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RESEARCH

Subtle Responses of Soil Bacterial Communities to Corn-Soybean-Wheat Rotation

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ABSTRACT

Crop rotational diversity can improve crop productivity and soil health, and boost soil microbial diversity. This research hypothesized that a 3-year rotation of corn-soybean-wheat (CSW), compared with a 2-year corn-soybean (CS) rotation, would result in a more diverse and more complex soil bacterial community, together with a greater abundance of beneficial bacteria. This was evaluated in a replicated experiment established in 2013 at two locations in Ohio (United States). The soil bacterial communities under soybean were compared between CS and CSW at both studied sites in 2018 and 2019, through 16S ribosomal DNA amplicon metabarcoding. Experimental site was the main driver of bacterial richness and evenness. Significant effects on bacterial community composition were observed in response to the interaction between site, rotational sequence, and year of study. Eight bacterial amplicon sequence variants were identified within all CSW treatments and were not present in CS. Several taxa were differentially abundant between rotation treatments, including the genera *Ralstonia* being more abundant in CS. Co-occurrence networks, including hub taxa, were generally different between rotation treatments and year, with more structure observed in CSW networks for one of the studied sites. Few bacterial genera were consistently identified as hubs across all networks, including an unidentified member of order *Acidobacteriales*, while other hubs were unique for CSW networks, including members of the family *Gemmatimonadaceae*. Finally, the composition of the bacterial communities at the northwestern site positively correlated with plant biomass and active carbon, whereas more recalcitrant pools (total carbon and organic water) correlated with the bacterial communities at the western site.

Keywords: crop rotation, diversified cropping systems, microbiome, soil health

The soil microbiome is defined as the community of microorganisms present within the soil environment, interacting between each other and with other components of the soil community (Marchesi and Ravel 2015). The soil microbiome performs key ecosystem functions with direct effects on plant growth; for example, by promoting carbon (C) and nitrogen (N) cycling, nutrient transport, and disease suppression (Jansson and Hofmockel 2018). The beneficial soil microbial community includes mycorrhizal fungi, N-fixing bacteria, plant growth-promoting rhizobacteria (PGPR), and many others, which enhance crop productivity and help plants to cope with stresses (Fierer 2017). Previous studies found that the soil bacterial communities are different across diverse biogeographical areas, biomes, soil types, and soil pH levels (Delgado-Baquerizo et al. 2018; Fierer and Jackson 2006; Zhang et al. 2018). In agricultural farmlands, the soil bacterial community could also be affected by cropping practices such as crop rotation and tillage intensity (Hartman et al. 2018; Wattenburger et al. 2019). Understanding how the soil microbiome is affected by cropping practices could improve the agricultural production system (Lakshmanan et al. 2014).

Crop rotation increases the diversity of root exudates through the presence of different plants species in a temporal sequence, which then affects the activity and diversity of the soil microbiome

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(Ai et al. 2018; Zhang et al. 2014). Indeed, having more than one crop in a rotation increases soil metabolic activity and improves plant growth (McDaniel et al. 2014). Similarly, higher microbial activity has been correlated with higher plant nutrient uptake from the soil and increased N and phosphorus (P) use efficiency (Anderson 2017). Crop rotational diversity also affects soil microbial diversity. Venter et al. (2016) focused on the effects of crop rotations on soil microbial diversity compared with monoculture, concluding that a higher crop diversity (three or more crops in the rotation) had greater soil microbial richness and diversity compared with monoculture. Crop rotation can also increase the richness of PGPR such as N-fixing and antibiotic producing bacteria (Dias et al. 2015; Peralta et al. 2018). Also, adding a nonhost crop into crop rotation helps break the soilborne disease cycle and reduces soilborne pathogen populations (Ratnadass et al. 2012). Thus, the application of higher crop rotational diversity can change the soil microbiota toward a more diverse and functional community to support crop growth.

In this study, we aimed to determine the impact of crop rotation on soil microbial communities, comparing a corn-soybean-wheat (CSW) rotation with a corn-soybean (CS) rotation. A decline in crop diversity has been observed worldwide (Ramankutty et al. 2018) and regionally (Aguilar et al. 2015; Becot et al. 2020). In the United States, increased trends toward 2-year CS rotations and soybean monoculture are observed. As a result of this, a reduction of 9.1 million ha of wheat planting over the past 10 years (USDA-NASS 2022) has been observed. To address the impacts of the loss of crop diversity in corn and soybean productions systems in Ohio, a replicated field experiment was established in 2013 at two research stations. As reported by Huo et al. (2022), at three environments (location–site combinations), significantly greater yield was observed in the CSW rotation compared with CS. In addition, after two 3-year rotation cycles, soil organic matter was greater in the CSW compared with the CS at both locations. Therefore, we hypothesize that soil bacterial diversity will be higher in the CSW rotation treatment than the CS rotation treatment, regardless of site and year; and that the bacterial communities in soils under CSW will be enriched in beneficial bacteria (and less plant pathogen abundance) compared with the CS rotation treatment.

MATERIALS AND METHODS

Field experiment description and sample collection. A crop rotation field experiment was established in 2013 at two Ohio State University research locations, Northwest Agricultural Research Station (NWARS) and Western Agricultural Research Station (WARS). The experimental treatments were a 2-year CS rotation compared with a 3-year CSW. The experiment was managed as a no-till system, with herbicide and fungicide applications as needed. At each field site, the experimental treatments were established as a randomized complete block design with four replicate plots per treatment. The experimental design included plots in which all phases of the rotation were planted each year. Experiment design and research location characteristics are further described by Huo et al. (2022) and in Supplementary Tables S1 and S2 and Supplementary Figure S1. For this experiment, soils were sampled at the soybean entries of the rotation during the 2018 and 2019 growing seasons (Supplementary Table S2). Soybean seedlings and their associated soils were sampled between stages V3 and V5 (three to five nodes on the main stem with fully developed leaves, beginning with the unifoliate node) (Fehr and Caviness 1977). The complete root and associated soil were sampled with a shovel at an approximate depth of 10 to 15 cm. Eight samples were collected within each plot and the shovels were cleaned with 70% ethanol between plots. Each sample was placed in a plastic bag and kept cool in a cold box during transfer from the field to the laboratory, and at 8°C until processing. Within 48 h after sampling, soils were separated from the root tissue by first removing large pieces of soils from the root ball, followed by a collection of soils loosely attached to the root (released after shaking). These soil samples represented a mix of soybean rhizosphere soil and bulk soil and were stored at −80°C until processing for DNA extraction.

DNA extraction and quantification. Given the differences in soil characteristics and precipitation between sites and collection times (Supplementary Table S1), soil samples were dried prior to weighing and DNA extraction. For this, soils were vacuum dried at 60°C for 30 min. This rapid air-drying method was chosen in the absence of freeze drying, given its minimal effects reported in the literature (Castaño et al. 2016; Tedersoo et al. 2019), and supported by observations that thermal degradation of DNA occurs after over 30 min of exposure at 170°C (Karni et al. 2013). The total soil DNA was extracted from 0.25 g of dried soils using the SurePrep Soil DNA Isolation Kit (Fisher BioReagents), according to manufacturer recommendations, and followed by a ribonuclease A (RNAse A) treatment. Samples were submitted for amplicon library preparation at an approximate concentration of 5 ng/μl. DNA quantification, prior to amplicon library preparation, was performed spectrophotometrically (Nanodrop 2000; Thermo Scientific, Waltham MA, U.S.A.) and through 1% agarose gel electrophoresis. For the latter, test samples were run for 45 min next to a GeneRuler 1-kb-Plus DNA ladder (0.5 μg/μl) (Thermo Scientific). The gel image was processed in ImageJ (Rueden et al. 2017) to quantify the DNA concentration using multiple GeneRuler ladders per gel as a reference.

Amplicon library preparation and sequencing. Bacterial community characterization through amplicon sequencing was performed at the Molecular and Cellular Imaging Center (The Ohio State University College of Food, Agricultural, and Environmental Sciences, Wooster Campus) using a two-step amplification protocol. In the first PCR, the primers 515F and 806R (Apprill et al. 2015; Parada et al. 2016) targeting the V3-V4 region of the 16S ribosomal DNA were used, followed by a double-indexing PCR approach. Samples were pooled into a single sequencing library based on equimolar concentrations and sequenced using an Illumina MiSeq (Illumina, San Diego, CA, U.S.A.) sequencing platform with the 300PE chemistry, with two separate runs for samples collected in 2018 and 2019, respectively. In total, 256 DNA samples from soybean-associated soils, corresponding to two treatments, four plots/treatment, and 8 samples/plot for two locations in Ohio and two growing seasons (2018 and 2019) were processed. In addition, positive controls, including the commercial ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, CA, U.S.A.) and a mixture of DNA of known soil isolates (Supplementary Table S3) and two types of negative controls (water and DNA-extraction controls: kitome, per Hornung et al. [2019]) were included in sequencing runs and analysis for both years of analysis.

Sequence data and statistical analyses. Raw data processing included four major steps: (i) combining 2018 and 2019 sequencing files; (ii) sequence quality control and preprocessing to remove primers and adapters using usearch (Edgar 2010) and cutadapt (Martin 2011), respectively; (iii) filtering, denosing, merging pairs, chimera detection, detection of amplicon sequence variants (ASVs), and taxonomy assignments using the DADA2 pipeline (version 1.14.1) (Callahan et al. 2016) and SILVA database version 132 (Quast et al. 2013); and (iv) statistical analysis, performed using R statistical software 3.5 (R Core Team 2013) using the packages *phyloseq* (McMurdie and Holmes 2013), *metagenomeSeq* (Paulson et al. 2013), *vegan* (Oksanen et al. 2019), *microbiome*

(Leo Lahti 2019), *ampvis2* (Andersen et al. 2018), and *picante* (Kembel et al. 2010). Prior to statistical analysis, all nonbacterial ASVs, including ASVs assigned to the order *Chloroplast* (within the phylum *Cyanobacteria*) and the family *Mitochondria* (within class *Alphaproteobacteria*, order *Ricketsialles*), ASVs classified as kingdom *Eukaryota*, and ASVs not classified to any kingdom (i.e., N/A assignment at the kingdom level) were removed. In addition, contaminants were assessed based on presence and dominance on negative control samples (water and DNA extraction controls), and those ASVs observed across control samples and years of study were considered contaminants and removed. Finally, two samples with <100 reads were removed from the analysis. The code used for the different steps of the pipeline, as well as resulting outputs (operational taxonomic unit tables, and taxonomic and fasta files) are available in Dryad.

We analyzed the effects of the rotation treatment on the bacterial communities using various approaches. Diversity of bacterial communities within rotation treatment samples were investigated using three different metrics: observed richness and Shannon diversity (H), as implemented in the 'estimate_richness' function of *phyloseq*; and evenness (E), estimated as E = H/ln(k), where k = number of observed ASVs per sample, according to Magurran (1988). Comparison of diversity indices was performed by fitting a linear mixed-effects model using the *lme4* package (Bates et al. 2015), specifying sampling site, sampling year, rotation treatment, and their interactions as fixed factors and block as random effect. Distances between samples from rotation treatments, in terms of bacterial community composition, were calculated using the Bray-Curtis distance metric estimated using the *phyloseq* distance method set as 'bray', and differences between sample groups (sampling site, sampling year, and rotation treatment) were inferred through a permutational multivariate analysis of variance (PERMANOVA) with the 'adonis2' function in *vegan*.

Identification of shared taxa across rotation treatments, years, and site of studies, was performed through core ASVs analysis (Leo Lahti 2019), with the 'core.taxa' function in *microbiome* package set as 90% ASV prevalence within each treatment combination (site–year–rotation). Core taxa comparisons were visualized with Venn diagrams generated with Venny (version 2.1) (Oliveros 2007). We assessed the impact of rotation treatment on genus read abundance using the 'mt' function in *phyloseq*, calculated for each site–year combination. To determine the relationship between bacterial community composition and differences in soil characteristics across sites and years of study, we performed canonical correspondence analysis (CCA), followed by fitting of soil and plant variables within the ordination. Soil and plant variables were obtained for the same sites and years of study and were previously reported in Huo et al. (2022). Specifically, ASV read abundance was first averaged per plot using the 'merge_sample' function in *phyloseq*, because soil and yield measurements were collected on a per-plot basis. Bacterial community ordination was constrained by rotation treatment and site of study, using the *ampvis2* implementation of CCA, through the function 'amp_ordinate', with the options of ordination method = 'cca', data transformation = 'hellinger', and the soil and plant data selected as "envfit_numeric". Vectors of fitted variables were plotted if $P < 0.1$, based on 999 permutations.

Network analysis was performed using the *NetCoMi* (Peschel et al. 2021) package in R. Networks were constructed and compared between rotation treatment (CS and CSW) per site and year of study. For this, taxa with the highest variance were selected for the analysis, networks were constructed using read counts, and taxa association were determined using the Sparcc correlation estimator (Peschel et al. 2021; Poudel et al. 2016). Network sparsification considered taxa with a threshold of 0.3 as connected. Associations were transformed into dissimilarities using the 'signed' option. Clusters were identified using the 'cluster_fast_greedy' option (greedy modularity optimization) and hub nodes were determined based on the 95% quantiles of the centrality measures of degree, betweenness centrality, and closeness centrality (Agler et al. 2016), as implemented in the 'hubPar' and 'hubQuant' options of 'net_Analyze' in the *NetCoMi* package. Network comparisons were performed using the 'netCompare' function and differential network analysis was performed using the 'diffnet' function, with Fisher's *z* test, considering up to 50 taxa with the highest variance.

RESULTS

Dataset description. A summary of sequencing reads and number of ASVs generated in this work is recorded in Supplementary Table S4 and Supplementary Figure S2. The raw sequenced reads for the 2018 and 2019 samples were 13,382,635 and 22,772,032, respectively, which, after pair-end merging and chimera removal, accounted for a combined number of 12,378,588 reads (average 33,235 and 50,788 reads/sample for 2018 and 2019, respectively). From these, in total, 29,328 bacterial and archaeal ASV were identified for both years.

Bacterial diversity and community structure across treatments and sites. Overall, site had the greatest effect on bacterial α-diversity metrics, including observed ASVs, Shannon diversity, and evenness (Table 1; Supplementary Table S5), with an average of 1,200 and 1,074 ASVs recovered from WARS and NWARS, respectively. When considering evenness, year of study had a significant interaction with site (Supplementary Table S5), with significantly greater evenness observed at WARS during the 2019 experiments (Table 1). No significant effect of rotation sequences, or their interaction with site and year of study, were observed for any of the diversity indexes analyzed. However, PERMANOVA, based on Bray-Curtis dissimilarity estimates, indicated significant interactions between site, year of study, and rotation sequence in bacterial community composition ($P = 0.031$) (Table 2; Supplementary Table S6). Therefore, results below focus on the effects of rotation for each site and year of study.

The analysis of core ASVs (ASVs present in at least 90% of the samples of a rotation–site–year combination) identified a unique set of eight bacterial ASVs found within the CSW rotation samples at both sites (Fig. 1; Supplementary Table S7). Furthermore, 9 core ASVs were uniquely recovered in CS rotation, and 57 core ASVs were uniquely found in CSW rotation when considering all ASVs present at both sites (Fig. 1). Ten core ASVs were shared between the four rotation-site groups, including bacteria of the genera *Bradyrhizobium*, *Bryobacter*, *Gaiella*, and *Candidatus Udaeobacter*, and unclassified *Nitrosomonadaceae*, *Burkholderiaceae*, *Gaillales*, *Acidobacteriales*, and *Betaproteobacteriales* (Supplementary Table S8).

Differentially abundant genera in CS rotation and CSW rotation samples. A subset of genera was differentially abundant between the CS rotation and CSW rotation samples at NWARS (8 genera) and WARS (24 genera) for both 2018 and 2019 (Tables 3 and 4). Most of these genera belong to the phyla *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Verrucomicrobia*, and *Firmicutes*. Most of the genera differentially abundant between rotation treatments were different across NWARS and WARS in 2018 and 2019. However, the genus *Ralstonia* was differentially abundant between the rotation treatments in more than one site–year combination. The genus *Ralstonia* had higher relative abundance in the CS than CSW rotation at NWARS 2018 and WARS 2019 (Tables 3 and 4). In addition, for NWARS, 6 of the 8 differentially abundant genera were more abundant in the CS treatment whereas, in WARS, 11 of

24 differentially abundant genera were more abundant in the CS treatment.

Relationship with soil characteristics and soybean vigor. In addition to characterizing bacterial community responses to rotational treatment, during the 2018 and 2019 sampling years, we collected data about soybean vigor (seedling stand, biomass, and yield) and soil characteristics (total C, total N, organic matter, and active C) (Huo et al. 2022). The relationship between these measured variables and bacterial community composition was determined by fitting the environmental vectors on the constrained ordination (Fig. 2). For 2018, the constrained analysis explained 20.2% of the variance, with 13.5% represented on the first constrained axis. In addition, plant yield ($r^2 = 0.39$, $P = 0.042$) and the C:N ratio ($r^2 = 0.43$, $P = 0.026$) were significantly correlated with the communities within the NWARS site, whereas soil C ($r^2 = 0.45$, $P = 0.019$), soil N ($r^2 = 0.50$, $P = 0.008$), and plant stand ($r^2 = 0.69$, $P = 0.003$) were correlated with the communities from WARS site. Similar results were observed in 2019, with greater separation observed between the CSW and CS bacterial communities along the second constrained axis. For 2019, the constrained analysis explained 22.6% of the variance, with 16.3% represented on the first axis. Plant vigor data (aboveground biomass, $r^2 = 0.57$, $P = 0.007$; and belowground biomass, $r^2 = 0.87$, $P = 0.001$) and active C ($r^2 = 0.42$, $P = 0.037$) correlated with the bacterial communities in NWARS, whereas seedling stand ($r^2 = 0.90$, $P = 0.001$) correlated with the bacterial communities in WARS and soil organic matter ($r^2 = 0.33$, $P = 0.09$) with the CSW communities.

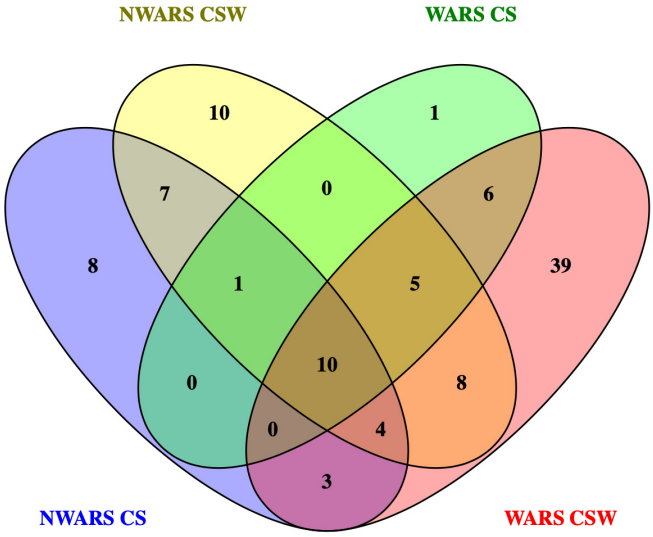

Fig. 1. Venn diagram of amplicon sequence variants present in >90% samples of the corn-soybean (CS) rotation and corn-soybean-wheat (CSW) rotation for NWARS and WARS, per year of study. NWARS = Northwest Agricultural Research Station and WARS = Western Agricultural Research Station, The Ohio State University.

Comparison of bacterial associations in CS and CSW rotation through network analysis. Association networks between bacterial ASVs recovered from CS and CSW rotation treatments were estimated for each site and year of study. The number of bacterial ASVs used in the analysis ranged between 310 and 361. In general, network metrics (Table 5; Supplementary Fig. S3; Supplementary Table S9) for CS and CSW samples differed between site–year combinations. For instance, in NWARS, CSW networks had more clusters, higher modularity, and lower edge density compared with CS, regardless of year. In contrast, in WARS, network

TABLE 2
Effect of year of study, location, and rotation sequence on bacterial communities, as determined by permutational multivariate analysis of variance on Bray-Curtis distance matrices[a]

	df	R^2	F	P
Year	1	0.0304	8.902	**0.001**
Site	1	0.0940	27.541	**0.001**
Rotation	1	0.0099	2.892	**0.001**
Year × Site	1	0.0106	3.093	**0.001**
Year × Rotation	1	0.0042	1.233	0.157
Site × Rotation	1	0.0057	1.661	**0.018**
Year × Site × Rotation	1	0.0054	1.581	**0.031**

[a] Values in bold represent P values < 0.05.

TABLE 1
Comparison of diversity estimates within rotation treatments based on α-diversity indices at NWARS and WARS in 2018 and 2019[a]

Year, site	Rotation[b]	Observed ASVs[c]	Shannon diversity (H)	Evenness (E)
2018				
NWARS	CS	1,073.09 ± 350.35	6.26 ± 0.41	0.904 ± 0.029
	CSW	1,105.00 ± 522.21	6.19 ± 0.89	0.898 ± 0.072
WARS	CS	1,036.90 ± 486.16	6.40 ± 0.43	0.941 ± 0.020
	CSW	1,222.66 ± 455.28	6.49 ± 0.41	0.925 ± 0.035
2019				
NWARS	CS	1,073.69 ± 423.78	6.28 ± 0.42	0.912 ± 0.018
	CSW	1,043.94 ± 394.75	6.24 ± 0.36	0.909 ± 0.019
WARS	CS	1,227.56 ± 360.10	6.45 ± 0.30	0.912 ± 0.028
	CSW	1,314.09 ± 366.74	6.55 ± 0.24	0.917 ± 0.009

[a] NWARS = Northwest Agricultural Research Station and WARS = Western Agricultural Research Station, The Ohio State University.
[b] CS = corn-soybean rotation and CSW = corn-soybean-wheat rotation.
[c] Mean and standard deviation. Observed richness is the number of different amplicon sequence variants (ASVs) in a sample; Shannon diversity considers the abundance or reads of each individual taxa (ASV) in a sample; and Evenness is calculated as E = H/ln(k), where k = number of observed ASVs per sample, according to Magurran (1988).

metrics tended to be similar (e.g., number of clusters) between rotations, or differences were not consistent between rotations across years. However, according to the Jaccard index comparison (Table 6), characteristics of node centrality and hub taxa were not similar between CS and CSW networks for all sites and years, except for the hub taxa in WARS 2018 and 2019, and eigenvector centrality in 2019. At the genus level, some bacteria were consistently observed as hubs within sites and rotation treatments (Supplementary Tables S10 and S11). For example, several *Acidobacteriales* ASVs were hubs in both CS in NWARS and WARS in both years. Similarly, *Gemmatimonas* and other *Gemmatimonadaceae* were hubs in all four CSW networks (and one CS network).

Differential association analysis was also performed to test for differences between specific bacterial associations across networks. Differential association analysis tests the strength of association between taxa in the network. The taxa shown in each plot (Fig. 3) corresponded to those which are differentially associated between CS and CSW treatments. The number of taxa showing differential association varied between WARS ($n = 8$) and NWARS ($n = 16$) in 2018 and WARS ($n = 21$) and NWARS ($n = 26$) in 2019. For all years and sites, ASV_5 (*Bradhyrhizobium*), ASV_6 (unknown *Chitinophagaceae*), and ASV_10 (*Nitrosomonadaceae* mle1-7 group) were differentially associated between rotations. In addition, ASV_13 (unknown *Acidobacteriales*), ASV_20 (*Rugosimonospora*), ASV_32 (*Pedosphaeraceae* ADurb.Bin063-1 group), ASV_34 (*Terrimonas*), and ASV_35 (unknown *Latescibacteria*) showed differential association between networks of CS and CSW rotations in both years at NWARS, and ASV_15 (*Betaproteobacteriales* TRA3-20) in both years at WARS.

TABLE 3
Differentially abundant bacterial genera observed between CSW and CS rotations at NWARS in 2018 and 2019[a]

Year, phylum	Genus[b]	CSW – CS[c]
2018		
Bacteroidetes	*Hymenobacter*	−14.75
Planctomycetes	Unknown *Rubinisphaeraceae*	15.25
Proteobacteria	Unknown *Sphingomonadaceae*	−24.75
Chloroflexi	*Herpetosiphon*	−16.5
Proteobacteria	*Ralstonia*	−238.75
2019		
Proteobacteria	*Aeromonas*	−23.25
Proteobacteria	*Klebsiella*	−138.50
Proteobacteria	*Tahibacter*	24.00

[a] Differential abundance between corn-soybean (CS) rotation and corn-soybean-wheat (CSW) rotation. Rotation was tested for all genera recovered, only the differentially abundant genera ($P < 0.05$ in mt test) are shown in this table. NWARS = Northwest Agricultural Research Station, The Ohio State University.
[b] Unknown = unidentified genus within the specified family.
[c] Difference in mean read counts for each bacterial genera between CSW and CS samples, $n = 8$ for all comparisons, except for NWARS CSW and WARS CS, for which $n = 7$.

DISCUSSION

In this study, the soil bacterial communities under CSW rotation were compared with those of soils under CS, during the soybean phase of each rotation. This study was performed at two field sites during two growing seasons, which provides the opportunity to evaluate the consistency of soybean-associated bacterial community responses to rotation treatment across sites, years, and environmental conditions. We hypothesized that the CSW rotation could influence the soil bacterial diversity and community composition compared with CS rotation, due to differences in root exudate characteristics from the wheat crop (Dias et al. 2015; Venter et al. 2016; Zhalnina et al. 2018), as well as influence of wheat aboveground and belowground biomass and its residue on soil characteristics (Huo et al. 2022; Gaudin et al. 2015; Zhao et al. 2019).

In this work, site had the greatest effect on bacterial richness and evenness, and bacterial richness and evenness were not signif-

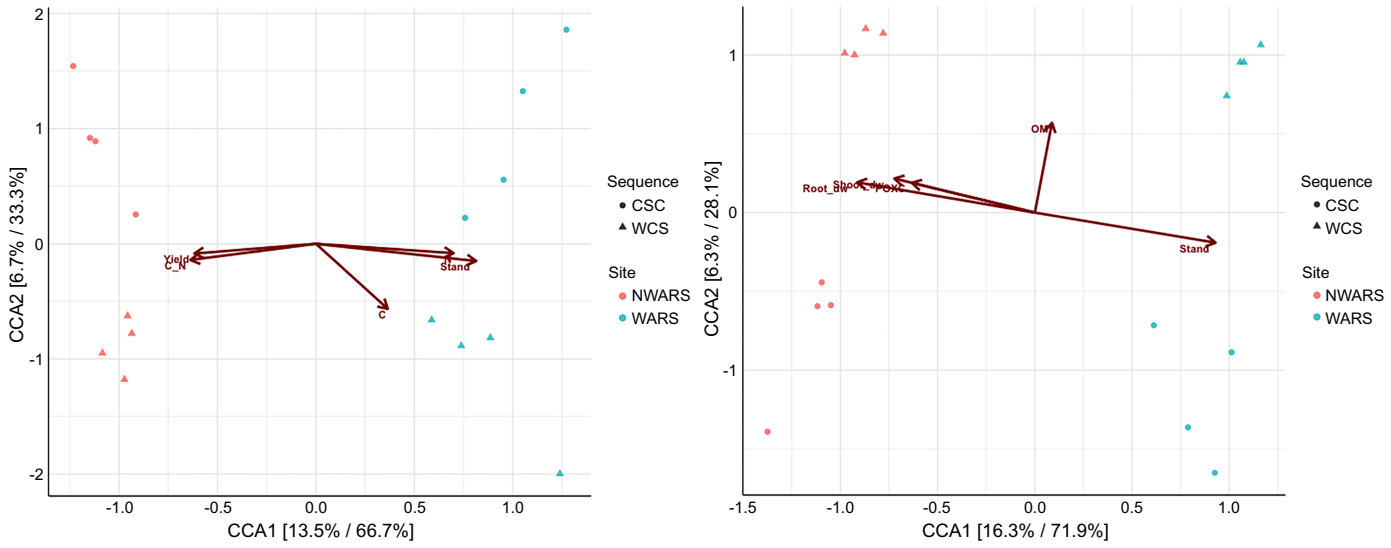

Fig. 2. Constrained correspondence analysis (CCA) ordination of bacterial amplicon sequence variants (ASVs) recovered from corn-soybean-corn (CSC) and wheat-corn-soybean (WCS) rotational treatments at two sites (NWARS and WARS) in 2018 and 2019. Red vectors indicate environmental variables and their correlations with bacterial ASV composition ($P < 0.1$). NWARS = Northwest Agricultural Research Station and WARS = Western Agricultural Research Station, The Ohio State University. C = total soil carbon, N = total soil nitrogen, Stand = seedling establishment, dw = dry weight, and POXC = permanganate oxidizable carbon method, used as an estimate of readily available active carbon. Soil and plant data from Huo et al. (2022).

icantly different between rotations. However, bacterial community composition varied in response to the interaction of year and site of study, with rotation sequence, as indicated by PERMANOVA and canonical correspondence analysis. The studied sites (NWARS and WARS) differ in soil characteristics and climate patterns. Soils at NWARS and WARS belong to different classes of silty clay loam, differing in their C content and drainage. For instance, WARS has higher C content, better drainage, and lower soilborne disease pressure than NWARS, but overall greater yields are observed in NWARS (Huo et al. 2022; Lal 1996). Furthermore, at NWARS, positive relationships were observed between bacterial communities and plant vigor measurements (biomass and yield). The site effect on bacterial community composition has also been found in other studies. For instance, Zhang et al. (2018) showed that the soil bacterial community composition in soybean fields was related to different environmental and spatial variables such as climate and latitude.

It is likely that the two sets of complete 3-year rotations did not result in measurable changes in soil bacterial richness and evenness, compared with three sets of 2-year rotations. Many of the studies that detect rotation effects on microbial community parameters compare monoculture against more diverse practices (e.g., Venter et al. 2016). In addition, the effect of no-till and the presence of corn and soybean residue could be stronger determinants of bacterial diversity in the studied sites, at a soil depth for which no-till is known to have influence in C dynamics (Salinas-Garcia et al. 1997). Consistent with this, Le Guillou et al. (2019) observed that, across various farming practices, tillage had the greatest effect on bacterial diversity and biomass, and Smith et al. (2016), described a greater effect of tillage than crop history on bacterial community structure.

A subset of bacterial ASVs were unique in soils under CSW compared with CS. From the eight unique core ASVs recovered in the CSW rotation, five were classified to genus level as *Skermanella*, *Nocardioides, Gemmatimonas, Steroidobacter*, and *Aetherobacter*; the others belonged to unknown taxa within the families *Pedosphaeraceae* and *Gemmatimonadaceae* and the phylum *Acidobacteria*. Bacteria within these taxonomical groups have been described as soil inhabitants in agricultural ecosystems. For example, soil bacteria of the family *Gemmatimonadaceae* have been found in soils under cotton (Fawaz 2013), members of the *Nocardioides* were described for their ability to degrade atrazine herbicides in soil (Topp et al. 2000), a novel species of *Steroidobacter* was recently described from a farmland region in China (Huang et al. 2019), and bacteria from the genus *Skermanella* are known for their potential for biological control activity against insect pests (Panneerselvam et al. 2018). Bacterial ASVs classified as *Gemmatimonadaceae* were also identified as hubs in CSW networks. The functions of these core bacterial ASVs in soils in general, and the CSW rotation in particular, are still unknown, and it remains to be determined whether similar bacteria are found associated with soybean or other diversified rotational cropping systems in the United States.

At the genus level, 32 bacterial taxa were differentially abundant between the CS and CSW rotation treatment. From these 32 genera, one was differentially abundant between rotations in more than one

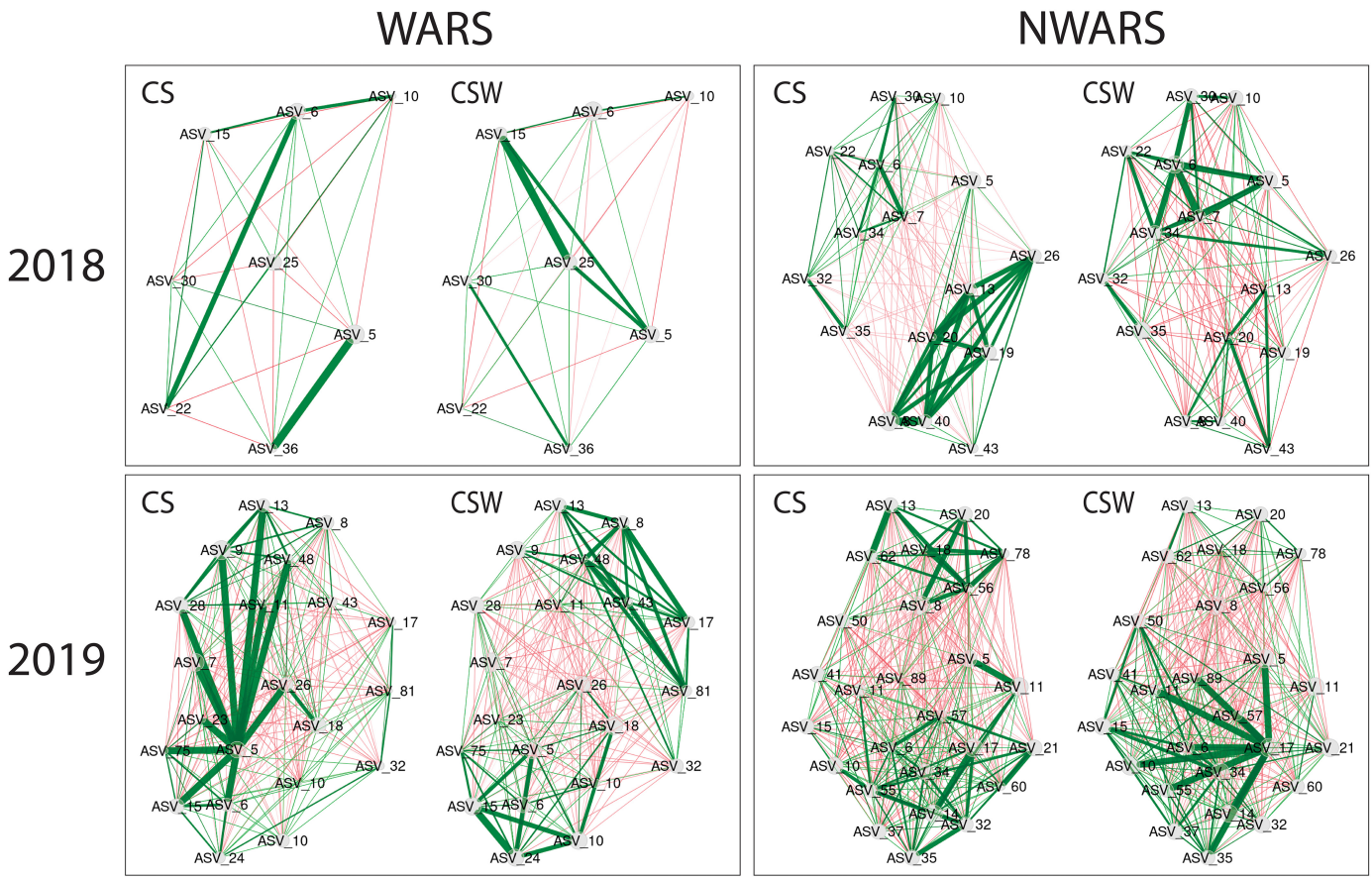

Fig. 3. Differential association of bacterial amplicon sequence variants (ASVs) recovered from corn-soybean (CS) and corn-soybean-wheat (CSW) rotations at two sites (WARS and NWARS) and year of study (2018 and 2019). Green color denotes positive associations, red denotes negative associations, and edge width = strength of the association. NWARS = Northwest Agricultural Research Station and WARS = Western Agricultural Research Station, The Ohio State University. Taxonomic affiliation of individual taxa in each plot is shown in Supplementary Tables S11 and S12.

site and year: the genus *Ralstonia. Ralstonia* had lower abundance in the CSW rotation compared with CS. Within the genus *Ralstonia*, *Ralstonia solanacearum* is a soilborne pathogen that causes vascular wilt disease in more than 200 crop species, including soybean (Ailloud et al. 2015; Harveson and Vidaver 2007; Sharma et al. 2022). In the United States, bacterial wilt of soybean, caused by *R. solanacearum*, is uncommon (Harveson and Vidaver 2007); and, to our knowledge, wheat and corn have not been reported as a host; instead, corn and wheat have been used to break the Ralstonia disease cycle in tomato crops (Adhikari and Basnyat 1998; Terblanche and de Villiers 1998; Yuliar et al. 2015). The importance of *Ralstonia* in the CS rotation has not been studied; however, other work suggested that, under CS rotation, more fungal plant pathogens can be recovered in 2-year rotations compared with more diverse rotations (Benitez et al. 2021). Similarly, most differentially abundant taxa identified in this study were more abundant in the CS treatment.

Network analysis allowed us to evaluate potential interactions within the communities of bacteria in the studied CSW and CS rotation. Even if the diversity of microorganisms recovered from both rotational treatments did not differ, various network characteristics differed in response to rotation, in particular at the NWARS site. For example, a greater number of clusters and higher modularity were observed in the CSW rotation, and hub taxa among rotations at NWARS were not similar. Also, more taxa with greater connectivity in the network (i.e., hub taxa) were observed in the NWARS CSW rotation. Crop rotation effects on microbial interaction in soybean rhizosphere were also observed by Chen et al. (2022), and microbial networks from soils that incorporate wheat straw also showed greater modularity (Banerjee et al. 2016). The higher modularity (and number of highly connected taxa) in CSW networks could represent different functional specialization, which could be linked to the presence of wheat in this rotation. Next steps should involve disentangling the members of individual clusters and generating hypothesis and experiments about functional contributions of these taxa in the CSW rotation (Zamkovaya et al. 2021).

Differential association analysis indicates that the number of positive relationships shown by a *Bradyrhizobium* ASV differ between rotations. We speculate that the differentially associated *Bradyrhizobium* form symbiotic associations with soybean; however, we do not know how differences in associations with other bacterial taxa could influence soybean growth in the CSW rotation. *Bradyrhizobium* has been detected as network hub in other crop diversity studies (Floc'h et al. 2022). Furthermore, a small subset of taxa, including unknown *Acidobacteria* and members of the family *Gemmatimonadaceae*, were consistently identified as hub (and core CSW taxa) in our analysis. These suggest the importance of bacterial taxa within the phyla *Acidobacteria* and *Gemmatimonadota* in our studied system. Members of the phylum *Acidobacteria* have been involved with nutrient cycling in the soil (Kalam et al. 2020), including decomposition of wheat straw (Banerjee et al. 2016).

Through a multianalysis approach, we observed that, even though bacterial richness and evenness did not respond to rotation, the structure of the bacterial community and interactions within were

TABLE 4
Differentially abundant genera observed between CS rotation and CSW rotation at WARS in 2018 and 2019[a]

Year, phylum	Genus[b]	CSW – CS[c]
2018		
Proteobacteria	*Legionella*	39.50
Verrucomicrobia	*Xiphinematobacter*	124.00
Proteobacteria	*Pseudomonas*	1,222.75
Firmicutes	*Sporosarcina*	64.25
Proteobacteria	*Rhodopseudomonas*	−29.00
Proteobacteria	*Rhodopila*	24.75
Proteobacteria	*Herminiimonas*	70.50
Fibrobacteres	Unknown *Fibrobacteraceae*	−47.00
Actinobacteria	*Kibdelosporangium*	67.00
Proteobacteria	*Captivus*	13.25
Actinobacteria	*Williamsia*	−58.00
Proteobacteria	*Diplorickettsia*	8.75
2019		
Firmicutes	*Ammoniphilus*	−22.75
Proteobacteria	Unknown *Hyphomonadaceae*	255.25
Proteobacteria	*Ralstonia*	−148.75
Acidobacteria	*Holophaga*	−36.00
Proteobacteria	*Leptothrix*	−120.00
Verrucomicrobia	Unknown *Pedosphaeraceae*	2,351.75
Proteobacteria	*Pseudogulbenkiania*	−112.50
Proteobacteria	*Massilia*	−254.25
Actinobacteria	*Actinospica*	−239.50
Firmicutes	*Ruminiclostridium_1*	168.25
Acidobacteria	Unknown *Blastocatellaceae*	1,378.50
Proteobacteria	*Chujaibacter*	−564.50

[a] Differential abundance between corn-soybean (CS) rotation and corn-soybean-wheat (CSW) rotation was tested for all genera recovered; only the differentially abundant genera ($P < 0.05$ in mt test) are shown in this table. WARS = Western Agricultural Research Station, The Ohio State University.
[b] Unknown = unidentified genus within the specified family.
[c] Difference in mean read counts for each bacterial genera between CSW and CS samples, $N = 8$ for all comparison.

TABLE 5
Comparison of network metrics and centrality measures between association networks of bacterial amplicon sequence variants recovered from corn-soybean (CS) rotation and corn-soybean-wheat (CSW) rotation treatments at NWARS and WARS in 2018 and 2019[a]

	2018		2019	
Metrics	CS	CSW	CS	CSW
NWARS				
Number of clusters	15	27	8	20
Path length	1.96	1.63	1.44	2.07
Clustering coefficient	0.45	0.36	0.48	0.37
Modularity	0.30	0.88	0.09	0.53
Edge density	0.024	0.002	0.102	0.014
WARS				
Number of clusters	13	13	9	9
Path length	1.13	1.91	1.51	1.54
Clustering coefficient	0.28	0.40	0.47	0.50
Modularity	0.79	0.27	0.14	0.28
Edge density	0.001	0.034	0.073	0.065

[a] Network statistics are defined in Supplementary Table S9. NWARS = Northwest Western Agricultural Research Station and WARS = Western Agricultural Research Station, The Ohio State University.

TABLE 6
Similarity of central nodes and hub taxa centrality measures between corn-soybean (CS) rotation and corn-soybean-wheat (CSW) rotation at NWARS and WARS in 2018 and 2019[a]

	NWARS				WARS			
	2018		2019		2018		2019	
Measures[b]	Jaccard[c]	*P* (≤ Jaccard)[d]	Jaccard	*P* (≤ Jaccard)	Jaccard	*P* (≤ Jaccard)	Jaccard	*P* (≤ Jaccard)
Degree	0.091	0	0.206	0.000593	0.108	0	0.238	0.013157
Betweenness centrality	0.072	0	0.259	0.039331	0.054	0	0.258	0.044031
Closeness centrality	0.103	0.000001	0.241	0.012137	0.108	0	0.248	0.024785
Eigenvector centrality	0.103	0.000001	0.259	0.039331	0.12	0.000002	0.289	0.176006
Hub taxa	0.083	0.053951	0.083	0.053951	0.182	0.234111	0.2	0.299141

[a] NWARS = Northwest Western Agricultural Research Station and WARS = Western Agricultural Research Station, The Ohio State University.
[b] Network statistics are defined in Supplementary Table S9.
[c] Jaccard index estimates for central nodes (centrality values above the 75% quartile) and hub taxa (centrality values above the 95% quartile). Values closer to zero indicate that samples are not similar.
[d] Probability that the Jaccard index is lower than or equal to estimated.

influenced by rotation strategy. As part of this, we identified a subset of bacterial taxa (representing core taxa, taxa hubs, and network clusters) that could potentially be relevant in CSW. These taxa should be the focus of future studies to understand the dynamics of microbial communities and microbial community interactions, in response to CSW across different temporal scales, and their relationship with wheat straw decomposition, crop growth, and soil health.

Data availability. Raw Illumina MiSeq reads generated in this work are available at Sequence Read Archives Bioproject ID PRJNA833126. The code used in this work as well as output files are available in Dryad (doi:10.5061/dryad.tht76hf2x).

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