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Long-Term Grazing and Nitrogen Management Impacted Methane Emission Potential and Soil Microbial Community in Grazing Pastures

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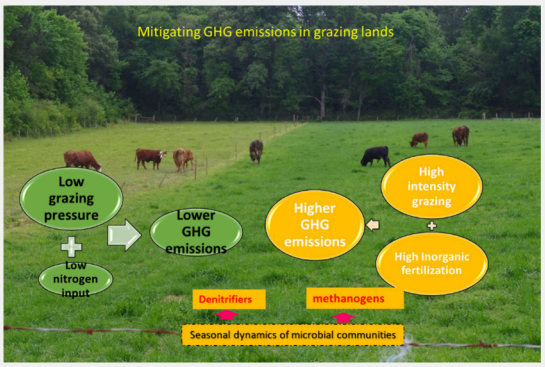
ABSTRACT: Achieving sustainable development in livestock agriculture by balancing livestock production, reduction of greenhouse gas (GHG) emissions, and effective utilization of nitrogen nutrient has indeed been challenging. This study investigated the long-term effects of continuous cattle grazing, stocking rates, and fertilization regimens on methane (CH_4) emissions, soil microbial communities, and soil organic carbon (SOC) stocks in Bermudagrass pastures in East Texas, USA. Pastures were subjected to high or low stocking rates for over 50 years, with further subdivision based on fertilization: nitrogen-based fertilizer application or no fertilizer but with the growth of annual clover. Seasonal soil cores (0−60 cm) were collected, and laboratory microcosm incubation studies revealed unexpectedly high *in vitro* CH_4 emissions in surface soils, particularly in the top 0−5 cm soil layer, reaching up to 300 nmol of CH_4 mL^{-1}. Higher CH_4 emissions and methanogen abundance, along with lower SOC stocks, were observed in pastures subjected to high stocking rates compared to those with low stocking rates and in clover pastures compared to those with N-fertilized ryegrass. On the contrary, in low-stocked, N-fertilized annual ryegrass pastures, methanogen abundance was lowest, CH_4 emissions were negligible, and SOC stocks were highest. Furthermore, animal excreta deposition significantly contributed to increased CH_4 emissions. Prokaryotic and potential methanotrophic taxa, as compared to fungi, exhibited greater responsiveness to N-fertilization than to cattle stocking treatments with higher levels of methanotrophs observed in pastures subjected to high stocking rates and clover growth. This study suggests that strategic management practices such as optimal grazing and nitrogen management could effectively mitigate CH_4 emissions in grazing lands.



KEYWORDS: *grazing lands, methane emission and methanogens, mcrA and pmoA genes, stocking rates, bermudagrass, clover and ryegrass*

1. INTRODUCTION

Grazing pastures as a significant component of agricultural landscapes worldwide, occupy about 25% of the world's land surface and 41% of the arable lands in the USA,[1] and serve as primary sources of forage for livestock production. However, the ecological dynamics within these pastures are intricate, influenced by various factors, such as grazing intensity, nutrient management practices, and interactions with soil microbial communities. Among the environmental concerns associated with grazing systems, methane (CH_4) emissions and alterations in soil microbial communities have garnered considerable attention due to their implications for climate change mitigation and ecosystem functioning. Grazing soils act as a sink for biological carbon and a source of greenhouse gases (GHG) with improved land management practices,[2] as the current soil organic carbon (SOC) stocks can be increased at a rate of 0.3 to 1.6 Pg CO_2 eq $year^{-1}$.[3] Consequently, grazing activities can significantly contribute to methane emissions, which impact the overall greenhouse gas balance of agricultural systems.

Nitrogen management practices, including fertilization regimes and grazing strategies, have been identified as key drivers shaping methane emissions and soil microbial communities in grazing ecosystems. Long-term grazing intensity and N-fertilization strategies impacted soil fertility, soil aggregates,[4] and vegetation cover,[5] which shifted many microbial functional groups[6] such as CH_4-cycling microbes.[7] Particularly, grazing practices can impact both methanogens and the methanotrophic community,[8] which are key drivers of

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net CH_4 emission potential.[9] Different grazing intensities and management practices exert varying impacts on soil microbial communities and associated methane fluxes, reflecting the complex interplay between agronomic practices and ecosystem processes. Thus, implementing best management practices to minimize GHG emissions and increase soil organic carbon (SOC) stocks is highly valuable for the sustainability of grazing lands. All methanogens are Archaeal, whereas methanotrophs are mostly *Proteobacteria* and *Verrucomicobia*. A majority of methanotrophs in arable soils are represented within *Alphaproteobacteria* (Type II) and *Gammaproteobacteria* (Type I), and a few are *Betaproteobacteria*.[10] These taxonomic groups have demonstrated niche adaptations in soils and unique responses to land-use practices.[11] Their responses to long-term grazing and N-management must be understood clearly to implement effective mitigation practices in grazing lands.

Loss of soil carbon through methanogenesis from grazing lands is a source of CH_4 emissions globally, although at a much smaller scale compared to wetlands, rice paddies or livestock enteric sources.[12] Nonetheless, many reports implicate grazing lands as a source of CH_4 emissions under some climatic and management conditions.[13,14] With the global warming potential significantly higher than that of CO_2, CH_4 is expected to increase from the current level of 1.77 ppm to 2.55 ppm within the next 50 years.[15] Methanogens are found in most environmental habitats including nonwetland agriculture soils, and become active under favorable conditions.[16] Methane emissions from arable soils are mostly noted in anaerobic niches within the soil aggregates and during soil saturation conditions.[17] These potential soil emission sources coupled with animal excreta addition in grazing lands have been noted to drive substantial CH_4 production during saturated conditions.[18] Likewise, soil zones enriched by animal excreta and N-fertilization could alter microbial community composition[19] and impact CH_4-cycling microbes.[8]

Impacts of grazing or N-management on the CH_4 cycling community and quantitative estimates of the methane emission potential are lacking from long-term study sites. Minimal data sets are available from pastures situated on humid tropical climates, a constraint for gauging methane emission potential under different cattle stocking rates and pasture N-management.[2,20,21] Measuring field CH_4-fluxes from actively grazed pastures is difficult and could also be confounded by livestock emissions.[22] Moreover, CH_4 emissions are driven by climatic factors such as rainfall and soil saturation, and thus fluxes are short-lived and highly variable in arable soils.[23] One way to address these challenges is to evaluate *in vitro* CH_4– emission potential using controlled lab incubations, which have been reliably correlated to field conditions.[24] Although laboratory incubation fluxes are not always comparable to field fluxes, they offer a rapid access and efficient method for comparing the CH_4-emission potential among systems. Moreover, *in vitro* emissions can be coupled with the quantification of functional genes associated with CH_4 flux to gain insights into these microbial community responses to long-term pasture management and potential implications on net emission potential. This knowledge could be useful for determining the right combination of stocking management and pasture N-management to identify avenues for mitigating emissions from grazing lands.[25]

Understanding the interactions among grazing, nitrogen management, methane emissions, and soil microbial communities is crucial for devising sustainable management strategies that mitigate greenhouse gas emissions while maintaining ecosystem services and agricultural productivity. Therefore, this study aims to investigate how grazing intensity and nitrogen management practices for over 50 years influence methane emission potential and soil microbial community composition in grazing pastures. By elucidating these relationships, we can advance our knowledge of the ecological mechanisms underlying greenhouse gas dynamics in grazing systems and inform the development of more effective mitigation strategies to enhance the environmental sustainability of livestock production. We hypothesized that long-term grazing and N-management impacted the soil microbial community structure and shifted the balance between methanogens and methanotrophs, which might increase CH_4 emission potential and impact SOC stocks from the soil profile.

2. MATERIALS AND METHODS

2.1. Study Sites, Experimental Plot Design, and Soil Sampling

The experimental stocked bermudagrass pastures have been maintained under different stocking rates and N-based fertilization or legume N fixation schemes (Figure S1) for the last 50 years in Overton, Texas, USA.[26] The experimental sites were situated on low fertile sandy profiles of grasslands representative of expansive regions within humid subtropical regions around the world. The experimental treatments used for this study were (1) high stocking rate with fertilizer-N (HSR_FN) application onto ryegrass and bermudagrass vegetation, (2) high stocking rate with legume-N supplementation (HSR_LN) onto clover and bermudagrass vegetation, (3) low stocking rate with fertilizer-N (LSR_FN) application onto ryegrass and bermudagrass vegetation, and (4) low stocking rate with legume-N supplementation (LSR_LN) onto clover and bermudagrass vegetation, as details shown in Figure S1. The field experiment design included completely randomized pastures for individual stocking rate treatments. Individual treatment pasture area ranged from 1 to 3 ha to impose two different stocking rates of cattle. We used four pseudoreplications within each pasture treatment for sampling replications.

A temporal sampling scheme was used for this study to explore the SOC and microbial community dynamics and seasonal changes in *in vitro* methane (iCH_4) emissions. A sampling area in the middle of each replicate plot was established to avoid border effects. Surface probe samples (0−20 cm) were taken during the months of August, September, October, November, and December of 2016 and February, April, June, and July of 2017. Additionally, soil core samples to a depth of 60 cm were taken once every season in duplicate from each replicate plot using a hydraulic probe (Giddings Machine Company, Inc.) with sterile plastic liners to avoid contamination. Sterile plastic liners with soil cores were capped at both ends and then transported on ice (4 °C) to the laboratory. Soil cores were cut into three depths of 0−20, 20−40, and 40−60 cm for SOC and microbial analyses. For iCH_4 emissions from different depths, the core samples were split to 0−5, 5−15, 15−30, 30−45, and 45−60 cm. Individual depth samples were mixed within a ziplock bag, and a subsample of approximately 10 g was used for microcosm studies and another 10 g without observable roots was taken from the composite for microbial and biochemical analyses and immediately frozen at −80 °C. For additional lab incubation experiments, soil samples were taken using a hand probe of 5 cm diameter from each replicate subplot. In each subplot, 12−15 repeated samples from surface soil (0−20 cm) were taken randomly from the treatment pastures. The samples were homogenized in clean sterile plastic zip bags on the site and immediately stored at 4 °C prior to incubation processing and analysis. *In situ* water used for incubations was

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obtained from a nearby freshwater creek and was sterilized prior to incubation.

2.2. Microcosm Incubation Experiments for Determining *In Vitro* CH_4 Emissions from Soil Samples Collected from the Experimental Treatments

Three separate soil microcosm experiments were conducted in an anaerobic chamber flushed with balanced N_2 and CO_2. Within 36 h of sampling, soil samples from each treatment plot were first homogenized well again in the chamber. Seven grams of each soil sample was added in a 20 mL glass vial (Microliter Wheaton) mixed with 3 mL of sterilized deoxygenated *in situ* water from one creek near the grassland plots. The vials with soil slurry were sealed with 20 mm gray butyl stoppers (Microliter) and incubated at 25 °C under dark conditions in the incubator within the anaerobic chamber. The vials were shaken once a day during the incubation period. All soil samples were incubated in triplicate for a period of 9 days along with a duplicate of gas blanks and a triplicate of kill controls. Kill controls were used for applying a background correction to iCH_4 emissions. Kill controls were made by autoclaving anaerobically prepared slurry incubation of surface samples from paddock under HSR_LN and HSR_FN pastures at 120 °C for 50 min. After incubation periods, all samples were stored at −80 °C to terminate microbial activities prior to GC analyses.

The preliminary microcosm experiment was conducted to determine the incubation time range of the *in vitro* CH_4 (iCH_4) emission assays to establish the optimal incubation time periods for the gas emission analyses. Based on the preliminary incubation tests, surface soils were sampled at 0−20 cm depths from different treatments, and were incubated in triplicate for periods of 2, 4, 6, 9, and 12 days. Although the highest yield of CH_4 was found in 12 days but the CO_2 yield started to significantly drop. The yields of the two gases on Day 9 were both significantly enhanced in comparison with Day 1 (see Supplemental Figure S2). Therefore, a period of 9 days of incubation was determined to be optimal for the iCH_4 emission analyses from the treatments.

***In Vitro* CH_4 Emissions from Different Soil Depths.** For the soil depth assay, soil samples from different depths (0−5, 5−15, 15−30, 30−45, and 45−60 cm) were taken from the HSR_LN and HSR_FN plots. Duplicates of soil cores at different depths from each treatment plot were homogenized prior to incubation. Soil samples were incubated in triplicate for an optimal period of 9 d as shown in the previous paragraph (Figure S2) along with a duplicate of gas blanks and a triplicate of kill controls. Kill controls followed the previous incubation experiment. After incubation periods, all samples were stored at −80 °C to terminate microbial activities prior to GC analyses.

***In Vitro* CH_4 Emissions from Seasonal Samples.** To explore the seasonal changes in iCH_4 emissions, surface soil samples from all grazing paddock treatments (i.e., HSR_FN, HSR_LN, LSR_FN, and LSR_LN) were taken during the months of August, September, October, November, and December of 2016 and February, April, June, and July of 2017. Soil samples taken from the depth of 0−20 cm were incubated in triplicate for a period of 9 d along with a duplicate of gas blanks and a triplicate of kill controls. Kill controls were prepared as the previous incubation experiment. After incubation periods, all samples were stored at −80 °C to terminate microbial activities prior to GC analyses.

***In Vitro* CH_4 Emissions in Response to Manure Amendment.** Surface soil samples from 0 to 20 cm depth were taken in February 2017 and March 2017 from HSR_FN and HSR_LN pastures. All soil slurry samples were prepared under anaerobic conditions and incubated. Manure amendments were prepared by adding sterilized fresh cow manure in 2% and 5% of the wet soil weight, respectively. Native soil samples without manure addition were prepared to serve as additional controls, along with the killed controls. Triplicate samples were incubated for 9 days.

Methane Assays. The headspace CH_4 and CO_2 levels of 20 mL glass vials with frozen incubation samples were analyzed by using a Shimadzu 2014 gas chromatograph (Kyoto, Japan) with flame ionization (FID), thermal conductivity (TCD), and electron capture (ECD) detectors, which was equipped with a Shimadzu AOC-5000 auto sampler with a 2.5 mL gastight syringe. Chromatograms were analyzed by integrating the peaks at known retention times and comparing them to the linear regression of integrals of known calibration gases run at the beginning of each analysis. Cumulative iCH_4 concentrations for 9 d of incubation were analyzed and reported as nmol mL^{-1}.

2.3. Geochemical Analyses of Soil Samples

Soil pH was measured in a 1:2.5 ratio of soil:water suspension using 2 g of frozen soil sample stored for chemical analyses. Soil moisture was determined for individual soil samples from all time points, based on gravimetric mass difference after drying the samples for 24 h at 105 °C. The moisture correction was applied to estimate all of the soil biochemical parameters on a dry mass basis. Soil organic carbon (SOC) and total nitrogen (TN) were determined by a dry combustion C/N analyzer (Elementar Inc.). Approximately 0.5 g of frozen soil was air-dried and used for determining SOC and TN. Measurements of the analyzer were calibrated using a set of two primary standards (USGS-40 glutamic acid for carbon and a glycine standard for nitrogen) between every 10−15 samples and two verified soil standards as check references (Soil ID-2014−108, North American Proficiency Testing Program). The quantified SOC as mg kg^{-1} was converted to SOC stocks estimates (Mg ha^{-1} in 0−60 cm soil profile) based on soil bulk density. Bulk density (BD) was measured individually for all treatments by collecting a separate soil core of 7.6 cm diameter for each depth (0−20, 20−40, and 40−60 cm), and by determining the gravimetric mass of air-dried soil. However, to avoid erroneous estimates of SOC and TN stocks (mass per unit area) due to differences in BD, we normalized (mean) BD across the treatments for individual soil depth and used this mean BD value to estimate "equivalent soil mass" in individual soil layers as previously described.[27] SOC stocks (Mg ha^{-1}) were computed for each soil layer (i) according to eq 1. Percent coarse fragments were in negligible quantity in all samples.

$$\mathrm{SOCi} = bi \times Ci \times Li \quad (1)$$

where SOCi refers to SOC stocks (Mg ha^{-1}), *bi* is bulk density for the soil layer, *Ci* is the SOC concentration, and *Li* is the length of soil layer.

A separate portion of soil was used for determining water extractable organic carbon (WeOC), nitrogen (WeN), cations, and anions (labile fractions). Approximately 5 g of frozen soil was shaken in 25 mL of DI H_2O for 1 h and was then centrifuged at 2,000 × g for 5 min to settle suspended soil particles. Samples were then filtered using Whatman 42 filter papers into 20 mL scintillation vials containing a drop of 12 mol L^{-1} HCl. Filtered samples were capped immediately and refrigerated prior to analysis (within 24 h) to maintain sample integrity. WeOC and WeN were determined by a wet combustion NPOC/NPN analyzer (Shimadzu Inc.). Check standards, blanks, and reference samples were used every 10−15 samples. Approximately 2 mL of this water extraction was used for analyses of cations and anions estimation using a dual channel ion chromatograph (Thermo Inc.). Standard curves were prepared using two primary standard stock solutions containing a suite of cations (Li, Na, NH_4, K, Mg, Ca) and anions (F, Cl, NO_2, Br, NO_3, PO_4, SO_4). The columns used for cation separation was a Dionex IonPac CS16−4 μm 3 × 250 mm and a Dionex IonPac AS19−4 μm 2 × 250 mm was used for anion separation. Standards, blanks, and reference samples were used every 10−15 samples for QA/QC. Additional soil properties at experimental pastures are presented in Table S1.

2.4. Microbial DNA Extraction, qPCR Analysis, and Gene Sequencing for Characterizing Microbial Community

Soil profile samples (0−60 cm) collected from experimental pastures in August were selected for characterizing the soil microbial community under long-term grazing and N-management. Additionally, several microcosm experiment soils after gas analyses were utilized for quantifying methanogen and methanotroph abundances in response to manure amendments. Microbial DNA from individual soil

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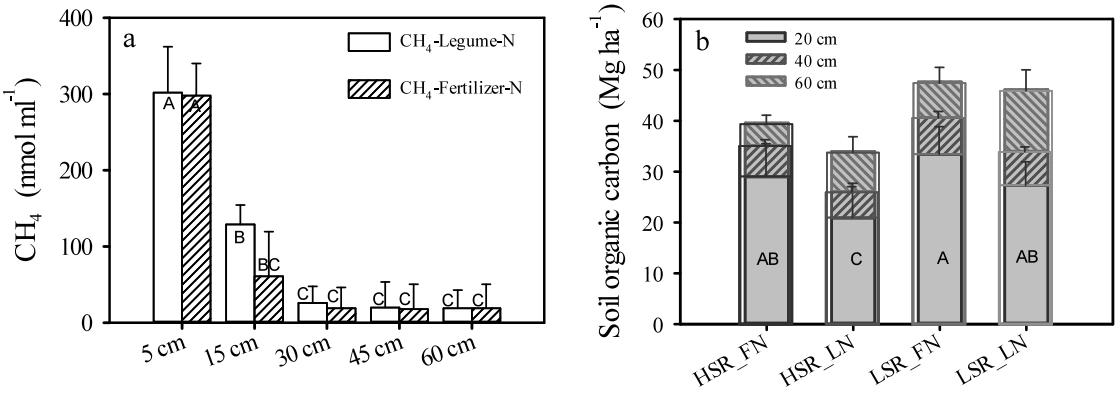

Figure 1. *In vitro* CH_4 missions of different layers of soil samples at high stocking rate (HSR) (a) and soil organic carbon (SOC) distribution for individual layers under the influence of stocking rates (b) in the two N-management systems. Data presented on the *y*-axis in panel (a) are for cumulative iCH_4 emissions for 9 days of incubation from soil samples taken from different depths. Letters within the bars in panel (b) represent a mean difference test for SOC in 0−20 cm among the treatments. Means with the same letter marked on the bar within one treatment are not significantly different at $p < 0.05$. Error bars are standard deviations. FN = Fertilizer-N, LN = legume-N. Low (or high) stocking rate is marked as LSR (or HSR).

samples were extracted using PowerLyser PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol, modified by starting with an initial soil aliquot of approximately 0.5 g and by use of a bead beater for cell lysing. Quality and concentration of extracted DNA were determined spectrophotometrically by using a ND-1000 Nanodrop (Thermo Scientific).

Abundance of methanogens, methanotrophs, and bacteria in the experimental samples was determined using qPCR assays. Methanogens were estimated by targeting the functional gene *mcrA* (encoding the alpha subunit of methyl coenzyme M reductase),[28−30] methanotrophs by targeting the functional gene *pmoA* (encoding the particulate methane monooxygenase),[31] and bacteria by targeting the 16S ribosomal subunit genes.[32] Each qPCR run was set up to include appropriate quality controls (positive, negative, no template controls, gBlock standards, and spikes). All qPCR reaction mixtures were made of 7.5 μL of SYBR Green (2x) Master Mix, 1.5 μL each primer (5 μmol L^{-1}), 2 μL DNA template, and 2.5 μL nuclease-free H_2O. Additional details on qPCR conditions and primers used are provided in Table S2. The qPCR analyses were performed using Corbett Rotor-Gene (model RG-6000) and Rotor-Gene 6000 Series Software 1.7.75 (Qiagen Inc.). Sample preparation for qPCR reaction plates was performed using a Corbett CAS1200 auto pipetting robot (Qiagen Inc.). Standards were made via a serial dilution of gBlock synthetic DNA sequences including the ones emulating *mcrA* and *pmoA*, manufactured by Integrated DNA Technologies, Inc. (Coralville, Iowa). Spikes were composed of equal parts of sample and middle standard gBlock synthetic DNA. All standards, samples, no template control (NTC), spikes, positive controls, and negative controls were run as triplicate. qPCR products were verified by using both melt curve analyses and checking the products on agarose gels. Melt curve analysis was produced by running a denaturing temperature ramp of 55−98 °C, increased by 1° for every 5 s. Data were accepted only after passing quality checking for reaction efficiency, standard curve r^2, gene copy numbers in controls, NTC, spikes, positive and negative controls. Additional details about qPCR assay primers and PCR conditions are presented in Table S2.

The soil microbial community in the soil profiles of grazing pastures under long-term grazing and N-management treatments were characterized using 16S gene sequencing. Bacterial diversity was estimated by sequencing the V4 region of the 16S rRNA genes amplified by primers 519F, 5′-CAGCMGCCGCGGTAA-3′, and 786R, 5′-TACNVGGGTATCTAATCC-3′ and fungal diversity by sequencing the intergenic transcribed spacer (ITS2) region with primers ITS4 5′- TCCTCCGCTTATTGATATGC −3′ and ITS7 5′-GTGAATCATCGAATCTTTG-3′.[33−35] Paired-end sequence data were generated on an Illumina MiSeq instrument using v3 600 cycle kits (Illumina, San Diego, CA) as described in the Illumina 16S metagenomic sequencing library preparation protocol. The raw sequencing reads were processed with a combination of QIIME 1.9.1[36] and USEARCH 8.0.1[37] software packages. Individual ITS sequence tags were compared to the UNITE fungal ITS sequence database[38] and individual 16S sequences were compared to the SILVA database 128[39] using UCLUST in order to pick referenced-based (prokaryotes) operational taxonomic units (OTUs) at 97% similarity. The OTU abundance data sets were further normalized using cumulative sum scaling (CSS) transformation[40] available on the QIIME platform. All of the sequence data have been deposited in the NCBI Genbank database under project number PRJNA 529502.

Statistical Analysis. All experimental data were analyzed to compare two stocking rates of high (HSR) and low (LSR) and two nitrogen sources (FN and LN), along with repeated measures of time (seasons) and soil depth as separate variables. Differences among the treatments for SOC and iCH_4, were determined using analyses of variance (ANOVA) in SAS software (SAS Inc.). The abundance of the OTU with taxonomy classification was used to prepare a graphical depiction of bacterial diversity among the experimental variables. A two-way permutation multivariate analysis of variance (PERMANOVA) was used to test the significant differences in the community structure between the experimental treatments based on Bray−Curtis distance measured between the groups.[41] Canonical correspondence analysis (CCA) was performed using PAST 3.1 software on OTU abundance data of the OTU with corresponding soil biochemical data as environmental variables for ordination axes. The implementation of CCA in PAST software follows the eigen analysis algorithm[42] with each environmental variable plotted as correlations with abundance scores.

3. RESULTS

3.1. *In Vitro* CH_4 Emissions, CH_4-Cycling Functional Gene Abundance, and Soil Organic Carbon in the Grazing soil

The first set of laboratory incubation studies of soil samples from different soil depths indicated that the cumulative 9-day iCH_4 emissions varied considerably from soil layers in both legume clover (LN) and ryegrass (FN) plots under high stocking rates (HSR_LN and HSR_FN treatments) (Figure 1a). *In vitro* CH_4 missions were mostly noted in the surface soil layer (0−5 and 5−15 cm), and highest iCH_4 emission were noted in the 0−5 cm soil layer, which was up to 300 nmol CH_4 mL^{-1}. The iCH_4 emissions from soils below the depth of 15 cm remained obviously low and did not differ significantly

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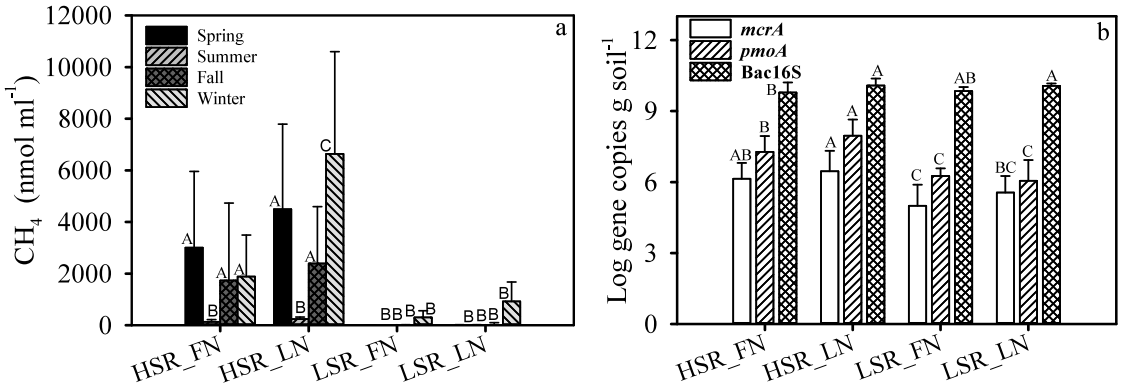

Figure 2. *In situ* CH_4 emissions and microbial functioning genes under long-term grazing and N-management treatments during different active grazing seasons. Data presented on the *y*-axis in panel (a) are for cumulative iCH_4 emissions for 9 days of incubation of surface soil samples (0−20 cm) taken from different seasons. Error bars are standard deviations. Gene abundance in panel (b) was estimated based on the gene numbers for *mcrA, pmoA*, and Bac16S in the surface soil (0−20 cm) collected in the winter season. Letters on the bars represent the mean difference test for the gene copy numbers between the treatments. Means with the same letter are not significantly different at $p < 0.05$. HSR = high stocking rate, LSR = low stocking rate, FN = Fertilizer-N, LN = legume-N.

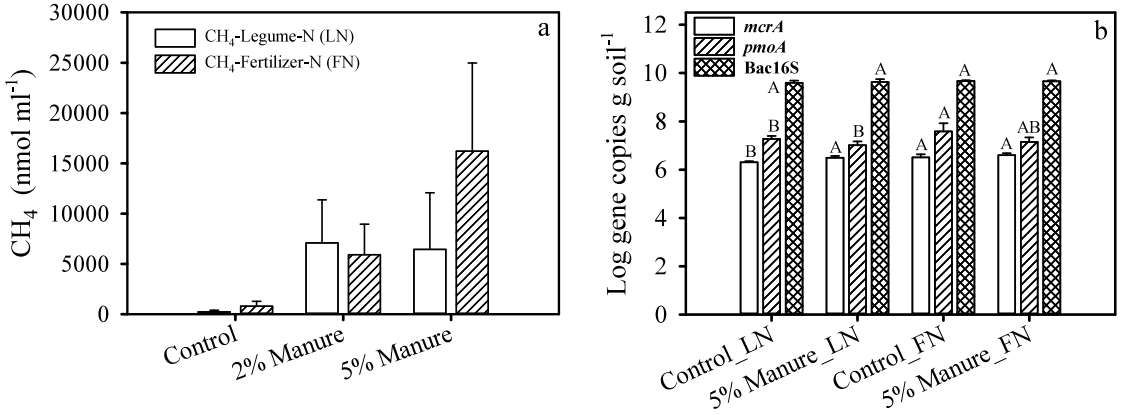

Figure 3. *In vitro* CH_4 missions (a) and abundance of methanogens, methanotrophs, and bacteria (b) in response to manure amendment under two N-management systems. Data presented on the *y*-axis of panel (a) are for cumulative iCH_4 emissions, while abundance on panel (b) was estimated based on the gene numbers for *mcrA*, *pmoA* and Bac16S for 9 days of incubation from topsoil samples (0−20 cm depth) at the high stocking rate taken in the spring season. Error bars are standard deviations. No significant differences were found in panel (a). Letters on the bars on panel b represent mean difference test results for the gene numbers between the treatments. Means with the same letter are not significantly different at $p < 0.05$. FN = Fertilizer-N, LN = legume-N.

among the layers from 15 to 30, 30−45, and 45−60 cm (Figure 1a).

The soil organic carbon showed a similar declining distribution profile as the CH_4 emission went down along the soil depth, with the dominant portion (up to 72%) of soil organic carbon storing in the top 0−20 cm soil layer (Figure 1b). Experimental factors of stocking rate (SR) and N-management practices significantly impacted the SOC stocks at the soil profile level (0−60 cm; Figure 1b). Average SOC stocks in the experimental treatments ranged between 34 and 49 Mg C ha^{-1} within the 0−60 cm profile. Results indicated that SOC stocks were significantly higher in LSR compared with HSR pastures under both N-management scenarios. Higher SOC stocks (about 10% higher at the soil profile level) were also noted in pastures that were continuously fertilized with inorganic-N fertilizers (FN treatment) compared to legume-N (LN treatment). The differences were larger in the surface soil (0−20 cm), as treatment of HSR_FN contained significantly higher SOC stocks (29 Mg C ha^{-1}) compared to HSR_LN (21 Mg C ha^{-1}), which was the lowest among all the treatments. Similarly, LSR_FN contained about 33% higher SOC stocks (36 Mg C ha^{-1}) compared to LSR_LN treatment (27 Mg C ha^{-1}). There was no significant interaction effect of SR × N on the SOC stocks. Based on these results, it was concluded that long-term grazing with low stocking and N fertilization resulted in higher SOC stocks in the soil profile, whereas legume-N controls under high stocking contained the lowest SOC stocks in the soil profile. Long-term grazing and N-management affected the biogeochemical characteristics of the soils (Table S1). There were differences for soil pH, NO_3 and total nitrogen (TN) levels. Continuous N fertilization (FN treatment) contained higher TN and NO_3 concentrations compared to LN treatments under both HSR and LSR. Soil pH was lower under FN compared to LN treatments, which indicates fertilizer-driven changes in soil pH.

Significant seasonal variations in *in situ* CH_4 emissions were observed in two grazing grasslands subjected to stocking rate and fertilization treatments, with elevated emissions generally noted during the winter (Figure 2a). CH_4 emissions were also significantly influenced by the stocking rate (SR) treatments over seasonal comparisons ($p < 0.03$, Figure 2a). *In vitro* CH_4-missions were remarkably higher in the high stocking rate

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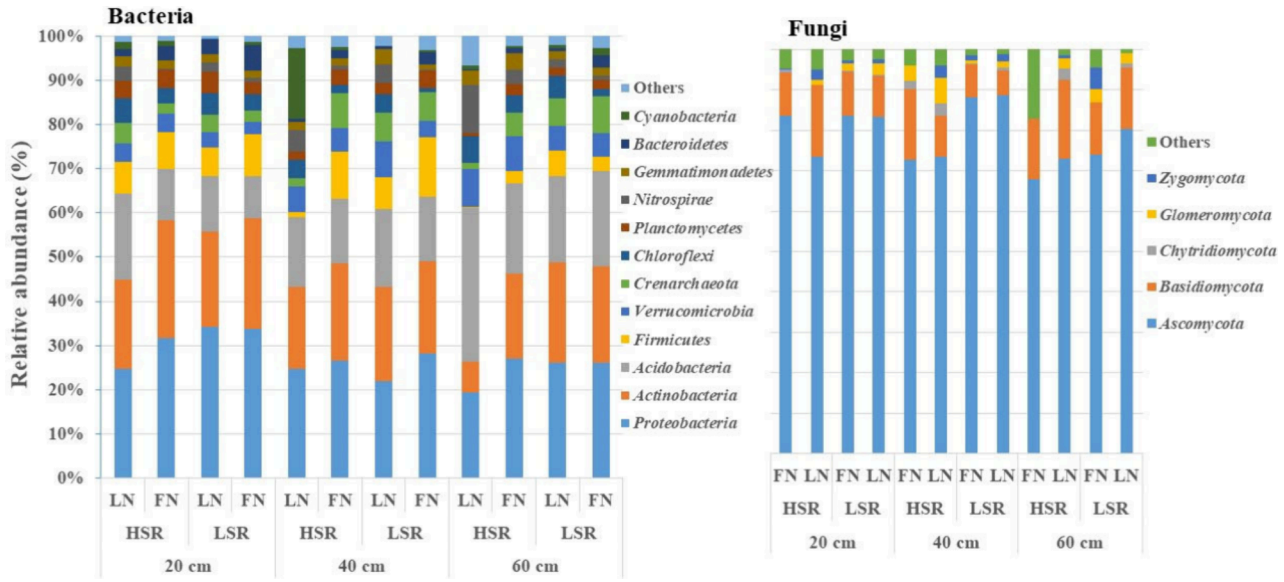

Figure 4. Relative phylum abundance of bacteria (left panel) and fungi (right panel) in soil samples taken from different layers (20, 40, and 60 cm) in response to nitrogen fertilization and stocking rate treatments. Different colors within the bars represent individual OTUs in varied phyla. LN is a label as legume (clover)-N, FN as fertilizer-N, HSR as high stocking rate, and LSR as low stocking rate.

(HSR) treatments compared to the low stocking rate (LSR) (Figure 2a). The seasonal iCH_4 emission in the clover plot with the HSR_LN treatment was the highest, reaching up to 3444 nmol iCH_4 mL^{-1}. Only up to 82 nmol of iCH_4 mL^{-1} was recorded in the ryegrass pastures on the LSR_FN treatment. It was also noted that iCH_4 emissions were higher in the HSR_LN treatment compared to those of HSR_FN in most seasonal samples.

To evaluate the methane-cycling gene abundance in relation to total bacterial abundance, seasonal soil surface samples collected from the experimental pastures in the winter showing generally the highest CH_4 emission rates were used for qPCR-based quantification of *mcrA* and *pmoA* genes and 16S gene numbers. ANOVA results indicated that abundance of methanogens (*mcrA* gene copies) was significantly influenced by the fertilization mode (N) and stocking rate (SR) treatments, but not by their interactions (Figure 2b). Log_{10} *mcrA* gene copy numbers were significantly higher in the biological-nitrogen fixation clover pasture (rather than N-addition) or LN treatment compared to the FN treatment, and in HSR compared to LSR treatments (Figure 2b). The highest *mcrA* gene numbers were recorded in the HSR_LN (log_{10} = 6.46) and the lowest were in the LSR_FN (log_{10} = 4.98). These trends were similar to those of seasonal average iCH_4 emissions from the same treatments. Methanotrophs (*pmoA* gene copies) were significantly influenced by SR treatment and N × SR interactions. Similar trends were also observed for the *pmoA* gene numbers in these treatments, as the highest numbers were recorded in HSR_LN (log_{10} = 7.95) and lowest in LSR_LN (log_{10} = 6.05). A significant positive correlation was noted between iCH_4 emissions and *mcrA* gene copies (Figure S3(a)), and between *pmoA* gene numbers (Figure S3(b)) and between *mcrA* and *pmoA* numbers (Figure S3(c)).

3.2. *In Vitro* CH_4 Emissions and CH_4-Cycling Functional Gene Abundance in Response to Manure Amendment

Results from laboratory incubation studies after examining iCH_4 emissions in response to the manure amendment indicated positive responses, as iCH_4 emissions mostly increased in manure amended soils compared to the unamended control soil samples (Figure 3a). However, only the fertilizer-amended treatment (FN) significantly influenced the iCH_4 emissions, as only in FN treatment there were positive linear responses at both 2% and 5% manure amendment rates. The highest iCH_4 emissions (up to 16208 nmol of CH_4 mL^{-1}) were recorded in the FN treatment that received 5% manure amendment.

Soil samples collected from the control and 5% manure amendment treatment were used for qPCR-based quantification of *mcrA, pmoA* and 16S gene numbers. ANOVA analyses indicated that methanogen abundance (*mcrA*) was significantly influenced by the fertilization management mode and manure amendment, but there was no significant interaction effect (FN × manure amendment) (Figure 3b). Numbers of *mcrA* genes significantly increased in response to manure amendment in the LN treatment of clover plots (log_{10} = 6.51) compared to control (log_{10} = 6.28), but not in FN treatments. Similarly, methanotrophs were significantly influenced by the N treatment mode but not by manure amendment. Numbers of *pmoA* genes for LN or FN separately slightly decreased in response to manure amendment (log_{10} = 7.3 and 7.6 in LN and FN, respectively) compared to unamended controls (log_{10} = 7.0 and 7.2 in LN and FN, respectively), but the differences were not statistically significant (Figure 3b).

3.3. Influence of Long-Term Grazing and Nitrogen Fertilization on Microbial Community Structure in the Soil Profile

The relative abundance of major bacterial phyla (Figure 4) in varied soil layers indicated that *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* constituted the major phyla in all the treatments. *Verrucomicrobia* was observed at a significantly lower relative abundance compared with that of dominant phyla described above. Some phyla appeared to be sensitive to soil depth, as *Actinobacteria* decreased, while *Verrucomicrobia* and *Crenarchaeota* increased in the subsoil. Similarly, when the most abundant OTUs (top 500) were subject to hierarchical clustering, the microbial community was different between the

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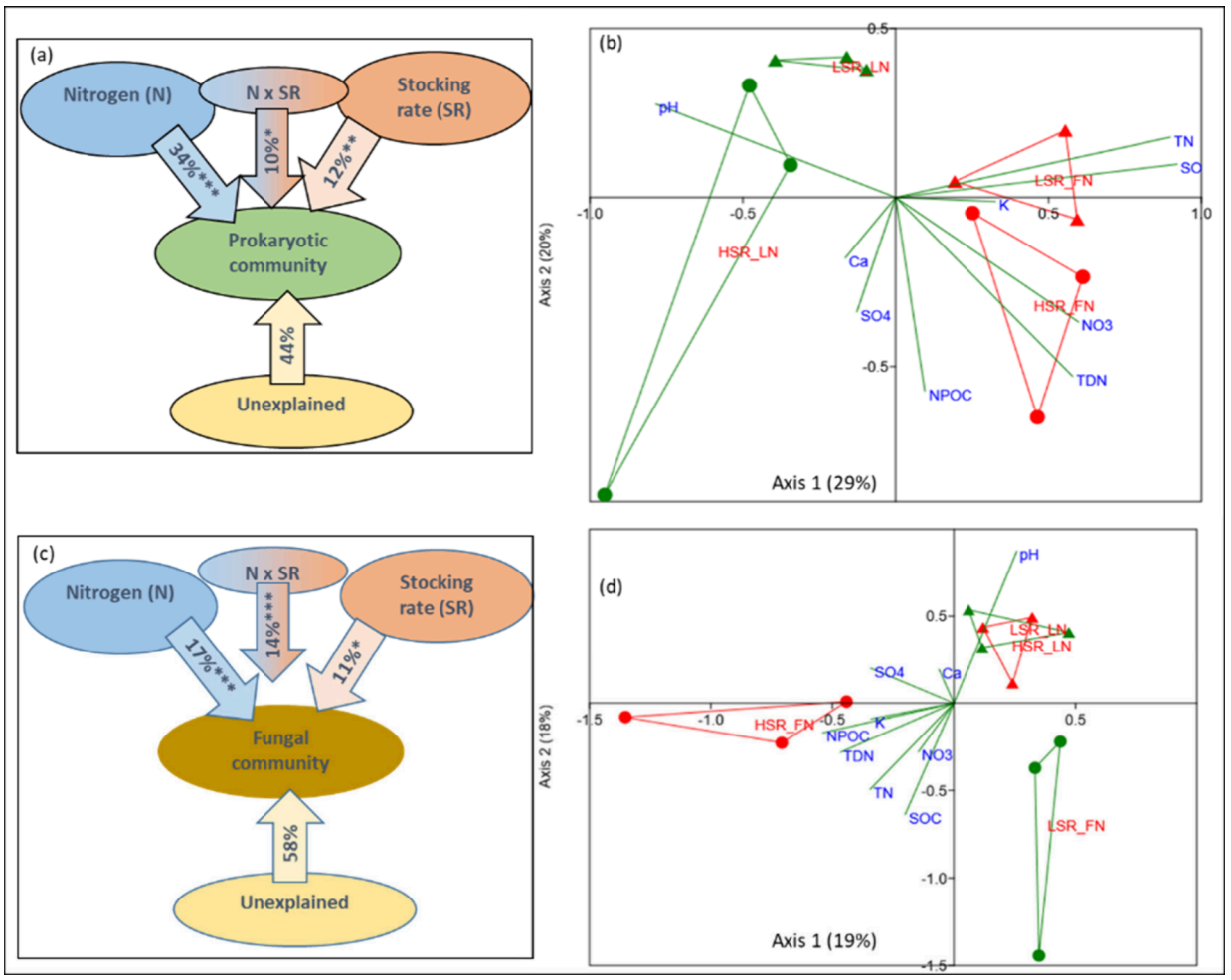

Figure 5. Permutational multivariate analyses of variances and effects on the prokaryotic (a) and fungal (c) communities in the grazing soil systems. Percentages presented within arrows are variance percentages explained by the factor or interactions. Asterisks beside percentage numbers represent p values at <0.01 (***), <0.05 (**), and <0.1 (*). Permanova p values are based on the Bray−Curtis distances. Scatter plots on the right are for the canonical correspondence analysis (CCA) of a distance matrix of prokaryotic (b) and fungal (d) OTU abundance with soil parameters, as influenced by experimental treatments of long-term grazing and nitrogen management. Soil parameters were used as environmental variables for correspondence correlation based on eigenvalue estimates.

two N treatment systems (Figure S4). Relative abundance plots of fungal phyla revealed that the most numerous fungi were *Ascomycota* (62 to 88% of total abundance) (Figure 4). Phylum *Glomeromycota*, which is composed almost entirely of arbuscular mycorrhiza fungi (AMF), was observed to have less than 3% of the relative abundance. As methanotrophs are mostly represented within *Alphaproteobacteria* (Type II) and *Gammaproteobacteria* and *Verrucomicrobia* (Type I), we further explored the relative abundances of these potential methanotrophic taxonomic groups among the treatments. The relative abundance of *Alpha-* and *Gammaproteobacteria* was higher in the fertilizer addition (FN) treatments, whereas *Verrucomicrobia* was higher in the LN clover treatments (Figure S5).

Two-way permutation multivariate analyses of variances (PERMANOVA) were performed on the Bray−Curtis distances for 16S and ITS sequence counts to compare the experimental treatment effects. It was revealed that prokaryotic community composition was significantly different ($p \leq 0.01$) between the two N-treatments, and between the SR treatments ($p \leq 0.05$), and their interactions at $p < 0.1$ (Figure 5(a)). The potential methanotrophic community was significantly influenced by the N-treatments ($p \leq 0.01$), but not by SR treatment or by interaction between SR and N treatments (Table S3). Canonical correspondence analysis (CCA) was performed on OTU abundances, which further indicated that the bacterial community was largely differentiated by N-management systems (FN vs LN), as indicated by a greater separation along axis 1, which represented a variation of 29% (Figure 5(b)). Among the soil properties (represented as biplot vectors), pH, NO_3, TN, and SOC were major drivers of bacterial community structure. The fungal community was significantly influenced by N-treatments ($p \leq 0.01$), and their interaction effect ($p \leq 0.01$), but to lesser extent ($p \leq 0.1$) by the SR treatment (Figure 5(c)). CCA analyses of fungal OTUs revealed that fungal communities exhibited major differences between the HSR_FN and LSR_FN treatments (Figure 5 (d)). Among the soil parameters compared for their influences on the fungal community composition, soil pH appeared to be the major driver of separation.

4. DISCUSSION

Lab incubation comparisons revealed higher iCH_4 emissions from the surface soil than those from deeper layers. The qPCR data indicated a greater abundance of methanogens in the

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surface soil than in subsoil. In grazing lands, methanogens mostly survive in the surface soil because of higher substrate availability and continuous repopulation through animal excreta.[43] Moreover, the subsoil was more acidic (pH < 4.5), which may have some inhibitory effects on methanogens.[44] These trends are contrary to the assumption that methanogens preferentially inhabit deeper soil layers where oxygen concentrations decrease.[45] Zhang et al.[46] reported that soil microbial biomass carbon (MBC) and nitrogen (MBN) decreased with increasing soil depth, and the 0−10 cm soil layer of grasslands had the highest MBC and MBN which generally include both aerobic and anaerobic microbes (e.g., methanogens). Nevertheless, the study results emphasize the value of subsoil SOC and support the notion that subsoil SOC is more stable[47,48] and may be less sensitive to gaseous CH_4-loss pathways.[49] Results from SOC analyses of the soil profile in this study indicated that SOC stocks in the soil profile of LSR pastures were significantly increased compared to HSR pasture soil profiles. Higher grazing intensity-induced differences in SOC were larger in the surface soil layer (0−20 cm), where SOC stocks decreased by up to 38% in the HSR compared to LSR pastures. In subsoil (20−60 cm), the differences were less than 10%. These results concur with reports that have clearly established how overgrazing induced loss of SOC stocks in many grazing systems.[50,14]

Our seasonal assays clearly showed higher iCH_4 emissions in soil samples originating from the HSR pastures compared to the LSR pastures. Substantial CH_4 emissions were noted in all four seasonal samples collected from HSR pastures but not in the LSR pasture soils. qPCR assays indicated an increased abundance of both *mcrA* and *pmoA* gene numbers in the HSR pastures. These results suggest that methanogenic activity surpass methanotrophy under anaerobic soil conditions, but only in HSR pasture soils. During soil saturation phases (after rainfall events), particularly from anaerobic soil aggregates, substantial iCH_4 emissions could be anticipated from overgrazed pastures. Thus, grazing management could be a potential mitigation avenue to minimize this phenomenon. Similar trends were noted in one previous study, where extensive grazing was shown to reduce the capacity of soils to consume CH_4 and stimulated CH_4 production potentials by shifting the balance in favor of methanogenic activities.[8] However, the long-term interactive effects of grazing on grassland soil GHGs has remained unclear.[21] For instance, Pan et al.[2] indicated that grazing in Inner Mongolia Grassland dominated by *Leymus chinensis* significantly decreased CH_4 emission and uptake. Ren et al. also reported that in Songnen meadow steppe, the grazing significantly decreased the CH_4 flux.[20] Therefore, our results showing the significantly positive correlation between the grazing and methane emission are quite important to understanding the East Texas grassland, likely due to the varied vegetation species and biogeochemical features. It is possible that grazing might induce soil compaction under high stocking rates, which likely increases potential CH_4 emission.[51,13] Another reason could be the increased availability of carbon substrates for methanogenesis, as it was shown that animal excreta was quickly mineralized, with some of which was lost as CH_4 emissions from soils.[52] Moreover, methanogen loaded through animal excreta under high density of animals can also contribute to increased potential CH_4 emissions.[13] qPCR assay results concurred with the previous studies, as the relative abundance of methanogens was significantly higher in the HSR compared to LSR. Additionally, results from the manure amendment experiments clearly indicated a positive correlation between manure addition and potential iCH_4 emission with higher methanogen abundance. Higher CH_4 emissions were recorded during winter and spring grazing of clover compared to summer grazing of bermudagrass, although vegetation type might influence CH_4-consumption and production.[53] These seasonal dynamics must also be considered in the design of effective grazing and N-management strategies and could be integrated with other pasture management technologies to mitigate CH_4 emissions and increase SOC stocks in grazing lands.

The findings additionally revealed higher CH_4 emissions in the HSR_LN compared to HSR_FN, suggesting that N-deficient legume-N paddocks under a high stocking rate further increased iCH_4 emission potential. Studies have shown that animal excreta deposition under continuous grazing can turn grass-legume pastures from net CH_4 consumers to net CH_4 emitters.[54,55] Methanogen gene numbers (*mcrA*) were lower in the HSR_FN than in HSR_LN, suggesting N-deficient systems favor methanogens, whereas N-fertilization probably reduced methanogenic activity in agreement with other reports.[56] Similarly, methanotrophs were responsive to N-fertilization as *pmoA* gene copies were lower in N-fertilized (HSR_FN) compared to legume-N pastures (HSR_LN), which was similar to trends noted in other studies.[57,58] There is some evidence to suggest that N-fertilization could inhibit methanotrophs under acidic conditions,[59] but other studies did not find this to be true.[8] Overall, results of this study supported our hypothesis that grazing intensity and nitrogen management were major drivers of microbial community structure in the soil profile, shifted the functional balance between methanogens and methanotrophs, and increased iCH_4 emission potential in overgrazed pastures.

The outcomes of this study highlighted significant alterations in soil microbial community structure in long-term grazing pastures subjected to two different stocking rates of cattle and N-management treatments. The soil prokaryotic microbial community was significantly influenced by N-fertilization treatments, whereas the fungal community was influenced by stocking rate management but mostly occurring in the N-fertilization (FN) treatment (HSR_FN vs LSR_FN). Studies have noted that N-fertilization[60] and grazing practices are major drivers of soil microbial community structure in grazing lands.[61] Continuous manure deposition largely favors copiotrophic taxa such as *Proteobacteria* and *Actinobacteria*,[62,63] *Nitrospira* and *Actinomycetes*,[64] which were increased in N-fertilized pastures compared to N-deficient legume pastures. Results further showed that N-management influenced specific taxa such as *Alphaproteobacteria* (potential Type II methanotrophs) and *Gammaproteobacteria* (potential Type I methanotrophs), which were generally higher in FN treatments, whereas *Verrucomicrobia* were higher in LN treatments. It is possible that methanotrophs from *Alpha-* and *Gammaproteobacteria* were stimulated by continuous N-fertilization compared to *Verrucomicrobia*. It has been shown that *Verrucomicrobia* are a slow growing microbial group that thrive under low substrate systems,[65] compared to most *Proteobacteria*, which are known to metabolize and grow faster (copiotrophic taxa) and proliferate under high nutrient inputs.[66] Moreover, soil pH was more acidic in FN compared to that in LN pastures, which might have diminished CH_4 cycling microbes. Acidic pH and N-fertilization are generally inhibitory to some methanotrophs and methanogens as

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suggested by previous reports.[67,11] For example, Type I methanotrophs were reduced under acidic soil pH, whereas Type II increased.[57]

5. CONCLUSIONS

In summary, this study demonstrated that higher stocking rates substantially increased methane emissions in both grazing soils dominated by nitrogen-fixing clover and those with ryegrass supplemented by nitrogen fertilizer across various long-term management scenarios. In contrast, lower stocking rates and reduced nitrogen and manure fertilization effectively suppressed methane production. CH_4 emissions also exhibited a consistent decrease with depth in both grazing soils, with the highest rates observed in the top 0−5 cm layer. Long-term management practices in these grazing systems appeared to influence soil properties, microbial communities (especially methanogens and methanotrophs), and, subsequently, methane emissions. However, grazing intensity and fertilization emerged as pivotal factors influencing CH_4 output.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.4c00138.

Detailed description of the study sites, experimental plot design, and soil sampling for the Materials and Methods part; supplemental tables including biochemical characteristics of grazing pastures, target genes and primers used in qPCR, PERMANOVA F and p values for microbial community abundance data; supplemental figures including sampling sites and layouts of experimental grazing plots, incubation time range finding assays with CH_4 and CO_2 production of clover and rye grassland soils, Spearman correlations between CH_4 emissions and *mcrA* gene copies, CH_4 emissions and *pmoA* gene copies, and *mcrA* and *pmoA* gene copies in first lab incubation study, hierarchical clustering of microbial communities based on relative abundance of predominant OTUs (top 500) in the grassland soil, and relative abundance of *Verrucomicorbia*, *Alphaprotebacteria*, and *Gammaproteobacteria* in the top 20 cm soil profile (PDF)

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Notes

The authors declare no competing financial interest.

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