe (III) and S0 reduction by *Pelobacter carbinolicus*. Applied and Environmental Microbiology. 1995;61(6):2132-8.

Park M-K, Park Y-J, Kim M, Kim M-C, Ibal JC, Kang G-U, et al. Complete genome sequence of a plant growth-promoting bacterium *Pseudarthrobacter* sp. NIBRBAC000502772, isolated from shooting range soil in the Republic of Korea. The Microbiological Society of Korea. 2020;56(4):390-3.

Albuquerque L, França L, Rainey FA, Schumann P, Nobre MF, da Costa MS. *Gaiella occulta* gen. nov., sp. nov., a novel representative of a deep branching phylogenetic lineage within the class Actinobacteria and proposal of Gaiellaceae fam. nov. and Gaiellales ord. nov. Systematic and Applied Microbiology. 2011;34(8):595-9.

Bae H-S, Rash BA, Rainey FA, Nobre MF, Tiago I, da Costa MS, et al. Description of *Azospira restricta* sp. nov., a nitrogen-fixing bacterium isolated from groundwater. International journal of Systematic and Evolutionary Microbiology. 2007;57(7):1521-6.

Bahulikar RA, Torres-Jerez I, Worley E, Craven K, Udvardi MK. Diversity of nitrogen-fixing bacteria associated with switchgrass in the native tallgrass prairie of Northern Oklahoma. Applied and Environmental Microbiology. 2014;80(18):5636-43.

Bomar M, Hippe H, Schink B. Lithotrophic growth and hydrogen metabolism by *Clostridium magnum*. FEMS Microbiology letters. 1991;83(3):347-9.

Dahal RH, Kim J. *Microvirga soli* sp. nov., an alphaproteobacterium isolated from soil. International Journal of Systematic and Evolutionary Microbiology. 2017;67(1):127-32.

Fujitani H, Momiuchi K, Ishii K, Nomachi M, Kikuchi S, Ushiki N, et al. Genomic and physiological characteristics of a novel nitrite-oxidizing *Nitrospira* strain isolated from a drinking water treatment plant. Frontiers in Microbiology. 2020;11:545190.

Norton JM, Klotz MG, Stein LY, Arp DJ, Bottomley PJ, Chain PS, et al. Complete genome sequence of *Nitrosospira multiformis*, an ammonia-oxidizing bacterium from the soil environment. Applied and Environmental Microbiology. 2008;74(11):3559-72.

Rivas R, Mateos PF, Martínez-Molina E, Velázquez E. *Paenibacillus xylanilyticus* sp. nov., an airborne xylanolytic bacterium. International Journal of Systematic and Evolutionary Microbiology. 2005;55(1):405-8.

Safronova VI, Kuznetsova IG, Sazanova AL, Belimov AA, Andronov EE, Chirak ER, et al. *Microvirga ossetica* sp. nov., a species of rhizobia isolated from root nodules of the legume species *Vicia alpestris* Steven. International Journal of Systematic and Evolutionary Microbiology. 2017;67(1):94-100.

Trautwein K, Kühner S, Wöhlbrand L, Halder T, Kuchta K, Steinbüchel A, et al. Solvent stress response of the denitrifying bacterium "*Aromatoleum aromaticum*" strain EbN1. Applied and Environmental Microbiology. 2008;74(8):2267-74.

Xiujie W, Weiqi W, Jing Z, Siyu W, Jun L. Dominance of Candidatus Saccharibacteria in SBRs achieving partial denitrification: effects of sludge acclimating methods on microbial communities and nitrite accumulation. RSC advances. 2019;9(20):11263-71.

Ortíz-Castro R, Valencia-Cantero E, López-Bucio J. Plant growth promotion by *Bacillus megaterium* involves cytokinin signaling. Plant Signaling & Behavior. 2008;3(4):263-5.

Slama HB, Cherif-Silini H, Chenari Bouket A, Qader M, Silini A, Yahiaoui B, et al. Screening for *Fusarium* antagonistic bacteria from contrasting niches designated the endophyte *Bacillus halotolerans* as plant warden against Fusarium. Frontiers in Microbiology. 2019;9:3236.

Jiménez-Gómez A, García-Estévez I, García-Fraile P, Escribano-Bailón MT, Rivas R. Increase in phenolic compounds of *Coriandrum sativum* L. after the application of a *Bacillus halotolerans* biofertilizer. Journal of the Science of Food and Agriculture. 2020;100(6):2742-9.

Gutiérrez-Mañero FJ, Ramos-Solano B, Probanza An, Mehouachi J, R. Tadeo F, Talon M. The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. Physiologia Plantarum. 2001;111(2):206-11.

Martínez-Hidalgo P, Flores-Félix JD, Sánchez-Juanes F, Rivas R, Mateos PF, Santa Regina I, et al. Identification of canola roots endophytic bacteria and analysis of their potential as biofertilizers for canola crops with special emphasis on sporulating bacteria. Agronomy. 2021;11(9):1796.

Sabir A. Improvement of grafting efficiency in hard grafting grape *Berlandieri hybrid* rootstocks by plant growth-promoting rhizobacteria (PGPR). Scientia Horticulturae. 2013;164:24-9.

Tanner S, Reighard G, Wells C. Soil treatments differentially affect peach tree root development and demography in a replant site. Acta Horticulturae. 2006; 713: 381-387.

CHAPTER 3 SHIFTS OF THE SOIL MICROBIOME COMPOSITION INDUCED BY PLANT–PLANT INTERACTIONS UNDER INCREASING COVER CROP DENSITIES AND DIVERSITIES[3]

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Synopsis

Interspecific and intraspecific competition and facilitation have been a focus of study in plant-plant interactions, but their influence on plant recruitment of soil microbes is unknown. In this greenhouse microcosm experiment, three cover crops (alfalfa, brassica, and fescue) were grown alone, in paired mixtures, and all together under different densities. For all monoculture trials, total pot biomass increased as density increased. Monoculture plantings of brassica were associated with the bacteria *Azospirillum* spp., fescue with *Ensifer adhaerens*, and alfalfa with both bacterial taxa. In the polycultures of cover crops, for all plant mixtures, total above-ground alfalfa biomass increased with density, and total above ground brassica biomass remained unchanged. For each plant mixture, differential abundances highlighted bacterial taxa which had not been previously identified in monocultures. For instance, mixtures of all three plants showed an increase in abundance of *Planctomyces* sp. SH-PL14 and *Sandaracinus amylolyticus* which were not represented in the monocultures. Facilitation was best supported for the alfalfa-fescue interaction as the total above ground biomass was the highest of any mixture. Additionally, the bulk soil microbiome that correlated with increasing plant densities showed increases in plant growth-promoting rhizobacteria such as *Achromobacter xylosoxidans*, *Stentotrophomonas* spp.,

and *Azospirillum* sp. In contrast, *Agrobacterium tumefaciens*, a previously known generalist phytopathogen, also increased with alfalfa-fescue plant densities. This could suggest a strategy by which, after facilitation, a plant neighbor could culture a pathogen that could be more detrimental to the other.

Introduction

The soil hosts interactions between the largest global biomass distribution of terrestrial plants and microbes (Bar-On et al., 2018). Plants shift the biotic environment in the soil to benefit themselves, their offspring, and other plant species (Hu et al., 2018). As such, plants and their microbiomes directly or indirectly impact one another through competition and facilitation (Zhou et al., 2021; Wagg et al., 2019). Understanding the fundamental underpinnings of plant-microbiome feedbacks that manipulate the soil environment would be invaluable for agriculturalists.

Cover cropping is an ancient agricultural technique where plants are grown for the purpose of improving soil health instead of being harvested for profit. Cover cropping can improve acquisition and retention of nutrients in the soil, prevent erosion, and control weeds and pathogens (Couëdel et al., 2019; Osipitan et al., 2018; Reeves, 2018; Lal, 2015; Boudreau, 2013; Franchini et al., 2004; Malik et al., 2000). Cover crops have successfully regenerated heavily used agricultural soils (Giller et al., 2021; Abdalla et al., 2019).

Exemplary cover crops are alfalfa (*Medicago sativa*), which can increase the levels of nitrogen in the soil14; mustard plants (*Brassica* sp.), which are known to produce powerful antimicrobials (Stanger & Lauer, 2008); and grasses (*Festuca* sp.), which prevent erosion, control weeds, and produce large amounts of fibrous roots that sequester organic carbon (Abdalla et al., 2019; MacLaren et al., 2019; Bergkvist et al., 2010; Stanger & Lauer, 2008; Malik et al.,

2000). Non-leguminous cover crops decrease nitrogen leaching and increase soil organic carbon; however, this may promote a yield reduction of the primary cash crop under certain circumstances (Abdalla et al., 2019). Thus, it has been posited that a mix of legume and non-leguminous cover crops is the best method for increasing cash crop yield (Abdalla et al., 2019). However, to effectively combine each plant-specific benefit for agricultural purposes, fundamental knowledge of plant co-existence is vital.

Plant intraspecific and interspecific competition make compatibility and density optimization of cover crops challenging (Neumann et al., 2009; Lotz et al., 1997). Plant density and diversity are linked to soil microbial community diversity, function, and interaction (Lamb et al., 2011). Although plant diversity may increase microbial activity and functionality (Maron et al., 2011) as different plant species recruit different beneficial microbes, microbe functionality could be distilled due to the lack of compatibility between different plant species.

In this study, the aim was to identify treatments and microbiomes that induce greater total cover crop biomass (not necessarily individual plant biomass), since the increased plant matter provides soil health attributes (MacLaren et al., 2019; Ghimire et al., 2017) Furthermore, it was proposed that plant–plant competition leading to a better occupation of space and increased total cover crop biomass could result from enhanced recruitment of beneficial microbes in the soil. Microbial analysis focused on the bulk soil microbiome, which is where the following primary cash crop will be established. This work aimed to use a significant increase of total plant biomass to identify bulk soil bacterial shifts related to plant-plant density or diversity situations.

Methods

Soil disinfection and cover crop seed density

Soil was collected from Colorado State University's Agricultural Research, Development and Education Center South. Large debris were removed from the soil using metal sieves (2 cm wide). Autoclaved soil was used to reduce soil microbial biomass and community complexity and to maximize the impact the plant had on the soil microbiome (DiLegge et al., 2022; Monohon et al., 2021; Li et al., 2019) Soil was homogenized and then autoclaved in batches of approximately 13.5 kg in 61 × 76 cm polyethylene autoclave bags using a STERIS steam autoclave (Mentor, Ohio, USA) for three 40 min liquid cycles at 121 °C. After soils were autoclaved, they were pooled to reduce any potential variability associated with each autoclave cycle. Different seed density maximums were tested prior to the experiment showing 1–3, 24, and 48 plant densities had high seedling survivability, and senescence started at week four.

Cover crop greenhouse experiment

Plants were grown for 32 days from August 1 to September 1, 2021, in Colorado State University's Horticultural Center Greenhouse Facility. A microcosm was its own "pot" (6 × 4.9 × 5.6 cm) taken from a 36-cell tray, and each microcosm was separated by ~ 2 cm (Fig. 6). Pots were lined with a double layer of Vigoro Weed Control Fabric Medium Duty to reduce soil runoff. There were 7 diversity treatments (1. alfalfa, 2. brassica, 3. fescue, 4. alfalfa-brassica, 5. alfalfa-fescue, 6. brassica-fescue, 7. alfalfa-brassica-fescue) and 3 density treatments (low: 1–3 plants, medium: 24 plants, and high: 48 plants) for a total of 21 treatments (Table 4). Each treatment had 12 replicates for a total of 252 pots. Random block design of 21 × 12 was configured by an online random block design generator (https://www.randomizer.org). There was one treatment type per column. The reference control for this plant-plant competition/facilitation study was a single cover crop species to exemplify a plant with no competition/facilitation. Cover crop seed mixes and densities were manually counted and placed

into microcentrifuge tubes. Each microcentrifuge tube was briefly vortexed to mix the seeds. Seeds were spread evenly into the pots with autoclaved soil using tweezers, which were washed with ethyl alcohol in between samples. To remedy seed germination failure, pregerminated seeds were planted into each pot 7 days into the experiment to reach the target densities. Plants were watered daily at water holding capacity with DI water to reduce the introduction of microbes and other contaminants. Additionally, DI water was used to mimic uncontaminated rainwater since cover crops are ideally not irrigated. As an aside, seeds were not sterilized as to prevent additional seed death and to maintain fundamental microbes on the surface of the seed coat for the respective plant. At the end of the experiment, the number of plants in each pot were counted.

Bulk soil collection

Bulk soil samples were collected at the end of the study, and prior to biomass harvest. Bulk soil refers to the surrounding soil, which has been influenced by an organism such as a plant but excludes the soil adhering to the roots which is known as the rhizosphere (Blouin & Jacquiod, 2020). Within each treatment, the top five replicates that best represented target densities were selected for bacteriome analysis. A core borer (1.5 cm diameter) was used to collect the surrounding bulk soil from the center of the pot without disturbing the above-ground biomass. The soil probe was sterilized between samples. Visible soil debris was scrubbed off the soil probe using a brush soaked in a tap water-Alconox (White Plains, New York, USA) solution. Next, the soil probe was rinsed with 2% bleach followed by 70% ethyl alcohol. Bulk soil cores were placed in a 15 ml falcon tube and immediately stored at − 20 °C. Bulk soil samples were taken over four days.

Plant biomass

Above ground biomass was measured for every sample (n = 235) and was harvested using scissors, which were surface sterilized between samples using a Bacti-Cinerator III (Monoject Scientific, St. Louis, Missouri. 63103, USA). For each pot, above ground biomass was separated from below ground biomass. If there was more than one plant was growing in the pot, then the above ground biomass was also separated by crop type as well. Plant biomass was oven dried for 72 + hours, and then weighed.

DNA extraction

Closely following Qiagen's protocol, total genomic DNA (gDNA) was extracted from 0.25 g of surrounding bulk soil in a Qiagen QIAcube instrument (Germantown, Maryland, USA) using Qiagen PowerSoil Pro ® DNA kits. Any roots and their respective adhering soil were removed from the bulk soil that was to be used for DNA extraction. Elution volume for extractions was of 100 µl. An Invitrogen Qubit fluorometer (Waltham, Massachusetts, USA) quantified DNA concentrations with high sensitivity assay solutions. Bulk soil samples (n = 103) taken from each of the 21 treatments had 4–5 replicates that were randomly selected for DNA extraction. Controls used were pre-extracted Zymo gDNA (Zymo Research Corporation, California, USA) (n = 2), extracted HPLC water (n = 2), PCR 2 HPLC water (n = 2), and pre-extracted and sequenced soil (n = 2).

Oxford nanopore library prep, sequencing, and bioinformatics pipeline

Extracted DNA was diluted 5 times with HPLC water based on Qubit concentrations (ng/µl). Bacterial primers used were Bact_27F-Mn (5′ – TTTCTGTTGGTGCTGATATTGCAGRGTTYGATYMTGGCTCAG—3′) and Bact_1492R-Mn (5′ACTTGCCTGTCGCTCTATC TTC TACCTTGTTACGACTT—3′). Polymerase chain reaction (PCR) settings were 98 °C for 30 s, 98 °C for 15 s, 50 °C for 15 s, and 72 °C for 1 min

for 25 cycles, and 72 °C for 5 min. After the first PCR, equal volumes of DNA and beads were mixed. A 96-pronged magnetic stand was used to move beads with adhering DNA into two 30 s rinses of 70% ethanol. DNA was eluted in a 96-well plate with 40 μL PCR grade water, and beads were removed using a magnetic stand. DNA was quantified using a Qubit with high sensitivity assay solutions. The second PCR settings were 98 °C for 30 s, 98 °C for 15 s, 62 °C for 15 s, and 72 °C for 1 min for 25 cycles, and 72 °C for 5 min. After the second PCR, DNA and barcodes (EXP-PBC-96) were pooled in AMPure bead solution in a 96-well plate. Wells with suspended DNA and barcodes were pooled into a clean Lo-Bind tube. MinION sequencer was loaded with a flow cell (R9.4.1). To prepare the flow cell, air (~ 20 μL) was removed using a pipette. The flow cell was then primed with flush buffer, and pooled DNA was loaded into the sampling port. MinKNOW software was used to sequence the pooled library for 48 h. Raw data was base-called and demultiplexed using Guppy v6.0.1 and reads were then filtered by quality (Filtlong minimum length: 1000; mean quality: 70) and length (Cutadapt: -m 1000 -M 2000). Bacterial taxa were identified using EMU NCBI Reference Database. Sequencing data was processed using DADA2 which removed all singletons by default. EMU error correction removed identified bacterial taxa based on alignment and abundance profiles, such that bacterial taxa with < 1 per 10,000 reads were removed. Sequencing data came from three separate sequence runs, which were pooled for data analysis.

Statistical analysis

Statistical analyses were run, and figures were made using RStudio Version 1.4.1103. Rarefaction curves show that samples plateaued (Fig. 7). Normality for the biomass was tested using the Shapiro–Wilk normality test for normality. Linear models of residuals were used to assess the equality of variance. One-way analysis of variance followed by the Tukey HSD test

were used to denote the compact letter display to indicate significance using emmeans, multcompView, and dplyr packages (Lenth, 2021; Wickham et al., 2020; Graves et al., 2019). PERMANOVA was used to find significant differences between treatments and visualized with a constrained Principal Coordinate Analysis (PCoA) with Bray–Curtis dissimilarity index used as a distance from the Vegan package (Oksanen et al., 2007). Betadisper from the Vegan package was used to measure the homogeneity of multivariate dispersions. Differential abundance analysis was based on bacterial species counts using log2 fold change with the Benjamini–Hochberg method (Hagenfeld et al., 2018) using the fdr (false discovery rate) function at an adjusted p-value threshold of 0.05. Alpha diversity was visualized using the Shannon diversity index through the phyloseq package using rarified data ($M = 31{,}960$, $SD = 11{,}508$, reads per sample) (McMurdie & Holmes, 2013).

Rights and permissions for research involving plants

This study did not require special right or permissions for plant material use. Seeds (fescue mixture from Vitality, ranger alfalfa and Mighty Mustard® Pacific Gold from Johnny's selected seeds) used in this greenhouse study were not from wild plants and were purchased and are not listed as an endangered species.

Results

Monoculture

In monoculture, total biomass of all three cover crops increased with crop density. Total biomass of alfalfa increased significantly within each density increment and had the highest biomass of any cover crop for densities of 24 plants and 48 plants (Fig. 8). Brassica above ground biomass did not statistically differ between 1 and 24 plants but required a density of 48 plants to raise the total biomass. Fescue biomass at a density of 24 plants was double the same at

a density of 1 plant; the biomass was not significantly different between 24 and 48 plants. For all three cover crop types, a single plant density yielded the largest individual plant, and the number of plants-to-biomass ratio was inversely proportional to density.

Surrounding bulk soil bacteriome analysis

Bacterial microbiome shifts in the surrounding bulk soil of the plants were assessed using alpha and beta diversities, and differential abundance of specific taxa. For Shannon Diversity Index, there was no consistent visual trend of an increasing alpha diversity measure by density or diversity (Fig. 9). PERMANOVA model with all data combined showed both density and diversity were significant factors along with their interaction (Fig. 10). Low to high plant densities of alfalfa induced significant ($p = 0.001$, $R^2 = 0.286$) shifts on the surrounding bulk soil bacteriome (Fig. 1b, Table 6). As an estimate of beta diversity, the average distance to the median for the bacterial bacteriomes at a density of a single alfalfa plant was 0.2893, for 24 alfalfa plants 0.3251, and for 48 alfalfa plants 0.2841. The density of 48 alfalfa plants had the highest clustering out of the three densities. Increasing densities of brassica induced a significant ($p = 0.01$, $R^2 = 0.183$) shift on the surrounding bulk soil bacteriome (Fig. 1d, Table 6). For brassica, average distance to the median for the bacteriomes had the highest clustering for the single (0.2861), 24 (0.3165), and 48 (0.3047) plant densities. The PERMANOVA test showed that when looking at fescue by increasing densities of 1, 24, and 48 plants, the shift induced on the soil bacteriome was significant ($p = 0.002$, $R^2 = 0.208$) (Fig. 1f, Table 6). For fescue, the average distance to the median for the bacteriome had the highest clustering for the single plant density with 0.2862, with 24 fescue plants at 0.3227, and 48 fescue plants at 0.3367. The increasing density of the alfalfa monocrop explained the highest variability (CAP1 + CAP2: 28.6) as compared to brassica (18.3) and fescue (20.8) (Fig. 1b,d,f).

Differential abundance comparisons of the bacteriome in the surrounding bulk soil were conducted when there was a significant difference in total plant biomass per pot as density increased (Table 1). Bacteria of interest were those which were highlighted by the differential abundance comparison in conjunction with an increase in total plant biomass. Alfalfa with a low density (one plant) was enriched for 22 bacterial taxa compared to medium (24 plants) and high density (48 plants) microcosms. Alfalfa with a medium density was enriched for 9 bacterial taxa compared with high density microcosms. Alfalfa with a high density was enriched for 13 bacterial taxa compared to both medium and low-density microcosms. Brassica with a low density (one plant) was enriched for 7 bacterial taxa compared to high density (48 plants) microcosms. Brassica with a medium density (24 plants) was enriched for 3 bacterial taxa compared to a high-density microcosm. Fescue with a low density (one plant) was enriched for 8 bacterial taxa compared to medium (24 plants) and high density (48 plants) microcosms. Fescue with a medium density was enriched for 4 bacterial taxa compared to low density microcosms. Fescue with a high density was enriched for 5 bacterial taxa compared to low density microcosms.

Mixtures of two plants

Alfalfa plant biomass increased when grown in polyculture with higher densities of brassica (Fig. 2a) or fescue (Fig. 3a). Fescue's biomass did not significantly increase in densities of 24 and 48 plants with either alfalfa or brassica in paired mixtures (Figs. 3b and 4b). However, fescue had the highest biomass in a cover crop mixture with just alfalfa (Fig. 3b). The trend of fescue biomass in a cover crop mixture with alfalfa was similar to fescue growing in monoculture (Figs. 1e, 3b, 4b), where there was a significant increase followed by a leveling off in crop biomass. Brassica's biomass in cover crop mixtures did not change with increasing

densities (Figs. 2b, 4a). Overall, the average total above ground dry biomass was highest in the alfalfa and fescue cover crop mixture at a density of 48 plants.

For Shannon Diversity Index, increasing plant-plant intra/inter specific competition did not increase microbial alpha diversity in the surrounding bulk soil (Fig. 9). The PERMANOVA test showed that alfalfa and brassica mixtures under increasing densities of 2, 24, and 48 plants, induced a significant shift on the soil bacteriome ($p = 0.01$, $R^2 = 0.332$) (Fig. 2d, Table 6). For alfalfa and brassica mixtures, the average distance to the median for the bacterial microbiomes at a densities of 2 (0.305), 24 (0.272), and 48 (0.2586) plant mixtures had the highest clustering for the 48-plant density (Fig. 2d). Bacteriomes of alfalfa and brassica mixtures showed decreasing dispersion as density increased. The PERMANOVA test showed that when looking at alfalfa and fescue mixtures, increasing densities of 2, 24, and 48 plants induced a significant shift on the soil bacteriome ($p = 0.02$, $R^2 = 0.192$, Table 6) (Fig. 3d). For alfalfa and fescue mixtures, the average distance to median for the bacterial microbiomes at a density of two plant mixtures (0.3073), 24 plants (0.3067), and 48 plants (0.3240) had the highest clustering for the 24-plant density. The PERMANOVA test showed that when looking at brassica and fescue mixture by increasing densities of 2, 24, and 48 plants, the shift induced on the soil bacteriome was significant ($p = 0.038$, $R^2 = 0.203$) (Fig. 4d, Table 6). For brassica and fescue mixtures, the average distance to median for the bacterial microbiomes at a density of two plant mixtures (0.2849), 24 plants (0.2571), and 48 plants (0.2755) had the highest clustering for the 24-plant density. The increasing density of the alfalfa and brassica crop mixtures explained the highest variability (CAP1 + CAP2: 33.2) as compared to alfalfa and fescue (19.1), and brassica and fescue (20.3) (Figs. 2d, 3d, 4d).

Differential abundance analysis of the bacterial microbiome in the bulk soil was performed only if there was a change in the total biomass of a crop within the mixture (Table 2). Alfalfa and brassica mixture in low density (2 plants) showed an enrichment of 26 bacterial taxa compared and high density (48 plants) microcosms whereas high compared to low densities showed an enrichment of 2 bacterial taxa. Alfalfa and fescue mixture in low density (2 plants) showed an enrichment of 8 bacterial taxa compared to high (48 plants) and medium density (24 plants) microcosms whereas high and medium densities showed an enrichment of 13 bacterial taxa as compared to low density microcosms. There was no biomass increase for the total biomass for brassica and fescue mixture, and the biomass change for fescue was used instead to highlight bacteria with significant differential abundances. Brassica and fescue mixture in low density (two plants) showed an enrichment of 8 bacterial taxa compared to high density (48 plants) microcosms whereas high densities showed an enrichment of 1 bacterial taxon as compared to low density microcosms. Brassica and fescue mixture in medium density (24 plants) showed an enrichment of 5 bacterial taxa compared to high density (48 plants) microcosms whereas high densities showed an enrichment of 6 bacterial taxa as compared to medium density microcosms.

Mixtures of three plants

The mixture with three different cover crops showed similar trends as when they were grown in the cover crop mixtures of just two crops. When all three plants were grown together, there was a higher biomass for alfalfa and fescue as density increased, while there was no increase in biomass for brassica (Fig. 5b). For alfalfa, this trend was different that the previous cover crop mixtures and in monoculture since the density increase of 24 to 48 plants did not show an increase in biomass. Fescue biomass remained as the lowest in the mixture of three

crops (Fig. 5c). The number of brassica plants did not influence the total amount of above ground biomass for brassica. In summary, the biomass trends of mixtures of three cover crops followed previous trends for the mixtures of two cover crops.

Surrounding bulk soil bacteriome analysis

Biomass of an individual plant was largest in the density of three plants and decreased as density increased (Fig. 3c) Mixtures with all crops of a low density (3 plants) to medium density (24 plants) both showed an enrichment of 1 taxon (Table 3). Mixtures with all crops of a low density (3 plants) to high density (48 plants) showed an enrichment of 11 taxa, while high densities had an increase of 2 taxa as compared to low densities (Table 3). The PERMANOVA test showed that when looking at fescue by increasing densities of 3, 24, and 48 plants, the shift induced on the soil bacteriome was not significant ($p = 0.069$, $R^2 = 0.183$) (Fig. 5e, Table 6). For the mixture of all three plants, the average distance to median for the bacterial microbiomes at a density of three plants (0.3123), 24 plants (0.3035), and 48 plants (0.3279) had the highest clustering for 24 plants.

Discussion

In this study, it was shown that *Azospirillum* sp. TSA2s and *Ensifer adhaerens* increased between single to and 48 plants, while *Devosia riboflavina* and *E. adhaerens* increased between alfalfa densities of 24 and 48 plants. *Azospirilum sp*. and *E. adhaerens* are free-living nitrogen fixers, and *D. riboflavina* is a weak nitrate reducer; these species play a role in the nitrogen cycle in legume inhabited soils (Gao et al., 2019; He et al., 2016; Zhou et al., 2013; Biró et al., 2000; Nakagawa et al., 1996). Alfalfa's monoculture exhibited the least dispersion at densities of 48 plants, suggesting that the surrounding bulk soil bacteriome is progressing towards a tailored microbiome for alfalfa as intraspecific competition increases.

Though brassicas are known to produce antimicrobials (Pacheco-Cano et al., 2018), the Shannon Index did not reflect a decrease in of bacterial taxa as density increased. Taye et al., 2020 found that *Brassica napus* recruited many bacterial taxa whose effects included disease suppression. Incidentally, the abundance of the antiprotozoal microbe *Oscillatoria nigro-viridis* increased between brassica densities of 24 and 48 plants (Simmons et al., 2008). It was previously found that intraspecific competition in *B. juncea* manifested as increased counts of stress induced inflorescences and bolting (Qin et al., 2020; Maboko, 2012). In the present study, brassica bolted at the 24 and 48 plant densities which could be attributed to increasing intraspecific competition. Nitrogen might be in demand for competing brassica plants, since increasing densities were correlated with nitrogen fixers like *Azospirillum* sp. (TSH58) between single and 48 brassica densities, along with *Azospirillum* sp. TSA2s and *Azospirillum lipoferum* for brassica densities of 24–48 (Gao et al., 2019; Rai & Gaur, 1982). *Azospirillum* sp. are also known to produce phytohormones (Cassán et al., 2016). These shifts in the bacterial composition could potentially lower plant intraspecific competition by increasing the availability of limited nutrients.

Previous studies found that under increased *Festuca* spp. densities, seed germination remained high while plant mortality decreased. The same study found that increased *Festuca* spp. density did not increase the total biomass, which could be explained by intraspecific competition (Deschênes, 1974). It has also been reported that *Festuca* sp. density was directly correlated with the infection rate of the fungal pathogen *Rhizoctonia solani* (Zhou et al., 2016). In contrast, beneficial bacteria identified in the present study, such as *Roseomonas aestuarii* (can produce indole from tryptophan), increased from single to 24 and 48 fescue densities (Ramana et al., 2010). From a density of a single fescue plant to 48 plants the nitrogen

fixer *E. adhaerens* increased similar to alfalfa. *Stenotrophomonas* sp. (DAIF1), a possible bacterial phytopathogen, also increased from single to 24 and 48 fescue densities (Adeleke et al., 2021; Mishra et al., 2017). However, *Stenotrophomonas* sp. has also been found to be beneficial by providing stress protection, growth promotion, and biocontrol for plants (Berg et al., 2010). While beneficial bacteria like *E. adhaerens* could reduce plant–plant intraspecific competition, the present study has shown that higher fescue densities fail to increase total fescue biomass, indicating that there are other factors such as an asymptomatic phytopathogens playing a role in plant health.

In a previous study, an intercropping of alfalfa (*Medicago sativa*) and *B. juncea* showed that alfalfa's biomass increased by 55.3–70.0% while *B. juncea* biomass decreased by 0.4–11.8% which was attributed to an increased uptake of cadmium by *B. juncea* and a decrease uptake by alfalfa as compared to when grown alone (Xin-Bo et al., 2009). The present study does not support an increase in alfalfa biomass compared to grown in monoculture. Regardless, alfalfa's biomass increased with plant density, while brassica's remained the same. Although plants in the Brassicaceae family are not known to form mycorrhizal fungal connections (Lankau et al., 2011), this study supports the possibility that brassica plants rely on bacterial nitrogen fixers like *Azospirillum* spp when grown alone. It was expected that *Azospirillum* spp. would have enriched in alfalfa and brassica plant mixtures, since *Azospirillum* spp. was enriched for both alfalfa and brassica bulk soil monocultures. Instead, *Pseudarthrobacter phenanthrenivorans*, which has been known to produce numerous phytohormones (abscisic acid, auxin, cytokinin, ethylene, gibberellins, jasmonic acid, and salicylic acid), and the denitrifier *Pseudomonas stutzeri* were enriched (Tshishonga & Serepa-Dlamini, 2020; Lalucat et al., 2006). This finding supports that plant-plant interaction influence microbial recruitment in its own manner.

Alfalfa (*M. sativa*) and tall fescue [*Schedonorus phoenix* (Scop.) Holub] mixtures have been found to have a higher above ground biomass accumulation and weed suppression as compared to respective monocultures in other studies (Tracy et al., 2016). In this study, alfalfa and fescue mixture at 24 and 48 densities produced the highest above ground biomass out of the three plant mixtures. *Achromobacter xylosoxidans*, which has been previously associated with grasses and is a known plant growth promoting rhizobacterium, was identified in the differential abundance analysis for the single pair densities to the 24 and 48 densities (Ho et al., 2013). *Stentotrophomona*s spp. (genus known as PGPR and nitrogen fixers) abundance increased as previously observed in fescue monocultures, and Azopirillum sp. abundance increased as previously observed in fescue monocultures (Ghosh et al., 2020). Nonetheless, increasing plant diversity has the potential to allow for generalist bacterial phytopathogens to transmit from one plant species to another (Lopes et al., 2009). This drawback could spur *Agrobacterium tumefaciens*, which was enriched in alfalfa-fescue mixtures and has been known to cause crown gall disease in numerous plants (Gohlke et al., 2014). Strains of *A. tumefaciens* have shown to be highly virulent on alfalfa (*M. sativa*) (Palumbo et al. 1998) and to infect *Fescue* spp. (Dong & Qu, 2005). The increase of a known generalist phytopathogen was unexpected within the alfalfa-fescue mixtures since this plant mixture had the highest total biomass. Alfalfa's biomass continued to increase and was not distinctly impacted by the potential phytopathogen, whereas a non-significant decrease was observed for the fescue biomass at the highest density.

Aqueous extracts of *B. juncea* were found to induce total inhibition of root and shoot growth in (barnyard) grass (Toosi, 2010). Similarly in the present study, fescue biomass was the lowest in mixtures with brassica, possibly due to interspecific competition. In brassica-fescue mixtures, *Pontibacter chitinilyticus* which has possible antifungal capabilities through chitin-

hydrolsis, was enriched (Chhetri et al., 2019; Karthik et al., 2015). The nitrogen fixer *Azospirillum brasilense* was also relatively enriched much like when brassica was grown alone (Tien et al., 1979). The reoccurring nitrogen fixing species *Ensifer adharenes* was correlated with the presence of fescue, and could be a bacterium which reduces plant-plant competition leading to the significant total biomass increase. Drawing from biomass trends and differential microbial abundance results, brassica did not seem to be influenced by the presence of fescue.

For the crop mixtures of all three plants, differential abundance highlighted bacterial taxa whose abundances were not different in the monocultures or two-plant mixtures. *Planctomyces* sp. SH-PL14, known for its chitinase ability, and *Sandaracinus amylolyticus*, which has exhibited both antimicrobial production and starch hydrolysis, were highlighted in differential abundance analysis when moving from the soil with three individual plants to a density of 24 or 48 total plants (Kulichevskaya et al., 2019; Sharma et al., 2016; Steinmetz et al., 2012). While *Planctomyces* sp. chitinase could benefit all plants by causing a decrease in fungal phytopathogens (Lankau et al., 2011), and in our study these bacteria may contribute to reducing beneficial mycorrhizal networks for both alfalfa and fescue. It is also interesting to note that this chitinase producing bacteria was not identified in monocultures of brassica, suggesting brassica may not have a need for chitinase since the plant does not promote mycorrhizal networks. It is thought that *Brassica* spp. reduce the growth of interspecific competitors by producing the allelochemical sinigrin, which reduces mycorrhizal abundance of surrounding soils. However, sinigrin production is costly, and this investment does not alleviate intraspecific competition (Lankau et al., 2011). The nitrogen fixing *Azospirillum spp.* and *E. adharenes* found in monocultures were not apparent in three-plant mixture soils. Co-existence

would have been supported if free-living nitrogen fixers, even species driven dependent, was found. Abundances of the generalist phytopathogen, *Agrobacterium tumefaciens*, was not enriched in the plant mixture of the highest density and diversity, suggesting the soil bacteriome may benefit by increasing the diversity of alfalfa-fescue mixtures. This would support the dilution effect, where an increase of biodiversity decreases pathogen exposure and transmission (Pélissier et al., 2023; Ferraguti et al., 2021). Even in the most competitive mixture and density, bacterial phytopathogens were not highlighted by differential abundance. Overall, the present study shows that, while increasing densities can further increase previously promoted bacterial taxa, increasing plant diversity does not simply increase bacterial diversity as different bacterial taxa can then be promoted.

Autoclaving the soil simplifies the microbial community and has been shown to magnify the effect of plants on the soil bacteriome compared to non-autoclaved soils (DiLegge et al., 2022). Bacteriome shifts in the surrounding bulk soil in the microcosms are not comparable to the greater space in the field where spatial variability and legacy effects may have an influence. Moreover, a plant's developmental stage can influence the recruitment and selection of bacteria (Chaparro et al., 2014). Additional studies are required to directly define the functionality of these bacteria which could play a moderator role in plant-plant competition. In summary, this study supports the notion that bacterial shifts in the soil could depend on plant–plant interactions. The surrounding bulk soil bacteriomes of polycultures did not completely overlap with the bacteriomes of monocultures. Thus, bacteriome functionalities are not expected to be a simple overlap when one plant species is planted with another.

Table 1 Differential abundance of monoculture.

Alfalfa	Enrich Group (bolded)	Brassica	Enrich Group (bolded)	Fescue	Enrich Group (bolded)
Tumebacillus flagellates	**A1** vs A24	*Bacillus mannanilyticus*	**B1** vs B48	*Devosia riboflavina*	**F1** vs F24
Altererythrobacter dongtanensis	**A1** vs A24	*Oscillatoria nigro-viridis*	**B1** vs B48	*Janthinobacterium* sp. LM6	**F1** vs F24
Paenarthrobacter nicotinovorans	**A1** vs A24	*Anabaena cylindrica*	**B1** vs B48	*Microvirga soli*	**F1** vs F24
Devosia riboflavina	**A1** vs A24	*Daejeonella composti*	**B1** vs B48	*Paenarthrobacter nicotinovorans*	**F1** vs F48
Yonghaparkia alkaliphile	**A1** vs A24	*Trichocoleus desertorum*	**B1** vs B48	*Bacillus subtilis*	**F1** vs F48
Janthinobacterium sp. LM6	**A1** vs A24	*Azospirillum* sp. TSA2s	**B1** vs B48	*Thermomonas* sp. SY21	**F1** vs F48
Paenarthrobacter histidinolovorans	**A1** vs A24	*Cyanothece* sp. PCC 7425	**B1** vs B48	*Pontibacter amylolyticus*	**F1** vs F48
Noviherbaspirillum agri	**A1** vs A24	*Azospirillum* sp. TSH58	**B24**-B1	*Azohydromonas australica*	**F1** vs F48
Fictibacillus phosphorivorans	**A1** vs A48	*Arthrobacter* sp. FB24	**B24**-B1	*Stenotrophomonas* sp. DAIF1	**F24** vs F1
Bacillus acidicola	**A1** vs A48	*Arthrobacter crystallopoietes*	**B24**-B1	*Roseomonas aestuarii*	**F24** vs F1
Geitlerinema sp. PCC 7407	**A1** vs A48	*Oscillatoria nigro-viridis*	**B24**-B48	*Ammoniphilus oxalaticus*	**F24** vs F1
Ammoniphilus oxalaticus	**A1** vs A48	*Azospirillum* sp. TSA2s	**B24**-B48	*Chryseolinea soli*	**F24** vs F1
Bacillus carboniphilus	**A1** vs A48	*Azospirillum lipoferum*	**B24**-B48	*Stenotrophomonas* sp. DAIF1	**F48** vs F1
Oscillatoria nigro-viridis	**A1** vs A48			*Ammoniphilus oxalaticus*	**F48** vs F1
Altererythrobacter dongtanensis	**A1** vs A48			*Telluribacter humicola*	**F48** vs F1
Paenisporosarcina indica	**A1** vs A48			*Roseomonas aestuarii*	**F48** vs F1
Ammoniphilus resinae	**A1** vs A48			*Ensifer adhaerens*	**F48** vs F1
Bacillus subtilis	**A1** vs A48				
Oxalophagus oxalicus	**A1** vs A48				
Roseimicrobium gellanilyticum	**A1** vs A48				
Lysobacter helvus	**A1** vs A48				
Anabaena cylindrica	**A1** vs A48				
Bacillus carboniphilus	**A24**-A48				
Geitlerinema sp. PCC 7407	**A24**-A48				
Glaciimonas singularis	**A24**-A48				

Paenisporosarcina indica	**A24**-A48
Larkinella harenae	**A24**-A48
Oscillatoria nigro-viridis	**A24**-A48
Roseimicrobium gellanilyticus	**A24**-A48
Brevifollis gellanilyticus	**A24**-A48
Bacillus subtilis	**A24**-A48
Telluribacter humicola	**A48**-A1
Ensifer adhaerens	**A48**-A1
Daejeonella oryzae	**A48**-A1
Azospirillum sp. TSA2s	**A48**-A1
Tumebacillus flagellates	**A48**-A24
Devosia riboflavina	**A48**-A24
Ensifer adhaerens	**A48**-A24
Paenarthrobacter nicotinovorans	**A48**-A24

Table 2 Differential abundance of two plant mixtures.

Alfalfa-Brassica	**Enrich Group (bolded)**	**Alfalfa-Fescue**	**Enrich Group (bolded)**	**Brassica-Fescue**	**Enrich Group (bolded)**
Achromobacter spanius	**AB2** vs AB48	*Paenarthrobacter histidinolovorans*	**AF2** vs AF24	*Paenibacillus agaridevorans*	**BF2** vs BF48
Achromobacter xylosoxidans	**AB2** vs AB48	*Telluribacter humicola*	**AF2** vs AF24	*Oscillatoria nigro-viridis*	**BF2** vs BF48
Achromobacter insolitus	**AB2** vs AB48	*Lysobacter helvus*	**AF2** vs AF24	*Vicinamibacter silvestris*	**BF2** vs BF48
Stenotrophomonas sp. G4	**AB2** vs AB48	*Chryseolinea soli*	**AF2** vs AF48	*Pontibacter brevis*	**BF2** vs BF48
Agrobacterium tumefaciens	**AB2** vs AB48	*Azospirillum* sp. TSA2s	**AF2** vs AF48	*Thermomonas* sp. SY21	**BF2** vs BF48
Geitlerinema sp. PCC 7407	**AB2** vs AB48	*Brevibacillus brevis*	**AF2** vs AF48	*Brevifollis gellanilyticus*	**BF2** vs BF48
Daejeonella oryzae	**AB2** vs AB48	*Sandaracinus amylolyticus*	**AF2** vs AF48	*Anabaena cylindrica*	**BF2** vs BF48
Paenarthrobacter nicotinovorans	**AB2** vs AB48	*Thermomonas* sp. SY21	**AF2** vs AF48	*Trichocoleus desertorum*	**BF2** vs BF48
Kaistia defluvii	**AB2** vs AB48	*Achromobacter insloitus*	**AF24** vs AF2	*Pontibacter brevis*	**BF24** vs BF48
Telluribacter humicola	**AB2** vs AB48	*Stenotrophomonas* sp. MYb57	**AF24** vs AF2	*Paenibacillus agaridevorans*	**BF24** vs BF48
Adhaeribacter swui	**AB2** vs AB48	*Achromobacter xylosoxidans*	**AF24** vs AF2	*Vicinamibacter silvestris*	**BF24** vs BF48

Paucimonas lemoignei	**AB2** vs AB48	*Stentotrophomonas* sp. DAIF1	**AF24** vs AF2	*Thermomonas* sp. SY21	**BF24** vs BF48
Roseomonas aestuarii	**AB2** vs AB48	*Agrobacterium tumefaciens*	**AF24** vs AF2	*Trichocoleus desertorum*	**BF24** vs BF48
Chryseolinea soli	**AB2** vs AB48	*Azospirillum* sp. TSH58	**AF24** vs AF2	*Pontibacter chitinilyticus*	**BF48** vs BF2
Altererythrobacter dongtanensis	**AB2** vs AB48	*Anabaena cylindrica*	**AF24** vs AF2	*Azospirillum brasilense*	**BF48** vs BF24
Vicinamibacter silvestris	**AB2** vs AB48	*Luteolibacter pohnpeiensis*	**AF24** vs AF2	*Paenarthrobacter nicotinovorans*	**BF48** vs BF24
Trichocoleus desertorum	**AB2** vs AB48	*Achromobacter insloitus*	**AF48** vs AF2	*Ensifer adharenes*	**BF48** vs BF24
Janthinobacterium sp. LM6	**AB2** vs AB48	*Achromobacter xylosoxidans*	**AF48** vs AF2	*Pontibacter chitinlyticus*	**BF48** vs BF24
Anabaena cylindrica	**AB2** vs AB48	*Agrobacterium tumefaciens*	**AF48** vs AF2	*Larkinella harenae*	**BF48** vs BF24

Table 3 Differential abundance of 3 plant mixtures.

Alfalfa-Brassica-Fescue	**Enrich Group (bolded)**
Paenisporosarcina indica	**ABF3** vs ABF24
Trichocoleus desertorum	**ABF3** vs ABF48
Stenotrophomonas sp. DAIF1	**ABF3** vs ABF48
Geitlerinema sp. PCC 7407	**ABF3** vs ABF48
Azospirillum brasilense	**ABF3** vs ABF48
Azospirillum sp. TSH58	**ABF3** vs ABF48
Devosia riboflavina	**ABF3** vs ABF48
Oscillatoria nigro-viridis	**ABF3** vs ABF48
Noviherbaspirillum denitrificans	**ABF3** vs ABF48
Arcticibacter svalbardensis	**ABF3** vs ABF48
Brevifollis gellanilyticus	**ABF3** vs ABF48
Azospirillum sp. TSA2s	**ABF3** vs ABF48
Planctomyces sp. SH-PL14	**ABF24** vs ABF3
Planctomyces sp. SH-PL14	**ABF48** vs ABF3
Sandaracinus amylolyticus	**ABF48** vs ABF3

Table 4: Experimental set up of the 21 different treatments

	Monoculture	Polyculture
Low Density (1-3 Plants per microcosm)	1. Alfalfa (1 Plant) 2. Brassica (1 Plant) 3. Fescue (1 Plant)	10. Alfalfa (1 Plant) and Brassica (1 Plant) 11. Alfalfa (1 Plant) and Fescue (1 Plant) 12. Brassica (1 Plant) and Fescue (1 Plant) 13. Alfalfa (1 Plant), Brassica (1 Plant), and Fescue (1 Plant)
Medium Density (24 Plants per microcosm)	4. Alfalfa (24 Plants) 5. Brassica (24 Plants) 6. Fescue (24 Plants)	14. Alfalfa (12 Plants) and Brassica (12 Plants) 15. Alfalfa (12 Plants) and Fescue (12 Plants) 16. Brassica (12 Plants) and Fescue (12 Plants) 17. Alfalfa (8 Plants), Brassica (8 Plants), and Fescue (8 Plants)
High Density (48 Plants per microcosm)	7. Alfalfa (48 Plants) 8. Brassica (48 Plants) 9. Fescue (48 Plants)	18. Alfalfa (24 Plants) and Brassica (24 Plants) 19. Alfalfa (24 Plants) and Fescue (24 Plants) 20. Brassica (24 Plants) and Fescue (24 Plants) 21. Alfalfa (16 Plants), Brassica (16 Plants), and Fescue (16 Plants)

Table 5: Analysis of Principal Coordinates Summary:

Alfalfa Monocrop

```
Permutation test for adonis under reduced model
Marginal effects of terms
Permutation: free
Number of permutations: 999

adonis2(formula = dist ~ Density, data = d, permutations = 999, by = "margin")
         Df SumOfSqs      R2      F Pr(>F)    
Density   2  0.51724 0.28586 2.2015  0.001 ***
Residual 11  1.29221 0.71414                  
Total    13  1.80945 1.00000                  
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

        Homogeneity of multivariate dispersions

Call: betadisper(d = dist, group = d$Density)

No. of Positive Eigenvalues: 13
No. of Negative Eigenvalues: 0

Average distance to median:
     1     24     48 
0.2893 0.3251 0.2841 

Eigenvalues for PCoA axes:
(Showing 8 of 13 eigenvalues)
  PCoA1   PCoA2   PCoA3   PCoA4   PCoA5   PCoA6   PCoA7   PCoA8 
0.48797 0.19715 0.18176 0.15724 0.14892 0.13327 0.10969 0.08664 
```

Brassica Monocrop

```
Permutation test for adonis under reduced model
Marginal effects of terms
Permutation: free
Number of permutations: 999

adonis2(formula = dist ~ Density, data = d, permutations = 999, by = "margin")
         Df SumOfSqs      R2      F Pr(>F)   
Density   2  0.33811 0.18302 1.4562   0.01 **
Residual 13  1.50923 0.81698                 
Total    15  1.84734 1.00000                 
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

        Homogeneity of multivariate dispersions

Call: betadisper(d = dist, group = d$Density)

No. of Positive Eigenvalues: 15
No. of Negative Eigenvalues: 0

Average distance to median:
     1     24     48 
0.2861 0.3165 0.3047 

Eigenvalues for PCoA axes:
(Showing 8 of 15 eigenvalues)
 PCoA1  PCoA2  PCoA3  PCoA4  PCoA5  PCoA6  PCoA7  PCoA8 
0.3096 0.2407 0.1880 0.1846 0.1462 0.1296 0.1092 0.1027 
```

Fescue Monocrop

```
Permutation test for adonis under reduced model
Marginal effects of terms
Permutation: free
Number of permutations: 999

adonis2(formula = dist ~ Density, data = d, permutations = 999, by = "margin")
         Df SumOfSqs      R2      F Pr(>F)
Density   2  0.40227 0.20776 1.5735  0.002 **
Residual 12  1.53394 0.79224
Total    14  1.93621 1.00000
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

        Homogeneity of multivariate dispersions

Call: betadisper(d = dist, group = d$Density)

No. of Positive Eigenvalues: 14
No. of Negative Eigenvalues: 0

Average distance to median:
     1     24     48
0.2862 0.3227 0.3367

Eigenvalues for PCoA axes:
(Showing 8 of 14 eigenvalues)
 PCoA1  PCoA2  PCoA3  PCoA4  PCoA5  PCoA6  PCoA7  PCoA8
0.3840 0.2502 0.2101 0.1960 0.1512 0.1317 0.1187 0.1011
```

Alfalfa-Brassica Mixture

```
Permutation test for adonis under reduced model
Marginal effects of terms
Permutation: free
Number of permutations: 999

adonis2(formula = dist ~ Density, data = d, permutations = 999, by = "margin")
         Df SumOfSqs      R2      F Pr(>F)
Density   2  0.59762 0.33196 2.9815  0.002 **
Residual 12  1.20265 0.66804
Total    14  1.80027 1.00000
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

        Homogeneity of multivariate dispersions

Call: betadisper(d = dist, group = d$Density)

No. of Positive Eigenvalues: 14
No. of Negative Eigenvalues: 0

Average distance to median:
     1     24     48
0.3050 0.2720 0.2586

Eigenvalues for PCoA axes:
(Showing 8 of 14 eigenvalues)
  PCoA1   PCoA2   PCoA3   PCoA4   PCoA5   PCoA6   PCoA7   PCoA8
0.56528 0.20124 0.18656 0.13755 0.12447 0.10007 0.09422 0.07858
```

Alfalfa-Fescue Mixture

```
Permutation test for adonis under reduced model
Marginal effects of terms
Permutation: free
Number of permutations: 999

adonis2(formula = dist ~ Density, data = d, permutations = 999, by = "margin")
         Df SumOfSqs      R2      F Pr(>F)
Density   2   0.3557 0.19184 1.4243   0.02 *
Residual 12   1.4985 0.80816
Total    14   1.8542 1.00000
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

        Homogeneity of multivariate dispersions

Call: betadisper(d = dist, group = d$Density)

No. of Positive Eigenvalues: 14
No. of Negative Eigenvalues: 0

Average distance to median:
     1     24     48
0.3073 0.3067 0.3240

Eigenvalues for PCoA axes:
(Showing 8 of 14 eigenvalues)
 PCoA1  PCoA2  PCoA3  PCoA4  PCoA5  PCoA6  PCoA7  PCoA8
0.3509 0.2404 0.1942 0.1724 0.1366 0.1211 0.1106 0.1053
```

Brassica-Fescue Mixture

```
Permutation test for adonis under reduced model
Marginal effects of terms
Permutation: free
Number of permutations: 999

adonis2(formula = dist ~ Density, data = d, permutations = 999, by = "margin")
         Df SumOfSqs      R2      F Pr(>F)
Density   2  0.27282 0.20319 1.4026  0.038 *
Residual 11  1.06983 0.79681
Total    13  1.34265 1.00000
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

        Homogeneity of multivariate dispersions

Call: betadisper(d = dist, group = d$Density)

No. of Positive Eigenvalues: 13
No. of Negative Eigenvalues: 0

Average distance to median:
     1     24     48
0.2849 0.2571 0.2755

Eigenvalues for PCoA axes:
(Showing 8 of 13 eigenvalues)
  PCoA1   PCoA2   PCoA3   PCoA4   PCoA5   PCoA6   PCoA7   PCoA8
0.26313 0.20227 0.13826 0.11958 0.09981 0.09393 0.08693 0.07620
```

Alfalfa-Brassica-Fescue Mixture

```
Permutation test for adonis under reduced model
Marginal effects of terms
Permutation: free
Number of permutations: 999

adonis2(formula = dist ~ Density, data = d, permutations = 999, by = "margin")
         Df SumOfSqs      R2      F Pr(>F)  
Density   2  0.34206 0.18294 1.3434  0.069 .
Residual 12  1.52768 0.81706                
Total    14  1.86974 1.00000                
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

        Homogeneity of multivariate dispersions

Call: betadisper(d = dist, group = d$Density)

No. of Positive Eigenvalues: 14
No. of Negative Eigenvalues: 0

Average distance to median:
     1     24     48 
0.3123 0.3035 0.3279 

Eigenvalues for PCoA axes:
(Showing 8 of 14 eigenvalues)
  PCoA1   PCoA2   PCoA3   PCoA4   PCoA5   PCoA6   PCoA7   PCoA8 
0.39936 0.24266 0.20532 0.17901 0.13841 0.12668 0.10176 0.09281 
```

Table 6: Aboveground plant biomass per individual plant by treatment

Alfalfa Treatment	Individual Plant weight (g)	Brassica Treatment	Individual Plant weight (g)	Fescue Treatment	Individual Plant weight (g)
Alfalfa 1	0.349	**Brassica** 1	0.344	**Fescue** 1	0.168
Alfalfa-brassica1	0.109	alfalfa-**Brassica** 1	0.316	alfalfa-**Fescue** 1	0.125
Alfalfa-fescue 1	0.334	**Brassica-**fescue 1	0.311	brassica-**Fescue** 1	0.011
Alfalfa-brassica-fescue 1	0.097	alfalfa-**Brassica**-fescue 1	0.249	alfalfa-brassica-**Fescue** 1	0.016
Alfalfa 24	0.037	**Brassica** 24	0.016	**Fescue** 24	0.014
Alfalfa-brassica 24	0.018	alfalfa-**Brassica** 24	0.023	alfalfa-**Fescue** 24	0.018
Alfalfa-fescue 24	0.048	**Brassica-**fescue 24	0.026	brassica-**Fescue** 24	0.004
Alfalfa-brassica-fescue 24	0.022	alfalfa-**Brassica**-fescue 24	0.028	alfalfa-brassica-**Fescue** 24	0.004
Alfalfa 48	0.031	**Brassica** 48	0.012	**Fescue** 48	0.010
Alfalfa-brassica 48	0.016	alfalfa-**Brassica** 48	0.013	alfalfa-**Fescue** 48	0.010
Alfalfa-fescue 48	0.041	**Brassica-**fescue 48	0.012	brassica-**Fescue** 48	0.004
Alfalfa-brassica-fescue 48	0.016	alfalfa-**Brassica**-fescue 48	0.013	alfalfa-brassica-**Fescue** 48	0.004

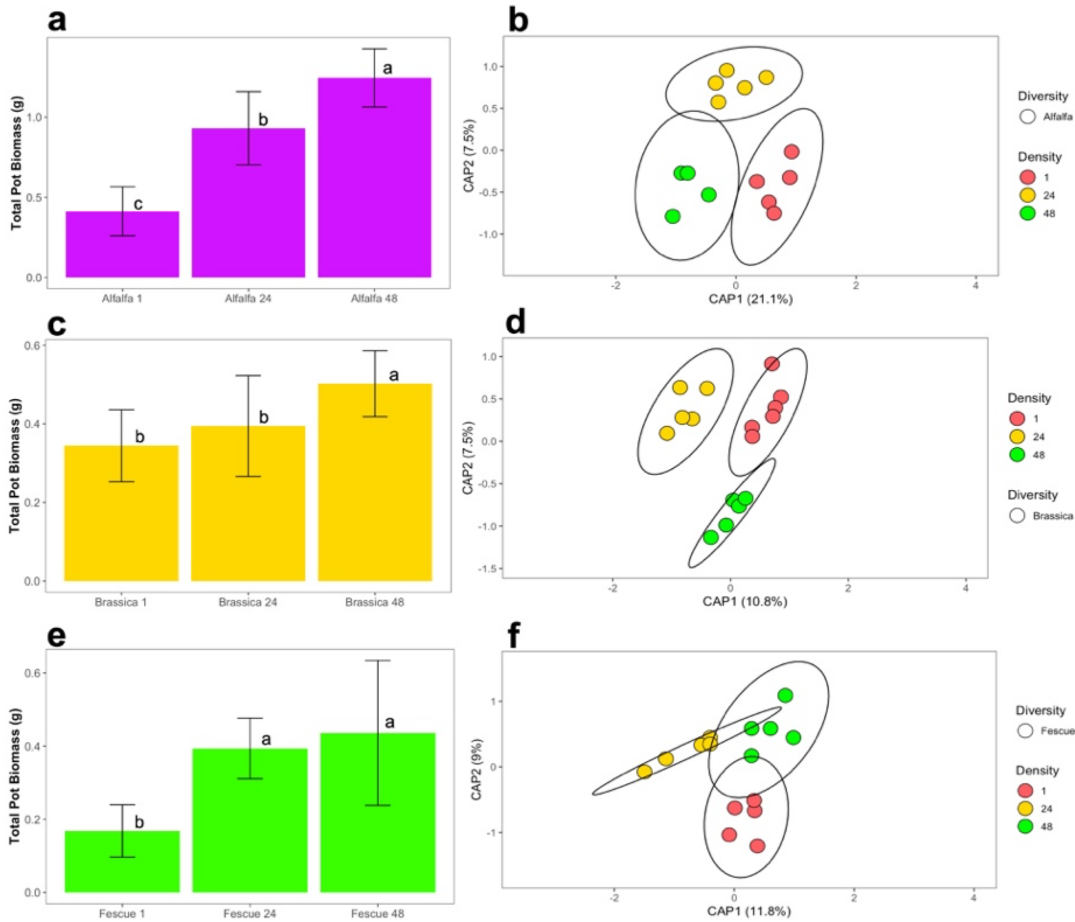

Figure 1. Above ground dry biomass in monoculture for crop densities of one plant total, 24 plants total, and 48 plants total. (**a**) Alfalfa dry biomass (purple), (**c**) brassica dry biomass (gold), and (**e**) fescue dry biomass (green). Constrained Principal Coordinate Analysis (PCoA) using Bray–Curtis distance for comparing bulk soil bacteriomes of increasing crop densities by each individual crop (**b**) alfalfa, (**d**) brassica, and (**f**) fescue. Colors were used to represent increasing densities from 1 plant (red), 24 plants (yellow), and 48 plants (green). Letters (a, b, and c) indicate significant differences between the mean values of plant biomass with (Tukey $P < 0.05$). Error bars are the SD.

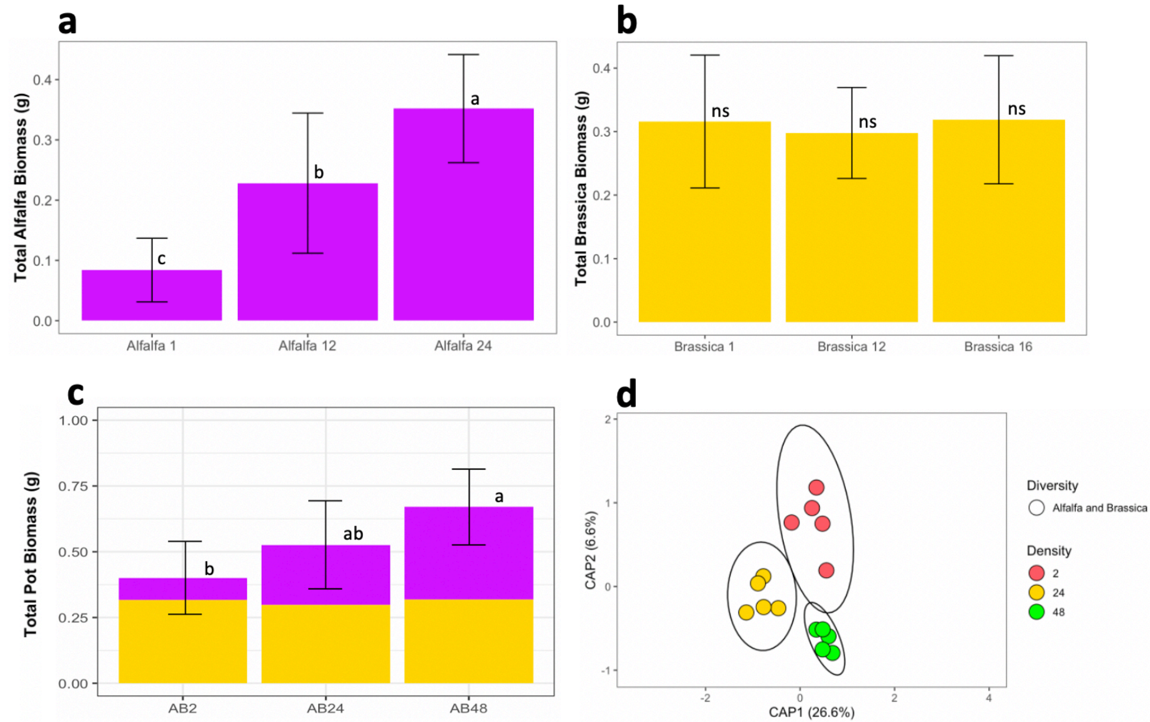

Figure 2. Above ground dry biomass for the alfalfa and brassica crop mixture densities of two plants total, 24 plants total, and 48 plants by (**a**) total alfalfa (purple), (**b**) total brassica (gold), and (**c**) total biomass (alfalfa and brassica). (**d**) Constrained Principal Coordinate Analysis (PCoA) using Bray–Curtis distance for comparing bulk soil bacteriomes of increasing crop densities of the alfalfa and brassica mixture. Colors were used to represent increasing densities from 2 plants (red), 24 plants (yellow), and 48 plants (green). Letters (a, b, and c) indicate significant differences between the mean values of plant biomass with (Tukey $P < 0.05$), and ns = not significant differences. Error bars are the SD.

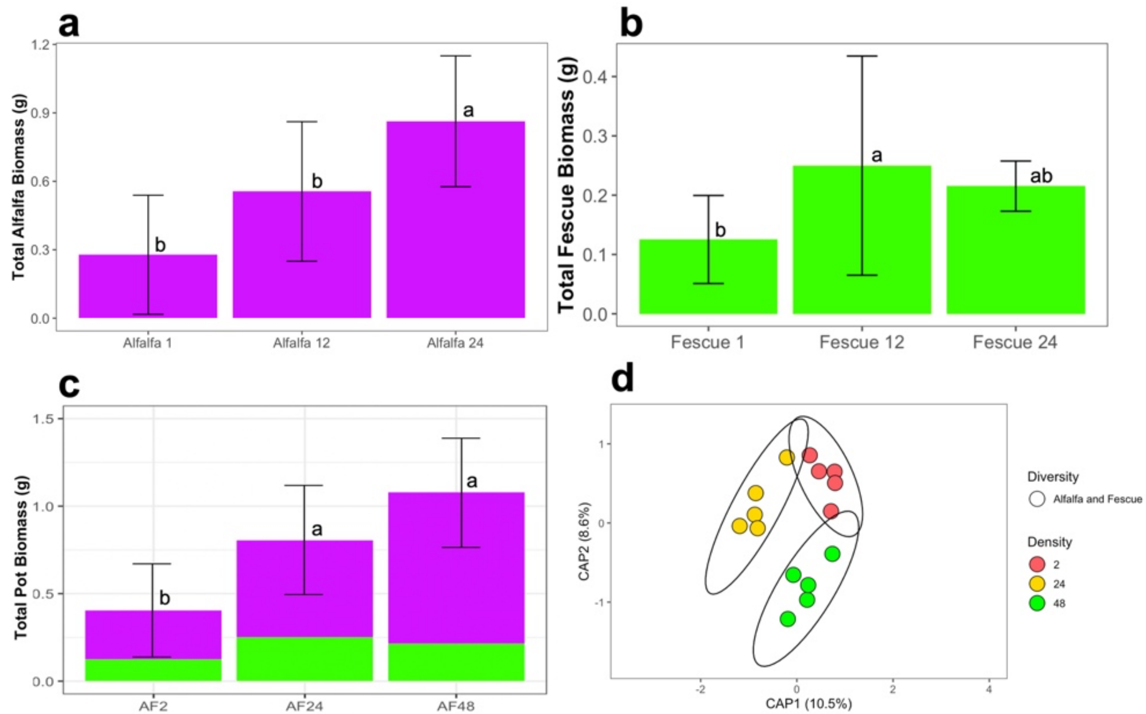

Figure 3. Above ground dry biomass for the alfalfa and fescue crop mixture densities of two plants total, 24 plants total, and 48 plants by (**a**) total alfalfa (purple), (**b**) total fescue (green), and (**c**) total biomass (alfalfa and fescue). (**d**) Constrained Principal Coordinate Analysis (PCoA) using Bray–Curtis distance for comparing bulk soil bacteriomes of increasing crop densities of the alfalfa and fescue mixture. Colors were used to represent increasing densities from 2 plants (red), 24 plants (yellow), and 48 plants (green). Letters (a, b, and c) indicate significant differences between the mean values of plant biomass with (Tukey $P < 0.05$). Error bars are the SD.

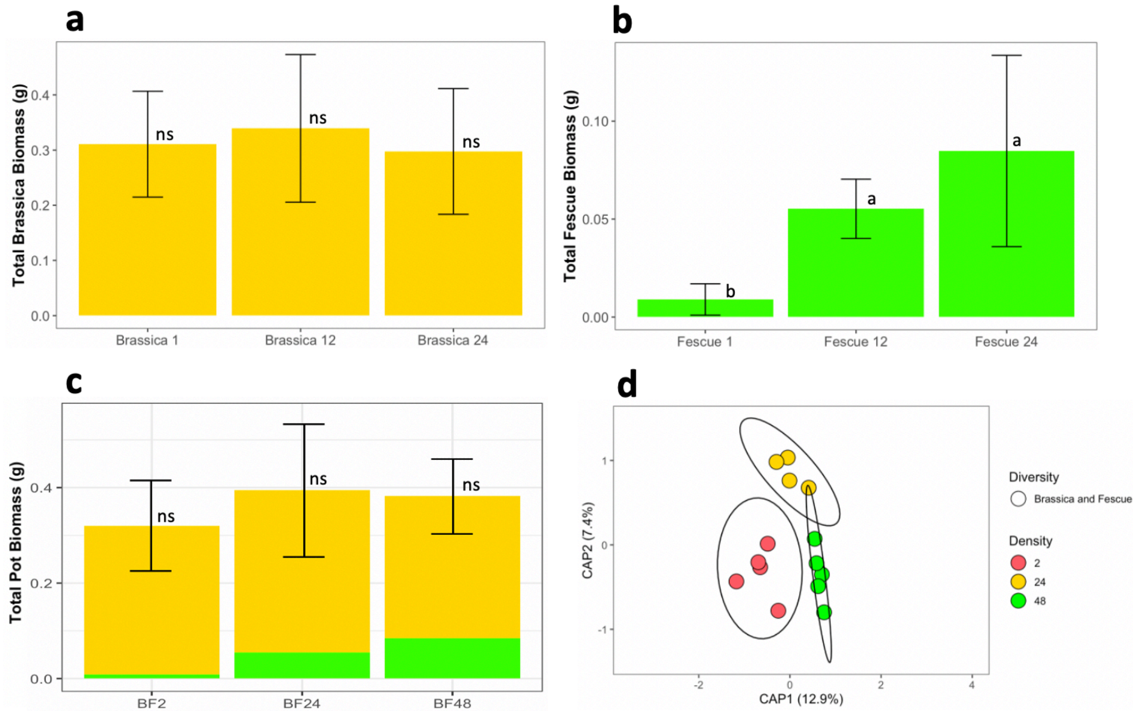

Figure 4. Above ground dry biomass for the alfalfa and fescue crop mixtures for densities of two plants total, 24 plants total, and 48 plants by (**a**) total brassica (gold), (**b**) total fescue (green), and (**c**) total brassica and fescue. (**d**) Constrained Principal Coordinate Analysis (PCoA) using Bray–Curtis distance for comparing bulk soil bacteriomes of increasing crop densities of the brassica and fescue mixture. Colors were used to represent increasing densities from 2 plants (red), 24 plants (yellow), and 48 plants (green). Letters (a, b, and c) indicate significant differences between the mean values of plant biomass with (Tukey $P < 0.05$), and ns = not significant differences. Error bars are the SD.

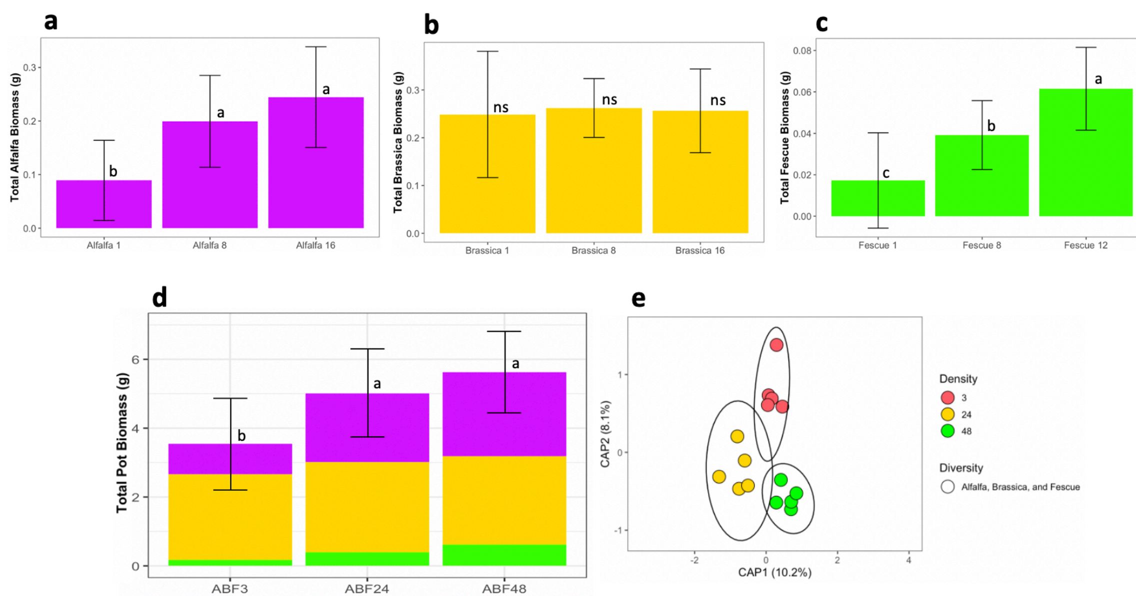

Figure 5. Above ground dry biomass for the alfalfa, brassica, and fescue crop mixture densities of three plants total, 24 plants total, and 48 plants shown separately by (**a**) total alfalfa (purple), total brassica (gold), total fescue (green), and (**b**) total biomass (alfalfa, brassica, and fescue). (**d**) Constrained Principal Coordinate Analysis (PCoA) using Bray–Curtis distance for comparing bulk soil bacteriomes of increasing crop densities of the alfalfa, brassica, and fescue mixture. Colors were used to represent increasing densities from 3 plants (red), 24 plants (yellow), and 48 plants (green). Letters (a, b, and c) indicate significant differences between the mean values of plant biomass with (Tukey $P < 0.05$), and ns = not significant differences. Error bars are the SD.

Figure 6: Picture of the microcosm experiment.

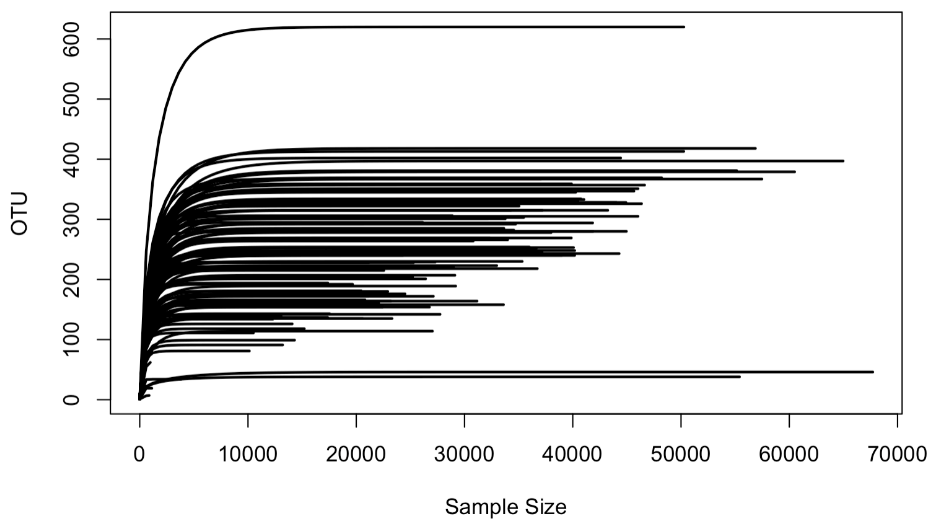

Figure 7: Rarefaction Curve for all samples.

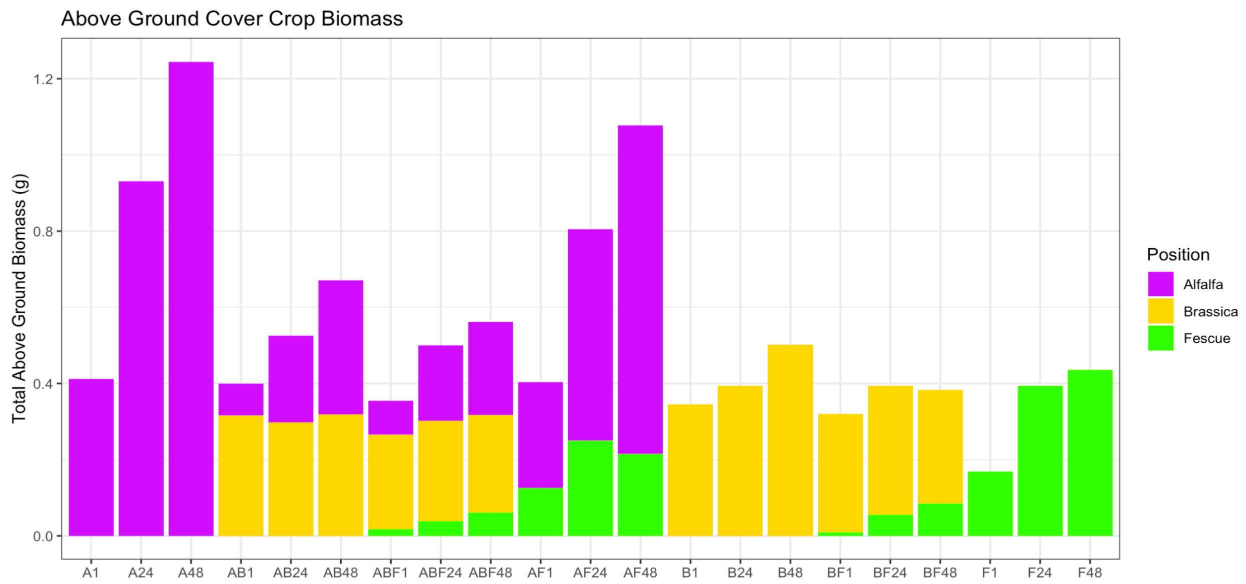

Figure 8: Total above ground cover crop biomass stacked by crop (alfalfa: purple, brassica: gold, fescue: lime green).

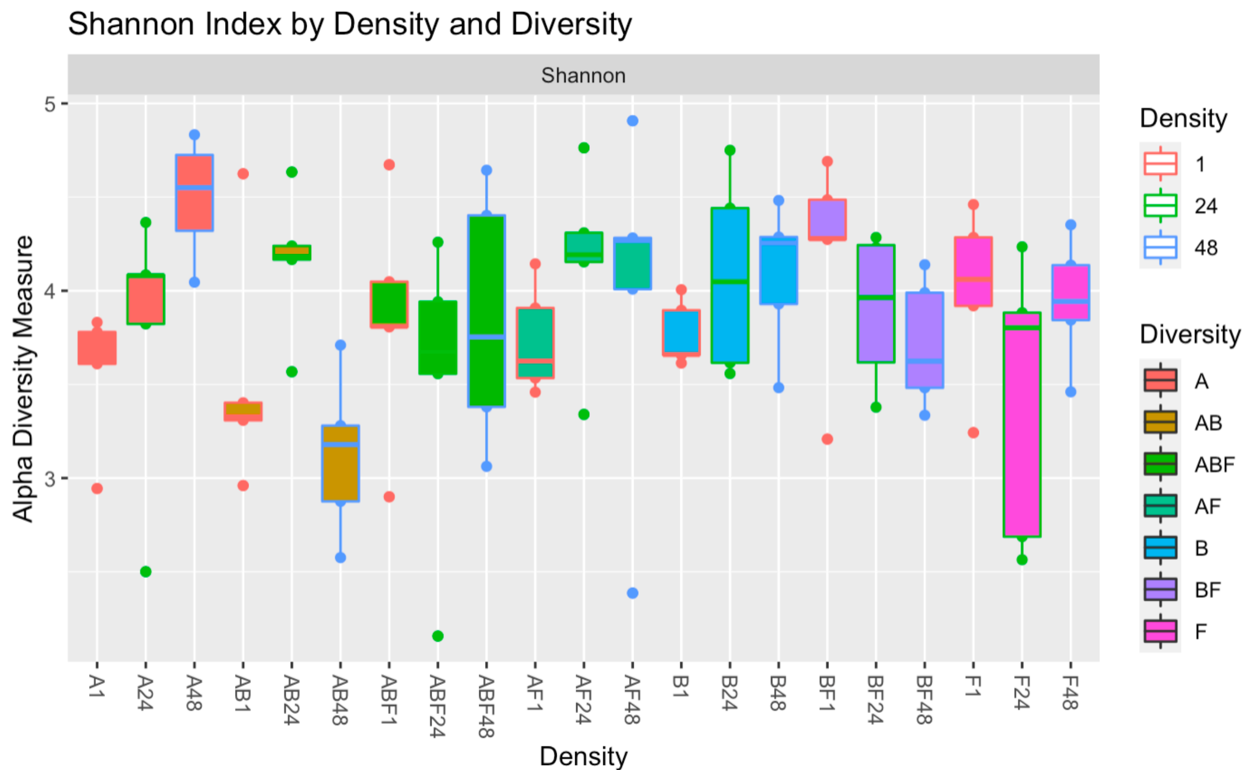

Figure 9: Alpha diversity of bacterial microbiomes denoted by increasing densities of monoculture and plant mixtures.

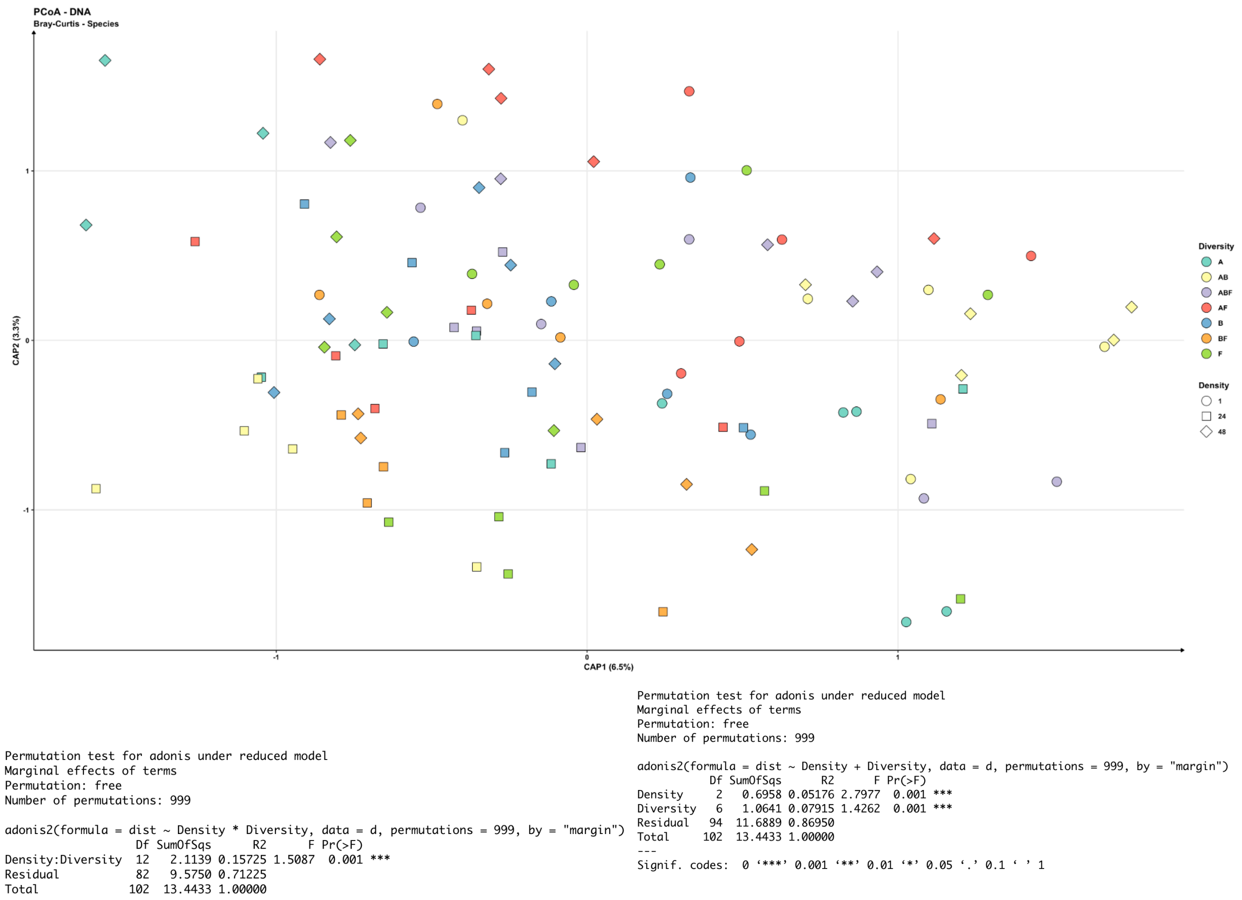

Figure 10: PERMANOVA model with all data combined of the interaction between plant diversity and density and the significance of both factors separately on the structure of bacteriomes

REFERENCES

Bar-On, Y. M., Phillips, R., & Milo, R. The biomass distribution on Earth. *Proceedings of the National Academy of Sciences*, **115(25),** 6506-6511, (2018).

Hu, L. *et al*. Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nature communications*, **9(1),** 1-13, (2018).

Zhou, X., Leite, M. F., Zhang, Z., Tian, L., Chang, J., Ma, L., ... & Kuramae, E. E. Facilitation in the soil microbiome does not necessarily lead to niche expansion. *Environmental microbiome*, **16(1),** 1-13, (2021).

Wagg, C., Schlaeppi, K., Banerjee, S., Kuramae, E. E., & van der Heijden, M. G. Fungal-bacterial diversity and microbiome complexity predict ecosystem functioning. *Nature communications*, **10(1),** 1-10, (2019).

Malik, R. K., Green, T. H., Brown, G. F., & Mays, D. Use of cover crops in short rotation hardwood plantations to control erosion. *Biomass and Bioenergy*, **18(6),** 479-487, (2000).

Franchini, J. C., Pavan, M. A., & Miyazawa, M. Redistribution of phosphorus in soil through cover crop roots. *Brazilian Archives of Biology and Technology*, **47(3),** 381-386, (2004).
Boudreau, M. A. Diseases in intercropping systems. *Annual review of phytopathology*, **51,** 499-519, (2013).

Lal, R. Soil carbon sequestration and aggregation by cover cropping. *Journal of Soil and Water Conservation*, **70(6),** 329-339, (2015).

Osipitan, O. A., Dille, J. A., Assefa, Y., & Knezevic, S. Z. Cover crop for early season weed suppression in crops: Systematic review and meta-analysis. *Agronomy Journal*, **110(6),** 2211-2221, (2018).

Reeves, D. W. Cover crops and rotations. *Crops residue management*, 125-172, (2018).
Couëdel, A., Kirkegaard, J., Alletto, L., & Justes, E. Crucifer-legume cover crop mixtures for biocontrol: Toward a new multi-service paradigm. *Advances in Agronomy*, **157,** 55-139, (2019).

Abdalla, M. *et al*. A critical review of the impacts of cover crops on nitrogen leaching, net greenhouse gas balance and crop productivity. *Global change biology*, **25(8),** 2530-2543, (2019).

Giller, K. E., Hijbeek, R., Andersson, J. A., & Sumberg, J. Regenerative agriculture: An agronomic perspective. Outlook on Agriculture, **50(1),** 13-25, (2021).

Stanger, T. F., & Lauer, J. G. Corn grain yield response to crop rotation and nitrogen over 35 years. *Agronomy journal*, **100(3),** 643-650, (2008).

Pacheco-Cano, R. D., Salcedo-Hernández, R., López-Meza, J. E., Bideshi, D. K., & Barboza-Corona, J. E. Antimicrobial activity of broccoli (Brassica oleracea var. italica) cultivar Avenger against pathogenic bacteria, phytopathogenic filamentous fungi and yeast. *Journal of applied microbiology*, **124(1),** 126-135, (2018).

Bergkvist, G., Adler, A., Hansson, M., & Weih, M. Red fescue undersown in winter wheat suppresses Elytrigia repens. *Weed Research*, **50(5),** 447-455, (2010).

MacLaren, C., Swanepoel, P., Bennett, J., Wright, J., & Dehnen-Schmutz, K. Cover crop biomass production is more important than diversity for weed suppression. *Crop Science*, **59(2),** 733-748, (2019).

Lotz, L. A. P., Groeneveld, R. M. W., Theunissen, J., & Van Den Broek, R. C. F. M. Yield losses of white cabbage caused by competition with clovers grown as cover crop. *Netherlands Journal of Agricultural Science*, **45(3),** 393-405, (1997).

Neumann, A., Werner, J., & Rauber, R. Evaluation of yield–density relationships and optimization of intercrop compositions of field-grown pea–oat intercrops using the replacement series and the response surface design. *Field crops research*, **114(2),** 286-294, (2009).

Lamb, E. G., Kennedy, N., & Siciliano, S. D. Effects of plant species richness and evenness on soil microbial community diversity and function. *Plant and Soil*, **338(1),** 483-495, (2011).

Maron, P. A., Mougel, C., & Ranjard, L. Soil microbial diversity: methodological strategy, spatial overview and functional interest. *Comptes rendus biologies*, **334(5-6),** 403-411, (2011).

Ghimire, R., Lamichhane, S., Acharya, B. S., Bista, P., & Sainju, U. M. Tillage, crop residue, and nutrient management effects on soil organic carbon in rice-based cropping systems: A review. *Journal of integrative agriculture*, **16(1),** 1-15, (2017).

Li, K., DiLegge, M. J., Minas, I. S., Hamm, A., Manter, D., & Vivanco, J. M. Soil sterilization leads to re-colonization of a healthier rhizosphere microbiome. *Rhizosphere*, ***12*,** 100176, (2019).

Monohon, S. J., Manter, D. K., & Vivanco, J. M. Conditioned soils reveal plant-selected microbial communities that impact plant drought response. *Scientific Reports*, **11(1),** 21153, (2021).

DiLegge, M. J., Manter, D. K., & Vivanco, J. M. Soil microbiome disruption reveals specific and general plant-bacterial relationships in three agroecosystem soils. *Plos one*, ***17*(11),** e0277529, (2022).

Blouin, M., & Jacquiod, S. Sampling the control bulk soil for rhizosphere and drilosphere microbial studies. *Geoderma*, **15(380)**, 114674, (2020).

Lenth, R. V. Estimated marginal means, aka least-squares means [R Package Emmeans Version 1.6. 0]. *Comprehensive R Archive Network (CRAN)*, (2021).

Graves, S., Piepho, H., Selzer, L., & Dorai-Raj, S. multcompView: visualizations of paired comparisons. R package, (2019).

Wickham, H., Romain F., & Lionel H. Müller K. dplyr: a grammar of data manipulation. 2020. (2020).

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., & Suggests, M. A. S. S. The vegan package. *Community ecology package* 10, **719**, 631-637, (2007).

Hagenfeld, D. *et al*. Do we treat our patients or rather periodontal microbes with adjunctive antibiotics in periodontal therapy? A 16S rDNA microbial community analysis. *PloS one*, **13(4),** e0195534, (2018).

McMurdie, P. J., & Holmes, S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one*, ***8*(4),** e61217, (2013).

Gao, N., Shen, W., Nishizawa, T., Isobe, K., Guo, Y., Ying, H., & Senoo, K. Genome sequences of two Azospirillum sp. strains, TSA2S and TSH100, plant growth-promoting rhizobacteria with N2O mitigation abilities. *Microbiology Resource Announcements*, **8(32),** e00459-19, (2019).

He, J. W., Hassan, Y. I., Perilla, N., Li, X. Z., Boland, G. J., & Zhou, T. Bacterial epimerization as a route for deoxynivalenol detoxification: the influence of growth and environmental conditions. *Frontiers in microbiology*, **7,** 572, (2016).

Zhou, G. C., Wang, Y., Zhai, S., Ge, F., Liu, Z. H., Dai, Y. J., ... & Hou, J. Y. Biodegradation of the neonicotinoid insecticide thiamethoxam by the nitrogen-fixing and plant-growth-promoting rhizobacterium Ensifer adhaerens strain TMX-23. *Applied microbiology and biotechnology*, **97,** 4065-4074, (2013).

Biró, B., Köves-Péchy, K., Vörös, I., Takács, T., Eggenberger, P., & Strasser, R. J. Interrelations between Azospirillum and Rhizobium nitrogen-fixers and arbuscular mycorrhizal fungi in the rhizosphere of alfalfa in sterile, AMF-free or normal soil conditions. *Applied Soil Ecology*, **15(2),** 159-168, (2000).

Nakagawa, Y., Sakane, T., & Yokota, A. Transfer of "Pseudomonas riboflavina"(Foster 1944), a gram-negative, motile rod with long-chain 3-hydroxy fatty acids, to Devosia riboflavina gen. nov., sp. nov., nom. rev. *International Journal of Systematic and Evolutionary Microbiology*, **46(1),** 16-22, (1996).

Taye, Z. M. et al. Core and differentially abundant bacterial taxa in the rhizosphere of field grown *Brassica napus* genotypes: implications for canola breeding. *Frontiers in Microbiology*, **10,** 3007, (2020).

Simmons, T. L. *et al*. A and B, lipodepsipeptides with antiprotozoal activity from the marine cyanobacterium Oscillatoria nigro-viridis. *Journal of natural products*, **71(9),** 1544-1550, (2008).

Maboko, M. M. Effect of plant density and harvesting frequency on yield components of hydroponically grown mustard spinach (Brassica juncea). *In II All Africa Horticulture Congress*, **1007,** 515-521, (2012).

Qin, X., Li, P., Lu, S., Sun, Y., Meng, L., Hao, J., & Fan, S. Phosphoproteomic analysis of lettuce (Lactuca sativa L.) reveals starch and sucrose metabolism functions during bolting induced by high temperature. *PLoS One*, **15(12),** e0244198, (2020).

Rai, S. N., & Gaur, A. C. Nitrogen fixation by Azospirillum spp. and effect of Azospirillum lipoferum on the yield and N-uptake of wheat crop. *Plant and Soil*, **69,** 233-238, (1982).

Cassán, F., & Diaz-Zorita, M. Azospirillum sp. in current agriculture: From the laboratory to the field. *Soil Biology and Biochemistry*, **103,** 117-130, (2016).

Deschênes, J. M. Intraspecific competition in experimental populations of weeds. *Canadian Journal of Botany*, **52(6),** 1415-1421, (1974).

Zhou, B., Bailey, A., Niblett, C. L., & Qu, R. Control of brown patch (Rhizoctonia solani) in tall fescue (Festuca arundinacea Schreb.) by host induced gene silencing. *Plant cell reports*, **35(4),** 791-802, (2016).

Ramana, V. V., Sasikala, C., Takaichi, S., & Ramana, C. V. Roseomonas aestuarii sp. nov., a bacteriochlorophyll-a containing alphaproteobacterium isolated from an estuarine habitat of India. *Systematic and Applied Microbiology*, **33(4),** 198-203, (2010).

Adeleke, B. S., Ayangbenro, A. S., & Babalola, O. O. Genomic assessment of Stenotrophomonas indicatrix for improved sunflower plant. *Current Genetics*, **67,** 891-907, (2021).

Mishra, S., Singh, B. R., Naqvi, A. H., & Singh, H. B. Potential of biosynthesized silver nanoparticles using Stenotrophomonas sp. BHU-S7 (MTCC 5978) for management of soil-borne and foliar phytopathogens. *Scientific reports*, **7(1),** 1-15, (2017).

Berg, G., Egamberdieva, D., Lugtenberg, B., & Hagemann, M. Symbiotic plant–microbe interactions: stress protection, plant growth promotion, and biocontrol by Stenotrophomonas. *Symbioses and stress: joint ventures in biology,* 445-460, (2010).

Xin-Bo, L., Jian-Zhi, X., Bo-Wen, L., & Wei, W. Ecological responses of Brassica juncea-alfalfa intercropping to cadmium stress. Yingyong Shengtai Xuebao, **20(7),** (2009).

Lankau, R. A., Wheeler, E., Bennett, A. E., & Strauss, S. Y. Plant–soil feedbacks contribute to an intransitive competitive network that promotes both genetic and species diversity. *Journal of Ecology*, **99(1),** 176-185, (2011).

Lalucat, J., Bennasar, A., Bosch, R., García-Valdés, E., & Palleroni, N. J. Biology of Pseudomonas stutzeri. *Microbiology and molecular biology reviews*, **70(2),** 510-547, (2006).

Tshishonga, K., & Serepa-Dlamini, M. H. Draft genome sequence of Pseudarthrobacter phenanthrenivorans Strain MHSD1, a bacterial endophyte isolated from the medicinal plant Pellaea calomelanos. *Evolutionary Bioinformatics*, **16,** 1176934320913257, (2020).

Tracy, B. F. *et al*. Evaluation of alfalfa–tall fescue mixtures across multiple environments. *Crop Science*, **56(4),** 2026-2034, (2016).

Ho, Y. N., Hsieh, J. L., & Huang, C. C. Construction of a plant–microbe phytoremediation system: Combination of vetiver grass with a functional endophytic bacterium, Achromobacter xylosoxidans F3B, for aromatic pollutants removal. *Bioresource technology*, **145,** 43-47, (2013).

Ghosh, R., Chatterjee, S., & Mandal, N. C. Stenotrophomonas. In Beneficial Microbes in Agro-Ecology. *Academic Press*, 427-442, (2020).

Lopes, J. R., Daugherty, M. P., & Almeida, R. P. Context-dependent transmission of a generalist plant pathogen: host species and pathogen strain mediate insect vector competence. *Entomologia Experimentalis et Applicata*, **131(2),** 216-224, (2009).

Gohlke, J., & Deeken, R. Plant responses to Agrobacterium tumefaciens and crown gall development. *Frontiers in plant science*, **5,** 155, (2014).

Palumbo, J. D., Phillips, D. A., & Kado, C. I. Characterization of a new Agrobacterium tumefaciens strain from alfalfa (Medicago sativa L.). *Archives of microbiology*, **169,** 381-386, (1998).

Dong, S., & Qu, R. High efficiency transformation of tall fescue with Agrobacterium tumefaciens. *Plant Science*, **168(6),** 1453-1458, (2005).

Toosi, A. F. Production ecology of Brassica Juncea (L.) czern. var. ensabi (Doctoral dissertation, University of Malaya), (2010).

Karthik, N., Binod, P., & Pandey, A. Purification and characterisation of an acidic and antifungal chitinase produced by a Streptomyces sp. *Bioresource Technology*, **188,** 195-201, (2015).

Chhetri, G., Kim, J., Kim, I., Kim, M. K., & Seo, T. Pontibacter chitinilyticus sp. nov., a novel chitin-hydrolysing bacterium isolated from soil. *Antonie van Leeuwenhoek*, **112,** 1011-1018, (2019).

Tien, T. M., Gaskins, M. H., & Hubbell, D. Plant growth substances produced by Azospirillum brasilense and their effect on the growth of pearl millet (Pennisetum americanum L.). *Applied and environmental microbiology*, **37(5),** 1016-1024, (1979).

Kulichevskaya, I. S., Naumoff, D. G., Ivanova, A. A., Rakitin, A. L., & Dedysh, S. N. Detection of chitinolytic capabilities in the freshwater planctomycete Planctomicrobium piriforme. *Microbiology*, **88,** 423-432, (2019).

Sharma, G., Khatri, I., & Subramanian, S. Complete genome of the starch-degrading myxobacteria Sandaracinus amylolyticus DSM 53668T. *Genome biology and evolution*, **8(8),** 2520-2529, (2016).

Steinmetz, H. et al. Indiacens A and B: prenyl indoles from the myxobacterium *Sandaracinus amylolyticus*. *Journal of Natural Products*, **75(10),** 1803-1805, (2012).

Pélissier, R., Ducasse, A., Ballini, E., Frouin, J., Violle, C., & Morel, J. B. A major genetic locus in neighbours controls changes of gene expression and susceptibility in intra-specific rice mixtures. *New Phytologist*, (2023).

Ferraguti, M., Martínez-de la Puente, J., Jiménez–Clavero, M. Á., Llorente, F., Roiz, D., Ruiz, S., ... & Figuerola, J. A field test of the dilution effect hypothesis in four avian multi-host pathogens. *PLoS Pathogens*, **17(6),** e1009637, (2021).

Chaparro, J. M., Badri, D. V., & Vivanco, J. M. Rhizosphere microbiome assemblage is affected by plant development. *The ISME journal*, ***8*****(4),** 790-803, (2014).

CHAPTER 4 PLANT NEIGHBORS BARE NO IMPACT ON THE STRONGLY CORRELATED RHIZOBACTERIA THAT A PLANT ENRICHES BUT DO INFLUENCE THE ENRICHMENT OF CONDITION SPECIFIC ASSOCIATED BACTERIAL TAXA.

Synopsis

Many root and rhizosphere studies have focused on analyzing single-plant microbiomes without considering potential influences neighboring plants might have on the target species' microbial recruitment. The rhizosphere microbiome of individual plants was analyzed in this microcosm study containing different combinations and densities of complementing cover crops *Medicago sativa*, *Brassica* sp., and *Fescue* sp.. Microbial beta diversity in the rhizosphere was promoted by increasing plant diversity around the target species and reduced by increasing plant density. Regardless of plant neighbor identity or density, a low number of bacteria were strongly associated with the target species. Alfalfa was shown to be strongly correlated with rhizobacteria that fix nitrogen, produce phytohormones, solubilize phosphorus, and are heavy metal resistant. Brassica was strongly correlated with rhizobacteria that produce auxin and other phytohormones. Fescue was strongly correlated with rhizobacteria that can fix nitrogen, solubilize phosphorus, and biosynthesize siderophores, which are nutrient chelators that protect against phytopathogens. However, a few bacterial taxa were shown to have conditional associations with their plant host since their enrichment was influenced by plant neighbor or density. For example, *Pseudarthrobacter oxydans* was enriched in monoculture and only in plant densities of 48 plants as compared to the single plant control. *P. oxydans* may have the ability to alleviate high competition pressure since it is able to synthesize indole-3-acetic acid, fix nitrogen (ammonia production), solubilize phosphorus, and is resistant to heavy metals. Overall, this study shows the

resiliency of bacterial recruitment by plants and demonstrates how the primary modules of the rhizosphere network shifts depending on the neighboring plant species.

Introduction

Roots and soil microorganisms have co-evolved for millions of years (Goh et al., 2013). Some root-recruited microorganisms are plant species-specific and provide services that are critical for the plant's survival (Berendsen et al., 2012; DiLegge et al., 2022). Due to these services, the plant host and associated microbial community become inseparable, collectively forming a holobiont (Lyu et al., 2021).

Although plants and microorganisms may compete for nutrients (Wallenstein, 2017), there is a mutualistic component to their association (Hillesland, 2018). Microorganisms have been shown to improve plant fitness by removing environmental stress, moderating plant development, mediating immune responses toward pathogens, and even indirectly influencing plant phenotypic plasticity (Berendsen et al., 2012; Goh et al., 2013). In exchange, plant roots secrete sugars, organic acids, phenolics, and amino acids, which microorganisms utilize as substrates or signals (Morgan et al., 2005; Azaizeh et al., 1995). Additionally, roots change the soil physical structure, absorb moisture, secrete phytochemicals, and slough off root cells, while senesced above-ground plant material increases organic carbon in the soil (Wallenstein, 2017; Pang et al., 2021; Walker et al., 2003). By altering the soil habitat of microorganisms, plants influence microorganisms (Wallenstein, 2017) and microbial behaviors (Goh et al., 2013).

The bulk soil surrounding plants serves as a reservoir of different microorganisms for the roots to recruit and culture based on the plant's developmental and environmental needs (Chen et al., 2023; Pantigoso et al., 2022; Monohon, et al., 2021; Chaparro et al., 2014). Different root exudates and architectures attract distinct microorganisms to the plant (Vives-Peris, 2020;

Pantigoso et al., 2020). After being drawn to the root exudates, microorganisms take residence in the rhizosphere, the narrow region where the roots interact with the soil (Singh & Singla, 2020; Walker et al., 2003). The following microbial compartment after the rhizosphere is the rhizoplane, which is the surface of the plant root or interface for recruited soil microorganisms (Bowen et al., 1999).

An important subset of the original bulk soil, which is first filtered in the rhizosphere, are known as endophytes (Compant et al., 2010; Marquez-Santacruz et al., 2010). Endophytes are microorganisms capable of bypassing the host's immune system and residing inside the organism without causing readily apparent harm; they have often been associated with promoting plant growth, a great service to the plant (Compant et al., 2010; Reinhold-Hurek & Hurek, 2011; Van Bael et al., 2005).

In nature, plants are often surrounded by several neighboring plants of either the same or different species. The strategies of coexistence in plant-plant interactions may be partially founded on tolerating the microorganisms recruited by neighboring plant species (Ke & Wan, 2023). According to the competitive exclusion principle, niche redundancy between microorganisms will eventually lead to some being outcompeted and consequently lost within the rhizosphere of different plant species (Martinuz et al., 2012; Navarro-Noya et al., 2012). Thus, microbial colonization in the shared rhizosphere of multiple plant species may follow a "first come, first served" strategy (Martinuz et al., 2012). Plants use multiple strategies to modulate their rhizosphere colonization, such as suppression or induction through secretion of secondary metabolites, competitive exclusion, induced resistance, or an amalgamation of these strategies (Martinuz et al., 2012). Furthermore, there is a possibility of microbe sharing. Similar to how the foundation of the human microbiome is established as the infant passes through the

birth canal (Costello et al., 2012), the seed represents an important reservoir of plant-beneficial endophytic and epiphytic microorganisms inherited by progeny plants (Nelson, 2018; Abdelfattah et al., 2023; Rodriguez et al., 2020). Just as human cohabitation (especially between couples) has been shown to promote shared skin microbiota (Song et al., 2013; Andreu-Sánchez et al., 2023), plants grown in proximity may also share microbiota not typically found when grown separately. However, further study is required to view how the colonization of shared microorganisms in the rhizosphere is influenced by plant-plant competition.

The present study aimed to provide the first glance into the effects plant density and diversity could have on the microbial composition of an individual plant's rhizosphere. This study used alfalfa (*Medicago sativa*), fescue (*Fescue* sp.), and mustard (*Brassica juncea*) to evaluate how interspecific and intraspecific plant-plant competition modifies the composition and functionality of the bacterial rhizosphere. These three plants have different growth strategies in terms of leave-shoot development, root architecture, and competitive abilities (Xu et al., 2022; Hannaway et al., 1999; Kirkegaard et al., 1993), and each recruit distinct microbial species in the root zone (Wang & Zhou, 2023; Li et al., 2022; Feng et al., 2021; Anwar et al., 2014; Liu et al., 2014; Asghar et al., 2002). The recruitment of bacteria is plant species-specific (Wang et al., 2020). The results from this study support the assertion that plant species-specific bacteria are recruited in the rhizosphere regardless of the identity or quantity of neighboring plants. The bacterial taxa that were consistently recruited by plant specific species are proposed to be strongly associated with those plants. However, the study identified another group of microbes that modify the rhizosphere of the target plant depending on the identity and density of the neighbors.

Methods

Soil Cover Crop Seed Preparation

Except for harvesting rhizosphere soil, the methods used in the present study were identical to those in Newberger et al., 2023. Soil collection occurred at the Agricultural Research, Development and Education Center South, which is owned by Colorado State University. Metal sieves (2 cm wide) were used to separate large debris from the soil. All soil was pooled prior to autoclaving. The purpose of autoclaving was to augment the plant's impact on the soil microbiome by decreasing soil microbial biomass and microbial community complexity (Li et al., 2019; Monohon et al., 2021; DiLegge et al., 2022; Newberger et al., 2023). Approximately 13.5 kg of soil per batch was autoclaved in 61 cm x 76 cm polyethylene autoclave bags using a STERIS steam autoclave (Mentor, Ohio, USA). Soils were autoclaved for three 40-min liquid cycles at 121°C. Soils were then pooled again.

Density and Diversity Greenhouse Experiment

The greenhouse experiment took place in Colorado State University's Horticultural Center Greenhouse Facility between August 1 to September 1, 2021 (31 days total). Microcosm was defined as an individual "pot" (6 cm x 4.9 cm x 5.6 cm) from a 36-cell tray, where pots were separated by approximately 2 cm. Microcosms were lined with two layers of medium duty weed fabric (Vigoro Corporation, Lake Forest, Illinois, U.S.A).

There was a total of 21 treatments. The diversity treatment was applied to 7 different combinations of alfalfa, brassica, and fescue plants (alfalfa, brassica, fescue, alfalfa-brassica, alfalfa-fescue, brassica-fescue, and alfalfa-brassica-fescue). For each diversity treatment, there were 3 density treatments (low: 1-3 total plants per pot, medium: 24 total plants per pot, and high: 48 total plants per pot). Each of the 21 different treatments had 12 replicates for a total of 252 microcosms. A 21 x 12 plot was configured using an online random block design generator

(<https://www.randomizer.org>). A single plant of each species was also grown in a mesocosm to serve as a control free from inter- and intra-specific competition or facilitation.

Plant seeds were manually counted for each treatment and dispersed evenly into the pots using autoclaved tweezers. Tweezers were washed with ethyl alcohol in between samples. To overcome seed germination failure, unsterilized pregerminated seeds were planted into each pot seven days into the experiment to reach the target densities. Pots were watered to holding capacity with DI water daily. This watering technique was employed to reduce the introduction of microorganisms and other substances. Prior to harvesting, the number of plants within each pot was verified.

Rhizosphere Soil Collection

Rhizosphere soil samples were collected over 4 days. Here, the rhizosphere soil is defined as the soil that remained adhered to the roots when the plant was gently removed from the pot. To ensure that the rhizosphere soil was collected from the target plant species, each individual plant was carefully pulled out of the pot leaving behind all bulk soil and overlapping roots. Each plant was separated by plant species per pot, and the attached shoot was used to identify the plant species of each root mass. The five replicates per treatment which plant counts most closely represented the target density and diversity treatment were selected for bacteriome analysis. Roots with rhizosphere soil were placed in 15 ml falcon tubes and immediately stored at -20°C.

DNA Extraction

Rhizosphere soil was removed from plant roots for total genomic DNA (gDNA) extractions. For each sample, 0.25 g of rhizosphere was extracted in a Qiagen QIAcube instrument using Qiagen PowerSoil Pro® DNA kits (Germantown, Maryland, USA) and the

manufacturer's protocol. Each DNA extraction was eluted to 100 µl. DNA concentrations were quantified using an Invitrogen Qubit fluorometer (Waltham, Massachusetts, USA) with high sensitivity assay solutions. Rhizosphere samples from each of the 21 treatments had 4-5 replicates. Some samples did not contain enough rhizosphere, or the DNA was not quantifiable after extraction. Pre-extracted Zymo gDNA (Zymo Research Corporation, California, USA) (n=2) and pre-extracted and sequenced soil (n=2) were used as positive controls. Extracted HPLC water (n=2), the first polymerase chain reaction (PCR) run (n=2), and the second PCR run (n=2) were used as negative controls.

Oxford Nanopore Sequencing and Bioinformatics Pipeline

Qubit concentrations (ng/µl) of DNA were used to calculate a 5x dilution with HPLC water. Primers for bacteriome analysis were Bact_27F-Mn (5' – TTTCTGTTGGTGCTGA TATTGCAGRGTTYGATYMTGGCTCAG – 3') and Bact_1492R-Mn (5' – ACTTGCCTGTC GCTCTATCTTCTACCTTGTTACGACTT – 3'). A Roche LightCycler® 96 (Basel, Switzerland) was used for PCR. The first PCR settings were 25 cycles of 98°C for 30 sec, 98°C for 15 sec, 50°C for 15 sec, and 72°C for 1 min followed by a single cycle of 72°C for 5 min. Following the initial PCR run, a 1:1 ratio of DNA and beads were combined. Beads with adhering DNA were magnetized to a 96-pronged magnetic stand and rinsed in 70% ethanol for 30 seconds twice. DNA was eluted with 40 µL of PCR grade water, and magnetic beads were removed using a magnetic stand. DNA concentrations were again quantified using Qubit with high sensitivity assay solutions.

The second PCR settings were 25 cycles of 98°C for 30 sec, 98°C for 15 sec, 62°C for 15 sec, and 72°C for 1 min followed by a single cycle of 72°C for 5 min. Following the second PCR run, DNA, barcodes (EXP-PBC-96), and the AMPure bead solution were mixed into a 96-well

plate. A cost-effective method of making the AMPure bead solution was used (Rohland & Reich, 2012). Samples were then pooled into a single Lo-Bind 2 ml centrifuge tube.

A R9.4.1 flow cell was loaded onto a MinION sequencer. For flow cell preparation, approximately 20 µL of air was drawn out of the flow cell. To prime the flow cell, the priming port was flushed with the buffer solution. Pooled DNA was loaded into the sampling port. The pooled library was sequenced for 48 hours. Guppy v6.0.1 was used to base-call and demultiplex raw data. Sequence reads were filtered by quality and length (Filtlong minimum length: 1000; mean quality: 70; Cutadapt: -m 1000 -M 2000). EMU NCBI Reference Database was used to identify bacterial taxa. EMU error correction identified and removed bacterial taxa using alignment and abundance profiles, during whichbacterial taxa with an abundance of < 1 per 10,000 reads were removed (Schoch et al., 2020; O'Leary et al., 2016; Stoddard et al., 2015). Sample replicates were equally sequenced over two sequence runs. The data from each run were pooled for data analysis.

Data Wrangling and Formatting

The taxonomic data were converted to relative abundances and wrangled into phyloseq (McMurdie et al., 2013) using a custom function for importing Oxford Nanopore sequencing data (emu_to_phyloseq in myFunctions.R). Relative abundances were converted to count data by multiplying by the final number of sequences reads in each sample.

Beta Dispersion and Differential Abundance

Betadisper (Vegan package) was used to measure the homogeneity of multivariate dispersions (Oksanen et al., 2022). Differential abundances between groups were calculated on the taxonomic species counts using the microbiomeMarker (Cao et al., 2022) package (relative log expression normalization, Benjamini-Hochberg adjustment, $p < 0.01$). Groups included

density comparisons (1 vs. 24 total plants and 1 vs. 48 total plants) and diversity comparisons (1 vs. 2 plant types and 1 vs. 3 plant types) within plant species (alfalfa, brassica, fescue). Additionally, similar density and diversity comparisons were made with plant species combinations. A table of significant markers was created for each comparison using the marker_table function. The relevant marker tables for density and diversity comparisons were combined, and the differentially abundant species were visualized using ggplot2 (Wickham, 2011).

Network Analysis

Network analyses and respective statistics were conducted on taxonomic species' relative abundances using the microeco and igraph packages (Liu et al., 2021; Csardi & Nepusz, 2006). Networks for each diversity treatment (alfalfa, brassica, fescue, alfalfa-brassica, alfalfa-fescue, brassica-fescue, and alfalfa-brassica-fescue) were created with densities combined for a total of seven networks. The trans_network function was used to calculate Spearman correlations with a filter threshold of 0.001. The networks were constructed using the cal_network function with a p-value threshold of 0.01 and correlation threshold of 0.5. Network modules were partitioned using the cal_module function and the "cluster fast greedy" method. The node properties, edge properties, and adjacency matrix were obtained for each network using the get_node_table, get_edge_table, and get_adjacency_matrix functions, respectively. Networks were formatted by the rgexf package (Yon, 2015) and then exported to Gephi for network visualization (Alam & Bhuiyan, 2012). In Gephi, networks were ran using Fruchterman Reingold with node partition colored by module, size set by relative abundance, and edges labeled by positive or negative correlations.

Results

The Effect of Intra- and Inter-specific competition on the Alfalfa Rhizosphere

Beta dispersion of alfalfa decreases when grown in monoculture and under increasing plant densities (Table 1). Bacteriome variation within the single-plant rhizosphere was inversely proportional to the proximity of neighboring alfalfa plants. Differential abundance comparisons of rhizospheres between alfalfa plants grown alone and alfalfa plants grown in monocultures of 24- and 48-plant densities showed that *Pseudarthrobacter* sp. NIBRBAC000502771, *Pseudarthrobacter phenanthrenivorans*, *Pseudarthrobacter oxydans*, *Neorhizobium* sp. SOG26, *Adhaeribacter swui*, *Arthrobacter* spp. UKPF54-2, and KBS0702 were consistently present in single individuals and increased in abundance with density (Figure 1). Where alfalfa was grown with either brassica or fescue (i.e., 2-plant species mixtures), beta dispersion of the alfalfa rhizosphere decreased as plant density increased (Table 1). When alfalfa was grown together with both brassica and fescue (i.e., 3-plant species mixtures), the beta dispersion of the alfalfa rhizosphere was higher compared to an alfalfa plant grown alone (Table. 1).

Differential abundance comparisons of alfalfa plant rhizospheres grown alone were compared to those grown in alfalfa-brassica mixtures, alfalfa-fescue mixtures, and alfalfa-brassica-fescue mixtures across the different densities. *Paucimonas lemoignei*, was enriched for every treatment apart from medium density alfalfa-brassica-fescue mixtures as compared to alfalfa grown alone (Figure 2). *A. swui* was present in the rhizosphere of alfalfa plants grown alone and significantly enriched in the alfalfa rhizosphere in all neighbor combinations (Figure 2). *Pseudarthrobacter* sp. NIBRBAC000502771 was present in the alfalfa rhizosphere in all neighbor combinations except low density (2- to 3- plant) alfalfa-brassica mixtures, low density (3-plant) alfalfa-brassica-fescue mixtures, and medium density (24) alfalfa-fescue mixtures

(Figure 2). *P. phenanthrenivorans* and *Arthrobacter* sp. KBS0702 were enriched in the alfalfa rhizosphere of all neighbor combinations except low density mixtures (Figure 2).

Following differential abundance comparisons, a network analysis was conducted to identify microbial interactions within the alfalfa rhizosphere. *Mesobacillus subterraneus* had the highest relative abundance in all four alfalfa bacteriome networks; however, it was correlated with only *Mesobacillus foraminis* for every network except alfalfa-brassica-fescue. The alfalfa monoculture network had two modules tied for the most prominent module (17.31%), one of which consisted mainly of *Mesobacillus* spp. *Bacillus* spp., the other most prominent module (17.31%), consisted mainly of *Cohnella* spp. and *Paenibacillus* spp. (Figure 3a). The third most prominent module (13.46%) in the network consisted of *Arthrobacter* sp. QXT-31, *Pseudarthrobacter* sp. NIBRBAC000502771, *Arthrobacter* sp. KBS0702, *Arthrobacter* sp. UKPF54-2, *Arthrobacter* sp. PGP41, and *Pseudarthrobacter phenanthrenivorans*. The alfalfa-brassica plant mixture most prominent module (13.43%) primarily consisted of *Microvirga* spp. and *Flavisolibacter* spp. (Figure 3b). The second most prominent module (8.96%) primarily consisted of *Bacillus* spp.. The third most prominent module (8.96%) primarily consisted of *Exiguobacterium* spp. and *Stenotrophomonas* spp.. The alfalfa-fescue plant mixture had the most prominent module (16.22%), which primarily consisted of *Mesobacillus* spp. and *Bacillus* spp. (Figure 3c). The second most prominent module (13.51%) consisted of *Stenotrophomonas* spp.. Two modules tied for the third most prominent module (8.11%), both of which consisted of *Massilia* spp.. The alfalfa-brassica-fescue plant mixture most prominent module (14.55%) contained *Massilia* spp.. (Figure 3d). The second most prominent module (12.73%) consisted of *Mesobacillus* and *Bacillus spp.*. The third most prominent module (10.91%) consisted of *Bacillus* spp..

The Effect of Intra- and Inter-specific competition on the Brassica Rhizosphere

Beta dispersion of brassica increases when grown in monoculture of increasing plant densities (Table 1). Differential abundance comparisons of the rhizosphere of a brassica plant grown alone compared to the rhizosphere of individual brassica plants grown in monoculture in higher densities showed an enrichment of *Nocardioides alpinus*, *Nocardioides cavernae*, and *Solibacillus silvestris* for the 24 and 48 density treatments (Figure 1).

In plant paired mixtures, the beta dispersion of brassica was lowest for medium plant densities (Table 1). For medium (24 plants) and high (48 plants) densities, the beta dispersion value was higher for plant mixtures of three plants than either plant mixtures with two plants (Table 1). Differential abundance comparisons were made between the rhizosphere of brassica plants grown alone and the rhizosphere of brassica plants grown with another plant. Grown plant mixtures showed an enrichment of *Nocardioides alpinus* in all plant mixtures and densities except for brassica-fescue at low density (2 plants) (Figure 4). *Nocardioides cavernae* (Figure 4) was present in the rhizosphere of brassica plants in all treatments except in low density brassica-alfalfa-fescue mixtures (3 plants). *Solibacillus silvestris* (Figure 4) was significantly present in the rhizosphere of all treatments except for low density brassica-alfalfa-fescue mixtures (3 plants) and medium density brassica-fescue mixtures. *Spirosoma linguale* (Figure 4) was enriched in low density brassica-alfalfa mixtures, high density brassica-alfalfa mixtures, high density brassica-alfalfa-fescue mixtures, and low density brassica-fescue mixtures.

Brassica network analysis displayed many of the same taxa as for alfalfa, but there were shifts in module connectivity and composition. In the brassica monoculture network, the most prominent module (22.41%) displayed mainly *Mesobacillus, Bacillus,* and *Paenibacillus* spp.. (4**a**). The second most prominent module (13.79%) mainly displayed *Cohnella* and *Tumebacillus*

spp.. Four networks tied for the third most prominent module in the network, mainly of *Exiguobactrium* spp., *Flavisolibacter*, *Bacillus* spp., or *Microvirga* spp.. Brassica-alfalfa mixture,showed the most prominent module (14.89%) displayed mainly *Bacillus* spp..(4**b**). The second most prominent module (12.77%) displayed *Mesobacillus* and *Bacillus* spp.. The third most prominent module (8.51) in the network displayed *Bacillus* and *Neobacillus* spp.. Brassica-fescue mixture showed the most prominent module (23.33%) mainly displayed *Mesobacillus, Cytobacillus, Bacillus,* and *Neobacillus* spp.. (Figure 5c). The second most prominent module (18.33%) mainly displayed *Massilia* and *Microvirga* spp.. The third most prominent module (11.67%) in the network mostly displayed *Exiguobacterium* spp.. Brassica-alfalfa-fescue plant mixture network's most prominent module (18.87%) mainly displayed *Mesobacillus* and *Bacillus* spp.. (Figure 5d). The second most prominent module (13.21%) only displayed *Massilia* spp.. The third most prominent module (7.55%) in the network mainly displayed *Bacillus* spp..

The Effect of Intra- and Inter-specific competition on the Fescue Rhizosphere

Beta dispersion of fescue increased when grown in monoculture of increasing plant densities (Table 1). Differential abundance comparison between the rhizosphere of a fescue plant grown alone and the rhizosphere of individual fescue plants grown in monoculture densities of 24 and 48 plants demonstrated an enrichment of *Adhaeribacter aerophilus*, *[Brevibacterium] frigoritolerans*, *Larkinella arboricola*, *Larkinella insperata*, and *Paenibacillus* sp. 37 for the higher density treatments (Figure 1). *Ensifer adhaerens* and *Dyadobacter sediminis* were enriched in medium and high fescue densities as compared to low plant densities (Figure 1).

In plant paired mixtures, beta dispersion of fescue decreased as plant density increased when grown with either alfalfa or brassica (Table 1). In mixtures with all three plants, the beta dispersion of microcosms of three plants (low density) was higher than microcosms of 48 plants

of three different species (Table 1). For medium (24 plants) densities, the beta dispersion value was higher for plant mixtures of three plants than alfalfa-brassica plant mixtures (Table 1). For high (48 plants) densities, the beta dispersion value was higher for mixtures of three plants than fescue-brassica mixtures (Table 1). *Larkinella arboricola* and *Larkinella insperata* was enriched in rhizospheres of all crop mixtures except for low density (three plants) fescue-alfalfa-brassica mixtures compared to the rhizosphere of a fescue plant grown alone (Figure 6). Differential abundance comparisons showed *Adhaeribacter aerophilus* was enriched in the rhizosphere of individual fescue plants grown in 24 fescue-alfalfa mixtures, 48 fescue-alfalfa mixtures, and all fescue-brassica/fescue-alfalfa-brassica mixtures compared to the rhizosphere of an individual fescue plant (Figure 6). Differential abundance comparisons showed *[Brevibacterium] frigoritolerans* was enriched in the rhizosphere of individual fescue plants grown in all medium density plant mixtures (fescue-alfalfa, fescue-brassica, fescue-alfalfa-brassica), and in high density fescue-brassica mixtures compared to the rhizosphere of an individual fescue plant (Figure 6). *Paenibacillus* sp. 37 (Figure 6) was enriched in the rhizosphere of fescue plants in all treatments except in low density fescue-alfalfa mixtures (two plants) and high density fescue-alfalfa-brassica mixtures (48 plants). Differential abundance comparisons showed *Ensifer adhaerens* was enriched in the rhizosphere of all fescue crop mixtures except for low densities of fescue-alfalfa-brassica mixtures and medium densities of fescue-brassica mixtures as compared the rhizosphere of an individual fescue plant (Figure 6). Differential abundance comparisons showed *Dyadobacter sediminis* was enriched in the rhizosphere of all fescue crop mixtures except for low densities of fescue-alfalfa mixtures as compared the rhizosphere of an individual fescue plant (Figure 6).

Network analysis was also run for the fescue rhizosphere for the different plant combinations. Fescue monoculture's most prominent module (12.5%) only consisted of *Microvirga* spp. (Figure 7a). The second most prominent module (9.38%) was tied, of which the first only consisted of *Exiguobacterium* spp.. The other second most prominent module (9.38%) in the network only consisted of *Noviherbaspirillum* spp.. For the fescue-alfalfa mixture network, the most prominent module (19.15%) mainly consisted of *Bacillus* and *Mesobacillus* spp. (Figure 7b). The second most prominent module (10.64%) only consisted of *Massilia* sp.. The third most prominent module (8.51%) in the network consisted of *Herbaspirillum* and *Noviherbaspirillum* spp.. In the fescue-brassica mixture, the most prominent module (20.73%) mostly consisted of *Bacillus*, *Cytobacillus*, and *Mesobacillus* spp. (Figure 7c). The second most prominent module (14.63%) mostly consisted of *Massilia* and *Microvirga* spp.. The third most prominent module (14.63%) in the network consisted primarily of *Achromobacter*, *Flavisolibacter*, and *Pontibacter* spp.. The fescue-alfalfa-brassica plant mixture network's most prominent module (20%) consisted mainly of *Bacillus* spp.. The second most prominent module (16.67%) consisted mostly of *Microvirga* spp.. The third most prominent module (10%) in the network was tied between five modules.

Discussion

It is well established that an individual plant's growth is affected by its plant neighbors through interspecific and intraspecific competition. Plant-plant competition in the form of growing longer roots to increase access to nutrients or growing taller to access higher quality sunlight (Craine et al., 2013) is a readily apparent form of plant competition. In determining if a plant species is dominant over other plant species, this dominance should also be measured by how influential the root exudates/rhizosphere microbial recruitment of a plant is on the

surrounding soil. Since the bacteriome of the rhizosphere is critical for the plant's development and stress tolerance (Park et al., 2023), it is important to acknowledge the influence that a plant neighbor has on rhizosphere colonization. In monocultures, there were inconsistent results. The variability of the rhizosphere increased for brassica and fescue as densities increased, but within alfalfa, rhizosphere variability decreased with increasing density. It is possible that alfalfa's intraspecific allelopathic ability could have had an influence in reducing the variability of the rhizosphere. Alfalfa secretes autotoxic chemicals which prevent the establishment of new alfalfa seedlings (Singh et al., 1999; Chon et al., 2006). Although these plant-derived chemicals do not appear to negatively impact adult alfalfa stands (Chon et al., 2006), these chemicals could negatively impact the soil health in the long term since they may increase pathogenic fungi and decrease beneficial microorganisms (Wang et al., 2022). For brassica, cover crop *Brassica juncea* was successfully used as a biocontrol since it decreased *Escherichia coli* populations to non-detectable levels in a greenhouse study (Zhao et al., 2023). *B. juncea*, when used as green manure for cucumbers, altered the mycobiome composition of the rhizosphere without changing the alpha diversity (Jia et al., 2020). This finding is reflective in this study as well, since brassica was did not decrease the variability of the rhizosphere's bacteriome but to cause a shift in its composition

Beta-dispersion analysis indicated that the variability of the plant's rhizosphere increased with plant species diversity. Additionally, as plant density increased within plant mixtures, the variability of the plant's rhizosphere decreased within each diversity treatment. As the number of types of plant neighbors increases, more types of bacteria are recruited and can colonize the rhizosphere. Additionally, as the density of plant neighbors increases, bacteria could be recruited even more. Overall, plant diversity influences the microbial richness of the rhizosphere while

plant density allows for more opportunities for microbial recruitment as shown by the beta dispersion.

Differential abundance analysis unveiled bacterial taxa that strongly associate with alfalfa, brassica, and fescue. However, bacterial taxa which were no longer enriched in the rhizosphere if density or diversity changed are considered to express conditional associations with their specific plant host. Each of these plant species tested have been known to affect the soil microbiome. When allelochemicals from alfalfa plants increase pathogenic fungi and decrease beneficial microorganisms (Wang et al., 2022), this microbial shift could negatively impact any plant neighbor. *Brassica juncea* is known to have antimicrobial and insecticidal properties (Munir et al., 2019). Since antimicrobials of brassica impede certain microorganisms (Wang & Zhou, 2023), it can potentially influence the recruitment of neighboring plants. It has been shown that *Festuca* sp. is allelopathic and can outcompete sweetgum by reducing sweetgum biomass (Walters & Gilmore, 1976). In addition, *Festuca* sp. has expressed competitive ability against red clover (*Trifolium pratense L.*) which is dependent on the colonization of *Neotyphodium* endophytes (Malinowski et al., 1999).

Differential abundance enrichment groups showed bacteria that strongly associated with alfalfa such as *Adhaeribacter swui* (enriched in all treatments), *Paucimonas lemoignei* (enriched in all treatments except for medium density alfalfa and medium alfalfa-brassica-fescue mixture), *Pseudarthrobacter* sp. NIBRBAC000502771 (enriched in all treatments except for lower density crop mixtures), *Pseudarthrobacter phenanthrenivorna* (enriched in all treatments except for low (2-3 plant) crop mixtures), and *Arthrobacter* sp. KBS0702 (enriched in all treatments except for low (2-3 plant) crop mixtures). Bacteria which were found to conditionally associate with alfalfa were *Pseudarthrobacter oxydans* (enriched in monoculture and only in plant densities of 48

plants), *Arthrobacter sp.* UKPF54-2 (enriched in monoculture and only in crop mixtures with brassica), and *Neorhizobium* sp. SOG26 (enriched in monoculture and inconsistently in plant mixtures). *Pseudarthrobacter* sp. NIBRBAC000502771*, Pseudarthrobacter phenanthrenivornans*, and *Arthrobacter* sp. KBS0702 requires multiple alfalfa plants, and is enriched despite whether the plant neighbor is a different species. For *Pseudarthrobacter oxydans*, density was more influential than diversity. Enrichment of *Arthrobacter sp.* UKPF54-2 and *Neorhizobium* sp. SOG26 could have been diversity dependent with enrichment of *A.* UKPF54-2 either negatively influenced by fescue or positively influenced by brassica. *N*. SOG26 was negatively influenced by brassica and fescue.

Past studies have shed some light onto the possible functions of the strongly correlated rhizobacteria of alfalfa. While not much is known about *Arthrobacter* sp. KBS0702 and *Adhaeribacter swui*, *A. swui* has tested positive for oxidase and catalase (Kim et al., 2018). *Neorhizobium* sp. SOG26 is an understudied organism but has sequencing data uploaded to UniProt (Submitted to EMBL/GenBank/DDBJ databases (DEC-2017) by Ghneim Herrera T. and Torres Bedoya E.) with possible plant functions like metal ion binding. *Paucimonas lemoignei* is flagellated, a potential nitrogen fixer with a few strains capable of producing gas from nitrate (Jendrossek, 2001). *Pseudarthrobacter* sp. NIBRBAC000502771 is capable of producing auxin (Indol-3-glycerol phosphate synthase) and has heavy metal (copper and arsenic) resistance (Park et al., 2019). *Pseudarthrobacter phenanthrenivornan*s can produce several phytohormones (abscisic acid, auxin, cytokinin, ethylene, gibberellins, jasmonic acid, and salicylic acid) (Tshishonga & Serepa, 2020). *Pseudarthrobacter oxydans* can synthesize indole-3-acetic acid, fix nitrogen (ammonia production), solubilize phosphorus, and is resistant to heavy metals (cadmium, copper, and nickel) (Bushra et al., 2023). *Arthrobacter sp.* UKPF54-2 has been shown

to promote growth for *Brassica* sp., fix nitrogen, produce acetolactate synthase (dihydroxy acid dehydratase and ketol acid reductoisomerase), and produce five genes attributed to antimicrobial properties (Shen et al., 2019). Of the eight bacterial taxa that were found to be differentially abundant between alfalfa grown alone and with a plant neighbor, three are capable of nitrogen fixation and three produce some form of phytohormone. Although there was some functional overlap, this redundancy does not fully explain the conditional association with *Pseudarthrobacter oxydans*, which can perform a wider range of functions as shown by the past aforementioned studies.

Brassica rhizosphere was strongly associated with *Nocardioides alpinus* (enriched in all treatments except for low density brassica-fescue), *Nocardioides cavernae* (enriched in all treatments except for low density brassica-alfalfa-fescue), and *Solibacillus silvestris* (enriched in all treatments except for low density brassica-alfalfa-fescue and medium density brassica-fescue). *Spirosoma linguale* was conditionally associated with brassica and was possibly diversity dependent since it was enriched in every crop mixture treatment with the exception of low density brassica-alfalfa-fescue and medium densities of brassica-fescue.

Previous studies have reviewed possible functions of these strongly associated brassica rhizobacteria. *Nocardioides alpinus* strains have been found to reduce nitrate and produce ammonia and indole-3-acetic acid (Alotaibi et al., 2022; Zhang et al., 2012). The purpose of *Nocardioides cavernae* recruitment is not indicated by the literature, since this taxon does not have nitrogen, phosphorus, or indole related activities (Han et al., 2017). *Solibacillus silvestris* may show N-Acyl homoserine lactone degrading activity which has quorum-quenching and biocontrol activities (Morohoshi et al., 2012). In addition, *Solibacillus silvestris* may produce phytohormones (indole-3-acetic acid, cytokinin, and gibberellin), fix nitrogen, and be resistant to

cadmium (Kaur & Karnwal, 2023). *Solibacillus silvestris* has shown strong utility for its possible functionality and was consistently recruited by the *Brassica* sp. as expected.

Within the fescue rhizosphere, strong correlations were seen for *Adhaeribacter aerophilus* (enriched for all treatments except low density fescue-alfalfa), *Larkinella arboricola* (enriched for all treatments except low density fescue-alfalfa-brassica), *Larkinella insperata* (enriched for all treatments except low density fescue-alfalfa-brassica), *Dyadobacter sediminis* (except for medium density brassica), *Paenibacillus* sp. 37 (enriched for all treatments except low density fescue-alfalfa and high density fescue-alfalfa-brassica), and *Ensifer adhaerens* (enriched for all treatments except for medium density brassica, low density fescue-alfalfa-brassica, and medium density fescue-brassica). Rhizobacterial enrichment even for the strongly correlated taxa needed more than one fescue plant to be present. [*Brevibacterium*] *frigoritolerans* (enriched in monoculture, all medium density polyculture, and high density fescue-brassica mixtures), *Trichormus azollae* (enriched in all medium densities except for fescue-brassica), and *Sinorhizobium meliloti* (enriched inconsistently, but only for mixtures including alfalfa) all showed fewer correlations and thus possibly a conditional association with the fescue rhizosphere. [*Brevibacterium*] *frigoritolerans* and *Trichormus azollae* may have been outcompeted by microbes from other plant species, especially when planted in higher densities. Plausible functions of the fescue rhizosphere bacterial taxa were searched for in the literature. *Adhaeribacter aerophilus* is oxidase positive and may hydrolyze starch (Weon et al., 2010). *Larkinella arboricola* has been found to produce indole (Kulichevskaya et al., 2009). *Larkinella insperata* bared no remarkable plant related characteristics (Anandham et al., 2011; Vancanneyt et al., 2006). *Dyadobacter sediminis* can produce catalase, oxidase, alkaline phosphatase, and acid phosphatase (Tian et al., 2015). *Paenibacillus* sp. 37 is a possible plant growth promotor due

to its ability for siderophore biosynthesis, antimicrobial production (paeninodin, bacitracin, paenilipoheptin, xenocoumacin, pellasoren, and octapeptin), and its phytohormone-associated genes (Garcia-Lemos et al., 2021). A strain of *Brevibacterium frigrotolerans* was shown to solubilize phosphate, produce indole-3-acetic acid, and produce siderophores (Tara & Saharan, 2017). *Trichormus azollae* has been found in ferns and is a nitrogen fixer (Gunawardana, 2020). *Ensifer adhaerens* is a nitrogen fixer and may produce indole-3-acetic acid, exopolysaccharides, ammonia, siderophores, salicylic acid (for abiotic stress), and even promote seed germination for soybean (Zhou et al., 2013). Most interestingly, another legume associated bacteria, *Sinorhizobium meliloti,* which is a nitrogen fixing symbiont of alfalfa, was found in the rhizosphere of the fescue sp. (Galibert et al., 2001). Although *E. adhaerens* and *S. meliloti* should have been highlighted in the alfalfa rhizosphere instead of the fescue rhizosphere, this could support the sharing of microbes between alfalfa and fescue. *E. adhaerens* was not enriched for only mixtures which included brassica, meanwhile *S. meliloti* was only enriched in mixtures which included alfalfa.

Networks did not show more edges and nodes as diversity increased, bacterial taxa with high abundance were not necessarily hub species (Lv et al., 2021), and networks overlapped with similar taxa with only alfalfa showing bacterial taxa which were also highlighted by the differential abundance analysis. Rhizobacterial networks which had the highest number of modules (Fb: 82, Ab: 71, Bf: 60) and edges (Fb: 136, Bf:99) were from plant mixtures which included brassica. The highest modularity for crop mixtures were for only paired mixtures. Modularity in crop mixtures was highest for alfalfa in alfalfa-brassica (0.898), for brassica in brassica-alfalfa (0.861), and for fescue in fescue-alfalfa (0.898). Increasing plant diversity could be considered as increasing agricultural intensification, which has shown to decrease microbial

network complexity (Banerjee et al., 2019; Karimi et al., 2019). *Mesobacillus subterraneus* was one of the largest modules by relative abundance, but was rarely highly connected to more than 1-2 bacterial taxa (Figures 3**a**, 3**b**, 3**c**, 5**c**, 7**b**, 7**c**, 7**d**), supporting that relative abundance alone does not serve as a good predictive measurement for a hub bacterial species and therefore is not expected to drive microbial shifts in the rhizosphere. The bacterial family Bacillaceae dominated networks. This dominance could possibly be explained by how many members of Bacillaceae are thermophiles (Coleri et al., 2017; Mandic-Mulec et al., 2016), and the methods by which the microbial complexity was reduced was an autoclave. Nevertheless, modules mainly composed of *Microvirga* sp. and *Massilia* sp. were still able to thrive and become the predominant modules over members of the Bacillaceae family, most likely due to plant host interaction. Since networks were often dominated by different taxa of the Bacillaceae family, it's possible that the plant had an influence on bacterial recruitment. Plants recruit a specific set of microorganisms as shown through differential abundance comparisons, however, by increasing plant diversity these recruited bacteria no longer played a major role in the rhizobacterial network. Alfalfa's third most prominent module in monoculture contained some of the same taxa that were shown to be strongly associated with the rhizosphere in our differential abundance analysis. These taxa included *Pseudarthrobacter* sp. NIBRBAC000502771, *Arthrobacter* sp. KBS0702, *Arthrobacter* sp. UKPF54-2, and *Pseudarthrobacter phenanthrenivorans*. Although interspecific competition introduced by the plant neighbor did not influence the recruitment of these bacteria, the plant neighbor of a different plant species did influence how these bacteria interacted within the microbial network. Further study is required to identify how plant neighbor influences the functionality of the rhizosphere, even if there is no change to bacterial recruitment.

Table 1: Beta Dispersion: Average distance to median for bacteriomes.
Bolded capitalized letter denotes plant species rhizosphere (A: alfalfa, B: brassica, F: fescue). Lowercase letter denotes neighboring plant species (e.g., Ab: alfalfa rhizosphere with brassica as a plant neighbor, Abf: alfalfa rhizosphere with brassica and fescue as plant neighbors). Number denotes density (i.e., plant count per pot; low density: 1-3 plants, medium density: 24 plants, high density: 48 plants).

A1	**A**24	**A**48	**A**b2	**A**b24	**A**b48	**A**f2	**A**f24	**A**f48	**A**bf3	**A**bf24	**A**bf48
0.460	0.458	0.451	0.500	0.460	0.423	0.484	0.475	0.471	0.493	0.494	0.488
B1	**B**24	**B**48	**B**a2	**B**a24	**B**a48	**B**f2	**B**f24	**B**f48	**B**af3	**B**af24	**B**af48
0.464	0.465	0.483	0.460	0.424	0.428	0.524	0.444	0.446	0.480	0.497	0.451
F1	**F**24	**F**48	**F**a2	**F**a24	**F**a48	**F**b2	**F**b24	**F**b48	**F**ab3	**F**ab24	**F**ab48
0.465	0.475	0.502	0.501	0.488	0.476	0.506	0.483	0.467	0.481	0.491	0.473

Table 2: Differential Abundance Comparison of Alfalfa 1 and Alfalfa Plant Densities. Enriched column shows which treatment the bacterial taxa is enriched (A1: single alfalfa plant, A24: 24 alfalfa plants, A48: 48 alfalfa plants). Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

A24

Bacterial Taxa	Enriched	Log Fold	P-adjust
Larkinella insperata	A1	-20.12	1.28E-03
Leptolyngbya sp. O-77	A1	-18.44	2.44E-03
Halomicronema hongdechloris	A1	-19.20	2.52E-03
Anabaena cylindrica	A1	-18.15	2.52E-03
Telluribacter humicola	A1	-18.03	2.52E-03
Adhaeribacter aerophilus	A24	12.61	2.52E-03
Arthrobacter sp. KBS0702	A24	9.46	5.63E-06
Arthrobacter sp. UKPF54-2	A24	9.16	3.68E-03
Arthrobacter sp. QXT-31	A24	5.15	7.57E-03
Adhaeribacter swui	A24	17.11	7.43E-18
Pseudarthrobacter sp. NIBRBAC000502771	A24	18.79	4.51E-16
Pseudarthrobacter phenanthrenivorans	A24	19.83	6.73E-22

A48

Bacterial Taxa	Enriched	Log Fold	P-adjust
Ammoniphilus oxalaticus	A1	-29.96	2.79E-24
Oscillatoria nigro-viridis	A1	-23.81	2.36E-12
Larkinella harenae	A1	-25.78	2.18E-11
Paenibacillus sp. 37	A1	-20.93	2.19E-10
Azospirillum brasilense	A1	-23.21	2.19E-10
Paenibacillus xylanexedens	A1	-21.14	2.00E-09
Larkinella rosea	A1	-24.28	6.07E-09
Adhaeribacter swui	A48	16.43	3.61E-15
Pseudarthrobacter sp. NIBRBAC000502771	A48	19.10	3.61E-15
Pseudarthrobacter phenanthrenivorans	A48	19.80	4.04E-20

Table 3: Differential Abundance Comparison of Alfalfa 1 and Alfalfa-Brassica Mixtures. Enriched column shows which treatment the bacterial taxa is enriched (A1: single alfalfa plant, Ab2: single alfalfa and brassica plant, Ab24: 12 alfalfa and brassica plants, Ab48: 24 alfalfa and brassica plants). Bacterial taxa which were enriched when alfalfa was grown alone as compared to multiple density treatments. Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched in more than one diversity treatment is highlighted in light sky blue. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

Ab2

Bacterial Taxa	Enriched	Log Fold	P-adjust
Dyadobacter sediminis	A1	-19.86	4.05E-09
Ensifer adhaerens	A1	-20.52	1.40E-04
Exiguobacterium aurantiacum	A1	-17.87	8.40E-08
Exiguobacterium mexicanum	A1	-29.94	4.45E-43
Larkinella harenae	A1	-28.32	1.43E-15
Larkinella rosea	A1	-24.96	1.82E-10
Lysobacter helvus	A1	-21.48	1.77E-03
Noviherbaspirillum suwonense	A1	-7.31	3.49E-04
Peribacillus simplex	A1	-16.77	8.34E-03
Pontibacter chitinilyticus	A1	-30.00	7.74E-30
Pontibacter rhizosphera	A1	-16.65	8.81E-05
Pseudomonas stutzeri	A1	-17.05	3.27E-03
Sinorhizobium fredii	A1	-18.50	1.95E-06
Paucimonas lemoignei	Ab2	17.31	8.34E-03
Gemmata massiliana	Ab2	15.96	8.01E-03
Adhaeribacter swui	Ab2	18.52	2.16E-21

Ab24

Bacterial Taxa	Enriched	Log Fold	P-adjust
Bacillus carboniphilus	A1	-26.49	2.35E-44
Bacillus mannanilyticus	A1	-6.39	6.33E-03
Devosia geojensis	A1	-22.10	5.95E-16
Azohydromonas australica	A1	-25.49	1.60E-03
Trichocoleus desertorum	A1	-21.31	1.61E-05
Gemmata sp. SH-PL17	Ab24	17.99	6.27E-03
Metabacillus indicus	Ab24	2.91	1.74E-03
Arthrobacter sp. KBS0702	Ab24	8.23	1.36E-04
Gemmata massiliana	Ab24	19.13	5.65E-04
Pseudarthrobacter sp. NIBRBAC000502771	Ab24	18.39	1.29E-15
Pseudarthrobacter phenanthrenivorans	Ab24	20.86	1.02E-24
Adhaeribacter swui	Ab24	17.54	4.30E-19

Ab48

Bacterial Taxa	Enriched	Log Fold	P-adjust
Achromobacter insolitus	A1	-29.96	1.32E-31
Luteolibacter pohnpeiensis	A1	-21.51	3.72E-07
Planomicrobium chinense	A1	-22.23	2.30E-09
Stenotrophomonas sp. MYb57	A1	-9.74	7.78E-03
Larkinella insperata	A1	-21.46	1.20E-04
Leptolyngbya sp. O-77	A1	-19.67	2.77E-04
Anabaena cylindrica	A1	-19.67	3.32E-04
Azohydromonas australica	A1	-26.41	4.47E-04
Trichocoleus desertorum	A1	-21.58	7.63E-06
Adhaeribacter aerophilus	Ab48	15.14	3.92E-05
Arthrobacter sp. KBS0702	Ab48	8.09	1.20E-04
Pseudarthrobacter sp. NIBRBAC000502771	Ab48	18.04	5.26E-15
Pseudarthrobacter phenanthrenivorans	Ab48	19.21	6.43E-21
Adhaeribacter swui	Ab48	17.16	2.44E-18

Table 4: Differential Abundance Comparison of Alfalfa 1 and Alfalfa-Fescue Mixtures. Enriched column shows which treatment the bacterial taxa is enriched (A1: single alfalfa plant, Af2: single alfalfa and fescue plant, Af24: 12 alfalfa and fescue plants, Af48: 24 alfalfa and fescue plants). Bacterial taxa which were enriched when alfalfa was grown alone as compared to multiple density treatments. Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched in more than one diversity treatment is highlighted in light sky blue. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

Af2

Bacterial Taxa	Enriched	Log Fold	P-adjust
Achromobacter insolitus	A1	-25.05	3.76E-21
Arthrobacter sp. Rue61a	A1	-17.25	7.55E-11
Azospirillum brasilense	A1	-22.15	3.85E-10
Azospirillum sp. TSH58	A1	-19.02	2.10E-04
Dyadobacter sediminis	A1	-19.09	4.62E-08
Larkinella rosea	A1	-23.79	4.13E-09
Luteolibacter pohnpeiensis	A1	-19.70	1.39E-05
Pontibacter rhizosphera	A1	-16.04	4.74E-04
Sphingoaurantiacus polygranulatus	A1	-21.37	3.34E-12
Paenibacillus sp. 37	A1	-19.93	3.85E-10
Paenibacillus xylanexedens	A1	-19.51	1.48E-08
Planomicrobium chinense	A1	-20.21	2.40E-07
Adhaeribacter swui	Af2	15.50	9.61E-14
Pseudarthrobacter sp. NIBRBAC000502771	Af2	17.93	8.12E-14

Af24

Bacterial Taxa	Enriched	Log Fold	P-adjust
Leptolyngbya sp. O-77	A1	-19.09	2.64E-03
Lysobacter helvus	A1	-21.19	6.71E-03
Methylophilus sp. TWE2	A1	-20.97	1.04E-03
Pseudomonas stutzeri	A1	-18.31	3.87E-03
Roseimicrobium gellanilyticum	A1	-20.03	3.31E-06
Telluribacter humicola	A1	-18.03	5.48E-03
Azospirillum sp. TSA2s	A1	-16.89	8.24E-03
Exiguobacterium acetylicum	A1	-21.95	1.10E-10
Exiguobacterium sp. U13-1	A1	-21.99	1.10E-10
Adhaeribacter aerophilus	Af24	15.77	9.40E-05
Arthrobacter sp. KBS0702	Af24	7.65	1.57E-03
Pseudarthrobacter phenanthrenivorans	Af24	17.83	5.07E-16
Adhaeribacter swui	Af24	17.16	5.07E-16
Pseudarthrobacter sp. NIBRBAC000502771	Af24	17.52	2.02E-12

Af48

Bacterial Taxa	Enriched	Log Fold	P-adjust
Massilia plicata	A1	-21.49	1.45E-10
Metabacillus indicus	A1	-3.36	2.17E-04
Peribacillus simplex	A1	-17.36	5.00E-03
Solibacillus silvestris	A1	-18.34	1.06E-06
Stenotrophomonas sp. G4	A1	-27.73	7.93E-36
Azospirillum sp. TSA2s	A1	-16.36	4.13E-03
Exiguobacterium acetylicum	A1	-23.24	6.13E-14
Exiguobacterium sp. U13-1	A1	-23.57	2.85E-14
Paenibacillus sp. 37	A1	-21.25	3.34E-12
Paenibacillus xylanexedens	A1	-21.19	9.35E-11
Planomicrobium chinense	A1	-21.48	6.42E-09
Flavisolibacter tropicus	Af48	3.44	5.00E-03
Paucimonas lemoignei	Af48	18.26	4.06E-03
Arthrobacter sp. KBS0702	Af48	7.72	3.21E-04
Pseudarthrobacter phenanthrenivorans	Af48	18.26	7.54E-19
Adhaeribacter swui	Af48	17.59	5.30E-19
Pseudarthrobacter sp. NIBRBAC000502771	Af48	18.09	5.80E-15

Table 5: Differential Abundance Comparison of Alfalfa 1 and Alfalfa-Brassica-Fescue Mixtures. Enriched column shows which treatment the bacterial taxa is enriched (A1: single alfalfa plant, Abf2: single alfalfa, brassica, and fescue plant, Abf24: 8 alfalfa, brassica, and fescue plants, Af48: 16 alfalfa, brassica, and fescue plants). Bacterial taxa which were enriched when alfalfa was grown alone as compared to multiple density treatments. Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched in more than one diversity treatment is highlighted in light sky blue. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

Abf3

Bacterial Taxa	Enriched	Log Fold	P-adjust
Exiguobacterium aurantiacum	A1	-19.23	7.32E-09
Larkinella insperata	A1	-21.27	3.07E-04
Halomicronema hongdechloris	A1	-20.27	1.23E-03
Adhaeribacter aerophilus	Abf3	16.67	5.19E-06
Adhaeribacter swui	Abf3	20.30	2.07E-25
Pseudarthrobacter sp. NIBRBAC000502771	Abf3	14.59	3.29E-09
Pseudarthrobacter phenanthrenivorans	Abf3	14.37	6.88E-11

Abf24

Bacterial Taxa	Enriched	Log Fold	P-adjust
Paenibacillus xylanexedens	A1	-19.80	4.56E-09
Planctomyces sp. SH-PL14	A1	-19.66	1.30E-05
Arthrobacter sp. KBS0702	Abf24	7.59	9.20E-04
Adhaeribacter aerophilus	Abf24	13.02	1.77E-03
Adhaeribacter swui	Abf24	17.74	2.78E-19
Pseudarthrobacter sp. NIBRBAC000502771	Abf24	19.22	6.70E-17
Pseudarthrobacter phenanthrenivorans	Abf24	18.53	2.78E-19

Abf48

Bacterial Taxa	Enriched	Log Fold	P-adjust
Azospirillum brasilense	A1	-24.73	3.48E-13
Azospirillum sp. TSH58	A1	-20.80	9.12E-06
Exiguobacterium sibiricum	A1	-5.99	9.10E-03
Exiguobacterium undae	A1	-6.34	3.74E-04
Leptolyngbya sp. O-77	A1	-19.31	4.38E-04
Methylophilus sp. TWE2	A1	-21.39	1.19E-04
Oscillatoria nigro-viridis	A1	-23.79	6.89E-14
Paenibacillus sp. 37	A1	-21.67	1.56E-12
Peribacillus simplex	A1	-17.70	4.17E-03
Planomicrobium chinense	A1	-21.86	5.45E-09
Roseimicrobium gellanilyticum	A1	-20.28	2.01E-07
Trichocoleus desertorum	A1	-20.95	1.77E-05
Halomicronema hongdechloris	A1	-20.09	7.63E-04
Planctomyces sp. SH-PL14	A1	-21.54	5.45E-07
Arthrobacter sp. KBS0702	Abf48	8.57	3.70E-05
Adhaeribacter aerophilus	Abf48	13.97	2.51E-04
Adhaeribacter swui	Abf48	16.98	1.66E-17
Pseudarthrobacter sp. NIBRBAC000502771	Abf48	17.66	5.31E-14
Pseudarthrobacter phenanthrenivorans	Abf48	18.40	1.33E-18

Table 6: Differential Abundance Comparison of Brassica 1 and Brassica Plant Densities. Enriched column shows which treatment the bacterial taxa is enriched (B1: single brassica plant, Ba2: single brassica and alfalfa plants, B24: 24 brassica plants, B48: 48 brassica plants). Bacterial taxa which were enriched when brassica was grown alone as compared to multiple density treatments. Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched in more than one diversity treatment is highlighted in light sky blue. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

B24

Bacterial Taxa	Enriched	Log Fold	P-adjust
Anabaena cylindrica	B1	-25.48	7.77E-03
Aneurinibacillus soli	B1	-22.03	2.20E-03
Halomicronema hongdechloris	B1	-24.41	1.36E-03
Leptolyngbya boryana	B1	-21.67	7.32E-03
Paenibacillus odorifer	B1	-20.30	3.97E-04
Paenibacillus sp. FSL H7-0357	B1	-19.47	9.55E-03
Nocardioides alpinus	B24	18.50	6.35E-04
Nocardioides cavernae	B24	19.14	7.50E-08
Solibacillus silvestris	B24	19.32	1.42E-04

B48

Bacterial Taxa	Enriched	Log Fold	P-adjust
Pontibacter populi	B1	-7.88	1.65E-04
Tumebacillus flagellatus	B48	7.75	1.24E-07
Nocardioides alpinus	B48	18.21	7.89E-04
Nocardioides cavernae	B48	18.51	1.67E-07
Solibacillus silvestris	B48	19.40	9.28E-05

Table 7: Differential Abundance Comparison of Brassica 1 and Brassica-Alfalfa Mixtures. Enriched column shows which treatment the bacterial taxa is enriched (B1: single brassica plant, Ba24: 12 brassica and alfalfa plants, Ba48: 24 brassica and alfalfa plants). Bacterial taxa which were enriched when brassica was grown alone as compared to multiple density treatments. Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched in more than one diversity treatment is highlighted in light sky blue. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

Ba2

Bacterial Taxa	Enriched	Log Fold	P-adjust
Aneurinibacillus soli	B1	-21.67	2.93E-05
Azospirillum sp. TSH58	B1	-.23.08	1.11E-10
Domibacillus robiginosus	B1	-18.17	1.35E-13
Exiguobacterium aurantiacum	B1	-24.54	9.03E-20
Pseudarthrobacter sp. NIBRBAC000502771	B1	-7.96	1.66E-04
Anabaena cylindrica	B1	-25.21	1.20E-04
Paenibacillus sp. FSL H7-0357	B1	-19.22	1.71E-04
Spirosoma linguale	Ba2	18.90	1.53E-04
Nocardioides alpinus	Ba2	17.23	1.89E-05
Nocardioides cavernae	Ba2	17.69	3.04E-09
Solibacillus silvestris	Ba2	19.39	4.24E-07

Ba24

Bacterial Taxa	Enriched	Log Fold	P-adjust
Adhaeribacter aerophilus	B1	-17.55	9.95E-13
Daejeonella composti	B1	-25.64	1.62E-08
Ensifer adhaerens	B1	-16.90	3.33E-04
Massilia plicata	B1	-20.09	3.13E-12
Methylotenera mobilis	B1	-7.26	3.07E-06
Paenibacillus xylanexedens	B1	-21.99	1.65E-16
Pirellula sp. SH-Sr6A	B1	-9.45	9.17E-05
Pontibacter populi	B1	-7.56	2.69E-06
Pontibacter rhizosphera	B1	-24.29	4.72E-10
Rhizobacter gummiphilus	B1	-22.21	1.86E-04
Roseomonas ludipueritiae	B1	-20.58	2.64E-04
Sinorhizobium fredii	B1	-18.59	6.57E-07
Telluribacter humicola	B1	-20.17	5.52E-06
Trichocoleus desertorum	B1	-25.81	2.57E-13
Halomicronema hongdechloris	B1	-24.22	1.31E-05
Larkinella arboricola	B1	-20.75	3.76E-04
Larkinella insperata	B1	-21.14	9.13E-06
Leptolyngbya boryana	B1	-20.96	1.42E-04
Lysobacter soli	B1	-24.72	3.74E-04
Paenibacillus sp. FSL H7-0357	B1	-18.94	2.12E-04
Metabacillus indicus	Ba24	4.38	1.07E-10
Pseudomonas stutzeri	Ba24	18.65	4.42E-04
Nocardioides alpinus	Ba24	15.74	1.04E-04
Nocardioides cavernae	Ba24	19.99	1.80E-11
Solibacillus silvestris	Ba24	20.04	1.71E-07

Ba48

Bacterial Taxa	Enriched	Log Fold	P-adjust
Exiguobacterium sibiricum	B1	-4.68	4.13E-03
Exiguobacterium sp. MH3	B1	-8.38	3.20E-03
Exiguobacterium undae	B1	-4.97	9.69E-04
Prosthecobacter fluviatilis	B1	-22.62	2.80E-07
Stenotrophomonas sp. G4	B1	-9.69	1.37E-03
Stenotrophomonas sp. MYb57	B1	-23.06	1.08E-13
Anabaena cylindrica	B1	-25.68	3.66E-03
Halomicronema hongdechloris	B1	-24.47	9.25E-04
Larkinella arboricola	B1	-21.22	9.75E-03
Larkinella insperata	B1	-20.92	9.25E-04
Leptolyngbya boryana	B1	-21.94	3.20E-03
Lysobacter soli	B1	-25.10	9.75E-03
Bacillus sp. Y1	Ba48	3.65	4.25E-04
Metabacillus litoralis	Ba48	2.44	9.75E-03
Spirosoma linguale	Ba48	19.90	3.20E-03
Nocardioides alpinus	Ba48	17.36	1.00E-03
Nocardioides cavernae	Ba48	17.33	1.15E-06
Solibacillus silvestris	Ba48	20.24	1.83E-05

Table 8: Differential Abundance Comparison of Brassica 1 and Brassica-Fescue Mixtures. Enriched column shows which treatment the bacterial taxa is enriched (B1: single brassica plant, Bf1: single brassica and fescue plants, Bf24: 12 brassica and fescue plants, Bf48: 24 brassica and fescue plants). Bacterial taxa which were enriched when brassica was grown alone as compared to multiple density treatments. Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched in more than one diversity treatment is highlighted in light sky blue. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

Bf2

Bacterial Taxa	Enriched	Log Fold	P-adjust
Aneurinibacillus soli	B1	-21.27	3.02E-03
Exiguobacterium aurantiacum	B1	-23.78	6.01E-16
Larkinella insperata	B1	-21.17	7.62E-04
Pontibacter populi	B1	-7.48	3.62E-04
Pontibacter rhizosphera	B1	-24.22	1.37E-07
Prosthecobacter fluviatilis	B1	-21.33	1.05E-06
Spirosoma linguale	Bf2	19.25	6.60E-03
Solibacillus silvestris	Bf2	15.39	4.98E-03
Nocardioides cavernae	Bf2	17.57	6.95E-07

Bf24

Bacterial Taxa	Enriched	Log Fold	P-adjust
Daejeonella composti	B1	-23.22891	1.48E-06
Paenarthrobacter nicotinovorans	B1	-19.0309	2.22E-09
Paenibacillus sp. 37	B1	-22.05469	1.92E-15
Paenibacillus xylanexedens	B1	-22.52285	2.56E-15
Nocardioides alpinus	Bf24	17.17779	5.64E-05
Nocardioides cavernae	Bf24	18.14108	8.26E-09

Bf48

Bacterial Taxa	Enriched	Log Fold	P-adjust
Anabaena cylindrica	B1	-25.74	5.50E-03
Halomicronema hongdechloris	B1	-24.49	8.58E-04
Oscillatoria nigro-viridis	B1	-26.77	4.80E-14
Rhizobacter gummiphilus	B1	-23.10	5.80E-03
Sphingoaurantiacus capsulatus	B1	-22.04	1.25E-17
Sphingoaurantiacus polygranulatus	B1	-22.36	1.95E-16
Prosthecobacter fluviatilis	B1	-22.69	9.91E-08
Nocardioides alpinus	Bf48	18.71	3.05E-04
Solibacillus silvestris	Bf48	16.33	1.56E-03
Nocardioides cavernae	Bf48	18.50	7.24E-08

Table 9: Differential Abundance Comparison of Brassica 1 and Brassica-Alfalfa-Fescue Mixtures. Enriched column shows which treatment the bacterial taxa is enriched (B1: single brassica plant, Baf1: single brassica, alfalfa, and fescue plants, Baf24: 8 brassica, alfalfa, and fescue plants, Baf48: 16 brassica, alfalfa, and fescue plants). Bacterial taxa which were enriched when brassica was grown alone as compared to multiple density treatments. Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched in more than one diversity treatment is highlighted in light sky blue. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

Baf3

Bacterial Taxa	Enriched	Log Fold	P-adjust
Daejeonella oryzae	B1	-23.20	1.23E-13
Exiguobacterium aurantiacum	B1	-20.41	1.03E-10
Gemmata massiliana	B1	-21.30	7.67E-09
Gemmata sp. SH-PL17	B1	-21.26	1.61E-05
Larkinella insperata	B1	-23.77	1.71E-05
Prosthecobacter fluviatilis	B1	-19.85	4.00E-06
Sphingoaurantiacus polygranulatus	B1	-20.69	2.49E-12
Daejeonella composti	B1	-20.21	1.25E-04
Pontibacter rhizosphera	B1	-19.68	1.42E-05
Nocardioides alpinus	Baf3	18.59	5.58E-05

Baf24

Bacterial Taxa	Enriched	Log Fold	P-adjust
Aneurinibacillus soli	B1	-20.95	4.56E-03
Daejeonella composti	B1	-25.20	7.92E-06
Pontibacter rhizosphera	B1	-24.00	4.18E-07
Tumebacillus flagellatus	Baf24	4.89	4.56E-03
Nocardioides cavernae	Baf24	16.46	8.50E-06
Solibacillus silvestris	Baf24	19.05	9.46E-05
Nocardioides alpinus	Baf24	18.27	6.21E-04

Baf48

Bacterial Taxa	Enriched	Log Fold	P-adjust
Azospirillum sp. TSH58	B1	-23.14	7.89E-09
Exiguobacterium acetylicum	B1	-22.59	5.47E-20
Exiguobacterium antarcticum	B1	-4.71	3.69E-03
Exiguobacterium sibiricum	B1	-4.44	7.68E-03
Exiguobacterium sp. MH3	B1	-7.89	6.12E-03
Exiguobacterium sp. U13-1	B1	-22.94	2.51E-19
Exiguobacterium undae	B1	-5.19	2.71E-04
Leptolyngbya boryana	B1	-21.33	4.00E-03
Paenibacillus odorifer	B1	-20.06	1.43E-04
Paenibacillus sp. 37	B1	-22.36	1.43E-15
Paenibacillus sp. FSL H7-0357	B1	-19.24	5.81E-03
Paenibacillus xylanexedens	B1	-22.42	5.93E-15
Pseudomonas koreensis	B1	-20.06	8.10E-03
Stenotrophomonas sp. MYb57	B1	-22.33	1.77E-13
Trichocoleus desertorum	B1	-26.03	1.49E-11
Spirosoma linguale	Baf48	19.37	4.00E-03
Nocardioides cavernae	Baf48	18.64	2.64E-08
Solibacillus silvestris	Baf48	18.12	1.43E-04
Nocardioides alpinus	Baf48	18.24	2.71E-04

Table 10: Differential Abundance Comparison of Fescue 1 and Fescue Plant Densities. Enriched column shows which treatment the bacterial taxa is enriched (F1: single fescue plant, F24: 24 fescue plants, F48: 48 fescue plants). Bacterial taxa which were enriched when fescue was grown alone as compared to multiple density treatments. Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched in more than one diversity treatment is highlighted in light sky blue. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

F24				**F48**			
Bacterial Taxa	Enriched	Log Fold	P-adjust	Bacterial Taxa	Enriched	Log Fold	P-adjust
Telluribacter humicola	F1	-19.97	5.94E-06	*Kaistia defluvii*	F1	-24.00	2.26E-08
Lysobacter helvus	F1	-22.62	7.67E-04	*Pseudomonas stutzeri*	F1	-22.96	2.03E-08
Lysobacter sp. TY2-98	F1	-21.69	2.12E-03	*Adhaeribacter terreus*	F48	8.53	7.45E-04
Metabacillus indicus	F24	2.64	7.35E-03	*Adhaeribacter swui*	F48	5.51	6.84E-03
Sinorhizobium meliloti	F24	7.52	7.08E-03	*Dyadobacter sediminis*	F48	18.80	1.23E-07
Trichormus azollae	F24	20.06	8.95E-03	*Ensifer adhaerens*	F48	16.76	7.45E-04
Adhaeribacter aerophilus	F24	14.77	5.94E-06	*Adhaeribacter aerophilus*	F48	15.30	1.25E-06
[Brevibacterium] frigoritolerans	F24	18.96	7.67E-04	*[Brevibacterium] frigoritolerans*	F48	20.12	1.60E-04
Larkinella arboricola	F24	19.42	3.03E-05	*Larkinella arboricola*	F48	18.29	8.47E-05
Larkinella insperata	F24	19.98	7.80E-07	*Larkinella insperata*	F48	18.63	2.32E-06
Paenibacillus sp. 37	F24	17.39	5.86E-10	*Paenibacillus* sp. 37	F48	17.15	9.81E-10

Table 11: Differential Abundance Comparison of Fescue 1 and Fescue-Alfalfa Mixtures. Enriched column shows which treatment the bacterial taxa is enriched (F1: single fescue plant, Fa1: single fescue and alfalfa plants, Fa24: 12 fescue and alfalfa plants, Fa48: 24 fescue and alfalfa plants). Bacterial taxa which were enriched when fescue was grown alone as compared to multiple density treatments. Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched in more than one diversity treatment is highlighted in light sky blue. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

Fa2

Bacterial Taxa	Enriched	Log Fold	P-adjust
Anabaena cylindrica	F1	-23.61	1.94E-09
Azospirillum brasilense	F1	-20.75	3.79E-08
Azospirillum sp. TSH58	F1	-20.48	3.72E-05
Calothrix sp. PCC 7507	F1	-17.47	2.44E-03
Halomicronema hongdechloris	F1	-18.51	5.70E-06
Peribacillus muralis	F1	-7.54	2.72E-03
Peribacillus simplex	F1	-17.07	9.67E-04
Sinorhizobium meliloti	Fa2	7.81	3.72E-03
Ensifer adhaerens	Fa2	16.80	1.24E-03
Larkinella arboricola	Fa2	17.97	2.08E-04
Larkinella insperata	Fa2	17.38	3.72E-05

Fa24

Bacterial Taxa	Enriched	Log Fold	P-adjust
Lysobacter helvus	F1	-21.88	7.45E-04
Lysobacter sp. TY2-98	F1	-20.78	2.58E-03
Noviherbaspirillum malthae	F1	-7.31	7.37E-03
Pararcticibacter amylolyticus	F1	-18.75	1.40E-05
Phenylobacterium haematophilum	F1	-8.10	9.63E-05
Pseudomonas stutzeri	F1	-21.91	7.67E-08
Pseudoxanthomonas mexicana	F1	-22.30	1.44E-05
Telluribacter humicola	F1	-18.64	2.04E-05
Trichormus azollae	Fa24	20.99	3.35E-03
[Brevibacterium] frigoritolerans	Fa24	15.99	6.63E-03
Paenibacillus sp. 37	Fa24	20.29	4.50E-14
Dyadobacter sediminis	Fa24	19.97	2.48E-08
Adhaeribacter aerophilus	Fa24	16.71	7.67E-08
Ensifer adhaerens	Fa24	19.30	4.07E-05
Larkinella arboricola	Fa24	18.57	4.60E-05
Larkinella insperata	Fa24	18.80	2.39E-06

Fa48

Bacterial Taxa	Enriched	Log Fold	P-adjust
Azospirillum sp. TSA2s	F1	-21.84	2.32E-05
Nostoc flagelliforme	F1	-21.72	6.09E-03
Stenotrophomonas sp. G4	F1	-22.37	6.45E-29
Sinorhizobium meliloti	Fa48	8.19	1.54E-03
Adhaeribacter aerophilus	Fa48	15.73	5.86E-07
Dyadobacter sediminis	Fa48	17.57	1.12E-06
Paenibacillus sp. 37	Fa48	16.81	1.47E-09
Ensifer adhaerens	Fa48	14.87	6.09E-03
Larkinella arboricola	Fa48	19.06	2.85E-05
Larkinella insperata	Fa48	19.82	5.58E-07

Table 12: Differential Abundance Comparison of Fescue 1 and Fescue-Brassica Mixtures. Enriched column shows which treatment the bacterial taxa is enriched (F1: single fescue plant, Fb1: single fescue and brassica plants, Fb24: 12 fescue and brassica plants, Fb48: 24 fescue and brassica plants). Bacterial taxa which were enriched when fescue was grown alone as compared to multiple density treatments. Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched in more than one diversity treatment is highlighted in light sky blue. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

Fb2

Bacterial Taxa	Enriched	Log Fold	P-adjust
Lysobacter helvus	F1	-21.99	1.23E-03
Lysobacter sp. TY2-98	F1	-20.69	4.37E-03
Azohydromonas australica	F1	-22.04	1.97E-03
Calothrix sp. PCC 7507	F1	-18.91	6.96E-04
Ensifer adhaerens	Fb2	20.85	1.35E-05
Adhaeribacter aerophilus	Fb2	15.95	8.42E-07
Dyadobacter sediminis	Fb2	18.17	8.42E-07
Larkinella arboricola	Fb2	17.29	5.05E-04
Larkinella insperata	Fb2	17.78	1.63E-05
Paenibacillus sp. 37	Fb2	20.05	1.26E-13

Fb24

Bacterial Taxa	Enriched	Log Fold	P-adjust
Anabaena cylindrica	F1	-24.97	2.26E-09
Azospirillum sp. TSA2s	F1	-22.24	1.01E-04
Azospirillum sp. TSH58	F1	-21.42	5.94E-05
Calothrix sp. PCC 7507	F1	-18.38	2.42E-03
[Brevibacterium] frigoritolerans	Fb24	20.90	3.08E-04
Adhaeribacter aerophilus	Fb24	15.25	1.38E-05
Dyadobacter sediminis	Fb24	14.96	4.12E-04
Larkinella arboricola	Fb24	15.13	8.77E-03
Larkinella insperata	Fb24	16.49	3.08E-04
Paenibacillus sp. 37	Fb24	16.27	8.37E-08

Fb48

Bacterial Taxa	Enriched	Log Fold	P-adjust
Anabaena cylindrica	F1	-24.75	3.70E-11
Leptolyngbya sp. O-77	F1	-23.95	2.27E-11
Nostoc flagelliforme	F1	-22.85	1.96E-03
Oscillatoria nigro-viridis	F1	-26.59	7.75E-23
Calothrix sp. PCC 7507	F1	-20.36	6.17E-05
Azohydromonas australica	F1	-22.99	4.75E-04
[Brevibacterium] frigoritolerans	Fb48	15.83	6.88E-03
Ensifer adhaerens	Fb48	19.07	4.32E-05
Adhaeribacter aerophilus	Fb48	15.45	5.67E-07
Dyadobacter sediminis	Fb48	18.89	7.28E-08
Larkinella arboricola	Fb48	20.09	5.48E-06
Larkinella insperata	Fb48	20.81	6.40E-08
Paenibacillus sp. 37	Fb48	14.65	2.32E-07

Table 13: Differential Abundance Comparison of Fescue 1 and Fescue-Alfalfa-Brassica Mixtures. Enriched column shows which treatment the bacterial taxa is enriched (F1: single fescue plant, Fab1: single fescue, alfalfa, and brassica plants, Fab24: 8 fescue, alfalfa, and brassica plants, Fab48: 16 fescue, alfalfa, and brassica plants). Bacterial taxa which were enriched when fescue was grown alone as compared to multiple density treatments. Bacterial taxa which were enriched in only one treatment of increasing plant density is highlighted in orange. Bacterial taxa which were enriched in more than one diversity treatment is highlighted in light sky blue. Bacterial taxa which were enriched all density treatment is highlighted in sky blue.

Fba3

Bacterial Taxa	Enriched	Log Fold	P-adjust
Planctomyces sp. SH-PL14	F1	-24.16	2.55E-10
Planomicrobium chinense	F1	-20.01	8.77E-09
Calothrix sp. PCC 7507	F1	-19.38	7.11E-04
Nostoc flagelliforme	F1	-22.24	9.13E-03
Adhaeribacter terreus	Fab3	8.42	2.50E-03
Adhaeribacter swui	Fab3	5.80	8.52E-03
Paenibacillus sp. 37	Fab3	19.72	5.57E-13
Adhaeribacter aerophilus	Fab3	18.42	2.54E-09
Dyadobacter sediminis	Fab3	17.29	3.99E-06

Fab24

Bacterial Taxa	Enriched	Log Fold	P-adjust
Daejeonella oryzae	F1	-19.11	5.81E-06
Leptolyngbya sp. O-77	F1	-23.64	1.29E-10
[Brevibacterium] frigoritolerans	Fab24	17.49	2.80E-03
Trichormus azollae	Fab24	20.50	7.39E-03
Paenibacillus sp. 37	Fab24	17.33	3.63E-10
Ensifer adhaerens	Fab24	19.02	7.16E-05
Larkinella arboricola	Fab24	18.96	4.23E-05
Larkinella insperata	Fab24	20.29	2.31E-07
Adhaeribacter aerophilus	Fab24	14.58	5.97E-06
Dyadobacter sediminis	Fab24	19.49	4.89E-08

Fab48

Bacterial Taxa	Enriched	Log Fold	P-adjust
Azospirillum brasilense	F1	-22.10	1.13E-09
Limisphaera ngatamarikiensis	F1	-3.43	3.00E-04
Oscillatoria nigro-viridis	F1	-25.25	2.55E-20
Calothrix sp. PCC 7507	F1	-19.37	2.68E-04
Nostoc flagelliforme	F1	-22.17	4.23E-03
Leptolyngbya sp. O-77	F1	-23.55	8.80E-11
Planomicrobium chinense	F1	-19.94	5.62E-09
Planctomyces sp. SH-PL14	F1	-24.22	1.02E-10
Sinorhizobium meliloti	Fab48	8.81	3.23E-04
Ensifer adhaerens	Fab48	19.73	2.81E-05
Larkinella arboricola	Fab48	17.71	1.74E-04
Larkinella insperata	Fab48	18.35	3.84E-06
Adhaeribacter aerophilus	Fab48	15.84	4.34E-07
Dyadobacter sediminis	Fab48	17.20	2.27E-06

Table 14: 1st, 2nd, and 3rd largest module by plant network.

Network	Module 1: 17.31%	Module 2: 17.31%	Module 3: 13.46%
A	*Alkalihalobacillus halodurans* *Bacillus infantis* *Bacillus methanolicus* *Bacillus* sp. S3 *Bacillus* sp. X1(2014) *Bacillus* sp. 1NLA3E *Mesobacillus subterraneus* *Mesobacillus foraminis* *Paenibacillus beijingensis*	*Bacillus circulans* *Cohnella abietis* *Cohnella phaseoli* *Cohnella massiliensis* *Cytobacillus oceanisediminis* *Limisphaera ngatamarikiensis* *Paenibacillus swuensis* *Paenibacillus chitinolvticus* *Paenibacillus tyrfis*	*Arthrobacter* sp. KBS0702 *Arthrobacter* sp. PGP41 *Arthrobacter* sp. QXT-31 *Arthrobacter* sp. UKPF54-2 *Pseudarthrobacter* sp. NIBRBAC000502771 *Pseudarthrobacter phenanthrenivorans*
Network	Module 1: 13.43%	Module 2: 8.96%	Module 3: 8.96%
Ab	*Flavisolibacter galbus* *Flavisolibacter ginsengisoli* *Flavisolibacter ginsenosidimutans* *Microvirga aerilata* *Microvirga ossetica* *Microvirga soli* *Microvirga subterranean* *Microvirga zambiensis* *Microvirga* sp. 17 mud 1-3	*Bacillus* sp. X1(2014) *Bacillus* sp. 1NLA3E *Bacillus* sp. S3 *Bacillus infantis* *Bacillus* sp. Y1. *Neobacillus niacin*	*Anabaena cylindrica* *Exiguobacterium antarcticum* *Exiguobacterium sibiricum* *Exiguobacterium undae* *Stenotrophomonas* sp. G4 *Stenotrophomonas* sp. MYb57
Network	Module 1: 16.22%	Module 2: 13.51%	Module 3: 8.11%
Af	*Bacillus infantis* *Bacillus mediterraneensis* *Bacillus methanolicus*	*Achromobacter xylosoxidans* *Ensifer adhaerens*	*Massilia agri* *Massilia oculi* *Massilia* sp. WG5

	Bacillus sp. 1NLA3E *Mesobacillus foraminis* *Mesobacillus subterraneus*	*Exiguobacterium mexicanum* *Stenotrophomonas* sp. G4 *Stenotrophomonas* sp. MYb57	
Network	Module 1: 14.55%	Module 2: 12.73%	Module 3: 10.91%
Abf	*Massilia agri* *Massilia albidiflava* *Massilia oculi* *Massilia putida* *Massilia timonae* *Massilia umbonata* *Massilia violaceinigra* *Massilia* sp. WG5	*Bacillus acidicola* *Bacillus carboniphilus* *Bacillus mediterraneensis* *Bacillus licheniformis* *Bacillus paralicheniformis* *Mesobacillus foraminis* *Mesobacillus subterraneus*	*Alkalihalobacillus halodurans* *Bacillus circulans* *Bacillus dafuensis* *Bacillus infantis* *Bacillus methanolicus* *Cytobacillus gottheili*
Network	Module 1: 22.41%	Module 2: 13.79%	Module 3: 4.9%
B	*Ammoniphilus resinae* *Alkalihalobacillus halodurans* *Bacillus mediterraneensis* *Bacillus methanolicus* *Bacillus infantis* *Bacillus* sp. 1NLA3E *Cytobacillus gottheilii* *Mesobacillus foraminis* *Mesobacillus subterraneus* *Oxalophagus oxalicus* *Paenibacillus beijingensis* *Paenibacillus chitinolyticus* *Paenibacillus vunnanensis*	*Cohnella abietis* *Cohnella candidum* *Cohnella massiliensis* *Cohnella phaseoli* *Cytobacillus oceanisediminis* *Tumebacillus algifaecis* *Tumebacillus ginsengisoli* *Tumebacillus soli*	4 way tie *Exiguobactrium* sp. *Flavisolibacter*, *Bacillus* sp. *Microvirga* sp.

Network	Module 1: 14.89%	Module 2: 12.77%	Module 3: 8.51%
Ba	Alkalihalobacillus halodurans Bacillus dafuensis Bacillus mediterraneensis Bacillus methanolicus Cytobacillus gottheilli Paenibacillus beijingensis Paenibacillus chitinolyticus	*Bacillus infantis* *Bacillus* sp. 1NLA3E *Bacillus* sp. Y1. *Cytobacillus* *Oceanisediminis* *Mesobacillus foraminis* *Mesobacillus litoralis*	*Bacillus* sp. S3 *Bacillus* sp. X1(2014) *Neobacillus mesonae* *Neobacillus niacin*
Network	Module 1: 23.33%	Module 2: 18.33%	Module 3: 11.67%
Bf	*Bacillus infantis* *Bacillus mediterraneensis* *Bacillus methanolicus* *Bacillus* sp. S3 *Bacillus* sp. X1(2014) *Bacillus* sp. Y1. *Bacillus* sp. 1NLA3E *Cytobacillus gottheilii* *Cytobacillus Oceanisediminis* *Mesobacillus foraminis* *Metabacillus litoralis* *Mesobacillus subterraneus* *Neobacillus niacin* *Neobacillus mesonae*	*Massilia armeniaca* *Massilia namucuonensis* *Massilia putida* *Massilia oculi* *Massilia umbonate* *Massilia violaceinigra* *Microvirga ossetica* *Microvirga subterranean* *Microvirga* sp. 17 mud 1-3 *Paracoccus* sp. Arc7-R13	*Exiguobacterium acetylicum* *Exiguobacterium antarcticum* *Exiguobacterium sibiricum* *Exiguobacterium undae* *Exiguobacterium* sp. U13-1 *Exiguobacterium* sp. ZWU0009 *Flavisolibacter* sp. 17J28-1
Network	Module 1: 18.87%	Module 2: 13.21%	Module 3: 7.55%
Baf	*Alkalihalobacillus halodurans* *Bacillus mediterraneensis* *Bacillus methanolicus*	*Massilia agri* *Massilia alkalitolerans* *Massilia oculi*	*Bacillus* sp. S3 *Bacillus* sp. X1(2014) *Bacillus* sp. 1NLA3E

	Devosia sp. A16 *Flaviaesturaribacter luteus* *Mesobacillus foraminis* *Mesobacillus stamsii* *Mesobacillus subterraneus* *Paenibacillus chitinolyticus* *Pontibacter populi*	*Massilia putida* *Massilia Umbonata* *Massilia violaceinigra* *Massilia* sp. WG5	*Neobacillus mesonae*
Network	Module 1: 12.5%	Module 2: 9.38%	Module 3: 9.38%
F	*Microvirga ossetica* *Microvirga soli* *Microvirga subterranean* *Microvirga* sp. 17 mud 1-3	*Exiguobacterium antarcticum* *Exiguobacterium sibiricum* *Exiguobacterium undae*	*Noviherbaspirillum aurantiacum* *Noviherbaspirillum massiliense* *Noviherbaspirillum soli*
Network	Module 1: 19.15%	Module 2: 10.64%	Module 3: 8.51%
Fa	*Alkalihalobacillus halodurans* *Bacillus ciccensis* *Bacillus infantis* *Bacillus mediterraneensis* *Bacillus methanolicus* *Cytobacillus gottheili* *Mesobacillus foraminis* *Mesobacillus subterraneus* *Paenibacillus chitinolvticus*	*Massilia agri* *Massilia oculi* *Massilia putida* *Massilia umbonate* *Massilia* sp. WG5	*Herbaspirillum* sp. meg3 *Herbaspirillum seropedica* *Noviherbaspirillum aurantiacum* *Noviherbaspirillum soli*
Network	Module 1: 20.73%	Module 2: 14.63%	Module 3: 14.63%
Fb	*Ammoniphilus resinae* *Bacillus infantis* *Bacillus mediterraneensis*	*Massilia albidiflava* *Massilia armeniaca* *Massilia namucuonensis*	*Achromobacter insolitus* *Achromobacter spanius*

	Bacillus methanolicus *Bacillus* sp. S3 *Bacillus* sp. X1(2014) *Bacillus* sp. 1NLA3E *Cytobacillus gottheili* *Cytobacillus oceanisediminis* *Mesobacillus foraminis* *Mesobacillus stamsii* *Mesobacillus subterraneus* *Oxalophagus oxalicus* *Paenibacillus mucilaginosus* *Pedobacter mongoliensis* *Roseisolibacter agri* *Rufibacter* sp. DG31D	*Massilia oculi* *Massilia putida* *Massilia umbonate* *Massilia* *Microvirga ossetica* *Microvirga subterrânea* *Microvirga zambiensis* *Microvirga* sp. 17 mud 1-3 *Ramlibacter tataouinensis*	*Achromobacter xylosoxidans* *Chthoniobacter flavus* *Flavisolibacter ginsenosidimutans* *Flavisolibacter tropicus* *Metabacillus litoralis* *Methylotenera versatilis* *Pontibacter chitinilvticus* *Pontibacter korlensis* *Pontibacter Populi* *Rubellimicrobium roseum* Fescue-alfalfa-brassica plant mixture
Network	Module 1: 20%	Module 2: 16.67%	Module 3: 10%
Fab	*Bacillus methanolicus* *Bacillus* sp. S3 *Bacillus* sp. X1(2014) *Bacillus* sp. 1NLA3E *Neobacillus mesonae* *Paenibacillus yunnanensis*	*Microvirga ossetica* *Microvirga soli* *Microvirga subterranean* *Microvirga zambiensis* *Rubellimicrobium roseum*	5 way tie

Table 15: Bacteriome Network Statistics. First capitalized letter denotes plant species rhizosphere; lower case letter denotes neighboring plant species.

Network	Node	Edge	Avg. Weight Degree	Graph Density	Connected Components	Modularity	Avg. Clustering Coefficient	Avg. Path Length
A	52	58	2.037	0.044	12	0.858	0.627	2.065
Ab	71	56	1.577	0.023	27	0.898	0.576	1.71
Af	42	26	1.141	0.03	19	0.876	0.515	1.537
Abf	55	49	1.597	0.033	18	0.873	0.636	1.54
B	58	80	2.454	0.048	13	0.714	0.678	2.88
Ba	47	38	1.454	0.035	15	0.861	0.525	2.184
Bf	60	99	2.993	0.056	10	0.761	0.688	2.942
Baf	53	54	1.822	0.039	15	0.819	0.676	2.139
F	32	21	1.206	0.042	14	0.898	0.926	1.087
Fa	47	35	1.34	0.032	16	0.874	0.473	1.775
Fb	82	136	2.948	0.041	10	0.682	0.478	5.331
Fab	30	26	1.556	0.06	9	0.835	0.562	1.452

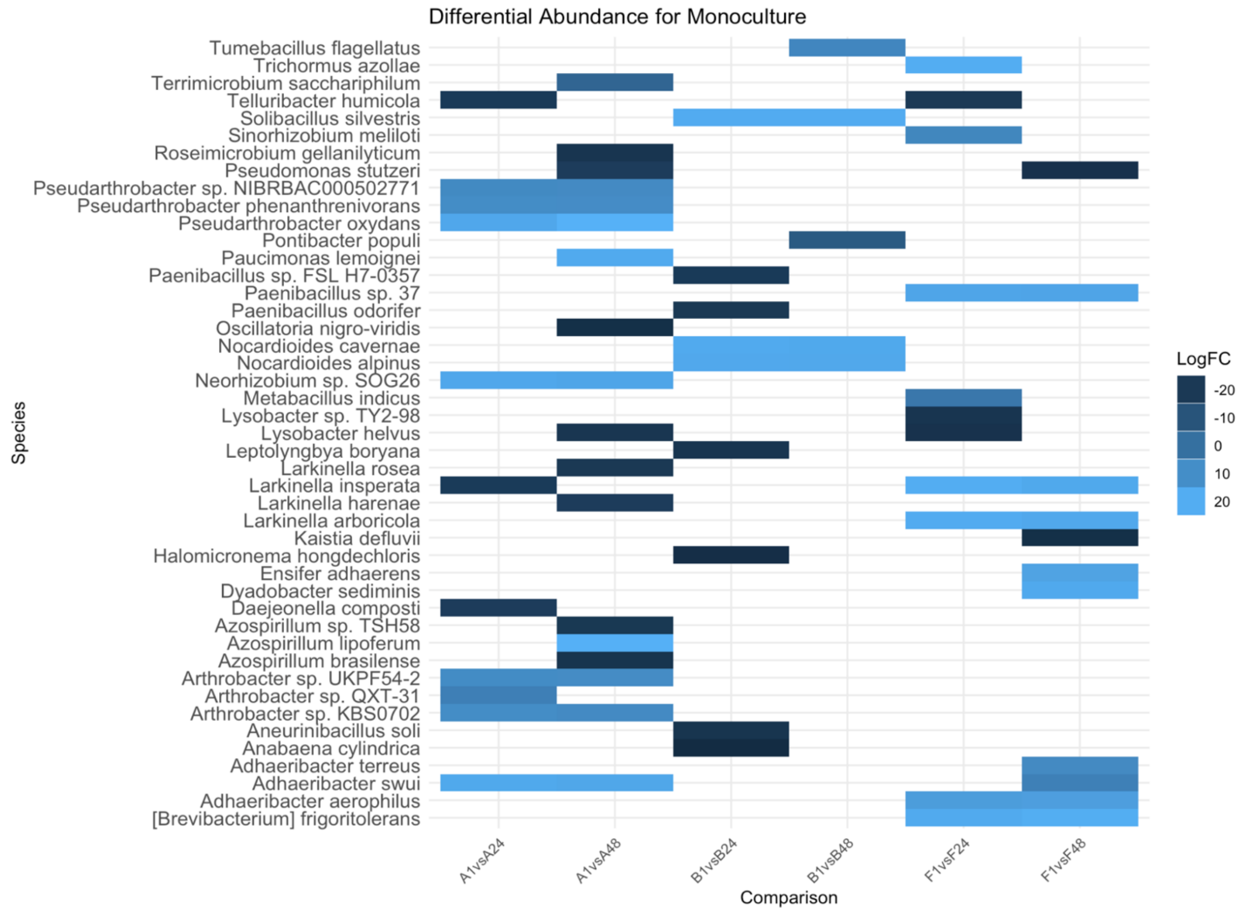

Figure 1: Differential abundance comparisons of individual plant (alfalfa, brassica, and fescue) rhizospheres when grown alone (1 plant) versus in medium (24 plants) and high (48 plants) density monocultures. On the horizontal axis, the first letter denotes plant species (A: alfalfa, B: brassica, F: fescue) and number denotes density (i.e. plant count per pot; low density: 1 plant, medium density: 24 plants, high density: 48 plants). Dark blue denotes a negative correlation, while light blue denotes a positive correlation.

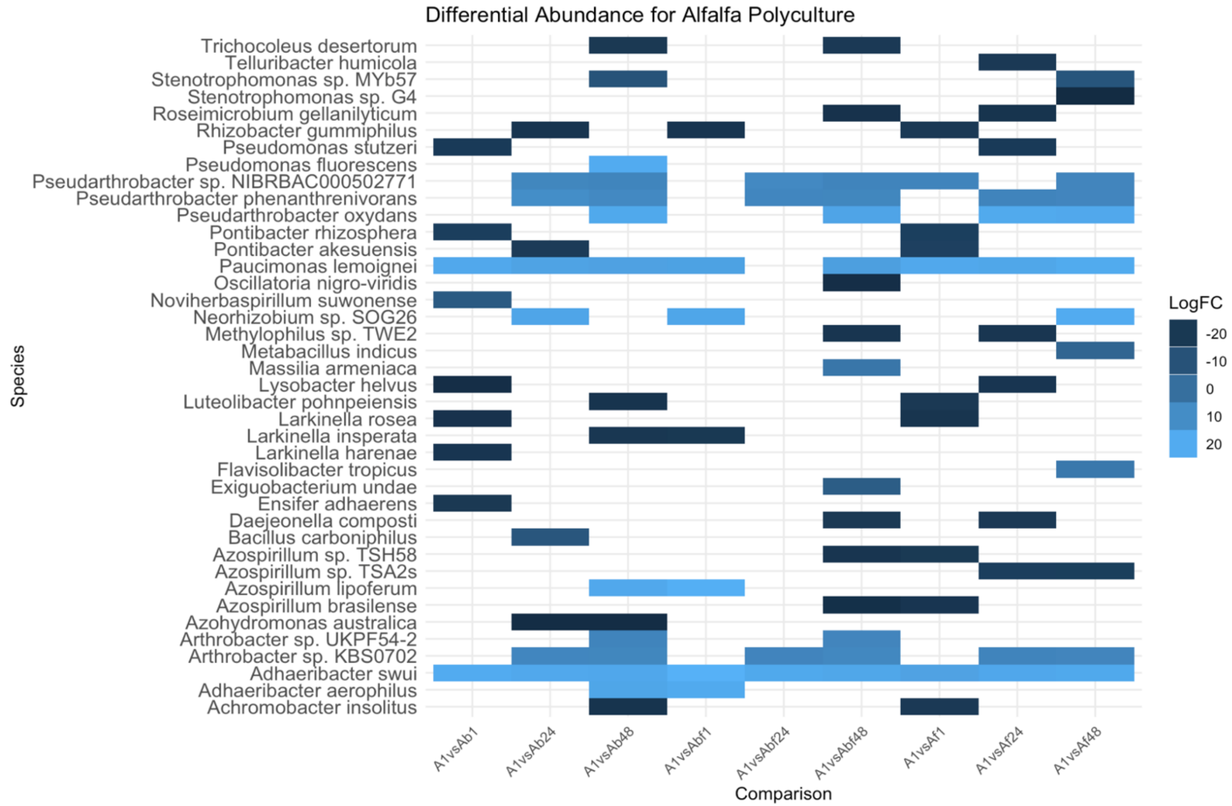

Figure 2: Differential abundance comparisons of alfalfa rhizospheres when grown alone (1 plant) versus in medium (24 plants) and high (48 plants) density polycultures. On the horizontal axis, the first letter denotes plant species (A: alfalfa rhizosphere), the lowercase letter denotes neighboring plant species (Ab: alfalfa rhizosphere with neighboring brassica, Af: alfalfa rhizosphere with neighboring fescue, Abf: alfalfa rhizosphere with neighboring brassica and fescue). Number denotes density (i.e., plant count per pot; low density: 1-3 plants, medium density: 24 plants, high density: 48 plants). Dark blue denotes a negative correlation, while light blue denotes a positive correlation.

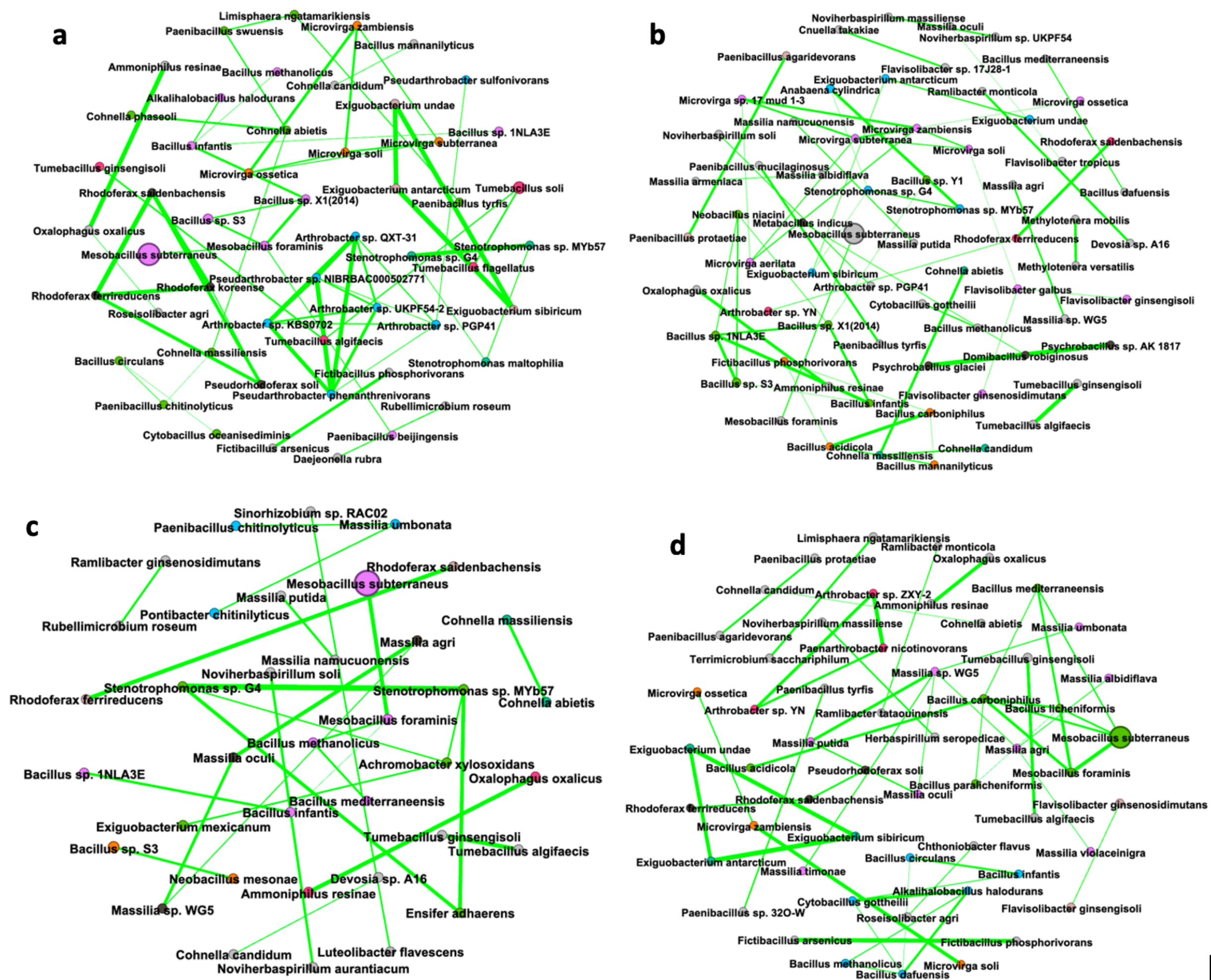

Figure 3: Bacteriome network of alfalfa in monoculture and plant mixtures. (**a**) Alfalfa monoculture (1 plant, 24 plants, 48 plants). (**b**) Alfalfa-brassica plant mixture (2 plants, 24 plants, 48 plants). (**c**) Alfalfa-fescue plant mixture (2 plants, 24 plants, 48 plants). (**d**) Alfalfa-brassica-fescue plant mixture (3 plants, 24 plants, 48 plants). Module color denotes module size (largest to smallest: lavender, lime green, sky blue, dark grey, orange, salmon, teal, grey); dot size denotes relative abundance (minimal size: 15, maximum size: 40); green edge denotes a positive correlation; red edge denotes a negative correlation.

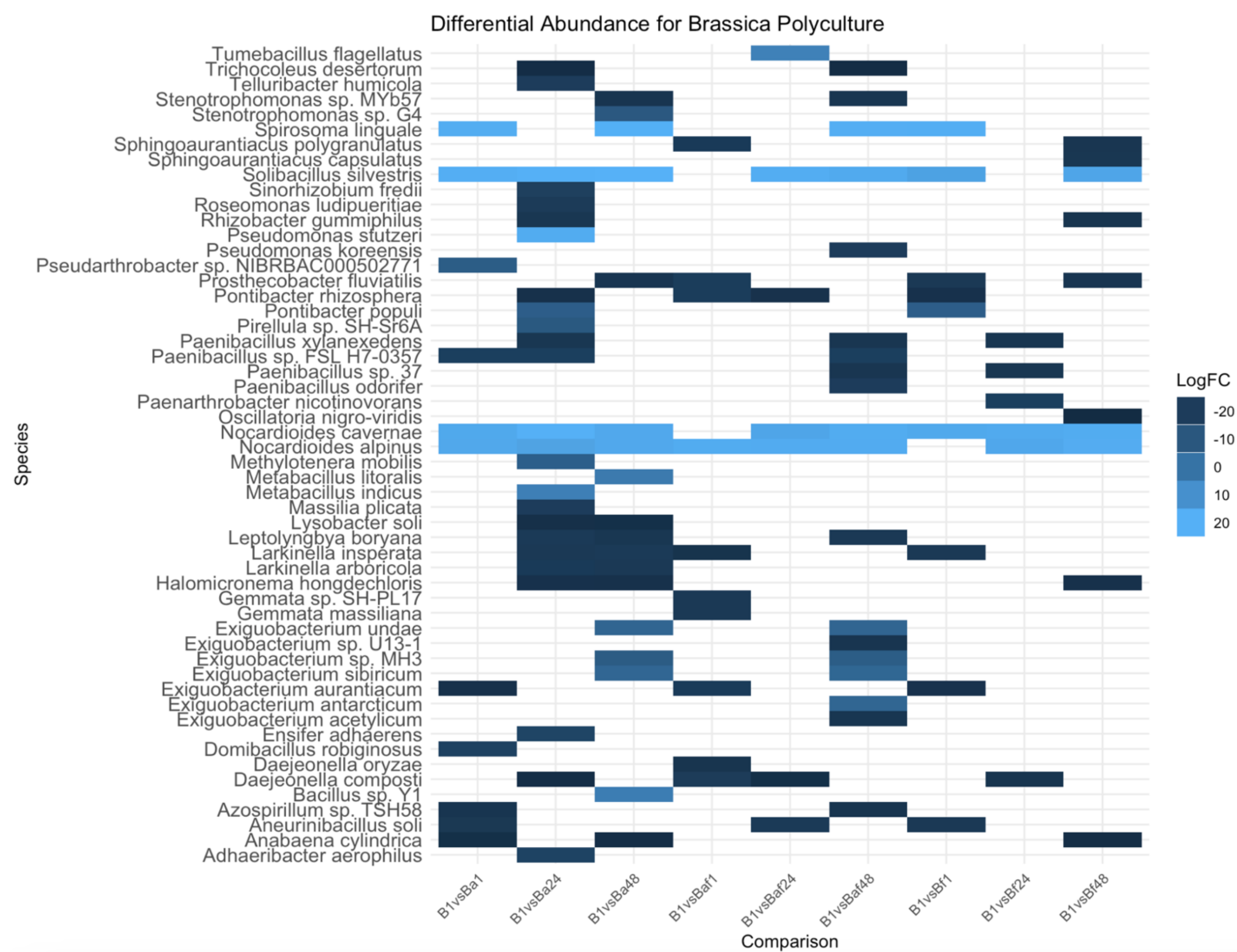

Figure 4: Differential abundance comparison of one brassica plant and medium (24 plants) and high (48 plants) densities in polyculture. First letter denotes plant species rhizosphere (B: brassica). Lower case letter denotes the plant species neighbor (e.g., Ba: brassica rhizosphere with alfalfa as a plant neighbor, Baf: brassica rhizosphere with alfalfa and fescue as plant neighbors). Number denotes density, or plant count per pot (low density: 1-3 plants, medium density: 24 plants, high density: 48 plants). Dark blue denotes a negative correlation while light blue denotes a positive correlation.

Figure 5: Bacteriome network of brassica in monoculture and plant mixtures. (**a**) Brassica monoculture (1 plant, 24 plants, 48 plants). (**b**) Brassica-alfalfa plant mixture (2 plants, 24 plants, 48 plants). (**c**) Brassica-fescue plant mixture (2 plants, 24 plants, 48 plants). (**d**) Brassica-alfalfa-fescue plant mixture (3 plants, 24 plants, 48 plants). Module color denotes module size (largest to smallest: lavender, lime green, sky blue, dark grey, orange, salmon, teal, grey), size of the dot denotes relative abundance (minimal size: 15, maximum size: 40); green edge denotes a positive correlation, and red edge denotes a negative correlation.

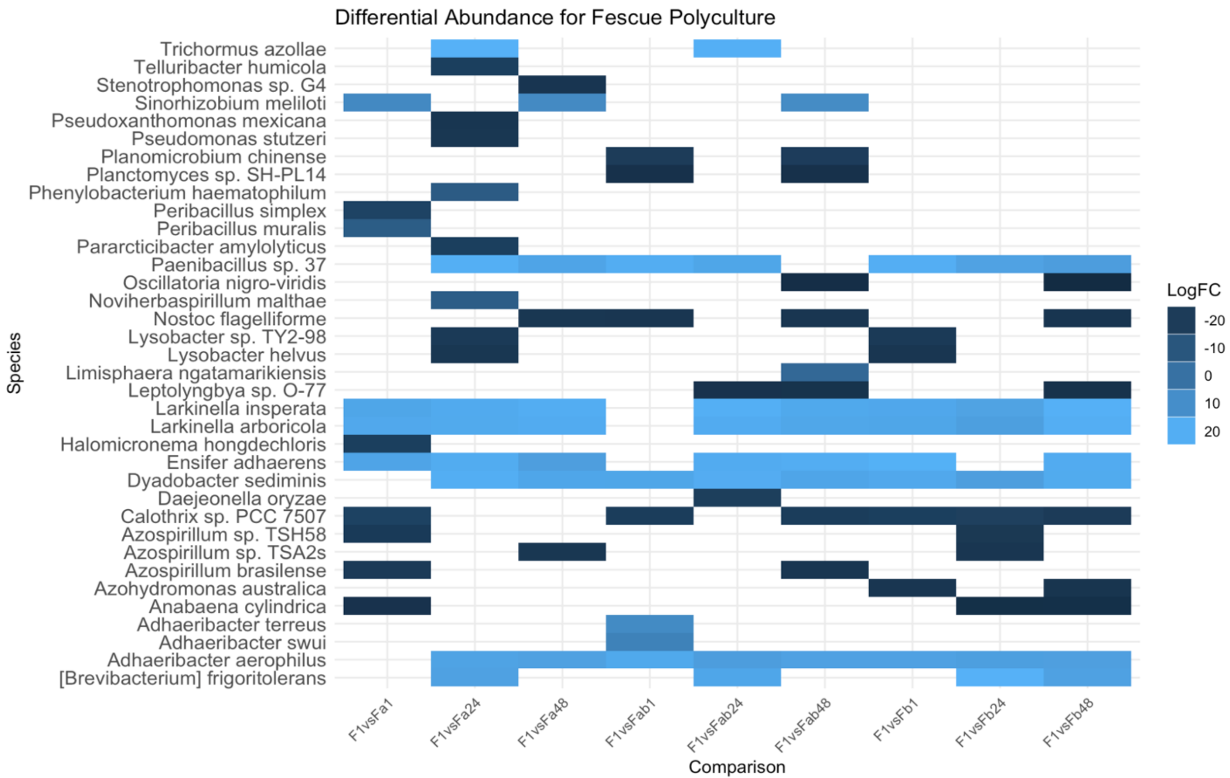

Figure 6: Differential abundance comparison of one fescue plant, and medium (24 plants) and high (48 plants) densities in polyculture. The first letter denotes plant species rhizosphere (F: fescue). The lower case letter denotes the plant species neighbor (e.g., Fa: fescue rhizosphere with alfalfa as a plant neighbor, Fab: fescue rhizosphere with alfalfa and brassica as plant neighbors). Number denotes density, or plant count per pot (low density: 1-3 plants, medium density: 24 plants, high density: 48 plants). Dark blue denotes a negative correlation while light blue denotes a positive correlation.

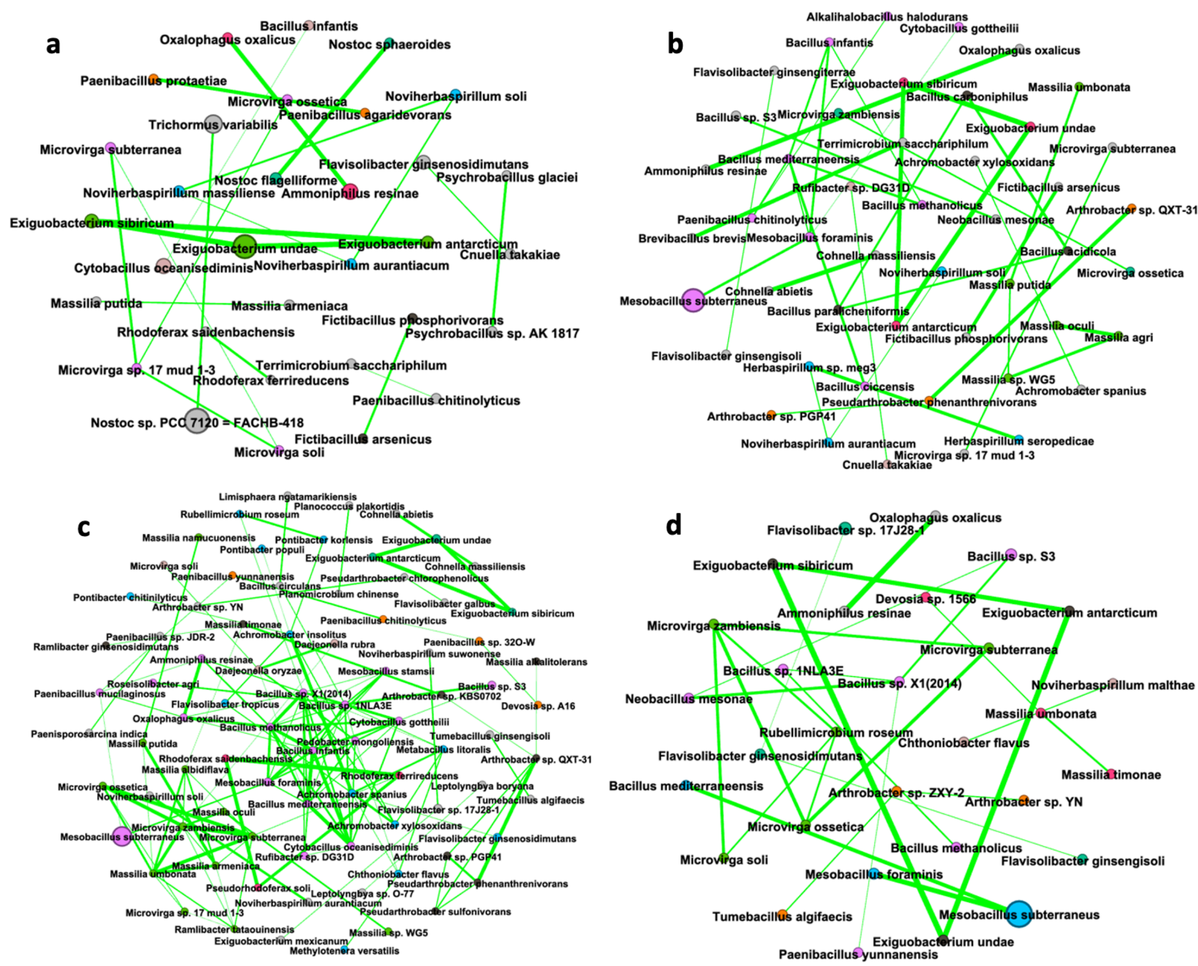

Figure 7: Bacteriome network of brassica in monoculture and plant mixtures. (**a**) Fescue monoculture (1 plant, 24 plants, 48 plants). (**b**) Fescue-alfalfa plant mixture (2 plants, 24 plants, 48 plants). (**c**) Fescue-brassica plant mixture (2 plants, 24 plants, 48 plants). (**d**) Fescue-alfalfa-brassica plant mixture (3 plants, 24 plants, 48 plants). Module color denotes module size (largest to smallest: lavender, lime green, sky blue, dark grey, orange, salmon, teal, grey), size of the dot denotes relative abundance (minimal size: 15, maximum size: 40); green edge denotes a positive correlation, and red edge denotes a negative correlation.

REFERENCES

Goh, C. H., Veliz Vallejos, D. F., Nicotra, A. B., & Mathesius, U. (2013). The impact of beneficial plant-associated microbes on plant phenotypic plasticity. Journal of chemical ecology, 39, 826-839.

Berendsen, R. L., Pieterse, C. M., & Bakker, P. A. (2012). The rhizosphere microbiome and plant health. Trends in plant science, 17(8), 478-486.

DiLegge, M. J., Manter, D. K., & Vivanco, J. M. (2022). Soil microbiome disruption reveals specific and general plant-bacterial relationships in three agroecosystem soils. Plos one, 17(11), e0277529.

Lyu, D., Zajonc, J., Pagé, A., Tanney, C. A., Shah, A., Monjezi, N., ... & Smith, D. L. (2021). Plant holobiont theory: the phytomicrobiome plays a central role in evolution and success. Microorganisms, 9(4), 675.

Wallenstein, M. D. (2017). Managing and manipulating the rhizosphere microbiome for plant health: a systems approach. Rhizosphere, 3, 230-232.

Hillesland, K. L. (2018). Evolution on the bright side of life: microorganisms and the evolution of mutualism. Annals of the New York Academy of Sciences, 1422(1), 88-103.

Morgan, J. A. W., Bending, G. D., & White, P. J. (2005). Biological costs and benefits to plant–microbe interactions in the rhizosphere. Journal of experimental botany, 56(417), 1729-1739.

Azaizeh, H. A., Marschner, H., Römheld, V., & Wittenmayer, L. (1995). Effects of a vesicular-arbuscular mycorrhizal fungus and other soil microorganisms on growth, mineral nutrient acquisition and root exudation of soil-grown maize plants. Mycorrhiza, 5, 321-327.

Pang, Z., Chen, J., Wang, T., Gao, C., Li, Z., Guo, L., ... & Cheng, Y. (2021). Linking plant secondary metabolites and plant microbiomes: a review. Frontiers in Plant Science, 12, 621276.

Walker, T. S., Bais, H. P., Grotewold, E., & Vivanco, J. M. (2003). Root exudation and rhizosphere biology. Plant physiology, 132(1), 44-51.

Chen, J. Z., Huang, X. L., Sun, Q. W., & Liu, J. M. (2023). Bulk soil microbial reservoir or plant recruitment dominates rhizosphere microbial community assembly: evidence from the rare, endangered Lauraceae species Cinmaomum migao. Ecological Indicators, 148, 110071.

Pantigoso, H. A., Newberger, D., & Vivanco, J. M. (2022). The rhizosphere microbiome: Plant–microbial interactions for resource acquisition. Journal of Applied Microbiology, 133(5), 2864-2876.

Monohon, S. J., Manter, D. K., & Vivanco, J. M. Conditioned soils reveal plant-selected microbial communities that impact plant drought response. *Scientific Reports*, **11(1),** 21153, (2021).

Chaparro, J. M., Badri, D. V., & Vivanco, J. M. (2014). Rhizosphere microbiome assemblage is affected by plant development. The ISME journal, 8(4), 790-803.

Vives-Peris, V., De Ollas, C., Gómez-Cadenas, A., & Pérez-Clemente, R. M. (2020). Root exudates: from plant to rhizosphere and beyond. Plant cell reports, 39, 3-17.

Pantigoso, H. A., Yuan, J., He, Y., Guo, Q., Vollmer, C., & Vivanco, J. M. (2020). Role of root exudates on assimilation of phosphorus in young and old Arabidopsis thaliana plants. PLoS One, 15(6), e0234216.

Singh, A. K., & Singla, P. (2020). Root phenolics profile modulates microbial ecology of rhizosphere. Plant Phenolics in Sustainable Agriculture: Volume 1, 555-578.

Bowen, G. D., & Rovira, A. D. (1999). The rhizosphere and its management to improve plant growth. *Advances in agronomy*, *66*, 1-102.

Compant, S., Clément, C., & Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry, 42(5), 669-678.

Marquez-Santacruz, H. A., Hernandez-Leon, R., Orozco-Mosqueda, M. D. C., Velazquez-Sepulveda, I., & Santoyo, G. (2010). Diversity of bacterial endophytes in roots of Mexican husk tomato plants(Physalisixocarpa) and their detection in the rhizosphere. Genetics and Molecular Research, 9(4), 2372-2380.

Reinhold-Hurek, B., & Hurek, T. (2011). Living inside plants: bacterial endophytes. Current opinion in plant biology, 14(4), 435-443.

Van Bael, S. A., Maynard, Z., Rojas, E., Mejia, L. C., Kyllo, D. A., Herre, E. A., ... & Arnold, A. E. (2005). Emerging perspectives on the ecological roles of endophytic fungi in tropical plants. *Mycology Series*, *23*, 181.

Ke, P. J., & Wan, J. (2023). A general approach for quantifying microbial effects on plant competition. Plant and Soil, 485(1-2), 57-70.

Martinuz, A., Schouten, A., & Sikora, R. A. (2012). Systemically induced resistance and microbial competitive exclusion: implications on biological control. Phytopathology, 102(3), 260-266.

Navarro-Noya, Y. E., Hernández-Mendoza, E., Morales-Jiménez, J., Jan-Roblero, J., Martínez-Romero, E., & Hernández-Rodríguez, C. (2012). Isolation and characterization of nitrogen fixing heterotrophic bacteria from the rhizosphere of pioneer plants growing on mine tailings. *Applied Soil Ecology*, *62*, 52-60.

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J., & Relman, D. A. (2012). The application of ecological theory toward an understanding of the human microbiome. *Science*, 336(6086), 1255-1262.

Nelson, E. B. (2018). The seed microbiome: origins, interactions, and impacts. *Plant and Soil*, *422*, 7-34.

Abdelfattah, A., Tack, A. J., Lobato, C., Wassermann, B., & Berg, G. (2023). From seed to seed: the role of microbial inheritance in the assembly of the plant microbiome. Trends in *Microbiology*.

Rodríguez, C. E., Antonielli, L., Mitter, B., Trognitz, F., & Sessitsch, A. (2020). Heritability and functional importance of the Setaria viridis bacterial seed microbiome. *Phytobiomes Journal*, *4*(1), 40-52.

Song, S. J., Lauber, C., Costello, E. K., Lozupone, C. A., Humphrey, G., Berg-Lyons, D., ... & Knight, R. (2013). Cohabiting family members share microbiota with one another and with their dogs. *elife*, *2*, e00458.

Andreu-Sánchez, Sergio, Jiafei Wu, and Jingyuan Fu. "Beyond personal space: Unveiling the transmission pattern of the human gut and oral microbiome." *iMeta* 2, no. 2 (2023): e98.

Xu, Z., York, L. M., Seethepalli, A., Bucciarelli, B., Cheng, H., & Samac, D. A. (2022). Objective phenotyping of root system architecture using image augmentation and machine learning in alfalfa (Medicago sativa l.). Plant Phenomics, 2022.

Kirkegaard, J. A., Angus, J. F., Gardner, P. A., & Cresswell, H. P. (1993). Benefits of brassica break crops in the Southeast wheatbelt. Proc. 7th Aust. Agron. Cons. Adelaide, 19-24.

Hannaway, D., Fransen, S. C., Cropper, J., Teel, M., Chaney, M., Griggs, T., ... & Lane, W. (1999). Tall fescue.

Wang, Y., & Zhou, X. (2023). Effects of green manures on rhizosphere fungal community composition of cucumber seedlings. Current Microbiology, 80(3), 87.

Zhao, Y., Haley, O. C., Xu, X., Jaberi-Douraki, M., Rivard, C., Pliakoni, E. D., ... & Bhullar, M. (2023). The Potential for Cover Crops to Reduce the Load of Escherichia coli in Contaminated Agricultural Soil. Journal of Food Protection, 86(7), 100103.

Li, Y., You, X., Tang, Z., Zhu, T., Liu, B., Chen, M. X., ... & Liu, T. Y. (2022). Isolation and identification of plant growth-promoting rhizobacteria from tall fescue rhizosphere and their functions under salt stress. Physiologia Plantarum, 174(6), e13817.

Feng, H., Fu, R., Hou, X., Lv, Y., Zhang, N., Liu, Y., ... & Zhang, R. (2021). Chemotaxis of beneficial rhizobacteria to root exudates: The first step towards root–microbe rhizosphere interactions. International Journal of Molecular Sciences, 22(13), 6655.

Anwar, M. S., Siddique, M. T., Verma, A., Rao, Y. R., Nailwal, T., Ansari, M., & Pande, V. (2014). Multitrait plant growth promoting (PGP) rhizobacterial isolates from Brassica juncea

rhizosphere: keratin degradation and growth promotion. Communicative & Integrative Biology, 7(1), e27683.

Liu, W., Hou, J., Wang, Q., Ding, L., & Luo, Y. (2014). Isolation and characterization of plant growth-promoting rhizobacteria and their effects on phytoremediation of petroleum-contaminated saline-alkali soil. Chemosphere, 117, 303-308.

Asghar, H., Zahir, Z., Arshad, M., & Khaliq, A. (2002). Relationship between in vitro production of auxins by rhizobacteria and their growth-promoting activities in Brassica juncea L. Biology and Fertility of Soils, 35, 231-237.

Wang, X., Sun, R., Tian, Y., Guo, K., Sun, H., Liu, X., ... & Liu, B. (2020). Long-term phytoremediation of coastal saline soil reveals plant species-specific patterns of microbial community recruitment. *Msystems*, *5*(2), 10-1128.

Newberger, D. R., Minas, I. S., Manter, D. K., & Vivanco, J. M. (2023). A Microbiological Approach to Alleviate Soil Replant Syndrome in Peaches. *Microorganisms*, *11*(6), 1448.

Li, K., DiLegge, M. J., Minas, I. S., Hamm, A., Manter, D., & Vivanco, J. M. Soil sterilization leads to re-colonization of a healthier rhizosphere microbiome. *Rhizosphere*, ***12*,** 100176, (2019).

Rohland, N., & Reich, D. (2012). Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. Genome research, 22(5), 939-946.

Stoddard, S. F., Smith, B. J., Hein, R., Roller, B. R. & Schmidt, T. M. (2015) rrnDB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. Nucl. Acids Res. 43, D593–D598.

O'Leary, N. A. et al. (2016) Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucl. Acids Res. 44, D733–D745.

Schoch, C. L. et al. (2020) NCBI Taxonomy: a comprehensive update on curation, resources and tools. Database (Oxford) 2020.

McMurdie, P. J., & Holmes, S. (2013). Package 'phyloseq'. *gan*, *2*(7).

Oksanen J, Simpson G, Blanchet F, Kindt R, Legendre P, Minchin P, O'Hara R, Solymos P, Stevens M, Szoecs E, Wagner H, Barbour M, Bedward M, Bolker B, Borcard D, Carvalho G, Chirico M, De Caceres M, Durand S, Evangelista H, FitzJohn R, Friendly M, Furneaux B, Hannigan G, Hill M, Lahti L, McGlinn D, Ouellette M, Ribeiro Cunha E, Smith T, Stier A, Ter Braak C, Weedon J (2022). _vegan: Community Ecology Package_. R package version 2.6-4, <https://CRAN.R-project.org/package=vegan>.

Cao, Y., Dong, Q., Wang, D., Zhang, P., Liu, Y., & Niu, C. (2022). microbiomeMarker: an R/Bioconductor package for microbiome marker identification and visualization. *Bioinformatics*, *38*(16), 4027-4029.

Wickham, H. (2011). ggplot2. *Wiley interdisciplinary reviews: computational statistics*, *3*(2), 180-185.

Liu, C., Cui, Y., Li, X., & Yao, M. (2021). microeco: an R package for data mining in microbial community ecology. *FEMS microbiology ecology*, *97*(2), fiaa255.

Csardi, G., & Nepusz, T. (2006). The igraph software package for complex network research. *InterJournal, complex systems*, *1695*(5), 1-9.

Yon, G. V., & Yon, M. G. V. (2015). Package 'rgexf'.

Alam, M., Arifuzzaman, S. M., & Bhuiyan, M. H. (2012). Large scale network visualization with gephi.

Craine, J. M., & Dybzinski, R. (2013). Mechanisms of plant competition for nutrients, water and light. Functional Ecology, 27(4), 833-840.

Park, I., Seo, Y. S., & Mannaa, M. (2023). Recruitment of the rhizo-microbiome army: assembly determinants and engineering of the rhizosphere microbiome as a key to unlocking plant potential. Frontiers in Microbiology, 14, 1163832.

Singh, H. P., Batish, D. R., & Kohli, R. K. (1999). Autotoxicity: concept, organisms, and ecological significance. Critical Reviews in Plant Sciences, 18(6), 757-772.

Chon, S. U., Jennings, J. A., & Nelson, C. J. (2006). Alfalfa (Medicago sativa L.) autotoxicity: Current status. Allelopathy Journal, 18(1), 57.

Wang, R., Liu, J., Jiang, W., Ji, P., & Li, Y. (2022). Metabolomics and Microbiomics Reveal Impacts of Rhizosphere Metabolites on Alfalfa Continuous Cropping. Frontiers in Microbiology, 13, 833968.

Jia, H., Rahman, M. K. U., Wu, F., & Zhou, X. (2020). Effects of rotation of Indian mustard on cucumber seedling rhizosphere fungal community composition. *Int. J. Agric. Biol*, *23*, 757-762.

Munir, A., Iqbal, S., Khaliq, B., Saeed, Q., Hussain, S., Shah, K. H., ... & Akrem, A. (2019). In silico studies and functional characterization of a napin protein from seeds of Brassica juncea. Int. J. Agri. Biol, 22, 1655-1662.

Walters, D. T., & Gilmore, A. R. (1976). Allelopathic effects of fescue on the growth of sweetgum. Journal of Chemical Ecology, 2, 469-479.

Malinowski, D. P., Belesky, D. P., & Fedders, J. M. (1999). Endophyte infection may affect the competitive ability of tall fescue grown with red clover. Journal of Agronomy and Crop Science, 183(2), 91-101.

Kim, D. U., Kim, K. W., Kang, M. S., Kim, J. Y., Jang, J. H., & Kim, M. K. (2018). Adhaeribacter swui sp. nov., isolated from wet mud. *International Journal of Systematic and Evolutionary Microbiology*, *68*(4), 1096-1100.

Jendrossek, D. (2001). Transfer of [Pseudomonas] lemoignei, a Gram-negative rod with restricted catabolic capacity, to Paucimonas gen. nov. with one species, Paucimonas lemoignei comb. nov. *International journal of systematic and evolutionary microbiology*, *51*(3), 905-908.

Park, M. K., Park, Y. J., Kim, M. C., Kang, G. U., Kim, M., Jo, Y., ... & Shin, J. H. (2019). Complete genome sequence of Pseudarthrobacter sp. NIBRBAC000502771 isolated from shooting range soil in Republic of Korea. *The Microbiological Society of Korea*, *55*(4), 419-421.

Tshishonga, K., & Serepa-Dlamini, M. H. (2020). Draft genome sequence of Pseudarthrobacter phenanthrenivorans Strain MHSD1, a bacterial endophyte isolated from the medicinal plant Pellaea calomelanos. *Evolutionary Bioinformatics*, *16*, 1176934320913257.

Bushra, R., Uzair, B., Ali, A., Manzoor, S., Abbas, S., & Ahmed, I. (2023). Draft genome sequence of a halotolerant plant growth-promoting bacterium Pseudarthrobacter oxydans NCCP-2145 isolated from rhizospheric soil of mangrove plant Avicennia marina. *Electronic Journal of Biotechnology*, *66*, 52-59.

Shen, W., Yu, X., Gao, N., Ota, S., Shiratori, Y., Nishizawa, T., ... & Senoo, K. (2019). Genome sequence of Arthrobacter sp. UKPF54-2, a plant growth-promoting rhizobacterial strain isolated from paddy soil. *Microbiology Resource Announcements*, *8*(45), e01005-19.

Alotaibi, F., St-Arnaud, M., & Hijri, M. (2022). In-depth characterization of plant growth promotion potentials of selected alkanes-degrading plant growth-promoting bacterial isolates. *Frontiers in Microbiology*, *13*, 863702.

Zhang, D. C., Schumann, P., Redzic, M., Zhou, Y. G., Liu, H. C., Schinner, F., & Margesin, R. (2012). Nocardioides alpinus sp. nov., a psychrophilic actinomycete isolated from alpine glacier cryoconite. *International journal of systematic and evolutionary microbiology*, *62*(2), 445-450.

Han, M. X., Fang, B. Z., Tian, Y., Zhang, W. Q., Jiao, J. Y., Liu, L., ... & Li, W. J. (2017). Nocardioides cavernae sp. nov., an actinobacterium isolated from a karst cave. *International Journal of Systematic and Evolutionary Microbiology*, *67*(3), 633-639.

Morohoshi, T., Tominaga, Y., Someya, N., & Ikeda, T. (2012). Complete genome sequence and characterization of the N-acylhomoserine lactone-degrading gene of the potato leaf-associated Solibacillus silvestris. *Journal of bioscience and bioengineering*, *113*(1), 20-25.

Kaur, M., & Karnwal, A. (2023). Screening of endophytic Bacteria from stress-tolerating plants for abiotic stress tolerance and plant growth-promoting properties: Identification of potential strains for bioremediation and crop enhancement. *Journal of Agriculture and Food Research*, *14*, 100723.

Weon, H. Y., Kwon, S. W., Son, J. A., Kim, S. J., Kim, Y. S., Kim, B. Y., & Ka, J. O. (2010). Adhaeribacter aerophilus sp. nov., Adhaeribacter aerolatus sp. nov. and Segetibacter aerophilus sp. nov., isolated from air samples. *International journal of systematic and evolutionary microbiology*, *60*(10), 2424-2429.

Kulichevskaya, I. S., Zaichikova, M. V., Detkova, E. N., Dedysh, S. N., & Zavarzin, G. A. (2009). Larkinella arboricola sp. nov., a new spiral-shaped bacterium of the phylum Bacteroidetes isolated from the microbial community of decomposing wood. *Microbiology*, *78*, 741-746.

Anandham, R., Kwon, S. W., Weon, H. Y., Kim, S. J., Kim, Y. S., Gandhi, P. I., ... & Jee, H. J. (2011). Larkinella bovis sp. nov., isolated from fermented bovine products, and emended descriptions of the genus Larkinella and of Larkinella insperata Vancanneyt et al. 2006. International Journal of Systematic and EvolutionaryMicrobiology, 61(1), 30-34.

Vancanneyt, M., Nedashkovskaya, O. I., Snauwaert, C., Mortier, S., Vandemeulebroecke, K., Hoste, B., ... & Swings, J. (2006). Larkinella insperata gen. nov., sp. nov., a bacterium of the phylum ‘Bacteroidetes’ isolated from water of a steam generator. *International journal of systematic and evolutionary microbiology*, *56*(1), 237-241.

Tian, M., Zhang, R. G., Han, L., Zhao, X. M., & Lv, J. (2015). Dyadobacter sediminis sp. nov., isolated from a subterranean sediment sample. *International journal of systematic and evolutionary microbiology*, *65*(Pt_3), 827-832.

Garcia-Lemos, A. M., Hennessy, R. C., Nielsen, T. K., Hansen, L. H., & Nicolaisen, M. H. (2021). Complete Genome Sequence of Paenibacillus sp. Strain 37, a Plant Growth-Promoting Bacterium Isolated from the Rhizosphere of Abies nordmanniana (Nordmann Fir). *Microbiology Resource Announcements*, *10*(8), 10-1128.

Zhou, G. C., Wang, Y., Zhai, S., Ge, F., Liu, Z. H., Dai, Y. J., ... & Hou, J. Y. (2013). Biodegradation of the neonicotinoid insecticide thiamethoxam by the nitrogen-fixing and plant-growth-promoting rhizobacterium Ensifer adhaerens strain TMX-23. *Applied microbiology and biotechnology*, *97*, 4065-4074.

Tara, N., & Saharan, B. S. (2017). Plant growth promoting traits shown by bacteria Brevibacterium frigrotolerans SMA23 isolated from Aloe vera rhizosphere. *Agricultural Science Digest-A Research Journal*, *37*(3), 226-231.

Gunawardana, D. (2020). An in silico study of two transcription factors controlling diazotrophic fates of the azolla major cyanobiont trichormus azollae. *Bioinformatics and Biology Insights*, *14*, 1177932220977490.

Galibert, F., Finan, T. M., Long, S. R., Pühler, A., Abola, P., Ampe, F., ... & Batut, J. (2001). The composite genome of the legume symbiont Sinorhizobium meliloti. Science, 293(5530), 668-672.

Lv, Z., Dai, R., Xu, H., Liu, Y., Bai, B., Meng, Y., ... & Zhang, J. (2021). The rice histone methylation regulates hub species of the root microbiota. *Journal of Genetics and Genomics*, *48*(9), 836-843.

Banerjee, S., Walder, F., Büchi, L., Meyer, M., Held, A. Y., Gattinger, A., ... & van der Heijden, M. G. (2019). Agricultural intensification reduces microbial network complexity and the abundance of keystone taxa in roots. *The ISME journal*, *13*(7), 1722-1736.

Karimi, B., Dequiedt, S., Terrat, S., Jolivet, C., Arrouays, D., Wincker, P., ... & Ranjard, L. (2019). Biogeography of soil bacterial networks along a gradient of cropping intensity. *Scientific Reports*, *9*(1), 3812.

Coleri Cihan, A., Karaca, B., Ozel, B. P., & Kilic, T. (2017). Determination of the biofilm production capacities and characteristics of members belonging to Bacillaceae family. *World Journal of Microbiology and Biotechnology*, *33*, 1-13.

Mandic-Mulec, I., Stefanic, P., & van Elsas, J. D. (2016). Ecology of bacillaceae. *The bacterial spore: From molecules to systems*, 59-85.