Red Clover Root-Associated Microbiota is Shaped by Geographic Location and Choice of Farming System

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Abstract

Background

Red clover (*Trifolium pratense*), a crop constrained by weak persistence, is the number one forage legume in Northern Europe. Whether plant loss is a sign of an increasing number of known or new pathogens, a decreasing occurrence of beneficial soil microorganisms or other factors is unclear. Here, we collected red clover root samples from Swedish conventional and organic farming sites located between latitudes 55°N and 66°N and analyzed their prokaryotic and eukaryotic communities using amplicon sequencing. We compared the organism diversity, differential abundance, hub taxa and impact of *Rhizobium* on fungal pathogens in relation to geographic location and farming practices.

Results

We found that microbial biodiversity was lowest in samples from southern Sweden and on conventional farms. *Rhizobium* was the most prevalent bacterial genus, followed by *Sphingomonas*, *Mucilaginibacter*, *Flavobacterium* and the unclassified *Chloroflexi* group KD4-96. The *Leptodontidium*, *Cladosporium*, *Clonostachys* and *Tetracladium* fungal genera known for endophytic, ectomycorrhizal, saprotrophic and mycoparasitic lifestyles were also frequently observed in all samples. Sixty-two potential pathogenic fungi were identified with a bias toward grass pathogens and a higher abundance in samples from conventional farms. To gain insights into the cooccurrence patterns of red clover root-associated communities, intrakingdom and interkingdom networks were constructed. Bacterial networks were dominated by known beneficial groups of bacterial species. The interaction between *Rhizobium leguminosarum* bv. *trifolii* and *Aeromicrobium* sp. was strongly positive in the north but negative in the south. Different co-occurrence responses were observed when fungal and bacterial data were combined, as exemplified by the interactions with *Clonostachys rosea*, a beneficial fungus that fluctuated in our dataset and was positively associated with species in *Enterobacterales* and *Aeromicrobium* in the north but negatively with species in the south.

Conclusions

We show that the microbial community is mainly shaped by geographic location and management procedures. *R. leguminosarum* bv. *trifolii* is a keystone species in samples from the northern region and Swedish organic farms. This *Rhizobium* species is negatively associated with all fungal pathogenic species recognized in this study. A number of previously unknown fungal species are identified, particularly those known to produce mycotoxins, which are of major concern for crop productivity and feed safety.

Background

The sustainable use of resources for food and feed production is of increasing importance to meet demands from growing populations, shrinking agricultural land and requests for high-quality and nutritious products [1, 2]. A number of factors determine the productivity of each crop, and different management regimes are applied worldwide. Intense production systems are commonly associated with high resource and economical inputs but also with high losses in terms of nutrient leakage and the release of greenhouse gases [3]. Conversely, yields are higher from conventional farming [4]. The balance between the choice of agricultural management (technical-based), biological preconditions (soil type, water, temperature, light) and farming traditions, among other factors, varies greatly among countries and regions.

The implementation of sustainable cropping systems highlights the importance of integrating legumes into crop rotation schemes [5]. *Fabaceae* is a species-rich plant family, but only a very small fraction of the approximately 19,000 species has been domesticated and used as a source for grains and forages. Concerns regarding climate change and the use of natural resources, such as in the production of mineral fertilizers, have revived interest in increasing the use of forage legume crops. Leys are associated with many advantages and ecosystem services, including nitrogen fixation, improved soil structure, and promotion of genetic diversity through insect pollination [6].

Red clover is a perennial species that is commonly grown intercropped with forage grasses to optimize the nutrient values of animal feed [7]. In Sweden, leys make up to 42% of the total acreage [8]; thus, red clover is by far the dominant legume crop. Soybean is not grown in northern Europe, and the import of soybean products is not regarded as a sustainable alternative to improve animal feed states. Current breeding goals of red clover comprise factors that influence yields, nutritional values, and persistence to withstand a range of stresses. Leys with red clover are aimed for two or three years of growth, hence the persistence of the red clover plants throughout the entire time period is important. The role of microbial soil biodiversity is here believed to be crucial but the knowledge of its potential status and impact is limited. Fungal diseases caused by *Sclerotinia trifoliorum* (clover rot) and *Fusarium* spp. (root rot) are observed in several locations [9] but not in all fields, highlighting the likelihood of new or hitherto unknown pathogenic microbes in soil.

In this work, we hypothesized that a higher microbial diversity is present in the southern part of the country, including an enrichment of potential pathogens and clear intra- and interkingdom competing patterns. The southern region of Sweden has a longer vegetation period than other parts of the country and hence an increased disease pressure. We further hypothesized that the choice of farm management would generate minor effects on the *Rhizobium* community. To address these questions regarding microbial community compositions and cooccurrence patterns in relation to geographic location and farming systems, red clover root samples were collected from south to north in selected places throughout Sweden.

Materials And Methods

Field sample collection

Eighty-nine field sites encompassing conventional and organic farms located between 55°50' N and 66°48' N were visited. In total, 260 samples were collected: 214 samples from 72 conventional and 46 from 17 organic farming sites (Additional file 1). From each field site, 2 to 4 samples were taken, separated by a maximal distance. Each sample consisted of 2 to 8 root systems of red clover with their surrounding soils. All samples were stored at -20°C until DNA extraction.

Sample preparation

The samples were prepared as previously outlined [10] with a few modifications. The soil attached to the root system was carefully removed without damaging the roots. Root systems with closely adhering soil particles (rhizospheric soil) were used for microbiota profiling. From 2 to 4 randomly chosen root systems per sample, 5 to 6 cm root pieces (crown part) were cut into two halves and transferred into 50-ml Falcon tubes (Additional file 2: Fig. S1). The root pieces were thoroughly rinsed 3 times each with 30 ml sterile ice-cold H₂O. The liquid fractions were collected and centrifuged for 15 min at 4500×*g* and 4°C, and the resulting pellets were used for DNA extraction. The samples represent the root-associated microbiota (RAM).

Microbial community profiling

DNA was extracted using a NucleoSpin Soil Kit (Macherey-Nagel, Germany) following the manufacturer's protocols. The quality and quantity of the sample DNA were checked on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). The 16S rRNA sequences were generated in two steps [11]. First, the PCR conditions and primers were applied as previously outlined [12, 13]. Duplicated reactions were performed to reduce PCR biases. Amplified products were cleaned using Sera-Mag beads, pooled and used as a template for the second PCR step using the same conditions [12]. Duplicated amplicons were purified using an EZNA CyclePure Kit (Omega Bio-Tek, USA) and pooled into two libraries. The quality and quantity of the libraries were measured using a Nanodrop, Qubit 4 Fluorometer (Invitrogen) and Agilent 2100 Bioanalyzer. Sequencing was performed on an Illumina MiSeq with paired-end 250-bp reads using the MiSeq reagent kit v2 at SciLife Laboratory, National Genomics Infrastructure (NGI), SNP&SEQ Technology Platform, Uppsala, Sweden. Demultiplexing of the reads was performed using Illumina software (version 2.6.2.1).

Eukaryotic ITS2 rDNA fragments were prepared by amplification using previously described conditions [14], including barcode information [15]. PCR was conducted in duplicate for each sample. Three purified amplicon pools were assembled by combining the barcoded DNA samples. The amplicon size distribution was prechecked using an Agilent 2100 Bioanalyzer. The libraries were prepared using the PacBio amplicon library protocol (Pacific Biosciences, Inc.) and sequenced on a Sequel instrument using three SMRT cells (SMRT Cell 1M v3) by SciLife Laboratory NGI (Uppsala, Sweden). PacBio circular consensus sequence reads were generated using SMRT Link v 8.0.0.79519 (Pacific Biosciences). For all sequencing primers, adapters and barcodes, see Additional file 2: Table S1 and Additional file 3.

16S rRNA gene and ITS2 rDNA read processing

Paired 16S rRNA amplicon sequencing reads were processed by applying the DADA2 method [16] in QIIME2 version 2020.8 (<https://qiime2.org/>) by filtering and trimming to a fixed length of 250 bp, including quality checking and chimera removal. Next, filtered sequences were clustered into amplicon sequence variants (ASVs) at 100% identity. Taxonomic assignment of ASVs was performed using SILVA database release 138 [17] in QIIME2 with the q2-feature-classifier plugin. The QIIME2-compatible preformatted SILVA reference sequence and taxonomy files were processed using RESCRIPt (<http://doi.org/10.5281/zenodo.3891931>). The output was used to train the taxonomic classifier by extracting the hypervariable V3-V4 region of the 16S rRNA gene. Multiple sequence alignment and phylogenetic reconstruction were carried out using MAFFT and FastTree ([18, 19]. Taxonomy-based filtering of ASV sequences was performed using the q2-taxa plugin to remove ASVs representing mitochondrial and chloroplast sequences. Samples were rarefied to an even depth of 11,184 reads to compensate for different sequencing depths.

ITS2 amplicon data were processed using various tools implemented in the PipeCraft v1.0 platform [20]. PacBio circular consensus sequence reads were quality filtered with Mothur 1.36.1 [21] (qwindowaverage = 30, qwindowsize = 50, minlength = 100, maxambig = 0, maxhomop = 12). The resulting reads were demultiplexed based on the unique sequence identifiers using Mothur by allowing one mismatch to the barcode (bdiff = 1) and two mismatches to the primer sequences (pdiff = 2). Putative chimeric sequences were removed using vsearch (v1.11.1; github.com/torognes/vsearch) *de novo* filtering and reference-based filtering against the UNITE reference dataset v7.1 [22]. Additionally, primer artifacts were filtered out using the PipeCraft built-in module. Full-length ITS2 sequences specific to fungi and oomycete kingdoms were extracted using ITS Extractor v1.0.11 [23] and clustered into operational taxonomic units (OTUs) at 97% sequence similarity using CD-HIT v4.6 [24]. Single OTUs were removed from further analyses. Prior to clustering, homopolymers > 8 bp were collapsed to 8 bp in all reads to reduce the weight to indels. Taxonomic assignment of OTUs was performed using BLASTn [25] (e-value = 10; word size = 7; reward = 1; penalty = -1; gap opening cost = 1; gap extension cost = 2) against the UNITE + INSD reference dataset [22, 26]. For unidentified OTUs, manual BLASTn searches were performed in the GenBank database (www.ncbi.nlm.nih.gov). OTUs with an e-value < e-50 of the BLASTn search results, BLAST id% >70% and query coverage > 55% were considered reliable for taxonomy assignments.

Statistical analysis

All statistical analyses were performed in R (<https://www.r-project.org>). QIIME2 and PipeCraft output and sample metadata files were imported into R v4.1.0. Microbial diversity and community composition were analyzed at the ASV or OTU level with the phyloseq [27] and vegan [28] packages in R. The Shannon index was calculated for bacteria/archaea and fungal/protist samples as a measure of the alpha diversity (within sample). Significant differences were tested using the Wilcoxon rank-sum test ($P < 0.05$). To estimate beta diversity (between-sample), nonmetric multidimensional scaling (NMDS) ordination of binary Jaccard dissimilarity matrices was used to identify differences between microbial communities sampled from different country regions and farming systems using PERMANOVA (with 999 permutations) and ANOSIM pairwise comparisons at $P < 0.001$. The relative abundance of taxa is depicted using a stacked bar representation with the ASV/OTU mean abundance values expressed as percentages by employing the phyloseq package. Shared taxa of bacteria, archaea, fungi and protists among country regions and farming systems were defined using the VennDiagram R package [29]. To identify taxa, geographical regions and farming systems, differential abundance analysis was carried out using the DESeq2 v1.32.0 package [30]. The FungalTraits database ($P < 0.01$) [31] was used to assign traits (trophic modes) to OTUs assigned to the fungal kingdom (Additional file 4). P values were corrected for multiple comparisons with Dunn's test using the false discovery rate (FDR) correction method [32].

Microbial co-occurrence network analysis

Co-occurrence networks were constructed and analyzed using the R package NetCoMi v1.0.2 [33] with the functions *netConstruct()* for network construction, *netAnalyze()* for network analysis, and *netCompare()* for network comparison. Associations were estimated based on Pearson's correlation coefficient [34] as an association measure with centered log-ratio (clr) transformation from the SpiecEasi package [35] as a normalization method and the multiplicative replacement (multRepl) method from the zCompositions package [36] to address zeros and missing values in the dataset. Data were filtered to include at least 50 sequences for both the 16S rRNA and ITS2 rDNA gene sequence datasets. Clusters were identified using the default "cluster_fast_greedy" algorithm [37]. Networks were generated using the 50 most abundant taxa to simplify correlation figures. Rhizobium–fungal pathogen cooccurrence networks were created using species-level data from the *Rhizobium* genus and potential pathogenic groups of fungal genera. Henceforth, potential pathogenic or potentially beneficial microbes are denominated pathogenic or beneficial in the text.

Rhizobium isolation

Pink nodules, 1–3 mm in diameter, were excised from frozen red clover root samples and rinsed in tap water. The nodules were surface sterilized by submersion in 95% ethanol for 30 s, followed by 10% sodium hypochlorite for 90 s and rinsing with autoclaved water. The following procedures was performed under sterile conditions. Sterilized nodules were suspended in 100 µl H₂O and crushed with a plastic rod until a milky suspension formed. This suspension was cultivated on tryptone yeast and selective yeast mannitol agar (YEMA) plates incubated at 28°C in the dark [38]. Colonies were picked after 48 and streaked onto YEMA and Jensen's nitrogen deficient media plates [39] with and without 0.025 g/l Congo red dye (Sigma–Aldrich) and incubated at 28°C in the dark. Isolates matching the criteria for growth on nitrogen-deficient media and lack of dye absorption were restreaked to obtain pure cultures.

Rhizobium DNA, PCR and sequencing

Single colonies were grown on YEMA plates, and DNA was isolated [40]. Two fragments of the 16S ribosomal RNA gene were amplified using Phusion High-Fidelity PCR (Thermo Fisher Scientific) with primers (Additional file 2: Table S1). Amplified products were sequenced using Sanger technology (Eurofins Genomics, Ebersberg, Germany). The obtained sequences were analyzed for quality using SnapGene software (from Insightful Science; available at snapgene.com) and assembled with Lasergene MegAlign Pro software (DNASTAR, Inc.). The taxonomy of the assembled sequences was determined using a BLASTN search at NCBI with a minimum of 99% identity.

Data availability

Amplicon sequencing raw datasets have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB49635. The sequence of a *Rhizobium leguminosarum* bv. *trifolii* isolate has been submitted to GenBank at NCBI under accession number MW980046.

Result

Samples from organic farms contain broad biodiversity

Two hundred and sixty red clover samples were collected from 89 Swedish field sites. Forty-six samples were derived from 17 organic farms, representing a similar proportion of organic farmland as the official statistics for the entire country (www.sjv.se). Each sample comprised 2 to 4 root systems. DNA isolation was performed mainly as previously described [10] to generate information on root-associated microbiota. In total, 9,944,450 high-quality amplicon sequences from the V3-V4 region of the 16S rRNA gene and 321,703 quality-filtered ITS reads were generated. The 16S rRNA gene sequences were sorted into 53,990 bacterial and 74 archaea amplicon sequence variants (ASVs) after rarefying to 11,184 sequences. The curated ITS dataset was assigned to 2756 fungal and 26 protist operational taxonomic units (OTUs). The dataset was further divided according to geographic origin. The southern region has intense agriculture similar to northern Germany. The northern region reaches the Arctic polar circle with few crops available for agricultural production and where leys are more common. Between those two regions are the central plains.

No significant differences were detected in the alpha diversity (within-sample diversity) of bacteria/archaea communities at the ASV level when comparing samples among the northern, central and southern regions of Sweden (Fig. 1a). The only exception was that the samples from the central region were more diverse than those from the northern and southern regions, which were equally diverse (Wilcoxon rank-sum test, FDR, $P < 0.05$). Furthermore, diversity was significantly greater in organic than in conventional farms (Additional file 2: Table S2). Fungal/protist diversity at the OTU level did not significantly differ between regions or between farming systems ($P < 0.05$). Nevertheless, samples from the southern region were less diverse than those from the northern and central regions, and samples from organic farms were more diverse than those from conventional farms in our dataset (Fig. 1a and 1b). The bacteria/archaea alpha diversity was approximately 2-fold higher than that of fungi/protists. Analysis of the beta diversity (between-sample diversity) using nonmetric multidimensional scaling (NMDS) ordination based on binary Jaccard distances revealed that bacteria/archaea communities in different regions and farming systems generally clustered together. Only a small fraction of the samples from the northern region and conventional farms were distinct from the corresponding central/southern regions and organic farm samples (Fig. 1c and 1d, Additional file 2: Table S3). Samples made up of fungal/protist communities showed a different pattern, where samples from the northern region were clearly distinct from those from the central and southern regions, whereas a large fraction of the samples from conventional and organic farms formed well-defined groups. Overall, microbial biodiversity was lowest in samples from conventional farms in southern Sweden. This finding may result in less available nutrients for the crops [12].

RAM composition is conditioned by geographic location and farming system

The root-associated microbiota (RAM) communities were further analyzed at the species level with an abundance value set to > 0.001 to assess the distribution of unique and shared species between different regions and farming systems. Bacterial species were more abundant, followed by fungal, archaea and protist species (Additional file 2: Fig. S2a and b). Likewise, most unique species representing these four main microbial groups were found in the northern region and in samples from conventional farmland.

Next, we examined the top 15 most abundant taxa for each microbial group at the phylum, class, genus and species levels (Additional file 2: Fig. S3 to Fig. S6). As expected, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (hereafter *Rhizobium*) was the most prevalent bacterial genus in all samples, followed by *Sphingomonas*, *Mucilaginibacter*, *Flavobacterium* and the unclassified *Chloroflexi* group KD4-96. *Rhizobium* was more abundant in samples from the north (56%) than in those from the central (50%) and southern regions (38%). No significant difference was observed in samples from the two farm management systems (Additional file 2: Fig. S6a). The unclassified *Rhizobium* enriched in the RAM samples (Fig. 2a) had 100% sequence identity to the *Rhizobium leguminosarum* bv. *trifolii* strain, which was independently isolated from nodules on the collected materials. The abundance of *Rhizobium leguminosarum* bv. *trifolii* gradually decreased from north (56%) through central (50%) to south (43%), but no major influence of farming systems was observed (Fig. 2b). Genera known to harbor both pathogenic and plant growth-promoting bacterial species, such as the unclassified groups *Sphingomonas*, *Pseudomonas*, *Flavobacterium*, *Mucilaginibacter*, and *Aquabacterium*, with the latter being present in water and soil, were abundant in all samples.

Among fungi, *Leptodontidium*, *Cladosporium*, *Clonostachys* and *Tetracladium* genera known for endophytic, ectomycorrhizal, saprotrophic and mycoparasitic lifestyles according to information from the FungalTraits database (Additional file 3) were frequently observed in all samples but with different distributions between regions and farming systems (Additional file 2: Fig. S6b). At the species level, *Leptodontidium* and *Cadophora* species were highly abundant in the northern region and *Cladosporium cladosporioides* in the central and southern regions (Fig. 2c). In comparison of the farming systems, the incidence of *Ceratobasidiaceae* sp. and *Cadophora* sp. were higher in organic samples, and *Leptodontidium* sp. were more prevalent in samples from conventional farms (Fig. 2d).

We also searched for the presence of pathogenic fungi (Additional file 2: Table S5, mean abundance value > 0.0001). A total of 62 pathogenic fungi from different genera were identified in our dataset. A greater number of pathogenic fungi were found less frequently in samples from the northern region and organic farms. Here, a gradient of increasing abundance from north to south was found for *Neonectria* sp., *Dactylonectria hordeicola*, *Didymella macrostoma*, and *Plectosphaerella pauciseptata* (Fig. 2c, Additional file 2: Table S5). These species were also more prevalent in samples from conventional farms. For example, *Neonectria* sp. and *Dactylonectria hordeicola* had approximately 1.5- and 2-fold higher incidences, respectively (Fig. 2d). Among archaea, the unclassified group of *Nitrososphaeraceae* sp. was highly abundant together with *Woesearchaeales* uncultured *euryarchaeote*, *Methanomicrobium* uncultured *Methanolacinia* and Group 1.1c uncultured *crenarchaeote* in samples from the northern region and organic farms (Additional file 2: Fig. S6a). *Cercozoa* sp. were the most prevalent protists representing the Rhizaria supergroup (Additional file 2: Fig. S6b). No oomycetes (*Stramenopiles*) were detected, which was unexpected since *Aphanomyces euteiches*, *Phytophthora pisi* and *Pythium* species, among others, are known as soil-borne pathogens in Sweden [43, 44].

To dissect microbial dynamics, our dataset was implemented in the DESeq2 package. A gradient of species enrichment was found from south to north (Additional file 2: Fig. S7 to S11, Additional file 4). In organic farms, no bacterial species were enriched, whereas only one fungal species (*Solicoccozyma terricola*) appeared in the analysis (Additional file 2: Fig. S12). We noticed that the bacterial species that were enriched or depleted in our samples mostly had plant growth-promoting or antibacterial properties. For example, *Streptomyces nogalater* was abundant in all geographic regions, particularly in the northern region, compared with the central and southern regions (Additional file 2: Table S4, Additional file 4). Similar distribution patterns of saprotrophs and endophytes were observed among the fungal communities. A higher number of saprotrophic or endophytic fungi and a lower number of pathogenic species were enriched in the northern region than in the central and southern regions (Additional file 2: Fig. S10, Table S6). In comparisons between the two farm management systems, no fungal pathogens were enriched in the samples from organic farms (Additional file 2: Fig. S12b, Table S6). Taken together, these results highlight two components that greatly impact the population shift between pathogenic and beneficial groups of microbes; geographic location (north) and organic farming are clearly associated with beneficial microbial communities.

***Rhizobium leguminosarum* bv. *trifolii* is a keystone species in the northern region and in organic farms**

To gain insights into the co-occurrence patterns of red clover root-associated communities in different regions and farming systems, intrakingdom and interkingdom networks were constructed using the top 50 most abundant species to simplify association networks. Bacterial networks from each region and farming system were more complex and densely connected compared with the fungal networks (Additional file 2: Fig. S13 to S16, Table S7). Three hub taxa were identified in each network. The clustering coefficient was higher in the bacterial networks, in addition to the number of nodes and edge density. The fungal network based on data from conventional farms deviated somewhat from these features with moderately high clustering coefficients (Additional file 2: Table S7). High modularity indicates more structured communities within a network and a greater number of connections within modules than between networks [45]. Thus, fungal networks were found to be more modular than bacterial networks, which were particularly prominent in the northern region. Bacterial networks were dominated by beneficial groups of bacterial species. The interactions among them were almost 50% positive (co-occurrence). When examining the northern and southern bacterial networks, two hub taxa were formed in the south by *Aeromicrobium* sp. and *Serratia* sp., whereas no such hub was detected in the north (Fig. 3a). A major difference between the two regions was the interaction between *R. leguminosarum* bv. *trifolii* and *Aeromicrobium* sp. It was strongly positive in the north but negative in the south. In a comparison between northern and central bacterial networks, *Actinobacteria* sp. was identified as a hub taxon in the north and was strongly associated with *Microbacteriaceae* and *Micrococcaceae* species but negatively associated with *R. leguminosarum* bv. *trifolii* (Additional file 2: Fig. S17a). Furthermore, *R. leguminosarum* bv. *trifolii* was also negatively associated with *Rahnella* 1 sp. in the south but positively in the central network (Additional file 2: Fig. S17b). Strong positive synergy was observed between *Rhizobium* sp. and *Sphingomonas* sp. in the south, an interaction that was lacking in the central network.

No hub taxa were detected in the fungal networks when comparing the northern and southern regions (Fig. 3b). In the north, several fungi were not associated with other species, whereas in the south, almost all species were connected, except *Gibberella tricineta* (former *Fusarium tricinctum*), a species associated with toxin production on cereals in Europe [46]. In the northern network, *G. tricineta* was positively associated with *Cladosporiaceae* sp. (Fig. 3b). When comparing the northern and central networks, *Solicoccozyma terricola* formed a fungal hub taxon in the north and with *Tetracladium furcatum* in the central region (Additional file 2: Fig. S18a). A strong positive association was observed between *G. tricineta* and the saprotroph *Cladosporium cladosporioides* in the north, whereas no such synergy was seen in the central region. Furthermore, the endophytes *Leptodontidium* sp. and *Cadophora* sp. were abundant and positively associated with each other in the fungal network from the north (Additional file 2: Fig. S18a). *C. cladosporioides* was positively associated with the pathogenic group of *G. tricineta* and *D. macrostoma* in the south, and this association was not seen in the central network (Additional file 2: Fig. S18b).

In a comparison between conventional and organic networks, no major difference was observed in the percentage of positive association in the bacterial networks. In contrast, the fungal network from conventional farms showed a higher positive association in comparison to the data from organic farms, indicating a higher number of negative interactions (strong competition) among pathogenic or beneficial groups of fungal species. Two bacterial hub taxa, *Microbacteriaceae* sp. and *Actinobacteria* sp. and two fungal hub taxa, *Solicoccozyma terricola* and *Cladosporium cladosporioides*, were detected in the organic networks (Fig. 4a and b).

Comparison of bacterial–fungal interkingdom networks showed that the bacteria formed most of the connections, predominantly with other bacteria. This result is also reflected by the greater abundance of bacterial ASVs compared with fungal OTUs in the samples. Only in the comparison between the northern and southern bacterial–fungal interkingdom networks was one fungal species, *Clonostachys rosea*, associated with bacterial species affiliated with *Rahnella* 1, *Curtbacterium*, *Pedobacter* and *Pseudomonas* (Additional file 2: Fig. S19). This fungus has antifungal properties [47], which may influence the surrounding microbes and bacterial clustering. Interactions with *C. roseae*

fluctuated in our dataset; they were positively associated with *Enterobacterales* and *Aeromicrobium* species in the north but negatively associated in the south.

Next, co-occurrence networks were constructed to gain insight into the association complexity between *Rhizobium* and potential pathogenic fungi in different regions and farming systems (Additional file 2: Table S5). To simplify the association networks, the top 50 most abundant species were included in the analysis. This approach generated different clusters, one with pathogen candidates and the others on *Rhizobium* species. Unexpectedly, the *Rhizobium* species were negatively associated with all pathogenic groups of fungi, suggesting competition between them. *R. leguminosarum* bv. *trifolii* was detected as the hub taxon in the northern and organic networks, whereas no hub taxon was found in the central, south and conventional networks (Fig. 5a and b). In central and southern Sweden, additional legume crops are grown, which most likely influences the *Rhizobium* community and explains the deviant diversity of *Rhizobium* species in those regions. Furthermore, a few fungal pathogens showed positive interactions with each other in the red clover root environment independent of the *Rhizobium* species. These species may play important roles in the persistence of this crop.

Discussion

Roots are important plant organs, besides providing physical anchoring, and nutrient storage, roots interact with surrounding soil and its inhabitants for water and nutrient uptake. Soil communities are incredibly variable and rich in prokaryotic and eukaryotic organisms. A plethora of factors impact their activities and the dynamic web of interactions between these organisms. Plant roots secrete exudates with a variable composition and microbial inhabitants due to host plant species, age and environmental factors [49, 50]. Plants have the ability to adapt and to some extent modify the composition of root exudates to suit the needs of individual microbes [51]. Microbial communities are also commonly assembled inside the root tissue as endophytes but become detrimental when colonized by soil-borne pathogens. Bacteria are currently a well-characterized part of the plant microbiome. Representatives of *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* comprise core phyla of different plant root microbiota across various soil types [52, 53]. These bacteria either colonize the root surface as epiphytes or as endophytes inside the root tissue [54]. A similar phenomenon was observed in our datasets, which were composed of approximately 50% *Proteobacteria*, 25% *Actinobacteria*, and 12% *Bacteroidetes* followed by *Verrucomicrobia*, *Chloroflexi* and *Patescibacteria*, each with a low relative abundance. In a study of soils from field trials located outside Helsinki, Finland, *Proteobacteria* (25%), *Actinobacteria* (20%), and *Bacteroidetes*, *Acidobacteria* and *Gemmatimonadetes*, each with a relative abundance of approximately 10%, were found with minor differences between samples from organic or fertilizer-treated soils of red clover and timothy [55]. Fewer *Proteobacteria* and more species from *Gemmatimonadetes* and *Acidobacteria* were identified in these field plots than in our data.

Studies of the *Lotus japonicus* and *Mesorhizobium loti* model systems have long been essential for our understanding of processes involving nodulation and nitrogen fixation. In *L. japonicus* – *M. loti* experiments using field soil from Cologne, Germany, a symbiosis-linked bacterial community was found to have strong plant growth-promoting activities attributed to two orders, *Flavobacteriales* and *Burkholderiales* [56]. We identified four other orders with the following main classes: *Alphaproteobacteria*, *Actinobacteria*, *Bacteroidia* and *Gammaproteobacteria*. In our case, *Alphaproteobacteria*, a class that includes *Rhizobium* species, had the highest ASV values in all samples, greatly impacting the taxonomic distribution. *Rhizobium* have been identified in the rhizosphere of a wide range of plant species and are considered members of the plant core microbiome [57]. The microbial community composition was analyzed under multiple plant–soil conditions. We learned that plant species, including legumes, secrete metabolites that influence the rhizosphere microbiome [56, 58]. There is also competition between various microbes to become attracted to and establish a community surrounding plant roots and for rhizobia to incite nodule formation followed by the transition to a nitrogen-fixing bacteroid [59, 60, 61]. The genus *Rhizobium* accommodates 112 species, of which several

are nonsymbiotic [62]. It is known that nodulating rhizobia harbor genes that are activated by quorum-sensing mechanisms [63]. Metabolites involved in quorum sensing and biofilm formation on root systems are also important community factors [64]. We can only speculate that the *Rhizobium* species network identified in this work could be a result of a joint population-density action to rapidly colonize red clover roots.

Previous studies have revealed that many members of the genera *Streptomyces*, *Sphingomonas*, *Pseudomonas*, *Flavobacterium* and *Mucilaginibacter* play vital roles in producing antibacterial compounds and modulating host performance to stimulate plant growth [65–69]. In the northern region where red clover is grown predominantly and conventional farming is less intense, an abundance of these five bacterial genera associated with plant beneficial traits was found, which may explain the low abundance of pathogenic fungi in these samples.

Organic farming is dependent on animal manure and organic fertilizers in addition to legume crops as major nitrogen sources. This practice is reflected among genera in archaea that dominate in samples from organic farms. Members of *Group 1.1c* are ammonia oxidizers of the superphyla TACK, comprising *Thaumarchaeota*, *Crenarchaeota* and *Korarchaeota*, which are known to play key roles in the nitrogen cycle [70, 71]. Other enriched archaea were *Methanomicrobium* in the Euryarchaeota phylum involved in methane production and anaerobic methane oxidation [72], and *Woesearchaeales*, a member of another archaeal superphyla (DPANN) present in diverse environments, which are believed to participate in methanogenesis [73]. Members of the above archaea were also more prevalent in the samples from the northern region. Mitigating methane, a very potent greenhouse gas, is presently prioritized in the agricultural sector worldwide. Biochar amendment in soils and paddies to reduce CH₄ emissions is a promising management practice [74, 75].

Basic information about soil fungi is limited in comparison to information about bacteria and archaea. Although many OTUs were not assigned a trophic mode in the FungalTraits database, a significant number of OTUs were still annotated to a trophic mode at the genus level. Many fungal species identified in our study belong to genera known to be endophytic, saprotrophic and mycoparasitic on species other than red clover [31]. This was also the case among the pathogenic group where most species were not previously reported on red clover in Sweden. For example, *Cylindrocarpon*-like species residing in *Neonectria*, *Dactylonectria* and *Ilyonectria* have commonly been associated with cankers, root rots, and decay on hardwood and coniferous trees [76, 77], and *Plectosphaerella* species are associated with root and collar rots of horticultural crops [78]. The frequently occurring clover rot pathogen *S. trifoliorum* had a very low sequence abundance in our samples. The sexual phase of this fungus occurs aboveground, where apothecia develop from germinating sclerotia shallowly buried in the topsoil. The airborne ascospores initiate infections on stems and leaves, preferably at low temperatures, followed by systemic growth toward the taproot [79]. This life cycle differs from the other pathogens detected in this study, likely explaining the low incidence of this fungus.

Microbial network analysis provides insight beyond microbial diversity *per se*, allowing us to reveal co-occurrence interactions among our microbial taxa. Keystone or central hub taxa are thought to frequently interact with many other taxa, thereby playing an important role in maintaining organism structure and function that could be crucial for each biological niche studied [80]. A study of the seed microbiome of *Brassica napus* revealed that a high bacterial diversity expressed by tight and complex bacterial networks affects the colonization ability of newcomers, including either symbionts or pathogens [81]. In the current study, we observed a high bacterial diversity in all samples, and *R. leguminosarum* bv. *trifolii* was identified as a keystone species in the constructed networks. We also identified independent sets of positively interacting fungi. *Fusarium oxysporum* is a common root-colonizing fungus with strains that can be neutral, have endophytic properties that induce defense against other soil-borne pathogens or are pathogenic [82]. Its impact on red clover is, however, unclear. In addition, several grass and small-grain cereal pathogens appeared in the network analysis, such as *Gibberella tricineta* (*Fusarium tricinctum*) and *Fusarium culmorum*, which

could indicate that red clover is commonly sown with companion forage species to establish leys during the first year. The potential effects of these fungal pathogens on red clover have not been fully clarified.

Conclusion

In this study, we aimed to map red clover root-associated microbiota throughout Sweden to improve understanding of the soil diversity and species dynamics involving *Rhizobium*. Several grass fungal pathogens were identified, among which a majority were previously unknown in Sweden. Fungal species with mycotoxin properties [83] are of major concern. The cooccurring network analysis demonstrated clear enrichment of *Rhizobium* sp. In the samples and extensive competition with fungal species, not the least of which was exerted by *R. leguminosarum* bv. *trifolii*. How such interkingdom interactions affect nodulation capacity under field conditions is unknown. A large share of *Rhizobium* genes is devoted to rhizosphere growth and root colonization, suggesting that successful competition is prioritized to succeed with the next step, symbiosis [84]. The datasets generated in this study, in addition to forming the basis for detailed examination of microbial interactions, may be useful as indicators to estimate future changes in soil microbe communities over time. The annual projected temperature rise is higher in Sweden than the global mean [85]. An estimated increase of up to 0.5°C per 10 years leads to rapid changes in climate zones and new constraints for food and feed production, including increasing challenging from pathogens.

Abbreviations

DPANN: acronym of the names of the first included phyla *Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanohaloarchaeota*, and *Nanoarchaeota* in the DPANN superphylum **RAM:** root-associated microbiota

Declarations

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Author's contributions

C.D. conceived the study and performed part of the field sampling. S.J. carried out all laboratory work and designed and performed most parts of the bioinformatic analysis. K.P.H. edited the R scripts used for data analysis. L.Ö. identified most field sites, and performed a large part of the sampling. C.D. and S.J. interpreted the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

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Availability of data and materials

Amplicon sequencing raw datasets have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB49635. The sequence of a *Rhizobium leguminosarum* bv. *trifolii* isolate has been submitted to GenBank at NCBI under accession number MW980046.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Microbial alpha (within-sample) and beta (between-sample) diversity across different geographic locations and management systems in Sweden. **(a)** Shannon's alpha diversity of both bacteria/archaea and fungal/protist communities in different regions. **(b)** Bacteria/archaea and fungal/protist alpha diversity in relation to management systems. The star indicates a significant difference between management systems (Wilcoxon rank-sum test, FDR $P < 0.05$). Beta diversity of bacteria/archaea and fungal/protist communities in relation to **(c)** geographic locations and **(d)** management systems. Beta diversity analysis was based on the nonmetric multidimensional scaling (NMDS) ordination estimated by binary Jaccard distances using PERMANOVA and ANOSIM pairwise comparisons, $P < 0.001$. Diversity analyses were conducted at the ASV/OTU level.

Figure 2

Relative abundances of bacterial and fungal species. Distribution of (a, b) bacterial and (c, d) fungal species in samples from different geographic locations and farming systems. “Triangle” denotes the potential pathogenic group of fungi. Low-abundance species (<1%) are not illustrated in the bar graphs.

Figure 3

Microbial cooccurrence networks. **(a)** Bacterial and **(b)** fungal intrakingdom cooccurrence network based on comparisons between samples from northern and southern regions. Network comparisons were created from species-level data based on the Pearson coefficient method [34] using the top 50 most abundant bacterial/fungal species to simplify the association network. Network properties are described in Additional file 2: Table S7. Nodes = species and edges = positive associations (green line) or negative associations (red line). Width/color intensity of lines = strength of positive or negative association. Nodes of the same color are clusters. Size of nodes = relative abundance of that species within a cluster. Hubs are nodes with a centrality value above the empirical 90% quantile and are identified by bold text.

Figure 4

Microbial co-occurrence networks. **(a)** Bacterial and **(b)** fungal intrakingdom cooccurrence network comparisons between samples from conventional and organic farming systems. Network comparisons were created from species-level data based on the Pearson coefficient method [34] using the top 50 most abundant bacterial/fungal species to simplify the association network. Network properties are described in Additional file 2: Table S7. Nodes = species and edges = positive associations (green line) or negative associations (red line). Width/color intensity of lines = strength of positive or negative association. Nodes of the same color are clusters. Size of nodes = relative abundance of that species within a cluster. Hubs are nodes with a centrality value above the empirical 90% quantile and are identified by bold text.

Figure 5

Rhizobium – fungal pathogen cooccurrence networks. Comparisons between samples from (a) northern and southern regions, (b) northern and central regions, (c) southern and central regions and (d) conventional and organic farming systems. Network comparisons were created from *Rhizobium* species and potential fungal pathogenic group of fungal species level data based on the Pearson coefficient method [34]. Network properties are described in Additional file 2: Table S7. Nodes = species and edges = positive associations (green line) or negative associations (red line). Width/color intensity of lines = strength of positive or negative association. Nodes of the same color are clusters. Size of nodes = relative abundance of specific species within a cluster. Hubs are nodes with a centrality value above the empirical 90% quantile and are identified by bold text.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1Samplemetadainformation.xlsx](#)
- [Additionalfile2SupplementaryfiguresandtablesfinalDix.docx](#)

- [Additionalfile3FungallifestyleinformationdeterminedbyqueryinggenusidentitiesagainsttheFungalTraitsdatabase.xlsx](#)
- [Additionalfile4Differentialabundanceofrootassociatedbacteriaandfungibetweentheregionsandfarmingsystems.xlsx](#)