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Quantifying the immediate response of the soil microbial community to different grazing intensities on irrigated pastures

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ABSTRACT

Grazing is known to affect soil microbial communities, nutrient cycling, and forage quantity and quality over time. However, a paucity of information exists for the immediate changes in the soil physicochemical and microbial environment in response to different grazing strategies. Soil microbes drive nutrient cycling and are involved in plant-soil-microbe relationships, making them potentially vulnerable to plant-driven changes in the soil environment caused by grazing. To test the hypothesis that variable grazing intensities modulate immediate effects on the soil microbial community, we conducted a grazing trial of three management approaches; high-intensity, short-duration grazing (HDG), low-intensity, medium-duration grazing (LDG), and no grazing (NG). Soil and vegetation samples were collected before grazing and 24h, 1 week, and 4 weeks after HDG grazing ended. Soil labile carbon (C) and nitrogen (N) pools, vegetation biomass, and soil microbial diversity and functional traits were determined, including extracellular enzymatic assays and high-throughput sequencing of the bacterial 16S rRNA and fungal ITS2 regions. We found that labile soil C and inorganic N increased following LDG grazing while C-cycling extracellular enzymatic activities increased in response to HDG grazing but both total extracellular enzymatic activity profiles and soil abiotic profiles were mostly affected by temporal fluxes. The soil fungal community composition was strongly affected by the interaction of sampling time and grazing treatment, while the soil bacterial community composition was largely affected by sampling time with a lesser impact from grazing treatment. We identified several key fungal taxa that may influence immediate responses to grazing and modulate plant-soil-microbe interactions. There was strong evidence of temporal influences on soil biogeochemical variables and the soil microbiome, even within our narrow sampling scheme. Our results indicate that the soil ecosystem is dynamic and responsive to different grazing strategies within very short time scales, showing the need for further research to understand plant-soil-microbe interactions and how these feedback mechanisms can inform sustainable land management.

1. Introduction

Grazing management has been the subject of a persistent global debate. Since grazing occupies as much as one-third of the earth's land surface area, the importance of understanding the ecological consequences of grazing management cannot be understated (Lal, 2002). Emerging research suggests adaptive grazing management strategies or systems may be beneficial for mitigating climate change (Lal et al., 2011) and reversing land degradation (Hillenbrand et al., 2019; Teague et al., 2011), while non-adaptive and inflexible grazing management can result in land degradation and reduce sustainability (Chen et al., 2011; Zhao et al., 2017). Yet to better understand the results found by rangeland scientists reported above, it is critical that we understand how grazing management impacts ecosystem functioning and related ecosystem services such as maintaining adequate forage quantity and quality through the lens of soil health which is the foundation for forage-based grazing enterprises (Derner et al., 2018).

Research has yielded conflicting results on the effects of high-intensity grazing on soil health and rangeland sustainability, forage productivity, and animal performance (Briske et al., 2008; Teague et al., 2013). Even though the soil microbial community is a major driver for nutrient availability and cycling (Nannipieri et al., 2002), there are

Abbreviations: C;, carbon; N;, nitrogen; DOC;, dissolved organic carbon; DON;, dissolved organic nitrogen.

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significant knowledge gaps in how soil microbes respond to grazing (Bagchi et al., 2017; Van Der Heijden et al., 2008), especially on short time scales (Vidal et al., 2020). Rapidly accelerating technology has unleashed new tools for analyzing soil health through the lens of microbial communities, and these methods can help us understand how grazing impacts plant-soil-microbe relationships. The debate over best management practices calls for statistically replicable, carefully constructed experimental designs to better understand the consequences of grazing intensities (Lal et al., 2011; Roche et al., 2015).

The major mechanisms by which grazing animals disturb rangeland are through physical trampling, excrement deposition and nutrient redistribution, and plant defoliation. These mechanisms affect soil pore space and microbial habitats, soil organic matter, and labile N (Damsama et al., 2015; Mikola et al., 2009; Schon et al., 2012; Schrama et al., 2013; Teague et al., 2011; Wang et al., 2006). Excrement deposition can directly deposit labile nitrogen (N) which encourages regrowth of high quality vegetation and enhances litter quality (Mikola et al., 2009; Schrama et al., 2013). Plant defoliation by grazing animals causes cascades of plant-soil-microbe interactions as plants mobilize resources to recover, which can shift below-ground resource allocation, affecting the microbial community involved in nutrient cycling. Effective grazing management has been found to increase organic matter decomposition rates (Bardgett et al., 2001; Dombos, 2001) and increase root exudation of labile carbon (C) (Hamilton et al., 2008), both of which can increase soil microbial biomass (Bardgett et al., 2001).

Many previous studies have oversimplified grazing as dichotomous (i.e. only comparing grazed or ungrazed) or have measured soil health responses on the timescales of years to decades (Cline et al., 2017; Hamonts et al., 2017; Medina-Roldán et al., 2012; Mueller et al., 2017). While the length and intensity of grazing vary widely and are known to affect plant communities and soil physicochemical properties (see reviews by Díaz et al., 2007; Byrnes et al., 2018), little is known about plant-soil-microbe interactions immediately following defoliation. This represents an important knowledge gap as soils research in other systems has found that the rhizospheric bacterial community composition responds to plant circadian rhythms (Hubbard et al., 2018) and pollution spikes (Thomas and Cébron, 2016) on the scale of hours to days. An additional study found that adding cattle manure to mesocosm soils affected the soil bacterial composition after approximately six months (Hu et al., 2016) and recent work has found that soil nitrogen and phosphorus were higher in pastures that were grazed within the past three months compared to grazing exclosures (Sato et al., 2019). As the soil microbial community is well characterized to play a major role in soil nutrient cycling (Fierer, 2017), there is a need to consider grazing at a series of intensities and at shorter time scales than has been previously characterized.

This study addressed the following research objectives: (1) quantify the effects of high-intensity, short-duration grazing (HDG), low-intensity, medium-duration grazing (LDG), and no grazing (NG) on soil physicochemical and biological parameters over a short time period, and (2) measure the pulse of microbial community dynamics immediately following grazing events. We hypothesized that plant-soil-microbe interactions result in measurable differences in soil nutrients and microbial activity and diversity, distinguishable by grazing intensity, within a single growing season. We quantified soil C and N pools and extracellular enzyme activity as a measure of soil microbial nutrient cycling capacity and microbial community diversity via high-throughput amplicon sequencing before grazing and 24 hours, 1 week, and 4 weeks after HDG grazing ended.

2. Methods

2.1. Field site description and experimental design

The grazing trials were conducted at the University of Wyoming's Agricultural Experiment Station – Laramie Research and Extension Center (UW AES LREC) in Laramie, Wyoming (41°18'13.5"N, 105°38'24.4"W), on twelve adjacent 0.2 hectare paddocks which have historically been flood irrigated and irregularly grazed (Fig. 1 A). Vegetation is dominated by creeping meadow foxtail (*Alopecurus arundinaceous Poir.*), an exotic C3 perennial grass. The soil type is a fine-loamy, mixed Borollic Haplargid (Soil Survey Staff, 2019).

Three grazing treatments were replicated four times in a randomized complete block design. The treatments were randomly assigned to paddocks/plots as: high intensity, short-duration grazing (HDG); low intensity, medium-duration grazing (LDG), and no grazing (NG) (Fig. 1B). Stocking density was planned in animal units (AU), defined as a 450 kg animal that consumes 12 kg of forage per day (Smith, 2016). Stocking density and subsequent forage utilization were planned to reflect high-intensity and medium-intensity grazing management systems that are realistic options for high-production, subirrigated pastures (Scasta et al., 2015). The stocking density for HDG was 29 heifers per paddock (56,020 animal kg hectare^{-1} which is equivalent to 124.5 AU hectare^{-1}), and for LDG was 3 heifers per paddock (5795 animal kg hectare^{-1} which is equivalent to 12.9 AU hectare^{-1}). The HDG treatment heifers grazed for 24 h, while the LDG treatment heifers grazed for 8 days to reach desired forage utilization (Fig. 1 C). Thus, stocking rates in terms of Animal Unit Months (AUMs) for HDG were 4.1 AUMs hectare^{-1}, and for LDG was 3.4 AUMs hectare^{-1}. Grazing durations were calculated from available forage measured before the grazing trial started (Elzinga et al., 1998). Heifers were provided by the UW AES LREC Beef Unit and were commercial Angus × Gelbvieh beef heifers (*Bos taurus*) with a mean weight of 386.3 kg. Animal use was approved by the University of Wyoming - Institutional Animal Care & Use Committee (IACUC protocol 20170508EB0061–02).

2.2. Vegetation biomass

Vegetation measurements were done at four intervals: 5–6 days before grazing (PRE), 24 h after HDG grazing completed (24H), 7 days after (1WK; 24 h after LDG completed grazing), and four weeks (4WK).

Rising plate pasture meter (RPM) measurements (Manual Platemeter, Jenquip, New Zealand) were used for estimation of vegetation biomass as a nondestructive alternative to clipping, drying, and weighing methods (Macadam and Hunt, 2015). RPM measurements were collected according to the manufacturer's instructions in a zig-zag pattern throughout each plot while maintaining a 1 m buffer zone around fences and water troughs, ensuring approximately 100 measurements per plot at each sampling time. Pre-calibrated regression equations for specific forage types have been shown to be unreliable outside of the specified vegetation, so RPM values were used as a relative vegetation biomass estimate (Sanderson et al., 2001).

2.3. Soil sampling and processing

Soil samples were collected at the same time as the vegetation measurements, between 6AM and 10AM. At each sample interval, five soil samples were collected within each plot along a 30 m transect at randomly determined distances to 5 cm depth with an ethanol (70%) sterilized bulb cutter (10 cm in diameter) and composited, resulting in one composite soil sample per plot per sample interval for a total of 48 samples (four time intervals x 12 plots). The 30 m transect was selected based on minimal distance to maintain a 1 m buffer from fences and water troughs which receive disproportional use and could confound results. Samples were stored on ice until transport to the laboratory for further processing the same day. Composite soil samples were homogenized, and roots were handpicked using sterilized tweezers. Roots were lightly shaken to remove non-rhizospheric soil, placed in a sterile 50 mL tube, and vortexed for 5 min, after which roots were removed and the remaining rhizospheric soil was frozen at − 20 °C until deoxyribonucleic acid (DNA) extraction. The remaining homogenized soil was sieved to < 2 mm and a subset used for physicochemical analyses or

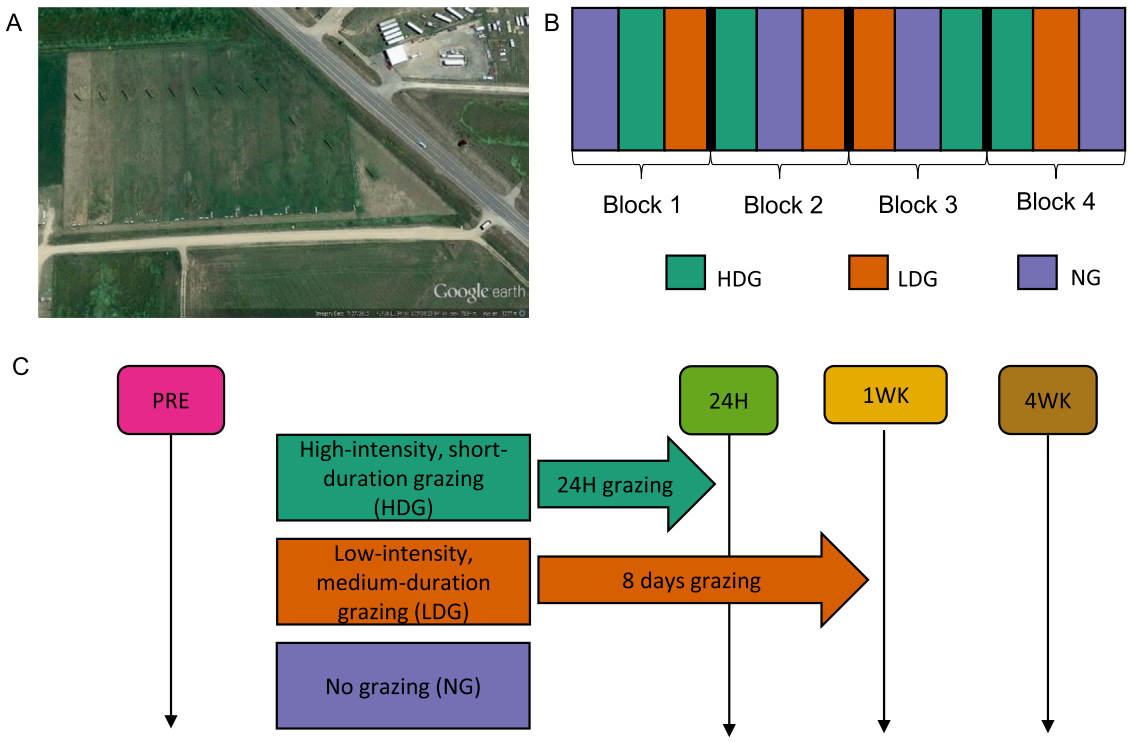

Fig. 1. The grazing trial was conducted in Laramie, Wyoming (USA) at the University of Wyoming's Agricultural Experiment Station – Laramie Research and Extension Center. (A) A satellite image of the twelve 0.2 hectare plots that were uniformly irrigated before grazing began. (B) A schematic of the treatment assignments. Treatments were randomly assigned, except for the westernmost and easternmost plots that were assigned to NG to minimize the risk of cattle jumping the fence to graze a neighboring alfalfa field. (C) A schematic of the sampling times. Samples were collected one week before grazing (PRE), and 24 h (24H), 1 week (1WK), and 4 weeks (4WK) after HDG grazing.

frozen at − 20 °C for extracellular enzyme assays and DNA extraction.

2.4. Soil physicochemical analyses

Soil analyses included gravimetric water content, pH, and C and N pools. Gravimetric water was measured by oven-drying field fresh soil at 105 °C for 24 h. Inorganic N (ammonium and nitrate) and dissolved organic carbon (DOC) and nitrogen (DON) were extracted from a ~10 g subsample of field fresh soil which was shaken for 30 min in 50 mL 0.5 M K_2SO_4 solution, filtered through Fisherbrand Q5 filter paper, and then frozen at − 20 °C until analysis. Ammonium concentrations were measured using a modified phenol-hypochlorite reaction according to Weatherburn (1967) and nitrate was determined using a modified method according to Doane and Horwath (2003). Ammonium and nitrate concentrations were read at 650 nm and 540 nm, respectively, using a BioTek Synergy HTX multi-mode reader (BioTek Inc., Winooski, VT, USA) and concentrations were converted to be expressed as mg kg^{-1} dry soil. Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were quantified via combustion catalytic oxidation (Shimadzu TOC-VCPH with TNM-1, Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). Dissolved organic nitrogen (DON) was calculated by subtraction of total inorganic N (ammonium and nitrogen) from TDN, and both DOC and DON were expressed as mg kg^{-1} dry soil.

2.5. Extracellular enzyme assays

Activities of carbon, nitrogen and phosphorus cycling enzymes were quantified either fluorometrically or colorimetrically following a modified version of Bell et al. (2013), Saiya-Cork et al. (2002), and van Diepen et al. (2015) as described in Custer et al. (2020). Optimal kinetics were determined by testing a range of substrate concentrations and incubation times according to German et al. (2011). Briefly, 1 g of freshly thawed soil was blended with 100 mL of 50 mM sodium acetate at 7 pH (mean pH of all soil samples). Then, 200 μL of soil slurry was pipetted into 96-well microplates, with four replicate wells per sample. The MUB- or AMC-linked enzyme-specific substrates (50 μL) were added to the assay wells, and samples were incubated at 20 °C for the predetermined incubation time. Standards and quench controls were run at the same time using the sodium acetate buffer, or soil slurries combined with 50 μL MUB- or AMC-linked standard (10 μM), respectively. Fluorescent assays of α-glucosidase (AG), β-glucosidase (BG), β-xylosidase (BX), cellobiohydrolase (CBH), leucine aminopeptidase (LAP), N-acetyl-β-glucosaminidase (NAG), and phosphatase (PHOS) were read at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using a BioTek Synergy HTX multi-mode reader (BioTek, Inc., Winooski, VT). The oxidative enzyme assays of peroxidase (PEROX) and phenol oxidase (PHENOX) were read for absorbance at 450 nm after addition of 50 μL L-DOPA (L-3,4-dihydroxyphenylalanine) with or without addition of 10 μL of 0.3% hydrogen peroxide, respectively, using a BioTek Synergy HTX multi-mode reader (BioTek, Inc., Winooski, VT). Due to practical limitations of field sampling, enzymatic assays were conducted on soil that was frozen at − 20 °C on the sampling day and thawed immediately before assay preparation. While freezing can affect enzymatic activity, it was not possible to perform enzymatic assays on fresh soil, but all samples were treated the same to allow direct sample comparisons (Peoples and Koide, 2012). Potential enzymatic activity is reported as nmol or μmol activity $hour^{-1}$ gram dry $soil^{-1}$.

2.6. DNA extraction and amplicon library preparation

DNA was extracted from ~0.25 g frozen soil using MoBio's Power Soil kit following the manufacturer's instructions (MO BIO, Carlsbad, CA) and DNA quantity and quality were quantified colorimetrically via Take3 software on the BioTek Synergy HTX multi-mode reader (BioTek, Inc., Winooski, VT). Extracted DNA was diluted 10x and stored at − 20 °C until further use. Indexed primers for the fungal ITS2 (Internal

Transcribed Spacer) region and V4 region of the bacterial 16S rRNA gene were coupled to an Illumina adaptor sequence. The forward primer 515f (5'-GTGYCAGCMGCCGCGGTAA-3') (Parada et al., 2016) and reverse primer 806R (5'-GGACTACNVGGGTWTCTAAT-3') (Apprill et al., 2015) were used for amplification of bacteria, and the forward primer fITS7 (5'-GTGARTCATCGAATCTTTG-3') (Ihrmark et al., 2012) and reverse primer ITS4 (5'-CCTCCGCTTATTGATATGC-3') (White et al., 1990) were used for fungi. Triplicate PCR was performed according to Custer et al. (2020) using Phusion High Fidelity polymerase, and correct amplification was checked on a 1.5% agarose gel. Triplicate PCR products were pooled and cleaned with Axygen's AxyPrep Mag PCR Clean-up Kit by manufacturer's instructions (Axygen Biosciences, Union City, CA). Concentrations of cleaned products were measured with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). An equimolar mix was prepared of the cleaned bacterial and fungal products separately for submission to the University of Minnesota Genomics Center, where sequencing was performed on the Illumina MiSeq platform with version 2 chemistry resulting in paired-end reads of 250 bp length.

2.7. Sequencing data processing

Bacterial and fungal paired-end reads were pre-processed differently to optimize merging parameters. Primers were removed from bacterial and fungal paired-end reads with cutadapt software v1.9 and bacterial paired-end reads were merged in USEARCH (Edgar, 2010; Martin, 2013). All other processing was done with the DADA2 R package (Callahan et al., 2016). Remaining bacterial and fungal paired-end reads were filtered for quality by truncating at Phred score 10, removing reads with more than two expected errors, and removing ambiguous (N) bases. Denoising, merging of paired-end reads (fungi), and chimera removal with the consensus method were done prior to grouping into amplicon sequence variant tables (ASV – hereon referred to as "taxa"). Taxonomy was assigned to bacterial ASVs using the Silva v132 database (Quast et al., 2012) and fungal ASVs using the UNITE database (Nilsson et al., 2018).

2.8. Statistical analyses

Data are presented as mean ± standard error. All analyses were performed at the plot level and in R (R Core Team, 2016). Significance was accepted at $p < 0.05$. Visual data exploration was performed prior to all statistical analysis to identify outliers and evaluate normality and heteroscedasity (Zuur et al., 2009). Forage consumption was calculated as the percent change of the immediate post-grazing sampling time (HDG, 24H; LDG, 1WK) compared to the pre-grazing baseline.

After exploratory analysis, block 4 was removed from the analysis based on the following factors; 1) irrigation water pooled in block 4 for several days longer than the rest of the plots, leading to a vegetation shift, 2) the NG plot of block 4 is shaped differently than the rest of the plots and has historically been used as loading area, which resulted in an increased amount of bare ground relative to the rest of the plots ($p < 0.05$), and 3) 100 sheep were placed in plot 12 the evening before the 4WK sampling interval, so data could not be collected.

Statistical analyses were comprised of 1) generalized linear models to determine changes in vegetation biomass estimates, soil physicochemical variables, extracellular enzymes, and alpha diversity indices, 2) permutational multivariate analysis of variance (PERMANOVA) tests to examine differences in soil bacterial and fungal community compositions, extracellular enzymatic profiles, and soil abiotic profiles, 3) Mantel correlation tests to examine directional changes between aforementioned profiles, and 4) differential abundance analysis to determine key taxa.

Soil physicochemical variables and extracellular enzyme activities were analyzed using a generalized linear model comparing grazing treatment, sampling time, and their interaction. Because of the heterogeneous nature of soil, data was converted to percent change from the pre-grazing baseline at the plot level and log-transformed where necessary to improve normality. Several outliers were identified in the nitrate data where transformation was not possible. Non-parametric Kruskal-Wallis tests were performed on nitrate data for treatment and sampling time differences; no significant differences were observed, and nitrate was removed from further analysis (Table S1). If the generalized linear model indicated significance, post hoc pairwise comparisons were made via estimated marginal means with the emmeans R package (Lenth, 2019).

Bacterial and fungal reads were filtered for reads assigned at the Bacteria or Fungi Kingdom level, respectively. Bacterial and fungal taxa were rarefied to the lowest sample read count for alpha diversity metrics while beta diversity was analyzed using standardized values (proportion of total reads by taxa for each sample). Alpha diversity indices were calculated for observed ASVs (number of taxa) and the Shannon's diversity index with the phyloseq package in R (McMurdie and Holmes, 2013). Alpha diversity indices were tested by three-way ANOVA (comparing bulk vs. rhizospheric soil, treatment, and sampling time) if sufficiently normally distributed, or the generalized linear model as described above. Bray-Curtis dissimilarity was calculated for the standardized soil bacterial ASVs, soil fungal ASVs, and extracellular enzymatic data, and Euclidean distance was calculated for the soil abiotic data to examine these variables as singular profiles. Permutational ANOVA (PERMANOVA; adonis) was performed on each dissimilarity matrix as a function of treatment, sampling time, the interaction of treatment and time, and soil type (bulk or rhizospheric) (Oksanen et al., 2019). PERMANOVA results were visualized using non-metric multidimensional scaling (NMDS) ordinations. Mantel correlations were performed between each dissimilarity matrix to test directional correlations between bacterial, fungal, extracellular enzymatic, and soil abiotic profiles with combined bulk and rhizospheric samples. Differential relative abundance analysis was performed with DESeq2 in bacterial and fungal soil microbial communities at the phylum and genus level to compare bulk and rhizospheric soil, and at the genus level to compare treatments (Love et al., 2014). The pairwise comparisons were made between grazing treatments (HDG and LDG) and the non-grazing control (NG) with the data aggregated from all sampling times except pre-grazing (PRE), which was removed to consider only grazing impact versus non-grazing impact.

The R code and raw data from these analyses are available at: https://github.com/EmilyB17/soils-micro. Sequencing reads are available at NCBI SRA accession PRJNA658147 and PRJNA681576.

3. Results

3.1. Vegetation biomass

LDG treatment forage consumption was 26 ± 4.50% while HDG was 50 ± 1.35%. Vegetation biomass increased over the sampling period in the grazing exclusion plots (NG) (16.8 ± 8.58% at 4WK compared to PRE baseline; Fig. 2 & Table S2). In accordance with the grazing intensities, relative vegetation biomass was significantly lower in each grazing treatment after grazing (HDG at 24H, LDG at 1WK) and did not recover to pre-grazing levels (PRE) by the end of sampling (Fig. 2, Table S2 and S3).

3.2. Soil physicochemical variables

Soil water content was strongly affected by sampling time regardless of treatment. Soil water content decreased from PRE to 24H, then increased between PRE and subsequent sampling times (1WK and 4WK) (Fig. 3A, Table S2 and S3). While DON did not have any significant effects, DOC was higher in the LDG than the HDG treatment at both 24H and 1WK sampling, but not different compared to NG (Figs. 3B and 3 C, Table S2 and S3). In addition to having lower DOC than LDG, HDG had a

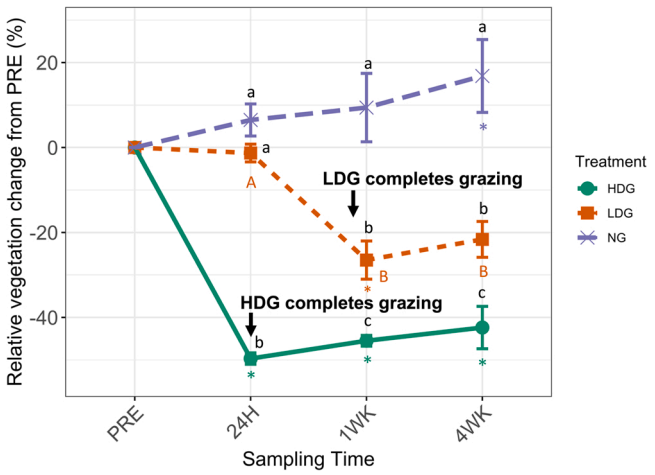

Fig. 2. Relative vegetation biomass changes over time by treatments. Data are presented as mean ± standard error of the percent change compared to pre-grazing (PRE), and black arrows show when high-density grazing (HDG) and low-density grazing (LDG) completed grazing. Different black lowercase letters indicate significant differences between treatments within a sampling time; different colored uppercase letters indicate significant differences between sampling times within a treatment; colored asterisks indicate a significant difference from the PRE baseline for a treatment at that sampling time. NG: No grazing treatment.

decrease in DOC from PRE to 1WK, while no changes between sampling times were observed in LDG or NG. Ammonium showed similar trends as DOC, with higher concentrations in the LDG than HDG treatment at 1WK sampling with no differences compared to NG, and ammonium levels in HDG decreased from 24H to 1WK (Fig. 3D, Table S2 and S3). These data suggest ammonium and labile C decreased immediately following HDG grazing while LDG and NG were largely unaffected, but by four weeks following the grazing trials ammonium and labile C concentrations were similar among all three treatments.

PERMANOVA revealed that the soil abiotic profile differed only over sampling times with no overall treatment effect (Sampling time $F_{24,35} = 7.15$, $p \leq 0.001$; Treatment $F_{24,35} = 0.50$, $p = 0.718$; Time by Treatment interaction $F_{24,35} = 0.91$, $p = 0.499$). Pairwise comparisons showed differences between PRE and 24H (Bonferroni-adjusted $p = 0.03$), 24H and 4WK (Bonferroni-adjusted $p = 0.012$), and 1WK and 4WK (Bonferroni-adjusted $p = 0.006$), suggesting that the soil chemical environment is dynamic and variable due to discrete temporal shifts that may be driven by soil water content within the short experimental time period.

3.3. Soil extracellular enzymatic activity

Extracellular enzyme activities were characterized by fluxes in response to sampling time in addition to a spike in enzymatic activity in HDG immediately following grazing. At 24H, HDG had higher activity in β-glucosidase (BG) and cellobiohydrolase (CBH) than the LDG and NG treatments, and N-acetyl-β-glucosaminidase (NAG) was higher in HDG and LDG compared to NG (Fig. 4A, Table S4 and S5). This spike was not detected at the 1WK sampling time when LDG had finished grazing (Fig. 4B).

Treatment-specific temporal shifts were also observed; specifically, β-glucosidase (BG), β-xylosidase (BX), and cellobiohydrolase (CBH), involved in cellobiose, hemicellulose, and cellulose degradation, respectively, decreased from 24H to 1WK and from 24H and 4WK in the HDG treatment (Table S4). In the LDG treatment, BG and BX decreased from PRE to 1WK and BX and CBH decreased from PRE to 4WK, while in

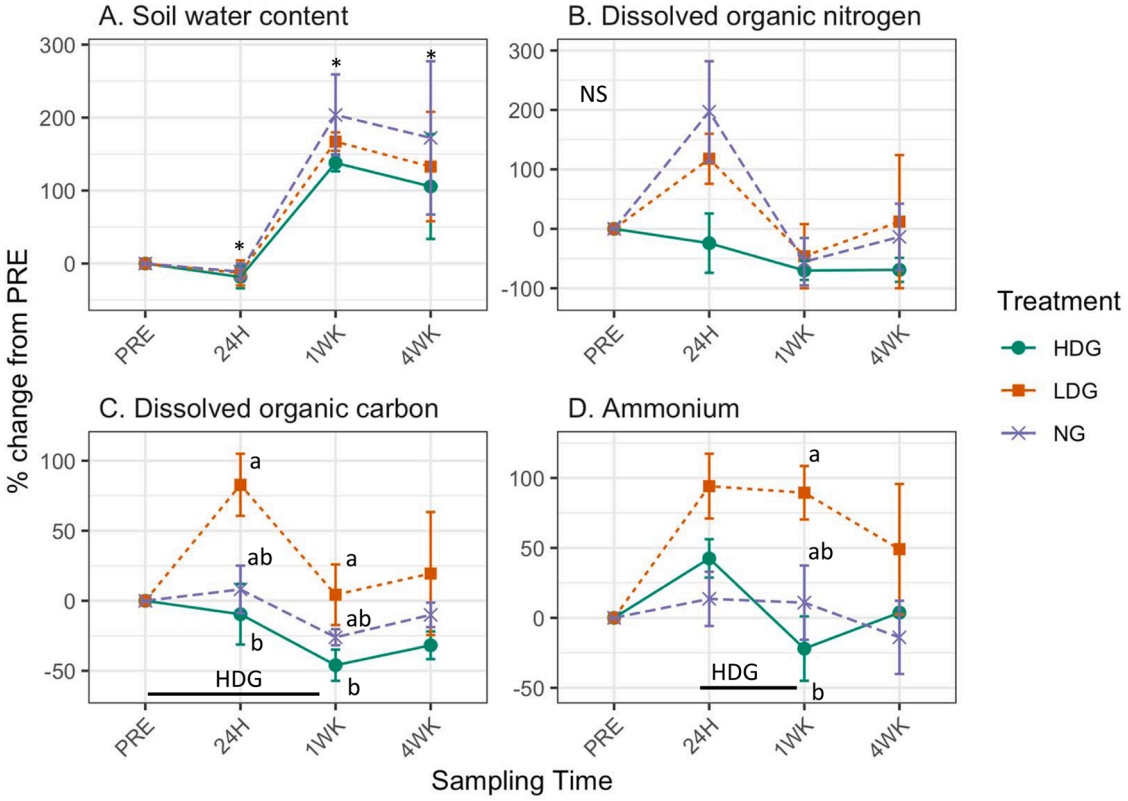

Fig. 3. Soil physicochemical variables in each grazing treatment over the sampling period. Data is shown as the mean of percent change from pre-grazing baseline (PRE) and error bars show standard error. Asterisks denote significant changes in all grazing treatments between the sample time and the pre-grazing sample time (PRE) (in soil water content only). Different letters indicate significant differences between treatments within a sampling time. Black bars show significance between two sampling times within a treatment (HDG only). NS: no significance within times or treatments (dissolved organic nitrogen only).

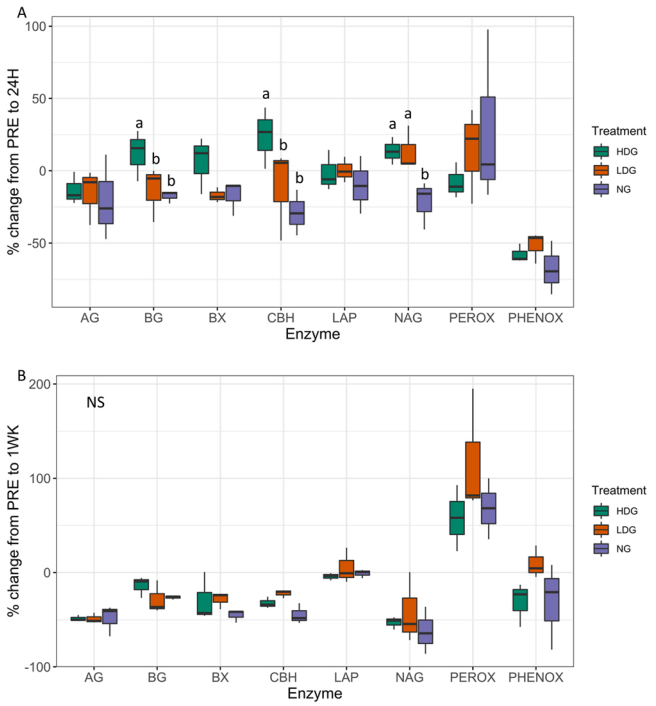

Fig. 4. Potential extracellular enzyme activity during (A) the 24H sampling period, when HDG had just finished grazing and (B) the 1WK sampling period, when LDG had just finished grazing. Data is shown as the percent change compared to pre-sampling baseline (PRE). The black line within each box is the median, and the whiskers show 1.58 times the inter-quartile range. Different letters denote significant differences between treatments for each enzyme. Phosphatase (PHOS) is not shown. AG, α-glucosidase; BG, β-glucosidase; BX, β-xylosidase; CBH, cellobiohydrolase; LAP, leucine aminopeptidase; NAG, N-acetyl-β-glucosaminidase; PEROX, peroxidase; PHENOX, phenol oxidase.

the NG treatment BG, BX, and CBH all decreased from both PRE to 1WK and PRE to 4WK. Thus, LDG and NG did not have the same spike as observed in the HDG treatment at 24H.

There was a strong temporal shift in α-glucosidase (AG – involved in starch hydrolysis) and BX regardless of treatment; all three treatments decreased from PRE to 1WK and from PRE to 4WK. There were no significant differences in phosphatase (PHOS), leucine aminopeptidase (LAP), peroxidase (PEROX), or phenol oxidase (PHENOX) enzymes, indicating that phosphorus cycling (PHOS), protein (LAP), and lignin degradation (PEROX and PHENOX) were largely resilient to temporal or treatment effects (Table S4 and S5).

Similar to the soil abiotic profile, PERMANOVA testing showed that the enzymatic activity profile differed over the sampling times with no overall treatment effect (Fig. 5; Sampling time $F_{24,35} = 2.64$, $p = 0.006$; Treatment $F_{24,35} = 0.48$, $p = 0.827$; Treatment & Time interaction $F_{24,35} = 0.70$, $p = 0.814$). Pairwise comparisons showed a significant difference specifically between 1WK and 4WK (Bonferroni-adjusted $p = 0.006$).

3.4. Soil microbial communities

We obtained a total of 4,984,182 fungal reads and 4,143,245 bacterial reads. After processing, 38% of fungal reads were retained (1,892,993 total, average 19,718 reads per sample) and 87% of bacterial reads were retained (3,588,527 total, average 37,380 reads per sample). Processing of fungal ITS reads was complicated by poor quality at the ends of longer sequencing fragments; shortening the fragments during quality processing possibly resulted in fewer ASVs and taxonomic assignments against the longer ITS region (approximately 550 bp compared to approximately 254 bp in the 16S V4 region) (Nilsson et al., 2015). A total of 3591 fungal ASVs were assigned and rarefied to 2701 ASVs, and 19,801 bacterial ASVs were assigned and rarefied to 9559 ASVs.

The alpha diversity in the soil microbial community was largely resilient to change. Fungal richness (observed ASVs) was higher in bulk

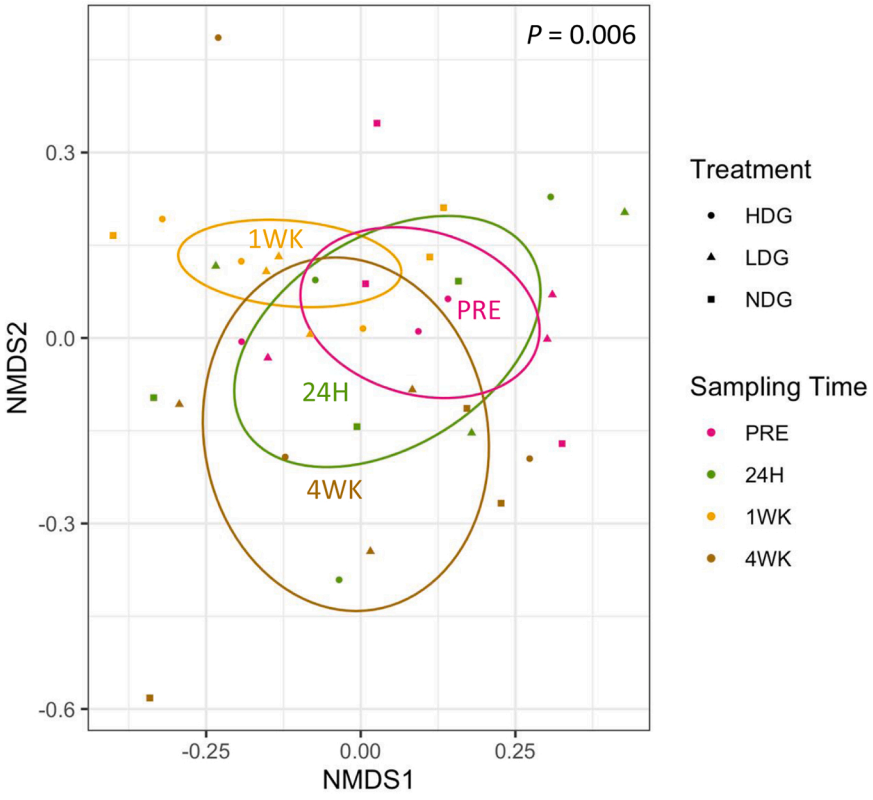

Fig. 5. Nonmetric multidimensional scaling visualization of the extracellular enzymatic profile over sampling time (stress = 0.137). Pairwise permutational ANOVA testing showed a significant difference between 1WK and 4WK with no overall treatment effects (Treatment $p = 0.827$). Sampling time PERMANOVA p value is reported on the figure. Ellipses represent 95% confidence intervals, shapes represent treatments (HDG – circle; LDG – triangle; NG – square), and colors represent sampling time (PRE – pink; 24H – green; 1WK – yellow; 4WK – brown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

than rhizospheric soil, but no differences were found in bacterial richness, or fungal and bacterial Shannon's diversity index between bulk and rhizospheric soil (Table S6). There were also no differences observed between treatments, sampling times, or their interaction, both in bulk and rhizospheric soil for fungal (Table S7) or bacterial alpha diversity indices (Table S8).

Bulk soil had distinct fungal and bacterial community compositions (beta diversity) compared to rhizospheric soil (Fig. S1, Table S9). These changes were further investigated with differential relative abundance analysis at the phylum level, which revealed that the fungal phylum Glomeromycota was relatively more abundant in rhizospheric than bulk soil ($\log_2$ fold change 0.739) and the bacterial phylum Cyanobacteria was significantly less relatively abundant in rhizospheric than bulk soil ($\log_2$ fold change −0.821) (Table S10). However, at the genus level there were 13 fungal genera and 33 bacterial genera that had different relative abundances in rhizospheric soil compared to bulk soil (Table S10). These findings suggest that shifts in the microbial community compositions between bulk and rhizospheric soil may be driven by many simultaneous changes across genera with only a few large shifts in phylum abundance.

Fungal community composition was more responsive to treatment (Fig. 6A), while the bacterial community strongly responded to time (Fig. 6D). PERMANOVA analysis of Bray-Curtis distance of fungal ASVs resulted in significant effects of treatment, sampling time, and soil type (bulk versus rhizospheric), but not the interaction between time and treatment (Figs. 6A and 6C, Table S11). Pairwise comparisons showed differences between all treatments, and between both 24H and 1WK and 24H and 4WK (Table S11).

Similarly to the fungal soil community, PERMANOVA on Bray-Curtis distance of bacterial ASVs resulted in significant effects of treatment, sampling time, and soil type (bulk versus rhizospheric), but not the interaction between treatment and time (Fig. 6B & 6D, Table S11). Pairwise comparisons showed a treatment effect only between NG and HDG, but there were strong differences between all sampling times (Table S11).

Differential relative abundance analyses of the bacterial and fungal communities confirmed that the soil fungal community responded to grazing pressure, while the soil bacterial community was largely resilient to grazing impacts (Table 1). Pairwise comparisons revealed 12 fungal genera that differed between HDG and NG, and 12 genera that differed between LDG and NG. In sharp contrast, there was only one bacterial genus that was significantly lower in the HDG treatment than NG, and there were no differences found between LDG and NG. The fungal genera *Antennariella*, *Knufia*, *Cyphellophora*, *Sclerostagonospora* (all Ascomycota phylum), and *Naganishia* (Basidiomycota phylum) had a negative effect size (decreased relative abundance) in both HDG and LDG treatments compared to NG and *Trichoderma* (Ascomycota phylum) had a positive effect size (increased relative abundance) in both HDG and LDG compared to NG (Table 1).

Mantel correlations between all dissimilarity matrices (soil abiotic profiles, extracellular enzymatic profiles, and bacterial and fungal community profiles) showed that only the bacterial and fungal communities were significantly correlated ($r = 0.312$, $p = 0.007$; Table S12). Thus, dynamic shifts within the soil chemical environment and soil microbial communities are not necessarily changing in similar directions.

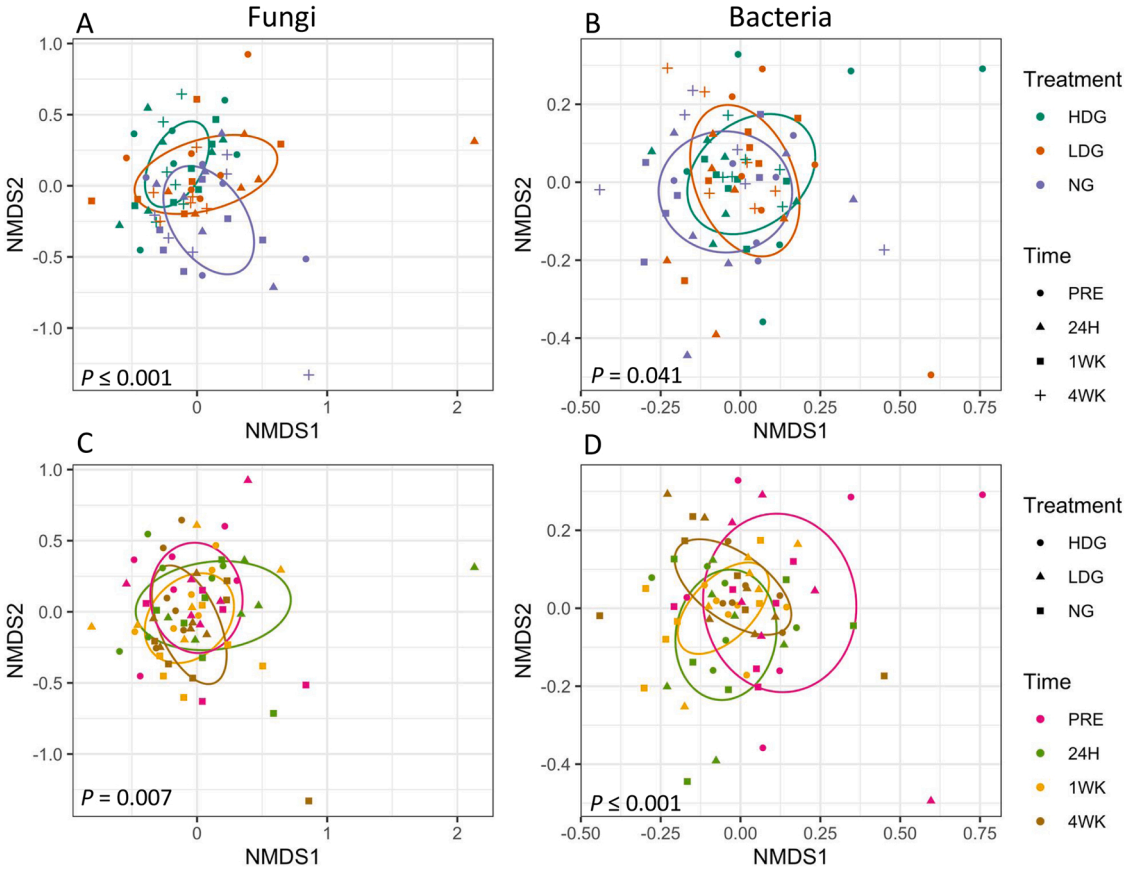

Fig. 6. Nonmetric multidimensional scaling visualization of Bray-Curtis dissimilarity of the soil fungal (A, C) and bacterial (B, D) communities by grazing treatment (A, B) and by sampling time (C, D). Panels A and C and panels B and D show the same ordinations (fungal and bacterial communities, respectively) with different colors for contrasts. The PERMANOVA p value is reported for each contrast shown by ellipses. NMDS stress is 0.21 for the fungal community and 0.22 for the bacterial community.

Table 1
Differential abundance analysis of soil fungal (A, B) and bacterial (C, D) genera. Mean relative abundance represents rarefied taxa relative abundance. Effect size represents the $\log_2$ fold change. Adjusted p values include Benjamini-Hochberg corrections. (**A**) and (**C**) show pairwise comparisons between HDG and NG where a negative log2 fold change represents higher relative abundance in the NG treatment compared to HDG; (**B**) and (**D**) show pairwise comparisons between LDG and NG where a negative $\log_2$ fold change represents higher relative abundance in the NG treatment compared to LDG. Rows are arranged based on effect size strength.

Mean relative abundance	Effect size ($\log_2$ change)	Adjusted p value	Family	Genus
A. Fungal HDG vs NG				
125.176	-9.115	0.029	*Capnodiaceae*	*Antennariella*
49.945	-7.403	0.000	*Trichomeriaceae*	*Knufia*
3.979	-5.800	0.033	*Sporidiobolaceae*	*Rhodotorula*
2.009	-4.943	0.039	*Wallemiaceae*	*Wallemia*
74.128	-4.391	0.006	*Cyphellophoraceae*	*Cyphellophora*
209.696	-4.080	0.001	*Didymellaceae*	*Ascochyta*
44.691	-2.279	0.000	*Filobasidiaceae*	*Naganishia*
104.417	-2.217	0.001	*Phaeosphaeriaceae*	*Sclerostagonospora*
78.397	-1.793	0.029	*Lophiotremataceae*	*Lophiotrema*
20.286	-1.759	0.033	*Holtermanniales*	*Holtermanniella*
54.080	2.367	0.017	*Pleosporaceae*	*Pyrenophora*
100.342	25.772	0.000	*Hypocreaceae*	*Trichoderma*
B. Fungal LDG vs NG				
125.176	-27.410	0.000	*Capnodiaceae*	*Antennariella*
5.905	-22.993	0.000	*Psathyrellaceae*	*Psathyrella*
10.959	-17.572	0.000	*Crepidotaceae*	*Simocybe*
49.945	-5.126	0.005	*Trichomeriaceae*	*Knufia*
8.643	-4.791	0.003	*Plectosphaerellaceae*	*Gibellulopsis*
74.128	-3.695	0.032	*Cyphellophoraceae*	*Cyphellophora*
104.417	-1.831	0.012	*Phaeosphaeriaceae*	*Sclerostagonospora*
44.691	-1.759	0.010	*Filobasidiaceae*	*Naganishia**
19.545	1.756	0.047	*Aureobasidiaceae*	*Aureobasidium*
262.686	3.706	0.000	*Pyronemataceae*	*Kotlabaea*
620.804	5.420	0.000	*Marasmiaceae*	*Marasmius*
100.342	23.228	0.000	*Hypocreaceae*	*Trichoderma*
C. Bacterial HDG vs NG				
52.826	-1.081	0.009	*Nannocystaceae*	*Nannocystis** *
D. Bacterial LDG vs NG (no significance)				

**Naganishia* is in the phylum Basidiomycota; all other genera belong to Ascomycota
* *Phylum Proteobacteria

4. Discussion

Grazing had an immediate effect on edaphic and microbial variables, which were detected within 48 h of the onset of grazing. We also found that over the total sampling period of 5 weeks, there was a strong temporal influence on the soil microbial community and abiotic parameters, and that some temporal effects were mediated by grazing treatment. Treatment-independent temporal fluxes were likely driven by precipitation patterns, as soil water content increased throughout time regardless of treatment.

Dissolved organic carbon (DOC) and ammonium increased while LDG were grazing (24H and 1WK) compared to the HDG treatment, while they decreased during and after HDG grazing during that same period. While previous research has shown that soil nutrients respond to grazing on the scale of weeks to months (e.g., Sato et al., 2019), our results show that the soil environment responds within days to grazing disturbance. These findings indicate that high-intensity grazing may have a negative impact on soil ammonium and DOC that is not reflected under low-intensity grazing conditions. The LDG treatment may have deposited more labile C and N through excrement deposition than the HDG treatment (Bardgett and Wardle, 2003; Schrama et al., 2013), or the lower-intensity defoliation could have shifted below-ground resource allocation to accelerate C and N cycling (Mikola et al., 2009). We expected the HDG treatment to deposit more excrement than the LDG treatment due to the higher number of cattle, but it is possible that since the LDG cattle were on the landscape for a longer period, they deposited more urine with labile N (Esch et al., 2013; Vidal et al., 2020).

Potential enzymatic activity (EEA) generally decreased through time, but three enzymes (BG, CBH, and NAG) were higher at 24H under the HDG treatment. At the 24H sampling time, HDG and LDG had grazed for approximately 24 h, so the rapid response of soil microbes to the HDG disturbance is consistent with current findings that soil EEA are time-sensitive on the scale of minutes (Baldrian, 2014). As such, EEA profiles may be the most appropriate method to detect short-term microbial responses to disturbance, even though EEA, like other biological parameters, are also highly dependent on soil abiotic conditions (Boeddinghaus et al., 2015). Soil microbes release enzymes in response to soil conditions, nutrient availability, and substrate complexity; however, the relationship between nutrient availability and EEA response is convoluted as microbes decrease energy-costly enzyme production when labile nutrients are readily available (Allison and Vitousek, 2005; Burns et al., 2013). This corroborates our finding that the LDG grazing treatment with the concurrent spike in labile nutrients did not show the same increase in EEA as the HDG treatment.

Previous studies that have examined long-term (years to decades) effects of grazing have found weak correlations when considering grazing impact as the driver of EEA fluxes (Esch et al., 2013; Francini et al., 2014; Hewins et al., 2016, 2015). We found that not only did the two grazing treatments have changes in EEA compared to the no grazing control, but that all treatments were additionally subject to temporal fluxes in EEA throughout only five weeks of sampling. The lack of EEA response to grazing in longitudinal studies could be a direct result of sampling in slightly different temporal conditions or because EEA reached a steady state after prolonged grazing pressure. The temporal changes that we detected may be due to shifts in belowground resource allocation as perennial grasses proceed through their growth stages over the growing season, as EEA are known to shift in response to changing plant communities (Bardgett and Wardle, 2003; Teague et al., 2011).

Soil bacterial and fungal richness (alpha diversity) were not strongly affected by grazing disturbance. This finding is similar to other studies that have examined the impacts of grazing compared to short-term (a few years) exclosures (Eldridge et al., 2017; Van Der Heyde et al., 2017), although one study found that bacterial richness increased in two-year-old grazing exclosures while fungal richness did not change (Epelde et al., 2017). Taken together, these findings illustrate that grazing may not affect short-term soil microbial richness, or the number of soil microbes present; rather, the flux of labile N from fecal and urine deposits may change resource competition and thereby drive restructuring of microbial community composition.

In contrast to alpha diversity, the soil microbial community composition (beta diversity) responded strongly to treatment, time after grazing, and the type of soil. In particular, the bulk and rhizospheric soil communities for both bacteria and fungi had strong differences in richness, composition, and differential abundance of key taxa. Since few previous studies have differentiated bulk and rhizospheric soil in the context of grazing pastures, comparing our findings to previous work is difficult. While we expected to observe different bacterial and fungal communities between bulk and rhizospheric soil, we hypothesized that rhizospheric soil would be more responsive to grazing intensity than bulk soil due to the immediate proximity to plant roots (LeBlanc et al., 2015; Zhang et al., 2017). However, there were no significant differences in treatment responses for Shannon's H index or community composition between bulk and rhizospheric soil communities, indicating that these communities responded similarly to grazing intensity and thereby disproving our hypothesis.

Generally, we detected stronger fluctuations in the soil fungal community based on grazing intensity and sampling time than the soil bacterial community. These results corroborate previous findings that indicate that soil fungi are more responsive to grazing impacts than the bacterial community (Eldridge et al., 2017; Hamonts et al., 2017). The stronger treatment response of fungi compared to bacteria could be

because some soil fungi are dependent on rhizodeposits for C assimilation (Eldridge et al., 2017). Thus, as plants shift belowground allocation to recover post-defoliation, the soil fungal community is immediately affected. Though bacteria are also dependent on these rhizodeposits, the strong temporal effects we observed may have marginalized the grazing effects.

Differential abundance analysis revealed that several fungal genera were responsive under both the HDG and LDG treatments compared to the NG control, and most were less abundant under grazing conditions. However, the fungal genera *Trichoderma* was found to be more abundant under the HDG and LDG treatments than in the NG control. This well-studied saprotroph is known for its agricultural importance as a biocontrol of pathogenic fungi and promotes plant growth and nutrient uptake (Lorito et al., 2010; Zhang et al., 2018). Neither *Trichoderma* nor its family *Hypocreaceae* have been studied in response to grazing impact, but our findings suggest that grazing may confer a short-term beneficial effect driven by the increase of *Trichoderma* under grazed conditions. The genus *Cyphellophora* has been previously correlated to long-term grazing exclusions (Yao et al., 2019). We corroborate these findings by reporting that *Cyphellophora* was less abundant in both the LDG and HDG treatments compared to NG. The other fungal and bacterial genera detected in our analyses have not been well studied in regard to plant-soil-microbe interactions or responses to grazing impact, and we therefore refrain from making further inferences.

This research is the first to describe the immediate pulse in the soil microbial community and soil-available nutrients following different grazing management strategies. Examining soil quality through the lens of soil microbial communities is an expanding field (Wall et al., 2015). While many studies analyze long-term changes in the soil microbial community, we showed that: (1) C-cycling EEA increased directly following HDG compared to the same length of LDG and NG, and (2) the total soil enzymatic profiles shifted significantly throughout short time periods regardless of grazing intensity. Concurrent fluxes in the soil chemical environment, specifically labile C and N, indicated that soil microbes respond to immediate changes in available nutrients that differed between grazing intensities. These novel findings are especially pertinent in high-production, subirrigated pastures where land managers may be able to leverage intensive management practices to improve soil health and forage production (Derner et al., 2018). High-intensity, short-duration grazing resulted in different patterns of edaphic and microbial fluxes throughout the following weeks than low-intensity, medium-duration grazing and no grazing; specifically, the soil fungal community had strong interactions between the grazing treatments and sampling periods.

In summary, this study found that cattle grazing at different intensities resulted in an immediate pulse of plant-soil-microbe interactions that were detectable within 24 h. The study design was limited by design to better control grazing intensities and further work is required to expand these findings to the ranch or rangeland scale. Since grazing has an immediate effect on the soil biota and available nutrients, future research should seek to determine if these immediate fluxes result in lasting effects on forage and soil quality and could therefore inform agroecosystem management strategies.

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USDA WSARE had no influence over experimental design, sample collections, or data analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2021.107805.

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