Inter-Kingdom Networks of Canola Microbiome Reveal Bradyrhizobium as Keystone Species and Underline the Importance of Bulk Soil in Microbial Studies to Enhance Canola Production.

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Research Article

Keywords: sequencing, plant microbiota, soil, rhizosphere, roots

Posted Date: July 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-700628/v1>

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Abstract

The subterranean microbiota of plants are of great importance for plant growth and health, as root-associated microbes can perform crucial ecological functions. As the microbial environment of roots is extremely diverse, identifying keystone microorganisms in plant roots, rhizosphere and bulk soil is a necessary step towards understanding the network of influence within the microbial community associated with roots and enhancing its beneficial elements. To target these hot spots of microbial interaction, we used inter-kingdom network analysis on the canola growth phase of a long-term cropping system diversification experiment conducted at four locations in the Canadian prairies. Our aims were: to verify whether bacterial and fungal communities of canola roots, rhizosphere and bulk soil are related and influenced by diversification of the crop rotation system; to determine whether there are common or specific core fungi and bacteria in the roots, rhizosphere, and bulk soil under canola grown in different environments and with different levels of cropping system diversification; and to identify hub taxa at the inter-kingdom level that could play an important ecological role in the microbiota of canola. Our results showed that fungi were influenced by crop diversification but not by bacteria. We found no core microbiota in canola roots but identified three core fungi in the rhizosphere, one core mycobiota in the bulk soil and one core bacteria shared by the rhizosphere and bulk soil. We identified two bacterial and one fungal hub taxa in the inter-kingdom networks of the canola rhizosphere, and one bacterial and two fungal hub taxa in the bulk soil. Among these inter-kingdom hub taxa, *Bradyrhizobium sp.* and *Mortierella sp.* are particularly influential on the microbial community and the plant. To our knowledge, this is the first inter-kingdom network analysis utilized to identify hot spots of interaction in canola microbial communities.

Introduction

Plant subterranean microbiota have often been described as a “black box” [1–4]—a term referring to an inherent complexity of inner workings that makes a system difficult to grasp in its entirety. We can define plant subterranean microbiota as composed of different biotopes, notably: the root interior, the rhizosphere, and the bulk soil. The root and rhizosphere microbiota are highly influenced by the plant. Rhizodeposits recruit root symbionts and shape the microbial community of the rhizosphere [5–7]. Microbial communities shaped by the plant in the rhizosphere and root interior can protect it against pathogens and enhance its growth and production [6, 8–11]. Bulk soil microbiota, while important to plant health, are less influenced by the plant due to their distance from the roots. However, the bulk soil microbiota are the inoculum from which the plant recruits its rhizosphere microbiota, and there is a direct link between bulk soil microbial composition and rhizosphere composition, as a substantial portion of the microbiota of these two biotopes interact [12–14]. In addition, the microbes in the bulk soil can produce volatile compounds that influence plant health and development [13, 15–17].

Due to their proximity, the biotopes of the plant subterranean microbiota should influence each other, and their microbes should interact. Microbial co-occurrence network analysis is a tool increasingly used to apprehend the complexity of microbial dynamics in plant and soil ecosystems [18–23]. Analysis of these networks helps identify microbial taxa of ecological interest, particularly those linked to other members of the microbial community: the hub taxa [24–28]. Hub taxa can modulate, via their shared interactions, the composition and diversity of the plant microbiota, affecting agronomic production, plant growth and productivity [25, 29, 30].

Network analysis at the inter-kingdom level can reveal different microbial dynamics that are complementary. Fungal and bacterial communities interact with each other, and, considered as a whole, can be used to identify hub taxa at a higher level of ecological complexity than previously reported [26, 31].

In this study, our targeted plant is canola (*B. napus*), a highly valuable crop grown by producers across the Canadian Prairies [32, 33]. Most studies about canola microbial ecology have targeted the root interior and the rhizosphere [27, 28, 34–37]. Little is known about the ecology of canola bulk soil microbiota or their overlap with root and rhizosphere microbiota. Within these three biotopes, aside from the hub taxa, other microbes may be ecologically important. Floc'h et al., (2020a) and (2020b) reported several fungi and bacteria in the canola rhizosphere that were always present despite variations in crop rotation and environmental conditions. These organisms, also reported in canola roots by Lay et al., (2018a) were identified as core fungi and core bacteria. According to Vandenkoornhuyse et al., (2015), the ever-present taxa associated with a given plant form its core microbiome and have a preferential interaction with their host.

We aimed to identify hub taxa and universal components of the core microbiota in canola roots, rhizosphere, and bulk soil. We used a gradient of crop diversification levels to create variability in these biotopes. Building on Floc'h et al., (2020a) and (2020b) who identified hub taxa in the fungal and bacterial fractions of the canola rhizosphere microbiota, respectively, we aimed to consider the canola subterranean microbiome as a whole in order to identify core fungi, core bacteria, and several inter-kingdom hub taxa that could be of ecological importance for canola health and production.

Material And Methods

Experimental Design and sampling

Our study was conducted in 2018 using a subset of plots from a long-term experiment initiated in 2008. The experiment, which was replicated at four locations in the Canadian Prairies, tested the effects of diversification in canola-based cropping systems, with all rotation phases present each year. Our study used the canola phases of three crop rotation systems. The canola grown was the glufosinate-tolerant variety L241C. The three crop diversification treatments used in this study were: (1) monoculture of canola, (2) wheat-canola, and (3) pea-barley-canola (Table 1). These treatments were applied in a randomized complete block design with four blocks at each of four experiment sites.

Table 1
Selected crop diversification treatments from a long-term experiment established in 2008 at three different sites in the Canadian prairies [39]. The rotation phases examined in this study in 2018 are underlined.

Cropping systems	
Diversification level	2008–2018
Monoculture	C ¹ -C-C-C-C-C-C-C-C-C
Low	C-W-C-W-C-W-C-W-C-W-C
Medium	P-B-C-P-B-C-P-B-C-P-B-C
¹ C. canola; W. wheat; P. pea; B. barley.	

The four experiment sites were located in three pedoclimatic zones of the canola-producing regions of Canada. Two sites were in Alberta: one in Lacombe (lat. 52.5°N, long. 113.7°W) and the other in Lethbridge (lat. 49.7°N, long. 112.8°W), and two were in Saskatchewan, in Melfort (lat. 52.8°N, long. 104.6°W) and Swift Current (lat. 50.3°N, long. 107.7°W). Crops were grown according to best management practices, as described in Harker et al., (2015). With the exception of the Lethbridge site, the growing season at all sites was characterized by more frequent rain in July just before sampling (Figure S4).

Root, rhizosphere, and bulk soil samples were collected at the end of the canola flowering period, at full flower after 50% of flowers on the raceme had opened. This occurred in the fourth week of July 2018. Three to four plants within each plot were randomly selected and uprooted with a shovel. The shoots were removed, and roots were placed in plastic bags and brought to the laboratory on ice in a cooler. About 5 g of rhizosphere soil per plot was collected by gently brushing the roots. The brushed roots were then gently washed with sterilized distilled water. The bulk soil was sampled with a 2 cm diameter soil probe at a 7 cm depth, exactly in between two seed rows. The samples were kept at 4°C before being shipped on ice to the laboratory in Québec City, Québec, where they were preserved at -80°C until DNA extraction.

More details on site description, experimental design and sampling methods are provided in Floc'h et al. (2020a).

DNA extraction and PCR amplification

DNA extraction was conducted as described in Floc'h et al. (2020a) with a QIAGEN DNeasy extraction kit. We constructed amplicon libraries for fungal ITS sequences by using target-specific PCR primers attached to Illumina overhang sequences for Nextera library preparation. The primer pairs were ACACTGACGACATGGTTCTACTTGGTCATTTAGAGGAAGTAA (ITS1F-Illu) and TACGGTAGCAGAGACTTGGTCTCTGCGTTCTTCATCGAT (5.8A2R-Illu). Each 25-µL PCR reaction consisted of 0.10 µL of forward and reverse primers, with 19.6 µL H₂O, 2.5 µL 25 mM MgCl₂, 12.5 µL KAPA HiFi Hotstart ReadyMix (Kapa Biosystem, Cape Town, South Africa) and 1 µL of sample DNA. The reaction conditions were as follows: 95°C for 5 min, 25 cycles of 94°C for 45 sec, 52°C for 60 sec, and 72°C for 30 sec with a final extension at 72°C for 7 min. PCR products were verified by electrophoresis on 1% agarose gels. Dual Nextera

indices were then attached to PCR products based on the protocol “16S Metagenomic Sequencing Library Preparation” provided by Illumina (part no. 15044223 rev. B). The final purified product was quantified by Qubit Fluorometric Quantitation (ThermoFisher Scientific, Waltham, MA). Libraries were pooled in equimolar amounts before sequencing in rapid paired-ends 250 bp (PE250) mode on an Illumina MiSeq system, using the 500-cycle MiSeq reagent kit v.2 in accordance with the manufacturer’s recommendations.

Amplicon libraries for bacterial 16S rRNA gene sequences were constructed by using target-specific PCR primers attached to Illumina overhang sequences for Nextera library preparation. The primer pairs were GTGCCAGCMGCCGCGGTAA (515F-Illu) and GGACTACHVGGGTWTCTAAT (806R-Illu). This primer set was selected because it is used by the Earth Microbiota Project (<http://www.earthmicrobiota.org/emp-standard-protocols/16s/>). Two PCR reactions were performed to prepare the amplicon library. In the first PCR reaction, the V4 hypervariable region of prokaryotic 16S RNA genes was amplified using the previously described primers (515F and 806R). The PCR reaction was performed in a 25-µL reaction mixture containing 1 µL of template DNA, 1 × PCR-buffer (Qiagen, Germantown, MD, USA), 1.8 mM MgCl₂, 1.25 µL of 5% dimethylsulfoxide (DMSO), 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Roche, Branford, CT, USA), and 0.6 µM of each primer. The 5’ ends of the forward and reverse primers were tagged with CS1 (ACACTGACGACATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTTGGTCT), respectively, which were used as anchors for the second PCR reaction. The conditions to amplify the prokaryotic 16S rRNA fragments consisted of an initial denaturation at 94°C for 2 min, 33 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 30 s, followed by a final elongation at 72°C for 7 min.

The second PCR reaction was used to add barcodes to each sample and the Illumina sequencing adapters. This PCR reaction was performed in a 20-µL reaction mixture, containing 1× PCR-buffer (Qiagen, Germantown, MD, USA), 1.8 mM MgCl₂, 1 µL of 5% DMSO, 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Roche, Branford, CT, USA), 2 µM of Nextera XT index primers (Illumina Inc., San Diego, CA, USA), and 1 µL of 1/150 dilution of the first PCR products. The PCR conditions were as follows: initial denaturation at 95°C for 10 min, 15 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 1 min followed by a final elongation at 72°C for 3 min. After the second amplification, PCR products were quantified using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Canada) and a Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (D-Mark, Canada). The amplicon library was purified using calibrated AMPure XP beads (Agencourt Bioscience, Beverly, MA), and the average size and quantity of each library were assessed on the LabChip GX (Perkin Elmer, Waltham, MA) instrument. The library was then sequenced on Illumina MiSeq using the paired-end 250 protocol at the Genome Québec Innovation Centre of McGill University (Montréal, Canada).

ASV determination and bioinformatic pipeline

Bioinformatics were used in QIIME2 environment version 2021.4 [40]. The bioinformatic pipeline used for the processing of our ITS and 16S rRNA gene sequences was DADA2 v1.18.0 [41]. First, we used Cutadapt 3.4 to remove the primer part of the ITS and 16S rRNA gene sequences with “minimum-length” at 50 and “p-error-rate” at 0.1. Then, we excluded the sequences with less than 220 bp with the command “-p-trunc-len”, as the base quality of the sequences tended to diminish below that threshold in our data. Next, the amplicon sequence variant (ASV) table was calculated, and chimeras eliminated, resulting in a sequence length ranging from 250 to 253 nucleotides. ASVs were then identified using the naïve Bayesian classifier method on the databases SILVA and RDP, and the identities of ASVs of interest were verified manually using BLAST on the NCBI nt

database. With the taxonomic resolution of the ITS and 16S RNA gene, it is generally not possible to identify a bacterium or fungus at the species level. Thus, the identifications at species level presented here must be considered with caution despite the fact that they perfectly match (100% similarity and coverage) the NCBI reference sequences.

The MiSeq sequencing data generated as part of this work are publicly available on Zenodo (<https://doi.org/10.5281/zenodo.5028181>).

Data processing and statistical analyses

To assess the variation induced in the canola rhizosphere by crop diversification systems, the datasets were filtered from their rare ASVs using QIIME2 with “-p-min-frequency” set to 17 and “-p-min-samples” set to 1.

The effect of crop diversification on bacterial and fungal community structure was assessed by permutational multivariate analysis of variance (PERMANOVA) [42], considering 16 blocks (four blocks per each of the four sites), using the function “adonis” of the vegan package v2.5.7 [43] in R v4.1.0, and the set of relative abundance data.

We then aimed to identify the universal fungal and bacterial components of the core microbiota and hub taxa in each biotope of the canola subterranean microbiome. We defined the core microbiota of a biotope as the set of microbial ASVs that were present in the microbiota of a particular biotope at all sites and plots. To assess the interactions among bacterial and fungal taxa in the microbiota, we created a co-occurrence inter-kingdom network using the package Spiec-Easi v1.1.0 in R 4.1.0 [21]. The analysis incorporated the roots, rhizosphere, and bulk soil fungal and bacterial community. The input data consisted of the raw abundance matrixes of the ITS and 16S ASVs. We first filtered the datasets to remove ASVs with a frequency lower than 20%. The Spiec-Easi run was conducted with the algorithm “mb” with the lambda min ratio set at 10^{-2} and 50 repetitions. We then imported the networks into Cytoscape 3.8.2 [44] for plotting and used the “organic” layout to draw the networks. Edges were defined as co-occurrences or mutual exclusion based on the positive or negative values of inverse covariance linking the nodes. Betweenness centrality, defined as the fraction of the shortest path between all other nodes in the network containing the given node, and degree score, highlighted central nodes and provided information about network architecture. A score of betweenness centrality and degree of connectivity greater than 95% of the network taxa suggested participation in multipartite interactions in the community and allowed us to flag the highly connected taxa as hub taxa.

Results

Bioinformatic yield and taxonomic profile of the microbiota of canola roots, rhizosphere, and bulk soil

Sequencing yielded 10,118,613 ITS and 10,273,048 16S reads. Our bio-informatic pipeline retained 6,934,809 (68.03%) non-chimeric ITS-reads and 7,157,985 (68.21%) non-chimeric 16S reads after filtering, trimming, denoising and merging. These reads were respectively assigned to 2140 ITS amplicon sequence variants (ASV) and 9814 16S ASV. Rarefaction curves indicated that read abundances reached saturation for all samples (Figure S1).

Fungal ASV in bulk soil belong mainly to three families: Chaetomiaceae (~ 16%), Mortierellaceae (~ 13%) and Nectriaceae (~ 11%) (Fig. 1D). In the rhizosphere, the most dominant fungal families were Olpidiaceae (~ 24%), Chaetomiaceae (~ 12%), Mortierellaceae (~ 10%) and Nectriaceae (~ 10%) (Fig. 1C). In the roots, the most dominant fungal families were Olpidiaceae (~ 54%) and Glomeraceae (11%) (Fig. 1B). Unknown Ascomycota were abundant in the bulk soil (~ 20%), rhizosphere soil (~ 13%), and roots (~ 14%) (Fig. 1B).

The most abundant bacterial higher taxa in the bulk soil were: Actinobacteria (~ 18%), Thermoleophilia (~ 15%) and Alphaproteobacteria (11%). In the rhizosphere, Actinobacteria (~ 24%), Gammaproteobacteria (~ 16%) and Thermoleophilia (~ 14%) were the dominant bacterial higher taxa and in the roots, Gammaproteobacteria (~ 60%), Bacili (~ 19%) and Actinobacteria (13%) were the most abundant (Fig. 2).

Principal coordinate analysis (PCoA) showed a site effect on both ITS and 16S communities (Figure S2 and S3). PERMANOVA thus conducted by site reported a significant effect of cropping system diversification on fungal communities in certain sites and microbiota compartments, but no significant effect of diversification on bacterial communities was detected in any compartment of the subterranean microbiota of canola (Table 2). Fungal communities were significantly affected by crop diversification in canola roots only in Lethbridge; in the rhizosphere at Melfort and Lacombe; and in the bulk soil at Lacombe and Lethbridge.

Table 2

Effects of crop diversification on the structure of Bacterial 16S and Fungal ITS communities in the canola subterranean microbiome. according to PERMANOVA ($\alpha = 0.05$, $n = 12$) for each site.

ITS communities				16S communities			
Scott							
		Root	Rhizosphere	Bulk soil	Root	Rhizosphere	Bulk soil
Factor	Df ¹	P	P	P	P	P	P
Cropping system	2	0.48	0.332	0.282	0.814	0.51	0.138
Residual	9						
Total	11						
Melfort							
Cropping system	2	0.444	0.044*	0.494	0.193	0.418	0.156
Residual	9						
Total	11						
Lacombe							
Cropping system	2	0.535	0.006**	0.035*	0.313	0.724	0.647
Residual	9						
Total	11						
Lethbridge							
Cropping system	2	0.002**	0.206	0.001***	0.252	0.354	0.586
Residual	9						
Total	11						
¹ DF, Degree of Freedom							
“*” = p < 0.05. “**” = p < 0.01 and “***” = p < 0.001, Level of significance.							

Shared microbiota and Core-microbiota of canola roots, rhizosphere, and bulk soil.

The canola roots, rhizosphere, and bulk soil biotopes shared 318 fungal ASV. These ASV represent 59%, 24% and 21% of the fungal communities of the roots, rhizosphere, and bulk soil. Thirty-three fungal ASV representing respectively 5.6% and 2.5% of the root and rhizosphere fungal communities were shared in these ecospheres, but absent in bulk soil. Forty-two fungal ASV found only in the bulk soil and roots made up 7.2% and 2.7% of these fungal communities, respectively. Five hundred and fifty-two fungal ASV representing 42% and 36% of the rhizosphere and bulk soil fungal communities, respectively, were absent in the roots (Fig. 3).

The 2405 bacterial ASV that were shared between the three compartments of the canola subterranean microbiota represented 69.4%, 29.9% and 29.2% of the bacterial communities of the roots, rhizosphere, and bulk soil, respectively. The 453 ASV shared exclusively between canola roots and rhizosphere soil represented 13% and 5.6% of the root and rhizosphere bacterial communities. The 268 ASV shared only between bulk soil and roots represented 7.7% and 3.2% of the roots and bulk soil bacterial communities, respectively. There were 4353 ASV shared exclusively between the rhizosphere and bulk soil. These shared ASV represented 54.2% and 53% of the rhizosphere and bulk soil bacterial communities, respectively (Fig. 4).

We were unable to detect a core microbiota in roots. However, three fungal amplicon sequence variants (FASV) were always present in the rhizosphere of canola, at all sites and regardless of crop rotation specifications. This fungal core microbiota was composed of FASV1 (*Trichocladium sp.*), FASV4 (*Fusarium sp.*) and FASV7 (*Cryptococcus sp.*). Only one fungal ASV, FASV2 (*Fusarium sp.*), was present in the bulk soil sample of all plots. Only one bacterial amplicon sequence variant (BASV) was present in the canola rhizosphere sample of all plots. This BASV, BASV46 (*Marmoricola sp.*), was also the only BASV present in the bulk soil sample of all plots (Table 3).

Table 3
Core fungi and Core bacterium of canola subterranean microbiome.

Core fungi in canola rhizosphere		
ASV	Identity	Confidence score
FASV1	<i>Trichocladium sp.</i>	100
FASV4	<i>Fusarium sp.</i>	100
FASV7	<i>Cryptococcus sp.</i>	100
Core fungi in bulk soil		
FASV2	<i>Fusarium sp.</i>	100
Core bacterium in both rhizosphere and bulk soil		
BASV46	<i>Marmoricola sp.</i>	99

Inter-kingdom network analysis of the subterranean canola microbiota.

Network analysis detected no potential inter-kingdom interaction in canola roots; thus, no network was drawn.

In the rhizosphere, the inter-kingdom network of putative interactions involved 77 ASV sharing 120 edges and included 33 mutual exclusions and 87 co-occurrences (Fig. 5a). This network, formed with the bacterial and fungal abundance datasets, revealed 3 hub taxa (Table 4), namely BASV45 (*Bradyrhizobium sp.*), BASV134 (*Pseudonocardia sp.*) and FASV21 (*Mortierella sp.*). BASV45 (*Bradyrhizobium sp.*) had a betweenness centrality score of 0.25. It was connected to 7 BASV and 3 FASV (Fig. 5b), including the hub taxon FASV21 (*Mortierella sp.*). FASV21 was connected to 6 BASV and 2 FASV (Fig. 5D) and had a betweenness centrality score of 0.25. The third inter-kingdom hub taxon, BASV134 (*Pseudonocardia sp.*), was highly connected with

FASVs (Fig. 5c), with 4 connections to FASV and 4 connections to BASV. BASV134 had a betweenness centrality score of 0.11.

Table 4

Interaction cohorts of inter-kingdom hub-taxa in canola subterranean microbiome. The taxa in bold are inter-kingdom hub-taxa.

Cohort of hub-taxa BASV45 inter-kingdom - Rhizosphere			Cohort of hub-taxa BASV69 inter-kingdom - Soil		
ASV	Identity	Confidence score	ASV	Identity	Confidence score
FASV8	<i>Chaetomium mareoticum</i>	100	FASV1	<i>Humicola nigrescens</i>	100
FASV21	<i>Mortierella sp.</i>	99	FASV5	<i>Ascomycota sp.</i>	89
FASV23	<i>Trichoderma pubescens</i>	99	FASV14	<i>Ascomycota sp.</i>	75
BASV43	<i>Sphingomonas sp.</i>	99.99	FASV25	<i>Alternaria metachromatica</i>	100
BASV60	<i>Bacillus sp</i>	99.89	FASV101	<i>Sordariomycetes sp.</i>	100
BASV77	<i>Micromonosporaceae sp.</i>	72.41	BASV27	<i>Rubrobacter sp.</i>	99.99
BASV84	Jatrophihabitans sp.	92.99	BASV47	<i>Chloroflexi KD4-96</i>	72.03
BASV134	<i>Pseudonocardia sp.</i>	90.75	BASV52	<i>Skermanella sp.</i>	99.99
BASV181	<i>Ilumatobacter</i>	98.24	BASV61	<i>Agromyces sp.</i>	85.99
BASV200	<i>Chitinophagaceae sp.</i>	99.99	BASV109	<i>Vicinamibacteraceae sp.</i>	94.91
Cohort of hub-taxa BASV134 inter-kingdom - Rhizosphere			Cohort of hub-taxa FASV8 inter-kingdom - Soil		
ASV	Identity	Confidence score	ASV	Identity	Confidence score
FASV7	<i>Cryptococcus fuscescens</i>	100	FASV7	<i>Cryptococcus fuscescens</i>	100
FASV9	<i>Mortierella hyalina</i>	98	FASV9	<i>Mortierella hyalina</i>	98
FASV109	<i>Microascales sp.</i>	98	FASV11	<i>Nectriaceae sp.</i>	99
FASV221	<i>Ilyonectria sp.</i>	100	FASV63	<i>Dendryphion sp.</i>	100
BASV43	<i>Sphingomonas sp.</i>	99.99	FASV104	<i>Mortierella sp.</i>	99
BASV45	Xanthobacteraceae sp.	99.99	FASV151	<i>Exophiala equina</i>	100
BASV84	Jatrophihabitans sp.	92.99	FASV160	<i>Tetracladium sp.</i>	98
BASV107	<i>Gemmatimonadaceae sp.</i>	72.1	BASV43	<i>Sphingomonas sp.</i>	99.99
Legends of the figures					

Cohort of hub-taxa BASV45 inter-kingdom - Rhizosphere			Cohort of hub-taxa BASV69 inter-kingdom - Soil		
Cohort of hub-taxa FASV21 inter-kingdom - Rhizosphere			BASV107	<i>Gemmatimonadaceae sp.</i>	72.1
ASV	Identity	Confidence score	BASV158	<i>Vicinamibacteraceae sp.</i>	99.99
FASV151	<i>Exophiala sp.</i>	100	Cohort of hub-taxa FASV114 inter-kingdom - Soil		
FASV286	<i>Mortierella sp.</i>	99	ASV	Identity	Confidence score
BASV7	<i>Micrococcaceae sp.</i>	99.99	FASV4	<i>Ascomycota sp.</i>	100
BASV45	Xanthobacteraceae sp.	99.99	FASV129	<i>Davidiella sp.</i>	100
BASV77	<i>Asanoa sp.</i>	72.41	FASV180	<i>Leotiomyces sp.</i>	100
BASV87	<i>Chitinophagaceae sp.</i>	73.38	FASV196	<i>Lecythophora sp.</i>	100
BASV94	<i>Chloroflexi JG30-KF-CM45</i>	92.33	FASV318	<i>Helotiales sp.</i>	100
BASV151	<i>Gemmatimonas sp.</i>	79.34	BASV9	<i>Pseudonocardia sp.</i>	99.99
BASV200	<i>Chitinophagaceae sp.</i>	99.99	BASV18	<i>Blastococcus sp.</i>	98.89
			BASV52	<i>Skermanella sp.</i>	99.99
			BASV59	<i>Sphingomonadaceae sp.</i>	99.99
			BASV75	<i>Pseudonocardia sp.</i>	99.99
			BASV87	<i>Bacteroidetes sp.</i>	73.38
			BASV484	<i>Iamia sp.</i>	74.13
			BASV962	<i>Rhodanobacteraceae sp.</i>	99.99
Legends of the figures					

In the bulk soil, the inter-kingdom co-occurrence network had the highest complexity, with 96 ASV and 149 edges (Fig. 6a). All three modules of the network were organized around 3 inter-kingdom hub taxa (Table 4). One of these, FASV8 (*Corynascella inaequalis*), shared 7 interactions with FASV and 3 with BASV (Fig. 6c) and had a betweenness centrality score of 0.24. The other two inter-kingdom hub taxa were FASV114 (*Mortierella sp.*) and BASV69 (*Bacterium sp.*). FASV114 shared connections with 5 FASV and 8 BASV (Fig. 6d) and had a betweenness centrality score of 0.29. BASV69 shared connections with 5 FASV and 5 BASV (Fig. 6b) and had a betweenness centrality score of 0.25.

Discussion

Our results support the existence of stable core bacterial and fungal components of the canola rhizosphere and in the bulk soil of canola-producing fields. Inter-kingdom network analysis revealed hub taxa in rhizosphere and bulk soil, but no significant potential interaction was found in canola roots. Hub taxa BASV45 (*Bradyrhizobium* sp.), BASV134 (*Pseudonocardia* sp.), and FASV21 (*Mortierella* sp.) in the rhizosphere and bulk soil hub taxa FASV8 (*Corynascella* sp.), FASV114 (*Mortierella* sp.) and BASV69 (*Bacterium* sp.) are strongly suspected to act as structuring factors in canola microbial communities and could exclude pathogens or enhance plant health.

Canola subterranean microbiota compartmentation and response to crop diversification

Crop diversification is known to increase canola yield by preventing the accumulation of pests and pathogens in soil [27, 28, 39, 45]. We have previously shown the insignificance of the crop diversification effect on the bacterial communities of the canola rhizosphere in the Canadian prairies [28]. In this paper, our results confirm that bacterial communities are insensitive to diversification of cropping systems not only in the canola rhizosphere, but also in its roots and bulk soil (Table 2). This could be explained by the fact that bacteria are more influenced by soil physical properties and weather conditions than by crop rotations [46].

A contrario, the composition of the fungal communities in canola roots, rhizosphere and bulk soil appeared to be sensitive to cropping systems diversification. The sensitivity of the rhizosphere fungal community was previously reported [27, 36]. Geographic location and soil physical properties appear to affect fungal community composition as the crop rotation effect was site-dependant (Table 2).

Crop rotation systems are known to influence microbial community structure in a wide range of crops [47–49]. Crop rotations can be used to modulate biological N-fixation and to modify soil structure, with feedback on microbial communities [50–52]. Changing the fungal microbiota of canola with crop rotation systems could thus be useful for suppressing pathogens or enhancing canola nutrient uptake. The shaping of canola microbial communities must also take into account the environmental conditions, as fungal communities are subject to substantial geographical variations [53–55].

The dominance of Olpidiomyota in the taxonomic profiles of our fungal communities was similar to that previously reported in canola [27, 28, 36, 56]. The dominance of Olpidiomyota in the fungal microbiota of the roots and rhizosphere is mainly due to the abundance of *Olpidium brassicae* [27, 57]. This particularity of a single dominant species spread across the roots and rhizosphere is usually linked to situations of pathogen infestations [58–60] or symbiosis [61] in other crops. The predominance of *O. brassicae* was reported to have little influence on canola yield [27, 57].

In the soil, compartments like roots, rhizosphere and bulk soil are known to show a concentration gradient of plant chemical compounds that attract microbes [5, 7, 62]. Thus, the colonization of these three different niches share certain similarities in terms of the composition of their microbial communities. Cordero et al. (2019) reported 77% similarity between bacterial communities in the roots and rhizosphere of canola. In our case, canola subterranean microbiota were similar in terms of the proportion of bacterial ASV shared between the different compartments, with a significant percentage of shared community between the bulk soil and

rhizosphere (Figs. 3 and 4). The proportion of bacterial species shared between the root interior and rhizosphere of canola (82.4%) was similar to the proportion reported by Cordero et al., (2019). To the best of our knowledge, this is the first report of the percentage of mycobiota shared between canola roots, rhizosphere, and bulk soil communities. We found the same trend in fungi as in bacteria. The fact that a significant part (30 ~ 60%) of the microbial communities are shared between these ecological niches could reflect their physical proximity. The fact that between the three biotopes, bacteria are shared more than fungi could be explained by the presence of filamentous fungi that allow bacteria to migrate following their hyphae, thus allowing a wide range of bacteria to navigate between the compartments.

Canola core-microbes in roots, rhizosphere, and bulk soil.

The canola root interior was devoid of any core fungi or bacteria. This lack of detection could be attributed to the fact that canola roots produce glucosinolates that are toxic to microbial life and lead to limited root colonization by fungi and bacteria [32, 36, 64]. *Olpidium brassicae*, an obligatory endophyte previously reported as a core fungi in canola rhizosphere in the Canadian prairies [27, 36], was present and dominant in canola roots (Fig. 1A) but not enough to be flagged as a core fungus according to our threshold of 99%. This difference between our results and results previously published can be explained by the difference in bioinformatics methods used. In this study, DADA2 allowed us to form ASVs (amplicon sequence variants) that are much more discriminating than the 97% identity threshold used in USEARCH to form OTUs. We can thus identify different genetic variants of the same species as different ASVs in DADA2 (Callahan et al., 2016). This permits more precise inference of the structure and ecology of microbial communities. *Olpidium brassicae* shows significant genotypic variation and similarity with other pathogenic species known to affect canola, such as *O. virulentus* [57]. Its significant genetic variability and abundance in the roots and rhizosphere of canola suggest that *O. brassicae* should be the target of population genetics studies in the near future.

In the rhizosphere, three fungi and one BASV were detected as core microbes: FASV1 (*Trichocladium sp.*), FASV4 (*Fusarium sp.*), FASV7 (*Cryptococcus sp.*) and BASV46 (*Marmoricola sp.*). These three fungi were previously reported by [27] to be part of the canola rhizosphere fungal core microbiota. Paired with the observations reported in the previous article, the findings of the present study illustrate the stability over time of the concept of core microbiota in the canola rhizosphere, reinforcing the need for long term studies with recurrent samplings.

In canola bulk soil, a core fungus and core bacterium were found: FASV2 (*Fusarium sp.*) and BASV46 (*Marmoricola sp.*), respectively. The latter was also present as a core bacterium in the canola rhizosphere. Regarding the former, fusaria are well known commensalists and pathogenic fungi, widely abundant in agricultural soils [65–67]. As no core microbiota has ever been identified in canola bulk soil, these results should be taken with caution. Core fungi and bacterium are subject to variation in presence and abundance over time and depending on weather conditions [27, 28].

We were able to identify hub taxa at the intra- and inter-kingdom levels that are known as inter-kingdom hub taxa (Table 4). Each of these could be of importance for canola production and manipulation of canola subterranean microbial communities.

BASV45 (*Bradyrhizobium sp.*) is a hub taxa that has been linked to other inter-kingdom hub taxa of the canola rhizosphere. *Bradyrhizobium* is a nitrogen-fixing bacterial genus known to nodulate Fabaceae such as soybeans (*Glycine max*), cowpeas (*Vigna unguiculata*), Bambara groundnuts (*Vigna subterranea*) and chickpeas (*Cicer arietinum*) [68–70]. Furthermore, these bacteria demonstrate other ecological functions as plant growth promoting rhizobacteria (PGPR) through hormone secretions and antagonism in non-legume plants [10, 71]. [72, 73], reported *Bradyrhizobium*-induced nodular structures on canola roots. Thus, our detection of BASV45 (*Bradyrhizobium sp.*) as an inter-kingdom hub-taxon in the canola rhizosphere highlights this taxon as a potential PGPR for canola production, and as an agent for community manipulation in canola microbial networks. Indeed, high connectivity microbes are potentially beneficial for the plant, particularly in the rhizosphere [30, 74]. Given how this taxon interacts with other plant species, ASV45 could be an important actor in the canola microbiome.

In the plant rhizosphere, microbes compete for space. One of the mechanisms used to compete with other microbes is the production of anti-microbial compounds [75–77]. Such is the case for the second most connected inter-kingdom hub taxon, BASV134 (*Pseudonocardia sp.*). This genus of actinobacteria is known as an important producer of antibiotics associated with leaf cutter ants [78–80]. This genus can be encountered in a broad range of environments from marine ecosystems to rhizosphere soil [81–83]. BASV134 was negatively connected to FASV221 (*Ilyonectria sp.*), a fungus known to cause root rot in a wide spectrum of hosts, including olive trees, panax ginseng, strawberries and avocados [84–87]. The fact that our network analysis revealed negative connectivity between these two ASVs could indicate a suppressive effect of BASV134 on FASV221 through antibiosis. It could also indicate that these two species target different exclusive ecological niches and, thus, are not frequently associated. Either way, the fact that BASV45 (*Bradyrhizobium sp.*) is negatively linked to BASV134 (Fig. 5C) suggests that *Bradyrhizobium sp.* may have biocontrol abilities in the canola rhizosphere.

The third cross-kingdom hub taxon in the canola rhizosphere was a fungus of the genus *Mortierella* FASV21. *Mortierella* is often reported as a plant growth promoting fungi (PGPF) enhancing plant phosphate nutrition [88–91]. In the canola rhizosphere, *Mortierella* was previously reported as a dominant genus of fungi [27, 36, 92]. FASV21, which was negatively linked to BASV45 (*Bradyrhizobium sp.*), and FASV151 (*Exophiala sp.*), is also a potentially beneficial organism for canola production. FASV151 (*Exophiala sp.*) was reported by [27] to be strongly positively linked to canola yield in the Canadian Prairies. *Exophiala* is a genus of fungi belonging to the dark septate endophytes (DSE) group, which hosts a broad range of plant growth promoting fungi [93, 94]. This mutual exclusion between potentially mutualistic organisms could be explained by these microbes competing for similar ecological niches and may feed on similar rhizodeposits [95–97].

Bulk soil is an important part of plant microbiota, but very few studies of canola-related microbiota have taken bulk soil into consideration [45, 98–100]. Despite the fact that bulk soil is important to plant health and rhizosphere microbiota, most studies of canola-related microbiota have been restricted to root and rhizosphere microbiota. Bulk soil microbial communities emit volatile compounds that can enhance plant growth, protect against pathogens and even influence plant root architecture [13, 15–17]. These microbial volatile compounds also have an influence on the structure of rhizosphere microbial communities [14, 101]. Compared to the rhizosphere microbial network, the bulk soil network showed a higher overall connectivity and a higher number of potential interactions between fungi and bacteria (Fig. 6). The bulk soil network had three modules, each

centered on a hub taxon. In canola bulk soil, we were able to identify three inter-kingdom hub taxa, two fungi and one bacterium: FASV8 (*Corynascella sp.*), FASV114 (*Mortierella sp.*) and BASV69 (*Bacterium sp.*). None of these taxa were linked with each other. This finding, coupled with the modularity of the networks, suggests a strong ecological differentiation between the canola bulk soil hub taxa.

FASV114 (*Mortierella sp.*) was the most connected hub taxon in the network, with a dominance of mutual exclusions. It was negatively linked with *Pseudonocardia* and with FASV129 (*Davidiella sp.*). The latter genus, the teleomorph form of *Cladosporidium*, is known to count among its ranks numerous plant pathogens [102, 103]. As we discussed previously, *Mortierella* can act as a PGPF. Its negative links with potential pathogens reinforce the need to investigate the impact this genus could have on canola production and health, as it is present as hub taxa in both the bulk soil and rhizosphere soil.

The potential ecological role of the two other hub taxa, FASV8 (*Corynascella sp.*) and BASV69 (unknown bacterium *sp.*), is rather difficult to attribute, as FASV8 (*Corynascella sp.*) is only reported in donkey dung from Iraq [104] and BASV69 is unknown. FASV8 was positively linked with DSE FASV151 (*Exophiala sp.*), which is known to be of interest for canola cropping. The position of these two hub taxa in bulk soil suggests their potential importance as microbes in canola production and thus the need for subsequent studies defining their ecological functions.

The fungal microbiota in canola roots, rhizosphere and bulk soil respond to cropping system diversification, but show different responses depending on geography and weather conditions. The microbiota in canola roots, rhizosphere and bulk soil demonstrate ecological interconnectivity and recruitment, as a significant part of the microbial communities of these biomes is shared. This first inter-kingdom network analysis of canola rhizosphere and bulk soil microbiota allowed identification of two particular microbes of interest for canola production: *Bradyrhizobium sp.* and *Mortierella sp.* The latter is a hub taxon in canola rhizosphere and roots and is linked to *Exophila sp.*, a taxon previously described as associated with canola and positively correlated with high canola yield. The hub taxa matching *Bradyrhizobium* and *Mortierella* at the genus level could be of potential interest for bioengineering of canola subterranean microbiota and enhancing canola production.

Declarations

Acknowledgements

We are grateful to the technical staff of the AAFC research centers in Lacombe, Lethbridge, Melfort and Swift Current for carrying out the sampling and providing useful advice.

Funding: This study was supported by funds the Natural Sciences and Engineering Research Council of Canada CRD grant, the Canola Council of Canada, the Agricultural Bioproducts Innovation Program (ABIP), the Prairie Canola Agronomic Research Program (PCARP), the Agriculture and Agri-Food Canada Canola Cluster Initiative, the Alberta Canola Producers Commission, the Saskatchewan Canola Development Commission, the Manitoba Canola Growers Association, and the Western Grains Research Foundation.

Conflicts of interest/Competing interests: authors do not declare any **access**.

Availability of data and material: The MiSeq sequencing data generated as part of this work are publicly available on Zenodo at the following link: <https://doi.org/10.5281/zenodo.5028181>

Code availability: not applicable

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Figures

Figure 1

(A) Abundance of fungal families in all samples. (B) Relative abundance of main fungal families in canola roots, (C) rhizosphere and (D) bulk soil

Figure 2

(A) Abundance of Bacterial class in all samples. (B) Relative abundance of main bacterial families in canola roots, (C) rhizosphere and (D) bulk soil

Figure 3

Venn diagram of the ASV of the fungal community shared between root, rhizosphere and bulk soil, taking all sites in account.

Figure 4

Venn diagram of the ASV of the bacterial community shared between root, rhizosphere and bulk soil, taking all sites in account.

A.

B.

C.

D.

Figure 5

(A) Network of inter-kingdom interactions between the bacteria and fungi forming the microbiome of canola rhizosphere. Node shades indicate the degree of connectivity: ASVs with warm colors are more connected with the other members of the network than the cold colored ones. Green edges indicate positive relationships and red edges, negative relationships. (B) Sub-network centered around BASV45. (C) Sub-network centered around BASV134. (D) Sub-network centered around FASV21.

Figure 6

(A) Network of inter-kingdom interactions between the bacteria and fungi forming the microbiome of bulk soil in canola field. Node shades indicate the degree of connectivity: ASVs with warm colors are more connected with the other members of the network than the cold colored ones. Green edges indicate positive relationships and red edges, negative relationships. (B) Sub-network centered around BASV69. (C) Sub-network centered around FASV8. (D) Sub-network centered around FASV114.

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