Frequency of Soybean in Rotation and Persistence of Rhizobia in Manitoba Soils

by

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Abstract

Soybean (*Glycine max*), along with canola and wheat, are some of Canada’s top agricultural exports. It is also one of the few crops that does not require industrial nitrogen fertilizer. Soybean gets nitrogen from forming a symbiotic relationship with a soil bacterium called *Bradyrhizobium japonicum*. *B. japonicum* is not native in Manitoba soils and must be inoculated into soybean fields. The objectives of this thesis are to examine the persistence of the *B. japonicum* inoculant in the soil, as well as to observe whether varying the crop rotation will affect its abundance. The overall bacterial communities will also be examined for compositional changes between crop rotation treatments (continuous soybean, canola–soybean, corn–soybean, diversified), timepoints (before planting, emergence, pod fill, and full maturity), and sites (Carman, Kelburn, Melita). The bacterial communities were analyzed using 16S rRNA sequencing, and *B. japonicum* quantification in the soil was measured using qPCR. *B. japonicum* was shown to persist in the soil years after the initial inoculation. It was also observed that there are native species of *Bradyrhizobium* present in Manitoba soils that cannot nodulate soybean. The crop rotation effect on *B. japonicum*, as well as the bacterial community, was minimal. The principal reason for observed differences seem to be the site locations and their soil properties, such as soil type and pH. Carman had significantly higher bacterial diversity, as measured by the Shannon index, than Kelburn and Melita. However, in all three locations, the majority of the bacteria were from three phyla: Proteobacteria, Actinobacteria, and Acidobacteria. It is the subdivisions within the phyla that varied greatly depending on the location. Observations made in this study can lead to a better understanding of the complex plant–microbe and microbe–microbe relationships for future research and applications.
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Dedication

To my Mom and Dad . . .

. . . for everything.
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<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BP</td>
<td>before planting</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming unit</td>
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<td>KSB</td>
<td>potassium solubilizing bacteria</td>
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<tr>
<td>MCP</td>
<td>monocalcium phosphate</td>
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<tr>
<td>MPSG</td>
<td>Manitoba pulse and soybean growers’ association</td>
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<tr>
<td>OTU</td>
<td>operational taxonomic unit</td>
</tr>
<tr>
<td>PCoA</td>
<td>principal coordinate analysis</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>Po</td>
<td>organic phosphate</td>
</tr>
<tr>
<td>PSB</td>
<td>phosphate solubilizing bacteria</td>
</tr>
<tr>
<td>spp</td>
<td>several species</td>
</tr>
<tr>
<td>R5</td>
<td>beginning seed</td>
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<tr>
<td>R8</td>
<td>full maturity</td>
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<tr>
<td>VE</td>
<td>emergence</td>
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Chapter 1:

Literature Review
1.1  *Glycine max (Soybean)*

There are six principal field crops grown in Canada: wheat, canola, corn, soybean, barley, and oats. According to Statistics Canada (2018), 31.8 million tonnes of wheat, 20.3 million tonnes of canola, 13.9 million of corn for grain, 7.3 million tonnes of soybean, 8.4 million tonnes of barley, and 3.4 million tonnes of oats were produced in 2018. These crops are valuable trade commodities. In 2016, canola, wheat, and soybean were three of the top five agricultural exports of Canada (Arora 2017). Both canola and wheat are long-established crops in the country, but soybean is a relatively new crop for western Canada.

1.1.1  History

Soybean arrived in Canada in the mid-1800s, but the first trials were mostly unsuccessful due to the harsh climate. It wasn’t until the 1920s that the commercialization of soybean was established. Commercialization, however, initially only occurred in southern Ontario, the Canadian region with the longest and warmest growing season. Studies around this time focused on looking for suitable soybean varieties that would thrive in other parts of the country. In the 1970s, the first varieties to grow outside southern Ontario were recorded. These varieties matured earlier and have improved tolerance for colder climates. Since the 1970s’ plant breeding trials, soybean cultivation has spread throughout Canada.

Ontario has produced soybean since the mid-1970’s but it was not grown in other provinces until 1986, when Quebec begun its own soybean fields (Cloutier 2017). Ontario’s other neighbour, Manitoba, did not have significant soybean production until 2001. However, since then Manitoba has had a strong upward trend of soybean production. The amount of acreage seeded with soybean, as well as its yield, has been increasing continuously for the past
decade (Statistics Canada 2017). Currently, these three provinces produce the large majority of Canadian soybean.

### 1.1.2 *Bradyrhizobium japonicum*

*Bradyrhizobium japonicum* is a soil bacterium that can form a symbiotic relationship with soybean. When in symbiosis, *B. japonicum* can fulfill soybean’s nitrogen requirements by turning N₂ into ammonia (NH₃) via biological nitrogen fixation. Otherwise, synthetic nitrogen fertilizers must be added to the soil. Nitrogen fertilizers are mainly made through the Haber–Bosch process, which artificially converts N₂ into NH₃ through a reaction with hydrogen (H₂) using a metal catalyst under high temperature and pressure (Pool *et al.* 2013). Unfortunately, this process produces substantial greenhouse gases and causes eutrophication (Rockstrom *et al.* 2009). A more natural input of nitrogen into agricultural systems would be preferred, such as the *B. japonicum*–soybean association.

Soybean provides *B. japonicum* with photosynthates, and in return *B. japonicum* provides soybean plants with fixed nitrogen. Symbiosis begins with the legumes releasing flavonoids to the rhizosphere, which trigger the synthesis of nodulation (Nod) factors in the rhizobia. Nod factors are lipochitin oligosaccharides with a chitin core, composed of three to six β1,4-linked N-acetyl-D-glucosamine (NAG) residues, and an acyl group at the non-reducing end of the core (Denarie *et al.* 1996). Structural differences in these factors determine host specificity, where only successful host recognition leads to the nodulation required for nitrogen fixation (Wang *et al.* 2011). In the *B. japonicum*–soybean symbiosis, one of the observed structural differences is the fucosylation of the reducing-end NAG residue by NodZ, an α1,6-fucosyltranferase (Stacey *et al.* 1994). The fucosyl residue allows *B. japonicum* to be recognized by the soybean host plant (Sanjuan *et al.* 1992). The soybean plant recognizes the modified Nod factor and triggers the
curling of the root hair around *B. japonicum*, which leads to the formation of nitrogen-fixing nodules (Stacey *et al*. 1995).

Since *B. japonicum* is not native to Manitoba soils, soybeans must be inoculated with products containing *B. japonicum* in order to meet its nitrogen requirements. The Manitoba Pulse and Soybean Growers (MPSG 2017) have a fertility fact sheet that recommends inoculation strategies based on field history. Currently, they recommend double inoculation for fields with little to no history of soybean, and single inoculation for those that have an established history.

### 1.2 Plant Nutrients and Soil Bacteria

There are 17 essential nutrients required for plant growth (Troeh & Thompson 1993). Aside for carbon (C), hydrogen (H), and oxygen (O) which are absorbed through air and water, these essential nutrients are obtained from the soil. There are three primary macronutrients – nitrogen (N), phosphorus (P), and potassium (K). These are needed by plants in large quantities and are typically the limiting nutrients in agricultural soils (Parikh & James 2012). Other macronutrients include calcium (Ca), magnesium (Mg), and sulfur (S). Iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), boron (B), molybdenum (Mo), chlorine (Cl), and nickel (Ni) are needed in trace quantities.

#### 1.2.1 Nitrogen (N)

Nitrogen is an abundant but inaccessible nutrient to most organisms, including plants. However, nitrogen is essential for plant growth since it is found in all plant cells, proteins, and hormones. It is also necessary for chlorophyll synthesis (Leghari *et al*. 2016). Nitrogen
deficiency can lead to smaller plants and chlorosis. Soybeans, and other legumes, have developed symbiotic associations with soil bacteria to ensure sufficient nitrogen supply.

Unlike plants, bacteria have developed mechanisms to utilize nitrogen’s various forms and are the driving factor of N cycling. There are bacteria that can turn atmospheric N\textsubscript{2} into biologically accessible forms like NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{-}. This process is called nitrogen fixation and can be carried out by both free-living and symbiotic bacteria. N fixation uses the nitrogenase enzyme, which is highly sensitive to the presence of oxygen, to reduce N\textsubscript{2} to NH\textsubscript{4}\textsuperscript{+} and so N-fixing bacteria have developed special mechanisms to provide the necessary low oxygen areas. Free-living bacteria from the genus *Azotobacter* intensifies their metabolism to reduce the oxygen concentration (Wani *et al.* 2015). In addition, it produces a nitrogenase-protective protein to facilitate N fixation (Maier & Moshiri 2000). As for symbiotic bacteria, they produce nodules on the plant roots where the environment can be controlled to suit N fixation. Most N fixing symbiotic bacteria associate with legumes, which are collectively called rhizobia. Rhizobia include several families from the order Rhizobiales and the Burkholderiaceae family (Weir 2016). Other non-legume symbiotic bacteria include *Frankia* and *Parasponia* (Santi *et al.* 2013). The nitrogen produced through these various N fixation processes is the second largest source of the total global N input (Schlesinger 2009).

Plant and microbial residues produce organic N in the soil, which can be digested by bacteria into NH\textsubscript{4}\textsuperscript{+} through the mineralization/ammonification process (Leifeld *et al.* 2002). *Bacillus, Clostridium, Proteus, Pseudomonas,* and *Streptomyces* are known to use this process and are called ammonifying bacteria (Bisen 2014). They play an important role in N cycling since the NH\textsubscript{4}\textsuperscript{+} form can be utilized by all organisms – plants, bacteria, and fungi (Merrick & Edwards 1995). NH\textsubscript{4}\textsuperscript{+} that is not taken up by plants remains in the plant–soil system, as its
positive charge is attracted by the negatively charged soil. The remaining soil NH$_4^+$ can then be subjected to further changes through nitrification.

Nitrification is the process of converting NH$_4^+$ into NO$_3^-$. Nitrifying bacteria from the genera *Nitrobacter*, *Nitrococcus*, and *Nitrosomonas* use the energy of reduced N compounds, NH$_4^+$ and NO$_2^-$, to produce organic compounds (Bisen 2014). In aerated soils, NH$_4^+$ is oxidized into nitrite (NO$_2^-$) and then to NO$_3^-$ (Myrold 1998). Since NO$_3^-$ is negatively charged it is not attracted to soil particles, which makes it highly soluble and easily assimilated by bacteria and plants.

The conversion of NO$_3^-$ back into N$_2$ is called denitrification. It is carried out by denitrifying bacteria in the *Alcaligenes*, *Bacillus*, *Paracoccus*, and *Pseudomonas* genera (Bisen 2014). This process usually occurs in waterlogged anoxic soils and depletes the pool of biologically available N in the soil (Rich *et al.* 2003).

1.2.2 Phosphorus (P)

Phosphorus is used in plants for the synthesis of nucleic acids and phospholipids. It is also critical for energy transfers in the cell, since phosphorus is part of the adenosine triphosphate (ATP). Phosphorus is also used for cell signaling by modifying enzymes through phosphorylation (Stone & Walker 1995). Phosphorus deficiency, unlike nitrogen, is difficult to diagnose due to a lack of visually unique signs. However, deficiency in phosphorus can lead to changes in root architecture, such as the growth reduction of primary roots and increased branching of lateral roots (López-Bucio *et al.* 2003). There is also a general stunting of growth in the early stages of plant development.
Phosphorus is present in both organic (Po) and inorganic (Pi) forms. It is most commonly found in the soil as phosphoric acid (H₃PO₄) and is absorbed by plants as either H₂PO₄⁻ or HPO₄²⁻ (Shen et al. 2011). Weathering of primary Pi minerals, such as apatites and variscites, in the soil is too slow to provide the necessary amount of P required by plants (Pierzynski et al. 2005). Soil Po exists as inositol phosphates and phosphonates (Harrison 1987). Po mineralization is mediated by soil organisms and phosphatase secretions (Turner 2007). Both Pi and Po transformations greatly influence soil P availability. However, natural P pools often fail to meet current agricultural requirements. In these cases, chemical P fertilizers are usually added to fields in order to improve crop growth and yield. Monocalcium phosphate (MCP) and monopotassium phosphate are the most common forms of P fertilizers. MCP generates large amounts of protons and phosphates that cause further precipitation of plant–useable Pi (Benbi & Gilkes 1987).

Only a small percentage of the chemical P fertilizer in the soil is used by plants. Most chemical P fertilizers are rapidly immobilized upon application (Zhang et al. 2018). The phosphate forms bonds with metal ions in the soil rendering it insoluble and inaccessible to plants. The main P solubilization mechanisms include the release of mineral dissolving compounds (e.g. organic acids, siderophores, etc.), the secretion of extracellular enzymes (biochemical P mineralization), and substrate degradation (biological P mineralization). Bacillus and Pseudomonas secrete organic acids, such as glycolic and acetic acids, that cause bond dissolution of phosphate groups (Richardson et al. 2009). The negative charge of organic anions can mobilize P from metals via ligand exchange (Filius et al. 1997). Further, the release of protons, which is associated with organic anion release, acidifies the soil pH thereby aiding P solubilization (Farhat et al. 2009). Biochemical P mineralization uses phosphatase enzymes that catalyze the hydrolysis of H₃PO₄. The most common phosphatase is phosphomonoesterase,
which can be acidic or alkaline depending on the soil condition (Renella et al. 2006). Phosphate solubilizing bacteria (PSB) can produce both kinds of phosphatases, and some even suggests that PSB phosphatases have a greater affinity for Po than those produced by plants (Tarafdar et al. 2001). Biological P mineralization is the release of P through cell degradation. Most of the P solubilized by PSB is used by those same bacteria, and so P is not released into the soil until the cells die (Turner et al. 2003). Cell death can occur due to various environmental conditions, starvation, or predation. Sudden high P availability can be attributed to the presence of microbivores (e.g. nematodes and protozoa) since they increase microbial cell lysis (Bowman & Cole 1978).

1.2.3 Potassium (K)

Potassium is predominantly found as a cation (K\(^+\)) in the cytoplasm and vacuoles of plant cells (Wyn Jones et al. 1979). K\(^+\) is a very mobile nutrient that can be transported between individual cells, as well as throughout the plant system. This high mobility is necessary for its role as an osmolyte regulating turgor pressure in plant cells (Wang & Wu 2017). K\(^+\) is also involved in other physiological and biochemical process within the plant, such as enzyme activation and pH stabilization (Marschner 1995). Most of the enzymes that K\(^+\) binds to and activate are involved in protein and carbohydrate synthesis (Hasanuzzaman et al. 2018). Therefore, K deficiency can lead to necrosis, chlorosis, and reduced growth.

Potassium is present in the soil as mineral K, non-exchangeable K, exchangeable K, and solution K. Most of the soil K, 90 – 98\%, cannot be utilized by plants (Sparks & Huang 1985). Mineral K becomes incorporated into the soil due to the weathering of mica and feldspar (Hillel 2008). K can also be found dissolved in soil water, in organic matter, or within minerals. K\(^+\) channels and transporters in plant roots facilitate the K\(^+\) uptake (Wang & Wu 2013). Leaching
from plant leaves by rainfall also accounts for some of the soil K. Total soil K concentration is typically higher than N and P concentrations, but most of it is unavailable for plants (Wang et al. 2010). Further, plant–available K is easily leached by runoff. Therefore, the concentration of plant–available soil K is typically the lowest out of all primary plant nutrients (Sardans & Peñuelas 2007). To address K deficiency, fertilizers are usually added to growing plants. Potash, or potassium-containing salts, are mined and purified to be used as fertilizers (Wang et al. 2010).

However, there are potassium solubilizing bacteria (KSB) that can solubilize minerals containing K and convert inaccessible K into plant–useable forms. KSB examples include species from Acidothiobacillus, Paenibacillus, Bacillus, and Burkholderia (Meena et al. 2016). There is currently few available information on how KSB solubilizes K-bearing minerals, but it is believed that there are several mechanisms for K solubilization (Etesami et al. 2017). Examples include K release through acidolysis, polysaccharides, complexolysis, and chelation (Parmar & Sidhu 2013). Similar to phosphorus, mineral K solubilization is primarily through acidolysis. Acidolysis refers to the release of protons and organic acids by KSB to directly dissolve mineral K into soluble forms (Meena et al. 2014). Hydrogen ions displace cations like K\(^+\), Mg\(^{2+}\), Ca\(^{2+}\), and Mn\(^{2+}\) from the soil thereby releasing more potassium. KSB can also release K\(^+\) by chelating Al\(^{3+}\), Fe\(^{2+}\), and Ca\(^{2+}\) associated with mineral K (Meena et al. 2014). Aside from their exudates, KSB in itself is a good source of K\(^+\) for plants. Microorganisms, including KSB, require K\(^+\) for their growth. Therefore, their biomasses contain significant K\(^+\) quantities that can be released upon their death (Jones et al. 2003).
1.3 Microbiomes

Microbiome is a term that refers to the genomes of all the microorganisms in a particular environment. Advances in high-throughput sequencing, in terms of technique and cost, has allowed for the study of entire microbial communities instead of a single microbe. The study of microbial communities has given a clearer understanding of the complex relationships that microorganisms have between each other and their environment, including those of the aforementioned soil bacteria. Currently, there are two approaches to next-generation sequencing of microbiomes: shotgun and amplicon sequencing.

1.3.1 Shotgun Sequencing

Whole metagenome shotgun sequencing allows for a comprehensive list of the genes present within all organisms in the experimental samples. The total genomic DNA of a sample is randomly sheared into small fragments which can then be sequenced. Sequences are reconstructed based on overlapping regions to form the genomes of the whole community.

Since shotgun metagenomics amplifies all genes present in the sample, it can identify the presence of organisms and the metabolic capabilities of the community (Segata et al. 2013). However, due to the random nature of shotgun metagenomics, it is difficult to detect the presence of all organisms (Ghurye et al. 2016). The most abundant organisms are sequenced frequently and are highly represented in the microbiome data. The under-represented organisms may require a significantly higher coverage of the genomic sample in order to be detected.

1.3.2 Targeted Amplicon Sequencing

Most microbiome studies use targeted amplicon sequencing as it is the more efficient approach for microbial identification. Amplicons are short DNA reads that have been amplified
to allow for sequencing. In microbiome studies, the amplicons are made up of conserved DNA regions, which are commonly found in ribosomal genes. Ribosomes are found in all living things and are structurally similar to each other since they are necessary for protein synthesis. Therefore, the detection of ribosomal genes can indicate the presence of organisms in an environment.

In prokaryotes, the ribosome is comprised of the 30S small subunit and the 50S large subunit. Most microbiome studies target a specific region of the 16S ribosomal RNA (rRNA), which is a component of the 30S small subunit. The 16S ribosomal DNA (rDNA) is composed of highly conserved regions that are flanked by nine hypervariable ones (V1 – V9; Gray et al. 1984). The primers used in amplicon sequencing correspond to the conserved regions of 16S rDNA such that the sequence that will be generated corresponds to an intervening hypervariable region. Through sequence comparison of these hypervariable regions with curated databases, such as SILVA (Pruesse et al. 2007) and RDP (ribosomal database project; Cole et al. 2014), the identity of microorganisms can be known. Since targeted amplicon sequencing only focuses on one gene, it cannot identify metabolic processes. However, it requires less sequencing depth to profile a microbial community than shotgun metagenomics.

1.3.3 General 16S rRNA Sequencing Workflow

Microbiome sequencing begins with the isolated genomic DNA of an environmental sample. The DNA is then sheared into small fragments, which can be modified to have adapters that act as identifiers during amplification, sequencing, and analysis. The modified DNA is attached to the bottom of a platform where nucleotides can bind to it and make copies of the DNA fragment. Nucleotides bind one at a time and, depending on the technology, the attachment causes the release of a hydrogen ion (Ion Torrent; Rothberg et al. 2011) or of a fluorescent tag.
(Illumina; Bentley et al. 2008) that can be detected by the sequencing machine. The sequence of the nucleotide binding is recorded for each fragment until the DNA is fully copied. These sequences are then processed and analyzed through computer software.

The most popular software used for 16S rRNA sequences are QIIME (Caporaso et al. 2010) and mothur (Schloss et al. 2009). There are generally three phases to sequence analysis: quality control, taxonomic assignment, and statistical analysis. The first phase of quality control filters the contigs based on quality, length, chimeras, and ambiguous base calls. The second phase sees the filtered contigs cluster into operational taxonomic units (OTUs) with a 97% similarity cut-off (Stackebrandt & Goebel 1994), which are then taxonomically classified using a curated reference database. The statistical analyses, which can be completed in numerous other software, show microbial community similarities or differences, abundances, and correlations with indicated variables.
Figure 1. Schematic workflow of 16S rRNA sequencing.
1.4 Crop Rotation

Crop rotation is the practice of alternating the plants grown in the same area over time, so that the preceding crop is different than its subsequent one. This is common practice in the agricultural industry and has proven beneficial in managing the overall field condition. It helps with weed (Brainard et al. 2008) and disease control (Peters et al. 2003). It is also a factor in microbiome compositions and soil nutrient cycling (Benitez et al. 2017; Havlin et al. 1990). Overall, the benefits of crop rotation lead to improved yield potentials.

1.4.1 Standard Rotations in Manitoba

In Manitoba, a common crop rotation is alternately growing wheat and canola (Kubinec 2012). In 2019, the seeded areas for wheat and canola were very similar to each other. Wheat was seeded on ~3 million acres, and Canola on ~3.2 million acres (Manitoba Agriculture 2019). These two remain as the most popular field crops, but soybean has also gained popularity in the past decade. Soybean is currently the third most planted crop in Manitoba, with ~1.4 million acres seeded in 2019 (Manitoba Agriculture 2019). It has been shown that soybean planted in wheat stubble has a yield of 102% compared to the average yield of all crops in the past five years (Kubinec 2018). Soybean in canola has no effect on yield, while continuous soybean for a second year only gives 93% of average yield. Conversely, wheat and canola planted in soybean stubble gives a 107% and 103% average yields. Therefore, there are definite advantages to rotating crops with soybean.

Other commonly grown crops in Manitoba – such as oat, barley, flax, and sunflower – all saw increases in their average yields when planted in soybean stubble (Kubinec 2018). The only exception that was reported is in field peas, which showed a 90% average yield. This decrease can be attributed to compounding factors, such as similar nutrient requirements and pathogens...
(Gan et al. 2015). On the other hand, when soybeans were planted into these crops’ stubbles there were no negative differences in average yield. The only crop stubble that result in an increase average soybean yield are canola, and as previously mentioned, corn.

Corn for grain is another popular crop in Manitoba. About half a million acres, one of the largest for field crops, were seeded with corn in 2019 (Manitoba Agriculture 2019). Corn preceding soybean gives a 101% average soybean yield, and soybean in corn stubble has a 103% average corn yield (Kubinec 2018). The corn and soybean rotation show beneficial results for both crops in Manitoba. This is also the case in the Midwest US, which is Manitoba’s nearest neighbour and generally has a similar climate. The soybean/corn rotation has been used for years and has allowed farmers to apply less nitrogen fertilizer to their corn crop (Lauer 2010). Recent studies did mention that this rotation can lead to long-term problems with soil organic matter (Hall et al. 2019).

1.5 Objectives

In this research project, the frequency of soybean in rotation with various crops (wheat, canola, and corn) will be observed for its effects on the soil microbiome. Specifically, the project aims to:

1. Quantify the amount of *Bradyrhizobium japonicum* in field soils and its persistence through the year(s).

2. Determine the similarities and differences between soil bacterial populations.
Chapter 2:

Materials and Methods
2.1 Crop Rotation Study

The crop rotation study was conducted at three locations in Manitoba: the Ian N. Morrison research farm near Carman (49.492225, -98.042452), Richardson International’s Kelburn farm near Howden (49.694073, -97.122946), and an on-farm site near Melita (49.246706, -101.016030). The study was a randomized complete block design with four replicates. It contains four rotation treatments (Table 1) that were started in 2014 at all sites. Plot sizes ranged from 6 m to 8 m in width by 8 m in length. Crops were managed with standard best management practices for the region (Manitoba Agriculture). Conventional tillage was used at Carman and Kelburn, and no-till at Melita.

2.1.1 Soybean Crop Year

In the fourth year of the crop rotation study, all the plots were planted with soybean (Table 2) at a seeding density of 47 plants/m² on a 38 cm row spacing. The soybean variety Dekalb 24-10RY was selected since it was adapted to each of the three regions. The seeds were treated with CellTech 1 inoculant (Monsanto BioAg). No fertilizer was applied to any of the plots. Weeds were controlled by best management practices, including the use of herbicides.

2.2 Sample Collection

2.2.1 Soil Samples

Soil samples were collected in 2017 during the soybean crop year. Ten 2x8 cm soil cores from each plot were homogenized to form one sample per plot. This was repeated four times during the season: before planting (BP), at emergence (VE), at beginning seed (R5), and at full maturity (R8). Samples were stored at 4°C, and subsamples were frozen at -80°C, within the
same day of sampling. Soil stored at 4°C were used for plant nodulation assays, while frozen samples were used for DNA extraction and subsequent bacterial assays.

At before planting (BP), each plot was also sampled four times using a Dutch auger from 0 – 15 cm. These were homogenized to form one sample per plot and were sent for soil nutrient analysis to Agvise Laboratories (Northwood, USA).

2.2.2 Plant Samples

Plant samples were collected at beginning seed (R5). Ten plants were harvested from every plot, which were then separated to roots and shoots. The roots were washed and used for nodule counts. The leaves were removed from the shoots and its stems were cut into smaller fragments. The cut-up stems were dried at 65°C for 2 days and weighed. They were then ground to pass through a 1mm screen mesh and used for ureide measurements (Unkovich et al. 2008).

During the R5 stage, plants within two rows of 1 metre each at both the front and back of the plots were also harvested for above-ground biomass. These plants were cut into smaller pieces and dried at 65°C for 2 days before being weighed. Plant samples were sent to Agvise Laboratories (Northwood, USA) for further analyses.
Table 1. Crops in the rotation study based on the year and treatment.

<table>
<thead>
<tr>
<th>Year</th>
<th>Treatment 1</th>
<th>Treatment 2</th>
<th>Treatment 3</th>
<th>Treatment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year 1 (2014)</td>
<td>Soybean</td>
<td>Canola</td>
<td>Corn</td>
<td>Wheat</td>
</tr>
<tr>
<td>Year 2 (2015)</td>
<td>Soybean</td>
<td>Soybean</td>
<td>Soybean</td>
<td>Canola</td>
</tr>
<tr>
<td>Year 3 (2016)</td>
<td>Soybean</td>
<td>Canola</td>
<td>Corn</td>
<td>Corn</td>
</tr>
<tr>
<td>Year 4 (2017)</td>
<td>Soybean</td>
<td>Soybean</td>
<td>Soybean</td>
<td>Soybean</td>
</tr>
</tbody>
</table>
Table 2. Planting and harvest dates of 2017 soybean crops in three Manitoba locations.

<table>
<thead>
<tr>
<th>Location</th>
<th>Planting date</th>
<th>Harvest date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carman</td>
<td>May 23, 2017</td>
<td>October 5, 2017</td>
</tr>
<tr>
<td>Kelburn</td>
<td>May 26, 2017</td>
<td>October 10, 2017</td>
</tr>
<tr>
<td>Melita</td>
<td>June 8, 2017</td>
<td>October 4, 2017</td>
</tr>
</tbody>
</table>
2.3 Nutrient Analyses

The soil samples sent to Agvise were analyzed for nitrate, phosphorus, and pH. Nitrate (NO$_3^-$) was extracted from the soil using 0.2 M KCl and the cadmium reduction method was used for determination (Gelderman & Beegle 2015). Phosphorus (P) was measured using the Olsen method (Olsen et al. 1954) as described by the Frank et al. (2015) protocol. Soil pH was measured using a 1:1 soil to water solution (Watson & Brown 2015).

The plant samples were analyzed for nitrogen and phosphorus uptakes by Agvise Laboratories. Nitrogen uptake was measured using a nitrogen combustion analyzer (Matejovic 1995), and an inductive coupled plasma spectrophotometer (Masson et al. 2010) was used for phosphorus uptake.

2.4 Bacterial Strain and Media

*Bradyrhizobium japonicum* USDA110 (Kaneko et al. 2002) was routinely grown at 28°C in YEM media (Vincent 1970). Yeast extract mannitol (YEM) was composed of 1 g yeast extract, 10 g mannitol, 0.5 g K$_2$HPO$_4$, 0.2 g MgSO$_4$, 0.1 g NaCl per litre of water. Additionally, solidified YEM contained 1.5% agar w/v.

2.5 Plant Nodulation Assay

Soybean seeds were sterilized in 1% sodium hypochlorite for 10 minutes, followed by sterile water rinses (Wacek & Brill 1976). Sterilized seeds were germinated on water agar at room temperature in the dark for 2 – 3 days. Seedlings were transplanted into Leonard jars with a 1:1 mixture of sand and vermiculite, and in sterile germination pouches. Nitrogen-free Jensen’s
media (Jensen 1942) was used to add other essential plant nutrients aside from nitrogen. Ten-fold dilutions (10⁶ to 10¹ cfu) of *B. japonicum* USDA110 (Kaneko *et al.* 2002) were used as plant inoculants, as well as 1/10 dilutions of the Melita BP (10¹ – 10⁴) soil samples from four different rotation treatments. Each dilution was added to plants in triplicate. Sterile water was added to the negative controls in lieu of the bacterial/soil dilution. Plants in the Leonard jars were grown in a 23°C growth chamber for 4 weeks before plant dry weights were measured. Plants in the germination pouches were grown for 3 weeks while nodules were counted.

### 2.6 DNA Extraction

DNA extractions from the frozen soil samples were completed using Qiagen’s DNeasy PowerSoil Kit and protocol (Qiagen, Hilden, Germany). Briefly, 0.20 – 0.25 grams of soil (Figure B1. *Optimization of the soil quantity needed for Qiagen’s DNeasy Powersoil Kit.*) were added to tubes containing beads for mechanical disruption and chemical agents for complete cell lysis. Reagents to precipitate non-DNA material were subsequently added and separated from the supernatant. A high salt solution was added to the supernatant to bind DNA to a silica membrane. An ethanol wash step further cleaned the membrane-bound DNA. Finally, an elution buffer that lacked salt allowed DNA to pass through the silica membrane to be collected and used for downstream applications. DNA samples were measured in a NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, USA) for quantity – absorbance at 260 nm – and quality – 260:280 absorbance ratio.
2.7 Quantitative Polymerase Chain Reaction (qPCR)

The amount of *Bradyrhizobium* spp. and *Bradyrhizobium japonicum* (*B. japonicum*) in the DNA samples were measured using the J16S and the nodZ primer sets respectively (Table 3). The J16S primer set targets the V3 – V4 region of the 16S rDNA (Yudistira 2016). NodZ amplifies a fucosyltransferase gene, which is necessary for soybean – bacteria recognition (Furseth *et al.* 2010). Each reaction mixture contained 12.5 μL of PowerUP SYBR Green Master Mix (Thermo Scientific, Waltham, USA), 4.5 μL ddH$_2$O, 2.5 μL of each primer, and 2 μL of the sample. The reaction mixtures were assayed in 96-well plates using the Bio-Rad CFX Connect Real-Time System (Bio-Rad, Hercules, USA). Initial denaturation occurred at 95°C for 150 s, and then 40 cycles of 95°C for 15 s, 58°C for 120 s, and 72°C for 30 s (Yudistira 2016).

2.7.1 Standard Curves

*Bradyrhizobium japonicum* USDA110 was used to make the standard curves for the quantification of select bacterial DNA from soil samples. The J16S and nodZ primers could recognize *B. japonicum* USDA110, and so both primers were used in the assay to compare their detection capabilities. The data points and line equations (Figure B2) of the J16S and nodZ primers, slopes of -0.2798 and -0.267 respectively, were comparable to each other. Further, both standard curves showed strong linear correlations between the amount of extracted bacterial DNA and the detected cycle threshold level. The correlations were stable until the $10^{-5}$ dilution, which were at \(~10^2\) colony forming units (cfu). The outliers were marked as such since they did not fit into the linear correlation; they designate when the amount of DNA is too low for proper detection by the thermocycler machine.
Table 3. Quantitative PCR primers.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J16S</td>
<td>AGGACCGGTGCAGAGAT</td>
</tr>
<tr>
<td></td>
<td>CCATTGTAGCAGTGAGTGC</td>
</tr>
<tr>
<td>nodZ</td>
<td>GGTTTGGCGACTGTCTGTC</td>
</tr>
<tr>
<td></td>
<td>TTCCACCATGTTGAAAGAATGGTCC</td>
</tr>
</tbody>
</table>
2.8  **16S rRNA Sequencing**

A volume of 20 μL of the DNA samples were sent to Metagenom Bio Inc. (Toronto, Canada) for 16S rRNA paired-end sequencing using an Illumina MiSeq. The 515FB–806RB primer set and procedures (Parada *et al.* 2016, Apprill *et al.* 2015, Caporaso *et al.* 2012) recommended by the Earth Microbiome Project was used to target the V4 region of the 16S rRNA.

As outlined by Cheng (2019) from Metagenom Bio Inc., the PCR was set in triplicates for 25 μL samples. Each reaction mixture contained 2.5 μL of 10 x standard Taq buffer, 0.5 μL of 10 mM dNTP, 0.2 μL of BSA (20 mg/ml), 5.0 μL of 1 μM forward primer, 5.0 μL of 1 μM reverse primer, 2.0 μL DNA, 0.2 μL of Taq DNA polymerase (5 units/μL) and 9.6 μL of PCR water. DNA was denatured at 95°C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 30°C for 30 sec, and 72°C for 50 sec, and then extended at 72°C for 10 min. Triplicate PCR products were pooled and resolved using a 2% TAE agarose gel. PCR products in equal amount of correct amplicons were pooled, gel purified and quantified using Qubit dsDNA HS Assay Kit. Indexed library DNA was sequenced with MiSeq Reagent Kit v2 (2 x 250 cycles). FASTQ files were generated for taxonomic analysis.

16S rRNA amplicon data (FASTQ files) was analysed using the mothur software package (v.1.40.5; Schloss *et al.* 2009), and a version of the MiSeq SOP (Kozich *et al.* 2013). Paired-end reads were assembled into contigs, which were then filtered to remove contigs that were larger than 300 bp, contained any ambiguous base calls, or had homopolymers > 8. Contigs were aligned and filtered against a V4-specific SILVA 132 database (http://www.arb-silva.de). Contigs that differed ≤ 2 bp were clustered together. Chimeras were identified using VSEARCH (Rognes *et al.* 2016) and subsequently removed. The RDP reference database (v.16; Cole *et al.*
2014) provided classification to the sequences, with 80% confidence score. Those identified as chloroplast, mitochondria, archaea, eukaryota, or unknown were removed from the data. Output files containing relevant data were used for downstream analyses.

2.8.1 Statistical Analyses

Data files from mothur, as well as a metadata file, were uploaded into Calypso (Zakrzewski et al. 2017) for statistical analyses and visualization. Samples with less than 1000 sequence reads were removed, and taxa that have less than 0.01 percent relative abundance across all samples were excluded. Data was normalized using cumulative sum scaling (CSS) and log2 transformed.

Alpha diversity was measured using the Shannon and Simpson diversity indices (Shannon 1948; Simpson 1949). Analysis of variance (ANOVA) was used to determine statistical significance, with the cut-off set at \( p < 0.05 \). Beta diversity was visualized via the principal coordinate analysis (PCoA) using the Bray–Curtis index (Bray & Curtis 1975). Correlations between taxa were based on Spearman correlation coefficients. Soil microbiome data was compared within and between sites.

2.9 Overwinter Soil Assay

Previously collected soil samples were subsampled into 15 mL falcon tubes with holes for water and nutrient exchange. Subsampled soils were re-inserted into the Carman site after harvest and left until the following spring, when they were re-isolated and re-sequenced. Soil used in the assay was from the first block, constituting of all four treatments, in Melita at the BP timepoint.
Chapter 3:

Results
3.1 Persistence of *Bradyrhizobium japonicum* in Manitoban Soils

3.1.1 Gradual Decline of *Bradyrhizobium japonicum* over the Years

The J16S and nodZ primers were used on soil samples collected before planting (BP) from three different Manitoba locations. At this sampling point, May 2017, the study was started three years ago. Treatment 1, which had continuous soybean, last had the *B. japonicum* inoculant one year ago (May 2016). Treatments 2 and 3, which have soybean every other year, were inoculated two years ago (May 2015). Treatment 4 has not had soybeans yet and so did not have inoculant added in at least 3 years. By sampling these plots before planting (BP), we were able to monitor the *B. japonicum* inoculant’s ability to stay in the soil over the course of multiple years.

In all three locations, there were obvious differences between the bacterial populations detected by the J16S and the nodZ primers (Figure 2). The J16S primers always quantified more *B. japonicum* than the nodZ primer pair. This suggests that the J16S primers may be amplifying *Bradyrhizobium* bacteria that do not contain the nodZ gene. Further, the amount of J16S-detected bacteria stayed consistent over the years. In comparison, the amount of nodZ-containing bacteria decreased as the years since inoculation increased.

Although the general trends were similar to each other, the amount of bacteria and the rates of decline were different for each location. In Carman (Figure 2a) the J16S population was at $10^7$ cfu/g soil, while Kelburn and Melita (Figure 2b and Figure 2c) have ten times less at $10^6$ cfu/g soil. As for the amount of nodZ-detected bacteria, the three locations had similar numbers from the plots that were inoculated one year ago. After one year though, the locations differed on how quickly the bacterial population declined. Kelburn (Figure 2b) had less of a decrease than the other two, which ended at a still substantial amount of $10^4$ cfu/g soil.
Figure 2. Quantification of *Bradyrhizobium* (J16S) and *B. japonicum* (nodZ) over the years. Each data point represents treatment quadruplicates from before planting (BP) soil samples. Errors bars denote the calculated standard deviations. Manitoba location: A) Carman, B) Kelburn, C) Melita.
3.1.2 Overall Increase of *Bradyrhizobium japonicum* in a Growing Season

The amount of *B. japonicum* over the growing season was also monitored using both the J16S and nodZ primers. The bacterial quantities that were detected by J16S did not change significantly over the season (data not shown) in any of the three locations. Also, Carman had more bacteria per gram of soil than Kelburn and Melita. These were similar to the trends seen in Figure 3, which looked at *Bradyrhizobium* changes over the years. As for the nodZ-containing bacteria, the overall trend showed that the amount of *B. japonicum* in all three locations increased from before planting (BP) to full maturity (R8). However, there were noticeable differences between both the timepoints and the locations (Figure 3).

In Carman, the initial population range of *B. japonicum* is from $10^3$ to $10^6$ cfu/g soil (Figure 3a). The BP timepoint showed that the longer it had been since the plots were inoculated, the lower the amount of *B. japonicum* detected. The trend remained the same throughout the growing season: continuous soybean had the highest population, followed by the corn – soybean, then the canola – soybean, and finally the diversified treatment had the least. The rapid increase of *B. japonicum* between emergence (VE) and pod fill (R5) in the diversified treatment accounted for the more similar population counts at R8 between the treatments. The slight decline of *B. japonicum* at R8 was also to be noted in most treatments, except for continuous soybean.

In Kelburn, the initial populations at BP were grouped close together (Figure 3b). The similarity in *B. japonicum* populations between all treatments proved to be consistent throughout the season. It is especially apparent at R8 when all treatments had $10^6$ cfu/g soil of *B. japonicum* detected in them. The only exception to this high degree of similarity was in the diversified
treatment at VE, where it had one order of magnitude less *B. japonicum* than the other treatments.

The sites at Melita had the widest range of initial populations (Figure 3c). The diversified treatment, which had never been inoculated with *B. japonicum* previously, started at $10^1$ cfu/g soil. The initial population in the continuous soybean treatment was $10^6$ cfu/g, which was the same as in other locations. The two treatments with soybean every second year clustered close to each other, with the soybean–corn treatment having a slight advantage due to a shaper population increase between VE and R5. From R5 to R8, there were population declines again for most treatments, except for diversified which stayed the same. This discrepancy led to *B. japonicum* populations to once again be more similar at R8 than at BP.

### 3.1.3 Overwintering of *Bradyrhizobium*

There were overall decreases in the bacterial populations after the winter months (Figure 4). However, in most cases the decrease was negligible. One of the exceptions was in the continuous soybean treatment, which had a significant decrease in *Bradyrhizobium*, as detected by J16S primers, and in *B. japonicum* populations, as detected by the nodZ primers, from C17 R8 to C18 BP. In the diversified treatment, the amount of *Bradyrhizobium* detected by J16S was also significantly lower in C18 BP after winter. In general, diversified had lower bacterial quantities than the other treatments. Although, at $10^5$ to $10^6$ cfu/g soil, all treatments still had substantial amounts of *Bradyrhizobium* even after the winter months.
Figure 3. *Bradyrhizobium japonicum* populations over the soybean growing season. The *nodZ* primers were used. Four plots per treatment were measured at each timepoint. Manitoba location: A) Carman, B) Kelburn, C) Melita.
Figure 4. Average *Bradyrhizobium* populations after a winter season. Samples were collected from the same plots at September 2017 (R8) and May 2018 (BP). Four plots per treatment were measured. Errors bars denote the calculated standard deviations. Asterisks denote significant changes, as measured by the student’s t-test ($p \leq 0.05$).
3.1.4 Biennial *Bradyrhizobium japonicum* Inoculation Sufficient for Nodulation

Soil from the four different treatments were diluted in water and used as inoculant for soybean plants to be grown in a growth chamber. The plants were grown for four weeks under Nitrogen limiting conditions and subsequently harvested. The harvested shoots were dried, and their dry weights recorded (Figure 5). The shoot dry weight of the negative control had an average of 0.3036 grams. The plants inoculated with soil from the continuous soybean plot had an average of 0.8215 g. Plants inoculated with soil from soybean – canola, soybean – corn, and diversified treatments had averages of 0.4379 g, 0.4772 g, and 0.4172 g respectively. The plants that were inoculated with soil taken from the continuous soybean treatment showed a significant difference in dry weight when compared with the control and the other treatments. There were no significant differences between the control and the other treatments.

Concurrent with the soybean plants grown in pots for dry weight measurements, additional soybeans were grown in clear germination pouches to monitor nodulation. Between the second week and third week, when nodules were counted, there were no significant differences (data not shown). Therefore, final nodule counts at three weeks were used in Figure 6 to demonstrate the differences between treatments. Plants that were inoculated with diluted soil extract from the continuous soybean treatment were found to have significantly higher nodule counts than all other treatments. The two treatments with soybean every other year had similar numbers of nodules (Figure 7). The diversified treatment, which had never been inoculated with *B. japonicum*, did not form nodules and resembled the negative control.
Figure 5. Average dry weight of soybean (Glycine max) with various soil inoculants. Plants were grown for four weeks in triplicate. Errors bars denote the calculated standard deviations. Asterisks denote significant changes, as measured by the student’s t-test (p ≤ 0.05).
Figure 6. Nodulation of soybean plants using different soil inoculants. Nodules were counted three weeks after germination. Plants were grown in triplicates. Error bars denote the calculated standard deviations. Letters denote significant changes, as measured by the student’s t-test (p ≤ 0.05).
3.2 Effects of Crop Rotation on Soil Microbiomes

3.2.1 Microbial Diversity in Manitoba Soils

Sequencing of amplicons derived from the samples of all three sites yielded a total of 8,294,238 assembled raw contigs. After filtering out low quality and chimeric sequences, there were 2,137,591 high quality sequences with an average length of 292 base pairs. These sequences were clustered into 12,923 OTUs based on 97% similarity. The average number of sequences per sample was 29,215 (range: 13,465 – 64,033). All samples were sequenced to a sufficient depth (Figure 7) and can be used for further analysis.

3.2.1.1 Diversity Indices

Alpha diversity

Diversity measures the number of taxa present in an environment, as well as their abundance. In this study, the diversity within sites was measured using the Shannon index. Figure 8 shows that there are significant differences between sites in terms of microbial diversity. Carman has the highest diversity, while Melita is the least diverse. Richness measurements were also plotted to show just the number of taxa present within the sites (Figure 9). Based on the measurements, Kelburn has significantly more taxa present in its soil than the other two sites. Chao1 places more importance on rare taxa (Figure 9b), while ACE focuses on abundant taxa (Figure 9c). There is a wider gap between Kelburn and the other two sites in the ACE measurements, which means that the difference in richness is largely due to abundant taxa in the soil.
Figure 7. Rarefaction curves of samples from A) Carman; B) Kelburn; C) Melita. OTU counts of all sampling timepoints (BP – R8) were used in the rarefaction analysis.
Figure 8. Comparison of site microbial diversity using the Shannon index. T-test with a cut-off at $p \leq 0.05$ was used for significance.
Figure 9. Richness measurements of the three sites using B) Chao1 and C) ACE metrics.
**Beta diversity**

The experimental data form three clusters in the PCoA based on the different sites (Figure 10). This suggests that all sites have their own distinct microbial community. The Carman cluster is further away from the other two sites suggesting that the microbial community in that site is more dissimilar. The Kelburn and Melita clusters are close to each other, but do not overlap. This suggests that the microbial communities in these two sites are similar to each other.

Further analysis using spring soil measurements showed that the pH is a key factor to the observed differences (Figure 11a). The soil pH in Carman is acidic at roughly pH 5, Kelburn has a neutral pH of 7, and Melita is slightly basic at pH 8. The pH values clustered very strongly into the different sites. Other measurements made at BP include soil nitrate (Figure 11b) and phosphorus (Figure 11c) content. These measurements have too much variation within each site to provide insight in the relationships between site clusters. However, on average, there is significantly less soil nitrate and phosphorus in Melita than in Carman or Kelburn. When agronomic measurements were done at the R5 sampling stage and mapped into the corresponding microbial communities (Figure 12), none of them showed the strong correlation that soil pH had with the different sites. Although, the overall trend is the same: Carman has the highest amount of biomass (Figure 12a), nodule counts (Figure 12b), nitrogen uptake (Figure 12c), and phosphorus uptake (Figure 12d). Carman has significantly higher biomass and nitrogen uptake values than both Kelburn and Melita. Kelburn has significantly less phosphorus uptake than Carman, and Melita has significantly less nodules than Carman. Lastly, yield data was collected at the R8 soybean stage (Figure 13). The correlated yield data shows the same pattern as the other previous field measurements where Carman has a significantly larger value than the other two sites. There are no distinct differences between Kelburn and Melita.
Figure 10. Principal coordinate analysis (PCoA) of all data points. Analysis was calculated using Bray-Curtis distances. Colours/symbols represent sites.
Figure 11. PCoA of BP samples in all sites overlaid with soil properties. Analysis was calculated using Bray-Curtis distances. Colours represent A) Soil pH measurements; B) Soil nitrate content (lb/ac); C) Olsen P measurements (ppm).
Figure 12. PCoA of R5 samples in all sites overlaid with plant measurements. Analysis was calculated using Bray-Curtis distances. Colours represent A) Biomass (kg/ha); B) Average nodules; C) Nitrogen uptake (kg/ha); D) Phosphorus uptake (kg/ha).
Figure 13. PCoA of R8 samples in all sites. Analysis was calculated using Bray-Curtis distances. Colours represent yield data, which was measured in kilogram per hectare.
3.2.1.2 Hierarchical Taxonomy

At the phyla level, all three sites have similar compositions (Figure 14, Figure 15, Figure 16). The majority of the microbial community is composed of three phyla: Proteobacteria, Actinobacteria, and Acidobacteria. Around 30% of the sequenced bacteria is from the phylum Proteobacteria. Within Proteobacteria, the α-proteobacteria represent 46% of it in Carman, 36% in Kelburn, and 34% in Melita. The larger presence of α-proteobacteria in Carman is to the detriment of δ-proteobacteria, which is only 10% of the Proteobacteria population. In Kelburn and Melita, the δ-proteobacteria makes up 21% of all Proteobacteria. The Rhizobiales family, which contains nitrogen-fixing bacteria, is part of the α-proteobacteria class. In Carman, the Rhizobiales population is about 10% less than the ones in Kelburn and Melita. Instead, there are more Rhodospirillales in Carman. Further, there is almost no Rubrobacterales in Carman even though it is present in the other two sites. The bacterial family of interest, Bradyrhizobiaceae, can be found at around 14% of Rhizobiales in Carman, 6% in Kelburn, and 4% in Melita. The difference between Bradyrhizobiaceae populations is balanced by Methylobacteriaceae, which consists of 2% of Rhizobiales in Carman, 7% in Kelburn, and 10% in Melita. The significance of the variations in Bradyrhizobiaceae populations within the three sites were also investigated (Figure 17). Carman had the highest abundance of Bradyrhizobiaceae, followed by Kelburn, and finally Melita had the least amount. The abundance differences between the three sites were all significant.

Similarly, Actinobacteria is roughly the same in all three sites: 13% in Carman, 18% in Kelburn, and 15% in Melita. One of the few differences is that there is about 10% more Actinomycetales in Carman (33% of Actinobacteria) than the other two sites (23% of Actinobacteria). Also, there is almost no Rubrobacterales in Carman.
Acidobacteria is also very similar at 14% in Carman, 13% in Kelburn, and 11% of the bacterial population in Melita. Within the Acidobacteria phyla, Gp1 is prevalent in Carman at 41%. In Kelburn and Melita, the prevalent class is Gp6 at 39% and 44% respectively. The second most numerous class is Gp3 (23%) for Carman, and Gp 4 (13% and 9%) for Kelburn and Melita. Gp 6 in Carman is the third most populous Acidobacteria at 6%. In Kelburn, it is Gp 3 at 7%. In Melita it is Gp 16 at 6%, and followed by Gp 3 at 5%. The class Gp 1 is almost non-existent in Kelburn and Melita, even though it is prevalent in Carman, as it only makes up for 1% and 0.4% of the Acidobacteria populations respectively.

A Venn diagram of all the bacterial taxa present in each site was generated to identify a core microbiome (Figure 18). The core microbiome of the three Manitoba sites consists of 393 taxa. Kelburn’s significant species richness can be seen with the further 32 other taxa it shares with one other site. Melita has 3 taxa more than Carman, which follows the trend shown by Figure 2. Furthermore, this Venn diagram shows us that there are 13 taxa that are exclusively found in Carman. These 13 taxa fit within 4 genera: Rhodanobacter, Ktedonobacter, Granulicella, and candidate_division_WPS2.
Figure 14. Hierarchical taxonomy of Carman using a Krona plot.
Figure 15. Hierarchical taxonomy of Kelburn using a Krona plot.
Figure 16. Hierarchical taxonomy of Melita using a Krona plot.
Figure 17. ANOVA plot of *Bradyrhizobiaceae* spp. in Manitoba soils. Analyzed using a t-test with a significance cut-off at $p \leq 0.05$. 
Figure 18. Venn diagram depicting the core microbiome (OTU level) between sites.
3.2.2 Changes in Microbial Communities Based on Rotational Treatments

The diversity of the microbial communities was measured using the Shannon index, and compared between treatments in each site (Figure 19). In Carman, the continuous soybean treatment is significantly more diverse than the other treatments (Figure 19a). In Kelburn, the diversities between treatments are very similar to each other (Figure 19b). In Melita, there are no significant differences between treatments (Figure 19c). However, the corn – soybean treatment is the most diverse, and continuous soybean has the least diversity. This is unlike the trend shown in Carman.

Samples from the BP timepoint of the 2017 growing season were also plotted and measured with the Shannon index (Figure 20). BP would be the closest timepoint from when various crops were planted in the experimental plots. These plots would also have had different inoculation times. Treatment 1 with continuous soybean would have had an inoculant added to it a year ago. Treatments 2 and 3 would have seen it two years ago. Treatment 4 would not have seen it for at least three years. Therefore, the effects of the previous crop and inoculation would be seen in the BP samples before any further disturbances are done to it in the other timepoints. Based on the BP samples, there are no differences between treatments in terms of diversity. In Carman, the continuous soybean treatment seems to be more diverse than the other treatments (Figure 20a). In Melita, the opposite is true, where the continuous soybean treatment has the least diversity (Figure 20c). None of these changes are significantly different from each other though, so it does not seem like the previous crop has a strong influence on the microbial population. However, the diversity trends shown in Figure 20 do highlight the difference between sites. As for the rest of the timepoints, there are mostly no significant changes between treatments in all sites (Figure 20).
Figure 19. Microbial diversity between treatments based on the Shannon index. Manitoba sites: A) Carman; B) Kelburn; C) Melita.
Figure 20. Microbial diversity at BP between treatments based on the Shannon index. Manitoba sites: A) Carman; B) Kelburn; C) Melita.
Figure 21. Microbial diversity at Carman, Kelburn, and Melita using the Shannon index.

Sampling timepoints: A) VE; B) R5; C) R8.

Carman
Kelburn
Melita

Shannon Index

A
B
C

OTU p=0.564 (anova)
OTU p=0.83 (anova)
OTU p=0.238 (anova)
OTU p=0.598 (anova)
OTU p=0.476 (anova)
OTU p=0.206 (anova)
The presence of *Bradyrhizobiaceae* at different treatments was also examined (Figure 22). In all three sites, the amount of *Bradyrhizobiaceae* is consistent and there were no significant differences between the treatments. If the *Bradyrhizobiaceae* genus is plotted at only BP, then there seems to be more variations (Figure 23). However, the scale indicates that the appearance of the figures is only due to small, insignificant abundance changes between treatments. This is consistent with the J16S qPCR data, which was mentioned in the previous chapter. In both figures, the amount of *Bradyrhizobiaceae* in Carman is more numerous than in Kelburn and Melita. Therefore, in general, there are more bacteria present in Carman. The 16S sequencing is not sensitive enough to differentiate between species, and so a comparison of *B. japonicum* would not be possible. Taking into consideration that Melita was a virgin field prior to this study, and there has yet to be inoculant added to it in the BP samples, there are native *Bradyrhizobium* in its soil and likely in the other sites as well.

Although there are no overall differences in microbial diversity between treatments, some treatments do contain unique bacteria. In Carman, there are 146 bacterial genera in the core microbiome shared by all treatments (Figure 24a). However, *Pedomicrobium* and *Lysobacter* are only found in the continuous soybean treatment. In Kelburn, there are 159 genera shared between treatments (Figure 24b). The only treatment with a unique bacterium, *Rhodanobacter*, is the diversified treatment. Further, *Mucilaginibacter* and *Jatrophihabitans* are found in all treatments, except in the Canola – Soybean rotation. In Melita, the diversified treatment contains two unique bacteria: *Rhodanobacter* and *Rhizomicrobium_unclassified* (Figure 24c). *Mucilaginibacter* spp. was only found in the Corn – Soybean and Diversified treatments in Melita. However, Gp1_unclassified is missing from the Corn – Soybean treatment, and *Jatrophihabitans* is not found in Melita at all.
Figure 22. Presence of *Bradyrhizobiaceae* spp. between various treatments. Manitoba sites: A) Carman; B) Kelburn; C) Melita.
Figure 23. Presence of *Bradyrhizobiaceae* spp. between various treatments at BP. Manitoba sites: A) Carman; B) Kelburn; C) Melita.
Figure 24. Core microbiome between treatments in A) Carman; B) Kelburn; C) Melita.
3.2.3 Microbial Changes over the Growing Season

The microbial community through the growing season was observed using a principal coordinate analysis with the different sampling timepoints highlighted (Figure 25). In Carman (Figure 25a), the before planting (BP) and the full maturity (R8) samples are on opposite sides of the graph and are different from each other. Emergence (VE) samples are more scattered and overlap with the other samples, especially the R5 samples. Beginning seed (R5) is in a cluster between BP and R8. Aside from VE, the other sampling timepoints form their own clusters that are distinct from the others. This implies that there are differences in the microbial communities between sampling timepoints. The wider variations within VE can be due to soil disturbances, like tilling, that occurred around this time.

In Kelburn (Figure 25b), the BP and R8 samples still form distinct clusters but they are not as far apart as the ones in Carman. The R5 samples are still in between BP and R8 although they overlap more with the samples from those two timepoints. The VE samples are the ones that are more obviously separate from the rest of the timepoints. VE samples are also more tightly clustered than the scatter in Carman.

In Melita (Figure 25c), the samples are all tightly clustered. There does not seem to be any difference between BP, R8, and R5. The VE samples are more scattered again, and separate from the other timepoints. There seems to be disturbances at VE that changes the microbial communities. Melita was a no-till site, and so the variation seen at VE must be due to other causes or disturbances other than tilling.
Figure 25. PCoA of all data points in A) Carman; B) Kelburn; C) Melita. Analysis was calculated using Bray-Curtis distances. Colour/symbols represent timepoints.
To look at how soybean may influence the microbial community, BP samples were compared to R8 samples. In Carman, there were less bacteria in all the treatments at R8 than there were at BP (Figure 26). The core microbiome at BP consisted of 147 bacterial genera, while R8 only had 142. However, the overall distribution showed that R8 had more bacteria shared between various treatments. Unclassified Thermomicrobia could only be found in the continuous soybean treatment at BP but was detected in all treatments at R8. *Anaeromyxobacter* also showed up in all treatments at R8, even though it was only in the continuous soybean and corn – soybean treatments at BP. Conversely, *Adhaeribacter* was in all treatments at BP but could not be detected at R8. The soybean plants might have released chemicals that encouraged the growth of Thermomicrobia and *Anaeromyxobacter*, and suppressed *Adhaeribacter*. Other noteworthy bacteria include *Ilumatobacter*, *Luteolibacter*, and Nannocystaceae_unclassified. These bacteria were not seen at BP but appeared in the continuous soybean treatment at R8. The presence of these bacteria might be due to repetitive soybean planting and should be monitored.

In Kelburn, the bacteria shared between all treatments increased from 154 to 157 genera (Figure 27). Unclassified Ruminococcaceae was found in the continuous soybean and corn – soybean treatments at BP but was in all treatments at R8. Unclassified Sphingobacteriaceae was detected in all treatments at R8 despite being only in the continuous soybean treatment at BP.

In Melita, there was one more bacterium shared between all treatments at R8 compared to BP, which was at 152 genera (Figure 28). The rest were redistributed and were shared between more treatments. Unclassified candidatus_Koribacter was in most treatments at R8, except corn – soybean, even though it was not present in any treatment at BP. *Burkholderia*, on the other hand, was not found at R8 despite being in all the treatments at BP. Therefore, soybeans do seem to influence bacterial growth within the soil.
Figure 26. Core microbial genera between treatments in Carman. Sampling timepoints: A) Before planting (BP); B) Full maturity (R8).
Figure 27. Core microbial genera between treatments in Kelburn. Sampling timepoints: A) Before planting (BP); B) Full maturity (R8).
Figure 28. Core microbial genera between treatments in Melita. Sampling timepoints: A) Before planting (BP); B) Full maturity (R8).
As the genus of interest, the presence of *Bradyrhizobiaceae* over the season was also investigated. In Carman, there were differences between timepoints in most treatments. The continuous soybean treatment was the exception (Figure 29a). The canola – soybean treatment showed an increase in *Bradyrhizobiaceae* from the BP to the R8 timepoint (Figure 29b). In the corn – soybean treatment there was a significant increase from VE to R8 (Figure 29c). In the diversified treatment, there were increases between BP and R8, as well as between BP and R5 (Figure 29d).

In Kelburn, all treatments had significantly more *Bradyrhizobiaceae* at R8 than at BP. In the continuous soybean treatment, the R8 samples had significantly higher abundances than all other timepoints (Figure 30a). In the canola – soybean and corn – soybean treatments, the BP and VE timepoints were different than R8 (Figure 30b & Figure 30c). There was also a significant increase from BP to R5 in the canola – soybean treatment. The diversified treatment only showed an increase from BP to R8 (Figure 30d).

In Melita, *Bradyrhizobiaceae* trends between timepoints differed for each treatment. In the continuous soybean and diversified treatments, no significant abundance changes were observed (Figure 31a & Figure 31d). In the canola – soybean treatment, there were significant increases from BP and VE to R5 and R8 timepoints (Figure 31b). In the corn – soybean treatment, R8 samples had more *Bradyrhizobiaceae* than BP and VE (Figure 31c).

In the Manitoba sites sampled, all treatments showed increases in the *Bradyrhizobiaceae* population from BP to R8, even if some of them were not significantly different. In the continuous soybean treatments, the difference between BP and R8 might not have been apparent due to the large amount of *Bradyrhizobiaceae* already in the soil from previous inoculations.
Figure 29. T-test with *Bradyrhizobiaceae* genus in Carman. T-test with a cut-off at $p \leq 0.05$ was used for significance. Treatments: A) Continuous Soybean; B) Canola – Soybean; C) Corn – Soybean; D) Diversified.
Figure 30. T-test with *Bradyrhizobiaceae* genus in Kelburn. T-test with a cut-off at $p \leq 0.05$ was used for significance. Treatments: A) Continuous Soybean; B) Canola – Soybean; C) Corn – Soybean; D) Diversified.
Figure 31. T-test with *Bradyrhizobiaceae* genus in Melita.

T-test with a cut-off at $p \leq 0.05$ was used for significance. Treatments: A) Continuous Soybean; B) Canola – Soybean; C) Corn – Soybean; D) Diversified.
3.2.3.1 Microbial Changes over the Winter

Samples from each treatment (M101 – M104) were put back into the ground at 2017’s harvest time and were re-extracted at spring 2018. Along with the treatments, a negative control and two positive controls were buried over the winter. The two positive controls contained varying concentrations of *Bradyrhizobium japonicum* USDA110. Both the negative and positive controls showed similar microbial communities by clustering together in the PCoA (Figure 32). The positive control with the lower bacterial concentration, $10^3$, clustered closer with the negative control. The effect of the site’s soil microbiome was stronger on samples with little to no bacteria in them. The treatment samples clustered tightly with each other, and away from all the overwintered data points. The overwintered treatments all clustered together, as well. There were microbial changes in the overwintered treatment samples as they were separate from the original treatment samples. The changes in the overwintered treatment samples would also be different from the changes in the controls, as they were clustered away from each other. Therefore, winter did affect the microbial communities of all the samples but in varied ways.

As for the presence of *Bradyrhizobiaceae*, there were increases in its abundance through the winter (Figure 33). The overwintered M102 (continuous soybean) and M104 (corn–soybean) treatments had significantly more *Bradyrhizobiaceae* than its counterparts. However, since the treatment samples came from Melita and were re-buried in Carman, the increase in *Bradyrhizobiaceae* could also be due to site differences. Nonetheless, the *Bradyrhizobiaceae* abundances were more similar between treatments after the winter rather than prior to it.
Figure 32. PCoA of overwinter samples. Analysis was calculated using Bray-Curtis distances. Colours/symbols represent sample names.
Figure 33. ANOVA plot of *Bradyrhizobiaceae* abundance in overwinter samples. Analyzed using a t-test with a significance cut-off at $p \leq 0.05$. 
Chapter 4:

Discussion
4.1 Persistence of *Bradyrhizobium japonicum* in Manitoban Soils

The two primer sets, J16S and nodZ, were not measuring the same bacteria as highlighted by the discrepancies in the DNA abundance detected by the qPCR assays. Although J16S was previously thought to amplify *B. japonicum* (Yudistira 2016), its consistently large numbers suggested that J16S measured the genus *Bradyrhizobium* while nodZ measured *B. japonicum* specifically (Furseth *et al.* 2010). The presence of non-symbiotic *Bradyrhizobium* in Manitoba has been previously suggested by Yudistira (2016) when no nodules were observed on soybean grown in uninoculated soil, even though the J16S primers detected the presence *Bradyrhizobium*.

4.1.1 Gradual Decline of *Bradyrhizobium japonicum* over the Years

Previous work showed that *B. japonicum*, strains 61A148 and 61A196, did not overwinter in Canadian prairie soils (Bailey 1989). Based on this paper and other observations, the Manitoba Pulse & Soybean Growers (MPSG) suggested that inoculation was necessary for soybean fields every year. Single inoculation for fields with soybean history, and double inoculation for those with little to no history of soybean (MPSG 2017). However, there was a widespread study in the United States, including Minnesota, which is one of Manitoba’s neighbours, that showed no benefit to inoculation in fields with soybean history (De Bruin *et al.* 2010). The result of this study was consistent with what was observed in this rotation study, where *B. japonicum* was still detected in plots that have not seen a soybean crop in years. The amount of *B. japonicum* observed in the soil was substantial even after winter, which showed that the population decline was gradual and that yearly inoculation may not be necessary to attain sufficient soybean nodulation.

Overall, Carman had more bacteria present in its soil than Kelburn and Melita. There’s no definitive reason for this discrepancy, but it had been shown that soil type can affect the
microbiome (Schreiter et al. 2014). Carman and Kelburn had similar field histories, but their soils were quite different. According to Manitoba Agriculture (2014), Carman had the soil type called Loamy Lacrustine (DNH) and Kelburn was a Clayey Lacrustine (SCY). The heavier clay texture of Kelburn could have been more difficult to process and breakdown thus resulting in less isolated bacteria. As for Melita, its soil was classified as Loamy Till (Nd) which was similar in texture with Carman, however herbicide was added to it to correct a seeding error. Herbicides target weeds primarily but has also been shown to affect non-target soil microorganisms (Thiour-Mauprivez et al. 2019), which could have caused the smaller detected bacterial population.

The *B. japonicum* rates of decline were quite varied between sites. Carman’s decline had a slope of -2.119. In comparison, Kelburn had a more gentle slope at -0.892, and Melita had a steeper one at -3.559. The clay texture of Kelburn’s soil could have made it more difficult for bacteria to be detected due to difficulty in processing and breaking it apart, which could explain Kelburn’s more consistent bacterial readings through the years. The sharp decrease in Melita could be due to its history of no previous *B. japonicum* inoculation.

### 4.1.2 Overall Increase of *Bradyrhizobium japonicum* in a Growing Season

In Carman, all treatments had similar *B. japonicum* abundance at the pod fill timepoint (R5). The diversified treatment showed a rapid increase in order to have similar values as the other three treatments. Previous work determined that the period between soybean growth stages R3 and R5 had the highest symbiotic nitrogen fixation (Zapata et al. 1987), and so the increased abundance of *B. japonicum* was likely due to the soybean plants’ higher nitrogen requirements. The decrease in *B. japonicum* at full maturity (R8) could be due to harvesting. Sugiyama et al. (2015) suggested that soybeans select for specific *Bradyrhizobium* species. The lack of plant exudates at R8 could mean that *B. japonicum* was not being enriched. As for the continuous
soybean treatment, the lack of a decrease in *B. japonicum* could be due to its more stable population which was built up over the years.

In Kelburn, the treatments were all similar to each other throughout the season. As suggested before, Kelburn’s soil composition could be making bacteria difficult to detect thus the closeness of all data points. Detection can be impaired by the large aggregates of soil particles as suggested by Sessitsch *et al.* (2001).

Melita was a unique site, since *B. japonicum* inoculants were never added to it prior to the start of this study. The absence of *B. japonicum* was apparent in the almost imperceptible number that nodZ detected. The slight amount detected could be due to a sampling cross-contamination, or the presence of a nodZ–containing native *Bradyrhizobium*. As for the lack of bacterial decline at R8 of the diversified treatment, it could indicate a threshold population that can be supported by the soil.

### 4.1.3 Overwintering of *Bradyrhizobium*

The overwintering experiment suggests that winter reverts the soil microbiome to a basal population. The results showed similar bacterial populations in all treatments after the winter months despite some previous significant differences. Around $10^6$ cfu per gram of soil was the observed bacterial amount that seemed capable of surviving prairie winters, which fell within other calculated bacterial concentrations in soil (Carson & Zeglin 2018).

### 4.1.4 Biennial *Bradyrhizobium japonicum* Inoculation Sufficient for Nodulation

Shoot dry weights showed that the continuous soybean treatment had enough *B. japonicum* to make a significant difference in the plants’ growth. The plant assays were completed with inoculants made from BP soil samples, which meant that the continuous soybean
treatment last had inoculant one year ago and yet it still had a sufficient *B. japonicum* concentration. Recent on-farm trials, in Manitoba fields with soybean histories, supported this study’s finding about the redundancy of *B. japonicum* inoculation every year (MPSG 2019). On-farm trials showed no yield or nodulation differences between fields with single inoculation vs no inoculation. As for nodulation in this study, there were nodules found in all treatments when soybeans were planted in soil. However, no nodules were seen at the diversified treatment when plants were grown in germination pouches. The lack of soil in the germination pouches meant that bacteria were more easily washed away from the plant roots. Therefore, a sufficient *B. japonicum* population cannot be established near the roots in the diversified treatment, which could be the reason for the observed lack of nodulation.

### 4.2 Effects of Crop Rotation on Soil Microbiomes

#### 4.2.1 Microbial Diversity in Manitoba Soils

The microbial diversity, as measured by the Shannon index (Shannon 1948), of all sites were similar to previously reported values in agricultural soils (Hill *et al.* 2003; Trivedi *et al.* 2016). Many factors affect microbial diversity, including the soil temperature and water content. However, these factors are not measured in this study, and so comparisons can only be made between microbial diversity and the reported soil pH. Carman, which had acidic soil (pH = 5), was significantly more diverse than the other sites. This contradicted some studies that showed neutral soils had the highest diversity and acidic soils had the least (Fierer & Jackson 2006). However, the study conducted by Fierer & Jackson focused on comparing grasslands and forests soils. When agricultural soils were used for comparison, the effect of pH varied between climates (Trivedi *et al.* 2016). In continental agricultural fields, which would be Manitoba’s classification,
there were lower microbial diversity in the neutral agricultural fields compared to the acidic natural soils. And so, Carman’s acidic soil might be a reason for its higher microbial diversity.

The majority of the soil microbiome were within three phyla: Proteobacteria, Actinobacteria, and Acidobacteria (Janssen 2006; Buckley & Schmidt 2003). Their presences were ubiquitous, but their abundances varied between different soils. Roughly 30% of the microbial community in this study was Proteobacteria, which aligned with the average of 39% calculated by Janssen (2006). Within Proteobacteria, the classes of α-proteobacteria and δ-proteobacteria were of interest due to their negative correlation. A-proteobacteria contains a lot of diverse bacteria that makes generalization difficult. However, δ-proteobacteria is a smaller class and most of them are myxobacteria or sulfate–/sulfur–reducing bacteria. Myxobacteria are gliding soil bacteria that degrade organic matter for nutrition, and form myxospores when in starvation conditions. They are studied for their secondary metabolites, which may be useful as antimicrobials (Reichenbach 2001). The negative correlation between α- and δ-proteobacteria implied that there were α-proteobacteria susceptible to secondary metabolites secreted by δ-proteobacteria.

Within the α-proteobacteria, Rhodospirales and Bradyrhizobiaceae were more abundant in Carman. Rhodospirales can be subdivided into two major kinds: acetic acid bacteria and purple nonsulfur bacteria. The presence of Rhodospirales could be contributing to the acidic nature of Carman’s soil (Adeleke et al. 2017). Bradyrhizobiaceae, the family of interest, contains a wide variety of bacteria. Some are plant-associated, some are animal-associated, and others are free-leaving. However, its higher abundance in Carman does correlate with the Bradyrhizobium qPCR data. Other bacteria that were found to be more abundant in Carman include Actinomycetales and Gp1. Actinomycetales are Gram-positive bacteria that have filamentous
and branching growth (Gillespie 1994). They can form associations with a wide variety of plants and have antifungal activity (Sardi et al. 1992; Tian et al. 2004). Gp1 is a subgroup of Acidobacteria, one of the most abundant soil bacteria, and has the most identified genera (Janssen 2006). It has been noted that Gp1 presence increases in acidic soil compared to neutral ones (Liu et al. 2019), which is consistent with its abundance in Carman. Furthermore, there were bacteria that were only found in Carman: Rhodanobacter, Ktedonobacter, Granulicella, candidate_division_WPS2. For the most part, all these Carman-only bacteria are well-suited to acidic soil. Rhodanobacter consists of acid-tolerant denitrifiers, which have been observed in acidic, nitrate-rich soil (Green et al. 2012). Ktedonobacter are aerobic, filamentous Gram-positive bacteria. They share similarities with actinomycetes but are classified within the Chloroflexi phylum. The optimal growth for Ktedonobacter has been measured at 28–33°C and pH 4.8 to 6.8 (Chang et al. 2011). Granulicella is a genus of Acidobacteria Gp1. They are classified as acidophilic and psychotolerant bacteria, whose optimal growth is at around pH 4 and 20°C (Pankratov & Dedysh 2010). There is not much known about candidate_division_WPS2. It has no cultured representative and only been detected using metagenomic analyses. Previous work did associate it with acidic soils, pH 4.2, which is similar to Carman (Wang et al. 2015).

In Kelburn and Melita, there were a few bacteria that were more abundant than in Carman: Methylobacteriaceae, Gp6, Rhodobacterales, and Rubrobacterales. Most Methylobacteriaceae are facultative methylotrophs that can grow on methanol and other one-carbon compounds (Kelly et al. 2014). Generally, methylotrophs prefer to be in neutral pH (Topp & Pattey 1997) which can explain their prevalence in Kelburn and Melita. Acidobacteria Gp6 was prevalent in Kelburn and Melita, instead of Gp1, and it is likely due to the neutral pH in
these soils. Gp6 was abundantly found in the soybean rhizosphere of neutral agricultural soil (Liu et al. 2019). Rhodobacterales have been found to be associated with a nitrite-reducing community found near plant roots (Ai et al. 2016). Rubrobacterales only has the Rubrobacter genus, which are aerobic, thermophilic, radiotolerant bacteria. Their growth has been measured to be between pH 6 to 11, with the optimum at 8, thus the apparent absence in Carman’s acidic soils. (Chen et al. 2004).

4.2.2 Changes in Microbial Communities Based on Rotational Treatments

There were few differences found between microbial communities based on rotational treatments in this study. However, the continuous soybean treatment did show significantly higher bacterial diversity than the other treatments. This observation was contrary to what was originally hypothesized, in which continuously planting soybean will select for specific beneficial bacteria thus lowering the overall bacterial diversity. Previous crop rotation work, albeit with corn, had shown that a monoculture had the same bacterial diversity as those in rotation with one or two other crops (Peralta et al. 2018). The finding of no differences between treatments is consistent with what was observed in the three sites of this study. The consistency of the continuous soybean treatment in Carman’s acidic soil could have been beneficial to some bacteria that might otherwise not have been able to survive. For example, *Pedomicrobium* and *Lysobacter* were only found in the continuous soybean rotation. *Pedomicrobium* is an α-proteobacteria, that has been isolated from both soil and water samples, and is mostly known for its ability to oxidize minerals like manganese at acidic pH (Sly et al. 1990). *Lysobacter* produces antimicrobial metabolites and have been abundant in soils suppressive against soybean pathogen, *Rhizoctonia solani* (Gómez-Expósito et al. 2015). Contrarily, Kelburn and Melita have *Rhodanobacter*, which consists of acid-tolerant denitrifiers as previously mentioned, only in their
diversified treatments. In Melita, *Jatrophihabitans* was not present at all. *Jatrophihabitans* consists of Gram-positive bacteria that have been isolated in soil and plant tissue. It is part of the Actinobacteria phylum and growth has been observed in a wide-range of temperature (10 – 37°C) and pH (5 – 10) (Gong *et al.* 2016). There does not seem to be an apparent reason for the absence of *Jatrophihabitans* in Melita.

### 4.2.3 Microbial Changes over the Growing Season

Changes within the soybean growing season were shown when microbial diversity was compared between the BP and R8 timepoints. In Carman, the soybean plants seem to be releasing chemicals that encourage the growth of Thermomicrobia and *Anaeromyxobacter*, while suppressing *Adhaeribacter*. Thermomicrobia are thermophilic bacteria that have optimal growth between 50 - 75°C (Houghton *et al.* 2015). Some thermophilic bacteria have been shown to be metabolically inactive in cooler temperatures then grow when the temperature gets warmer (Marchant *et al.* 2008), which was likely the reason for the increase of Thermomicrobia as the growing season progressed. *Anaeromyxobacter* are aryl-halo-respiring facultative anaerobic bacteria. They have been previously isolated using acetate as an electron donor and 2-chlorophenol as an electron acceptor (Sanford *et al.* 2002). It possible that the reason for their increase through the season is chlorophenol-like soil contamination that can occur through the use of pesticides and herbicides. *Adhaeribacter* is part of the Bacteriodetes phylum and consists of Gram-negative bacteria that produce extracellular fibrillar material (Zhang *et al.* 2009). There are a few species of *Adhaeribacter* characterized so very little is known. However, that it is negatively correlated with soybean growth needs further investigation.

In Kelburn, unclassified Ruminococcaceae was only found in the continuous soybean and corn – soybean treatments at BP, but was in all rotations at R8. Ruminococcaceae has been
linked to microbial communities with the ability to degrade plants using methanogenesis (Wegner & Liesack 2016). Unclassified Sphingobacteriaceae was also detected in all rotations at R8, despite being only in the continuous soybean treatment at BP, suggesting that it may be selected for in the presence of soybean. Sphingobacteriaceae are part of the Bacteriodetes phylum which contains several genera of environmental bacteria that are characterized by the presence of sphingolipids (Prasad et al. 2013). There is not a clear explanation why it appears to be associating with the presence of soybean.

In Melita, unclassified candidatus_Koribacter was in most rotations at R8, except corn – soybean, even though it was not present in any rotations at BP. Koribacter was originally isolated from a ryegrass/clover pasture in Australia, but it can be found widely distributed in the soil (Joseph et al. 2003). Koribacter has been linked to polymer degradation, carbon monoxide oxidation, nitrogen reduction, and iron redox reactions (Ward et al. 2009). Burkholderia, on the other hand, was not found at R8 despite being in all the rotations at BP. This genus contains both pathogenic and beneficial bacteria, which thrive in acidic soils (Stopnisek et al. 2014). The absence of Burkholderia at R8 could be due to further alkalinization of the soil.
Chapter 5:

Conclusions and Future Directions
The aim of this study was to determine the effects of the frequency of soybean in rotation on the soil microbial community, especially on the soybean symbiont *Bradyrhizobium japonicum*. The assays conducted with qPCR allowed for the quantification of *B. japonicum*, which showed that it does persist and survive in the soil years after the initial inoculation. Further, there are native species of *Bradyrhizobium* present in Manitoba soils that cannot nodulate soybean as well as *B. japonicum*. In terms of the crop rotation effect, the continuous soybean treatment showed the highest abundance of *B. japonicum* as expected, although the degree of difference varied between the three Manitoba sites.

The microbial community, as a whole, is less affected by the different crop rotation treatments. There were minimal differences between treatments, and they were typically site-specific. Differences between timepoints were more apparent, but the overall microbial community depended mostly on the site. The three Manitoba sites had varying soil properties, including soil type and texture, but the soil pH seemed to be the driving factor in choosing the composition of the microbial community.

Future work would focus on the isolation of bacteria that were noticeably unique or different between treatments, timepoints, and sites. Examples would include *Lysobacter*, which is characterized as a soybean pathogen suppressor, and *Adhaeribacter*, which is largely unknown but is negatively correlated with soybean growth. Also, the culturing and isolation of native *Bradyrhizobium* should be completed. There may be native *Bradyrhizobium* that can contain the *nodZ* gene and be better candidates for future inoculants.

Another future experiment would be to use the soil samples to grow soybean, canola, corn, and wheat. Growing these crops in a controlled setting would allow for more definitive
answers to any observed anomalies in their microbial communities. The compounding factor of soil types and pH can be corrected to see if that would yield similar microbial communities. The differences between the microbiomes of the bulk soil, rhizosphere, and endosphere could also be examined. The soil in the pots, the soil pressed right against the roots, and the roots themselves can be extracted for genomic material and sequenced. This would show the bacteria ubiquitously present in the soil, those that are attracted and perhaps enriched for by the plants, and those that has had direct interactions with the plants.

Lastly, the functional profiles of the microbial communities, which could be predicted by programs like PICRUSt, must be completed. This study shows the composition of the microbiome, which allows for observations of bacteria’s presence and abundance, but provides no information on what these bacteria are doing in the soil. The differences observed in taxonomic profiling does not necessarily mean that the microbial community is behaving differently, since bacteria can perform multiple tasks depending on the situation. Comparing functional profiles may provide better insight on the current state of the microbial communities.
Appendices

Appendix A – mothur Batch File

List of commands used to process raw sequencing reads in the mothur software.

#!/bin/bash

pcr.seqs(fasta=silva.bacteria.fasta, start=11894, end=25319, keepdots=F)
system(mv silva.bacteria.pcr.fa sta silva.v4.fasta)
#change *.files name to your data name
make.contigs(file=C16S_17.files, processors=8)
screen.seqs(fasta=current, group=current, maxambig=0, maxlength=300)
unique.seqs()
count.seqs(name=current, group=current)
align.seqs(fasta=current, reference=silva.v4.fasta)
screen.seqs(fasta=current, count=current, maxambig=0, maxlength=300)
classify.seqs(fasta=current, count=current, reference=trainset9_032012.pds.fasta,
taxonomy=trainset9_032012.pds.tax, cutoff=80)
remove.lineage(fasta=current, count=current, taxonomy=current, taxon=Chloroplast-
Mitochondria-unknown-Archaea-Eukaryota)
cluster.split(fasta=current, count=current, taxonomy=current, splitmethod=classify, taxlevel=4,
cutoff=0.03)
make.shared(list=current, count=current, label=0.03)
classify.otu(list=current, count=current, taxonomy=current, label=0.03)
make.biom(shared=current)
Figure B1. Optimization of the soil quantity needed for Qiagen’s DNeasy Powersoil Kit. Four soil samples of 0.015 – 0.30 grams (increments of 0.05) were used to test Qiagen’s recommended 0.25 grams of soil for DNA extraction.
**Figure B2.** Standard curves with *B. japonicum* USDA110 dilutions $10^0$ to $10^{-8}$. Colours represent primers used for assay.
Literature Cited


