

The wheat seed phytomicrobiome as a potential source of resistance to the fungal disease, Fusarium head blight

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Masters of Science in Life Sciences in

In Plant Pathology, Physiology and Weed Science

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May 08, 2020

Blacksburg, VA

Keywords: Wheat, phyllosphere microbiome, Fusarium head blight, genotype.

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ABSTRACT

Plant-associated microbes (collectively the microbiome) are important contributors to plant health. They are known to play roles in increasing yield via improving stress tolerance, promoting growth, and suppressing the activity of plant pathogens. We investigated the wheat seed-head microbiome (phytomicrobiome) as a potential source of resistance to Fusarium head blight (FHB), or scab. FHB is a devastating disease in wheat, and other cereal grains, that causes losses in both quantity, through reduced yield, and quality of grain, through the production of toxins such as Deoxynivalenol. Efforts to combat FHB have focused primarily on breeding cultivars with resistance and applying fungicides. However, new resources for combatting FHB may lie in microbiome-plant interactions. To explore host-microbiome-pathogen interactions, we used field trials to characterize the seed head bacterial community (16S rRNA gene amplicons) across planting locations, host resistance genotypes, varieties, and plant development stages. We identified bacterial amplicon sequence variants (ASVs) present in each sample and then examined ASV community composition based on our variables. Characterizing bacterial relative abundance across samples, we identified 9,063 ASVs. These ASVs clustered according to plant developmental stages or maturity plant, location, and host genotype, but not by variety or maturity group. First, comparing plants at the pre-flowering versus mature

grain-head stage, we found that both bacterial community richness and evenness changed significantly. In addition to these developmental changes, we found that bacterial community structure changes across locations, even between locations. Finally, we found that, in the presence of the pathogen, ASVs cluster by host resistance genotype, and that there are important taxonomic groups that are differentially abundant in the presence of the pathogen. Overall, we found that the wheat grain-head microbiome is shaped by environment-host-pathogen interactions, and that these interactions lead to differential abundance of particular community members that may be important in the management of FHB.

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GENERAL AUDIENCE ABSTRACT

Plant associated microbes are important contributors to plant health. They are known to play roles in increasing yield via improved stress tolerance, promoting growth, and suppressing plant disease. We investigated the wheat grain-head microbial communities as a source of disease resistance. The disease is called *Fusarium* Head Blight (FHB) and is caused by *Fusarium graminearum*. FHB is a devastating disease in wheat and other cereals, causing losses, through reduced yield and quality through the production of toxins that prohibit use of the grain. To combat FHB, research has focused on developing plants that have resistance and the application of chemical fungicides. However, new resources for combating FHB may lie in the interactions between plants and microbes. This research is focused on identifying microbes that naturally interact with the plant, and how the pathogen, *Fusarium*, interacts with these beneficial microbes. In field trials, we characterized the microbial community by DNA sequencing technologies across locations, wheat with varying levels of genetic resistance, and wheat developmental stages. First, between the wheat kernel samples of pre-flowering and maturity, we found significant differences in microbial community. Consistent with other studies we found that the largest changes in microbial community composition across different growing locations. Finally, we found an interaction between the grain head microbiome and host

resistance state when plants were exposed to the pathogen. Overall, we find that the wheat grain head microbiome is shaped by growing location and through interactions with the plant host and pathogen.

Acknowledgements

This thesis represents the product of two years of lab work, study and perseverance and I would like to immensely thank my advisor, Dr. David Haak, for giving me the opportunity to work with him, introducing me to the world of plant molecular biology, and for his patience too. I would also thank him for introducing me to a world of exciting, innovative and competitive research. It was a challenge to immerse myself on microbial analysis. I grew comfortable engaging in discussion, ideation, research, and communication in a productive and rewarding way.

I also thank the rest of my thesis committee members, and my committee members Dr. Lisa Belden, Dr. David Schmale and Dr. Boris Vinatzer for encouragement and guidance. I would also like to thank Ariel Heminger, for being very supportive since the first day I was in the laboratory, and for translating some English words for me. Thanks to all the members of David's lab group, Cheiming, Haidong, Suzanne.

Thanks to my parents, Lucida Diaz and Modesto Gonzales, for the continuous support and encouragement. Without their advice, support and encouragement, this thesis would not have become a reality. Thanks also to William and Mary Lee Hendricks, for being supported in my career and for inviting me to Thanksgiving and Christmas every year I would also like to thank my former advisor in Peru (Juan Carlos Cabrera), for encouraging me during my undergraduate to study abroad, also my friends Zisi Montero (titi), Lucelia Pereira, Katya Castillo and Deivis Garay. Studying Plant pathology, physiology and weed science has been a fascinating and fulfilling experience.

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Chapter 1. Introduction to the phytomicrobiome as an agricultural management tool.

1.1 The plant host associated microbiome

Global plant productivity depends on symbiotic relationships with microbes that are found in and on plants—the phytomicrobiome. Such host associations among root or rhizosphere inhabiting microbes are well known in agriculture. For example, rhizobia bacteria and arbuscular mycorrhizal fungi mediate nutrient acquisition (Bakker et al. 2012). Another important, but less recognized, example is the use of microbial metabolic products for plant protection via external application or through genetic modification of the host (e.g., *Bacillus thuringiensis* produced δ -endotoxins; Woo and Pepe 2018). While the identification of many of these microbes that can improve plant health have come from culturing microbes (e.g., Kloepper *et al.* 1980, Hayat *et al.* 2010), there is also hope that the previously uncultured diversity of microbes revealed by next-generation sequencing approaches will lead to new microbial applications that support plant health (Bakker et al. 2012). Recent work has identified important contributions from the soil and above ground (phyllosphere) microbiome, including plant growth promoting microbial communities from the rhizosphere (Lau and Lennon 2012) and endophytic microbes that confer salt and heat stress tolerance in grasses (Rodriguez et al. 2008).

Collectively, the plant-associated microbiome is termed the "phytomicrobiome" and, importantly, this refers to the full complement of microbes (e.g., bacteria, fungi, archaea, etc.) present in and on the tissues (Mueller & Sachs, 2015). The phytomicrobiome is then spatially subdivided into the phyllosphere, which are those communities that live in

the aerial part of the plant (Turner, James et al. 2013), and the rhizosphere, the root-associated community of microbes (Levy, Conway, Dangl, & Woyke, 2018). In practice, most of the above ground plant tissues and organs receive specific recognition of their microbiome (e.g., floral or fruit microbiome) (Ottensen et al., 2016). The phyllosphere, on the other hand, is often used to refer specifically to the leaf, stems and flowers, these organs dominate the above ground portion of the plant (Bailey et al., 2006; Ruinen, 1956). Leaves provide microhabitats, such as stomata, trichomes, hydathodes, that each offer unique spaces for the growth and development of microbial communities that vary with plant species (Remus-Emsermann and Schledchter. 2018). Thus, the phyllosphere harbors diverse microbial communities, which include organisms such as, bacteria, fungi, algae and protozoa (Agler et al., 2016). Bacteria are the most abundant microorganisms in the phyllosphere (Morris and Kinkel. 2002), with a density up to 10^8 cells/cm² (Leveau., 2018) (Lindow et col., 2003). Fungi are the second most abundant microorganisms in the above ground portion of the plant (Voriskova et al, 2013), (Gurdeep Rastogi, Coaker, & Leveau, 2013), and Archaea represent a minor component of the phyllosphere microbiota (Knief et al., 2012).

Phyllosphere microbiomes are comprised of both surface and interior microbiota (Newton *et al.*, 2010). These microorganisms are classified as endophytes or epiphytes. Epiphytic microorganisms are defined as microbes that live on the surface of the plant tissues (Mano & Morisaki, 2008), (Berg, Grube, Schloter, & Smalla, 2014), while endophytic microorganisms are those that live inside plant tissues (Sessitsch et al., 2012)Turner, James et al. 2013). These microbes, endophytes in particular, are sometimes

integrated into the plant host life cycle via vertical transmission (Rossmann, Sarango-Flores, Chiaramonte, Kmit, & Mendes, 2017). Close associations with the host, host life cycle, and interactions with the host genome, mean that the microbiome can serve as a reservoir of genes for the host, impacting the health and fitness of plants (Bertelsen et al., 2001).

1.2 Host benefits from microbes

Early work identified the importance of microbes in plant host nutrient acquisition (Jacoby et al. 2017). In addition to nutrient acquisition, recent work has shown that microbes, both individually and as microbial consortia, can confer abiotic and biotic stress resistance in their host plants. For example, bacterial strains of *Achromobacter piechaudii* confer drought stress in pepper and tomato (Kloepper, Ryu, and Zhang 2004), In *Arabidopsis* plants, a set of microbes has the ability to confer drought tolerance (Zolla et al. 2013), and endophytic bacteria have the same effect on maize (Naveed et al. 2014). This work extends to the systems level, wherein Lau and Lennon (2012) demonstrated different rhizosphere microbes aid in plant adaptation to extreme environments, such as hypersaline conditions for grasses (Rodriguez et al. 2008). Some members of the bacterial genus *Pseudomonas* confer protection against salt stress in cotton (Yao et al. 2010). Other microbes improve the growing plant to confer salt tolerance in *Salicornia*, member of *Amaranthaceae* family (Mapelli et al. 2013), seepweed Suaeda (Yuan et al. 2016). Microbes can also confer tolerance to extreme temperatures. For example, some quinoa microbiomes perform better at high temperatures, which buffers plant health at high

temperatures (Yang et al. 2016). Conversely, some endophytic bacteria strains of *Burkholderia phytofirmans* help provide tolerance to cold temperatures in *Arabidopsis thaliana* (Su et al. 2015) and grape *Vitis vinifera* (Theocharis et al. 2011). The activity of microbes also helps plants remove toxic metals from contaminated soils (Rajkumar et al. 2010) (Feng et al. 2017). *Pseudomonas putida* showed biodegradation of contaminants of phenanthrene by promoting root growth (Khan et al. 2014).

Much of the research on biotic interactions between plants and microbes has focused on negative interactions between hosts and pathogens. However, plant-microbe interactions are not only negative (Mendes, Garbeva, and Raaijmakers 2013)-- they can provide benefits to their host plants as well. One case is in the form of disease suppression, wherein host-associated microbes can suppress pathogen abundance. For example, when tobacco (*Nicotiana attenuate*) germinates, the plant pathogen *Fusarium alternaria* normally attacks the plant; however, this pathogen induces the plant to recruit beneficial bacteria against this pathogen (Santhanam et al., 2015). The same result was found where the secretion of compounds from *Burkholderia cenocepacia* inhibits *Phytophthora cinnamomic* growth on *Trifolium subterraneum* (Colavolpe et al. 2020). At the microbiome level, researchers found that the microbial consortia present on the leaf surface of *Arabidopsis thaliana* can contribute to *Botrytis cinerea* resistance (Ritpitakphong et al., 2016). Similarly, research in wheat also shows suppression against pathogens, where members of the wheat microbiome can reduce the virulence of *Fusarium graminearum* isolates *in vitro* (Chen et al. 2018). In addition to above ground microbial activity, we also know that there are certain mixtures of soil microbial communities that have been

associated with suppression of soil borne diseases, including *F. graminearum* (Klein, Ofek, Katan, Minz, & Gamliel, 2013). Therefore, the plant microbiome is an important determinant of plant health and productivity.

1.3 Agricultural management and the microbiome

Agricultural production is replete with examples of successful implementation of biological amendments for improved plant health. One such example is the use of the bacteria *Bacillus thuringiensis* (Bt) either directly applied or with its genes inserted into the host plant. Bt is a gram-positive bacteria that forms crystal proteins during the stationary phase of its growth cycle. These proteins (Cry) are toxic to insect pests of plants (Schnepf et al. 1998), (JY et al. 2007). Moreover, Cry proteins exhibit negative effects on the germination of fungal spores (Betz, Hammond, and Fuchs 2000). Some researchers have demonstrated this effect *in planta*. For example, strains of Bt induce systemic resistance against Fusarium wilt (*Fusarium oxysporum lycopersicum*) (Akram, Mahboob, and Javed 2013). The gene for expressing the Cry proteins has already been transferred into various crops, including corn. In addition to controlling insect pests, Bt corn has also been shown to reduce the levels of different toxins produced by fungal pathogens in the *Fusarium* group: deoxynivalenol (DON), zearalenone (ZEA) (Ostry et al. 2010) and fumonisins (FUM), reducing some of them up to 68% (Hammond et al. 2004) (Munkvold, Hellmich, and Showers 1997). Other stellar examples abound; for example, the addition of beneficial microbes as a seed coating (i.e., *Bradyrhizobium spp.*) to promote nutrient

acquisition. Thus, agricultural management appears well poised to adopt practices that would support beneficial microbial consortia.

This support could be through identification of native microorganisms that provide some benefit to the host and then enhancing community stability to promote the growth of these microbes (Haas and Keel 2003). Another method would be direct delivery of a probiotic community, which could be accomplished with the addition of a probiotic mixture applied to seed at the time of planting (Woo and Pepe 2018). In addition to reducing the use of chemicals (fertilizers, pesticides) in agriculture, these mixtures applied to seeds can also provide disease resistance (Berlec, 2012). For example, in the tobacco plant, a set of five bacteria was found that provided resistance to *Fusarium alternaria* at the time of germination (Santhanam et al. 2015).

1.4 The plant disease ‘quadrangle’

Despite the successes in identifying microbial consortia that suppress disease, direct applications under agronomic conditions have had limited success (Sergaki et al. 2018). There are several reasons for this, most of which center on the sheer scale of diversity of the microbial community (e.g., 10^6 - 10^9 bacterial taxa in 1 teaspoon of soil) and the interdependencies of host-microbiome-environment interactions. While above ground plant tissues harbor fewer microbial taxa, the assemblages tend to be tissue-specific (Ottensen et al. 2013), adding to the complexity of the interactions. Traditional models of plant disease development incorporate three components: plant host, pathogen, and the environment, which collectively, is called the disease triangle (Gurdeep Rastogi et al.,

2013). While this model is quite useful for establishing the presence of disease, increasingly we recognize its limitations for describing the interactions that drive disease prevalence (Müller & Ruppel, 2014).

1.5 Importance of host genotype

While the disease triangle model is quite useful for establishing the presence of disease, increasingly we recognize its limitations for describing the interactions that drive disease prevalence (Müller & Ruppel, 2014). Microbiome composition is strongly affected by plant pathogenic microbes (Trivedi et al., 2012). However, plant genotype plays an important role. *Arabidopsis thaliana*, a popular model plant, had different microbial communities across different genotypes (Trivedi et al. 2012; Agler et al., 2016). Also, under laboratory conditions, the same hypothesis of differences of microbial community across genotypes was also corroborated in *Arabidopsis* (Bodenhausen et al., 2014). Similar findings were reported for the study of 26 different cultivars of lettuce that grew in the same field of cultivation (Hunter, Hand, Pink, Whipps, & Bending, 2010). Investigation in bean showed that the bacterial communities of the phyllosphere three different cultivars of *Phaseolus vulgaris* were different, and thus, it was concluded that different cultivars might form different communities of organisms (de Oliveira Costa et al. 2012). Similar conclusions about the importance of host genotype have come from studies of berries leaves and fruits (Singh et al. 2018), cotton (Adams and Kloepper 2002) and tomatoes (Correa et al. 2007), and some non-commercial plants, including the tree *Ombrophilous* (Lambais, Lucheta, and Crowley 2014).

2.1 Wheat-*Fusarium* pathosystem as a model for microbiome-mediated disease management

A plant pathosystem consists of the plant host, pathogen, and environmental factors (Lucas and Campbell 2012). This is a system because it relies on the communication of the three components (Ansar and Ghatak 2019). *Arabidopsis* has been used as a model *Arabidopsis-Fusarium* pathosystem. In this model, after the plant is infected with the *Fusarium* pathogen, the pathogen spreads in seeds. This serves as a good plant model for investigating *Fusarium* disease because the majority of the *Fusarium* infection of wheat is also in the seeds (Urban et al. 2002). However, *Fusarium* does not naturally infect *Arabidopsis*, and infection symptoms in the flowers differ in *Arabidopsis* and wheat (Chen et al. 2009). For that reason, the ideal plant model should be closer to wheat. Other researchers use another pathosystem model, using *Brachypodium distachyon*, which is related to grass species, this new plant model showed similar spikelet infection as wheat (Peraldi et al. 2011). The plant pathosystem is very important in epidemiology development (Savary et al. 2006). That is why in order to understand more, this research is going to integrate wheat and *Fusarium graminearum* under the different developmental stages.

2.2 Summary

The wheat-*Fusarium* pathology model is very important for the development of agricultural management of *Fusarium* and can help us understand when intervention strategies should be applied. Knowing when the plant is under pathogen attack and the environmental conditions that favor disease development is an important part of disease management. However, it is also important to investigate the beneficial microbial communities that are present in wheat and to understand if the wheat genotype plays a role in the microbial structure.

Chapter 2. Evaluating associations between location, host genotype, developmental stage, disease state, and microbial community structure.

2.1 Abstract

Fusarium head blight (FHB or scab) is a common disease on wheat, causing losses in both yield and grain quality. To combat FHB, the integration of many disciplines is required. In this research, we focus on using the advantages that plant microbiomes confer to the plant, we characterized the composition of the grain head microbiome of wheat, and how it is affected by different factors such as host genotype, geographic location, developmental stages and the pathogen. To assess these associations, we characterize the seed head bacterial community (16S rRNA gene amplicons), between pre-flowering and mature wheat kernel samples and host genetic resistance state, in three locations across Virginia. Overall, we found that growing location significantly impacted bacterial community composition. Bacterial community richness and evenness changed significantly in a single location (Mt. Holly) between pre-flowering and mature grain-head samples. Finally, we find that the structure of the wheat grain head bacterial communities are shaped by the interaction of local conditions, developmental growth stage and interactions between host genetic resistance and pathogen.

2.2 Introduction

Wheat is one of the most important cereal crops in the world, including in the United States, which is among the top five wheat producers globally, producing 820 billion bushels of winter wheat in 2016/17 (USDA, 2019). However, across portions of the U.S.

growing region, wheat is commonly plagued by Fusarium head blight (FHB) caused by the fungus *Fusarium graminearum* (*sensu stricto*) (Rawat et al., 2016). This pathogen can cause reductions in grain yield via kernel abortion, and losses in grain quality because it produces mycotoxins, such as deoxynivalenol (DON) (Bai and Shaner 2004), that are harmful to human and animal health (Agler et al., 2016), and thus, the allowable ppm of DON in grain samples is tightly regulated (Pestka., 2007). *Fusarium* is largely dispersed by rain, air, and insects, and spreads more easily with high humidity. The critical timing for pathogen infection is during anthesis (flowering), with infection maintained through grain maturity (Pritsch et al. 2000, Voigt et al. 2007). During anthesis, the anthers produce stimulants that promote the growth of the fungus (PARRY, JENKINSON, and McLEOD 1995). Once established on the spikelet, the pathogen moves into the developing kernel, which is then aborted.

Plant breeders have focused on developing cereal grain varieties with genetic resistance to FHB, using diverse approaches, such as phenotypic selection, marker-assisted selection, and genomic selection (Steiner et al. 2017). While this work has led to the development of lines with resistance to FHB, no varieties have been identified with complete resistance, and therefore, the application of chemical fungicides remains an important approach to control FHB (Blandino et al., 2012). The integration of different methods, such as the use of resistant varieties with the use of fungicides and the application of best management practices, have been effective at reducing disease severity (Purdue extension. 2015; Takemoto et al. 2018). However, the continued application of fungicides increases the chances of resistance evolving in the pathogen. In addition, broad application

of fungicides has negative impacts on the environment. Together, these factors are leading the push to find alternatives, such as biological control, to help manage FHB.

Biological control can be used as part of an integrated management strategy for control of FHB. Multiple biological control agents (BCAs) have antagonistic activity against *Fusarium* spp., including *Bacillus* spp. (Zhao et al. 2014), *Pseudomonas* spp. (Schisler et al. 2006), *Glomerata*, *Aureobasidium proteae*, and *Sarocladium kiliense* (Comby et al. 2017). Mixtures of antagonistic strains can also control *Fusarium* spp. under laboratory conditions, and are capable of reducing FHB during anthesis (Baffoni et al. 2015). Despite these successes, few BCAs have made it to market, typically because the effectiveness of control was not uniform across environments and/or genotypes (Schisler et al. 2002, McMullen et al. 2012). Thus, researchers have turned to natural microbial communities as potential sources of environmentally-stable BCAs (Berg et al. 2017).

To develop effective biological control practices using stable microbial communities, we must identify the factors that influence microbial community structure (Vorholt, 2012). For example: climatic drivers, such as rain, influence microbial dispersal through mechanisms such as rain splash water movement, and carry by insects (Kinkel 1997, Vorholt 2012, G. Rastogi et al., 2012). Another abiotic factor that influences the composition of microbial communities is the geographic location of the host plant. In one experiment, phyllosphere communities among 3 different tree species (*Tamarix* spp.) were assessed to discern the impact of geography on microbiome structure across broad spatial scales (Finkel et al. 2011). The researchers found that microbial communities were more similar among tree

species from the same location than across locations (Finkel et al, 2011). In lettuce leaf microbiomes, when the distance increased between cultivars, the microbial community became more diverse in microbial species (G. Rastogi et al., 2012). Similar research conducted at five different vineyards found that the microbial communities of the phyllosphere were affected by geographic location even at small distances (5 km) (Zarraonaindia et al. 2015).

We also know that biotic interactions, for example between the host and microbial community, can influence microbial community structure. Studies finding differences in the microbiome among host genotypes suggest that the role of the host is important. For example, an investigation that assessed 27 varieties of corn showed differences in the rhizosphere microbial communities (Peiffer et al. 2013), and in rice, it was determined that 15 varieties showed highly significant variation in epiphytic bacterial communities (De Costa et al. 2006). In winter wheat (*Triticum aestivum*), winter and spring barley (*Hordeum vulgare*), oat (*Avena sativa*), rye (*Secale cereale*), and triticale (*Triticum* × *Secale*) host genotype at both the species and cultivar level was found to be important for shaping phyllosphere fungal communities (Sapkota et al. 2015). In addition to host genotype, host developmental stage can also impact microbiome structure. Many studies have found these changes from seedling to plant maturity (Chaparro, Badri, and Vivanco 2013), and they can also occur at finer scales. For instance, in apple, investigators found significantly different microbial communities from unopened flowers, open flowers, and senesced flowers (Shade et al. 2013).

Host-microbiome-pathogen interactions can influence both the composition and abundance of the microbes in the community. For example, the absence or presence of a *Phytoplasma* was found to shape the microbial community on grapes (Bulgari et al. 2011). It has also been observed in potatoes that microbial diversity increases when host plant has either infected or inoculated previously i.e. bacteria *Erwinia carotovora* (Reiter et al. 2002) or *Pseudomonas syringae* showed an increasing PsJN, plant growth promoters (Chaparro, Badri, and Vivanco 2013). These suggest that presence of pathogens triggers shifts in bacterial communities' composition.

The goal of this study was to identify the abiotic and biotic factors shaping bacterial communities in the wheat-FHB pathosystem across Virginia state. Our objectives were to characterize the interactions between 1) Location, wheat variety, and wheat resistance genotype (susceptible, moderate resistance, resistance), 2) Developmental stage, variety, and resistance genotype, and 3) Pathogen, variety, and resistance genotype, and how they alter the composition of the grain-head microbiome. To accomplish these objectives, we measured changes in the bacterial communities from wheat head samples of three varieties collected at two development stages (pre-flowering and mature) across three growing locations in Virginia, using 16S rRNA amplicon sequencing to assess the bacterial community. The varieties we examined varied in resistance genotype (susceptible, moderate resistance, resistance).

2.3 Materials and methods

Field design

Wheat was grown in three different locations, which are all Virginia Tech owned agricultural stations (Figure 01): Kentland Farm, Virginia Tech, Whitehorne, VA (37°12'00.1"N 80°33'49.9"W), Mount Holly, VA (38°05'31.4"N 76°43'25.7"W), and Warsaw, VA (37°59'11.1"N 76°46'44.4"W). Each field experiment consisted of a total of 19 plots, with 3 replicate plots of 6 resistance genotypes that were either early or late maturing varieties (susceptible (S), moderate resistance (MR) and resistance (R) x Early or Late maturing), and 1 check plot (yield) (Table 1). Samples were collected either during pre-flowering (Mt. Holly only) or when mature (all three sites). The Mount Holly location serves as the 'scab nursery', wherein plants were inoculated with *Fusarium*-infected corn around the base of the plant, when the spikelets were opened and favorable conditions such as humidity irrigated the field. to *Fusarium* growth are maintained by. The research farm at Warsaw is just 19 Km away and has similar edaphic and environmental conditions (e.g., favorable conditions for the growth of the pathogen), but Warsaw plots were not inoculated with the pathogen. Finally, the Kentland research farm is geographically isolated from Warsaw and Mount Holly (19 km West). Environmental conditions at Kentland are typically not favorable for pathogen growth, and our attempt to establish infection using corn inoculum failed.

Sampling, processing, and DNA extraction

The samples were taken from two different wheat development stages: Pre-flowering samples were taken only from Mt. Holly, since this is the scab nursery, and mature grain samples were taken from all three locations (Table 1; Figure 1 and 2). For each of the 19 plots located at the three sites, five subsamples wheat head samples were collected, from every subsamples, five wheat-head were taken. (total N=5). The pre-flowering (spikelet) and mature grain-head (spikelet) was collected using disposable gloves rinsed with 70% ethanol and flame sterilized instruments. The grain-head was separated from the rest of the plant by cutting from at the base of the wheat-head. Immediately after collection, five spikelet samples were placed inside sterile 25 x 32 cm Nasco Whirl-Pak® bags, and stored at -80C until processing. For DNA extraction, frozen tissue was ground using a mortar and pestle under liquid nitrogen. To prevent sample warming during grinding, additional liquid nitrogen was added to the mortar, as needed. Pulverized tissue was transferred into 50 ml sterile Falcon® tubes and stored at -80C. 0.20 g were taken as a representative sample to do DNA extraction, it was performed using the DNeasy PowerSoil® Kit (QIAGEN USA catalog no. 12888-100), according to the manufacturer's instructions.

Table 1. Sampling dates across developmental stages in three locations in Virginia, where S= Susceptible, R=Resistant and MR= Moderately resistant.

Sample ID	Location	Variety	Genotype	Maturity	Collection Growth stage	Collection date
102	Kentland	Pioneer 26R46	S	Early	Maturity	June 29, 2018
103	Kentland	Coker 9835	S	Late	Maturity	June 29, 2018
104	Kentland	L11541 (Fhb1)	R	Early	Maturity	June 29, 2018
105	Kentland	Jamestown	R	Late	Maturity	June 29, 2018
106	Kentland	VA11W-279	MR	Early	Maturity	June 29, 2018
201	Kentland	Tribute	MR	Late	Maturity	June 29, 2018
202	Kentland	Pioneer 26R46	S	Early	Maturity	June 29, 2018
203	Kentland	Coker 9835	S	Late	Maturity	June 29, 2018
204	Kentland	L11541 (Fhb1)	R	Early	Maturity	June 29, 2018
205	Kentland	Jamestown	R	Late	Maturity	June 29, 2018
206	Kentland	VA11W-279	MR	Early	Maturity	June 29, 2018
301	Kentland	Tribute	MR	Late	Maturity	June 29, 2018
302	Kentland	Pioneer 26R46	S	Early	Maturity	June 29, 2018
303	Kentland	Coker 9835	S	Late	Maturity	June 29, 2018
304	Kentland	L11541 (Fhb1)	R	Early	Maturity	June 29, 2018

305	Kentland	Jamestown	R	Late	Maturity	June 29, 2018
306	Kentland	VA11W-279	MR	Early	Maturity	June 29, 2018
101	Warsaw	Pioneer 26R46	S	Early	Maturity	June 12, 2018
102	Warsaw	Coker 9835	S	Late	Maturity	June 12, 2018
103	Warsaw	L11541 (Fhb1)	R	Early	Maturity	June 12, 2018
104	Warsaw	Jamestown	R	Late	Maturity	June 12, 2018
105	Warsaw	VA11W-279	MR	Early	Maturity	June 12, 2018
106	Warsaw	Tribute	MR	Late	Maturity	June 12, 2018
201	Warsaw	Pioneer 26R46	S	Early	Maturity	June 12, 2018
202	Warsaw	Coker 9835	S	Late	Maturity	June 12, 2018
203	Warsaw	L11541 (Fhb1)	R	Early	Maturity	June 12, 2018
204	Warsaw	Jamestown	R	Late	Maturity	June 12, 2018
205	Warsaw	VA11W-279	MR	Early	Maturity	June 12, 2018
206	Warsaw	Tribute	MR	Late	Maturity	June 12, 2018
301	Warsaw	Pioneer 26R46	S	Early	Maturity	June 12, 2018
302	Warsaw	Coker 9835	S	Late	Maturity	June 12, 2018
303	Warsaw	L11541 (Fhb1)	R	Early	Maturity	June 12, 2018
304	Warsaw	Jamestown	R	Late	Maturity	June 12, 2018

305	Warsaw	VA11W-279	MR	Early	Maturity	June 12, 2018
306	Warsaw	Tribute	MR	Late	Maturity	June 12, 2018
01-M	Mt. Holly	Pioneer 26R46	S	Late	Maturity	June 12, 2018
02-M	Mt. Holly	Coker 9835	S	Early	Maturity	June 12, 2018
03-M	Mt. Holly	L11541 (Fhb1)	R	Late	Maturity	June 12, 2018
04-M	Mt. Holly	Jamestown	R	Early	Maturity	June 12, 2018
05-M	Mt. Holly	VA11W-279	MR	Late	Maturity	June 12, 2018
06-M	Mt. Holly	Tribute	MR	Early	Maturity	June 12, 2018
07-M	Mt. Holly	Pioneer 26R46	S	Late	Maturity	June 12, 2018
08-M	Mt. Holly	Coker 9835	S	Early	Maturity	June 12, 2018
09-M	Mt. Holly	L11541 (Fhb1)	R	Late	Maturity	June 12, 2018
10-M	Mt. Holly	Jamestown	R	Early	Maturity	June 12, 2018
11-M	Mt. Holly	VA11W-279	MR	Late	Maturity	June 12, 2018
12-M	Mt. Holly	Tribute	MR	Early	Maturity	June 12, 2018
13-M	Mt. Holly	Pioneer 26R46	S	Late	Maturity	June 12, 2018
14-M	Mt. Holly	Coker 9835	S	Early	Maturity	June 12, 2018
15-M	Mt. Holly	L11541 (Fhb1)	R	Late	Maturity	June 12, 2018
16-M	Mt. Holly	Jamestown	R	Early	Maturity	June 12, 2018

17-M	Mt. Holly	VA11W-279	MR	Late	Maturity	June 12, 2018
18-M	Mt. Holly	Tribute	MR	Early	Maturity	June 12, 2018
01-P	Mt. Holly	Pioneer 26R46	S	Early	Pre-Flowering	May 2, 2018
02-P	Mt. Holly	Coker 9835	S	Late	Pre-Flowering	May 2, 2018
03-P	Mt. Holly	L11541 (Fhb1)	R	Early	Pre-Flowering	May 2, 2018
04-P	Mt. Holly	Jamestown	R	Late	Pre-Flowering	May 2, 2018
05-P	Mt. Holly	VA11W-279	MR	Early	Pre-Flowering	May 2, 2018
06-P	Mt. Holly	Tribute	MR	Late	Pre-Flowering	May 2, 2018
07-P	Mt. Holly	Pioneer 26R46	S	Early	Pre-Flowering	May 2, 2018
08-P	Mt. Holly	Coker 9835	S	Late	Pre-Flowering	May 2, 2018
09-P	Mt. Holly	L11541 (Fhb1)	R	Early	Pre-Flowering	May 2, 2018
10-P	Mt. Holly	Jamestown	R	Late	Pre-Flowering	May 2, 2018
11-P	Mt. Holly	VA11W-279	MR	Early	Pre-Flowering	May 2, 2018
12-P	Mt. Holly	Tribute	MR	Late	Pre-Flowering	May 2, 2018
13-P	Mt. Holly	Pioneer 26R46	S	Early	Pre-Flowering	May 2, 2018
14-P	Mt. Holly	Coker 9835	S	Late	Pre-Flowering	May 2, 2018
15-P	Mt. Holly	L11541 (Fhb1)	R	Early	Pre-Flowering	May 2, 2018
16-P	Mt. Holly	Jamestown	R	Late	Pre-Flowering	May 2, 2018

17-P	Mt. Holly	VA11W-279	MR	Early	Pre-Flowering	May 2, 2018
18-P	Mt. Holly	Tribute	MR	Late	Pre-Flowering	May 2, 2018

Library preparation and sequencing

Extracted DNA concentrations and quality (260/280 and 260/230 values) were determined using a NanoDrop™ One/Onec (Thermo Scientific). DNA plants were extracted again if the quality score fell below 1.8 or about 2.2. Plant organelles (mitochondria and chloroplast) are an important source of contamination when investigating the bacterial community associated with plants. This is because organelles are derived from bacteria (Gould, Waller, and McFadden 2008) and, therefore, they contain 16S rRNA genes that amplify with the same polymerase chain reaction (PCR) primers used for bacteria (Sakai and Ikenaga 2013). To avoid amplifying organelle 16S sequences, an initial locked nucleic acid (LNA) PCR was used (Yu et al. 2016), (Ikenaga et al. 2015). The LNA PCR mixture contained 12.5 µL Premix Hot Start Accustart II Supermix, 1.0 µL primer 63f-mod (20 pmol µL/1), 1.0 µL of primer 1492r (20 pmol µL/1), 1.0 µL of LNA-Mit63 (20 pmol µL/1), 1.0 µL of LNA-Mit1492 (20 pmol µL/1), 1.0 µL LNA-Pla63a (20 pmol µL/1), 1.0 µL LNA-Pla 1492a (20 pmol µL/1) and 5.5 µL of sterile water . To bring the final volume to 25 µL, 1.0 µL DNA template was added (Table 2). LNA PCR was performed in sets of 8, with 7 samples and one negative control without template DNA per run. The amplification conditions were as follows: 94°C for 3 min (initial denaturation), followed by 30 cycles of 94°C for 1 min, 70°C for 1 min (annealing step of LNA oligonucleotides), 54°C for 1 min (annealing step of primers), and 72°C for 2 min, with

the final extension step at 72°C for 10 min. PCR products were visualized under ultraviolet (UV) light on a 1.5% agarose gel stained with GelRed® nucleic acid stain 10000x in water to ensure the expected size for the targeted sequence (**See Appendix**).

Table 2. LNA oligonucleotide sequences from Ikenaga et al. (2015).

LNA oligonucleotide	Sequence
Mod 63F	5'-YRKG CYT WAYACATGCAAGTC-3'
1492r	5'-GGYTACCTTGTTACGACTT-3'
LNA-Pla63a	5'-GTCGAACGGGAAGTGGTp-3'
Pla1492a	5'-CTTCACTCCAGTCGCAAGCp-3'
LNA-Mit63	5'-GTCGAACGTTGTTTTTCGGp-3'
Mit1492	5'-CTTCACCCCAGTCGAAGAp-3'

LNA PCR was followed with amplification of the V4 region of the 16S rRNA gene using primers 515f and individually-barcoded 806R (Caporaso *et al.*, 2011) and addition of Illumina adaptors. PCRs were run in duplicate along with a negative control that did not contain template DNA. The PCR mixture contained 12.65 µL UltraClean PCR grade H₂O, 10 µL of 5 Prime Hot Master Mix, 0.5 µL of Forward primer IL 515F and 0.5 µL of Reverse primer + barcode IL 806R. and 6 µL of DNA samples. The amplification condition was as follows: 94°C for 3 min (initial denaturation), followed by 30 cycles of 94°C for 45 sec, 50°C for 1 min, 72°C for 1.5 min, and 72°C for 10 min, with the final extension step at 72°C for 10 min. Duplicate samples were combined following PCR, and concentrations of each sample were determined using a Qubit Fluorometer 2.0 with a HS DNA kit. Equimolar

amounts of each sample were then pooled into a final sample for sequencing, which was cleaned using the Qiaquick PCR purification kit (Qiagen, Canada), following the manufacture's protocol. The pooled sample was shipped to the Sequencing Facility at the Dana Farber Cancer Institute at Harvard University and was sequenced on an Illumina Mi-Seq with a 250bp single-end strategy.

Identifying amplicon sequence variants (ASVs)

Amplicon sequence data is typically analyzed by identifying operational taxonomic units (OTUS) (Schlee, 1975), which is clustering the sequences at an arbitrary threshold, typically 97% similarity. This clustering approach was devised to mitigate errors associated with PCR and next generation sequencing, at the cost of resolution (particularly at the strain level). A new approach (DADA2) allows users to resolve sequences, post error correction, at 100% identity and retain amplicon sequence variants (ASVs ; (Callahan, McMurdie, and Holmes 2017). The primary departure from the OTU approach is that errors are modeled from the sequence data and then error correction is applied. Thus, individual sequences can then be used to represent an organism in the original sample rather than clustered reads (E. V. M. Michael J. Cox, Markus J. Ege 2019). ASV methods have demonstrated sensitivity and specificity that exceeds OTUs (Needham, Sachdeva, and Fuhrman 2017). Thus, we chose to use ASVs in the present study.

Statistical analysis

Raw data were read into R (version 1.2.5001) and using a package called Bioconductor (Callahan et al, 2016), this is the most common package for bioinformatic analysis with inclusion of packages such as DADA2 (Callahan et al., 2016), phyloseq (McMurdie and Holmes, 2013), DESeq2 (Love et al., 2014), ggplot2 (Wickham, 2008) and vegan which allows to visualize, test, and compare microbial data samples.

Only forward reads were used in this study. Raw reads were demultiplexed by the sequencing center. All datasets were prepared following procedures outline in the DADA2 tutorial (Callahan et al., 2016). Reads were visualized using quality profiles “plotQualityProfile”. In order to improve the overall quality of the sequences, the reads were filtered and trimmed using the “filterAndTrim” function implemented in DADA2. To remove low quality bases at the end of the reads, the “truncLen”. Since DADA2 relies on a parametric error model, we used the “learnErrors” function to evaluate error rates from the data and to visually confirm that the resulting error rate estimates provided a good fit to the observed rates using plotErrors (Callahan et al. 2016). The “derepFastq” function was used to combine identical sequence reads into a unique sequence. Construction of a sequence table was done through the “makeSequenceTable” function. We removed chimeric reads using “removeBimeraDenovo” using the consensus method. ASVs were assigned taxonomy using the DADA2 function “assignTaxonomy” and the SILVA v123 database (Quast et al., 2013). We used phyloseq v1.22.3 (McMurdie and Holmes, 2013) for downstream analysis, identifying 9063 ASVs from 69,369 unique input sequences

Richness and β diversity measures and associations

Comparisons of ASV relative abundance were conducted using the alpha diversity indices Shannon (H), Simpson and inverse Simpson and visualized via the phyloseq package in R. Alpha diversity is a reflection of the number of bacterial species and/or how evenly their relative abundances are distributed in a sample (Holloway 1977). Shannon's index emphasizes the richness component of diversity, Simpson's index (D) represents the evenness component. It is also called a dominance index because it gives weight to the most common species (Gardener 2014). Simpson's index can be modified so that when all species are equally abundant the value is equal to the species richness; this is called the Inverse Simpson index. The Inverse Simpson ($1/D$) is often more intuitive than Simpson's index in that increasing values indicate higher levels of diversity (Miller, Hauer, and Werner 2015). Differences between the tree location were tested using alpha diversity metrics, Kruskal-Wallis tests, Differences between early and late varieties (heading) and developmental stages were tested using alpha diversity metrics Student's T test.

To evaluate differences in bacterial community composition among the three locations, variables we assessed β diversity. β diversity is generally defined as variation in the identities or abundances of species among sites, and is typically measured as a distance matrix (Whittaker, 1960). We used several standard measures of β diversity, including the Bray-Curtis dissimilarity metric and Jaccard similarity index. Bray-Curtis measure relative abundance and Jaccard is presence/absence based, if the results are the same, the results only showed as a Bray-curtis.

Bray-Curtis dissimilarity creates a matrix of pairwise distances between samples based on relative abundance of the ASVs. This matrix handle large proportion of zeroes typical in microbiome data wherein species are absent from samples (Xia, Sun, and Chen 2018). Differences in Bray-Curtis dissimilarities were assessed using PERMANOVA (Anderson et al. 2011). The data were visualized using Multidimensional scaling (MDS) plots. Analyses and visualization were conducted using the phyloseq package in R.

To evaluate ASV differential abundance between two wheat head samples (susceptible and resistant genotype), we used DESeq2 (version 1.20.0) from Bioconductor in R. The p-values were adjusted by Bonferroni correction. Graphics were created with ggplot2 (version 2.1.0) in the R environment.

2.4 Results

We sampled the bacterial diversity of 86 wheat grain-head samples growing in three locations across the state of Virginia (Kentland Farm, Mt. Holly and Warsaw). At the genus level, the dominant bacteria at the three sites were classified into *Pantoea*, *Pseudomonas*, *Hymenobacter*, *Sphingomonas*, *Methylobacterium*, *Chryseobacterium* and *Rhizobium* (Figure 3). *Pantoea* had the highest relative abundance, followed by *Pseudomonas*, with the two of these accounting the diversity, followed by the others.

Bacterial diversity analysis

Based on the investigated variables, alpha diversity was estimated separately using Shannon diversity, Simpson's index, and inverse Simpson (1-D), as implemented in phyloseq. Where patterns were consistent statistical tests were applied using only Shannon diversity. Across all metrics, changes in Alpha diversity were only associated with plant maturity (Students' t-test was significant ($t=4.49$, $p\text{-value} < 0.001$; Figure 4). Shannon alpha diversity was not significantly different between early and late heading, richness was higher in early maturing varieties (Student's t-test $t = 0.67$, $p\text{-value} = 0.5$; Figure 5), or across locations (Kruskal-Wallis $H = 6.54$, $p = 0.08$; Figure 6)

Changes in bacterial community structure (beta diversity) were estimated using Bray-Curtis dissimilarity and Jaccard similarity of ASVs and visualized using MDS ordination. Significant differences were observed in microbial community structure among the locations (pairwise PERMANOVA, $F=6.46$, $p = 0.0001$; Figure 7). The MDS plot revealed clear clustering by location with the greatest separation between Kentland (red circles, Figure 7) and both other locations, which reflects the largest geographic distance. Additionally, MDS was able to separate Mt. Holly (green circles, Figure 7) and Warsaw (blue circles, Figure 7), even though these locations are less than 12 miles apart.

Next, we investigated differences in microbial community structure by host resistance genotype, focusing on two environmentally similar locations, Mt. Holly and Warsaw, that experienced different treatments. In Mt. Holly, plants were inoculated with FHB and kept under conditions favorable for the growth of the pathogen. Whereas in Warsaw only naturally occurring FHB was present. Ordination via MDS revealed

clustering between susceptible and resistance genotypes in Mt. Holly (Figure 8A), while there was no evidence of clustering Warsaw (Figure 8B). This clustering in Mt. Holly was confirmed by PERMANOVA ($p = 0.008$), whereas in Warsaw there was no significant separation (PERMANOVA, $p = 0.3$).

Differential abundance

To gain some mechanistic insight into the differences among taxa by host genotype, we examined the differential abundance of bacterial taxa, defined at the genus level, in the Mt. Holly location, using DESeq2 (Figure 9). Comparing susceptible versus resistance genotypes, we find 30 ASVs that are differentially abundant at the adjusted p-value of 0.05 and a \log_2 fold change > 2 . The highest relative abundance genera in the susceptible genotype samples were *Massilia*, *Hymenobacter*, *Novosphingobium*, *Spirosoma* and *Pseudomonas*. Resistant genotype plants had a higher relative abundance of ASVs from the genera *Pedobacter*, *Sphingomonas*, *Massilia*, *Hymenobacter*, *Novosphingobium*, *Roseomonas* and *Spirosoma*.

2.5 Discussion

We investigated the impacts of location, host resistance, variety, and plant growth stage on the structure of the grain-head microbiome. We found: 1) that local conditions are important for shaping the structure of the bacterial communities in wheat grain-heads, even between locations that are separated by $<20\text{km}$. 2) Bacterial community richness and evenness changed significantly between pre-flowering and mature grain-head samples. But

not based on other factors, such as variety or heading date. 3) Host resistance genotype was only associated with changes in bacterial community structure in the presence of FHB. Here, there appears to be an interaction between pathogen, host and microbiome that impacts the abundance of particular ASVs.

Location effects

Geographic location had a stronger impact on shaping bacterial community structure than did host genotype (Oyserman et al. 2019), which suggests, genotype by environment interactions contributed to the complexity of microbiome assembly. Microbiome and environment may interact to alter host independent of the genotype. According with (Roman-Reyna et al. 2019) environmental locations can alter the effect of plant genotype in *Vitis Vinifera* on the association microbiota, where the impact of the genotype was low compared to the different locations where the samples were, which suggests that the genotype-by-environment interactions model, contributed to assemble the microbial communities. In addition, increasing distance between cultivars on lettuce, showed that the leaf microbial community becomes more diverse (G. Rastogi et al., 2012) this may explain why Kentland and Mt. Holly, the clustering of microbial community on beta diversity was separate. Likewise (Zarraonaindia et al. 2015) was found that microbial communities vary within 5 km distance in 5 different vineyards.

Developmental stage effects

Differences were observed in alpha diversity between pre-flowering and at maturity wheat samples. Mature samples recruit significantly more microbial diversity than pre flowering samples. In a study conducted by Copeland et al. (2015), observed that plant developmental stage can influence leaf microbiome structure in beans. As plants grow and change through their different developmental stages, their microbial diversity increases. This could be due to mature plants produce complex metabolites that leads to different microbial responses, as Gdanetz and Trail (2017) showed differences between early flowering and late developments on wheat.

Genotypic effects

It was found that microbial communities were genotypically grouped more in Mt. Holly samples (Figure 8), being this the scab nursery, than Warsaw samples. According with (Peiffer and Ley 2013) maize rhizosphere microbiota is heritable, if is heritable it may mean that every genotype shape its own microbiome. Other research also confirms that the genotype influences the configuration of this microbiome (Peiffer and Ley 2013). Similar results were found in *Boechera stricta* leaves microbiomes, where they used a genotype-by-site interaction model, it was shown that microbial communities in host genotypes separate more widely when interacting with site (Wagner et al. 2016).

Pathogen effects

Another explanation is that plants could be affected by certain groups of microbiomes, which may be present in different quantities (Wagner et al. 2016),

plant pathogens play a role and can influence the increase or reduction of bacterial communities. According with Whipps et al. 2008, the pathogens trigger the plant a defense mechanism in which, they cause beneficial bacteria to increase. The beneficial microbial community varies genotypes, such as wild and modern *Phaseolus vulgaris*, recurring different beneficial bacteria (Pérez-Jaramillo et al. 2017). In the figure 4, see Mt. Holly, the scab nursery, which the presence of the pathogen, influenced the formation of microbial communities according to the genotype. This explanation has been reported using the plant model *Arabidopsis thaliana*, which under the stress of the pathogen *Hyaloperonospora arabidopsidis*, the plant promotes the three bacterial species as a defense in roots (Berendsen et al. 2018). It has been show during aerial infection by the pathogen *Pseudomonas syringae* pv *tomato* led to the recruitment of the rhizobacteria *B. subtilis* FB17 on *Arabidopsis thaliana* roots (Rudrappa et al. 2008). In order to corroborate either the infection the plant pathogen exert in the increasing of microbial community, Henry (1931) used two pathogen *Pseudomonas syringae* DC3000 and *Xanthomonas campestris* and one bacteria beneficial *Bacillus cereus*. They were inoculated and corroborate with uninoculated controls in *Arabidopsis thaliana*. They found the plant grew faster under the pressure of the pathogens rather than the one who was inoculates with beneficial bacteria. This is probably because the pathogen induce the defense (Saleem et al. 2017). In our experiment (Figure 7), the presence of the pathogen, influenced the formation of microbial communities. It has been demonstrated that continues cultivation of wheat under stress, can induce soil suppressive on roots (Henry, 1931).

Differential abundance

Differential abundance points out that differentiation in recruitment of bacteria (Figure 9). One of the investigations indicates that different plant genotypes recruit different microbes (Redford et al. 2010). According to (Guo et al. 2018) it is pointed out that resistant and susceptible genotypes of mulberry recruited different microorganisms, this indicates that the resistant genotypes exhibit different microbes to control that disease than the susceptible ones.

In our investigation one of the bacteria found in the susceptible genotypes was *Pseudomonas*. *Pseudomonas* has been shown to secrete antifungal components against *Fusarium* (Müller, 2016); it could be deduced that the susceptible genotypes recruit this type of bacteria to be able to counteract the attack of fusarium. It has also been observed that different genotypes of *Leptospermum scoparium* and bacteria found in the phyllosphere may coevolved together in order to survive (Noble, 2018).

The genus *Pseudomonas* in susceptible wheat samples and not in resistant samples. It may be because bacteria in resistant variants do not need *Pseudomonas*. On the other hand, the absence of the bacterial the genres *Roseomonas*, *Novosphingobium*, *Sphingomonas* and *Pedobacter* is observed in the samples of susceptible wheat. For example, the *Roseomonas* genus is not associated with grasses, (Loreti et al. 2009), and the genus *Novosphingobium* was only detected in *Arabidopsis thaliana* rhizosphere (Lin et al. 2013). The genus *Pedobacter*, was found in resistant wheat samples, but not in the susceptible ones. This bacterium does not exert any biological control over fusarium, rather

it is only part of wheat leaf enriched with chitin (Zhang et al. 2018). The genus *Sphingomas* is found in large proportions in maturity samples, where it has been seen that this genus reduces the fusarium population (Wachowska et al. 2013)

It was also observed that *Spirosoma*, *Hymenobacter* and *Masilia* were found in both susceptible and resistant samples. *Spirosoma* was found to be greater in the susceptible samples, however, no information was found about *Spirosoma* that had been presented in plants. The genus *Massilia*, was detected in part of the microbial community in soil suppression in *Fusarium oxysporum f. sp. radicis-cucumerinum* in cucumber plants, which is not considerate bacterial control in scab (Klein et al. 2012). In summary, phyllosphere may recruit different microbial communities according to plant genotype.

Microbial abundance

In this study also, wheat head bacterial communities comprised 9063 ASVs, which represents a comprehensive analysis of the wheat phyllosphere to date, applying ASVs. According to our data (Figure 5), the prevalence of Genus *Pantoea*, has been identified as an antagonist of many plant pathogens belonging to bacteria and fungi, as a result of antibiotic production, competition mechanisms or induction of plant resistance (Dutkiewicz et al. 2016). Previous data related the members of the genus *Pantoea* shown inhibitory activity against the growing of *Fusarium graminearum* and the virulence of mycotoxin under lab conditions (Pandolfi et al. 2010), (Comby et al. 2017) and (Dreo et al. 2018).

In our samples we have also detected the genus *Pseudomonas*, a commonly found in the aerial part of wheat, living either healthy or infected spikelet tissues (Yoshida et al. 2012), it has been detected that this non-pathogenic bacteria promote plant antagonistic activity over plant pathogens (Müller et al. 2016). *Pseudomonas* isolates obtained from infected spikelet tissues were highly associated with the FHB pathogen and has potential to be biological control against FHB under laboratory and field conditions (Yoshida et al. 2012) (Alimi, Soleimani, and Taghinasab 2012), (Müller et al. 2016), (Hu et al. 2014) (Javad, Reza) and (Khodakaramian 2006), In addition *Pseudomonas* can reduce the levels of mycotoxin (Chen et al. 2018).

In smaller composition are microbial communities of and that also help reduce the fusarium index, the genus *Methylobacterium* (Yoshida et al. 2012), *Sphingomonas* reduced the population size of *fusarium*, (Wachowska et al. 2013), (“Microorganisms as Biological Control Agents against *Fusarium Pathogens* in Winter Wheat” 2013). In some cases degrading the DON into less toxic compounds (He et al. 2017). Some strains like *Chryseobacterium* reduced symptoms of *F. graminearum* in the seeds under controlled environmental conditions (Khan et al. 2006), (Domenech et al. 2006).

2.6 Conclusions

The analysis of the wheat head microbiomes has resulted in the introduction to novel approach called Amplicon sequence variants (ASV) to characterize microbial community on wheat, this provide a better resolution provide better resolution than OTUs.

We found that locations influence microbial community composition on wheat, despite sites being a short distance apart. Developmental stages also influence, microbial community composition as well as the presence of the pathogen. Finally, we found that the presence of the pathogen influences the recruitment of beneficial microbial communities that have deleterious actions against Fusarium head blight.

Understanding the factors that influence microbiomes can give us an idea regarding the different factors that we must take into account for an integrated pest management. However, trying to understand the wheat head microbiome continuum requires more investigation in order to applied to the agriculture system. Future steps might include the manipulation of microbiomes to determine how different factors relate to each other.

Figure 1. Research plot locations across Virginia and sample collection at two different developmental stages.

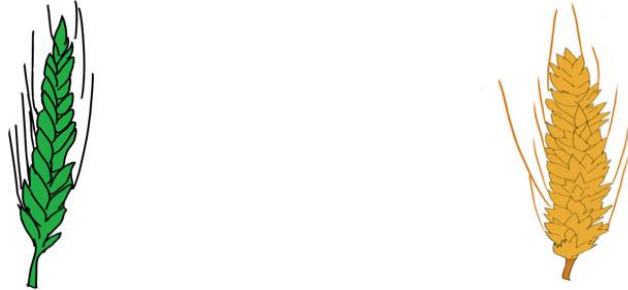


Figure 2. sample collection at two different developmental stages.



Figure 3. Relative abundance of bacteria at the genus level of wheat head microbiome of ASVs. Location samples are represented along the horizontal axis, and relative abundance is denoted by the vertical axis.

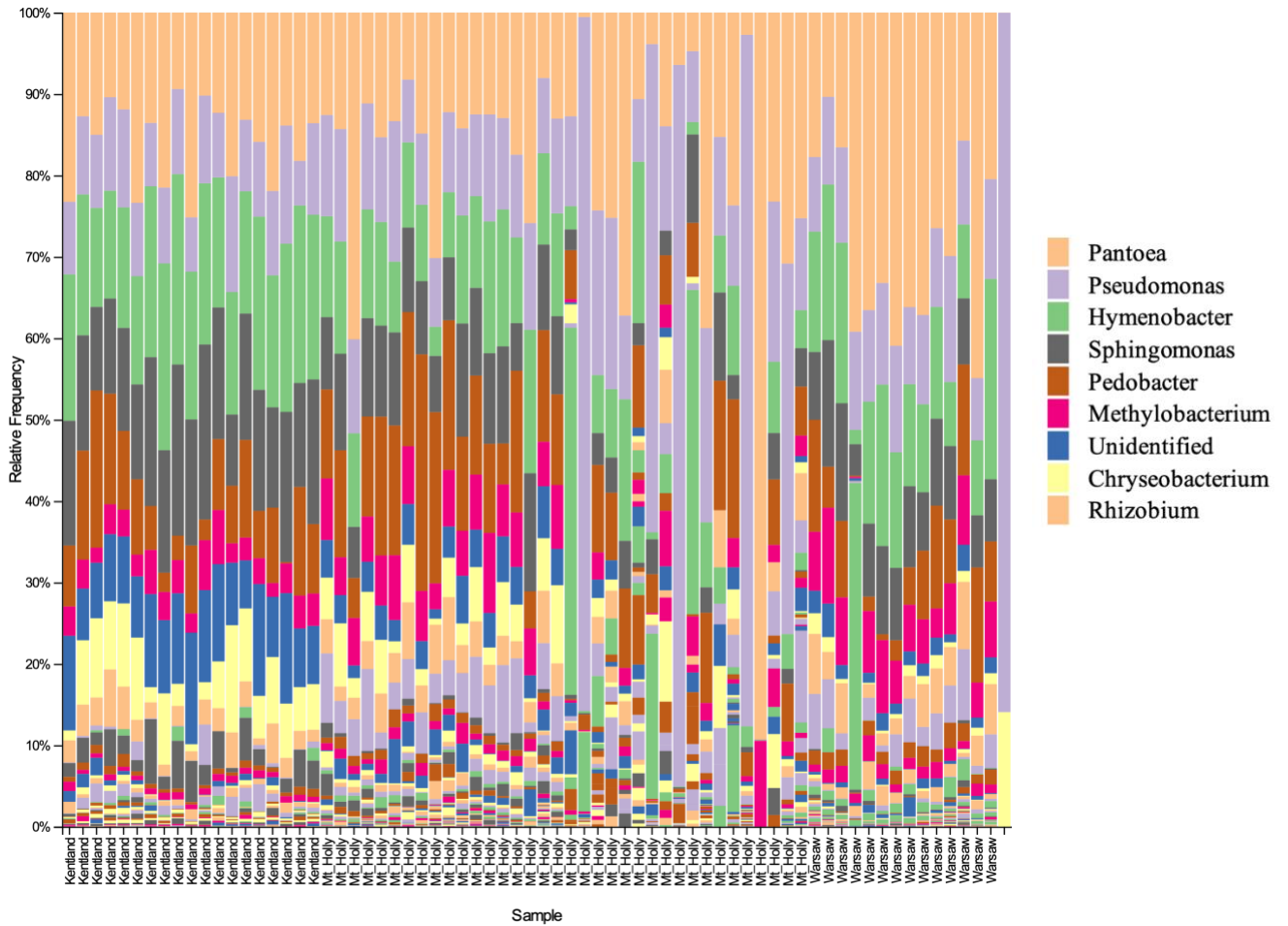


Figure 4. Scatter plots illustrating alpha diversity indices (Shannon diversity, Simpson's index and Inverse Simpson for maturity and pre-flowering wheat grain-head microbiome samples.

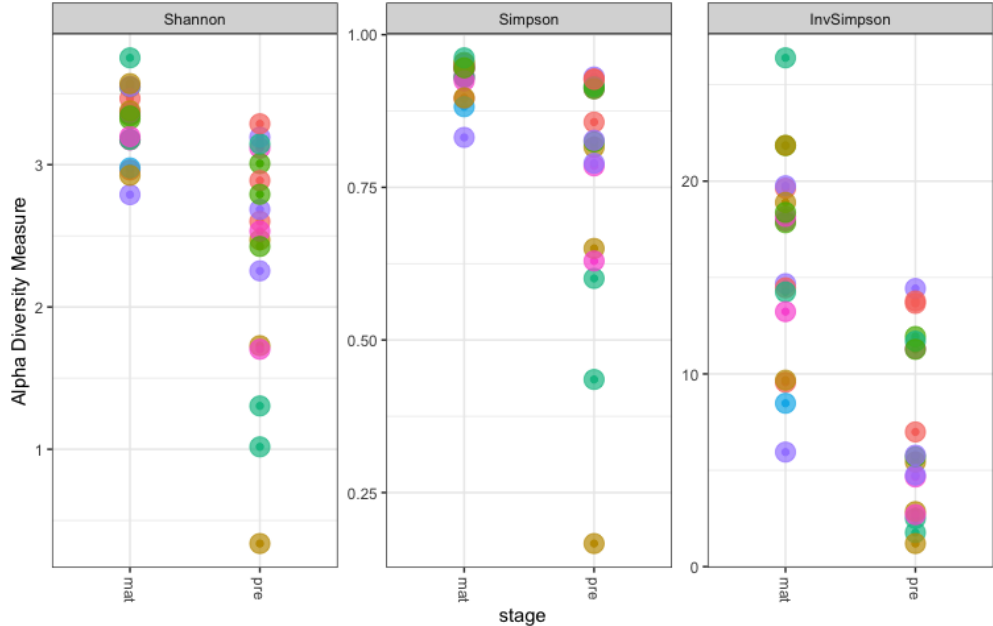


Figure 5. Scatter plots illustrating alpha diversity indices (Shannon diversity, Simpson index and Inverse Simpson) in wheat bacterial microbiomes between early and late maturing wheat. A Students' t-test across diversity indices indicated no significant differences between early and late maturing wheat samples.

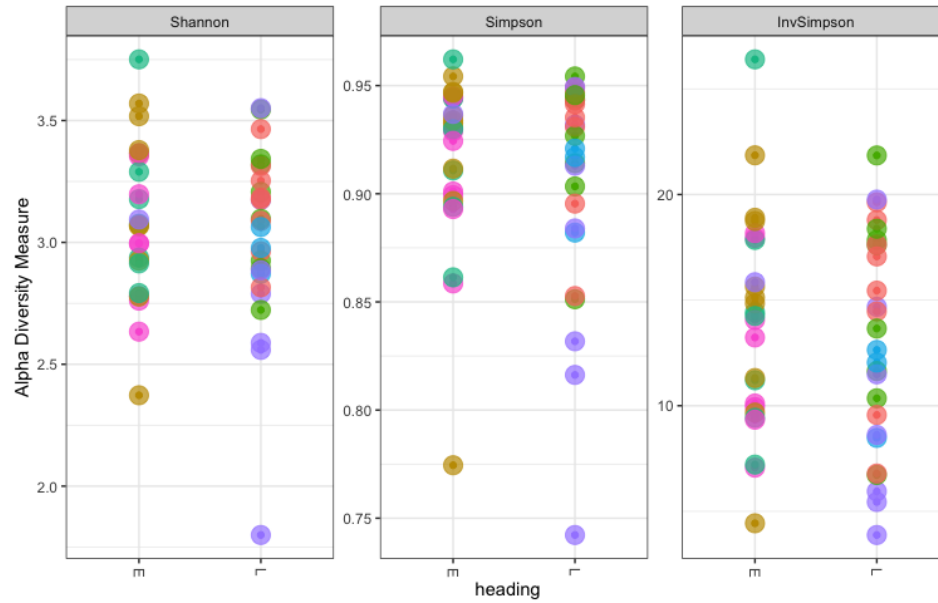


Figure 6: Scatter plots illustrating alpha diversity indices (Shannon diversity, Simpson index and Inverse Simpson) in wheat bacterial microbiomes across the three different locations. Kruskal-wallis was applied ($H=6.54$, $p\text{-value} < 0.08$), resulting no significant between locations samples.

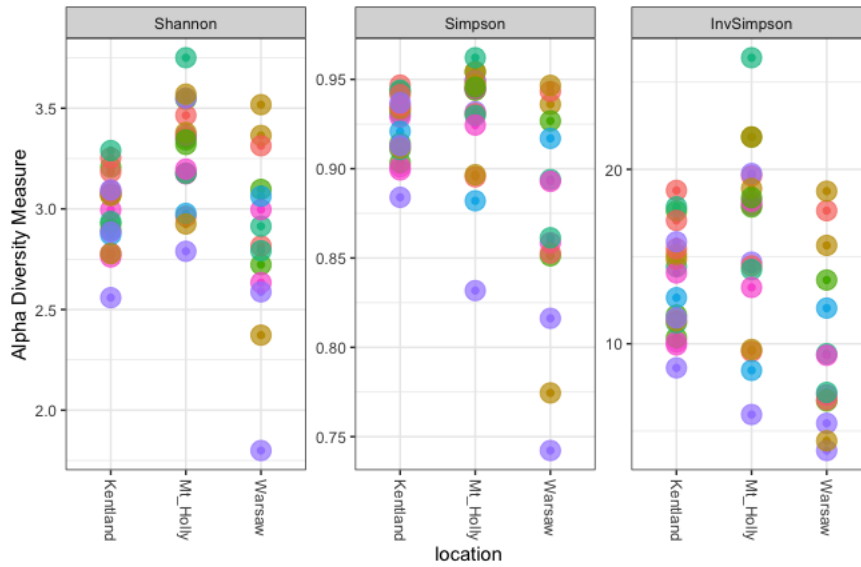


Figure 7. Beta diversity across three locations. Multidimensional scaling plots was used to determine distance between wheat head microbiome by locations. Each point represents a sample, which are wheat microbiome sample from different locations with colors indicating the location. Points that cluster nearer to one another indicate bacterial community structure is more similar. The axes indicate the percentage of the variation in the data for the bacterial communities. ($F=6.46$, $p = 0.0001$ by PERMANOVA). The X and Y axis are the first and second components respectively, both make 43 % out of the total information from the original data.

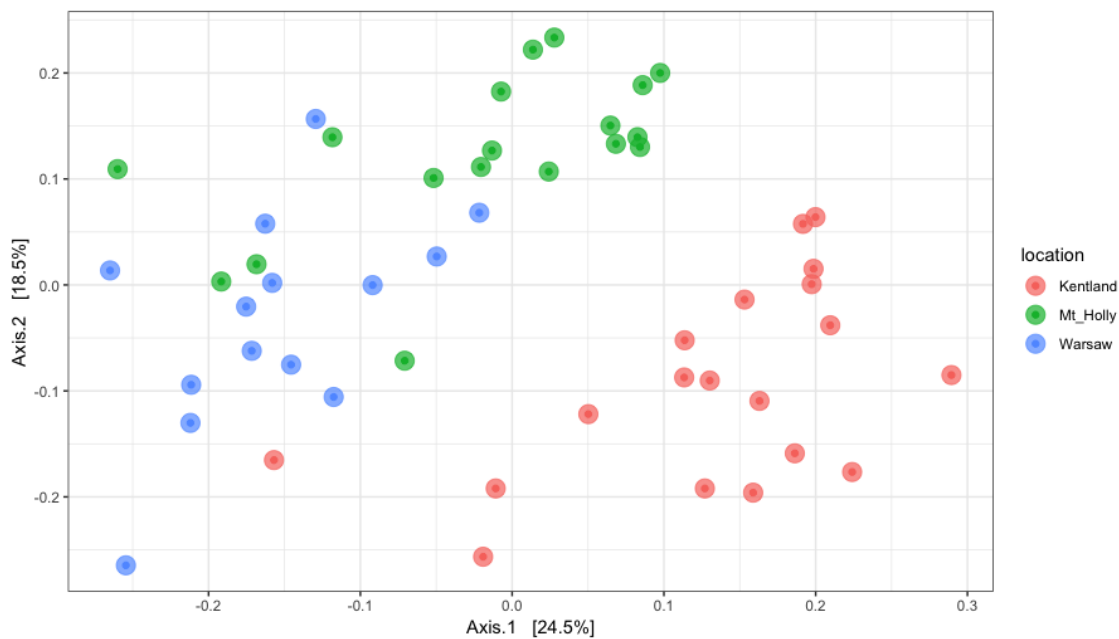


Figure 8. Genotypic differences in beta diversity. Multidimensional scaling plots were used to visualize distances between, susceptible (blue), moderate resistance (red), and resistance genotypes (green) grown in two locations: Mt. Holly (A) and Warsaw (B). Points that cluster nearer to one another are more similar to one another. The axes indicate the percentage of the variation explained by that dimension. For Mt. Holly $p = 0.008$ (A) by PERMANOVA and for Warsaw $p = 0.3$ (B) by PERMANOVA. The X and Y axis are the first and second components respectively, both make 49.5 % and 60% out of the total information from the original data respectively.

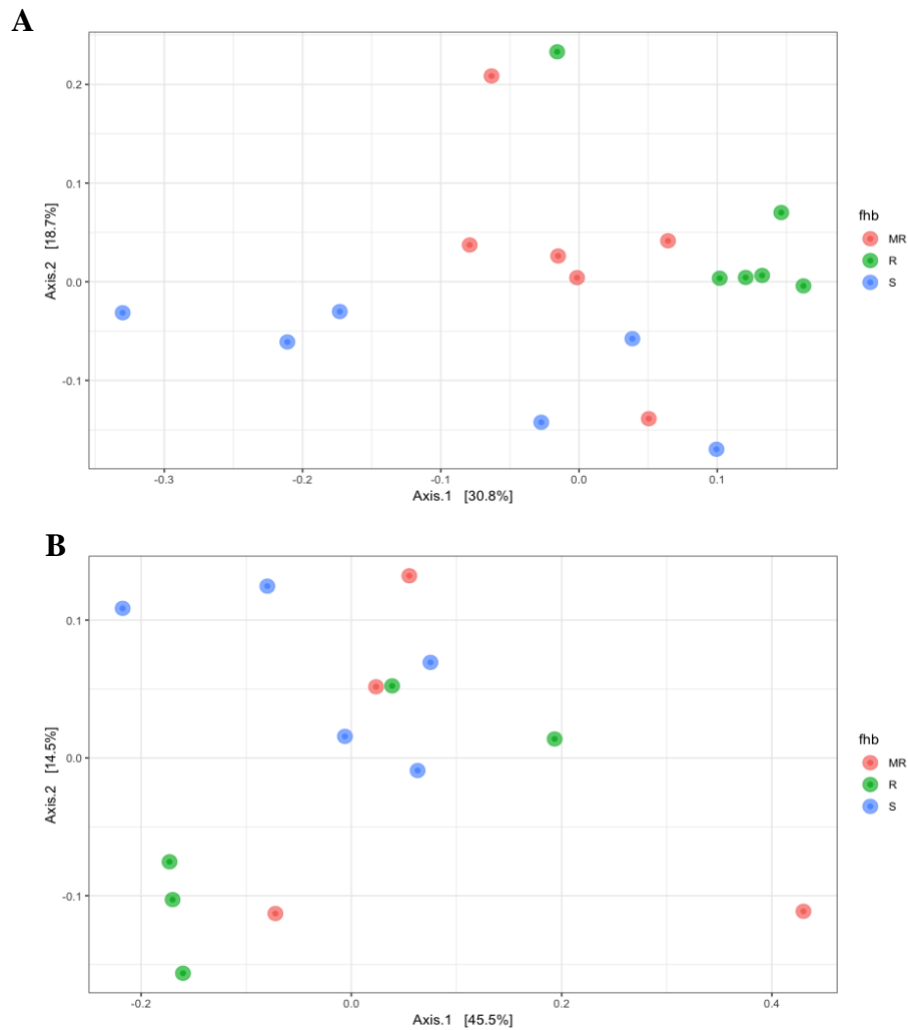
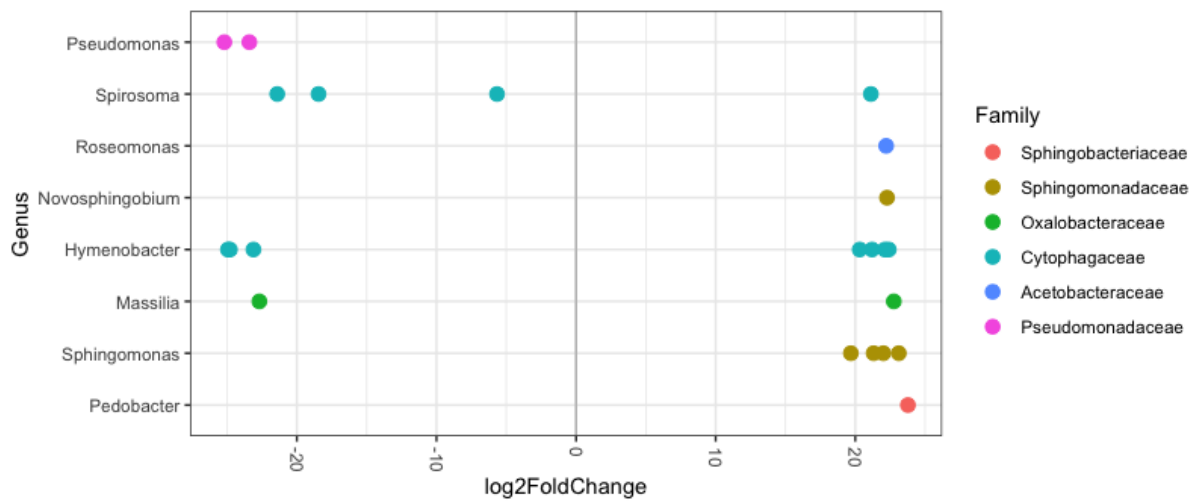


Figure 9. DESeq2 analysis results indicating the log₂fold change of bacterial genera in wheat communities of susceptible and resistant samples treatments. The plot shows changes for genus-level bacterial (y-axis) identified as significantly different along with the log₂fold change (X-axis). Each data point represents an ASV. ASVs to the right of the zero line are more abundant and ASVs to the left of the zero line are less abundant between groups.



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Appendix

Culture-dependent techniques

Many of the first investigations to characterize microbial communities have been dependent of culture-dependent methods (Amanda J. Redford, Bowers, Knight, Linhart, & Fierer, 2010), (Kisand and Wikner 2003). these techniques have allowed isolate microbes in pure cultures, and get their genes sequences to be studies in details (Turner, James, & Poole, 2013). However, microorganism cultures only allow organisms that were adapted to grow in laboratory conditions (Rappé and Giovannoni 2003), As far as the microorganisms that are recognized, were not all that were originally at the beginning which these method may not estimate the diversity of microbes (Rappe and Giovannoni, 2003).

Next generation sequencing

With the advent of Next generation sequencing (NGC) technology, culture-independent techniques such as PCR it is possible to isolate and sequence DNA and RNA and amplification of 16S rRNA (16S ribosomal RNA) for bacteria (Turner et al., 2013) and ITS (Internal Transcriber Spacer) for fungi (Schoch et al. 2012) directly from environmental samples, providing a clearer picture of relative abundance of microbiota in the environment (Lindow & Brandl, 2003), (Turner et al., 2013).

Microbial community characterization

Ribosomal ribonucleic acid (rRNA), often called amplicon, it is the RNA component of the ribosome, and it is essential for protein synthesis in all living organisms. It consist as a small region of microbial DNA (Müller and Ruppel 2014) that is why it is used to characterize bacterial organisms (Bhagavan and Ha 2015). 16S rRNA is widely used to identify bacterial species and do taxonomic studies (Petti, Polage, and Schreckenberger 2005). Because its estimated substitution rate is ~7000 times higher for the hypervariable regions than the highly-conserved ones and these genetic differences have been considered to reflect, for most bacteria genome (Rosselli et al. 2016). This mechanism started to be used since 1970, which has shown great progress in the identification an classification (Rosselli et al. 2016). Now a days, PCR allow to sequencing 16S rRNA. Which this region of genes, covers almost all species of bacteria, giving a large database of genes (Yoon et al. 2017).

The Full-length 16S rRNA gene is nearly 1600 base pairs long and it has nine hypervariable regions of varying conservation called: V1-V9 (Kim, Morrison, and Yu 2011). These nine hypervariable regions are very conserved for bacteria (Petti, Polage, and Schreckenberger 2005), which it can be used for taxonomic identification of bacterial and archaeal strains (Chakravorty et al. 2007), (Tringe and Hugenholtz 2008). Therefore, each region corresponds to its specific primers, which help to study microbial communities for each different experiment.

The V4 sub region of 16S rRNA gene is commonly targeted in microbiome studies with the widely used 515F/806R universal primers (Caporaso et al. 2011). These primers

are recommended in the Earth Microbiome Project's Illumina NGS protocol (16S Illumina Amplicon Protocol: Earth Microbiome Project. (2020))

The V4 sub region is the ideal region for designing universal primers with resolution for bacterial phyla (Yang, Wang, and Qian 2016). Many studies for identification of bacteria in wheat have been targeted V3–4 (Prudence et al. 2019), (Chen et al. 2018), others only using V4 (Mavrodi et al. 2018) and (Gdanetz and Trail 2017). However, there is no agreement on the most suitable region to target for wheat.

The 16S rRNA gene is universal and it is presented in all bacteria (Fox *et al.* 1980). Thus 16S rRNA gene is the most use molecular marker in microbial experiments (Copeland et al. 2015). However, the rRNA of chloroplast, mitochondria and bacterial are all similar. This similarity makes the amplification of the genes and sequencing difficult. It is difficult to difference between DNA from mitochondrial, chloroplasts and bacteria. The mitochondrial DNA involves the DNA of bacterial organism when occurs the extraction, and chloroplast sequences are most closely related to nitrogen-fixing unicellular of Cyanobacteria and often cannot be differentiated when there is a sequencing of 16S rRNA gene (Falcon et all, 2010) that is why the amplification and analysis make difficult (Hanshew et al. 2013).

LNA protocol

The contamination of plant organelle (mitochondria and plastid) genes in the DNA extraction step becomes an important problem in investigating the bacterial community

with is associated with host plants. This is because organelle genes are amplified by polymerase chain reaction (PCR) with the same primers that it is used for bacteria (Sakai and Ikenaga 2013). The first organelle: mitochondria. Mitochondria being descended from eukaryotic cells, so these carry 16S rRNA genes, that is why this is problem for 16S rRNA analysis (Fricker, Podlesny, and Fricke 2019). On the other hand, chloroplast are evolutionarily descended from bacteria (Hanshew et al. 2013). This type of organelle descends from bacterial endosymbiont, which is related to cyanobacteria. That is why it also presents problems to the sequencing reads (Gould, Waller, and McFadden 2008). Therefore, a different PCR technique are required, in which they allow us to remove these contaminants. This kind of PCR is locked nucleic acid (LNA). When soybeans and corn were sequenced using LNA oligonucleotides. The percentage of chloroplast and mitochondrial sequences were 0%, in the other hand, the percentage of these organelles, when the sequencing was not use LNA oligonucleotides. This percentage was high (YU et al. 2016). Other experiment using 0.5 μM of LNA oligonucleotides, the intensity of mitochondrial genes was significantly decreased, this decreased even more when it was increased to 3.0 μM , making the mitochondria genes imperceptible when detected, when 4.0 μM was used there were no products of mitochondrial genes in soy bean (Ikenaga et al. 2015).

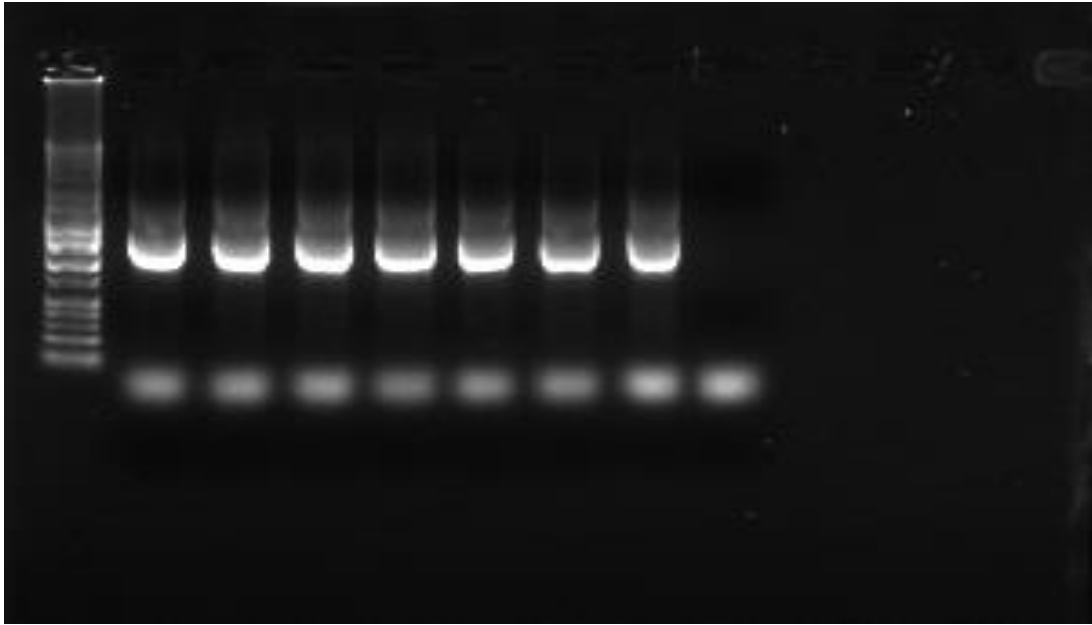


Figure 01. PCR amplified products were run on 1% agarose gel in TAE Buffer and stained with GelRed® nucleic acid stain 10000x in water. First lane indicates the DNA ladder (Hi-Lo™DNA Marker 50-10000 base pair, Minnesota Molecular). Markers with high. Lanes 1 to 7 indicate the PCR amplified 16S rRNA gene of the respective bacterial isolates. Lane 8, negative control without DNA to ensure that there was no outside contamination. DNA bands shows that the amplification of conserved regions of the 16S rRNA gene were amplified by the 63F and 1492R primer pair.

Illumina PCR

Next Generation Sequencing (NGS) platforms suitable for 16S rRNA gene analyses include: Illumina HiSeq and MiSeq; Roche 454, which is phasing out; and Thermo Fisher

Ion Torrent)(Jo, Kennedy, and Kong 2016), (Pichler et al. 2018). High-throughput sequencing of the 16S rRNA gene on the Illumina platform is commonly used to assess microbial diversity in environmental samples (Sinclair et al. 2015) (Allali et al. 2017). Illumina Miseq platform is used because of these advantages rapid turnaround time, cost-effective and gene expression studies, that is why MiSeq has become one of the most widely used next generation sequencing platforms. (Ravi, Walton, and Khosroheidari 2018) (Illumina, 2020). In addition Illumina MiSeq platform produces paired sequence reads up to 300 bp long (Holm et al. 2019).

The ribosome complex is responsible for translating mRNA into proteins. it is a highly conserved complex present in all living organisms. The complex is formed by many proteins as well as RNAs called ribosomal RNAs (rRNA). In particular, one rRNA subunit of the ribosomal complex, called the 16S rRNA subunit in prokaryotes and the 18S rRNA subunit in eukaryotes. (Hadziavdic et al. 2014). The conserved regions allow the design of “universal” (kingdom-specific) primers, while the variable regions targeted by the amplification are sequenced and used for phylogenetic analysis. A large body of literature documents the development and use of distinct ‘universal primers’, which typically target the amplification of one or two variable regions of the 16S rRNA gene.

In this research we use multiplexed sequencing (multiplexing), which allows to sequence DNA from multiple samples in a single run (Wright and Vetsigian, 2016). This process use a unique index or barcodes sequences, which is attached to each sample, also into the sequences of the universal primers, with this methodology, multiple samples are pooled

together for sequencing in the same run (Bhagavan and Ha 2015). Then Individual samples can then be identified during the analysis of the sequencing data based on the unique barcode (index) sequence.

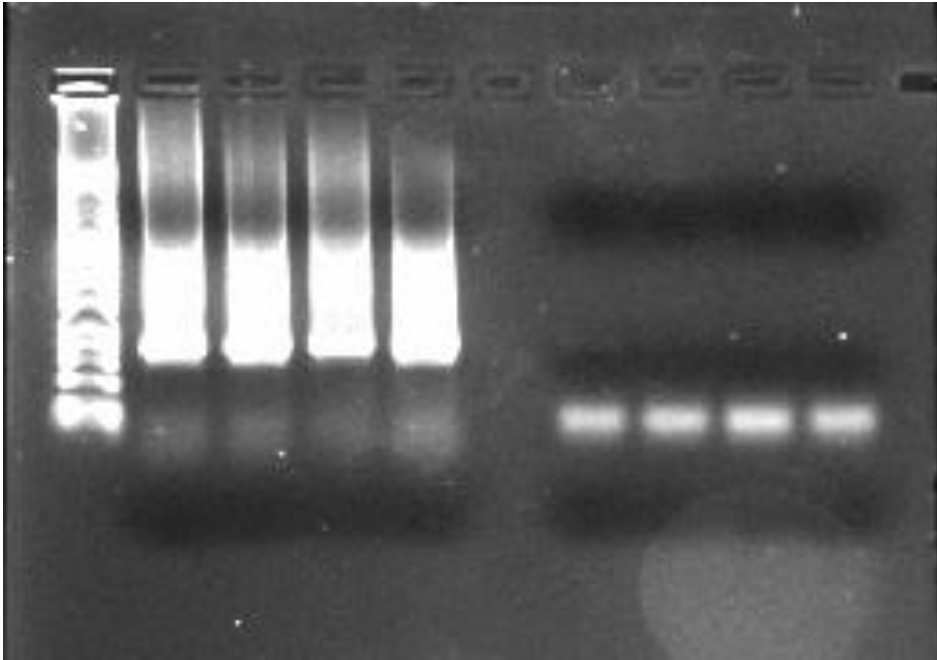


Figure 02. PCR amplified products were run on 1% agarose gel in TAE Buffer and stained with GelRed® nucleic acid stain 10000x in water. First lane indicates the DNA ladder (Hi-Lo™DNA Marker 50-10000 base pair, Minnesota Molecular). Lanes 1 to 4 indicate amount of DNA. Lanes from 5 to 8 indicates the amplification of the primers.

PCR products from duplicates were visualized on a 1% agarose gel (Figure 3) and quantified using a Qubit 2.0. PCR products of each DNA sample were pooled to make a composite sample and cleaned with the QIAquick PCR Purification Kit (Qiagen, Valencia, California). The final pooled sample was sent for sequencing on an Illumina Mi-Seq instrument.

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