



# Forage Grasses Steer Soil Nitrogen Processes, Microbial Populations, and Microbiome Composition in A Long-term Tropical Agriculture System

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## ABSTRACT

Forage grasses used in cropping no-till systems in tropical regions alter soil chemical properties, but their long-term impact on soil microbial processes of the nitrogen (N) cycle and microbial community abundance, composition and structure are unknown. Here, microbial functions related to nitrogen fixation, nitrification and denitrification as well as bacterial, archaeal and fungal populations were evaluated in a long-term field experiment in which tropical forage grasses palisade grass (*Urochloa brizantha* (Hochst. Ex A. Rich.) R.D. Webster) and ruzigrass (*U. ruziziensis* (R. Germ. and C.M. Evrard) Crins) were cultivated with or without N fertilization. Uncultivated soil was used as a control. Forage grasses, especially palisade grass, increased soil bacterial and fungal abundances, whereas the archaeal population was highest in uncultivated soil. In soils cultivated with forage grasses, N fertilization favored N-cycle-related genes; however, cultivation of palisade grass increased the abundances of *amoA* bacteria (AOB) and *amoA* archaea (AOA) genes associated with soil nitrification and decreased the abundances of genes *nirS*, *nirK* and *nosZ* genes related to denitrification, compared to ruzigrass and control, regardless of N input. In addition, abundances of total bacteria and total fungi were associated with the N cycle and plant biomass in soils cultivated with forage grasses. Forage cultivation clearly benefitted the soil nutrient environment ( $S-SO_4^{2-}$ ,  $Mg^{2+}$ , total-C and -N,  $N-NO_3^-$  and  $N-NH_4^+$ ) and microbiome (bacteria and fungi) compared with uncultivated soil. In soil cultivated with palisade grass, the microbial community composition was unresponsive to N addition. The high N uptake by palisade grass supports the competitive advantage of this plant species over microorganisms for N sources. Our results suggest that palisade grass has advantages over ruzigrass for use in agriculture systems, regardless of N input.

## 1. Introduction

Investigations of biogeochemical nitrogen (N) processes and microbial communities in tropical agricultural soils have aroused interest in production chains that unite sustainability, soil diversity, and food production at the micro- and macro-scales (Sadras et al., 2020; Sun et al., 2020; Tiwari et al., 2019). In tropical regions, extensive agricultural areas are cultivated with forage grasses for production, for grazing in integrated crop-livestock systems, as cover crops in rotation with cash crops, or in intercropping systems (Moraes et al., 2019). Forage grasses promote soil quality and nutrient content as well as crop productivity (Baptistella et al., 2020), but little is known the effects of these grasses

on microorganisms in tropical soils and their role in soil chemistry, especially under N input (Bossolani et al., 2020; Rocha et al., 2020).

The aggressive, deep root systems of forage grasses improve soil organic matter (SOM) by providing high biomass production and nutrient cycling (Fisher et al., 1995; Galdos et al., 2020). Compared to abandoned soils (fallow), forage grasses decrease the risk of soil erosion and protect the soil surface. Cultivation of forage grasses is especially beneficial in regions with dry winters and low rainfall, where most winter crops cannot be cultivated, leaving the soil exposed (Paul et al., 2020). The absence of soil coverage results in nutrient leaching by rainfall to deeper soil layers and can lead to desertification (Yang et al., 2019). Among forage cover crop genera such as *Pennisetum*, *Megathyrsus*

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(syn. *Panicum*) and *Urochloa* (syn. *Brachiaria*), *Urochloa* is notable for its low soil fertility demands and great adaptability in tropical regions (Canisares et al., 2021; Crusciol et al., 2020; Namazzi et al., 2020). *Urochloa* spp. exhibit high growth performance and benefits for cropping systems and food production, including improved soil aggregation, microbial biomass, organic matter and nutrient cycling processes (Crusciol et al., 2019; Momesso et al., 2021, 2019). Cultivation of *Urochloa* spp. has been expanding rapidly in Latin America, Asia, and Africa to combat global soil degradation, greenhouse gas emissions and hunger (Costa et al., 2020; Ondabu et al., 2017; Ramírez-Restrepo et al., 2020; Rao, 1998).

The justification for forage grass introduction is to provide services to soil agro-ecosystems and improve agricultural yields (Baptistella et al., 2020; Foley, 2005). The two most commonly cultivated grasses, palisade grass (*Urochloa brizantha*) and ruzigrass (*U. ruziziensis*), impact soil N and crop yield, particularly when palisade grass is grown as a cover crop (Momesso et al., 2020, 2019; Tanaka et al., 2019). N is a key nutrient for forage production and maintenance and is provided by inorganic and organic fertilization, and animal urine (Boddey et al., 2004; Byrnes et al., 2017; Durango Morales et al., 2021; Momesso et al., 2020). As an important management practice for crop production, N fertilization significantly influences the nitrifying and denitrifying microbial communities, and these changes are at the heart of the environmental and sustainability issues faced by agriculture (Cassman et al., 2019; Chen et al., 2019; Lourenço et al., 2020). Forage grasses have the potential to tighten the N cycle and avoid N losses through ammonia volatilization, nitrate leaching and denitrification in the soil, which often lead to reduced N use efficiency in agricultural ecosystems (Norton and Ouyang, 2019; Subbarao et al., 2017). Although few relevant studies have been performed under tropical conditions, differences in microorganisms related to N microbial processes (N fixation and nitrification) have been observed in soil cultivated with ruzigrass (Rocha et al., 2020). However, information on the impact of N fertilizer applied over forage species on the soil N cycle and microbiome composition is limited, and the potential advantages of forage grass cultivation compared with unplanted soil have not been established.

Long-term plant cultivation usually alters soil microbial communities and soil properties (Boeddinghaus et al., 2019). The soil microbiota and plant responses are interlinked through changes in soil pH and sources of N and carbon (C), which can reduce competitive barriers for beneficial microbes (Trivedi et al., 2020; Wagg et al., 2019). Plant growth alters the chemical and physical properties of the soil environment (Tiritan et al., 2016), and the improved soil aggregation and increased organic matter in soils cultivated with grasses promote actinomycetes, gram-positive bacteria, arbuscular mycorrhizal fungi (AMF), and fungi (Sarto et al., 2020; Teutscherova et al., 2019b, 2019a). Plant-microbe competition for nutrients, especially N, also plays an important role in changes in bacterial and fungal communities (Cassman et al., 2016; Chen et al., 2019). Finally, plant cutting, such as mowing and grazing, alters root exudates and therefore soil microbial properties (Bardgett et al., 1998). Many studies have focused on the potential relationships and interactions of microorganisms with the environment (Prosser, 2020), but the links between above- and below-ground changes under tropical grass cultivation in the field conditions have not received as much attention.

The goal of the present study was to investigate the effects of two tropical forage grasses and N fertilizer application on soil N-cycle genes and microbiome responses to the altered environment in a long-term field experiment in a tropical no-till system. We hypothesized that (i) cultivation of forage grasses increases N fixers and decreases nitrifiers and denitrifiers compared to uncultivated soil, (ii) application of N fertilizer to forage grasses increases the fungi:bacteria ratio in the soil by the stimulated plant biomass production, and (iii) cultivation of forage grasses consequently benefits soil properties and soil fungal communities, especially organic matter decomposers, compared with uncultivated tropical soil, regardless of the cultivated grass species.

Understanding the responses of both the N cycle and the microbial community to tropical grass cultivation and N fertilization may aid the development or improvement of strategies for avoiding N losses via microbial processes and promoting potential beneficial microorganisms in sustainable agricultural ecosystems.

## 2. Materials and Methods

### 2.1. Experimental field plots, aboveground and soil sampling

We selected three field experimental areas located at the Experimental Farm Station in Botucatu, Sao Paulo State, Southeastern Brazil (48° 26' W, 22° 51' S, 769 m). The experimental site has a typical climate with dry winters and warm, wet summers, classified as Cwa type according to the Köppen classification. The long-term mean minimum and maximum temperatures are 15.3 and 26.1 °C and the mean rainfall is 1358 mm (50-year average). The seasonal precipitation and temperature during the experiment are shown in Supplementary Figure 1. The soil is classified as a clayey, kaolinitic, thermic Typic Haplorthox according to the USDA soil taxonomy (Soil Survey Staff, 2014). The average soil texture in the top 0.10 m is 9% silt, 28% sand, and 63% clay. Before setting up the experiment, chemical characteristics of the topsoil (0–0.10 m) were determined in each area (Supplementary Table 1).

We set up the experiment in two field areas abreast cultivated with forage grasses (palisade grass and ruzigrass) for a long-term in no-tillage system (10 years). The crop rotation was composed by different crops during previous growing season and further details of crop rotation are shown on Supplementary Table 2. The forage grasses were sown at a density of 10 kg seed ha<sup>-1</sup> (34% viable seed) and zero-fertilizer application in April 2017, the grasses used only residual fertilization from previous crops. The forage cultivation was during seven months in 2017 and, due to stimulation of plant growth and N uptake, forage grasses were managed by mechanically mowing at 0.30 m above soil level. No herbicides were applied for weed control. The third selected area was 150 m distant from forage areas and a non-agricultural area as a control without forage with weed growth (*Raphanus raphanistrum* L., *Conyza bonariensis*, *Avena strigosa*, *Avena sativa*, *Leonotis nepetaefolia* Benth, *Commelina benghalensis* L., *Panicum Maximum* Jacq., *Ipomoea* spp.). The three field areas had similar soil properties (Supplementary Table 1).

In a randomized complete block design, the experiment had four treatments + control: ruzigrass (Ruzi), ruzigrass receiving N (Ruzi +N), palisade grass (Palisade), palisade grass receiving N (Palisade +N), and control (no-agriculture, i.e., no forage grasses). The rate of N fertilizer applied was 120 kg N ha<sup>-1</sup>, as ammonium sulfate, that was applied at surface broadcast on palisade grass and ruzigrass in October 2017, except in the control. Each treatment was replicated four times in experimental plots sized 10 × 5 m in a randomized complete block design with 1 m buffer zone between the plots.

The forages have not received any mineral fertilizer at seeding. Approximately 30 days before N fertilizer application, the palisade grass and ruzigrass were cut 0.30 m above the soil level by mechanical mowers. Samples of biomass and soil were collected 5 days after N fertilizer application (October 2017), which is sufficient for forage response and for adequate biomass production in agricultural systems observed in previous studies (Momesso et al., 2020, 2019; Tanaka et al., 2019). For aboveground residues, two samples of the plant material present on the soil surface from a central area of 0.25 m<sup>2</sup> in each plot were combined and dried in oven at 65 °C for 72 h. From sample of each plot, subsamples of biomass were used for carbon (C) and N determination by an elemental analyzer (LECO-TruSpec® CHNS). Subsequently, N accumulated was extrapolated to Mg ha<sup>-1</sup> of biomass and C:N ratio was calculated. For soil samples, five subsamples (0.0–0.10 m depth) were collected at random from each plot to form a composite sample. The samples were separated into three batches. For the Batch 1, samples were dried in oven at 40 °C for 72 h and sieved with 10-mesh sieves. In each sample, the soil pH was measured in 0.01 M CaCl<sub>2</sub> (1:2.5

soil/solution). Phosphorus (P) and exchangeable  $K^+$ ,  $Ca^{+2}$  and  $Mg^{+2}$  cations were extracted using exchange resins; P was determined by colorimetry, and cations by atomic absorption spectrometry (van Raij et al., 2001). Soil sulfur-sulfate ( $S-SO_4^{2-}$ ) extraction were determined by calcium phosphate extraction at  $0.01 \text{ mol L}^{-1}$  in a 1:2:5 soil/solution ratio and later determined by turbidimetric method using  $BaSO_4$  (Vitti, 1989). The  $NH_4^+$  and  $NO_3^-$  contents (soil inorganic N) were determined by extraction with KCl and distilled water (Keeney and Nelson, 1982). The C and N contents in the soil were determined on an elemental analyzer (LECO-Trup® CHNS). For the Batch 2, samples were dried at  $105^\circ\text{C}$  for 24 h to determine the soil moisture and the Batch 3 was stored at  $-80^\circ\text{C}$  for further molecular analyses.

## 2.2. DNA extraction

Soil samples (0.0–0.10 m top layer) were collected at 5 days after N fertilizer application, the same time of aboveground sampling. For each plot, subsample from sample collection was separated into two batches. Soil DNA was extracted from 0.25 g of soil (stored at  $-80^\circ\text{C}$ ) using the MoBioPowerSoil™ DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), following the manufacturer's protocol. The quantity and quality of DNA extracts were determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, DE, USA) and a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). The DNA integrity was checked by electrophoresis on agarose gel (1% w/v). The DNA samples were used to quantify the functional N genes, bacteria, archaea and fungi total population, and to determine the bacterial, archaeal and fungal communities in the soil samples.

## 2.3. Quantitative real-time PCR

Quantification of the copy number (abundances) of populations of total bacteria and archaea (16S rRNA gene) and total fungi (18S rRNA gene) as well as genes encoding the N fixation (*nifH*), nitrification (*amoA* bacteria, AOB; *amoA* archaea AOA), and denitrification (*nirS*, *nirK* and *nosZ*) were performed by quantitative real-time PCR (qPCR). The determination of abundances was performed out in a 96-well plate (Bio-Rad) using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Primer sets and the PCR conditions of each gene are detailed in Supplementary Table 3. The confirmation of amplicon sizes was carried out by separation on agarose gels (1%). Standard curves were constructed using serial 10-fold dilutions of a known amount of plasmid DNA ( $10^9$  to  $10^1$  gene copies) containing the gene fragment of interest. Negative controls were performed with water instead of template DNA. Reaction efficiencies for all assays ranged from 80% to 101%, and  $R^2$  values ranged from 0.91 to 0.99.

## 2.4. Illumina sequencing of bacterial, archaeal and fungal phylogenetic markers

DNA extracted from soil samples was used for amplification and sequencing of the 16S rRNA gene and ITS region. The PCRs of bacterial and archaeal 16S rRNA gene was targeted the variable V4 regions gene with the primer set of 515 F (forward primer 5'-GTGC CAGCMGCCGCGGTAA-3') and 806 R (reverse primer 5'-GGAC TACHVGGGTWCTAAT-3') with barcodes. The PCR amplification of fungal ribosomal internal transcribed spacers (region ITS2) was performed using ITS9F (5'-GAACGCAGCRAAIIGYGA-3') and ITS2R (5'-TCCTCCGCTTATTGATATGC-3') primers with barcodes. Sequencing was carried out on the Illumina MiSeq System at Genome Quebec (Quebec, Canada).

## 2.5. Data statistical and bioinformatic analyses

Most of the statistical analyses were conducted in R v4.02 (Team, 2019) using different packages. The results of soil, plant and soil genes abundances were log-transformed, and the normal distribution of residues and variance stability were confirmed (ggpubr package). First, single effect of forage species and N input factors, as well as their interactions were tested. Because there was effect of factors interaction, factors were combined as forages (forage species + N input) and the calculations were performed with forages as fixed factor. The data were subjected to analysis of variance (ANOVA), and the means were compared by Tukey's test ( $P \leq 0.05$ ) (agricolae package). Ratios of total archaea:bacteria, total fungi:bacteria, and total archaea:fungi were calculated. The ratios of the *nifH*, AOB, *nirK*, *nirS*, *nosZ* genes to 16S rRNA gene of bacteria were calculated by dividing the copy number of each N cycle gene by the copy number of total bacteria. For the AOA gene ratio, the copy number of the AOA gene was divided by the copy number of total archaea. The calculated ratios were graphed in a box-plot. Additionally, redundancy analysis (RDA) was applied to determine the correlation between soil and plant analysis (environmental factors) with the microbial phylogenetic markers and N cycle functional genes (biological factors) using Canoco 4.5 (Biometrics, Wageningen, the Netherlands). Monte Carlo permutation test were applied with 999 random permutations to verify the significance of environmental factors on genes responses. Data were subjected to analysis of one-way PERMANOVA (Anderson, 2017) to group the treatments for similarity in Past4 (version 4.0).

For bacterial and fungal communities, the bioinformatics pipeline and subsequent analyses were performed using the R programming language version 4.10. Forward and reverse PCR primers were removed from the MiSeq reads by using the cutadapt plugin v2.10 (Martin, 2011) (dada2 package). The DADA2 pipeline was used to data processing and taxonomy assignment (Callahan et al., 2016). Forward and reverse reads were trimmed to 240 base pairs and 200 base pairs, respectively, and at the location of the first occurrence of a base calls or containing greater than or equal to 18 estimated errors, and merged with a minimum overlap of 12 bases. Chimeric sequences were discarded and merged reads dereplicated. Taxonomy was assigned to amplicon sequence variants (ASVs) using the SILVA v138 database (McLaren, 2020).

Sequencing data from the DADA2 analysis was first filtered to remove chloroplast reads and unknown taxa at Phylum level. After that, all the ASV's abundance were summarized at Genus taxonomic level. Low-occurring microbes (presence in less than 5 samples) were aggregated into a single variable named 'others' (this was performed for bacterial and fungal groups, separately). After that, data was transformed to the center log ratio (CLR) using the Bayesian-multiplicative replacement of count zeros (CZM method). Gjam package (Clark et al., 2017a) was used to estimate the effects of control (no-agriculture), ruzigrass (Ruzi), ruzigrass receiving N (Ruzi + N), palisade grass (Palisade), and palisade grass receiving N (Palisade + N) in the soil microbial community together with the soil and plant variables (pH, SOM, total-N,  $NH_4^+$ ,  $NO_3^-$ , P,  $K^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$  and  $SO_4^{2-}$ , and biomass, N content and C/N ratio of aboveground plants) and the abundance of bacteria, archaea, and fungi, obtained via qPCR. Because of the high variability within the qPCR data and biomass, those variables were log-transformed before entering in the GJAM model (Clark et al., 2017b). From the model we extracted the regression coefficients from each treatment to identify shifts in the microbial community and in the other variables (Leite and Kuramae, 2020). Model diagnosis evaluated the Markov Chain Monte Carlo (MCMC) to check when the estimated coefficients reached a stable value (after 2000 simulations with a burn-in of 500). The regression coefficients obtained were visualized via heatmaps and clusters tree using WARD distance. Regression coefficients were used on PCA plot to explore communities' similarities between treatments (gplots, cowplots, BiocManager and devtools packages).

### 3. Results

#### 3.1. Forage grasses production, leaf N content and C:N ratio

Nitrogen fertilization significantly increased the aboveground plant biomass in Palisade +N (Supplementary Fig. 2). Biomass production in Palisade was 17% lower than Palisade +N, but Palisade and Ruzi +N reached similar production of biomass. Leaf N content and C/N ratio resulted in different pattern of that observed in biomass production. Leaf N content was improved by forages cultivation (Palisade +N, Palisade, Ruzi +N and Ruzi) compared to control. Meanwhile, Palisade +N, Palisade and Ruzi +N decreased the C/N ratios. Control promoted the lowest biomass, leaf N content and the highest C/N ratio.

#### 3.2. Quantification of bacteria, archaea, fungi and N-cycle gene abundances

The cultivation of forage grasses under N fertilization significantly increased the abundances of bacteria and fungi in the soil while decreased the abundances of archaea (Fig. 1). Bacterial abundance decreased in the following order: Palisade +N > Palisade > Ruzi +N and Control > Ruzi (Fig. 1A). The abundance of archaea was highest in uncultivated soil (Control) and was lower but similar among the treatments with forage with or without N fertilization (Fig. 1B). In contrast to the bacterial and archaeal abundances, the fungal abundance was greatest in Palisade and Palisade +N and decreased from Ruzi and Ruzi +N to the Control (Fig. 1C). The total archaea:bacteria and total archaea:fungi ratios were greatest in the Control (Fig. 1D, and 1 F) whereas the total fungi:bacteria was lowest in the Control (Fig. 1E).

To determine whether ruzigrass or palisade grass and N fertilization alter soil N cycling under tropical conditions, we quantified the abundance of N-cycle genes related to  $N_2$  fixation (*nifH*), nitrification (AOA and AOB), and denitrification (*nirS*, *nirK* and *nosZ*). Soil *nifH* abundance was higher in Ruzi than in the other treatments (Control, Ruzi +N, Palisade and Palisade +N) (Fig. 1G). The abundances of *amoA* bacterial (AOB) and *amoA* archaeal (AOA) genes were higher in the soil cultivated with forage grasses than in the Control. AOB were highest in Palisade and Palisade +N; while AOA were more abundant in Palisade and Ruzigrass than in Palisade +N and Ruzi +N (Figs. 1H and 1I). The soil abundance of the denitrification gene *nirS* was highest in Ruzi and lower but similar among the Control, Ruzi +N, Palisade and Palisade +N (Fig. 1J). The soil abundance of the denitrification genes *nirK* and *nosZ* (Figs. 1L and 1M) was highest in the Control and was lower in the treatments with forage grasses, regardless of N fertilization (Ruzi, Ruzi +N, Palisade, Palisade +N).

Correlation analysis of gene abundances and soil and plant factors is shown in Fig. 2A. Plant biomass and soil  $S-SO_4^{2-}$  and  $N-NH_4^+$  contents were positively correlated with the abundances of AOB, soil  $S-SO_4^{2-}$  content was positively correlated with abundance of bacteria, and  $N-NH_4^+$  content positively correlated with abundance of fungi. By contrast, soil  $N-NH_4^+$  was negatively correlated with the abundances of *nirK* and *nosZ*, soil  $S-SO_4^{2-}$  content negatively correlated with abundances of *nifH*, *nirS*, and *nosZ* abundances, plant biomass negatively correlated with *nirS* and *nosZ* abundances, and plant N content negatively correlated with *nifH* and *nosZ* abundances. Redundancy analysis (RDA) revealed that environmental factors explained 91.5% of the total variability in the abundances of total bacteria, total archaea and total fungi and in the relative abundance of N-cycle genes (Fig. 2B). The treatments were segregated into the following four distinct groups by PERMANOVA analysis ( $p < 0.001$ ): group 1 consisting of the Control (uncultivated soil); group 2 represented by Ruzi and Ruzi +N; group 3 composed of Palisade but with similarity to Ruzi +N belonging to group 2; and group 4 composed of Palisade +N. Furthermore, Monte Carlo permutation analysis showed significant correlations between environmental factors and genes abundances analyses. Soil availability of  $S-SO_4^{2-}$  ( $F = 10.8$ ;  $p = 0.007$ ),  $Mg^{2+}$  ( $F = 4.19$ ;  $p = 0.038$ ), total-N ( $F =$

$2.94$ ;  $p = 0.049$ ),  $N-NO_3^-$  ( $F = 3.25$ ;  $p = 0.042$ ) and  $N-NH_4^+$  ( $F = 3.72$ ;  $p = 0.046$ ) and aboveground biomass production ( $F = 6.61$ ;  $p = 0.026$ ) were the main environmental factors responsible for changes in the abundances of bacteria and fungi and in the relative abundances of nitrification-related genes (AOA and AOB) in the forage grass treatments. By contrast, the Control treatment strongly correlated with archaeal abundance and  $Ca^{2+}$ .

#### 3.3. Bacterial/archaeal and fungal communities

The soil bacterial/archaeal and fungal communities in the Control (uncultivated) differed from those of the treatments with forage grasses, regardless of N fertilization (Fig. 3). Heatmap analysis of bacteria and fungi and plant and soil factors clustered the Control and forage grass treatments into two groups by similarity (Fig. 3).

Group I was separated into 3 subgroups (1, 2 and 3). Subgroup 1 was composed of soil  $Ca^{2+}$ , which was positively correlated with the Control, unlike soils cultivated with forage grasses. Subgroup 2 comprised bacterial and fungal species, plant C:N ratio and total-N, soil  $NO_3^-$ ,  $Mg^{2+}$ , and P content and SOM, which were negatively correlated with the Control but mostly positively correlated with the forage grass treatments regardless of N fertilization. Subgroup 3 comprised mainly bacterial and fungal members that responded positively in the Control and negatively in the soils cultivated with forage grasses.

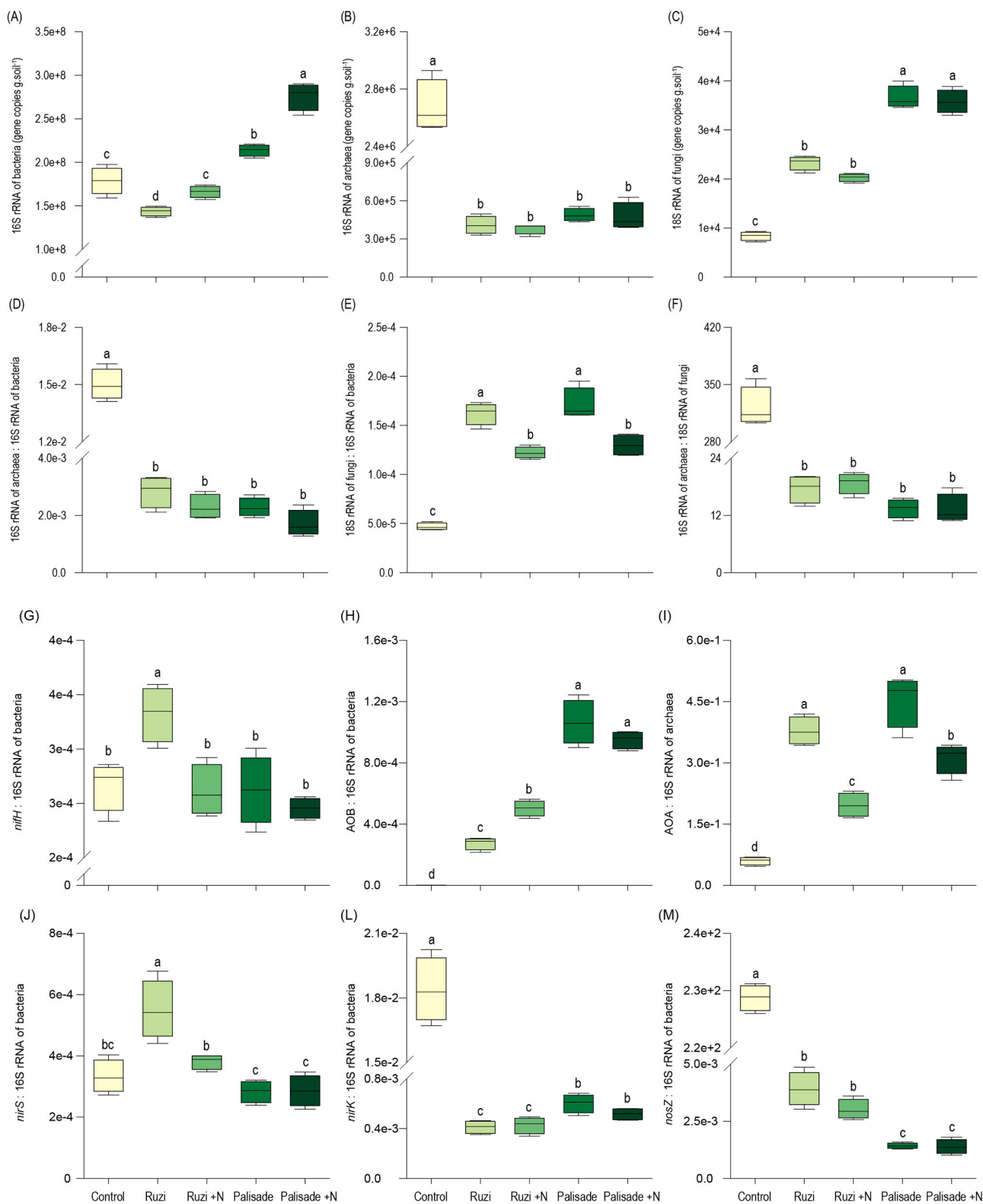
In Group II, soil  $S-SO_4^{2-}$ ,  $NH_4^+$  and total-C content were negatively correlated with the Control but mostly positively correlated with the forage grass treatments regardless of N fertilization. A negative correlation of soil  $S-SO_4^{2-}$  content was observed only in Ruzi (no N fertilization); in Ruzi +N, a positive correlation of  $S-SO_4^{2-}$  was observed, similar to the responses observed in Palisade and Palisade +N.

Principal component analysis (PCA) explained 63% of the differences among the treatments (Fig. 4). The Control treatment differed from the treatments cultivated with grasses, however more different from Ruzi and Ruzi +N than from Palisade and Palisade +N. The Control treatment exhibited a stronger influence of  $Ca^{2+}$  content, total archaea, *Fusarium* ud and low presence of *Pressia* ud and *Elsterales* in the soil. In Ruzi and Ruzi +N, the three most influential factors were *Nitrosomonadaceae*, *Clavaria* ud, and *Myrothecium gramineum*; by contrast, the contributions of *Amycolatopsis* ud, *Chloroflexi*, *Pyremochaetopsis* ud, *Arnim* ud, and *Scytalidium* ud were reduced in these treatments. In Palisade and Palisade +N, *Acidobacteriaceae* (subgroup 1), *Pyrenochaetopsis leptos* and *Chaetosphaeria fusiformes* were the most influential factors, whereas the two least influential factors were soil pH and *Helotiales*.

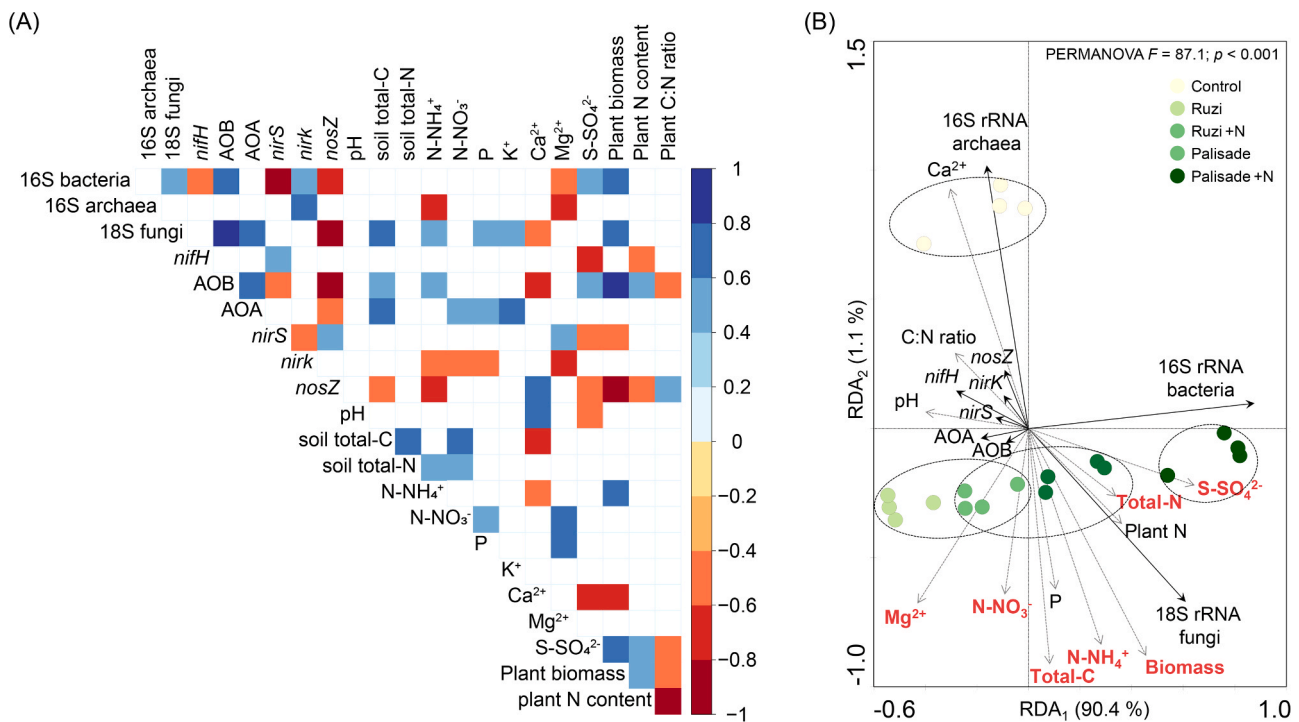
We assessed which microbes were responsible for the differences among the treatments based on regression coefficients (Figs. 5 and 6). A total of 49 amplicon sequence variants (ASVs) in the bacterial community were highly responsive among the treatments (Fig. 5). No effect of the Control was found for 26 ASVs, and most of the ASVs were significantly negatively influenced by Ruzi (unidentified *Pajaroellobacter*, unidentified *Edaphobacter*, unidentified *Opitutus*, *Isosphaeraceae*, unidentified *Solirubrobacter*, unidentified *Rhodoplanes*, *Phenylobacterium mobile*, unidentified *Amycolatopsis*, *ADurb.Bin063-1*, *Burkholderiaceae*). Many of the 15 significantly positive ASVs in the Control were decreased in Ruzi (unidentified *Jatrophihabitans*, unidentified *Bacillus* and unidentified *Conexibacter*), Ruzi +N (*Ktedonobacteria* and unidentified *Solibacter*), and Palisade (unidentified *Quadrifphaera*, *Acidimicrobiia*, unidentified *Planosporangium* and unidentified *Gemmata*); however, none of these ASVs were affected by Palisade +N. Eight ASVs were reduced in the Control, but cultivation of forage grasses had the opposite effect on these bacteria, with increases in *Rhodospirillales* in Ruzi, *Devosia* and *Terrimonas* in Ruzi +N, and *Devosia* and *Koribacter* in Palisade +N.

A total of fungal 66 ASVs differed among the treatments (Fig. 6). The Control had no effect on 33 ASVs, and most of these ASVs were increased in Ruzi, Ruzi +N, Palisade, and Palisade +N. In general, the patterns of





**Fig. 1.** Total bacterial, archaeal and fungal populations (A-C); gene abundance ratios for total archaea (16S rRNA gene):total bacteria (16S rRNA gene), total fungi (18S rRNA gene):total bacteria (16S rRNA gene) and total archaea:total fungi (D-F); and N-cycle-related gene abundance ratios (G-M) in uncultivated soil (Control) and soils cultivated with ruzigrass or palisade grass without N fertilization (Ruzi; Palisade) or with N fertilization (Ruzi +N; Palisade +N). Different letters denote significant difference between treatments (Tukey,  $P \leq 0.05$ ).



**Fig. 2.** Spearman correlation analysis (A) and redundancy analysis (RDA) (B) of the relative abundances of N-cycle genes, total bacteria, total archaea and total fungi (gene copies per g dry soil) and plant and soil variables in uncultivated soil (Control) and soils cultivated with ruzigrass or palisade grass without N fertilization (Ruzi; Palisade) or with N fertilization (Ruzi +N; Palisade +N). The arrows indicate correlations between factors. Black arrows indicate gene abundances in the soil. Grey arrows correspond to plant and soil factors. Significant correlations as determined by the Monte Carlo permutation test are indicated in red ( $p \leq 0.05$ ). The circles with dashed lines indicate significant clusters by permutation analysis (PERMANOVA,  $p \leq 0.05$ ).

changes in fungi were similar to those of bacteria: among the 18 fungal ASVs that were positively affected by the Control, 12 were reduced in soil cultivated with forage. By contrast, some of the 15 significantly negative fungal ASVs in the Control were increased in Ruzi (*Phaeosphaeriaceae* and *Bipolaris*), Palisade (*Cyphellophora fusarioides* and *Chaetosphaeria fusiformis*), and Palisade +N (*Penicillifer martinii*, *Staphylotrichum boninense*, *Fusarium solani*, *Nectriaceae*).

## 4. Discussion

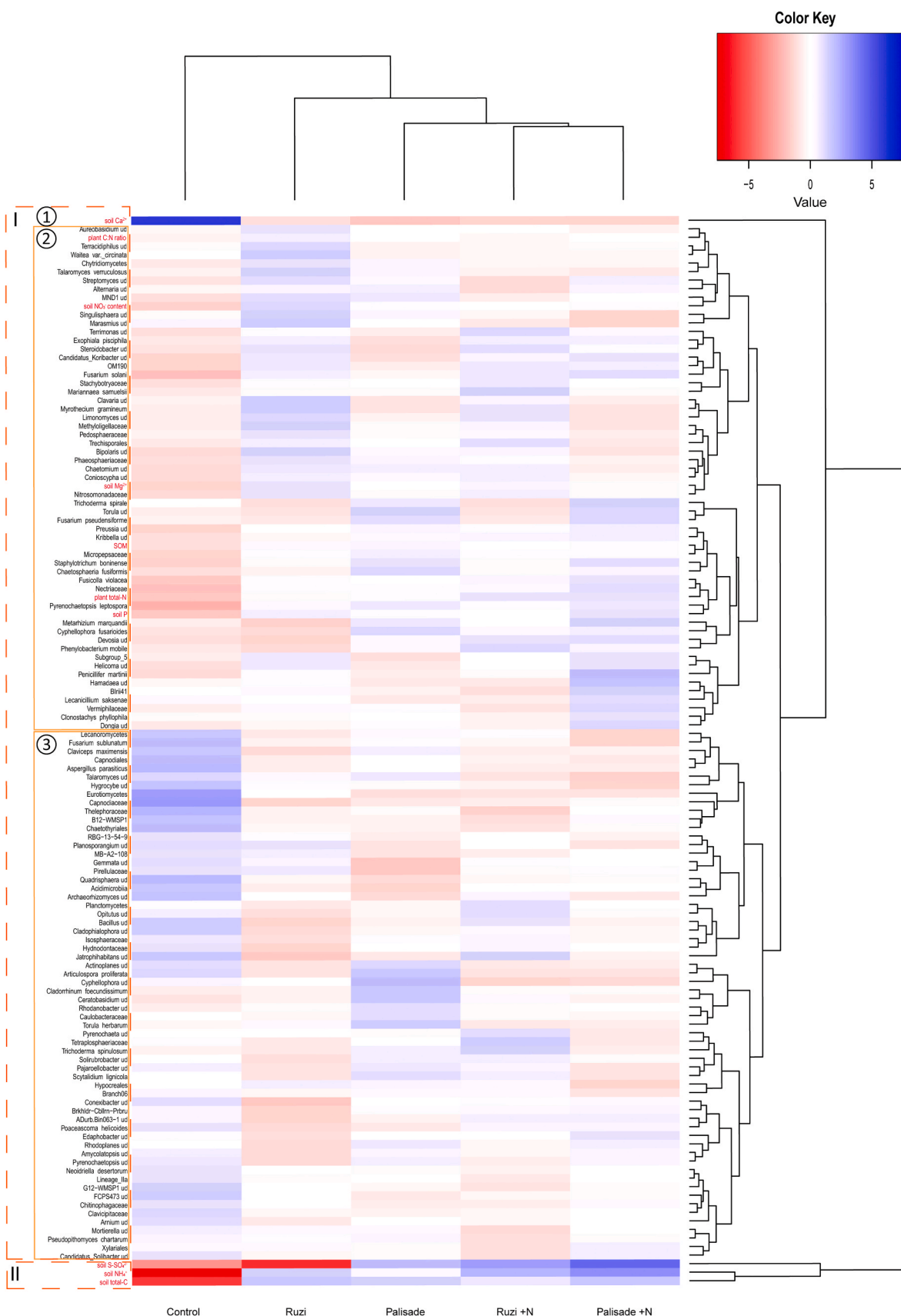
### 4.1. Effects of forage cultivation under N fertilization on microbial populations

The tropical soil investigated in this study had undergone long-term cultivation with  $C_4$  perennial grasses before initiation of the experiment. Targeted real-time PCR analysis of microbial genes can provide useful insights on the abundances of total bacteria, archaea and fungi as well as key genes of N-cycle processes in agricultural systems. The cultivation of tropical forage grasses with or without N fertilization under no-till management in this study altered the chemical properties of the soil and consequently impacted the abundances of total bacteria, total archaea, total fungi and N-cycle genes in the bulk soil.

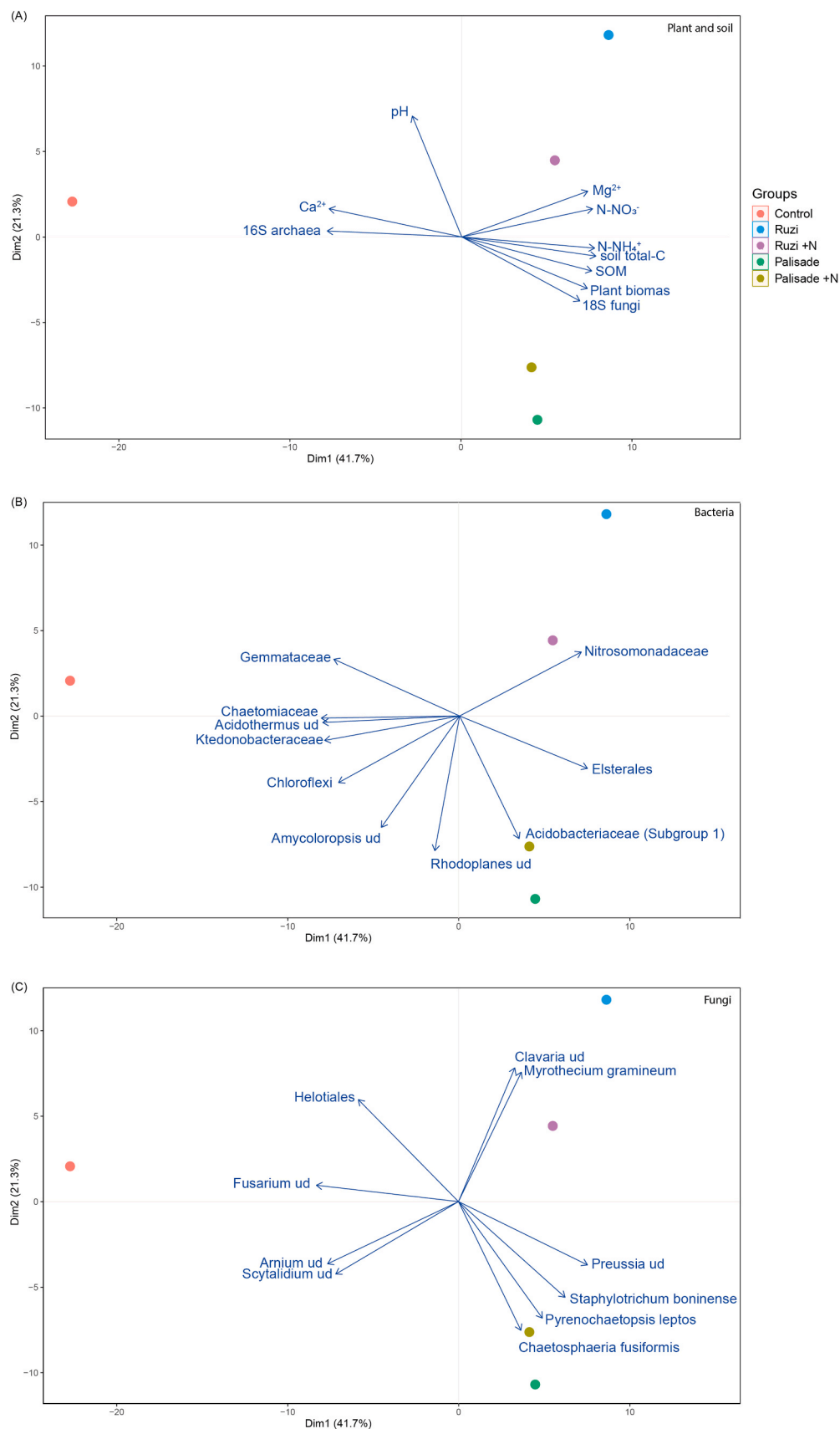
**Bacteria and archaea.** qPCR analysis revealed that bacterial abundance increased under forage grass cultivation and N fertilization, especially palisade grass. The greater availability of resources such as carbon (total C, root exudates and biomass decomposition) and N were the main factors responsible for increasing the bacterial population (Leite et al., 2017). In addition, bacterial abundance was correlated positively with  $S-SO_4^{2-}$  content and biomass production. By contrast, forage grass cultivation decreased archaeal abundance compared with uncultivated soil, and negative correlations were observed between archaea and  $NH_4^+$  and  $Mg^{2+}$  levels. Archaea are capable of living in a wide range of environments, including low-fertility soils

(Martens-Habbena et al., 2009) like those in the uncultivated soil of the Control treatment. Thus, soils with greater availability of resources tend to have lower archaeal abundance and diversity (Merloti et al., 2019), consistent with the observed ratios of archaea to bacteria (Fig. 1D).

**Fungi.** Fungal abundance was more responsive to cultivation of forage grasses, particularly palisade grass, than to N fertilization, which somehow boosted the microorganisms in the soil by low amount of sulfate from N source. N fertilization reduced the fungi:bacteria ratio (Fig. 1E), suggesting that N fertilization favors the bacterial over the fungal community and plant biomass production did not stimulate the increase of fungi:bacteria ratio as affirmed in our hypothesis. These changes can be attributed to increased substrate heterogeneity and immobilization of available N (Bardgett and Shine, 1999) and the N uptake by plants is facilitated by the combination and assimilation of sulfur (Bona and Monteiro, 2010; Li et al., 2019a, 2019b). Changes in the relationship between fungi and bacteria can be considered an indicator for production systems: increases in the fungi:bacteria ratio suggest that SOM decomposition and consequent mineralization of N dominate the supply of nutrients to crops, indicating low nutrient losses to the environment (Leite et al., 2017). The higher ratio of archaea to fungi in the Control treatment compared with the treatments with forage grasses may be related to higher occupation of niches by archaea in environments with low resource availability (Korzhnikov et al., 2019). Additionally, the cultivation of forages under no-till has a potential soil legacy effect on increasing nitrifying communities and the competition between  $K^+$  and  $NH_4^+$  for cation exchange sites through slight pH difference (0.5) compared to Control (fallow). Nitrification rates are relatively low in acidic soils such as autotrophic microbial group (AOA) by adaptation of low substrate concentration ( $NH_3$ ), selecting soil specific microorganisms involved in nitrification process and consequently affecting the ecosystem services; while an increased availability of high demand cations ( $NH_4^+$  and  $K^+$ ) in acidic soil generates a soil competitive cation exchange for sorption sites, resulting in  $NH_4^+$  and  $K^+$  release from

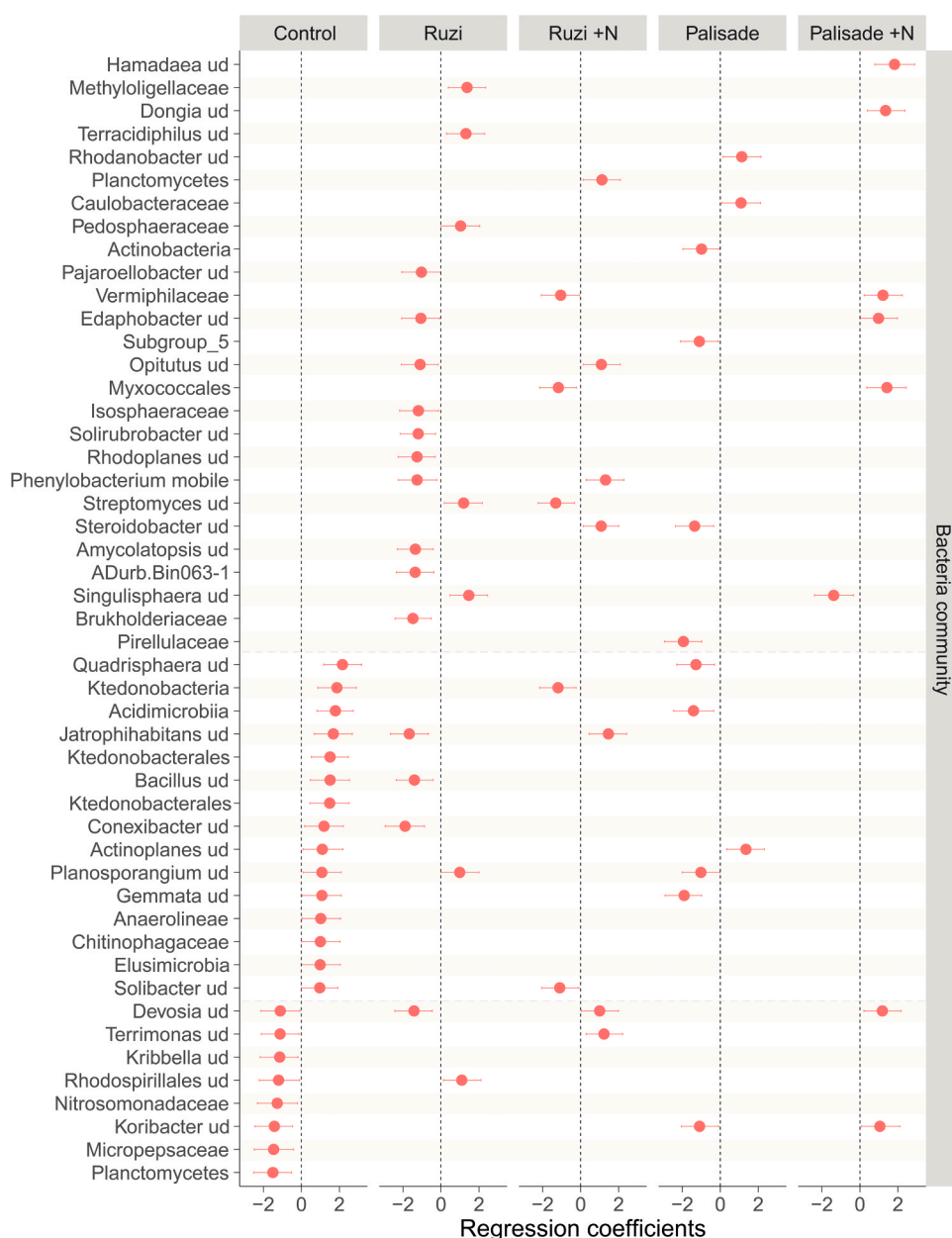


**Fig. 3.** Heatmap of uncultivated soil (Control) and soil cultivated with ruzigrass or palisade grass without N fertilization (Ruzi; Palisade) or with N fertilization (Ruzi +N; Palisade +N). Roman numbers in dashed-line rectangles and ordinal numbers in continuous-line rectangles denote groups and subgroups, respectively, with similar environmental factors (soil and plant) and bacterial and fungal species. Environmental factors are indicated in red font. Blue blocks represent positive correlations, while red blocks represent negative correlations. White blocks are non-significant relationships. Ud = unidentified.



**Fig. 4.** Principal component analysis (PCA) based on plant and soil factors (A), bacterial 16S rRNA gene (B) and fungal ITS (C) in uncultivated soil (Control) and soil cultivated with ruzigrass or palisade grass without N fertilization (Ruzi; Palisade) or with N fertilization (Ruzi +N; Palisade +N). The arrows indicate the ten main correlations among plant and soil factors and microbial profiles. Ud means unidentified.





**Fig. 5.** Regression coefficients showing differential abundances in the soil bacterial community among uncultivated soil (Control) and soil cultivated with ruzigrass or palisade grass without N fertilization (Ruzi; Palisade) or under N fertilization (Ruzi +N; Palisade +N). The black dashed lines correspond to a regression coefficient of 0. The grey dashed line separates bacterial genera with neutral regression coefficients for the Control (above) from those with positive or negative regression coefficients (below). Values less than zero indicate a decrease in the bacterial species due to the treatment, and values greater than zero indicate an increase in the bacterial species; 95% confidence intervals are shown. Ud means unidentified.

soil and availability to plant.

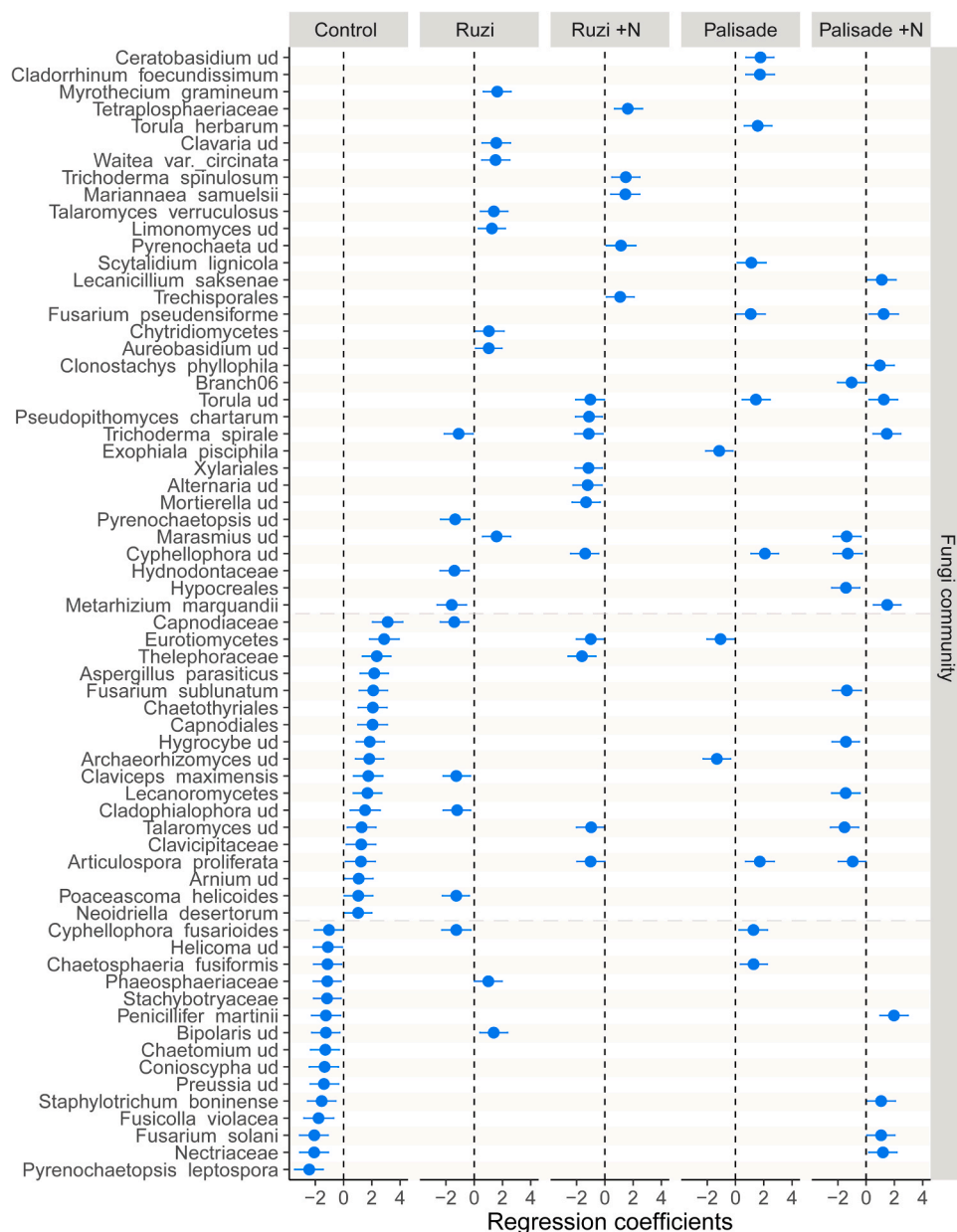
#### 4.2. Effects of forage cultivation under N fertilization on N-cycle genes

The abundances of N-cycle genes relative to the archaeal and bacterial populations depended on the forage grass species and N fertilizer application. The high copy number of *nifH* confirmed that only in soil cultivated with ruzigrass this species provided adequate conditions to increase N fixation in the absence of N input. The low soil mineral N level coupled with the absence of fertilizer application forced ruzigrass to acquire the necessary N for growth from other sources, such as N fixers. Previous studies of N fixers have reported variations in *nifH* abundance and responses of the microbial community to changes in the local environment depending on forage grass species and soil N (Gupta et al., 2019; Rocha et al., 2020), but there are no reports on estimated amount of  $N_2$  fixed in soil cultivated with species of forages and relevant questions on the increase in the association of ruzigrass roots with N-fixing bacteria deserve further investigations. In this study, ruzigrass clearly responded quickly to N fertilization, suggesting a high turnover

time and a depressed *nifH* in the soil; while the high total-N content, sulfur and biomass in soil cultivated with palisade grass minimized the populations of N fixers, reducing the efficiency of utilization of N resources (Reis et al., 2001).

Contrary to our hypothesis, forage grasses increased soil populations of AOA and AOB, which are responsible for nitrification, but different effects of palisade grass and ruzigrass were observed. Palisade grass enhanced the abundance of AOB regardless of N fertilization. In the study system, palisade grass and ruzigrass were cultivated during the fall and winter and cut 35 days before sample collection, leaving the biomass on the soil. The resulting increases in C and N sources may favor higher populations of AOB, since senescence of the leaves of mowed forage and decomposition of their residues in the soil stimulate microbial processes of mineralization and provide  $NH_4^+$  to the soil.

The relative abundance of AOA genes (AOA:16S rRNA archaea ratio) was higher when forage grasses were grown without N fertilization compared with N fertilization (Fig. 1). Most archaea are oligotrophic and capable of surviving/adapting in different types of environments (Chroňáková et al., 2015). The relatively large archaeal population in



**Fig. 6.** Regression coefficients showing differential abundances in the soil fungal community among uncultivated soil (Control) and soil cultivated with ruzigrass or palisade grass without N fertilization (Ruzi; Palisade) or with N fertilization (Ruzi +N; Palisade +N). The black dashed lines correspond to a regression coefficient of 0. The grey dashed line separates fungal genera with neutral regression coefficients for the control (above) from those with positive or negative regression coefficients (below). Values less than zero indicate a decrease in the fungal species due to the treatment, and values greater than zero indicate an increase in the fungal species; 95% confidence intervals are shown. Ud means unidentified.

soil without N input (low N source) was associated with an increased contribution of AOA microorganisms to nitrification (Momesso et al., 2022). The soil chemical and microbial compositions of the treatments with forage grasses clearly differed from those of the Control, and these differences were mainly attributable to soil  $S-SO_4^{2-}$ ,  $Mg^{2+}$ , total-N,  $N-NO_3^-$  and  $N-NH_4^+$  levels and plant biomass (Fig. 2). In addition, palisade grass (regardless of N fertilization) was associated with the highest relative abundances of AOA and AOB (Fig. 1H and I), as further supported by RDA and correlation analysis (Fig. 2). The treatment in which N was applied to ruzigrass resembled the palisade grass treatment without N fertilization. Fertile soils provide nutrients that function as cofactors of numerous proteins and enzymes related to the N cycle (Godfrey and Glass, 2011). In general, the relative abundances of AOA and AOB increased in the forage grass treatments, compared with the Control. This difference is probably attributable to the low availability of carbon sources (low total-C) in the Control. The roots of forage grasses exude large amounts of C (Fisher et al., 1995), which, in addition to decomposition of forage grass residue, serves as a source for increasing the populations of archaea and bacteria (Flemming and Wuerzt, 2019),

including those related to soil nitrification.

The relative abundances of denitrifier genes (*nirS*, *nirK* and *nosZ*) were lower in palisade grass and both forages with N addition than in the Control. Although *nirS* and *nirK* participate in the same process ( $NO_2^- \rightarrow NO$ ), the relative abundance of *nirK* was higher than that of *nirS*. This difference indicates that the absence of forage grasses increases genes related to denitrification, as supported by the lower relative abundance of *nosZ* in the forage grass treatments. Ligi et al. (2014) reported that the abundances of *nirK* and *nosZ* genes are strongly influenced by  $NH_4^+$  and  $NO_3^-$ , the same environmental parameters observed in our findings. Notably,  $NO_3^-$  and  $NH_4^+$  levels were lower in the Control treatment than in the forage grass treatments; forage grasses compete strongly with soil microorganisms for soil N, thus reducing substrates for denitrification (Bossolani et al., 2020). Additionally, the abundance of these genes based on DNA can be from active as well as dormant or dead soil microbes present in the soil. Forage grass cultivation is an important management for increasing C and N content by aboveground input to the soil, retaining N in soil microbial biomass (N immobilization) and exuding residues and substances from roots as a potential role in N cycle

control (Rosolem et al., 2017; Vázquez et al., 2020). Consequently, soil N (N available + SOM mineralization) is less prone to be lost as greenhouse gas to the atmosphere and  $\text{NO}_3^-$  by leaching into groundwater (Subbarao et al., 2017). Additionally, our findings point out that ruzigrass is inefficient in harnessing N by plant uptake and cycling N by stimulating microbiome involved in nitrification process. Moreover, forage grasses took up inorganic N from soil, causing high N levels in leaves and thus increases in biomass of forage grasses, especially in palisade grass.

#### 4.3. Differential responses of the soil microbiome to cultivation with forage grasses

This study provides a novel perspective on opportunities to promote soil bacterial and fungal communities that improve the sustainability of food production based on the selection of an appropriate forage grass under N fertilization in tropical no-till systems. Microbial characterization is important for assessing the effects of long-term forage cultivation under no-till on the soil microbiome and the interactions among microorganisms, soil properties and plant characteristics in tropical soil. Intensive plant cultivation and nutrient management practices alter soil properties and microbial communities (Chen et al., 2019; Kim et al., 2020; Pan et al., 2014; Suleiman et al., 2019) and, in turn, chemical cycles and reactions in the soil ecosystem, especially nutrient cycling. The results of the current study clearly show that forage grass cultivation as part of intensive agriculture impacts the soil environment and microbiome compared with uncultivated soil. Cropping practices have been shown to influence soil microbes (Edwards et al., 2019; Hartman et al., 2018), and plants partially drive these changes in the soil microbiota and thus can be a tool for developing microbiota management strategies for agriculture.

Our results revealed that forage grass cultivation in winter is advantageous in tropical fields, which are commonly left fallow and unplanted during winter. In addition to increasing the abundances of soil microbial communities, grasses enhance organic matter, total-C and -N,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , P,  $\text{Mg}^{2+}$ , and  $\text{S-SO}_4^-$  content. Grasses and their residues from mowing improve the soil environment, including soil aggregation, via root growth and provide resources to the soil biota through nutrient cycling (Bani et al., 2018; Brunetto et al., 2011). Plant-soil interactions modulate microbial activity and community composition involved in N cycle and enhance soil N available (from litter mineralization and SOM) to plants uptake (Kuzakov, 2010; Meier et al., 2017; Rosolem et al., 2017). Although only bulk soil was analyzed in the present study, the root architecture of forage grasses of the genus *Urochloa* permits exploration of the entire soil area (Galdos et al., 2020), and it is possible that plant root growth was responsible for some of the effects observed in the analyzed soil.

Similar effect of N fertilization on soil microbiome composition under forage species cultivation was somewhat unexpected (Fig. 3) since N fertilization decreases bacterial diversity in grassland soils (Li et al., 2019a, 2019b; Soares et al., 2016) and negatively impacts the soil activities of microbial communities (Chen et al., 2019). The properties of soil cultivated with forage were similar within the same species with or without N fertilization. Some studies have reported that microbial community composition is unresponsive to N addition in crop soils or that fertilization weakens plant-microbe networks (Huang et al., 2019; Roberts et al., 2011). The high N uptake by palisade grass and ruzigrass (Supplementary Fig. 2) indicates that these plants have an advantage over microorganisms in competition for N fertilizer and can increase the N use efficiency of fertilizer in agricultural systems (Heijboer et al., 2016; Momesso et al., 2020). In addition, nutrient use efficiencies are improved with forage cultivation of *Urochloa* genus by the AMF in environments with C supply from roots in exchange for mineral nutrients such as P and N ( $\text{NH}_4^+$ ), as well as AMF presence has the potential to decrease the denitrifying bacteria (Teutscherova et al., 2019b, 2019a; Veresoglou et al., 2012).

While a negative response of soil  $\text{S-SO}_4^{2-}$  content to ruzigrass cultivation was observed without N fertilization (Fig. 3 and Supplementary Fig. 3 and 4), the application of N fertilizer on ruzigrass resulted in a positive response of soil  $\text{S-SO}_4^{2-}$  content. These data suggest that low soil  $\text{S-SO}_4^{2-}$  content disfavors N uptake by ruzigrass cultivated in N-poor soil since the synergism of N and S maximizes nutrient uptake and use efficiency (Marschner, 2012). Ruzigrass produces less above- and belowground biomass than palisade grass. Probably the low SOM contributes to reduced  $\text{S-SO}_4^{2-}$ , consequently, reduction of sulfur plant uptake (Anderson, 1975; Ma et al., 2020). N losses and reduced crop yields have been observed after ruzigrass cultivation in agricultural systems, indicating negative effects of this forage on soil and food production (Momesso et al., 2020; Rocha et al., 2019). Excess N and S in the soil, i. e., nutrients not taken up by the plant, remain available in the soil for microorganisms. However, plant-microbe competition is weak since both ruzigrass and ruzigrass receiving N fertilizer were positively linked with soil  $\text{NO}_3^-$  and *Nitrosomonadaceae* abundance. Based on these results, we can conclude that N sources are available in soil cultivated with ruzigrass for *Nitrosomonadaceae*, which are involved in nitrification via autotrophic conversion of  $\text{NH}_4^+$  to  $\text{NO}_3^-$  (Fig. 4).

The soil microbial communities and soil environment clearly separated and explained the variations between the uncultivated control and the soils cultivated with grasses (Fig. 4), regardless of N fertilization. Studies of other plant species in tropical soils have also observed distinct features of cultivated soils (Goss-Souza et al., 2017; Mendes et al., 2015; Schlemper et al., 2018). During growth, plants recruit a microbiome from the larger soil microbiome (Mendes et al., 2014; Schlemper et al., 2018). Thus, the composition of the soil microbiome is the most influential factor determining the composition of the plant microbial community (Schlemper et al., 2018), and the response of the soil microbiome is dependent on the plant species (Schlemper et al., 2017). The experimental site in this study has been managed under no-till with crop rotation for 10 years, and the cultivation of grasses allows nutrients to be returned to the soil surface from deeper soil layers compared with a fallow plot. The crop residues left on the soil surface becomes resources for soil microbial communities.

In this study, palisade grass and ruzigrass cultivation significantly shifted the soil microbiota towards a microbial community composition favorable to agricultural production by favoring microbial taxa involved in the decomposition of soil organic matter, nutrient cycling and thus, improvement of soil properties compared to uncultivated tropical soil (Fig. 3). We evaluated changes in soil microorganisms associated with the N cycle or organic matter decomposition. Additionally, we investigated whether forage grass cultivation resulted in differences in bacteria and fungi in the soil community compared with fallow management (Figs. 5 and 6). The differences in soil properties and microbial communities between the control and the treatments cultivated with forage grasses were larger than any of the differences between the forage grass treatments themselves. Uncultivated soil is typically not subjected to management practices such as soil acidity correction, fertilizer addition, plant seeding, and crop rotation (Garcia-Torres et al., 2003). The acid nature of the soil in the current study may have contributed to the higher  $\text{Ca}^{2+}$  content and archaeal population in the uncultivated Control due to the lower availability of resources and lower pH, which disfavors plant nutrient uptake. Unclassified *Fusarium*, which play diverse roles in organic matter and cellulose decomposition and steroid and lignin metabolism and as plant pathogens and endophytes (Kavroulakis et al., 2018; Skiada et al., 2019; Went and de Jong, 1966), were more abundant in uncultivated soil. By contrast, *Preussia*, endophytic and saprobic fungi that decompose dead organic matter in the soil and are essential for continuous ecosystem functioning (Gonzalez-Menendez et al., 2017; Mapperson et al., 2014), was less abundant in uncultivated soil, as was *Elsterales*, a genus positively related to N, especially  $\text{NH}_4^+$  and  $\text{NO}_3^-$ , consistent with a previous report by Wang et al. (2021). These results clearly indicate low activity of SOM decomposers and consequently low nutrient cycling. The soil microbiome under ruzigrass cultivation was



explained by the presence of *Clavaria*, a genus of saprotrophs that play essential roles in cycling soil N in grasslands (Marí et al., 2020), and *Myrothecium gramineum*, which degrades leaf litter and lignin and contributes to N<sub>2</sub>O emissions (Pandey et al., 2020). The soil microbiome of palisade grass was composed mainly of *Acidobacteriaceae*, which can enhance soil aggregation and maintain soil moisture (Costa et al., 2018); *Pyrenochaetopsis leptos*, which contributes to CO<sub>2</sub> emissions (Bai et al., 2019; Yu et al., 2018); and *Chaetosphaeria fusiformes*, an endophytic fungus that modifies HBOA chemical compound in biotransformation and decomposes lignin-containing substrate (Shankar Naik, 2019). This microbiome composition reflects the characteristics of the environment under palisade grass cultivation, which include acid soil, high moisture and high soil coverage by biomass.

## 5. Conclusions

Based on the results of the present study, we conclude that cultivation of forage grasses combined with N fertilization is a better soil management option than leaving areas fallow or abandoned. The benefits of ruzigrass and palisade grass cultivation were assessed by evaluating N-cycle-related genes, bacterial and fungal populations, and the soil microbiome, with a focus on close plant-soil-microbe interactions. Palisade grass cultivation was associated with greater fungal decomposer abundance in the soil, regardless of N fertilization. Palisade grass promoted soil nitrification and decreased denitrification while strongly competing with microbes for N uptake. In addition, this forage species promoted an increase in the abundance of microbes involved in the N cycle, organic matter decomposition, and soil nutrient levels to improve plant-soil-microorganism balance. Our results based on single point observations suggest that appropriate forage species selection is a management strategy for improving the chemical and biological properties of soil. Long-term impact of forage grass genotypes and N fertilization on soil microbial processes and bacterial and fungal communities deserve further investigations over time, focusing on mechanisms of N cycling by grasses and N fertilization to specific microbial groups. In this study, palisade grass cultivation shaped the microbiota beneficially independently of N input. Although N fixers increased in soil cultivated with ruzigrass, the resulting microbial community composition and N-cycle processes might contribute to N losses such as NO<sub>3</sub><sup>-</sup> leaching and N<sub>2</sub>O emissions. Future studies investigating the effects of N fertilizer forms applied on tropical agriculture with forage grasses cultivation are needed to determine the impacts on soil microbial N processes and microbial communities as well as N losses.

## CRedit authorship contribution statement

L.M., C.A.C.C. and E.E.K. designed the research; L.M. and J.B. conducted the experiments; L.M. and J.B. conducted the soil chemical analysis; L.M. conducted the qPCR analyses; L.M. and M.F.A.L. performed the bioinformatics and statistical analyses; L.M. wrote the paper. All authors reviewed the manuscript.

## 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.

## Availability of data

The raw sequences of 16S rRNA gene and ITS were submitted to the European Nucleotide Archive (ENA) under study accession number PRJEB41425.

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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.2021.107688.

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