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Research Article

Restoration age and reintroduced bison may shape soil bacterial communities in restored tallgrass prairies

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Abstract

Knowledge of how habitat restoration shapes soil microbial communities often is limited despite their critical roles in ecosystem function. Soil community diversity and composition change after restoration, but the trajectory of these successional changes may be influenced by disturbances imposed for habitat management. We studied soil bacterial communities in a restored tallgrass prairie chronosequence for >6 years to document how diversity and composition changed with age, management through fire, and grazing by reintroduced bison, and in comparison to pre-restoration agricultural fields and remnant prairies. Soil C:N increased with restoration age and bison, and soil pH first increased and then declined with age, although bison weakened this pattern. Bacterial richness and diversity followed a similar hump-shaped pattern as soil pH, such that the oldest restorations approached the low diversity of remnant prairies. β-diversity patterns indicated that composition in older restorations with bison resembled bison-free sites, but over time they became more distinct. In contrast, younger restorations with bison maintained unique compositions throughout the study, suggesting bison disturbances may cause a different successional trajectory. We used a novel random forest approach to identify taxa that indicate these differences, finding that they were frequently associated with bacteria that respond to grazing in other grasslands.

Keywords: soil microbial community, ecosystem restoration, grassland, bison, random forest, prescribed fire

Introduction

Grasslands historically comprised >40% of global terrestrial habitats (Gibson 2009), but their conversion to agriculture and over grazing has led to widespread degradation (Bardgett et al. 2021) and biodiversity loss (Lark et al. 2020). There are efforts to restore grassland communities and the critical ecosystem functions and services that they provide (Kiehl et al. 2010, Bengtsson et al. 2019, Zhao et al. 2020), and these projects are taking on particular importance during this UN Decade on Ecosystem Restoration (Török et al. 2021). Soil microorganisms are important drivers of grassland ecosystem functions, including decomposition of organic matter, nutrient cycling, and C sequestration (Conant et al. 2017, Mackelprang et al. 2018, Yang et al. 2019). Thus, successful grassland restoration includes the establishment of soil microbial communities that can carry out these functions (Kardol and Wardle 2010), but there is limited knowledge of how restoration and ongoing management influence soil microbes.

Soil communities in grasslands restored from previously cultivated agricultural land will be shaped by the interactions between postagricultural changes in abiotic soil conditions and turnover in plant communities. Where prior agricultural practices included intensive fertilization, the cessation of nutrient inputs and transition to perennial plants can cause significant changes in soils, including rapid decline in N content followed by gradual accrual of N, C, and microbial biomass over time (Baer et al. 2002, 2010, Bach et al. 2010, Jangid et al. 2010, Klopf et al. 2017). Soil microbial communities also may change with these environmental shifts; for example, N inputs have been shown to drastically alter soil communities (Ramirez et al. 2012, Leff et al. 2015). These altered nutrient regimes will lead to differences in diversity and composition on the scale of years to decades, which may take a similar time to restore (Jangid et al. 2010, Barber et al. 2017a, Martinović et al. 2021). Along with altered nutrient regimes, the termination of tillage practices greatly reduces soil disturbance and can significantly alter microbial community composition (Mbuthia et al. 2015). Finally, increased biodiversity due to the transition from monocultures to complex prairie polycultures will drastically alter the carbon inputs, in both quantity and complexity, to soil, which will shape the composition of the microbial communities (Wang et al. 2019). These altered soil microbial communities will play a large role in the restoration of vital ecosystem services (Coban et al. 2022).

Grassland restoration often requires ongoing management and the application of disturbances that mimic historical disturbance regimes to maintain structure, physiognomy, and plant diversity (Collins and Adams 1983, Knapp et al. 1999, Fuhlendorf and Engle 2004, Bond and Keeley 2005). In addition to the indirect effects of management on soil microbes via changes in plant growth and composition (Kitchen et al. 2009), management disturbances like prescribed fire, grazing, and mowing can more directly influence soil organisms through changes in the soil environment (Wagle and Gowda 2018). Frequent fires reduce soil C, N, and N mineralization (Blair 1997, Kitchen et al. 2009) and can reduce soil moisture and increase soil temperature by eliminating thatch (Hulbert 1969, Dickson 2019), although soil bacterial communities do not necessarily differ in diversity or composition when comparing an-

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nually burned vs. unburned prairies (Coolon et al. 2013). Grazing mammals, on the other hand, can increase soil N availability and N mineralization, redistribute nutrients in labile forms in their feces and urine (Knapp et al. 1999, Johnson and Matchett 2001), and increase soil moisture (O'Keefe and Nippert 2017). They may also act as dispersal vectors of microbial communities through feces deposition (Hawkins and Zeglin 2022).

Chronosequence approaches, in which sites varying in age, such as time since restoration activities began, can reveal how communities change over time. However, single-time snapshots of microbial communities within a chronosequence can be misleading because community abundance, diversity, and composition can be influenced by short-term changes like recent precipitation (Evans and Wallenstein 2012). Additionally, the assumption that chronosequence sites differ only in age is often questioned (Johnson and Miyanishi 2008, Lem et al. 2022). For these reasons, longer-term monitoring is necessary to better understand changes in soil microbial diversity and composition, and to identify the taxa driving community changes that potentially shape functional outcomes (Martinović et al. 2021).

Here, we used long-term repeated sampling of a tallgrass prairie restoration chronosequence to understand how soil bacterial community diversity and composition change following restoration and under management with prescribed fire and reintroduced large native mammal grazers. Examining multiple restorations simultaneously is more robust than many restoration studies that follow a single restoration and allows us to assess whether communities follow a common pattern of postrestoration succession. We also compare communities to pre-restoration agricultural sites and original uncultivated remnant grasslands. Previous studies have found that restored prairie soil communities can come to resemble but remain distinct from remnant soil communities (Jangid et al. 2010, Barber et al. 2017a). We predict that bacterial communities will decline in diversity over time, with composition becoming more similar to remnants (and thus having high similarity among older-aged restorations), as found previously in a single-year class-level analysis at the same site (Barber et al. 2017a). We hypothesize that grazing disturbance and its resulting environmental heterogeneity will lead to distinct, and more diverse, communities, while the effects of frequent prescribed fire will be weak or nonexistent. Finally, we employ a machine-learning approach to identify taxa most strongly associated with community differences related to restoration age and management. We expect that taxa involved in nutrient cycling, especially N cycling, will be associated with change over time and grazing effects because of the influences of vegetation turnover and dung inputs (Coolon et al. 2013).

Methods

Site description and experimental design

Nachusa Grasslands is a 1600-ha preserve in north-central Illinois, USA, owned and operated by The Nature Conservancy. It consists mostly of restored tallgrass prairie and smaller areas of remnant hilltop prairies (never plowed or cultivated for crops), wetlands, and woodlands. Restorations have been carried out since the 1980s, primarily on former agricultural land that was cultivated in rowcrops for decades. In general, following the last harvest, native plant seeds from dozens of species are distributed, and ongoing management includes regular spot-spraying of aggressive invasive weeds and the application of prescribed fire. Sites receive prescribed fire approximately every 2 years, although the exact fire return interval for a site varies depending on weather. Restoration and management techniques and frequency are consistent across time. We studied a chronosequence of 13 restored prairies planted between 1987 and 2013, plus two remnant prairie sites that have never been converted to rowcrops and two agricultural sites that have been in continuous corn/soy cultivation for at least 40 years. Remnants also receive prescribed fire, but agricultural sites do not. Each site is >2 ha in size, and soil texture at all sites are loams, with some areas of sandy loams or silt loams. In late 2014, a herd of American bison (*Bison bison*) were introduced to a portion of the site, including five of our selected restorations, and by 2016 had access to seven restorations and one remnant. For additional site details, see Barber et al. (2017a, b, c).

We collected soil from study sites from 2013 to 2018, although the timing and sites varied slightly from year to year based on weather and site access. In 2013, we sampled sites at the end of the growing season (October). From 2014 to 2018, most sites were sampled in spring (April or May), summer (July or August), and fall (September or October) to correspond to the beginning, height, and end of the growing season. Table S1 provides details of sites' history and sampling events. Soil was collected from the top 5 cm in five locations within ~1 ha at each site, pooled, and homogenized. Any thatch or other detritus was moved prior to soil collection, and we avoided sampling directly from a plant's root mass. Samples were transported on ice and stored at −80°C within 2–3 h from sampling. We measured soil moisture, pH, total C, total N, and C:N ratio (ECS 4010 CHNSO Analyzer, Costech Instruments; Delta Plus Advantage IRMS, Thermo Fisher) from each sample.

DNA extraction, sequencing, and data processing

We extracted DNA from 0.2 g of soil from each sample using the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Venlo, Netherlands). We amplified 16S rRNA genes in preparation for Illumina sequencing using 515f/806r primers (Caporaso et al. 2012). rDNA was sequenced using 2 × 150 bp paired-end amplicon sequencing on Illumina MiniSeq (2013–14, 2017–18) or MiSeq (2015–16) platforms at the University of Illinois at Chicago Core for Research Informatics. We processed each sampling year independently using QIIME2 (Bolyen et al. 2019) and the DADA2 pipeline (Callahan et al. 2016) to filter forward reads for quality, trim primers, and remove chimeras. Total sequences and mean sequences per sample (before and after denoising) are presented in Table S2. DADA2 was also used to quantify amplicon sequence variants (ASVs), which we then classified to taxonomic groups with a naïve Bayesian classifier trained on the V4 region of the Silva 132 database (Quast et al. 2012). We used this classification to remove mitochondria and chloroplast sequences and unassigned sequences and produce ASV-based community tables for each sampling year. Features were collapsed to D5 level (genus) using QIIME2 plugin q2-taxa with command "collapse," and all analyses for this study were carried out at genus level. We also verified no singletons existed at this genus level. The number of ASVs and genera for each year are shown in Table S2.

Alpha diversity and soil characteristics

We calculated alpha diversity measures (genus richness and Shannon diversity) with rarefaction within each sampling year, using the minimum number of sequences in a sample (range 16 154–88 782) after dropping samples with very low sequence counts (Table S1). Total number of samples used for alpha diversity was 188 from restorations, 30 from remnants, and 27 from agricultural sites. We analyzed rarefied alpha diversity measures of restorations using linear mixed effect models (R package lme4, Bates et al. 2014), with site and year as partially crossed random factors and season (spring, summer, and fall) as a fixed factor. The inclusion of year accounts for both annual variation in communities and variation due to sample batch preparation and sequencing each year. Other fixed factors were site age (number of growing seasons since planting, centered), bison presence (if bison had access to a site at the time of sampling or not), and fire (if prescribed fire was used on the site in the previous dormant season). We used AIC to compare models that included these factors to models that also included age × bison and age × fire interactions and models that included a quadratic term for age. For both alpha diversity measures, a model containing the quadratic term but no interactions was the highest-ranked model (DAIC > 3.05 for all other models), so we retained this model. Fixed factors were evaluated with "Type II" tests using the Anova() function in the R car package (Fox and Weisberg 2018). To compare alpha diversity measures among agricultural sites, restorations, and remnants, we used the same linear mixed model structure but with only site type and season as fixed factors. Differences among site types were evaluated using emmeans with Tukey correction for multiple comparisons (Lenth 2021). This same approach was used to analyze soil characteristics (moisture, pH, C, N, and C:N).

Community composition

To determine how restoration characteristics affected community composition, we used perMANOVA with the adonis function in vegan (Oksanen et al. 2022). Each year was analyzed independently to avoid nonindependence of repeatedly sampled sites in a single analysis, although 2013 samples were combined with 2014 because of the limited sample size of soil collections in that year. Within each year, we removed all genus-level features that represented <0.1% of the sample to prevent rare taxa from exerting excessive influence on the analysis. We then calculated Bray–Curtis dissimilarities among all samples and scaled them to range from 0 to 1. PerMANOVA tests included restoration age, bison, fire, and season as predictor variables, using 999 permutations to estimate *F*, *P*, and the percent of variation in composition explained by each predictor (R^2). Because young restorations with bison had significantly different bacterial composition (see the section "Results"), we also analyzed each year using perMANOVA with sites classified as young restorations with bison (≤3 years old at the start of the study), old restorations with bison (≥4 years old), and non-bison restorations; these models also included fire and season as predictor variables. We visualized composition using principal coordinates analysis (PCoA), an unconstrained ordination technique that is particularly useful in situations where there are many more taxa than samples (Borcard et al. 2011).

Focal taxa

Features (genera) that most strongly drive classifier models were identified by assessing the information gain ratio for each feature used to construct individual decision trees. Those ASVs with an average abundance <0.1% in the entire dataset, as well as those that provided no information gain for any of the random forest decision trees, were removed from further analysis. Of the top 50 features with highest average information gain ratio across all 5 years (minimum 0.142), we selected those with >0.5% average relative abundance as focal taxa and analyzed their relative abundance in linear mixed effect models with restoration site class (bison young, bison old, or bison absent) and season as fixed factors, and site and year as random factors. Significant differences among

Table 1. Results of generalized linear mixed models of genus-level alpha diversity vs. site age, bison presence, prescribed fire, and sampling season.

	Rarefied richness		Shannon diversity		Soil moisture		Soil pH		Soil C		Soil N		Soil C:N	
Factor	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
Age	**9.11**	**0.003**	**20.95**	**<0.001**	1.50	0.220	**18.35**	**<0.001**	**4.03**	**0.045**	0.68	0.409	**25.20**	**<0.001**
Age^2	**9.79**	**0.002**	**19.73**	**<0.001**			**17.58**	**<0.001**					**8.36**	**0.004**
Bison	3.28	0.070	0.01	0.921	0.28	0.599	1.65	0.198	1.53	0.216	0.14	0.707	**9.97**	**0.002**
Prescribed fire	0.21	0.646	0.00	0.977	**5.14**	**0.023**	**12.37**	**<0.001**	0.32	0.574	0.28	0.596	0.08	0.778
Season	0.21	0.901	1.71	0.426	**117.11**	**<0.001**	4.68	0.096	**6.14**	**0.046**	5.36	0.069	4.95	0.084
Age × Bison							**6.88**	**0.009**						
Age × Fire							0.42	0.515						

Bold values indicate significant factors.

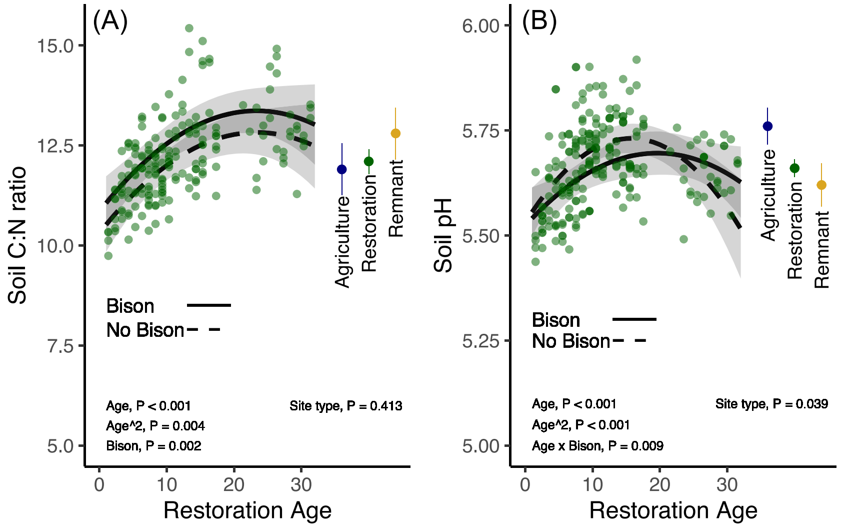

Figure 1. Effects of restoration age and bison presence (left side of each panel) and site type (agriculture, restoration, or remnant; right side of each panel) on (A) soil C:N ratio and (B) soil pH. In restorations, there was a significant quadratic relationship with site age, controlling for variation from repeated sampling of sites within and across years. Bison increased C:N and weakened the quadratic relationship with age for soil pH. Remnant prairies had higher C:N ratio than either prairie restorations or agricultural fields, while pH was highest in agricultural fields.

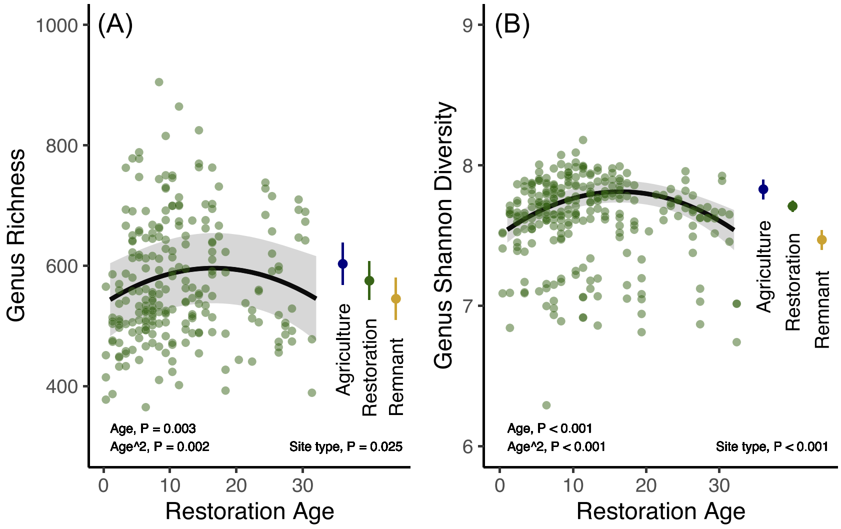

Figure 2. Relationships between restoration age and site type (agriculture, restoration, or remnant) and genus-level alpha diversity metrics. (A) Richness; and (B) Shannon diversity. For both metrics, there was a significant quadratic relationship with site age, controlling for variation from repeated sampling of sites within and across years. Remnant prairies had lower alpha diversity metrics than either prairie restorations or agricultural fields.

site class were examined using emmeans with Tukey correction for multiple comparisons. We also identified taxa that exhibited a large increase or decrease in information gain ratio following bison introduction. We analyzed their relative abundances in linear mixed models that were similar but included sample year as a fixed factor and a site class by year interaction. A significant interaction would indicate that relative abundance differences among site classes for the taxa changed during the study, such as after bison reintroduction.

Results

Soil characteristics. Soil moisture was reduced by fire and varied across a growing season, with moisture highest in spring, lowest in summer, and intermediate during fall (Table 1). Soil C increased with restoration age and was highest in fall and spring; soil N was unaffected by restoration characteristics but tended to show a similar seasonal pattern to C. C:N ratio had a quadratic relationship with age, with highest values (∼13) in mid-age and older restorations, and increased with bison presence by 0.54 (Fig. 1). Soil pH had a similar hump-shaped relationship, but was also slightly increased by fire (by 0.04), and there was a weak age × bison interaction such that the hump-shaped relationship was weaker when bison were present (Fig. 1). Site type also affected soil characteristics, with reduced soil moisture in agricultural fields and increased soil C and N in remnants (Table 2). Site types significantly differed in soil pH, with agricultural soils highest and remnant soils lowest pH, but post-hoc tests were nonsignificant.

Table 2. Results of generalized linear mixed models of genus-level alpha diversity vs. site type (agriculture, restoration, and remnant) with all years combined, and estimates of each metric of alpha diversity ± 1 SE.

	Rarefied richness		Shannon diversity		Soil moisture		Soil pH		Soil C		Soil N		Soil C:N	
Factor	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
Site type	**7.41**	**0.025**	**17.71**	**<0.001**	**14.65**	**<0.001**	**6.47**	**0.039**	**15.91**	**<0.001**	**11.09**	**0.004**	1.77	0.413
season	0.10	0.952	1.48	0.478	**125.13**	**<0.001**	3.66	0.160	5.67	0.059	**6.57**	**0.037**	4.19	0.123
Estimates:														
Agriculture	603 ± 35.3^{a}		7.83 ± 0.07^{a}		0.12 ± 0.02^{a}		5.76 ± 0.04^{a}		1.39 ± 0.31^{a}		0.12 ± 0.03^{a}		11.9 ± 0.65	
Remnant	545 ± 35.0^{b}		7.47 ± 0.07^{b}		0.20 ± 0.02^{b}		5.62 ± 0.05^{a}		2.97 ± 0.31^{b}		0.23 ± 0.03^{b}		12.8 ± 0.65	
Restoration	$575 \pm 32.3^{a,b}$		7.71 ± 0.04^{a}		0.17 ± 0.02^{b}		5.66 ± 0.02^{a}		2.05 ± 0.12^{a}		0.17 ± 0.01^{a}		12.1 ± 0.31	

Superscripts of estimates indicate significant differences post-hoc pairwise tests.

Alpha diversity. Genus-level rarefied richness and Shannon diversity were positively correlated, and both showed a significant hump-shaped relationship with age, with diversity increasing to mid-age restorations, then declining in older restorations (Table 1, Fig. 2). Neither bison presence nor prescribed fire significantly affected alpha diversity measures, and diversity did not vary consistently with season. Prairie remnants tended to have lower alpha diversity than either agriculture sites or restorations (Table 2, Fig. 2).

Community composition. Restoration age and bison presence were significant predictors of bacterial community composition in all years, and fire explained additional variation in 2015, 2017, and 2018 (Table 3). Bison status explained 17%–38% of variation in community composition, even in 2013 and 2014, before bison were introduced. This is because young restorations (in their third growing season or earlier) had very different composition than older restorations prior to bison introduction. When we reclassified sites as young bison, old bison, or no bison restorations, this age/bison category was significant in all years and explained 35%–53% of community variation, with young bison sites remaining different even when 5–7 years old (Fig. 3). Fire was significant only in 2015 (explaining only 5% of variation).

Focal taxa. Ten genera (Table 4) that had a high relative abundance and consistently provided high information gain to RF classifiers distinguishing bison/age classes were identified as focal taxa for further descriptive and statistical analyses. For all ten genera, site class was highly significant (Table 5). In most cases, bison absent and old bison restorations did not differ, while relative abundance in young bison restorations was either significantly greater (uncultured Micropepsaceae, *Gemmatimonas*) or lower (*Pseudonocardia*, *Gaiella*, *Solirubrobacter*, *Rhodoplanes*) (Fig. 4). Bison absent and old bison sites only differed in abundance of *Candidatus* Solibacter and *Acidothermus*, with reduced abundances in bison absent sites for both genera.

Five taxa showed large increases or decreases in information gain ratio after bison reintroduction. Three of these had zero information gain prior to bison (2013–2015) but relatively high gain (>0.374) after. These included *Rhodoplanes* and Ellin6067, as well as an uncultured genus from family Methyloligellaceae (also commonly discussed as Hyphomicrobiaceae) in class Alphaproteobacteria. Two uncultured genera, from family Desulfarculaceae in class Deltaproteobacteria and family *SC-I-84* in class Betaproteobacteria, had high information gain in early years but no gain in the last three or four years of the study. Ellin6067, uncultured *SC-I-84*, and the uncultured Methyloligellaceae all had significant site class by year interactions, while the other two taxa significantly differed by site class and year, but there was no interaction (Table S4, Fig. 5). However, none of these taxa showed clear divergence in abundances between bison and no bison sites after reintroduction. Rather, the uncultured Desulfarculaceae and Ellin6067 increase in abundance in young bison sites across the study period, becoming similar in abundance to or greater than other site classes, respectively. *Rhodoplanes* also increased in all site classes, but the increase was greatest in young bison sites. The uncultured *SC-I-84* differed among site classes in the first 2 years and then converged in abundance.

Discussion

Ecosystem restoration seeks to repair degraded habitats to support biodiversity and the functions they drive. We used long-term sampling to show that tallgrass prairie restoration and its ongoing management are associated with changes in the soil environ-

Table 3. Results of genus-level perMANOVAs on Bray–Curtis dissimilarity.

	Age			Bison			Fire			Season		
Year	F	P	R^2	F	P	R^2	F	P	R^2	F	P	R^2
2013–2014	**11.58**	**0.001**	0.193	**14.62**	**0.001**	0.244	1.40	0.212	0.023	1.66	0.109	0.055
2015	**5.79**	**0.004**	0.096	**9.91**	**0.001**	0.165	**3.97**	**0.014**	0.066	**3.75**	**0.005**	0.162
2016	**7.14**	**0.001**	0.092	**16.52**	**0.001**	0.212	2.01	0.127	0.026	**9.63**	**0.001**	0.247
2017	**6.47**	**0.003**	0.103	**12.72**	**0.001**	0.203	**3.92**	**0.020**	0.062	**3.82**	**0.001**	0.122
2018	**16.76**	**0.001**	0.159	**40.27**	**0.001**	0.383	**3.57**	**0.033**	0.034	**5.78**	**0.002**	0.110

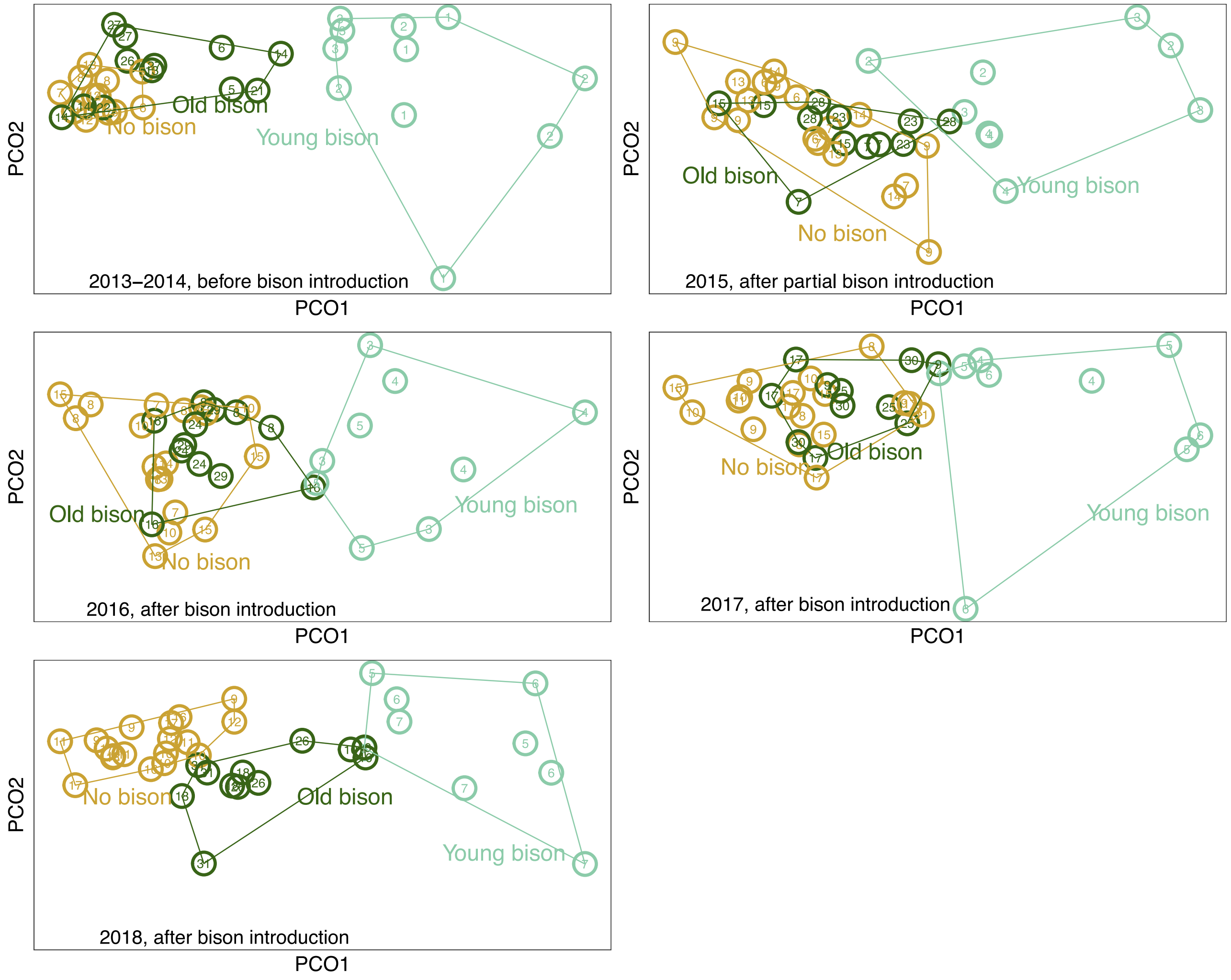

Figure 3. PCoA plots of soil bacterial community composition in restorations by year, with sites classified by age and bison status (young bison sites ≤ 3 years old; old bison sites ≥ 4 years old; and non-bison sites). Each point represents a soil sample, and the number on each point is the age of the restoration.

ment and bacterial diversity compared to pre-restoration agricultural fields and cultivated prairie remnants where bacterial diversity is high and low, respectively. The presence of reintroduced American bison did not influence bacterial richness or diversity, but was correlated with soil community composition. Younger restorations with early succession influenced by the presence of bison remained distinct from older restorations and those with no bison access, although directly attributing these differences to bison presence is not possible because younger restorations were already distinct before bison were introduced (see Fig. 3). Our novel approach to finding taxa that help distinguish between these restorations identified several genera, including some with abundance differences related to management in other grasslands around the world.

Restoration and management affect the soil environment, which in turn will impact the microbial communities. The reconversion of agricultural soils back into grassland communities increased soil moisture, C, and N content, while decreasing soil disturbance. As these restorations age, they further gain C and thus increase soil C:N ratio. Total C and N also increased over time in a restoration chronosequence at Konza Prairie (Rosenzweig et al. 2016). Although this did not translate into increased C:N ratio

Table 4. Focal genera identified as distinguishing among site class (bison young, bison old, and bison absent sites), with mean information gain ratio and mean abundance across all years of the study.

Phylum	Class	Order	Family	Genus	Mean GR	Mean abundance
Acidobacteria	Acidobacteriia	Solibacterales	Solibacteraceae Subgroup 3	*Candidatus* Solibacter	0.399	0.56
Acidobacteria	Blastocatellia Subgroup 4	Pyrinomonadales	Pyrinomonadaceae	*RB41*	0.348	1.42
Actinobacteria	Actinobacteria	Frankiales	Acidothermaceae	*Acidothermus*	0.428	0.69
Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	*Pseudonocardia*	0.290	0.62
Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiella*	0.397	0.84
Actinobacteria	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	*Solirubrobacter*	0.394	0.61
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas*	0.406	1.43
Proteobacteria	Alphaproteobacteria	Micropepsales	Micropepsaceae	(uncultured)	0.530	0.72
Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	*Rhodoplanes*	0.340	0.56
Proteobacteria	Betaproteobacteria	Betaproteobacteriales	Nitrosomonadaceae	Ellin6067	0.314	1.81
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methyloligellaceae	(uncultured)	0.152	0.12
Proteobacteria	Deltaproteobacteria	Desulfarculales	Desulfarculaceae	(uncultured)	0.228	0.26
Proteobacteria	Betaproteobacteria	Betaproteobacteriales	*SC-I-84*	(uncultured)	0.254	0.34

Rhodoplanes, Ellin6067, and taxa below the line showed large changes in information gain ratio during the study and were examined in more detail (see the section "Methods").

Table 5. Results of linear mixed models of focal genera relative abundance vs. site class (young bison, old bison, and bison absent) and season.

Genus	Site class		Season		Relative abundance in young bison sites
	χ^2	P	χ^2	P	
Candidatus Solibacter	**111.73**	**<0.001**	**8.39**	**0.015**	↑
Acidothermus	**108.85**	**<0.001**	0.20	0.905	↑
Pseudonocardia	**21.22**	**<0.001**	5.31	0.070	↓
uncultured Micropepsaceae	**123.92**	**<0.001**	0.85	0.654	↑
Rhodoplanes	**53.35**	**<0.001**	1.54	0.464	↓
RB41	**14.41**	**<0.001**	**4.21**	**0.012**	↓
Ellin6067	**9.60**	**0.008**	0.72	0.699	↑
Gemmatimonas	**48.26**	**<0.001**	**8.96**	**0.011**	↑
Gaiella	**19.76**	**<0.001**	**6.17**	**0.046**	↓
Solirubrobacter	**24.91**	**<0.001**	1.59	0.452	↓

Arrows in the final column indicate whether the relative abundance of that genus was higher or lower in young bison sites than the other site classes.

at Konza, the highest C:N values there were in the oldest restoration and in remnant soil. Cultivation reduces soil organic C (Six et al. 2000), so the increase in C:N after restoration is expected (Barber et al. 2017a, Li et al. 2021). We found that agricultural soils had higher pH than restored or remnant prairies, which is surprising because long-term fertilization was expected to acidify soils (Barak et al. 1997, Karlen et al. 1999, Russell et al. 2006). There was an initial reduction in soil pH following restoration, which increased in mid-age restorations, similar to Rosenzweig et al. (2016) findings, although it then declined in old restorations. This increase in C and C:N, and decreased pH, resulted in old restorations that more closely resemble, but do not match, the prairie remnants where soil C and N are both high. Bison tended to reduce the magnitude of this hump-shaped relationship. Fecal and urine inputs may have reduced the high pH values otherwise seen in mid-age restorations (Clegg 2006, Zhang et al. 2017). Although fire influences soil conditions (Johnson and Miyanishi 2008), and this may drive changes in soil microbial diversity (Zhu et al. 2021), the high prescribed burn frequency (approximately every 2 years) in our sites means few differences were apparent between sites that were burned in the previous dormant season and those that were not: fire slightly reduced soil pH and reduced soil moisture, likely by eliminating the thatch layer that otherwise covers the soil surface and reduces insolation and evaporation (Hulbert 1969). Although these are short-term effects of fire, there are also likely long-term impacts of repeated fires. Older restorations have received more prescribed fires, but these effects are encompassed by age, which includes all long-term changes in the biotic and abiotic environment, and are not distinguishable among sites because all restoration sites (and remnants) receive fire at similar frequency.

The hump-shaped pattern in soil C:N and pH with restoration age was mirrored by soil bacterial alpha diversity, for which both genus richness and Shannon diversity showed a weak increase in 10–15 years following restoration, then somewhat declined. A positive correlation between soil pH and bacterial richness occurs in other prairies and cultivated soils (Rousk et al. 2010, Zhu et al. 2021). The lower diversity of young restorations particularly contrasts with the relatively high richness and diversity of agricultural fields. This reduction in diversity likely follows the immediate environmental changes from re-conversion of agricultural fields to new restorations, including the cessation of tillage and fertilizer inputs, the addition of a diverse plant seed mixture, and

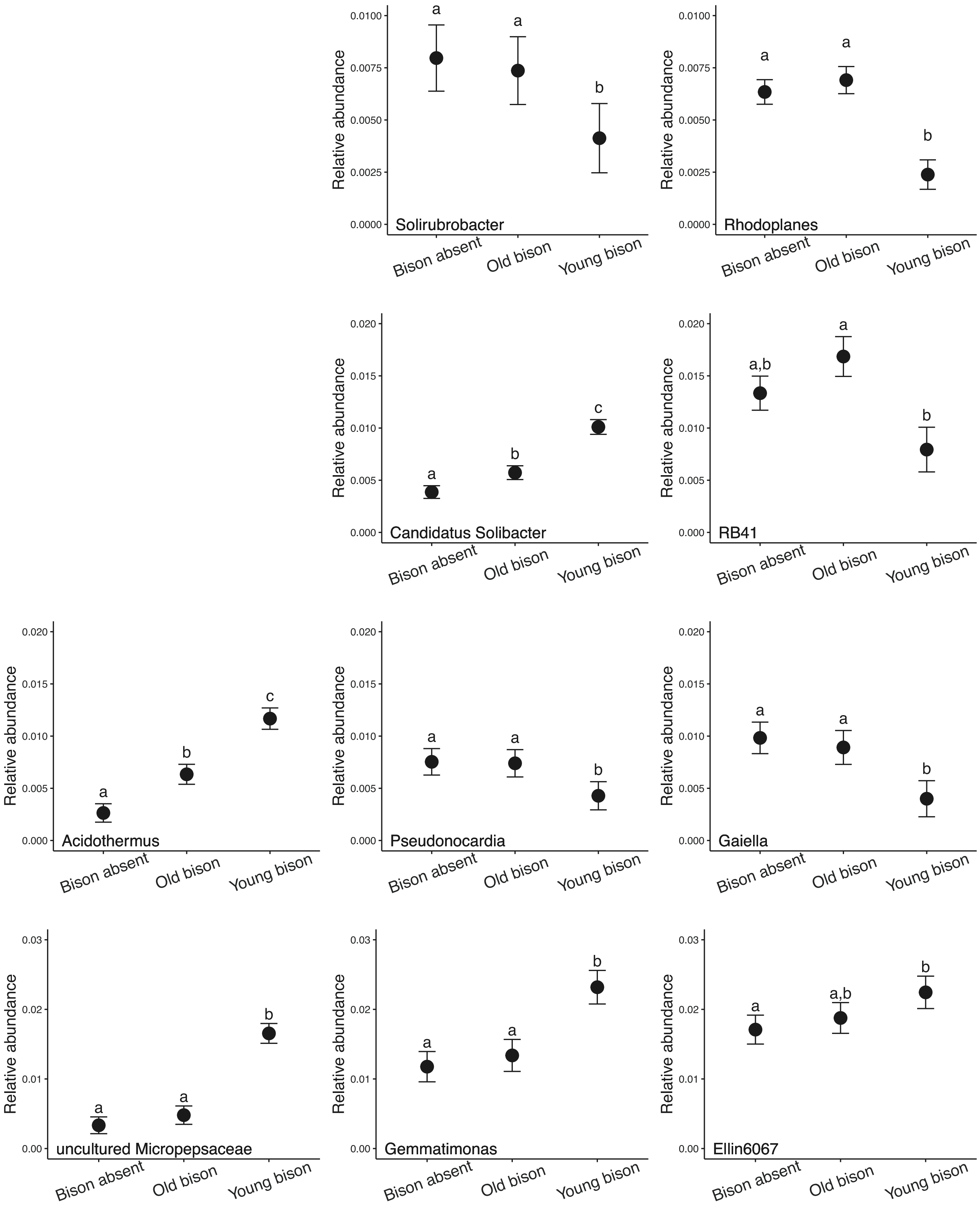

Figure 4. Estimated marginal mean relative abundances (± 1 SE) of focal genera by site class (bison absent, old bison, or young bison). Letters above each bar indicate significant differences among the three site types.

dense plant cover by both seed native species and agricultural weeds that will ultimately be outcompeted in the following years. On the other hand, the low alpha diversity of the oldest restorations is similar to diversity in prairie remnants, in agreement with previous studies that have found somewhat depauperate communities in unplowed native prairie (Barber et al. 2017a, Mackelprang et al. 2018). Thus diversity does eventually decline in restorations, perhaps due to gradual declines in plant richness as restorations age (Sluis 2002, Grman et al. 2013, Hansen and Gibson 2014), reducing the diversity of organic matter inputs (El Moujahid et al.

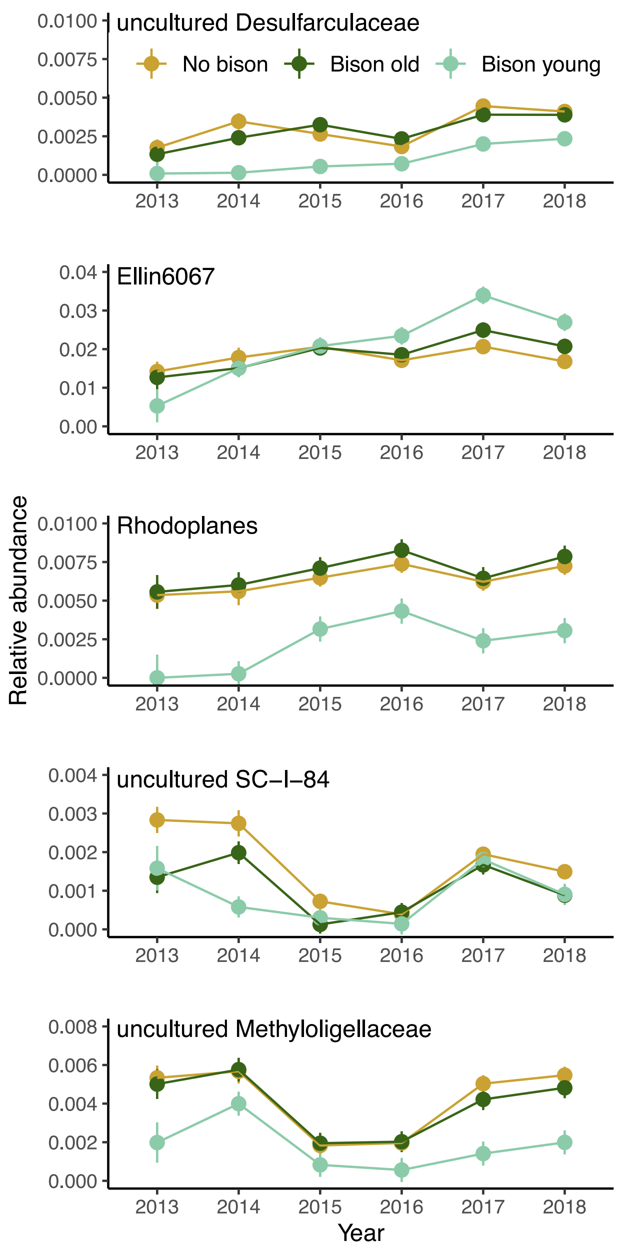

Figure 5. Estimated marginal mean relative abundances (± 1 SE) of focal genera by site class (bison absent, old bison, or young bison) across study years for species that showed large changes in information gain ratio.

2017). Our prediction that soil bacterial communities would be more diverse in the presence of bison was incorrect, although bison grazing, wallowing, defecating, and other activities does seem to result in community compositions distinct from ungrazed soils.

The early sampling years in this study, and an initial class-level analysis (Barber et al. 2017a), suggested a common pattern of postrestoration succession, in which bacterial composition in soils 1–3 years after restoration are distinct but then converge with older, more established restorations by the time they are 5–7 years old. However, our sampling over the following years showed that these young communities did not converge with those in other sites, instead remaining compositionally distinct by the end of our study period, when they reached 5–7 years old. One possible explanation is that these young sites, because they are somewhat spatially clustered, simply represent a different local community or species pool than other sites. But one of the oldest restorations (13–18 years old) nearby these same sites consistently differed in composition, with only one or two samples ever clustering near the young sites. Another nearby site (5–10 years old) also remained distinct, until 2018, when old bison restorations separated from non-bison sites and more closely resembled the young bison communities. Further, Mantel tests comparing Bray–Curtis dissimilarity to spatial distance for spring, summer, and fall 2018 sampling all found no significant relationship (all $|r| < 0.15$, $P > 0.7$). These patterns suggest another potential explanation, that the presence of bison is driving succession along a different trajectory in these young sites, perhaps through both their direct impacts on the soil environment and indirect effects on plant communities (Knapp et al. 1999, Blackburn et al. 2020). This is important because it could mean bison reintroduction adds new heterogeneity to the environment, bolstering total biodiversity across the entire preserve, and possibly supporting variation in soil function as well. Alternatively, another characteristic shared by the young sites could explain their consistently different composition, such as planting year weather (Groves and Brudvig 2019) or restoration methods. This seems unlikely, however, because these sites were planted over three years that were not consistently wet or dry, and restoration methods using locally collected and processed seed are standardized by the managers. These results also emphasize how chronosequences need to be used with caution when other environmental drivers than age differ among sites, as others have stressed (Johnson and Miyanishi 2008, Walker et al. 2010, Lem et al. 2022). Continued monitoring of these soils, paralleling plant community monitoring (Bach and Kleiman 2021), will help reveal if bison activities are indeed causing successional trajectories that vary with age, or if all bison-impacted sites will eventually converge. This latter scenario is possible given the demonstrated ability of bison dung to disperse soil bacteria and homogenize soil communities (Hawkins and Zeglin 2022).

We used a random forest classification approach to identify focal taxa that distinguished between the three restoration site classes apparent in our community analyses, as well as those whose importance in distinguishing among site classes changed after bison were reintroduced. Not surprisingly, most of these taxa showed differences in relative abundance in the young bison sites compared to the other site classes. Some of these were significantly more abundant in young bison sites (*Candidatus* Solibacter, *Acidothermus*, uncultured Micropepsaceae, *Gemmatimonas*, and Ellin6067), while others had decreased abundance (*Solirubrobacter*, *Rhodoplanes*, *Pseudonocardia*, RB41, *Gaiella*, uncultured Desulfarculaceae, and uncultured Methyloligellaceae). The taxa increasing in abundance include bacteria found to both increase (*Gemmatinmonas*) and decrease (*Candidatus* Solibacter) in response to grazing in steppe (Wang et al. 2022). *Candidatus* Solibacter is a member of phylum Acidobacteria with cellulase genes (Ward et al. 2009) that has been found to be the most abundant near the soil surface (Zhang et al. 2022); bison grazing might reduce litter inputs, reducing cellulose resources for these and similar bacteria. Ellin6067 is a Gammaproteobacteria in the family Nitrosomonadaceae that has been documented in tallgrass prairie soils (Coolon et al. 2013, Zhu et al. 2021). Florian et al. (2021) found Ellin6067 to increase in abundance with pH in soils across Switzerland, and its increasing abundance in young bison sites matches the increasing pH we saw over the decade following restoration. However, Nitrosomonadaceae are often ammonia oxidizers whose abundance spikes following fertilization (Clark et al. 2021), so it was surprising that we saw Ellin6067 increase when nutrients were not being added, unless the dung and urine inputs from bison are in a form that it can take advantage of. This may apply to Micropepsaceae, which can also be abundant in fertilized soils (Megyes et al. 2021, Yang et al. 2021).

The taxa that were consistently more abundant in non-bison sites and old bison sites represent several groups regularly found in perennial grasslands and in higher abundance than in arable cropland, such as *Gaiella* and *Solirubrobacter* (Armbruster et al. 2021). These two genera, as well as *Pseudonocardia*, are known or predicted CO_2-fixers that may be more common in oligotrophic soils (Zhao et al. 2018, Severino et al. 2019). *Gaiella* and *Solirubrobacter*, as well as RB41, also decreased in grasslands that were heavily or overgrazed (Wang et al. 2022), as did Deltaproteobacteria in general, which includes the uncultured Desulfarculaceae that we detected. Although the old bison restorations are subject to grazing, the density of the herd is low; over the course of the study, the herd varied from about 30–130 animals with access to >600 ha, so prairie restorations are unlikely to experience heavy overgrazing common in livestock management. The similar communities and abundances of these bacteria in non-bison and old bison sites indicates that bison did not have immediate strong effects on these soil communities, but the differing compositions by 2018 might mean the abundances of some of these or other taxa are diverging.

A noteworthy absence from our focal taxa are members of the phylum Verrucomicrobia, which have repeatedly been identified as indicators of remnant prairies and mature restored grasslands (Kuramae et al. 2010, Fierer et al. 2013, Brewer et al. 2016, Armbruster et al. 2021). An earlier study of our sites also found a strong trend of increasing Verrucomicrobia relative abundance as restorations aged, with the phylum representing a large proportion (8%–23%) of remnant prairie soil communities (Barber et al. 2017a). Some members of Verrucomicrobia were positively correlated with soil organic matter in pristine calcareous grasslands (Armbruster et al. 2021), so the absence of any genera as indicators of site class differences was surprising. In a closer examination of *Udaeobacter*, a common genus in grassland soils, we found that abundances did not differ among site classes ($\chi^2 = 4.53$, $P = 0.104$), averaging 1%–2% of the community. Defining features of *Udaeobacter* include its reduced genome size, increase in transporter genes responsible for importing various nutrients associated with soil organic matter, and lack of biosynthetic pathways responsible for synthesizing necessary amino acids and vitamins that may be freely present in soil environments and excreted by other microbes (Brewer et al. 2016). Such "auxotrophies" may confer energetic advantages that allow it to compete in oligotrophic environments like remnant and postrestoration prairies (Brewer et al. 2016). It may be that once a grassland habitat is established and agriculture-related disturbances cease, *Udaeobacter* quickly establishes regardless of grazing or other bison disturbances.

Three future directions will help further elucidate how tallgrass prairie restoration and management shape soil microbial communities. The first is to incorporate fungal assemblages into community studies given their abundance and importance to soil C and nutrient cycles (Averill et al. 2014, Tedersoo et al. 2020). Second, to tease apart the potential direct effects of bison on soil microbes from their indirect effects via changes to plant communities, ecologists will need to jointly examine plant and soil microbial communities at a finer scale than the meters-to-hectares scale studied here. Finally, understanding the functional consequences of community variation will provide grassland managers with more precise information about how their management strategies and actions, through impacts on soil microorganisms, affect the functions and services that often are the ultimate goals of ecosystem restoration.

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Supplementary data

Supplementary data is available at *FEMSEC* online.

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