



# Cropping sequence affects the structure and diversity of pathogenic and non-pathogenic soil microbial communities

M. Nazrul Islam ·  
M. Alejandra Oviedo-Ludena ·  
H. Randy Kutcher · Oscar Molina · Xiben Wang

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## Abstract

**Aims** Current understanding of how cropping sequence affects pathogen-suppressive microbiomes in soil is limited. We investigated the effects of several cropping sequences from the 2020–2021 growing seasons, including cereals, pulses, and an oilseed, on microbial communities in rhizosphere and bulk soils in two western Canadian field locations.

**Methods** The fungi and bacteria were characterized by Internal Transcribed Spacer (ITS) and 16S rRNA gene sequencing, respectively. The QIIME 2™ bioinformatic

pipeline was used to measure the diversity and abundance of microbial species. Additionally, the concentration of the soil mineral chemicals, including macro and micro nutrients, was determined by colorimetric analysis. **Results** Ascomycota (62.5%) was the most common fungal phylum, followed by Glomeromycota (11.1%), Mucoromycota (8.9%), and Basidiomycota (6.8%). Pseudomonadota (35.0%), Actinomycetota (21.1%), and Bacillota (10.1%) were the three most common bacterial phyla. Fungal OTU richness and phylogenetic diversity were highest in the cereal-pulse cropping sequencing, and bacterial OTU richness was highest in the pulse-oilseed sequences. *Fusarium* was the fungal genus most commonly associated with cereal-cereal monoculture and least common in the oilseed-pulse cropping sequences. The fungi (*Mortierella*, *Funneliformis*, and *Diversispora*) and bacteria (*Rhizobium*, *Bradyrhizobium*, *Flavobacterium*, and *Candidatus*) were higher in the cropping sequences involving pulses. The most prevalent bacteria were *Streptomyces* in cereal-related sequences and *Solirubrobacter* and *Pseudomonas* in oilseed-related sequences. Among soil mineral chemicals, nitrate-nitrogen, copper, calcium, potassium, and chlorine were associated with a number of beneficial fungal and bacterial genera but not with pathogenic fungal genera.

**Conclusions** The results highlight the consequences of crop species selection in cropping sequences and the management of agrochemicals in the agricultural production system.

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M. N. Islam (✉) · O. Molina · X. Wang (✉)  
Agriculture and Agri-Food Canada, Morden Research  
and Development Centre, Morden, MB, Canada  
e-mail: nazrul.islam@usask.ca

X. Wang  
e-mail: xiben.wang@agr.gc.ca

O. Molina  
e-mail: oscar.molina@agr.gc.ca

M. A. Oviedo-Ludena · H. R. Kutcher  
College of Agriculture and Bioresources, University  
of Saskatchewan, Saskatoon, Saskatchewan, Canada  
e-mail: alejandra.oviedo@usask.ca

H. R. Kutcher  
e-mail: randy.kutcher@usask.ca

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## Introduction

Crop rotation, a common farming practice, is the successive cultivation of different crop species. It is one of the earliest agricultural practices with an emphasis on sustainability (White 1970; Howard 1996). Since the discovery of nitrogen-fixing bacteria (*Rhizobium* spp.) associated with legumes/pulses in 1896 (Nobbe and Hiltner 1896), crop rotation has been utilized in agricultural production to encourage beneficial microbes for plant development or protection against pests. Crop rotation has been shown to be a safe and efficient management approach that can improve soil productivity and crop yields while reducing pathogens and controlling plant soil-borne diseases in comparison to monocropping (Emmond and Ledingham 1972; Frank and Murphy 1977; Larkin and Halloran 2014; Moshynets et al. 2019; Ashworth et al. 2020).

Crop rotation is an essential component of integrated disease management in Western Canada, which reduces soil-borne diseases by reducing the survival of soil-borne plant pathogens and allelochemicals (Krupinsky et al. 2002; Jin et al. 2019). Cereals and oilseeds are the two most economically important crop types in this region. Due to the capacity to fix atmospheric nitrogen via biological nitrogen fixation, the incorporation of legumes/pulses, including pea, lentil, chickpea, and soybean, into cereal- or oilseed-based rotations has been widely adopted by growers. For many years, growers in western Canada have been practicing short or intensive rotations of only two or three crops, reduced fallow, and no-till production due to pressures related to commodity prices, agronomic management, and changes in the local climate (Peng et al. 2016).

Crop diversity and sequence in the rotation system can have a significant impact on the abundance and structure of the soil microbial community, including plant pathogens. Monoculture with a susceptible host can result in the increase of specific pathogens with a wide host range or pathogens that produce long-lived survival structures in soil (Huisman and Ashworth Jr 1976). On the contrary, a diverse crop rotation can lead to a decline in pathogen populations due to

natural mortality and the antagonistic activities of co-existing soil microorganisms (Ghorbani et al. 2008). Several studies show crop diversity in rotations can effectively reduce the abundance of soil- and residue-borne plant pathogens by increasing the diversity of soil microorganisms that are involved in disease suppression, plant growth promotion, and nutrient cycling (Emmond and Ledingham 1972; Frank and Murphy 1977; Achatz et al. 2010).

Recently, the diversity of soil microbial communities and the interactions between beneficial (symbiotic taxa) and pathogenic microbes have received extensive attention as indicators of potential pathogen-suppressive and productive agricultural soils (Berendsen et al. 2018). Substantial volumes of organic exudates are produced by plant roots in the microenvironment of the soil rhizosphere. Plant root exudates impact the diversity of soil microbial communities, which are essential parts of the soil rhizosphere and are affected by various activities such as water and nutrient uptake and exudation (White and Broadley 2003; White et al. 2007). Additionally, cropped soils are known to leave a “legacy effect,” including the preceding years’ crop root exudates, that modify the soil’s chemical, physical, and biological environment (Berendsen et al. 2018). For example, the previous year’s roots from legume monocultures contribute a low C/N ratio and increased N content to subsequent non-legume crop soils. This soil nutrient legacy results in a significant increase in the abundance of key microbial taxa in the soil, including arbuscular mycorrhizal fungi (AMF) and symbiotic bacterial communities (Acidobacteriota, Actinomycetota, and Pseudomonadota) in the following cover crop (cowpea) and wheat rotation (Somenahally et al. 2018).

Limited research has been conducted on the soil microbiome in conventional tillage (non-organic) cropping systems. The effect of cropping sequence in the rotation on the native soil microbial communities that promote plant growth and disease control in various Canadian soil ecozones is currently unknown. In this study, we conducted a two-year cropping sequence study to test the effect of rotating cereals (wheat and barley), pulses/legumes (pea and soybean), and an oilseed (canola) on soil microbial communities using metagenomics analysis based on Internal Transcribed Spacer (ITS) and 16S ribosomal RNA (rRNA) amplicon sequencing. It is hypothesized that the diversity and structure of pathogenic

and pathogen-suppressive soil microbiomes may respond differentially to various cropping sequences. We assessed the impact of the cropping sequence on the following biotic and abiotic variables in soil: (1) diversity of soil fungal and bacterial communities; (2) structure (abundance and composition) of pathogenic and non-pathogenic fungal taxa; (3) structure of non-pathogenic bacterial taxa; and (4) relationships between pathogenic and beneficial non-pathogenic communities and major soil chemical parameters (macro and micro nutrients).

## Materials and methods

### Experimental site, design and treatment

A diverse cropping sequence study was initiated by the Department of Plant Sciences, University of Saskatchewan, in 2019 across the six field sites located in the western Canadian Prairies to determine optimum cropping sequences that minimize *Fusarium* head blight, common root rot, and leaf spot diseases of cereal crops. This soil microbiome experiment was designed based on the diverse crop sequence study at two sites: (1) Saskatoon (W 52° N and 106° W), Saskatchewan; and (2) Morden (49° 11' 22" N and 98° 5' 19" W), Manitoba. The replicated cropping sequence experiment, which comprised a variety of crops, including cereals, pulses, and oilseeds, was carried out using a randomized complete block design (RCBD). In the 2021 cropping season, soil samples (rhizosphere and bulk) were collected from replicated cropping sequence plots, including barley, durum, pea, soybean, and canola, from both sites. The nine cropping sequence treatments were considered for two (2020–2021) growing seasons as follows: (1) cereal-cereal (CR-CR), (2) cereal-pulse (CR-PS), (3) cereal-oilseed (CR-OL), (4) pulse-pulse (PS-PS), (5) pulse-cereal (PS-CR), (6) pulse-oilseed (PS-OL), (7) oilseed-oilseed (OL-OL), (8) oilseed-cereal (OL-CR), and (9) oilseed-pulse (OL-PS). Due to work restrictions during the pandemic in 2020, all plots at the Morden site were seeded with cereal (barley), resulting in unequal treatment combinations between two sites. As a result, the first three treatments represent the Morden field site, while the remaining six treatments correspond to the Saskatoon field sites. Supplementary Table S1A provides detailed additional

information regarding the crop and soil sample types, sites, treatment combinations, and replicated plots. The soils of the Saskatoon and Morden sites are in the Black Chernozem and Dark Brown Chernozem soil ecozones, respectively (Soil Classification Working Group 1998). Agronomic management, including chemical fertilization, pest control, and tillage operations, was consistent at both experimental sites and followed conventional grain production practices across the prairies (Dai et al. 2014).

### Soil chemical analysis

Bulk soil samples (approximately 400 g per plot) from the 0–20 cm depth were collected using a 2.5 cm diameter soil probe (JMC Backsaver N-2; Clements Associates, Inc., IA 50,208, USA) during crop harvest (2021) at two sites. Twenty-seven bulk soil samples (for Saskatoon, 6 treatments × 3 replicates = 18; for Morden, 3 treatments × 3 replicates = 9) were collected and stored in zip-lock bags at -20 °C. Each of the 27 bulk soil samples was subjected to colorimetric analysis to determine the concentration of soil macronutrients, including nitrate-nitrogen (NO<sub>3</sub>-N), phosphate-phosphorus (P), potassium (K), and sulfate-sulfur (S). The concentrations of micronutrients Ca (calcium), Mn (manganese), Mg (magnesium), Fe (iron), Cl (chlorine), Cu (copper), B (boron), and OM (organic matter) and the above-mentioned macronutrients were determined according to standard protocols (Jones Jr 1999; Sillanpää 1982) at Farmers Edge soil testing laboratories (<https://www.farmersedge.ca/laboratory-ca/>).

### Soil metagenomic analysis

Representative soil sampling at two crop growth stages was performed, including rhizosphere soil during the flowering stage (July 2021) and bulk soil during crop harvest (August 2021) at both field sites. Microbiome sampling was conducted during crop flowering and maturity phases, as the higher diversity and activity of microbiomes are demonstrated during these stages (Edwards et al. 2018; Marag and Suman 2018). Fifty-four soil samples total, 27 bulk and 27 rhizosphere (6 treatments × 3 replicates in Saskatoon = 18; 3 treatments × 3 replicates in Morden = 9) were used for the microbiome analysis. For rhizosphere soil (ca. 100 g), the loose soil was shaken

from the roots (4 plants per plot), and the remaining soil adhering to the roots was carefully brushed off, pooled, and homogenized as one rhizosphere soil sample per plot. Bulk soil samples (ca. 100 g) from each replication were collected using the JMC Back-saver N-2 soil probe at a depth of 0 to 20 cm below the soil surface. The soil probes were alcohol-sterilized between samplings of the two plots. Field-moist soils were transported to the lab with an ice pack in a cooler and immediately frozen at  $-80^{\circ}\text{C}$  for DNA extraction and further metagenomic analysis.

#### Fungal ITS and bacterial 16S RNA gene sequencing

Metagenomic DNA was isolated from 54 soil samples (27 rhizosphere and 27 bulk) using the Omega Bio-Tek Mag-Bind® Environmental DNA Kit (Norcross, GA) according to the manufacturer's protocol. DNA concentration was measured using the QuantiFluor® dsDNA system on a Quantus™ Fluorometer (Promega, Madison, WI, USA). The ITS1 and ITS2 regions within the ribosomal transcript were amplified using the primer pair containing the gene-specific sequences and Illumina adapter overhang nucleotide sequences. The full-length primer sequences were ITS amplicon PCR forward and reverse primers (ITS1F: 5'-CTTGGTCATTTAGGAAGTAA and ITS4: 5'-TCCTCCGCTTATTGATATGC) (Bellemain et al. 2010). The V3-V4 region of the bacterial 16S rRNA gene was amplified using the primer pair containing the gene-specific sequences and Illumina adapter overhang nucleotide sequences. The full-length primer sequences were 16S amplicon PCR primers (forward 5'-CCTACGGGNGGCWGCAG and reverse 5'-GACTACHVGGGTATCTAATC) (Klindworth et al. 2013). ITS and 16S amplicon PCR were used to amplify the DNA templates and the insert size was about ~400 bp for both ITS and 16S platforms. Briefly, each 25  $\mu\text{L}$  of PCR reaction contained 12.5 ng of sample DNA as an input template, 12.5  $\mu\text{L}$  2x KAPA HiFi Hot-Start Ready-Mix (<https://kapabiosystems.com/>, Wilmington, MA), and 5  $\mu\text{L}$  of 1  $\mu\text{M}$  of each primer. PCR reactions were carried out using the following protocol: an initial denaturation step at  $95^{\circ}\text{C}$  for 3 min, followed by 25 cycles of denaturation ( $95^{\circ}\text{C}$ , 30 s), annealing ( $55^{\circ}\text{C}$ , 30 s), and extension ( $72^{\circ}\text{C}$ , 30 s), and a final elongation of 5 min at  $72^{\circ}\text{C}$ . The PCR product was cleaned up from the reaction mix with Mag-Bind RxnPure Plus

magnetic beads (Omega Bio-Tek, Norcross, GA). A second index PCR amplification, used to incorporate barcodes and sequencing adapters into the final PCR product, was performed in 25  $\mu\text{L}$  reactions, using the same master mix conditions as described above. Cycling conditions were as follows:  $95^{\circ}\text{C}$  for 3 min, followed by 8 cycles of  $95^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s. A final, 5-min elongation step was performed at  $72^{\circ}\text{C}$ . The libraries (~800 bases for ITS and ~600 bases for 16S) were normalized with the Mag-Bind® EquiPure Library Normalization Kit (Omega Bio-Tek, Norcross, GA) and then pooled. The pooled library was checked using an Agilent 2200 TapeStation and sequenced (2×300 bp paired-end read setting) on the Miseq-PE300 (Illumina, San Diego, CA) platform. The sequencing output of 16S/ITS was from ~60–100 K reads per sample (30–50 K in each direction) in PE300 format.

#### Bioinformatics

FastQC was used to check for adaptor sequences and to remove ambiguous nucleotides (average quality score < 30) (Huse et al. 2010) from the initial raw sequence reads. QIIME™ 2 (Quantitative Insights into Microbial Ecology), the next-generation microbiome bioinformatics platform, carried out downstream analysis. The major output files from 54 soil samples within nine treatments of the soil microbiome bioinformatics framework included core diversity and taxonomic analyses. Briefly, the reads were denoised, which involved removing chimeric sequences and excessively long and short homopolymers (singletons and doubletons) from the dataset and dereplicating them using the DADA2 algorithms. After denoising and dereplication, the reads were compared with the UNITE database (fungal/ITS) and Greengenes-16S rRNA (<http://greengenes.lbl.gov>) reference sequences at 99% similarity and assigned taxonomy by the QIIME 2 machine-learning based classifier.

Clustering of sequences into OTUs (operational taxonomy units) using q2-Vsearch from dereplicated, quality-controlled data and feature representative sequences that were generated using the qiime.deblur.denoise command. OTUs for both bacteria and fungi were retrieved from the 16S and ITS data sets, respectively, by pooling the sequences generated from the two sampling events (rhizosphere and bulk soil) (Supplementary Table S1B for number

of inputs, clean, and combined sequence reads). A sequence similarity threshold of 97% was set to ensure the identification of microbial taxa at genus or species levels under the BLAST search tools. The OTUs matrix aligned with replicated samples was created to determine the relative abundance of each of the OTUs present in replicated treatment samples. The pathogenic and non-pathogenic fungal taxa from ITS sequences were grouped based on plant disease PFP-ITSS databases (<http://unite.ut.ee>).

The core diversity analysis performed included alpha-rarefaction, beta-diversity analysis, and visualization. Diversity within samples, referred to as “alpha-diversity,” such as OTU richness, phylogenetic (PD), and Shannon (SD) diversity of fungi and bacteria, was measured with a UniFrac distance matrix using the QIIME 2™ pipeline. An unrooted phylogenetic tree for both ITS and 16S sequences was constructed using q2-phylogeny plugins aligned to tree-mafft-fasttree action in Qiime™ 2 to measure phylogenetic diversity/distance between taxa. Shannon diversity consists of the richness index (observed number of OTUs/species) and species evenness index (homogeneity of the species) within a sample (Shannon 1948). The 16S and ITS sequences were grouped into phylum, family, and genus levels aligned with nine crop sequence treatments. The latest revised phyla name as per the rules of the International Code of Nomenclature of Prokaryotes, the ending “-ota,” was used in this study (Oren et al. 2021), except Cyanobacteria. The uncultured Candidatus taxa whose phyla status has yet to be de facto established in the academic literature and databases by ICNP (Pallen 2021). In this study, the relative abundance of the 99 OTUs (16S) representing 11 bacterial genera and one uncultured taxon (Candidatus) was calculated collectively and presented.

#### Phylogenetic analyses of fungal and bacterial OTUs

For taxonomic identification, the representative sequences of each OTU were compared with GenBank reference sequences using BLAST search against the NCBI nucleotide collection database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The OTUs and GenBank reference sequences (97% or above similarity) were aligned using ClustalW (Tamura et al. 2013). Two separate phylogenetic trees were constructed with 71 fungal and 99 bacterial OTUs

using the maximum likelihood method and the Kimura 2-parameter model in MEGA X (Kimura 1980). The bootstrap replication method was set at a confidence level of 1000 with the Kimura 2-parameter model.

#### Statistical analysis

To estimate the percent relative abundance (PRA) of microbial sequences, the number of absolute sequence reads (ASR) present in each sample OTU was divided by the total number of ASR from the total OTUs in that sample and multiplied by 100. Before statistical analysis, the microbial abundance and diversity data were subjected to a normality test to ascertain skewness and kurtosis and were transformed (i.e., square root and arcsine) when required. We used SAS (version 2.0.5) to calculate the *p* values for the fixed and random effects using the GLM procedure to test hypotheses for mixed model analysis of variance. The GLM mixed model ANOVA (Type III expected mean square) separated the 2021 sampling year cropping sequence effects from 2020/previous year crop effects on selected dependent variables (relative abundance of 24 fungal and bacterial genera/taxa and six diversity indices). We were primarily interested in tracking the impact of three crop types (cereal, oilseed, and pulse) in the sequences in the 2021 sampling year based on microbial abundance and diversity, which was fit as a fixed effect in the GLM proc mix ANOVA model. Block and cropping sequences in 2020 were treated as random effects in this model because the reported changes in microbial taxa were the direct and immediate result of the 2021 sampling year crop sequences (fixed factor). The least significant difference (LSD) test was also used to rank the effects of the three crop types (CR, PS, and OL) on each of the dependent variables during the previous (2020) and the following crops in the sequence (2021). The ANOVA model is presented in Supplementary Table S8. Tukey’s HSD (honestly significant difference) test at  $p \leq 0.05$  and  $p \leq 0.01$  was used to rank the nine crop sequence treatment effects on microbial diversity (Table 1) and soil chemicals (Table S5). The multivariate statistical package PC-ORD Vol. 7 (McCune and Mefford 1999) was used for hierarchical clustering/dendrogram-heatmaps using unweighted pair grouping by

**Table 1** Analysis of variance (ANOVA) for alpha diversity indices of soil fungal and bacterial communities for nine cropping sequence treatments

<sup>β</sup> Cropping sequence (2020–2021)	Fungal diversity indices			Bacterial diversity indices		
	OTU richness	Phylogenetic diversity	Shannon diversity	OTU richness	Phylogenetic diversity	Shannon diversity
CR-CR	480 ± 21 b	126 ± 5 b	6.1 ± 0.5	839 ± 33 b	69 ± 6	9.9 ± 0.2
CR-PS	578 ± 18 a	145 ± 9 a	7.0 ± 0.2	1041 ± 72 a	87 ± 7	9.5 ± 0.5
CR-OL	433 ± 21 b	100 ± 8 b	6.0 ± 0.3	1089 ± 79 a	86 ± 9	9.7 ± 0.6
PS-PS	434 ± 15 b	115 ± 4 b	6.8 ± 0.4	691 ± 78 c	80 ± 9	8.9 ± 0.5
PS-CR	530 ± 17 a	142 ± 8 a	6.5 ± 0.5	730 ± 37 b	73 ± 7	9.2 ± 0.5
PS-OL	450 ± 19 b	150 ± 7 a	6.4 ± 0.5	1085 ± 81 a	88 ± 6	9.0 ± 0.3
OL-OL	490 ± 19 b	120 ± 6 b	6.2 ± 0.2	835 ± 74 b	98 ± 5	9.7 ± 0.7
OL-PS	470 ± 16 b	115 ± 5 b	7.0 ± 0.5	775 ± 53 b	72 ± 5	8.9 ± 0.4
OL-CR	450 ± 18 b	122 ± 7 b	6.0 ± 0.6	969 ± 48 a	85 ± 8	9.0 ± 0.4
<i>p</i> value	*	*	ns	**	ns	ns

<sup>β</sup>Cropping sequence treatment abbreviations: CR, cereals; OL, oilseeds; and PS, pulses. Mean values ± standard deviation ( $n=3$ ) are shown. Different lowercase letters within the column indicate significance shown by \* and \*\* ( $p \leq 0.05$  and  $p \leq 0.01$ , respectively); “ns” refers to non-significant differences among treatments. GLM proc mixed model ANOVA with Type III expected mean squares (EMS) was applied. The crop types (CR, PS, and OL) sequence effects (the 2021 sampling year) were fixed factors and separated from the random effects (block and 2020 crop types sequence). The Least Significant Difference (LSD) was also used to rank the fixed factors (CR, PS, and OL) against each of the variables (microbial abundance). Details of the mixed model for the GLM proc ANOVA and EMS are shown in Table S8.

the arithmetic mean (UPGMA) and principal component analysis (PCA) using the Bray-Curtis (dis) similarity matrix for the above-mentioned 24 microbial genera. A bi-plot principal coordinate analysis (PCoA) based on the Euclidian algorithm using fungal and bacterial relative abundance in matrix 1 and soil mineral chemical contents in matrix 2 was performed. To validate relationships between the soil components (nutrients and microbiomes) presented in a distance-based PCoA ordination plot, the Spearman correlation coefficients ( $\rho$ ) were also determined.

#### Submission of nucleotide sequences

The unique sequences representing 71 fungal and 99 bacterial OTUs analyzed in this study were deposited in the GenBank submission portal (<https://submit.ncbi.nlm.nih.gov/subs/>) under the accession numbers SUB13789011 for ITS and SUB13791987 for 16S. The data will be available once the manuscript is published. The clusters of the OTUs and their taxonomic IDs are also presented in the phylogenetic trees (Supplementary Figs. S1 and S2).

## Results

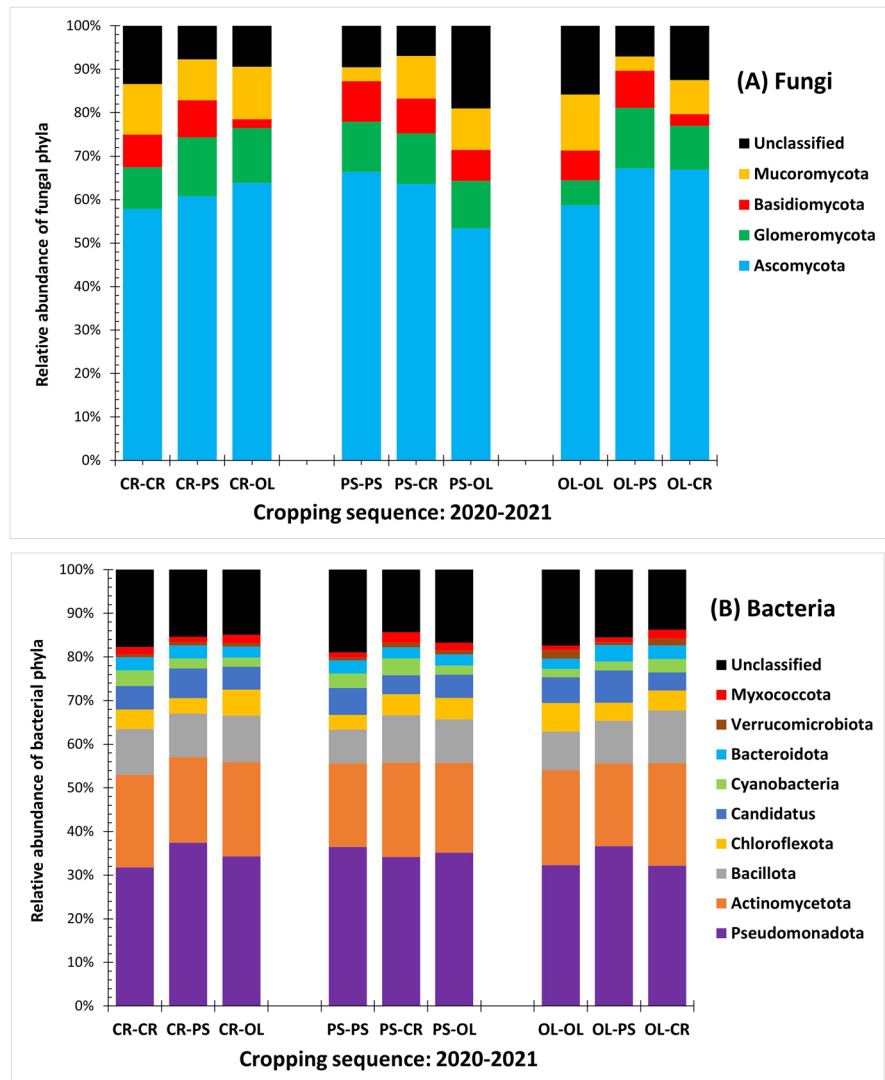
### Characterization of fungal and bacterial OTUs

The clean ITS sequences clustered into 71 unique OTUs of fungi belonging to 4 phyla, 22 families, and 33 genera (Fig. S1 and Table S3). Similarly, 16S rRNA sequences clustered into 99 unique OTUs of bacteria belonging to 9 phyla, 30 families, and 37 genera including uncultured Candidatus taxa (Fig. S2 and Table S4). Only OTUs and taxa with a relative abundance of 1% or higher were considered in this investigation. The alpha-rarefaction curve of the OTUs observed flattened out over time, showing that all fungal and bacterial species in the sample have been covered by the sequencing depth (Figs. S3 and S4).

### Effect of cropping sequence on soil fungal and bacterial phyla

Ascomycota (62.5%) were the most prevalent fungal phylum in soil, followed by Glomeromycota (11.1%), Mucoromycota (8.9%), and Basidiomycota (6.8%) (Fig. 1A and Table S2). The relative abundances of

**Fig. 1** Stacked column graphs showing the composition of fungal (A) and bacterial (B) phyla in the soil samples from nine cropping sequence (2020–2021) treatments. Crop type abbreviations: CR, cereals; OL, oilseeds; and PS, pulses. The values are the average of three replicates for each treatment



Ascomycota and Basidiomycota were the highest in the soils from the monoculture of PS-PS (69.5% and 9.7%, respectively). The abundance of Mucoromycota was the highest in the OL-OL monoculture (13.0%). In comparison, Glomeromycota, which contains many mycorrhizal fungi, was the most abundant in soils from the CR-PS and OL-PS sequences, both accounting for 13.8%, and its abundance was the lowest in the OL-OL monoculture (5.8%) (Fig. 1A and Table S2).

Pseudomonadota (35.0%) was the predominant bacterial phylum in soils, followed by Actinomycetota (21.1%), Bacillota (10.1%), Candidatus (5.8%), Chloroflexota (4.7%), Bacteroidota (2.9%), Cyanobacteria (2.7%), Myxococcota (1.6%), and Verrucomicrobiota

(1.0%) (Fig. 1B and Table S2). Among the top four bacteria phyla based on relative abundance, Pseudomonadota (28.6%) and Actinomycetota (19.2%) were the lowest in the CR-CR monoculture, whereas Bacillota (8.2%) had the lowest abundance in the PS-PS monoculture. In comparison, Candidatus was less abundant in treatments involving CR and OL compared to those involving PS (Fig. 1B).

Effect of cropping sequence on diversity of soil fungal and bacterial communities

OTU richness, phylogenetic, and Shannon diversity indices were used to visualize the variation among soil fungal and bacterial communities. For the soil

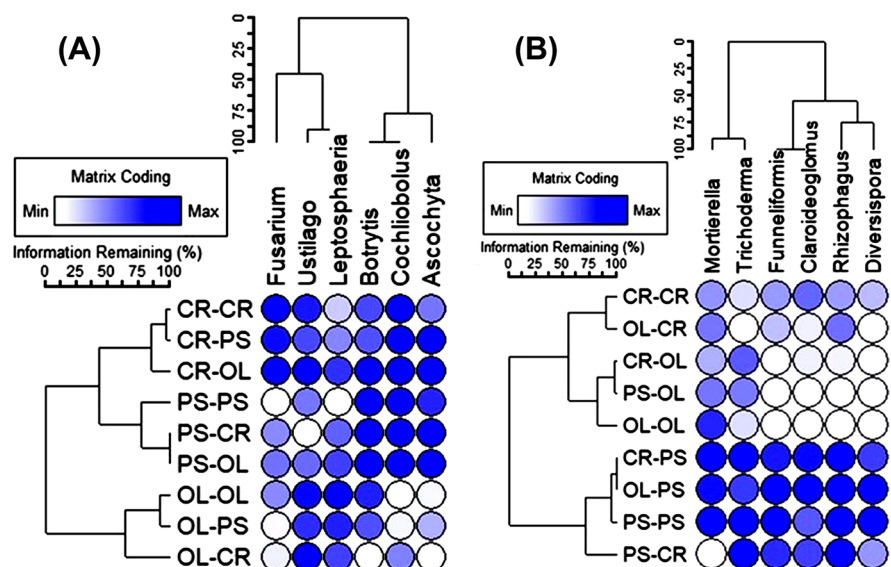
fungal community, OTU richness and phylogenetic diversity were significantly impacted by the crop types in the sequence ( $p \leq 0.05$ ) (Table 1). In the three cereal-based sequences (CR-CR, CR-PS, and CR-OL), these two indices were the highest in the CR-PS. In three pulse-based sequences, the highest OTU richness was detected in the PS-CR, compared to PS-PS and PS-OL. By comparison, the phylogenetic diversity was higher in the PS-CR and PS-OL sequences than in the PS-PS monoculture. OTU richness and phylogenetic diversity were similar in three oilseed-based sequences (OL-OL, OL-PS, and OL-CR).

For the soil bacterial community (Table 1), cropping sequences had a significant impact on OTU richness. Among the three cereal-based sequences, the bacterial OTU richness in the CR-CR monoculture was 839 and lower than in the CR-PS and CR-OL. Similarly, the PS-PS monoculture resulted in a lower bacterial OTU richness in the soil compared to the PS-CR and PS-OL. In the three oilseed-based sequences, the bacterial OTU richness in the OL-OL monoculture was higher than in the OL-PS but lower than in the OL-CR. There was no appreciable variation in the Shannon diversity of the bacterial and fungal communities in the soil from different cropping sequence treatments.

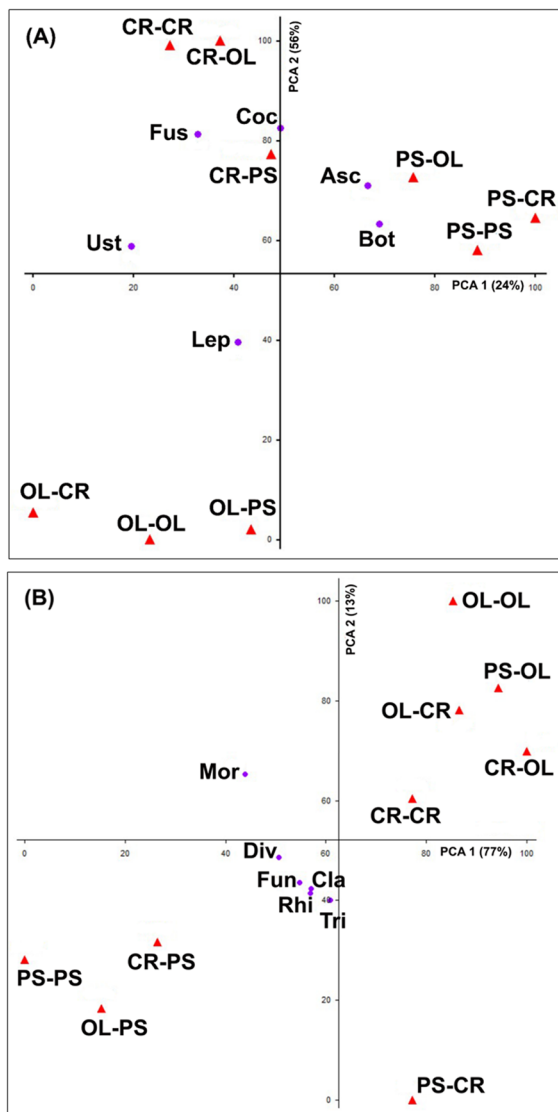
## Effect of cropping sequence on abundance and composition of soil fungal genera

The fungal genera derived from the ITS dataset were divided into two groups: pathogenic and non-pathogenic. Based on the relative abundance, we identified the six most abundant pathogenic fungal genera: *Fusarium*, *Ustilago*, *Leptosphaeria*, *Botrytis*, *Cochliobolus*, and *Ascochyta*. The hierarchical clustering (Fig. 2A) and PCA ordination analyses (Fig. 3A) based on the abundance of the pathogenic fungal genera divided the cropping sequence treatments into three clusters: the first cluster included CR-CR, CR-PS, and CR-OL; the second consisted of PS-PS, PS-CR, and PS-OL; and the third was comprised of OL-OL, OL-PS, and OL-CR. The presence of pulses in the cropping sequence appeared to have an association with certain groups of pathogenic fungal genera. For instance, when the pulse was rotated with cereals or oilseeds, a higher relative abundance of *Botrytis* and *Ascochyta* was detected. We also identified the six most abundant non-pathogenic fungal genera: *Mortierella*, *Trichoderma*, *Funneliformis*, *Claroideoglossum*, *Rhizophagus*, and *Diversispora*. The clustering of cropping sequences based on the relative abundance of these non-pathogenic fungal genera showed a different pattern. The hierarchical clustering (Fig. 2B) and PCA ordination analysis (Fig. 3B) grouped CR-PS, OL-PS, PS-PS, and PS-CR into

**Fig. 2** Hierarchical clustering (heatmap) showing the effect of cropping sequence on the abundance of pathogenic (A) and non-pathogenic (B) fungal genera in soil. Crop rotation (2020–2021) abbreviations: CR, cereals; OL, oilseeds; and PS, pulses. The dendrograms depicting the hierarchical clustering of cropping sequence treatments/fungal genera are based on the abundance of fungal communities using the arithmetic mean of unweighted pair groupings







**Fig. 3** Principal component analysis (PCA) using Bray-Curtis dissimilarity showing the clustering pattern of pathogenic (A) and non-pathogenic (B) fungal genera in the soil affected by the cropping sequence (2020–2021) treatments. The percentages of variance explained by each PCA axes 1 and 2 are shown in parentheses. Crop type abbreviations: CR, cereals; OL, oilseeds; and PS, pulses. Pathogenic fungi abbreviations: Fus, *Fusarium*; Ust, *Ustilago*; Bot, *Botrytis*; Coc, *Cochliobolus*; Asc, *Ascochyta*; and Lep, *Leptosphaeria*. Non-pathogenic fungi abbreviations: Mor, *Mortierella*; Fun, *Funneliformis*; Tri, *Trichoderma*; Rhi, *Rhizopagus*; Cla, *Claroideoglomus*; and Div, *Diversispora*

one cluster and separated them from the rest of the treatments.

The ANOVA indicated that the presence of cereals in the cropping sequence affected the abundance of *Fusarium* ( $p \leq 0.01$ ) and *Cochliobolus* ( $p \leq 0.05$ ) (Table 2). The effects of pulses on the abundances of *Botrytis* ( $p \leq 0.05$ ) and *Ascochyta* ( $p \leq 0.05$ ) and oilseeds on the abundance of *Leptosphaeria* ( $p \leq 0.05$ ) were also evident. Additionally, the inclusion of pulses in the cropping sequences affected the abundance of *Mortierella* ( $p \leq 0.01$ ), *Funneliformis* ( $p \leq 0.05$ ), and *Diversispora* ( $p \leq 0.05$ ) (Table 2).

Of all the ITS reads from 33 pathogenic and non-pathogenic fungal genera ( $> 1\%$  of the relative abundance), the top six pathogenic genera produced 23.4% of the sequence reads and the top six non-pathogenic genera produced 22.2% of the sequence reads; 14.4% of reads were for unclassified sequences at the genera level (Table S3).

#### Effect of cropping sequence on abundance and composition of bacterial genera

The hierarchical clustering based on the relative abundance of the top 12 bacteria genera/taxa divided the cropping sequences into two clusters (Fig. 4). Three cropping sequences where PS was planted as the 2nd year crop (CR-PS, OL-PS, and PS-PS) were placed in one cluster and separated from the CR-CR, CR-OL, OL-OL, PS-CR, PS-OL, and OL-CR. The PCA also showed the CR-PS, OL-PS, and PS-PS clustered in the same quadrant and separated from other cropping sequences. Additionally, the OL-OL, PS-OL, and CR-OL were plotted in a different quadrant than the PS-CR, CR-CR, and OL-CR (Fig. 5).

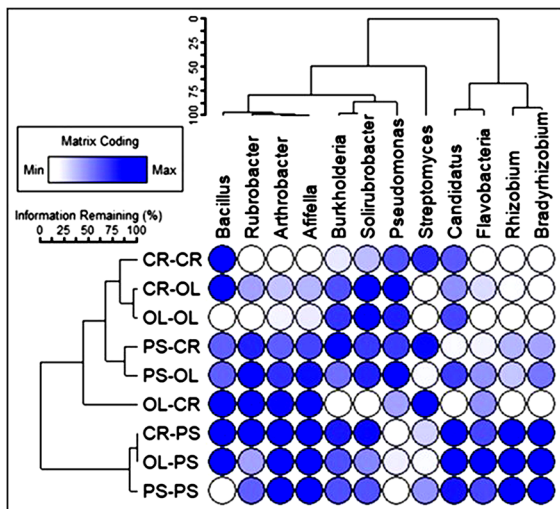
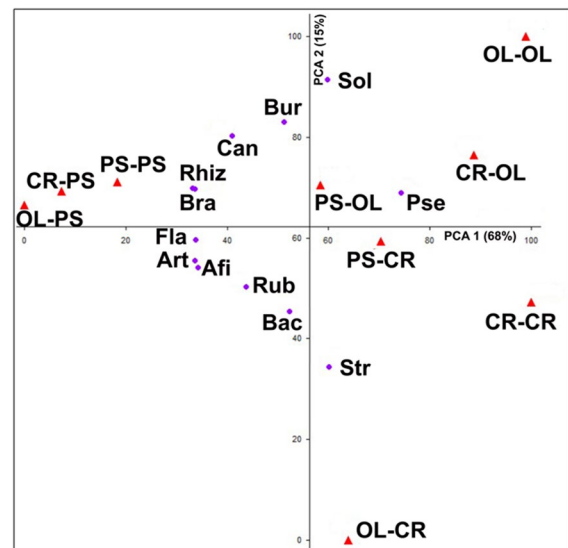
Pulses in the cropping sequences increased the relative abundance of *Rhizobium*, *Bradyrhizobium*, *Candidatus*, *Flavobacterium*, and *Arthrobacter* (Table 2). The abundances of *Rhizobium* and *Bradyrhizobium* were most affected ( $p \leq 0.01$ ). Additionally, the presence of cereals in the cropping sequences enhanced the abundance of *Streptomyces* ( $p \leq 0.05$ ), whereas *Solirubrobacter* and *Pseudomonas* were more prevalent when an oilseed was added to the cropping sequence ( $p \leq 0.05$ ) (Table 2).

Of all the 16S reads from 37 bacterial genera including one uncultured Candidatus taxa ( $> 1\%$  of the relative abundance), the top 12 taxa produced

**Table 2** The  $p$  values from the GLM PROC MIXED ANOVA (SAS) for the effects of crop types in sequences on the relative abundance of fungi and bacteria detected in the rhizosphere and bulk soils of nine cropping sequence treatments

$\beta$ Source of variation/Crop type	$p$ value																
	Percent relative abundance of soil microbiomes (fungi and bacteria)																
	Fungal pathogenic genera						Fungal non-pathogenic genera			Bacterial non-pathogenic genera/taxa							
	FUS	UST	BOT	COC	LEP	ASC	MOR	FUN	DIV	CAN	RHI	BRA	FLA	SOL	ART	PSE	STR
Cereal (CR)	**	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	*
Pulse (PS)	-	-	*	-	-	*	**	*	*	*	**	**	*	-	*	-	-
Oilseed (OL)	-	-	-	-	*	-	*	-	-	-	-	-	-	*	-	*	-

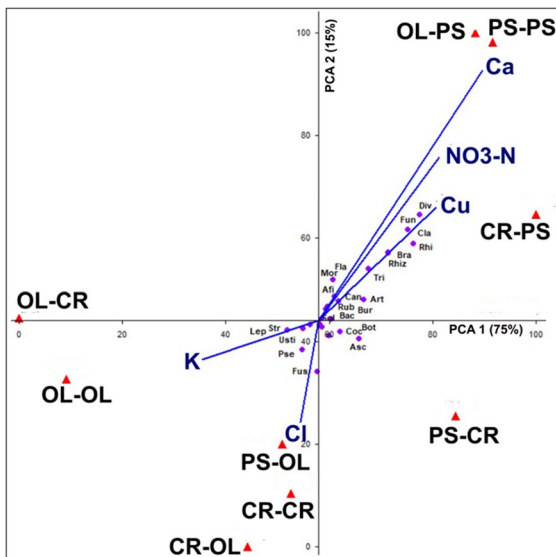
Values with significant effects shown by \* and \*\* are significantly different at  $p \leq 0.05$  and  $p \leq 0.01$ , respectively. The sign (-) indicates non-significant effects. Abbreviations of genera: FUS, *Fusarium*; UST, *Ustilago*; BOT, *Botrytis*; COC, *Cochliobolus*; LEP, *Leptosphaeria*; ASC, *Ascochyta*; MOR, *Mortierella*; FUN, *Funneliformis*; DIV, *Diversispora*; BAC, *Bacillus*; CAN, *Candidatus*; RHI, *Rhizobium*; BRA, *Bradyrhizobium*; FLA, *Flavobacterium*; SOL, *Solirubrobacter*; ART, *Arthrobacter*; PSE, *Pseudomonas*; and STR, *Streptomyces*. Relative abundance refers to the percent (%) relative abundance of the ITS (fungi) and 16S (bacteria) sequence reads.  $\beta$ Source of variation refers to the effect of cropping sequences (groups) on the abundance of soil microbiomes in 2021 soil samples. GLM proc mixed model ANOVA with Type III expected mean squares (EMS) was applied. The crop types (CR, PS, and OL) sequence effects (the 2021 sampling year) were fixed factors and separated from the random effects (block and 2020 crop types sequence). The Least Significant Difference (LSD) was also used to rank the fixed factors (CR, PS, and OL) against each of the variables (microbial abundance). Details of the mixed model for GLM proc ANOVA and EMS are shown in Table S8.

**Fig. 4** Hierarchical clustering (heatmap) showing the effect of cropping sequence on the abundance of the bacterial communities in soil. cropping sequence (2020–2021) abbreviations: CR, cereals; OL, oilseeds; and PS, pulses. The dendrograms depicting the hierarchical clustering of cropping sequence treatments/bacterial genera/taxa are based on the abundance of bacterial communities using the arithmetic mean of unweighted pair groupings**Fig. 5** Principal component analysis (PCA) using Bray-Curtis dis(similarity) shows the clustering pattern of the top 12 most abundant bacterial genera affected by the cropping sequence treatments (2020–2021 cropping seasons). The percentages of variance explained by PCA axes 1 and 2 are each shown in parentheses. Crop type abbreviations: CR, cereals; OL, oilseeds; and PS, pulses. Bacterial genera abbreviations: Bac, *Bacillus*; Can, *Candidatus*; Rhiz, *Rhizobium*; Bra, *Bradyrhizobium*; Fla, *Flavobacterium*; Sol, *Solirubrobacter*; Rub, *Rubrobacter*; Bur, *Burkholderia*; Art, *Arthrobacter*; Pse, *Pseudomonas*; and Str, *Streptomyces*

43.3% of the sequence reads; 17.5% of reads were for unclassified sequences at the bacterial genera level (Table S4).

Relationship between cropping sequences, soil mineral chemicals, and microbiomes

To gain a better understanding of the interaction among soil nutrients, the soil microbial community, and cropping sequences, we measured macro- and micronutrients in soil samples representing the nine cropping sequences (Table S5) and determined correlations with the abundance of fungal and bacterial



**Fig. 6** A biplot ordination plot derived from a principal coordinate analysis (PCoA) using Euclidean dissimilarity showing the clustering pattern of soil nutrients and soil fungal and bacterial communities (genera level) as observed under nine cropping sequence treatments. The percentages of variance explained by axes 1 and 2 are shown in parentheses. Each point represents the average of three replicate samples per treatment. Blue lines in PCoA indicate the strength and direction of the association between treatments, microbial communities, and soil chemical parameters (nitrate-nitrogen/NO<sub>3</sub>-N, potassium/K, calcium/Ca, chlorine/Cl, and copper/Cu). The joint plot cutoff and vector scaling were 0.40 and 90%, respectively. Crop type abbreviations: CR, cereals; OL, oilseeds; and PS, pulses. Fungal genera abbreviations: Fus, *Fusarium*; Ust, *Ustilago*; Bot, *Botrytis*; Coc, *Cochliobolus*; Asc, *Ascochyta*; Lep, *Leptosphaeria*; Mor, *Mortierella*; Fun, *Funneliformis*; Tri, *Trichoderma*; Rhi, *Rhizopagus*; Cla, *Claroideoglomus*; and Div, *Diversispora*. Bacterial genera abbreviations: Bac, *Bacillus*; Can, *Candidatus*; Rhiz, *Rhizobium*; Bra, *Bradyrhizobium*; Fla, *Flavobacterium*; Sol, *Solirubrobacter*; Rub, *Rubro-bacter*; Bur, *Burkholderia*; Afi, *Affifella*; Art, *Arthrobacter*; Pse, *Pseudomonas*; and Str, *Streptomyces*

**Table 3** Spearman’s rank correlation coefficients ( $\rho$ ) between soil chemical parameters and relative abundance of soil fungal and bacterial taxa

Soil Parameter	NO <sub>3</sub> -N	K	Ca	Cl	Cu
Non-pathogenic fungi					
<i>Mortierella</i>	0.87**	-	-	-0.89**	0.70*
<i>Rhizophagus</i>	0.74*	-	-	-	-
<i>Claroideoglomus</i>	-	-0.72*	-	-	-
Non-pathogenic bacteria					
<i>Candidatus</i>	0.91**	-	0.86**	-0.78**	-
<i>Rhizobium</i>	-	-	0.73*	-	-
<i>Bradyrhizobium</i>	0.70*	-0.69*	0.76*	-	-

Values with significant correlations shown by \* and \*\* are significantly different at  $p \leq 0.05$  and  $p \leq 0.01$ , respectively. The sign (-) indicates non-significant correlation coefficients. The nonparametric Spearman rank correlation coefficient ranges from negative (-1 to 0) to positive (0 to 1). Soil property abbreviations: NO<sub>3</sub>-N (nitrate-nitrogen); K (potassium); Ca (calcium); Cl (chlorine); and Cu (copper).

genera/taxa in soil using PCoA (Fig. 6). Spearman rank coefficient analysis was used to assess whether the association was positive or negative as well as the strength of the association between the two variables (microbes and nutrients) (Table 3).

In the biplot ordination plot derived from the PCoA, the concentrations of NO<sub>3</sub>-N, Ca, and Cu in soil were directly aligned with the PS-PS, OL-PS, and CR-PS and groups of non-pathogenic fungal and bacterial taxa (Fig. 6). However, K and Cl showed a different pattern and were directed toward the PS-OL, CR-CR, CR-OL, and OL-OL and groups of pathogenic fungal and bacterial taxa.

According to the Spearman rank coefficient (Table 3), *Mortierella* was positively associated with NO<sub>3</sub>-N ( $p \leq 0.01$ ,  $r = 0.87$ ) and Cu ( $p \leq 0.05$ ,  $r = 0.70$ ) but negatively correlated with Cl ( $p \leq 0.01$ ,  $r = -0.89$ ). *Rhizophagus* was positively affected by NO<sub>3</sub>-N ( $p \leq 0.05$ ,  $r = 0.74$ ), whereas *Claroideoglomus* was negatively affected by K ( $p \leq 0.05$ ,  $r = -0.72$ ). Nitrate-nitrogen (NO<sub>3</sub>-N) was positively associated with *Bradyrhizobium* ( $p \leq 0.05$ ,  $r = 0.70$ ) and *Candidatus* ( $p \leq 0.01$ ,  $r = 0.91$ ). Positive correlations were found between Ca and *Candidatus* ( $p \leq 0.01$ ,  $r = 0.86$ ), *Rhizobia* ( $p \leq 0.05$ ,  $r = 0.73$ ), and *Bradyrhizobium* ( $p \leq 0.05$ ,  $r = 0.76$ ), and negative correlations between Cl and *Candidatus* ( $p \leq 0.01$ ,  $r = -0.78$ ) and between K and *Bradyrhizobium* ( $p \leq 0.05$ ,  $r = -0.69$ ) (Table 3).

## Discussion

### Soil microbial diversity

To date, evidence regarding the impact of cropping sequence/rotation on above- and below-ground microbial diversity is conflicting. Among 27 studies on the effect of crop diversity on soil microbial populations, 14 report a neutral effect on microbial diversity and nine report a positive effect (Venter et al. 2016). In our study, most cropping sequences resulted in higher bacterial OTU richness in soil compared to monoculture production (Table 1), suggesting monoculture production reduces bacterial diversity and/or cropping sequence has a positive effect on bacterial diversity in soil. Woo et al. (2022) show continuous pulse-pulse monoculture results in a lower level of bacterial OTUs in soil compared to a cereal-pulse (wheat-pea) cropping sequence and fallow treatment after wheat monoculture. On the contrary, low pulse frequency rotations (pea-wheat-wheat-wheat) are reported to significantly increase the bacterial OTU richness in the rhizosphere or bulk soil compared to high pulse frequency rotations (pea-pea-pea-wheat) (Yang et al. 2021).

OTU richness and phylogenetic diversity of the fungal community were impacted by cropping sequence in our study. For instance, significantly greater fungal OTU richness, in PS-CR and CR-PS, and fungal phylogenetic diversity, in PS-CR, CR-PS, and PS-OL, were noted compared to monoculture cropping (PS-PS and CR-CR) (Table 1). This result is in slight misalignment with the results of Yang et al. (2021) and Hamel et al. (2018), who found a low pulse frequency (pea-wheat-wheat-wheat) rotation considerably boosted fungal OTU richness in the rhizosphere or bulk soil compared to a high pulse frequency rotation (pea-pea-pea-wheat). Those authors suggest the number of fungal OTUs (richness) may decline as the frequency of pulse rotation increases. The discrepancy between their results and ours with respect to the effect of crop rotation on soil fungal diversity is likely due to the difference in sampling techniques; they evaluated fungal diversity based on the fungal endophyte community present in wheat roots (Hamel et al. 2018; Yang et al. 2021), whereas the diversity and relative abundance of the soil microbial community were calculated in this study by combining microbial sequences from bulk and

rhizosphere soil DNAs. Additionally, the results for microbial diversity in our study were obtained from 2-year cropping sequences, whereas Hamel et al. (2018) and Yang et al. (2021) conducted a 4-year rotation study.

### Effect of cropping sequence on soil pathogenic fungal genera

*Fusarium* was the predominant pathogenic fungal genus in soil and its abundance was strongly impacted by crop rotation. The quality and types of organic carbon inputs, such as the vegetative debris of cultivated crops, are well known to affect the availability of nutrients for soil microbes (Woo et al. 2022). In the Canadian prairies, small grain cereals, including wheat and barley, have been intensively cultivated for the past century, and *Fusarium* head blight, mainly caused by *F. graminearum*, is the most important disease related to these crops. Therefore, the high level of *Fusarium* in the soils observed in our study is not surprising. The occurrence of *Fusarium* head blight and other cereal diseases at two study sites and years are partially shown in the Supplemental Tables S6–S8 and further detailed in a graduate dissertation (Oviedo Ludena 2022) submitted to the University of Saskatchewan.

Planting OL or PS after CR in the cropping sequences decreased the abundance of *Fusarium* in the soil compared to the CR-CR monoculture (Fig. 2A and Table S2). This result agrees with the outcome of previous rotation studies for managing *Fusarium* head blight, in which crop rotation with hosts susceptible to *F. graminearum* often leads to an elevated inoculum level and higher disease severity (Dill-Macky and Jones 2000; Karlsson et al. 2021). These results suggest cereals are the key crop type that promotes *Fusarium* populations in Canadian prairie soils, and *Fusarium* inoculum present in the cereal residues from the previous growing season plays a much bigger role in building up the *Fusarium* population in the soil than the infected root tissues of pulse and oilseed crops.

The presence of a susceptible host in the cropping sequences also has a similar effect on the abundance of *Leptosphaeria*, *Botrytis*, *Cochliobolus*, and *Ascochyta* in soil. For example, *Leptosphaeria* was most abundant in the OL-OL monoculture. The cropping sequences of OL after CR or PS resulted in the

reduction of *Leptosphaeria* (Fig. 2A and Table S2). In Kutcher et al. (2013), the frequency of canola in the rotation affected blackleg caused by *L. maculans*, with the severity of infection and amount of infected residue increasing as the use of canola in the rotation intensified.

Many oomycetes such as *Pythium* and *Aphanomyces* are important soil-borne plant pathogens that cause devastating diseases in prairie pulse crops such as pea. The fungal-specific universal primers (ITS1/ITS4) employed in this experiment lacked specificity or had low sensitivity to detect oomycete species (phylogenetic tree, Fig. S1). Taheri et al. (2017) effectively sequenced pea root rot pathogens (*A. euteiches*) in soil metagenomic samples from Alberta using oomycete-specific ITS-targeted primers (modified ITS6/ITS7). Both of our study sites experienced extremely dry conditions during the study years (2020–2021). This could have significantly reduced oomycete propagules because the flagellated zoospores of oomycetes require soil moisture to swim and infect plant roots.

#### Effects of cropping sequence on soil non-pathogenic fungal and bacterial genera

Canola in the cropping sequence had a negative effect on fungal genera of AMF (Fig. 2B and Table S2). AMF are beneficial soil microbes due to their ability to improve nutrient uptake of host plants and protect plants against multiple plant pathogens (Azcón-Agular et al. 1996). Glucosinolates secreted by the roots of brassica crops, including canola, can inhibit the establishment of symbiosis between AMF and plants (Glenn et al. 1988) because brassica roots lack a diffusible stimulant critical for the hyphal proliferation of AMF in the rhizosphere and the colonization of AMF.

The incorporation of pulses in the cropping sequence had positive effects on the abundance of several N-fixing bacteria, including *Rhizobium* and *Bradyrhizobium* (Fig. 4 and Table S2). It is well established that soil microbiota can be actively selected by the root exudates produced by host plants. These root exudates, which differ in quantity and chemical composition, will affect the structure and abundance of the associated microbes (Hu et al. 2018). Becard et al. (1995) demonstrate that flavonoids in pulse root exudates can activate the symbiotic genes in *R. meliloti*

that are essential for root nodulation and simultaneously stimulate the root colonization of mycorrhizal fungi. Bais et al. (2006) also show isoflavones produced by soybean roots increase the abundance of beneficial *Bradyrhizobium* species in soil.

#### Effects of cropping sequence on pathogen-suppressive soil microbes

Several studies show crop diversity can lead to disease suppression in agricultural soils by fostering antagonistic soil microbial communities, particularly biocontrol and growth-promoting symbiotic bacterial or fungal groups (van der Putten et al. 2016; Peralta et al. 2018). We observed an association between pulses in the cropping sequences and the abundance of *Mortierella* in the soil (Table 2). Furthermore, the inclusion of cereals and oilseeds in the cropping sequences affected the abundance of *Streptomyces* and *Pseudomonas*, respectively. *Mortierella* is a common soil fungus that has been detected in the rhizosphere and bulk soils of many plants (Zhang et al. 2011; Tong et al. 2021). *Mortierella* species have been related to soil disease inhibition, such as those caused by *Fusarium* pathogens (Wang et al. 2022; Zhu et al. 2022). Both *Streptomyces* and *Pseudomonas* communities contain promising biocontrol agents that can suppress the growth of various plant pathogens (Lahlali et al. 2022).

*Streptomyces* as a fungal endophyte can colonize above-ground parts of cereal plants (Patel et al. 2022) and is enriched in the root microbiomes of various cereal crops (Newitt et al. 2019). Some *Pseudomonas* strains can colonize the roots of canola and enhance root elongation, which increases their survivability in the rhizosphere (Pallai et al. 2012). Although a few *Pseudomonas* species, including *P. syringae*, have been identified as plant pathogens (Passera et al. 2019), our sequencing approach did not detect any OTUs that represented *P. syringae*. Instead, we detected *P. fluorescence* which is frequently used as a biocontrol inoculant to suppress several plant pathogens (OTU-95; see phylogenetic tree, Fig. S2). The majority of the OTUs were not identified up to the species level, hence it is uncertain whether the bacterial taxa discovered by amplicon sequencing were antagonistic to fungal plant pathogens. In fact, a growing body of research has shown that the manipulated beneficial microbes serve as an indirect layer of

the plant immune system by functioning as a deterrent to pathogen invasion or by developing systemic resistance in plants (Liu et al. 2021; Li et al. 2021). Plants could specifically alter and recruit advantageous microbial communities through root-type-specific metabolic capabilities, favorably modify their rhizosphere microorganisms, and promote the suppression of specific soil pathogens. The exact role of *Mortierella*, *Streptomyces*, and *Pseudomonas* in the inhibition of soil-borne plant pathogens identified in our study is currently unclear and is certainly an interesting area that deserves more investigation in the future.

#### Interactions between soil mineral chemicals and microbes

Soil mineral nutrients are important for the growth and development of both plants and soil non-pathogenic microbes, as well as to control plant pathogens/diseases (Agrios 2001; Dordas 2008). In our study,  $\text{NO}_3\text{-N}$  and Ca in soils were positively correlated with the abundance of several symbiotic bacterial and fungal genera when pulses were included in the cropping sequences (Table 3; Fig. 6). This result is in line with other studies (Lupwayi 2016; Hamel et al. 2018) that also demonstrate a positive correlation between symbiotic soil bacteria and fungi and nutrients involved in pulse nodulation (e.g., N). Strong evidence suggests the involvement of macronutrients (N, P, K, and S) in the metabolism of nitrogen-fixing bacteria under pulse rotations (O'Hara 2001). Moreover, lignocellulose-degrading fungi, including *M. isabelline* and *T. harzianum* can enhance the concentrations of several macronutrients in the soil by decomposing crop roots and residues (O'Hara 2001; Wachowska and Rychcik 2023).

A surprising finding was that potassium was negatively correlated with the abundance of several fungal (*Claroideoglomus*) and bacterial (*Candidatus* and *Bradyrhizobium*) taxa (Table 3). Potassium is an essential nutrient for plant growth and plays an important role in supporting the growth of soil microbes. Zhang et al. (2021) show potassium selectively stimulates the growth of AMF, which may be due to its compensation for Na-induced ionic imbalance. Potassium is a naturally abundant element in soil but can be present in an inaccessible form due to the binding of the potassium cation ( $\text{K}^+$ ) to negatively

charged soil particles, rendering it unavailable to plants and soil microbes. Potassium cation fixation in the soil is frequently caused by shifts in soil pH (Sparks and Huang 1985; Akinsanmi et al. 2006). The negative correlation between potassium and specific soil microbes is likely due to indirect effects caused by different rotation treatments, which result in changes in soil pH or an abundance of microbes that can solubilize potassium in soil.

To date, information on the effects of Cl on soil microbiomes has been very limited. In Truchado et al. (2018), the relative abundance of bacterial communities (Pseudomonadaceae and Enterobacteriaceae) in soil decreased when low residual chlorine dioxide ( $\text{ClO}_2$ ) concentrations were used to treat irrigation water. On the other hand, continuous chlorine treatment had no effect on soil microbial structure and composition (Yu et al. 2023). In our study, Cl concentrations were negatively correlated with the abundance of *Mortierella* and *Candidatus* (Table 3). This result suggests the effectiveness of Cl on soil microbes is non-specific, i.e., Cl could have negative effects on both pathogenic and beneficial soil microbes.

#### Soil sampling strategy, limitation on amplicon-sequence classification and future research direction

Microbes are less active in bulk soil but exhibit greater activity in the rhizosphere, also referred to as a microbial hot spot. However, the diversity of microbes in bulk soil may be more significant as the resource library for the microbiome in the rhizosphere (Schloter et al. 2018). Recent studies (Yang et al. 2021; Woo et al. 2022) showed soil microorganisms in the bulk and rhizosphere compartments responded differently to crop rotation. During soil metagenome analysis, it is critical to comprehend the combined responses of microorganisms present in the soil matrix and the bias inherent to soil metagenome processing (Lombard et al. 2011). In this study, we aim to investigate the effect of cropping sequence on overall microbial populations in soil, including both rhizosphere and bulk soil microbiomes. The tillage operations carried out during each cropping season (after harvest or before seeding) also broke up the hardpan and mixed microorganisms from all soil compartments. So, combining the responses

of microorganisms from bulk and rhizosphere soils rather than evaluating the microbiome in the two types of soil samples individually is likely more suitable for our objective. Therefore, the microbial sequences obtained from the bulk and rhizosphere soils in each cropping sequence were combined in our analysis to estimate the overall microbial diversity and relative abundance.

In pathogen-suppressive soil metagenomic investigation, it is crucial to identify plant pathogens down to the species level. In our study, the majority of fungal OTUs (70%) and a minority of bacterial OTUs (20%) matched up to species levels of the short-length (400–500 bp) Illumina (ITS and 16S) sequences (Figs. S1 and S2). There is no benchmark available for species- or genus-level identification of highly variable ITS or 16S rRNA gene regions with short-read classification methods (Porter and Brian 2011). For example, we identified four OTUs belonging to different *Fusarium* species that were highly abundant in the ITS data set, such as OTUs 28 (*F. venenatum*), 25 (*F. graminearum*), 27 (*F. equiseti* or *falcatum*) and 29 (unidentified *Fusarium* spp.) (Figs. S1 and S2). We also provided a complementary data set containing *Fusarium* species culture isolates (*F. graminearum*, *F. poae*, *F. avenaceum*, and *F. sporotrichioides*) isolated from experimental soil and tissue samples and identified by PCR with species-specific primers and morphological methods (Table S8). A few more plant pathogenic species (*Cochliobolus sativus* and *Pyrenophora teres*) were also isolated from experimental plots. This culture-dependent identification supported the presence of the aforementioned plant pathogenic fungal species in the ITS data set. Most 16S sequences, however, were only classified to the genus level, which restricted our ability to comprehend the abundance of bacterial plant pathogens or biocontrol species present in metagenomic samples. To overcome the above limitation, qPCR or dd-PCR-based analysis will be needed to validate the abundance of soil microbes at the species level. Third-generation sequencing techniques (such as PacBio, single-molecule real-time/SMRT, and nanopore), which generate longer contigs, more complete genes, and better genome binning than short-reads next-generation sequencing, can also assist in the analysis of soil microbes at the species level. These long-read DNA sequencing techniques effectively classify plant pathogens at species or strain levels in soil metagenomic

analysis (Eenjes et al. 2021; Johnson 2023). Furthermore, unique molecular identifiers (UMIs) and appropriate bioinformatic classifier approaches can also minimize error-prone short and long-read sequences and increase resolution in the analysis at the species level (Tedersoo et al. 2021).

## Conclusion

Our study demonstrated that a two-year cropping sequence can alter the structure and diversity of soil bacteria and fungi. The crop species in cropping sequences differentially impacted pathogenic and beneficial-symbiotic soil microbes. Additionally, the concentrations of soil mineral chemicals, including  $\text{NO}_3\text{-N}$ , K, Ca, Cl, and Cu, also influenced the abundance of soil microbes. These findings highlight the consequences of crop species selection in cropping sequences and their effects on the management of agrochemicals in the agricultural production system. Further investigation into the functional metagenomics network connecting root metabolites, important pathogens, keystone symbiotic taxa, and pertinent soil inputs will provide a better understanding of interactions between crop management practices, soil nutrients, and the function of microbial communities, which is critical for ensuring the sustainability of agriculture production systems.

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**Author contributions** Conception and design: MNI, XW and HRK, Data analysis: MNI, Soil sampling and material preparation: MNI and MAOL, Investigation: MNI and XW, Resources: HRK, XW and OM, Visualization: MNI, Data curation and validation: XW, Original draft author: MNI, Review and edition: XW, HRK, and OM.

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**Data availability** Sequences are deposited at <https://submit.ncbi.nlm.nih.gov/SUB13789011>.

## Declarations

**Informed consent** All authors have read and agreed to the published version of the manuscript.

**Conflict of interest** The authors declare no conflict of interest.

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