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Effect of Integrated Crop–Livestock Systems on Soil Properties and Microbial Diversity in Soybean Production

Namita Sinha [1], Brett R. Rushing [2], Aniruddha Acharya [3] and Shankar Ganapathi Shanmugam [1,4,*]

[1] Department of Plant and Soil Sciences, Mississippi State University, Starkville, MS 39762, USA; ns485@msstate.edu
[2] Coastal Plain Branch Experiment Station, Mississippi State University, Newton, MS 39345, USA; jbr93@msstate.edu
[3] Department of Biological & Earth Sciences, Arkansas Tech University, Russellville, AR 72801, USA; aacharya2@atu.edu
[4] Institute for Genomics, Biocomputing and Biotechnology, Mississippi State University, Starkville, MS 39762, USA
* Correspondence: sg383@igbb.msstate.edu

Abstract: Integrated crop and livestock systems (ICLSs) have been considered an important management-based decision to improve soil health by carbon sequestration. A two-year study (2019–2021) at CPBES in Newton, MS, was conducted to evaluate the effect of an ICLS on soil microbial diversity in the south-eastern region of the USA, representing agroclimatic conditions that are warm and humid. Amplicons targeting bacterial 16S rRNA genes and fungal ITS2 regions were sequenced. Taxonomic assignment and characterization of microbial diversity were performed using QIIME2®. Soil fungal diversity pattern showed significant difference (alpha diversity, $p = 0.031$ in 2020 and beta diversity, $p = 0.037$ in 2021). In contrast, no significant differences were observed in bacterial diversity. However, there were several beneficial bacterial phyla, such as *Proteobacteria* and *Actinobacteria*, and fungal phyla such as *Ascomycota*, which were dominant in both years and did not show significant differences due to cover crop treatments. Canonical Correspondence Analysis (CCA) and Mantel test showed significant influence on fungal diversity due to carbon (rm = 0.2581, $p = 0.022$), nitrogen (rm = 0.2921, $p = 0.0165$), and electrical conductivity (rm = 0.1836, $p = 0.0583$) in 2021, and on bacterial diversity due to EE-GRSP (rm = 0.22, $p = 0.02$) in 2020. However, the results showed that there were no significant differences between the cover crop treatments that were consistent over a two-year study period. However, the mix of different cover crops such as oats (*Avena sativa* L.), crimson clover (*Trifolium incarnatum* L.), and tillage radish (*Raphanus sativus L.)* demonstrated higher positive correlation and lower negative correlation with different bacterial and fungal phyla. Long term study of ICLS is suggested to understand the shift in microbiome that would help in understanding the role of cover crops and grazing in improving crop production sustainably.

Keywords: ICLS; ITS2 region; soil properties; soil health; 16S rRNA; sustainable agriculture

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1. Introduction

Soil health across the globe faces significant challenges due to adverse effects of intensive land cover changes due to farming, recreation, and retirement [1,2]. Microbial diversity, carbon (C) and nitrogen (N) content, water stable aggregates (WSAs), and enzyme activity change with change in land use [3]. Sustainable agriculture and implementing proper conservation practices in agriculture can restore soil health by sequestering soil organic carbon (SOC) [4,5]. Systematic reviews by Vida et al., 2020 [6] and Norris and Congreves, 2018 [7] show that alternative management practices such as usage of cover crops (CCs), minimum disturbance to soil, and soil amendments improve soil's chemical, physical, and biological properties, thereby improving soil health. According to USDA-NRCS, "Soil health is the continued capacity of soil to function as a vital living ecosystem

that sustains plants, animals, and humans". The assessment of soil health would require measuring microbial diversity, in addition to the physical and chemical properties of soil [8], because there is a direct correlation between high microbial diversity and soil health [9]. The presence of high biodiversity in soil helps in suppressing plant pathogens and displays resistance to disturbances and stresses [10]. However, soil biodiversity is threatened due to anthropogenic effects such as changes in land cover, climate change, elevated levels of chemical fertilizers, pesticides, and recalcitrant chemicals in soil [11]. A systematic review by Vukicevich et al. 2016 [12] suggests that crop yield could increase by manipulating soil microbial diversity through growth and management of selective cover crop combinations.

Integrated crop and livestock systems (ICLSs) can increase land productivity while conserving natural resources [13]. Before industrialization, farmers worked with both crops and livestock systems together for meeting global food demands and economic gain [14]. A three-year study estimating the effect of grazing and CC involving oats (*Avena sativa*) and pea/oats (*Pisum sativum*/*Avena sativa*) in grazed (sheep) and non-grazed systems in Manitoba, Canada, showed higher N concentrations (NO^3) in the grazed system [15]. Furthermore, there were no negative effects on the crop yields of wheat (*Triticum aestivum*) and rye (*Secale cereale*) that were cultivated in subsequent years. Soil microbial diversity is deemed crucial to improve and revive degraded soil and can thus augment crop production and ecosystem services [16]. The farmer's income can be boosted through the utilization of livestock that can be raised by incorporating cattle into grazing on CCs, while improving crop yield and soil health [17,18]. The direct effect of an integrated crop and livestock system (ICLS) on microbial diversity has not been extensively explored, thus, it necessitates further study.

Cover crops are plants grown in a fallow field, which are used to alleviate the weeds organically, diminish soil erosion, and elevate biomass, which changes the soil's properties. In addition to providing ground cover that helps prevent soil erosion, CCs incorporate carbon through carbon sequestration, balances temperature, aeration, and soil aggregation in the soil [19–21]. The use of CCs in row crop agriculture increases taxonomic/ phylogenetic richness and diversity of soil microbiota when compared with conventional farming practices [22]. Spatial and temporal variation in management practices promotes building better agroecosystems that improve the soil microbiome [23]. A study by Nair et al., 2012 [24] demonstrated that CC combinations such as rye (*Secale cereale*) and rye/vetch (*Vicia sativa*) did not significantly contribute to changes in soil properties when the effect of compost was compared. Moreover, the nitrogen-fixing leguminous crops such as alfalfa (*Medicago sativa*), pea (*Pisum sativum*), white clover (*Trifolium repens*), and crimson clover (*Trifolium incarnatum*), when cultivated on coarse sandy loam soil, have shown to improve organic matter content and change the active bacterial community [25]. Cover crops have been studied extensively for enriching soil properties and organic matter, but their capacity to affect soil microbial diversity remains unexplored.

Moreover, grazing can directly or indirectly affect the community and diversity of soil microbes by organic additions such as slurry and biogas residues that help the functional capability of the microbial community to improve soil health [26]. Trampling and deposition of urine and manure by cattle can affect the soil's physical and chemical properties (decrease in soil pH and increase in soil nitrogen), as well as soil microbial community [27,28]. An increase in grazing intensity decreases soil carbon, which in return increases bacterial diversity by reducing the dominant phyla *Actinobacteria and* decreases fungal diversity by increasing the dominant fungal phyla *Ascomycota* [29,30].

Multiple studies have been conducted to analyze above-ground diversity, but the below-ground microbial diversity is not properly understood [31]. Thus, the interactions of soil, crops, and microbes need further investigation, since the effects of incorporating grazing with CCs in regaining and retaining soil fertility are areas that have significant knowledge gaps [10]. However, there are limited studies quantifying the interaction between microbial diversity, soil, plants, and sustainable ecosystems [32]. An ICLS can provide opportunities to understand crop production in relation to agroecosystems and,

thus, can be utilized to postulate strategies to reduce the effects of agriculture on the environment [33]. Therefore, interactions affecting microbial diversity due to ICLSs and their functionality for sustainable agriculture were studied in the soil of East-Central Mississippi, and it was hypothesized that a mixture of cover crops such as oats (*Avena sativa*), incorporating C, and crimson clover (*Trifolium incarnatum*), incorporating N, integrated with grazing will improve helpful microbial richness and diversity.

2. Materials and Methods

2.1. Study Site and Crop Husbandry

The primary field operations from 2019–2020 (first year) and 2020–2021 (second year) included CC establishment, cattle management, and soybean (*Glycine max*) management, conducted at the Coastal Plain Branch Experiment Station (CPBES) in Newton, MS, USA (32°20′05.11″ N, 89°05′09.60″ W). The soils in the paddocks were classified as a Boswell fine sandy loam (fine, mixed, active, thermic Albaquic Paleudalfs) and Shubuta clay loam (fine, mixed, semiactive, thermic Typic Paleudults). The study site and experimental design are shown in Figure 1.

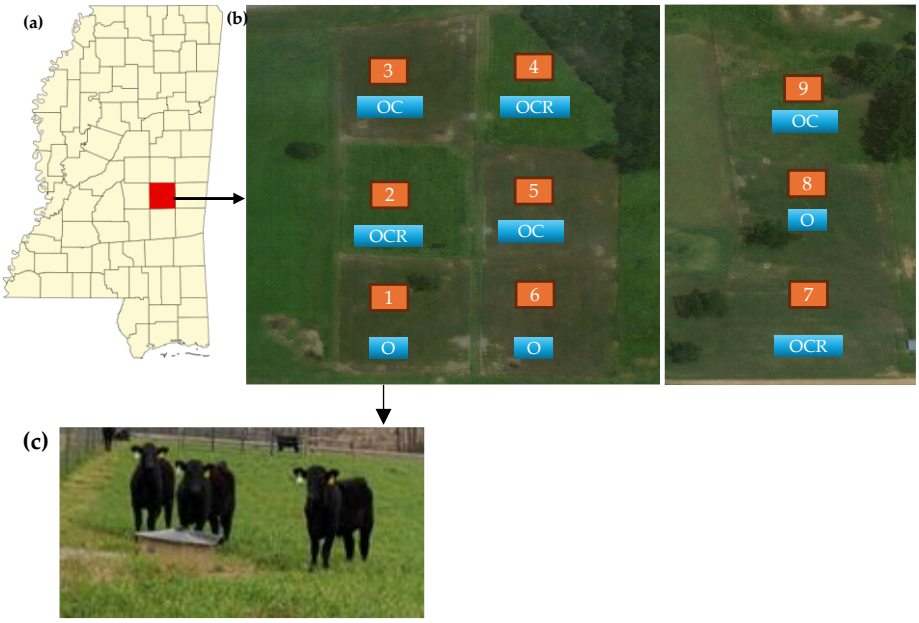


Figure 1. The study site and experimental design to study the effect of an ICLS on microbial diversity; (**a**) map of Newton in Mississippi; (**b**) paddock with different cover crop treatments, where O = Oats; OC = mix of oats and crimson clover; OCR = mix of oats, crimson clover, and tillage radish, Orange boxes represents different replications, and blue boxes represents the cover crop mixes in each replication; (**c**) cattle grazing on cover crops.

Cover crops, including oats, crimson clover, and tillage radish (*Raphanus sativus* var. oleifer), under no-tillage, were used in a randomized complete block design (RCBD) with three replications on nine paddocks of approximately 0.80 ha (2.0 ac) area [34]. Perennial grasses (warm-season) were grown in paddocks under no-tillage for grazing three years prior to the onset of the experiment. The CC treatments included monoculture of oats (O), mix of oats + crimson clover (OC), and mix of oats + crimson clover + tillage radish (OCR). The seeding rate of CCs and cultivars is shown in Table 1. The CC treatments were seeded by a no-till drill with 19 cm (7.5 in.) row spacing. Paddocks with O treatments were fertilized with 56.04 kg N ha^{-1} (50 lb. N ac^{-1}). Paddocks with OC and OCR were fertilized with 28.02 kg N ha^{-1} (25 lb. N ac^{-1}). For grazing, 36 weaned Angus crossbred steers (*Bos taurus*) were used to graze CC paddocks. A set stocking rate of 1345 kg ha^{-1} (1200 lb. ac^{-1}) was used throughout the trial, and no additional animals were added to any paddocks [35].

Grazing commenced each winter once CC heights reached approximately 25 cm (10 in). All paddocks were continuously grazed, and steers were removed once forage heights reached approximately 10 cm (4 in). Once CC height returned to approximately 25 cm (10 in), steers were reintroduced into the paddocks. Mean total animal days (sum of the number of days animals remained in each paddock divided by the total size of the paddock) were 47, 48, and 48 d for the O, OC, and OCR treatments, respectively. Weed control and other field operations are mentioned in Supplementary Table S1 [34].

Table 1. Cultivars and seeding rate for cover crop treatments in yr. 2019 and 2020.

Cover Crop Treatment	Species	Cultivars	Seeding Rate (lb ac^{-1})
Oats (O)	*Avena staiva* L.	Bob	80
Oats +	*Avena staiva* L.	Bob	80
Crimson clover (OC)	*Triticum incarnatum* L.	AU Sunrise	10
Oats +	*Avena staiva* L.	Bob	80
Crimson clover +	*Triticum incarnatum* L.	AU Sunrise	10
Tillage Radish (OCR)	*Raphanus sativus* L.	Daikon	5

The CCs and weeds were desiccated using N-phosphonomethyl glycine3 (glyphosate) (one qt ac^{-1}) applied in the month of April for both years. Each paddock was fertilized using potash (0-0-60) at 224.17 kg K ha^{-1} (120 lb. K ac^{-1}) during the first year. Soybeans (Invictus A4618 maturity group 4.6) were planted on 76 cm rows (30 in) in all paddocks at a rate of 48,500 seeds ha^{-1} (120,000 seeds ac^{-1}). Soybeans were harvested in the fall of each year, and CC treatments were subsequently no-till planted in each paddock and managed accordingly for the next year.

2.2. Soil Sampling and Processing

Three soil cores from each paddock were collected from the top to a depth of 15 cm (0–15 cm) on 2 June 2020, and 14 May 2021. These soil cores were composited, homogenized, and transferred to 1-gallon Ziplock bag for various physical and chemical characterization of the soil and 50 mL centrifuge tubes for genomic DNA extraction. The soil samples were kept on ice before returning to −20 °C (Ziplock bags) and −80 °C freezers (50 mL tubes). The soil from the −20 °C was air-dried before grinding using a mortar and pestle, then passed through a 2.0 mm (No. 10) sieve.

2.3. Soil Physicochemical and Biological Assay

Water stable aggregates were measured by the wet sieving method [36]. Soil pH was measured using a method described by Mclean [37]. The electrical conductivity was measured using an Oakton CON 510 bench conductivity/TDS meter. Air-dried and sieved soil samples were sent to Mississippi State University's (MSU) soil testing lab to analyze the total C and N using the Vario max cube organic elemental analyzer from Elementar® (Langenselbold, Germany). Easily extractable glomalin-related soil proteins (EE-GRSPs) were extracted from soil [38] using the bicinchoninic acid (BCA) assay [39]. The detailed methods for above soil property analysis have been provided in supplementary materials.

2.4. DNA Extraction and Amplicon Sequencing

A measured 0.5 g of soil was used to extract the total genomic DNA using the DNeasy® Power Soil ®Kit (Qiagen, Hilden, Germany). The DNA extractions were performed according to the manufacturer's instructions with minor modifications. Extracted genomic DNA was sent to Novogene® (Sacramento, CA, USA) advancing genomic services to sequence for the V4 region of bacterial 16S rRNA [40,41] using 515F (GTGCCAGCMGCCGCG-GTAA) and 806R (GGAC-TACHVGGGTWTCTAAT) primers, and the internal transcribed spacer 2 (ITS2) region [42,43] of fungal nuclear ribosomal RNA between 5.8S and 26S rRNA with a primer set of ITS3F (GCATCGATGAAGAACGCAGC) and ITS4R (TCCTC-

CGCTTATTGATATGC) [44]. MiSeq amplicon sequencing has been used to determine microbial diversity by using universal marker genes such as 16S ribosomal ribonucleic acid (16S rRNA) [45] and internal transcribed spacer 2 (ITS 2) region [46].

2.5. Bioinformatics and Statistical Analysis

The 16S rRNA and ITS2 raw sequence results were processed and analyzed by establishing a pipeline at the High-Performance Computing Collaboratory (HPC2) core facility at Mississippi State University, Starkville, MS. The Quantitative Insights into Microbial Ecology version 2 (QIIME2®) helped in transferring raw sequence data of 16S rRNA into taxonomic and phylogenetic profiles by denoising the data [47]. Primers and chimeras were trimmed from raw DNA sequences using QIIME2® [44]. Trimmed DNA sequences were sorted based on the barcodes to analyze the data to describe and compare microbial communities. After filtering and quality check, amplicon sequence variants (ASVs) were generated by running DADA2 version (2021.11.0). Taxonomy alignment was performed using SINA [48] taxonomy in QIIME. Microbiome Analyst, an online tool, was used to perform comprehensive analysis such as compositional analysis, biodiversity analysis, and correlation analysis based on data from 16S rRNA and ITS2 processed sequences. Abundance profiles and metadata files for both bacteria and fungi were uploaded as the BIOM format with QIIME as taxonomy labels on marker data profiling in https://www.microbiomeanalyst.ca/ (accessed on 17 May 2022) [49]. Biodiversity analyses (alpha and beta diversity) were performed on feature-level taxonomy for different operational taxonomic units (OTUs) (based on 97% similarity). Statistical significance was calculated using Analysisis of variance (ANOVA) and Permutaional ANOVA (PERMANOVA) at the level of 0.05 for alpha and beta diversity, respectively.

Ordination techniques such as principal component analysis (PCA), principal coordinate analysis (PCoA), and canonical correspondence analysis (CCA) [50] were used to perform multivariate analysis based on a distance matrix such as Bray Curtis and Euclidean distance. Principal coordinate analysis (PCoA) was performed to analyze each sample point in 2D for beta diversity. Feature-level taxonomy was used for alpha and beta diversity. Phylum as the taxonomy level was used to perform correlation analysis such as pattern search using Pearson r correlation coefficient t-test. Univariate analysis at an adjusted p-value cut-off of 0.05 was performed using ANOVA. Paleontological Statistics (PAST®) software (version 4.08) was used to analyze the relationship between approx. 300 generated amplicon sequence variants (ASVs) and physicochemical soil properties on CC treatments. Canonical coordinate analysis (CCA) was performed to study the correlation between different soil properties and 300 ASVs. The Mantel test was performed at p-value < 0.05 using PC-ORD software (version 6.22; MJM Software, Gleneden Beach, OR USA) to determine the correlation connection between two matrices, microbial communities and soil properties.

The PROC GLM procedure was used to study fixed effects using SAS version 9.4® (Cary, NC, USA) statistical software. Replication and year were considered random effects. Soil properties (pH, total C, total N, electrical conductivity, WSA, and EE-GRSP) were compared across the cover crop treatments O, OC, and OCR. Mean comparisons of all soil properties were analyzed using Tukey's protected least significant difference (LSD) at a significance level of 0.05.

3. Results

3.1. Soil Characterization

Different biophysiochemical measurements such as pH, total C, total N, C: N ratio, electrical conductivity, EE-GRSP, and WSA are shown in Table 2. A significant difference was observed in the C: N ratio due to the CC treatment in 2020 (Table 2). A significantly higher C: N ratio was observed in the CC treatment OC followed by OCR. There were no significant differences observed in other soil properties. However, the electrical conductivity, an estimate for measuring salt content, decreased in 2021 in each CC treatment, where

OC and OCR showed a much lower EC of 113.6 µS cm^{-1} in 2021 compared to 240 µS cm^{-1} in 2020. Moreover, WSA in OC increased from 75.8% to 83.37% in 2021 (Table 2).

Table 2. Soil biophysiochemical properties for 2020 and 2021. Different letters and * shows significant difference.

CC	pH	EC (µS cm^{-1})	Total C (%)	Total N (%)	C:N (%)	WSA (%)	EE-GRSP (mg g^{-1})
			2020				
O	6.28 ± 0.15 ^{a}	169.07 ± 17.00 ^{a}	2.63 ± 0.23 ^{a}	0.28 ± 0.03 ^{a}	10.72 ± 0.21 ^{b}	77.37 ± 5.98 ^{a}	2.02 ± 0.16 ^{a}
OC	6.43 ± 0.17 ^{a}	240.80 ± 19.29 ^{a}	3.06 ± 0.27 ^{a}	0.26 ± 0.02 ^{a}	11.66 ± 0.13 ^{a,*}	75.80 ± 3.90 ^{a}	2.06 ± 0.18 ^{a}
OCR	6.28 ± 0.13 ^{a}	238.63 ± 31.14 ^{a}	3.34 ± 0.19 ^{a}	0.28 ± 0.02 ^{a}	11.31 ± 0.30 ^{ab}	70.92 ± 7.98 ^{a}	1.78 ± 0.13 ^{a}
			2021				
O	6.48 ± 0.08 ^{a}	135.86 ± 11.86 ^{a}	3.37 ± 0.24 ^{a}	0.29 ± 0.02 ^{a}	11.39 ± 0.36 ^{a}	73.05 ± 2.72 ^{a}	1.73 ± 0.19 ^{a}
OC	6.59 ± 0.07 ^{a}	113.83 ± 11.39 ^{a}	3.16 ± 0.32 ^{a}	0.26 ± 0.03 ^{a}	12.16 ± 0.36 ^{a}	83.37 ± 2.06 ^{a}	1.69 ± 0.10 ^{a}
OCR	6.42 ± 0.12 ^{a}	113.60 ± 10.32 ^{a}	2.99 ± 0.20 ^{a}	0.26 ± 0.03 ^{a}	11.75 ± 0.62 ^{a}	76.36 ± 4.63 ^{a}	1.67 ± 0.12 ^{a}

Abbreviations: CC = cover crop treatments; O = oats; OC = mix of oats and crimson clover; OCR = mix of oats, crimson clover, and tillage radish; EC = electrical conductivity; C = carbon; N = nitrogen; C:N = carbon–nitrogen ratio; WSA = water stable aggregates; EE-GRSP = easily extractable glomalin-related soil protein; µS cm^{-1} = micro siemens per cm; mg g^{-1} = milligram per gram.

3.2. Effect of ICLS on Microbial Community Composition

3.2.1. Bacterial Distribution

There were 2,559,050 total read counts with a minimum count of 42,578 and a maximum count of 1,08,126 from 27 soil samples in 2020. In 2021, 2,014,657 total read counts with a minimum count of 51,266 and a maximum count of 87,239 were found. The maximum relative abundance of *Proteobacteria* (31%), *Actinobacteria* (29%), *Acidobacteria* (13%), and *Firmicutes* (11%) were found in 2020 (Figure S1). In 2021, the relative abundance of *Proteobacteria* was 34% with presence of *Actinobacteria* (18%), *Acidobacteria,* and *Firmicutes* (14%) (Figure S2). The presence of other bacterial phyla such as *Chloroflexi*, *Planctomycetes*, *Fibrobacteres*, *Gemmatimonadetes*, and *Nitrospirae* was observed in both years. Some phyla were present as not assigned in 2020.

3.2.2. Fungal Distribution

There were 1,908,356 total read counts from 27 soil samples with a minimum count of 25,023 and a maximum count of 1,10,830 in 2020 (Figure S3). There were 22,12765 total read counts with a minimum count of 49,700 and a maximum count of 103,506 in 2021 (Figure S4). The maximum relative abundances of the phylum *Ascomycota*, 85% and 88%, were found in 2020 and 2021, respectively. The presence of other phyla such as *Basidiomycota*, *Mortierellomycota*, *Rozellomycota*, *Mucoromycota*, and *Glomeromycota* were also found. Some phyla were present as not assigned and unidentified.

3.3. Effect of ICLS on Microbial ALPHA Diversity

Alpha diversity is the measure of variation within samples. The indices Chao1 (richness) and Shannon (richness and evenness) diversity index were used to analyze alpha diversity. The richness and evenness of different OTUs are measured by Shannon diversity index, which are randomly sampled [51].

3.3.1. Bacterial *ALPHA* Diversity

There were no significant differences found in richness (Chao1) with p value = 0.33 and p value = 0.52 for 2020 and 2021, respectively (Figure 2a,b), and evenness (Shannon) in both years with p value = 0.33 and 0.08 for 2020 and 2021, respectively (Figure 2c,d).

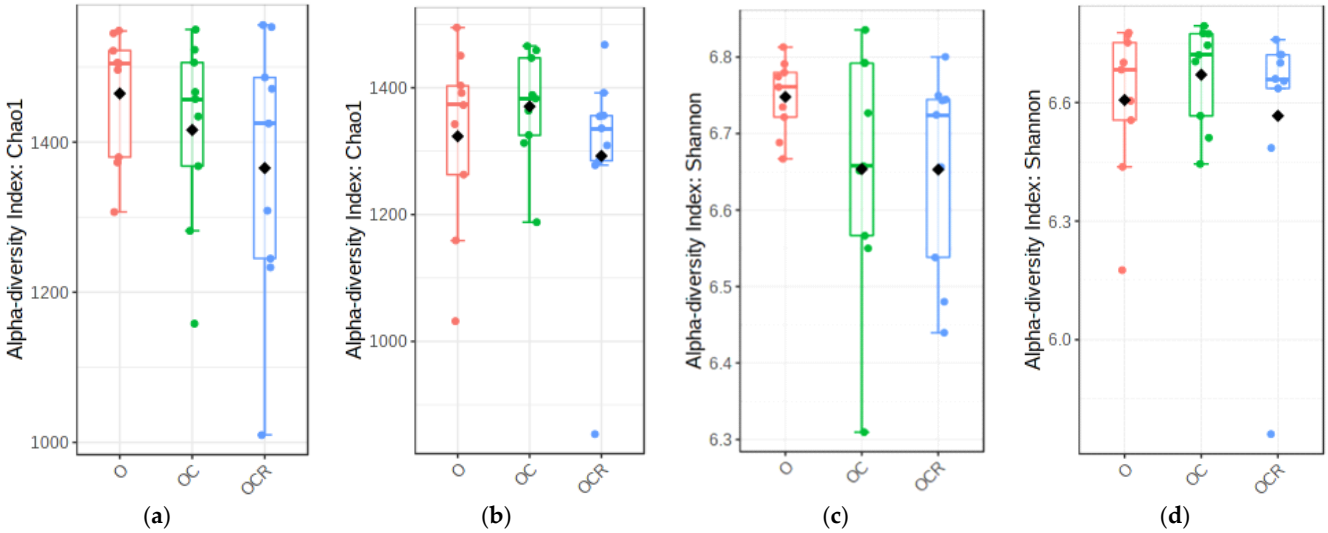


Figure 2. Box plots showing bacterial community richness index, Chao1 in (**a**) 2020 (p-value = 0.33), (**b**) 2021 (p-value = 0.52), and Shannon diversity index in (**c**) 2020 (p-value = 0.19) and (**d**) 2021 (p-value = 0.08) across cover crop treatments. O = oats, OC = mix of oats and crimson clover, and OCR = mix of oats, crimson clover, and tillage radish. Significant differences were observed by ANOVA at the level of 0.05. The black diamond shows mean, and different colored circles are individual OTUs for cover crop treatments. Red boxplot denotes O, green boxplot represents OC, and blue boxplot represents OCR.

3.3.2. Fungal *ALPHA* Diversity

The box plots shown in Figure 2 represent alpha diversity with indices Chao1 and Shannon in fungi. The ANOVA test showed p-values = 0.031 and 0.43 for fungal richness (Chao1 index) in 2020 and 2021, respectively (Figure 3a,b). The richness and diversity were not significantly different in both years (Figure 3c,d). Moreover, the fungal richness increased from 295 to 415 OTUs in CC treatment O, 270 to 400 OTUs in OC, and 315 to 420 OTUs in OCR (Figure 3a,b).

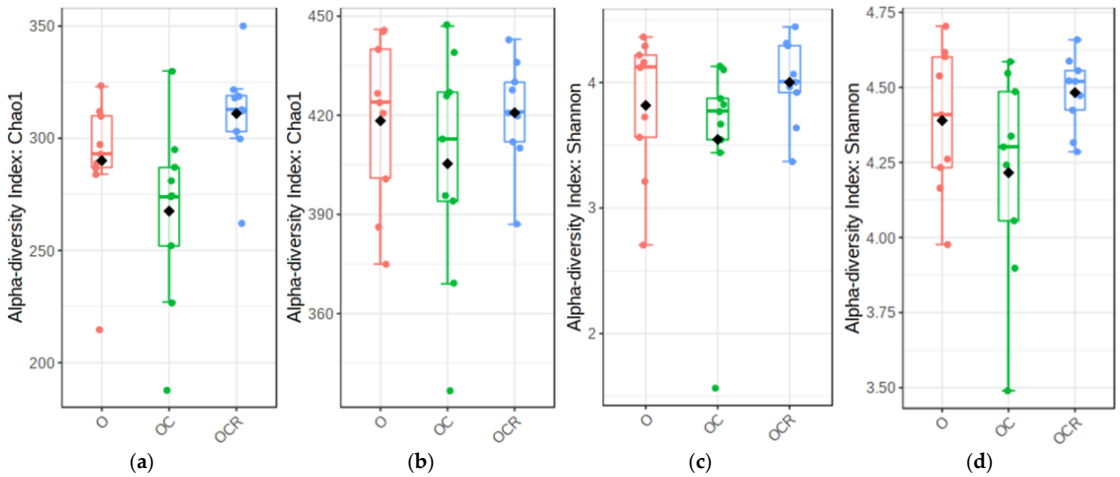


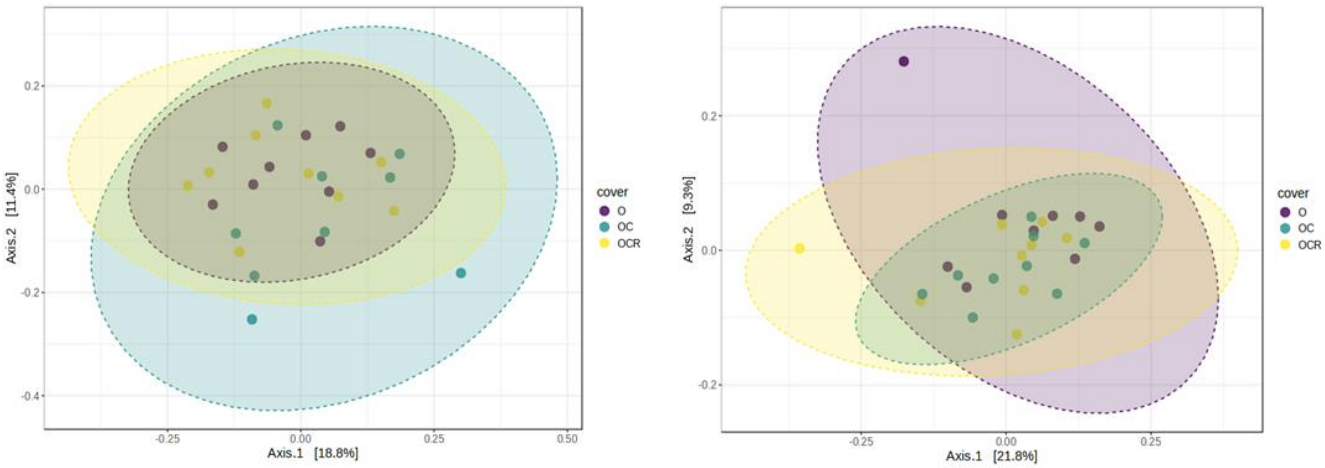
Figure 3. Box plots showing fungal community richness index, Chao1 in (**a**) 2020 (p-value = 0.031), (**b**) 2021 (p-value = 0.43), and Shannon diversity index in (**c**) 2020 (p-value = 0.27) and (**d**) 2021 (p-value = 0.10) across cover crop treatments. O = oats, OC = mix of oats and crimson clover, and OCR = mix of oats, crimson clover, and tillage radish. Significant differences were observed by ANOVA at the level of 0.05. The black diamond shows mean, and different colored circles are individual OTUs of different cover crop treatments. Red boxplot denotes O, green boxplot represents OC, and blue boxplot represents OCR.

3.4. Effect of Cover Crops and Grazing on Microbial Beta Diversity

β diversity is the measure of variation between samples. Principal coordination analysis is an ordination technique to depict large sets of data in 2D format, where each point represents a sample and diversity is determined by distance matrix, Bray Curtis dissimilarity. Samples close together show similarity, and samples far from each other show dissimilarity.

3.4.1. Bacterial *Beta* Diversity

Permutational ANOVA showed p-value < 0.366 and <0.301 in 2020 and 2021, respectively. The major axis (x-axis) showed percent variations of 18.8% in 2020 and 21.8% in 2021. The minor axis (y-axis) had percent variations of 11.4% and 9.3%, respectively, in 2020 and 2021. As shown in Figure 4a,b, all sample points have clustered together and p values are more than 0.05, representing no significant difference in bacterial beta diversity for cover crop treatments.



(**a**) (**b**)

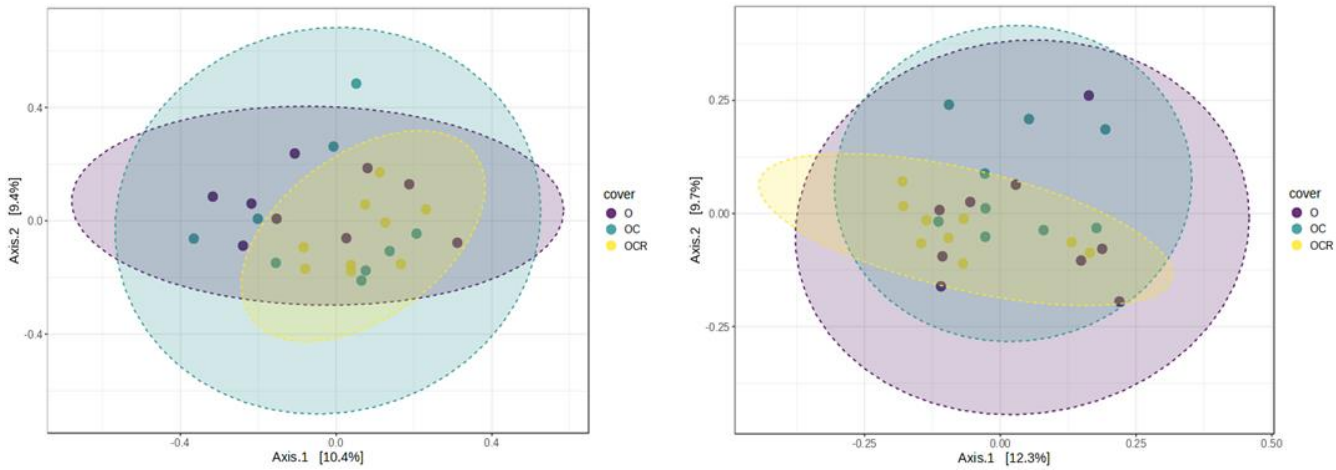
Figure 4. Principal coordinate analysis (PCoA) showing bacterial beta diversity based on the Bray Curtis dissimilarity matrix for cover crop treatments (O = oats, OC = mix of oats and crimson clover, and OCR = mix of oats, crimson clover, and tillage radish) in (**a**) 2020, R-squared: 0.080084; p-value < 0.366, and (**b**) 2021, R-squared: 0.082693; p-value < 0.301. PERMANOVA was conducted at significance level of 0.05. Each dot represents a sample point with several OTUs, where purple dot is cover crop treatment, O, green dot represents OC, and yellow dot represents OCR. Axis 1 on x-axis is major axis and axis-2 on y-axis is minor axis.

3.4.2. Fungal *Beta* Diversity

Permutational ANOVA showed p-value < 0.139 and < 0.037 in 2020 and 2021, respectively. The major axis (x-axis) showed percent variations of 10.4% in 2020 and 12.3% in 2021. The minor axis (y-axis) had percent variations of 9.4% and 9.7%, respectively, in 2020 and 2021 (Figure 5a,b). There was no significant difference in 2020. However, fungal beta diversity was significant in 2021, as separation of the sample points is visible in Figure 5b.

3.5. Correlation of Cover Crops and Grazing on Beneficial Microbial Phyla

The clustering analysis of bacterial phyla with different cover crop treatments provided the knowledge of which cover crop treatment had higher and positive correlations with beneficial bacterial phyla. The correlation was determined by distance measure, Pearson r correlation coefficient.

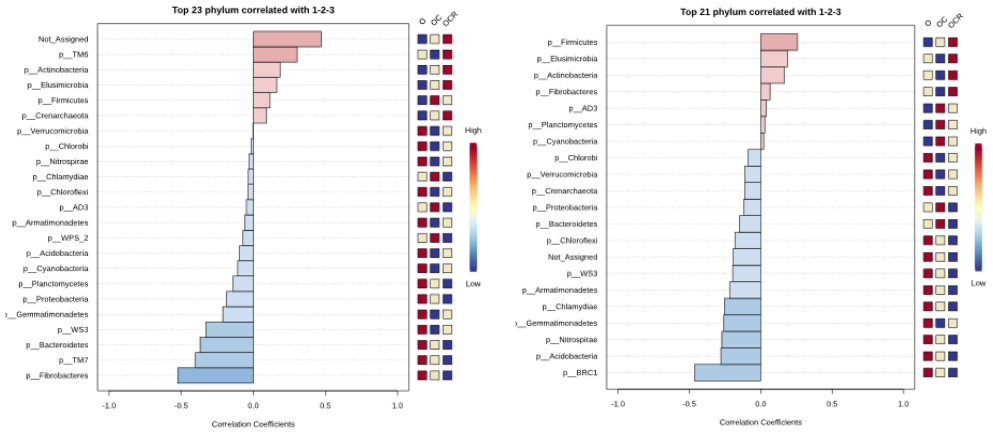


(**a**) (**b**)

Figure 5. Principal coordinate analysis (PCoA) showing fungal beta diversity based on the Bray Curtis dissimilarity matrix for cover crop treatments (O = oats, OC = mix of oats and crimson clover, and OCR = mix of oats, crimson clover, and tillage radish) in (**a**) 2020, R-squared: 0.08645; *p*-value < 0.139, and (**b**) 2021 (R-squared: 0.0986; *p*-value < 0.037). PERMANOVA was conducted at significance level of 005. Each dot represents a sample point with several OTUs, where purple dot is cover crop treatment, O, green dot represents OC, and yellow dot represents OCR. Axis 1 on x-axis is major axis and axis-2 on y-axis is minor axis.

3.5.1. Bacterial Correlation Analysis

In yr. 2020, 24 dominant phyla were correlated with O, OC, and OCR. Phyla such as *TM6*, *Actinobacteria*, *Elusimicrobia*, and *Firmicutes* were positively correlated with the CC treatment OCR, whereas *Fibroacteres*, *TM7*, *Bacteriodetes*, and *Acidobacteria* were negatively correlated with O, as shown in Figure 6a. Similarly, in 2021, 21 dominant phyla were found. *Firmicutes*, *Elusimicrobia*, and *Actinobacteria* were positively correlated with OCR, whereas *BRC1*, *Acidobacteria*, *Nitrosporae*, and *Gemmatimonadetes* were negatively correlated with cover crop O (Figure 6b).



(**a**) (**b**)

Figure 6. Impact of cover crop treatments (O—oats, OC—mix of oats and crimson clover, and OCR—mix of oats, crimson clover, and tillage radish) on different bacterial phyla based on Pearson r correlation coefficient shown by pattern search and heat map in (**a**) 2020 and (**b**) 2021. X-axes show correlation coefficients from −1 to +1, and Y-axes show different bacterial phyla. The pink bar shows positive correlation as it is higher than 0, and the blue bar shows negative correlation as it is lower than 0. The mini heat map on right shows high and low correlation of cover crop treatments, where blue is low, yellow is medium, and red is high correlation.

3.5.2. Fungal Correlation Analysis

The presence of the top 10 fungal phyla in 2020 and top 12 phyla in 2021 were found to be correlated based on distance measure, Pearson r correlation. The phyla *Chytridiomycota*, *Mortierellomycota*, and *Rozellomycota* were found to be positively correlated with the CC treatment OCR in 2020, as shown in Figure 7a. However, the presence of phyla *Blastidiomycota* and *Glomeromycota* was found to be positively correlated with OC treatment in 2021. The phylum Ascomycota was negatively correlated with O treatment in 2020 (Figure 7a), and positively correlated with OCR treatment in 2021 (Figure 7b).

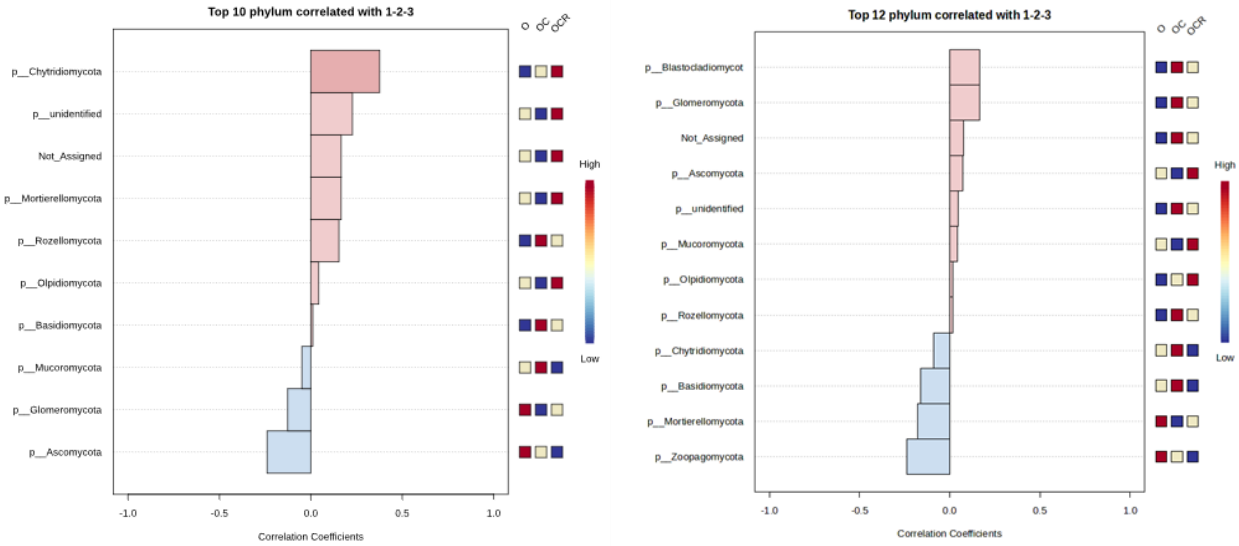
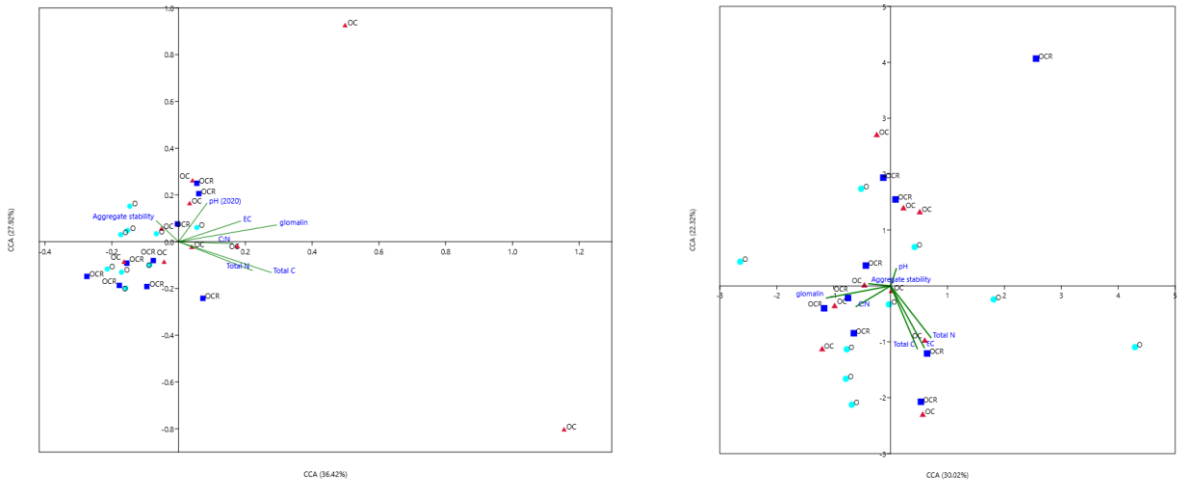


Figure 7. Impact of cover crop treatments (O = oats, OC = mix of oats and crimson clover, and OCR = mix of oats, crimson clover, and tillage radish) on different fungal phyla based on Pearson r correlation coefficient in (**a**) 2020 and (**b**) 2021. X-axes show correlation coefficients from −1 to +1, and Y-axes show different fungal phyla. The pink bar shows positive correlation as it is higher than 0, and the blue bar shows negative correlation as it is lower than 0. The mini heat map on the right shows high and low correlation of cover crop treatments, where blue is low, yellow is medium, and red is high correlation.

3.6. Effect of Biophysiochemical Properties on Microbial Communities

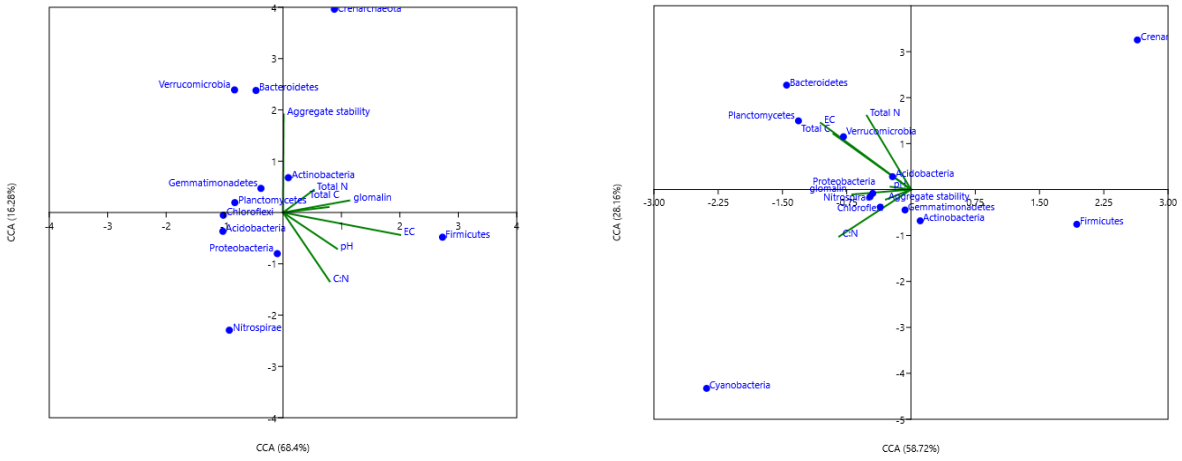
3.6.1. Bacterial Communities

Soil properties were correlated with CC treatment using CCA analysis and the Mantel test. In Figure 8a, WSA is positively correlated with the CC treatment O in 2020. There was no significant correlation in 2021 (Figure 8b). The phylum Firmicutes was significantly present and positively correlated with EC and pH in 2020, as shown in Figure 9a. The phylum *Actinobacteria* was also present and significantly correlated with total C, N, and glomalin. In 2021, several bacterial phyla such as *Acidobacteria* and *Verrucomicrobia* were strongly correlated with EC. *Plantomycetes* and *Bacteridetes* were correlated with total C and N. Glomalin has an effect on *Nitrosporae*, and the C: N ratio on *Chloroflexi* (Figure 9b). *Proteobacteria* was also correlated with pH. The Mantel test showed no overall significant correlation between bacterial diversity and soil properties in either year. However, a significant correlation was seen between bacterial diversity and EE-GRSP in 2020 (Table 3).



(**a**) (**b**)

Figure 8. Canonical correspondence analysis showing the correlation between different soil properties and bacterial communities across different cover crop treatments in (**a**) 2020 and (**b**) 2021. O is represented by blue circles, red triangles for OC, and dark blue squares for OCR. Green line represents different physicochemical properties such as pH, total C, total N, C:N ratio, EC, aggregate stability, and glomalin. The length of the line shows the strength of the relationship. Abbreviations: O = oats; OC = mix of oats and crimson clover; OCR = mix of oats, crimson clover, and tillage radish; EC = electrical conductivity; C = carbon; N = nitrogen; C:N = carbon–nitrogen ratio, glomalin = easily extractable glomalin-related soil protein.



(**a**) (**b**)

Figure 9. Canonical correspondence analysis shows the correlation between soil properties and bacterial abundance in (**a**) 2020 and (**b**) 2021. Points represents different bacterial phyla. Green lines represent different physicochemical properties such as pH, total C, total N, C:N ratio, EC, aggregate stability, glomalin The line length indicated the strength of variable responsible for relationship. Abbreviations: EC = electrical conductivity; C = carbon; N = nitrogen; C:N = carbon–nitrogen ratio, glomalin = easily extractable glomalin-related soil protein.

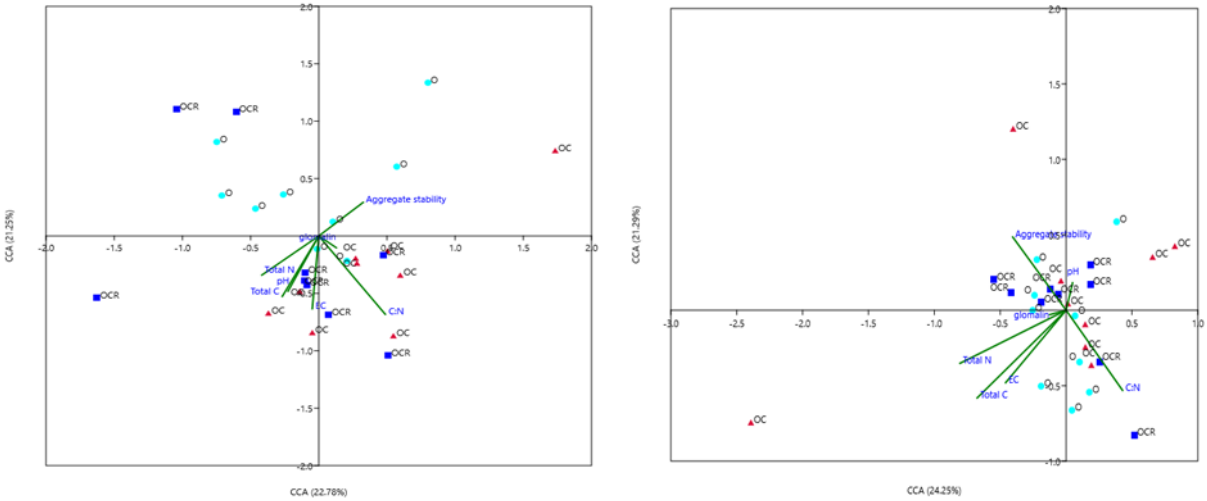
Table 3. Correlation analysis between different soil properties and bacterial communities (Mantel test) in 2020 and 2021; * represents a significant difference.

	2020		2021	
Soil Properties	**rM**	***p*-Value**	**rM**	***p*-Value**
pH	−0.06	0.50	−0.13	0.33
C	−0.001	0.98	−0.04	0.66
N	0.005	0.95	−0.07	0.55
C:N	0.083	0.38	−0.07	0.57
WSA	0.217	0.07	0.05	0.64
EC	−0.032	0.77	0.04	0.63
EE-GRSP	0.22	0.02 *	0.13	0.33

Abbreviations: EC = electrical conductivity, C = carbon, N = nitrogen, C:N = carbon–nitrogen ratio, WSA = water stable aggregate, EE-GRSP = easily extractable glomalin-related soil protein, rM = partial Mantel statistic, below 0 represents negative correlation, between 0 and 1 represents positive correlation.

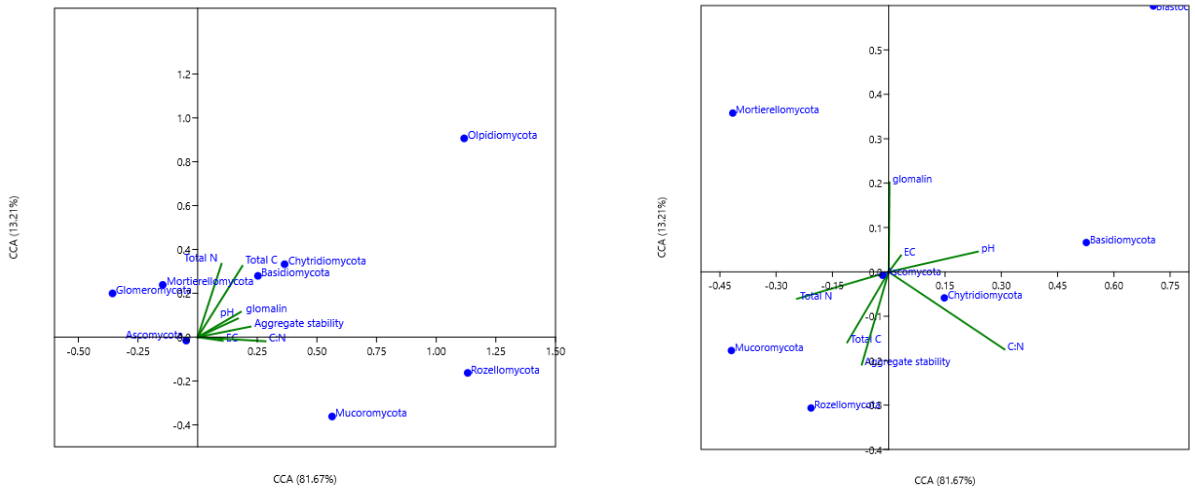
3.6.2. Fungal Communities

As a result of cover crop treatment, OC was significantly correlated with C:N ratio in yr. 2020, and OCR was significantly correlated with aggregate stability in 2021, as evident from Figure 10a,b, respectively. In 2020, the presence of the phyla *Mucoromycota* and *Rozellomycota* changed due to aggregate stability. *Chytridiomycota* was highly correlated with the C: N ratio in 2020 (Figure 11a).



(**a**) (**b**)

Figure 10. Canonical correspondence analysis shows the correlation between soil properties and fungal communities over different cover crop treatments in (**a**) 2020 and (**b**) 2021. O is represented by a blue circle, a red triangle for OC, and a blue square for OCR. The arrow length indicates the strength of the variable responsible for the relationship. The bottom x- and y-axes are scales for sample points. The top x- and y-axes represent plotted arrows. Lines represent different physicochemical properties. Abbreviations: O = oats; OC = mix of oats and crimson clover; OCR = mix of oats, crimson clover, and tillage radish; EC = electrical conductivity; C = carbon; N = nitrogen; C:N = carbon–nitrogen ratio, EE-GRSP = easily extractable glomalin-related soil protein.



(**a**) (**b**)

Figure 11. Canonical correspondence analysis shows the correlation between different soil properties and fungal communities in (**a**) 2020 and (**b**) 2021. Blue points represent different fungal phyla. Green lines represent different physicochemical properties such as pH, total C, total N, C:N ratio, EC, aggregate stability, and glomalin. The line length indicates the strength of the variable responsible for the relationship. Blue point represents different fungal phyla. Green line represents different physicochemical. Abbreviations: EC = electrical conductivity; C = carbon; N = nitrogen; C:N = carbon–nitrogen ratio, glomalin = easily extractable glomalin-related soil protein.

In 2021, several fungal phyla such as *Chytridiomycota* and *Basidiomycota* were significantly correlated with total C (Figure 11b). The Mantel test showed no overall significant correlation between fungal diversity and soil properties in 2020. However, a significant correlation was observed between fungal diversity and total C (r_M = 0.25, *p*-value = 0.02), N, and EC in 2021 (Table 4).

Table 4. Correlation analysis between different soil properties and fungal communities (Mantel test) in 2020 and 2021.

	2020		2021	
Soil Properties	**rM**	***p*-Value**	**rM**	***p*-Value**
pH	0.03	0.69	−0.06	0.64
C	0.16	0.18	0.25	0.02 *
N	0.13	0.15	0.29	0.01 *
C:N	0.07	0.44	−0.002	0.98
WSA	−0.09	0.43	0.05	0.68
EC	0.01	0.91	0.18	0.05 *
EE-GRSP	−0.18	0.06	−0.04	0.72

Abbreviations: C = carbon; N = nitrogen; C:N = carbon–nitrogen ratio, WSA = water stable aggregate; EC = electrical conductivity; EE-GRSP = easily extractable glomalin-related soil protein, rM = partial Mantel statistic, below 0 means negative correlation, between 0 and 1 means positive correlation. * represents significant difference at the level od 0.05.

4. Discussion

The long-term goal of this study was to understand how microbial diversity due to an ICLS could help in improving soil health. The knowledge gained can be utilized towards sustainable agriculture, thus reducing the negative effects of agriculture on the environment. The effects of an ICLS on microbial diversity were investigated using Illumina paired-end MiSeq sequencing. The effects of different soil properties on microbial diversity were also studied.

4.1. Effect of ICLS on Different Soil Biophysiochemical Properties

Soil health, which is interchangeably used with soil quality and soil fertility, is dependent on the physical, chemical, and biological properties of soil [52]. The WSA in OC increased from 75.8% to 83.37% after one year of ICLS. This might be due to the extracellular protein released from the hyphae of arbuscular mycorrhizal fungi (AMF), known as glomalin-related soil protein (GRSP), which promotes the development of soil aggregates [53]. Moreover, soil aggregates are small clusters of soil bound together due to the presence of minerals and organic carbon that remains intact even after mechanical disruption and wetting by rainfall [54]. A significantly higher C: N ratio was observed in OC followed by OCR, in contrast with a study by Wang et al., 2022 [55], where moderate grazing significantly reduced the C: N ratio in *Leymus chinensis* shoots. A previous study has also shown that cover crop C: N ratio is negatively related to availability of inorganic N [56]. By studying soil physical, chemical, and biological properties, and comparing them with microbial diversity, a robust understanding of soil health can be acquired [57,58].

4.2. Improved Fungal Richness Due to Cover Crop Treatments

There was a decrease in bacterial phyla total read counts from 2,55,9050 to 2,01,4657 in 2021 due to different CC treatments. However, the fungal community counts increased from 1,90,8356 to 2,21,2765 in 2021, which was contrary to a study by Eldridge et al., 2017 [30]. The increase in fungal community count might be due to hyphae formed by arbuscular mycorrhizal fungi (AMF) under no-tillage. Proteobacteria (31–34%), *Actinobacteria*, *Acidobacteria*, and *Firmicutes* were the major bacterial phyla. *Ascomycota* (85–88%) was the major fungal phylum out of 12 different fungal phyla. Cover crop treatments (O, OC, and OCR) did not show any different trend in either bacterial or fungal community composition.

A significant difference was found in fungal richness (p-value = 0.031) in 2020. There was increased fungal richness and diversity in each cover crop treatment. There was a significant difference (p-value = 0.037) in fungal beta diversity due to CC treatment in 2021 contrary to bacterial beta diversity, where a separation in clusters was found due to cover crop treatments OC and OCR. The significant increase in fungal abundance and diversity by cover crops compared to bacterial diversity can be explained because the grazing trial was performed on no-tillage, where soil was not disturbed. A previous review study has shown that the fungal community grows better in no-till due to hyphal formation and growth in fungi [59].

4.3. Differentiation of Different Bacterial and Fungal Phyla Due to ICLS

The positive correlation of beneficial bacteria such as TM6, *Actinobacteria*, *Elusimicrobia*, and *Crenarchaeota* was observed with CC treatment OCR, in agreement with a study by Wang et al., 2020 [60]. The bacterial phyla such as *Firmicutes*, *Planctomycetes*, and *Cyanobacteria* were also positively correlated with cover crop treatment OC. Previous studies have shown the presence of similar bacterial phyla in other CC treatments [61]. The bacterium *Actinobacteria* improves soil health through decomposition of organic matter, plant growth promotion, bioremediation, and resistance to pathogens [62–64]. *Crenarcaheota* acts as an ammonium oxidizer in thermophilic regions, which can help with nitrogen cycling [65]. The presence of Cyanobacteria and *Planctomycetes* in CC treatment OC can be attributed to the leguminous CC, crimson clover, in accordance with a study by Li et al. [66]. *Cyanobacteria* helps with N fixation, and *Planctomycetes* helps in C and N cycling [67]. In fungal phyla, *Chytridiomycota* helps with the decomposition of organic matter and is highly abundant in high-elevation regions like alpine regions [66], and Chytridiomycota, *Mortierellomycota*, *Blastoclmidomycota*, and *Ascomycota* were positively correlated with CC treatment OCR. *Mortierellomycota* is a saprophyte and lives on dead leaves, which can help in improving organic matter [68]. *Glomeromycota and Mucoromycota* were positively correlated with CC treatment OC, which might be due to the formation of arbuscular mycorrhizae from crimson clover that would help in extracting N from soil and plant

growth [69–71]. These fungi are dominant in soil due to different edaphic factors such as pH and moisture content [72].

4.4. Microbial Diversity Differentiation Due to Waterstable Aggregates, TotalC, Total N, and Electrical Conductivity

The Mantel test between bacterial communities and soil physicochemical properties showed a significant correlation between bacterial community composition and EE-GRSP (rM = 0.23, *p*-value= 0.02). Water stable aggregates also showed strong correlation with bacterial community (rM = 0.22, *p*-value = 0.07). A previous study has shown that that EE-GRSP and WSA are correlated, as glomalin helps in the formation of better soil aggregates [73]. Interestingly, there was a significant correlation between fungal community composition and total carbon, nitrogen, and electrical conductivity in 2021, since EE-GRSP also promotes soil organic carbon [74]. Higher carbon levels can also be explained by the incorporation of organic residues from grazing cattle in the treatment plots, in agreement with Chahal et al., 2020 [75]. Moreover, a previous study has shown that the addition of organic nitrogen affects the fungal community composition and diversity in undisturbed forests [76]. We can interpret that cover crop mixes improved levels of carbon, nitrogen, WSA, and EE-GRSP, which in turn helped in improving the microbial diversity; such observations are in agreement with a study by Chu et al., 2017 [77].

Canonical correspondence analysis revealed that total N, total C, aggregate stability, and glomalin were responsible for the abundance of *Actinobacteria* and *Crenarchaeota* in 2020. On the other hand, pH, C: N ratio, and electrical conductivity were responsible for the abundance of *Firmicutes*, with 68.4% as the major axis. The *Firmicutes* bacterial phylum can survive in extreme conditions such as high temperature and high salinity [78]. In 2021, total C, total N, and EC were responsible for the abundance of *Acidobacteria*, *Planctomycetes*, and *Verrucomicrobia*. Since carbon provides a substrate for microbial growth, there was an abundance of different bacteria and fungi. The abundance of the fungal phyla *Ascomycota* and *Chytridiomycota* could be explained by the C: N ratio, and complements the results of Y. Wang et al., 2020 [60]. Electrical conductivity and pH were responsible for the abundance of *Basidiomycota*. In 2021, *Basidiomycota* and *Chytridiomycota* were influenced by total C and total N levels, which is in agreement with a study by Di Lonardo et al., 2020 [79].

5. Conclusions

This study was undertaken to understand the effect of an ICLS on improving microbial community composition and diversity. We studied bacterial and fungal diversity using targeted metagenomic sequencing through Illumina MiSeq paired-end sequencing. Our results showed that there were no significant differences between the cover crop treatments that were consistent over the two years of the study period. However, the broad conclusion from this study would be that cover crop and grazing integration does help in improving soil properties through changing the microbial diversity. The presence of beneficial microbiota will help in promoting plant growth through nutrient cycling, nitrogen fixation, photosynthetic ability, degradation of organic matter, and promoting plant growth. The bacterial abundance of *Actinobacteria*, *Firmicutes*, *Planctomycetes,* and *Cyanobacteria* would help in the decomposition of organic matter, C/N cycles, and photosynthesis. The fungal abundance of *Chyridiomycota*, *Mucoromycota*, *Ascomycota,* and *Glomeromycota* would help in improving soil health by enhancing pathogen resistance and increasing mycorrhizal growth. It is conclusive that microbial functional diversity can be used as an indicator of management-induced changes to soil quality due to the presence of these beneficial microbiota. Knowledge of the management system, through which we can improve the microbial diversity and soil properties, would help us in gaining more confidence in incorporating grazing and cover crops in agriculture systems. The ICLS will help in improving the degraded ecosystem and pave a path for sustainable agriculture. However, targeted amplicon sequencing using16S rRNA and ITS2 genes has a limited ability in studying functionally active microbes present in the soil. Therefore, further studies using whole

genome sequencing techniques would help predict functional roles (genes present, genes transcription abundance) of the active microbiome in these systems. This study also suggests that long-term study of an ICLS can help us understand the shift in the microbiome that would help in understanding the role of cover crops, grazing, and tillage in improving crop production sustainably.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applbiosci3040031/s1, Figure S1: Relative abundance of bacterial communities across cover crop treatments; O (Oats), OC (Oats + crimson clover), OCR (Oats + crimson clover +tillage radish in 2020; Figure S2: Relative abundance of bacterial communities across cover crop treatments; O (Oats), OC (Oats + crimson clover), OCR (Oats + crimson clover + tillage radish in 2021; Figure S3: Relative abundance of fungal communities across different cover crop treatments; O (Oats), OC (Oats + Crimson clover), OCR (Oats + Crimson clover + tillage radish) in 2020; Figure S4: Relative abundance of fungal communities across different cover crop treatments; O (Oats), OC (Oats + Crimson clover), OCR (Oats + Crimson clover + tillage radish) in 2021; Table S1: Timeline of field operations in ICLS trial.

Author Contributions: Conceptualization, S.G.S. and B.R.R.; methodology, S.G.S.; investigation, S.G.S., N.S. and B.R.R.; writing—original draft, N.S.; writing—review and editing, S.G.S., N.S. and A.A.; supervision, S.G.S.; data curation and visualization, S.G.S. and N.S.; formatting and submission, S.G.S.; funding acquisition, S.G.S. All authors have read and agreed to the published version of the manuscript.

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