Unraveling the soil microbiome in *Brachiaria* pastures: exploring varietal influences and nitrogen fertilizers

Luis Fernando Merloti

Thesis presented to obtain the degree of Doctor in Science. Area: Agricultural Microbiology

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Unraveling the soil microbiome in *Brachiaria* pastures: exploring varietal influences and nitrogen fertilizers

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Dedication

I dedicate this thesis to the remarkable women in my life.

To Aparecida Cardoso de Souza, my beloved mother, who with nothing more than a bucket and a broom, raised a doctor.

To Anita Cardoso Tonioli de Souza, my dear aunt, who has always ensured my well-being.

And to my grandmother, Maria Lourdes dos Santos (in memoriam), who, despite never having finished her studies, was one of the wisest individuals I’ve ever known.

This thesis is dedicated to all of you.
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Epigraph

Com a cabeça erguida e mantendo a fé em Deus
O seu dia mais feliz vai ser o mesmo que o meu
A vida me ensinou a nunca desistir
Nem ganhar, nem perder mas procurar evoluir
Podem me tirar tudo que tenho

Só não podem me tirar as coisas boas que eu já fiz
pra quem eu amo
E eu sou feliz e canto e o universo é uma canção e
eu vou que vou

História, nossas histórias
Dias de luta, dias de glória

Charlie Brown Jr.
## SUMMARY

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RESUMO

Desvendando o microbioma do solo em pastagens com *Brachiaria*: explorando influências varietais e fertilizante nitrogenados

A degradação das pastagens representa um desafio crítico para o Brasil, país de destaque na produção mundial de carne bovina. Esta degradação, causada por práticas como o pastoreio intensivo e o manejo inadequado do solo, resulta na diminuição da produtividade agropecuária e em impactos ambientais negativos, tais como a degradação do solo, desmatamento e o aumento das emissões de carbono. Manejos agropecuários eficazes são fundamentais para enfrentar esses desafios, promovendo a resiliência e a produtividade das pastagens enquanto se minimiza o impacto ecológico. Esta tese avaliou o efeito de variedades de *Brachiaria* geneticamente aprimoradas e de diferentes fertilizantes nitrogenados no microbioma e nas características físico-químicas do solo, seguido de seus efeitos no desenvolvimento de plantas e potenciais consequências para o meio ambiente. O Capítulo 1 examinou os efeitos de quatro variedades de *Brachiaria* nas comunidades microbianas do solo e ciclagem de nutrientes, utilizando uma combinação de técnicas moleculares e análises físico-químicas do solo. O estudo revelou que essas variedades melhoram significativamente a qualidade do solo ao aumentar a porosidade, o conteúdo de carbono orgânico e a disponibilidade de fósforo, a passo que também moldaram as comunidades procarióticas e fúngicas do solo, especialmente aquelas envolvidas no ciclo de N. Além disso, as variedades de *Brachiaria* recrutaram comunidades microbianas benéficas para a liberação de nutrientes, resistência a patógenos e outros estresses, com a variedade Ipyporã obtendo um destaque para melhorar a qualidade do solo, e surgir como uma alternativa promissora para sistemas de cultivo intercalar. O Capítulo 2 explorou a reabilitação de pastagens brasileiras degradadas através da fertilização com N, destacando seu potencial para modificar o microbioma do solo e melhorar o crescimento das plantas. Por meio da aplicação de diferentes fontes de N, incluindo sulfato de amônio, ureia e torta de mamona, o estudo avaliou seu impacto no microbioma do solo, nitrificação e biomassa de plantas. Para isso, utilizou-se uma combinação de técnicas moleculares e análises físico-químicas do solo. Os resultados indicaram que, o sulfato de amônio, fertilizante mais eficaz em liberar N ao sistema, promoveu o crescimento de procariontes em detrimento de fungos, favorecendo funções relacionadas ao ciclo N. Entretanto, a ureia e a torta de mamona favoreceram o crescimento fúngico, especialmente aqueles ligados a doenças de plantas e reduziram funções relacionadas ao ciclo do N. O sulfato de amônio afetou significativamente a nitrificação, provavelmente devido seu impacto nas bactérias oxidantes de amónia. Todos os fertilizantes promoveram o crescimento radicular de plantas. De maneira geral, esta tese destacou a interação complexa entre a seleção genética de variedades forrageiras, práticas de fertilização e comunidades microbianas do solo em ecossistemas de pastagens tropicais. Foi ressaltada a importância da seleção de variedades de *Brachiaria* e a adequada fonte do fertilizante nitrogenado para melhorar as características físicas e químicas do solo, apoiar a produção agropecuária sustentável, e mitigar os impactos ambientais da degradação das pastagens brasileiras.

Palavras-chave: Comunidades procarióticas, Comunidades fúngicas, Potencial funcional, Sequenciamento de DNA, Ciclo do nitrogênio, Pastagens brasileiras, Fertilizantes nitrogenados, Gene 16S rRNA, Gene ITS, Oxidantes de amónia
ABSTRACT

Unraveling the soil microbiome in *Brachiaria* pastures: exploring varietal influences and nitrogen fertilizers

Degradation of pasturelands is a critical issue confronting Brazil, a country with a prominent role in global beef production. Such degradation, resulting from practices such as overgrazing and inefficient soil management, leads to reduced land productivity and adverse environmental impacts, including soil degradation, deforestation, and increased carbon emissions. Efficient management strategies are essential to address these challenges, enhancing pasture resilience and productivity while minimizing ecological footprints. This thesis assessed the effect of *Brachiaria* varieties and different N fertilizers on the soil microbiome and physicochemical characteristics, followed by their effects on plant development and potential environmental consequences. Chapter 1 examined the effects of four *Brachiaria* varieties on soil microbial communities and nutrient cycling, utilizing a combination of molecular techniques and traditional soil analyses. The study revealed that *Brachiaria* varieties significantly enhance soil quality by improving porosity, organic carbon content, and phosphorus availability, while also influencing the soil's prokaryotic and fungal communities, especially those involved in the N cycle. Notably, the *Brachiaria* varieties were found to selectively enhance microbial communities beneficial for increase the availability of soil nutrients, pathogen resistance, and stress resilience, with the Ipyporã variety showing a distinct potential for improving soil quality and supporting intercropping systems. Chapter 2 explored into the rehabilitation of degraded Brazilian pastures through N fertilization, highlighting its potential to modify the soil microbiome and improve plant growth. Through the application of different N sources, including ammonium sulfate, urea, and castor meal, the study assessed their impact on the soil microbiome, nitrification processes, and *Brachiaria* plant growth. Findings indicated that while all N sources increase soil N availability and alter soil chemistry, ammonium sulfate is particularly effective in promoting prokaryotic over fungal growth, thus maintaining N related functions. Conversely, urea and castor meal appear to favor fungal communities associated with plant diseases, suggesting that the choice of N fertilizer profoundly affects soil microbial balance, nitrification efficiency, and plant root development. Overall, this thesis underscores the complex interplay between genetic selection of forage varieties, fertilization practices, and soil microbial communities in tropical pasture ecosystems. It highlights the importance of selecting appropriate *Brachiaria* varieties and N fertilizers to enhance soil health, support sustainable livestock production, and mitigate the environmental impacts of pasture degradation.

Keywords: Prokaryotic communities, Fungal communities, Potential functions, High throughput DNA sequencing, Nitrogen cycle, Brazilian grasslands, Nitrogen fertilizers, 16S rRNA gene, ITS gene, Ammonia-oxidizers
1. GENERAL INTRODUCTION

1.1. Brazil's pastures: importance and challenges

Brazil is characterized as the second main beef producer on the global stage, accounting for 9.75 million tons in 2021, behind the United States of America (USA) with 12.73 million tons (Food and Agriculture Organization of the United Nations; 2023). The amount produced in 2021 was equivalent to 12% of the global production but most of it was exported to China, the USA and the European Union, respectively (ABIEC, 2023). The raising of cattle for meat production in Brazil is based almost exclusively on the use of pastures. It is estimated that about 177 million hectares of Brazilian territory are covered with pastures or 21% of the national total territory (UFG, 2022). However, the cultivation of pastures in Brazil is predominantly extensive and without significant investments and maintenance. Overgrazing, inadequate weed control and lack of fertilization were pointed out as the main factors responsible for the Brazilian pasture degradation (Feltran-Barbieri and Féres 2021). Due to that, approximately 62% of Brazil's total pasturelands are affected by some degree of degradation. Within this, it is estimated that 41% demonstrate medium vegetation vigor, indicative of an intermediate level of degradation, whereas 21% display low vigor, pointing to a severe level of degradation (UFG, 2022).

The lack of adequate vegetation cover on degraded pasturelands carries significant environmental consequences, including soil loss via runoff and erosion (Gonçalves et al. 2023). Furthermore, grassland degradation aids in the contamination of adjacent water bodies as particles are transported by leaching (Merten and Minella 2013). In the context of climate change, degraded pastures increase soil organic matter degradation, transforming soils from potential carbon sinks into sources of atmospheric CO$_2$ (Bieluczyk et al. 2023). Additionally, the reduced capacity of these areas to support livestock and the deterioration of soil nutrients, have escalated the demand for new agricultural lands, which often results in deforestation (Barona et al. 2010; Feltran-Barbieri and Féres 2021). To address these problems, ranchers face substantial challenges in rehabilitating these degraded pastures due to financial constraints and the absence of governance support for the adoption of innovative and sustainable technologies (Feltran-Barbieri and Féres 2021; Bragança et al. 2022).

Enhancing pasture areas with more sustainable and efficient land practices offers a viable solution to stimulate economic growth and reduce deforestation. It is estimated that, from 1975 to 2006, improvements in pasture productivity have prevented the deforestation of 147.5 million hectares in key biomes such as the Cerrado and Amazon (Valentim and de Andrade 2009). Furthermore, Dias-Filho (2014) suggested that for every hectare of pasture restored, an additional three hectares could be allocated for non-agricultural purposes such as reforestation and conservation, without adversely affecting national livestock production levels. More recently, Feltran-Barbieri and Féres (2021) highlighted that a more efficient use of Brazilian pastures for beef production could meet the national Forest Code requirements and increase the cattle area by 9 million hectares. Additionally, the authors conclude that revitalizing these pastures is a beneficial strategy that could enhance livestock farming and reduce deforestation, and should be a key priority for the Brazilian agribusiness sector.
1.2. Revitalizing pastures: *Brachiaria* varieties and nitrogen fertilization

The *Brachiaria* genus (Syn. *Urochloa*) is composed of grass species with their natural occurrence, or gene centre, in the African continent (Boonman 1993). The plant species developed aggressive root systems and high biomass production, thriving in tropical climates. Also, most *Brachiaria* have good seed germination, rapid soil coverage and good nutritional quality to sustain cattle production (Lapointe and Miles 1992). For these reasons, *Brachiaria* also holds significant importance in the agricultural landscapes of South America, particularly in Brazil, where it underpins the vast majority of livestock production systems (Pedreira et al. 2015).

The genus was brought to America during the 18th and 19th centuries and used as bedding on ships, but later on, due to its tropical adaptation and productivity, was introduced to improve cultivated grasslands (Lapointe and Miles 1992). For the past 45 years, *Brachiaria* has been the focus of extensive breeding programs aimed at enhancing its resilience to environmental stressors like drought, pests, and nutrient-deficient soils (Pizarro et al. 1996). These efforts have yielded numerous varieties with improved stress tolerance, higher nutritional values, and better digestibility for cattle, thus bolstering their appeal for livestock farming (Euclides et al. 2016). Thus, grasslands in Brazil have transitioned from being solely a subsistence crop with low use of technology and investment by farmers to being managed with more advanced and conservation-oriented agricultural practices (Dias-Filho, 2016; Baptistella et al. 2020).

Recently, *Brachiaria* has also begun to be utilized in sustainable agriculture practices, such as cover cropping, intercropping, and crop rotations, by improving soil physical properties and fertility (Oliveira et al. 2019, 2020; Galdos et al. 2020). Its biomass production can contribute to a positive legacy on soil moisture and organic matter accumulation for subsequent crops (Canisares et al. 2021). Moreover, *Brachiaria* species can employ various mechanisms to optimize N uptake and accumulation in the soil solution, such as biological nitrification inhibition (BNI) or by enhancing fine roots and soil porosity to reduce N leaching (Subbarao et al. 2009; Galdos et al. 2020). However, the results varied depending on the *Brachiaria* variety chosen in combination with different or subsequent crops (Oliveira et al. 2019; Canisares et al. 2021). When integrated with crop-livestock (ICL) and crop-livestock-forestry systems (ICLF), *Brachiaria* can optimize gains in nutrient cycling and enhance savings in fertilizer inputs, being regarded as a fundamental approach to enhance sustainable agriculture (Vilela et al. 2021).

To optimize the *Brachiaria* development and its recovery after grazing, N fertilization emerged as vital management. Over the years, cattle grazing extracts a great part of the N present in the grass. Part of the N ingested by the animal is exported off-site when animals are moved, or products such as milk are taken from the site (Dubeux and Sollenberger 2020). However, the majority of N consumed is lost from animal urine that is deposited in rest areas where there is no vegetation, increasing the N losses through denitrification and leaching (Boddey et al. 2004). It is estimated that N losses from animal urine in pasture lands could represent 35 -80% of the total N (Boddey et al. 2004). Thus, N supplementation became essential to maintain and recover degraded pastures, and increase the number of animals per area, optimizing the land use.

The selection of the optimal N source for fertilizing and restoring degraded pastures remains a complex challenge in agriculture. Various options, including organic and mineral sources, have different responses, depending on factors like soil conditions, climate, and production goals (Zhong et al. 2010; Francioli et al. 2016; Allam et al. 2022). Additionally, the interaction between the chosen N source and soil microorganisms influences the effectiveness of this agronomic practice (Zhong et al. 2010). Most N fertilizers improve grasslands through enhanced biomass production and by increasing nutritional characteristics (Heinrichs et al. 2013; Teixeira et al. 2020).
However, the efficiency of these fertilizers can be compromised by losses through nitrification, denitrification, and leaching, depending on the amount applied and interactions with the soil microbiome, with potential consequences for water bodies and greenhouse gas emissions (Canisares et al. 2021). Therefore, the decision on which N source to use continues to be a critical consideration for producers aiming to enhance pasture productivity and sustainability.

1.3. Exploring the soil microbiome in pasture ecosystems

The microorganisms living in the soil, also known as the soil microbiome, are essential for the maintenance of ecosystem processes. They contribute to the recycling of soil organic matter, assist in forming soil aggregates (Wilpiszeski et al. 2019), and regulate biogeochemical cycles such as C and N (Smith et al. 2015). Furthermore, the soil microbiome contributed to plant production by enhancing the availability of nutrients in the soil solution and facilitating their uptake by plants (Crecchio et al. 2018). They can also provide plant resistance to abiotic stress (Rolfe et al. 2019) and suppress pathogens (Mendes et al. 2018), underlining their critical role in both plant and soil health.

The advancement of next-generation sequencing technologies (NGS) combined with microbial databases has significantly enhanced our comprehension of the soil microbiome, often referred to as the ”soil black box” (Mishra et al. 2023). Utilizing methods that analyze the total DNA of soil, enabled to access the microbial composition and its diversity in different ecosystems (Zhu et al. 2023). Despite the current limitation in the number of species cataloged in microbial databases, DNA-based sequencing techniques have been fundamental in illustrating how soil diversity contributes to biosphere functions (Mishra et al. 2023). Moreover, genomic studies have extensively identified and characterized a vast array of genes associated with microbial metabolism, biogeochemical cycles, antibiotic resistance, among other critical functions (Quince et al. 2017). Through the identification of these genes, researchers are now able to predict the soil’s capacity for nutrient cycling, which is vital for supporting plant growth (Salam and Obayori 2019). Consequently, these advancements not only facilitate the identification of microbial communities but also their classification based on functional traits, representing a notable advancement in our comprehension of soil ecology (Nguyen et al. 2016; Louca et al. 2016).

More specifically in agriculture, the study of the microbiome allows us to understand the implications of different soil uses and agricultural management practices on these communities, with consequences for crop productivity and the environment (Salam and Obayori 2019). For instance, exploring soil community diversity has led to the identification of disease-suppressive soils, highlighting the importance of the entire microbial community in crop development (Schlatter et al. 2017). Additionally, research into soil communities aids in identifying the operational mechanisms and benefits of sustainable management practices like organic amendments, reduced tillage, cover cropping, and crop rotations (Bossolani et al. 2021; Cerecetto et al. 2021). These practices positively affect both plant communities and soil microorganisms, enhancing the overall health and productivity of agricultural systems.

Furthermore, soil microorganisms are closely linked to soil characteristics, depending on them for essential survival resources. Soil microbiomes are influenced by various soil properties, including pH and Al concentration, which can be toxic to most soil taxa (Lammel et al. 2018). Porosity plays a critical role as well, determining the level of oxygenation and thus influencing the balance between aerobic and anaerobic microbial communities (Wilpiszeski et al. 2019). Additionally, plant roots exude carbon-based substances that not only provide nutrients for soil microbes but also have a role in recruiting specialized microbes that assist in plant nutrition,
resistance to stress and diseases (Philippot et al. 2013). The C/N ratio is another soil component affecting microbial activity, with C serving as an energy source and N as essential for microbial structural components. This ratio has significant implications for the decomposition of organic matter and nutrient cycling within the soil ecosystem (Hoffland et al. 2020).

Interactions among soil microbes are critical for elucidating the assembly of microbial communities within soil ecosystems. Soil microorganisms, through a range of interactions from mutualistic to predatory, contribute to the formation of a complex interaction network, termed the soil food web (Morriën 2016). This network plays a key role in ecosystem processes by facilitating nutrient cycling and energy flow between aboveground and belowground systems (Morriën 2016). Bacterial and fungal taxa within these communities are pivotal in recycling organic matter and driving biogeochemical cycles, essential for ecosystem functioning (Cui et al. 2018; Wang et al. 2022). Specifically, bacteria are known to metabolize simpler substrates, whereas fungi possess the capability to decompose more complex, recalcitrant materials (Koranda et al. 2014; Cui et al. 2018). This functional differentiation underscores the complexity of soil ecosystems and highlights the significance of microbial interactions in maintaining soil health and productivity.

The relationship between Brachiaria species and the soil microbiome has been the subject of research in recent years, primarily focusing on optimizing soil nutrient acquisition by these plants. Brachiaria varieties have been shown to possess the capacity to inhibit soil-nitrifying microorganisms (also known as Biological Nitrification Inhibition - BNI) (Subbarao et al. 2009). This attribute may provide valuable opportunities for managing soil N emissions, thereby contributing to climate change mitigation efforts. The inhibition of soil nitrifiers affords Brachiaria species a competitive advantage in the contest for N available in the soil, potentially reducing N losses through the denitrification process (Subbarao et al. 2009; Momesso et al. 2022). Furthermore, the association of Brachiaria varieties with arbuscular mycorrhizal fungi (AMF) and phosphorus-solubilizing bacteria (PSB) has been frequently documented, highlighting it as an effective strategy to enhance P availability in tropical soils (Merlin et al. 2016; Oliveira et al. 2019; Baptistella et al. 2020). This synergy underscores the significant role of Brachiaria species in sustainable agriculture, particularly within tropical agroecosystems.

1.4. Thesis structure

Taking into account all the aforementioned points, this thesis evaluates the effects of Brachiaria varieties (Chapter 1) and N fertilizers (Chapter 2), considered key tools in the recovery of degraded pastures, on soil microbial communities, soil characteristics, and their implications for plant development and the environment.

Study 1 - Investigating the Effects of Brachiaria (Syn. Urochloa) Varieties on Soil Properties and Microbiome

**Aim:** To investigate the impact of different Brachiaria varieties on prokaryotic (archaeal and bacterial) and fungal communities and their potential functions, focusing on the N-cycle.

**Hypothesis:** We hypothesize that the diverse Brachiaria varieties will induce distinct changes in soil chemical parameters, leading to significant impacts on the microbiome composition and microbial functional traits.

**Methodology:** To test this hypothesis comprehensively, we employed a combination of molecular techniques, such as quantitative PCR (qPCR) and amplicon sequencing, alongside conventional soil and plant chemical analyses.
Specific Questions Addressed:

(i) What are the impacts of different Brachiaria varieties on soil physical-chemical characteristics, and which specific factors are affected?
(ii) How does the soil microbiome vary among different Brachiaria varieties?
(iii) Which soil characteristics show associations with the observed variations in the soil microbiome caused by different Brachiaria varieties?
(iv) To what extent do Brachiaria varieties influence the microbial potential functions and marker genes associated with the N-cycle in the soil?
(v) Is there a particular Brachiaria variety that stands out in its ability to enhance soil properties and foster a beneficial microbiome for plant growth?

Study 2 - N Fertilizers in Brachiaria Grasslands: Effects on Soil Microbiome Dynamics and Nitrification

Aim: To explore how different N fertilizer sources affect the soil microbiome, especially those involved in the nitrification process, and their subsequent impact on plant growth in Brachiaria grasslands.

Hypothesis: We hypothesize that variations in N fertilizer sources will change soil nutrient availability, influencing the competition dynamics for resources among prokaryotic and fungal communities, as well as among ammonia-oxidizing organisms. These changes are anticipated to affect plant growth and development in Brachiaria grasslands.

Methodology: To test this hypothesis comprehensively, we employed a combination of molecular techniques, such as quantitative PCR (qPCR) and amplicon sequencing, alongside conventional soil and plant chemical analyses.

Specific Questions Addressed:

(i) How do different N fertilizers affect soil characteristics?
(ii) How do prokaryotic and fungal communities, and their potential functions, vary with different N fertilizers?
(iii) To what extent do N fertilizers affect nitrification and its microbial agents in the soil?
(iv) What are the implications for Brachiaria development?

Through addressing these questions, we aim to enhance the understanding of the complex interplay between N fertilizers and the soil microbiome, contributing to sustainable Brachiaria grassland management in Brazil.

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2. INVESTIGATING THE EFFECTS OF Brachiaria (SYN. Urochloa) VARIETIES ON SOIL PROPERTIES AND MICROBIOME


Resumo

Investigando os Efeitos das Variedades de Brachiaria (Sin. Urochloa) nas Propriedades do Solo e Microbioma

A Brachiaria spp. (sinônimo de Urochloa) é uma das principais espécies de gramíneas utilizadas na produção de gado no Brasil e tem sido foco de programas genéticos de melhoramento para aumentar sua resistência à seca, inundação e pragas, além de aprimorar sua palatabilidade para os animais. No entanto, há uma compreensão limitada de como o melhoramento genético pode afetar o microbioma do solo e suas funções potenciais. Assim, este estudo teve como objetivo investigar o impacto de quatro variedades diferentes de Brachiaria nas comunidades procarióticas e fúngicas do solo, com foco no ciclo do nitrogênio. Utilizamos técnicas moleculares, como PCR quantitativo e sequenciamento em larga escala para caracterizar as comunidades procarióticas e fúngicas, além de análises químicas tradicionais do solo e das plantas. Os tratamentos foram compostos pelas variedades de B. brizantha cv. Marandu (BM), B. ruziziensis (BR), Brachiaria spp. cv. Ipyporã (BI), B. brizantha cv. BRS Paiguá (BP) e controle sem plantas. Nossas descobertas revelaram que todas as variedades melhoraram a porosidade do solo, conteúdo de fósforo, carbono orgânico e funções potenciais de grupos microbianos como quimio-heterotróficos, aeróbios-quimio-heterotróficos e fitopotagênicos-saprófitos. A acidez do solo, a disponibilidade de nutrientes e a porosidade foram os principais impulsionadores das comunidades microbianas. O estudo também identificou a capacidade de cada variedade em recrutar fixadores de nitrogênio microbianos e oxidantes de amônia. Destacamos que as variedades de Brachiaria podem favorecer comunidades microbianas do solo relacionadas à liberação de nutrientes, resistência a patógenos e estresse ambiental. Além disso, a variedade BI mostrou um maior potencial para melhorar a qualidade do solo ao aumentar a porosidade do solo e os potenciais fungos arbusculares micorrízicos. Por fim, todas as variedades mostraram algum potencial para beneficiar sistemas de consorciação e rotação de culturas.

Palavras-chave: Comunidades procarióticas; Comunidades fúngicas; Funções potenciais; Sequenciamento em larga escala; Ciclo do nitrogênio; Pastagens brasileiras

Abstract

The Brachiaria sp. (synonymous with Urochloa) is one of Brazil’s main grass species used in livestock production and has become the focus of breeding genetic programs to enhance its resistance to drought, flooding, and pests, as well as improving its palatability to animals. However, there is a limited understanding of how genetic breeding can affect the soil microbiome and its potential functions. Thus, this study aimed to investigate the impact of four different Brachiaria varieties on the soil prokaryotic and fungal communities, with focus on the N-cycle. We combined molecular techniques, such as quantitative PCR and amplicon sequencing, to target prokaryotic and fungal communities and traditional soil and plant chemical analyses. The treatments were composed of the
varieties of B. brizantha cv. Marandu (BM), B. ruziziensis (BR), Brachiaria spp. cv. Ipyporã (BI), B. brizantha cv. BRS Paiaguás (BP) and control without plants. Our findings revealed that all varieties improved soil porosity, P content, organic carbon, and potential functions as Chemoheterotroph, Aerobic-Chemoheterotroph, and Pathotroph-Saprotroph groups. Soil acidity, nutrient availability, and porosity were the main drivers of the microbial communities. The study also identified the ability of each variety to recruit microbial nitrogen-fixers and ammonia-oxidizers. We highlighted that Brachiaria varieties can favor soil microbial communities related to the release of nutrients, resistance to pathogens, and environmental stress. Also, the BI variety showed a higher potential to improve soil quality by increasing soil porosity and potential AMFs. Besides that, all varieties showed some potential to benefit intercropping and crop rotation systems.

**Keywords:** Prokaryotic communities; Fungal communities; Potential functions; High throughput sequencing; N-cycle; Brazilian grasslands

### 2.1. Introduction

The *Brachiaria* sp. (synonymous with *Urochloa*) is one of the main grass species used in livestock production lands in South America (Pizarro et al. 1996) and Brazil (Dias-Filho, 2016). The C4 plant has aggressive root growth and significant biomass production and can grow in soils with low fertility (Dias-Filho, 2016). Those characteristics make this species suitable for tropical climates and farms with low technology and investment. However, the lack of grassland maintenance and investments also created a land degradation scenario, and nowadays, more than 60% of the Brazilian grasslands have some level of degradation (Project MapBiomas, 2022). In this sense, over the past 40-45 years, this grassland species started to be the target of breeding genetic programs to meet different goals (Pizarro et al. 1996). As a result, different varieties have emerged as resistant to various stressors, including drought, fire, leafhoppers, and low-fertility soils. Additionally, these varieties offer a high nutritional value and are easily digestible by the cattle, making them an attractive option for livestock production (Euclides et al. 2016). Thus, grasslands in Brazil have transitioned from being solely a subsistence crop with low use of technology and investment by farmers to being managed with more advanced and conservation-oriented agricultural practices (Dias-Filho, 2016; Baptistella et al. 2020).

*Brachiaria* has recently become a popular choice for intercropping and crop rotation systems due to the growing demand for sustainable agriculture and the implementation of conservation practices (Klutheouski et al. 2000). Using *Brachiaria* in these systems benefits soil characteristics such as soil organic matter, porosity, protection against erosion, and suppression of weeds (Almeida and Rosolem 2016; Eri et al. 2020; Bieluczyk et al. 2020; Silva et al. 2021). Together, those characteristics can improve crop yield production in consortium or rotation with *Brachiaria* (Brandan et al. 2017; Cruscio et al. 2021). However, despite the potential benefits of using *Brachiaria* in intercropping and crop rotation systems, little is known about how the genetic breeding of these plants impacts the selection of the soil microbiome.

Agricultural soils represent a significant source of non-CO$_2$ greenhouse gases (GHGs), contributing approximately 37% of all agricultural emissions, which accounts for 10-14% of the global GHG budget (Paustian et al., 2016). Consequently, agricultural soils can play a dual role in both contributing to and mitigating climate change impacts. To address this issue, implementing climate-smart agricultural land management practices becomes crucial in achieving climate targets. Notably, certain *Brachiaria* varieties have demonstrated the capacity to inhibit soil nitrifiers, a process known as biological nitrification inhibition, as identified by Subbarao et al. (2009). This characteristic may offer valuable opportunities for managing soil emissions and, therefore, contribute to climate change mitigation efforts. The inhibition gives a competitive advantage to this plant in the competition for the N available in the soil against microbes, which can also potentially decrease the N losses through the denitrification process (Subbarao et al. 2009; Momesso et al. 2022). Recent studies have shown that *Brachiaria* has a complex relationship with the soil microbiome, involving competitive
advantages in nutrient acquisition and mutualistic associations with arbuscular mycorrhizal fungi (AMF). Additionally, a study conducted by Teutscherova (2019) showed that high-BNI varieties of Brachiaria had higher levels of AMF colonization on roots, these findings suggest that the use of different varieties of Brachiaria grasses could have implications for soil health and nutrient cycling in tropical agricultural systems.

A study conducted by Mutai (2017) has provided valuable insights into the influence of Brachiaria plants on soil microbial communities, revealing significant shifts in composition and diversity. Another study was focused specifically on the microbial community associated with Brachiaria decumbens, uncovering pronounced alterations in soil microbiome composition, nutrient cycling, and soil health. This study also found that the variety exhibits a preference for promising rhizobacteria during rhizoremediation (Uribe et al. 2022). Despite the contributions of the currently available literature, a critical research gap remains regarding the specific changes induced by Brachiaria varieties on the soil microbiome and its implications for the nitrogen cycle.

In this study, we aimed to investigate the impact of different Brachiaria varieties on prokaryotic (archaeal and bacterial) and fungal communities and their potential functions, focusing on the N-cycle. We hypothesize that the diverse Brachiaria varieties would induce distinct changes in soil chemical parameters, leading to significant impacts on the microbiome composition and microbial functional traits. To test this hypothesis comprehensively, we employed a combination of molecular techniques, such as quantitative PCR (qPCR) and amplicon sequencing, alongside conventional soil and plant chemical analyses. Throughout the investigation, we aimed to address the following key questions: (i) What are the impacts of different Brachiaria varieties on soil physical-chemical characteristics, and which specific factors are affected? (ii) how does the soil microbiome vary among different Brachiaria varieties? (iii) Which soil characteristics show associations with the observed variations in the soil microbiome caused by different Brachiaria varieties? (iv) To what extent do Brachiaria varieties influence the microbial potential functions and marker genes associated with the N-cycle in the soil? (v) Is there a particular Brachiaria variety that stands out in its ability to enhance soil properties and foster a beneficial microbiome for plant growth?

2.2. Methodology

2.2.1. Field characteristics and experiment design

The field experiment was carried out at the Experimental Farm Station from State Sao Paulo State University (UNESP) in Botucatu, southern São Paulo State, Brazil (22° 83′ 3″ S, 48° 42′ 04″ W, elevation 765 m). The field area was used for agriculture for the last ten years, and the land-use history is presented in Supplementary Table 1.1. The region’s soil type was classified as sandy clay loam kaolinitic and thermic Typic Haplorthox (USDA, 2022). The region’s climate is Cwa type, according to the Köppen–Geiger climate classification system The region’s long-term (1956-2020) annual average temperature is 20.7°C (maximum of 26.1°C and minimum of 15.3°C). The annual rainfall average is 1360 mm. The soil texture from the field experiment was classified as medium texture (309 g kg⁻¹ of clay; Supplementary Table 1.2).

The experiment was initiated in January 2019. The previous crop grown in the area was soybean with no-till management. After the soybean harvest, the soil was prepared by adding P and K in the seeding line based on the soil fertility analysis and following fertilization recommendations for the São Paulo State (Cantarella et al. 1997). The following varieties of Brachiaria were sown in a density of 5 kg ha⁻¹ of viable seeds: (1) Brachiaria brizantha cv. Marandu (treatment BM); (2) Brachiaria ruziziensis (treatment BR); (3) Brachiaria spp. cv. Ipyporã - BRS RB331
(treatment BI and a hybrid of *Brachiaria ruziziensis* × *Brachiaria brizantha*); (4) *Brachiaria brizantha* cv. BRS Paiaguás (treatment BP). In addition to the varieties, a control treatment (Ctrl) was added, being composed of plots without plans (bare soil). The BM and BI are recent varieties available in the Brazilian seed market (2017), while the BR and BP are considered the most used varieties in Brazil. More information about the chosen *Brachiaria* varieties’ characteristics is available in Supplementary Table 1.3.

The experimental design was a randomized block with four replications. A total of 20 plots were carried out ((4 varieties of *Brachiaria* + 1 control) × 4 replicates). Each plot had 15 m × 20 m and a space of 2 m between each plot (details are available in Supplementary Figure 1.1). Fifteen months after the sowing (mature forage grasses established in the field), plants and soil were sampled (April 2020).

### 2.2.2. Soil sampling

Five points were sampled in each plot (1 central point and 4 in each corner) in the 0-10 cm layer (details in Supplementary Figure 1.1). The sampled soil layer contained many *Brachiaria* roots. To ensure the capture of the plants’ influence on the soil communities, filtering was performed by shaking the sampled roots to remove soil that had not adhered to the roots, and the remaining soil was then sampled. After that, the samples were mixed to form one composite sample per plot. Approximately 600 g of soil were sampled to perform soil chemical and texture characteristics. Undisturbed samples were carried out using an auger and metal rings (100 cm³). After removal, the samples were rolled up and immobilized on cling film, delicately stored in cardboard boxes, and transported to the laboratory for analysis of density and porosity. Lastly, 50 mg was sampled in falcon tubes, immediately frozen in nitrogen (N) liquid, and stored in a -20°C freezer for further DNA extraction.

### 2.2.3. Soil chemical and physical analysis

The soil macro- and micronutrients were measured based on the methodology proposed by Cantarella et al. (1998) for Brazilian tropical soils. Details about each method were described by Bossolani et al. (2020). The analyses were based on homogenized soil using a 2 mm sieve, air-dried, and weighed according to the need for each analysis (gravimetric method). Briefly, soil pH and the soil organic carbon (SOC) were measured using 0.01M CaCl₂ and Walkley–Black method, respectively. P-phosphate (P), potassium (K), calcium (Ca²⁺), and magnesium (Mg²⁺) were extracted by anion exchange resin and determined by calorimetric method. S-Sulfate was extracted by 0.01 M calcium phosphate solution and quantified by the turbidimetric method using BaSO₄. Manganese (Mn), zinc (Zn), copper (Cu), and iron (Fe) were extracted using DTPA and determined by atomic absorption spectrometry. Aluminum (Al) was extracted using KCl and measured by titration. The Potential acidity (H⁺Al) was determined by the Shoemaker-McLean-Pratt method. Ammonium (NH₄⁺) and nitrate (NO₃⁻) were extracted using KCL and quantified by the calorimetric method. Based on the results, the sum of bases (SB) was calculated by the sum of the cations K, Ca²⁺, and Mg²⁺; cation exchange capacity (CEC) by the sum of H⁺Al and cations; base saturation (V%) was determined by SB/CEC and Al saturation (m%) by Al/CEC. Finally, the sum of NH₄⁺ with NO₃⁻ resulted in the N-Inorganic, and the difference between N-Total and N-Inorganic calculated the N-Organic.

The soil porosity analysis was based on Klute and Dirksen (1986) and Smith and Mullins (2001) methodologies. First, undeformed soil samples were saturated with water for 48 hours. After that, samples were
weighed and taken to Richard's pressure chamber on porous plates under -0.006 MPa tension until they stabilized. Then, samples were weighed and dried in a forced-air oven at 105 °C for 60 hours. The total porosity was calculated by the difference between the weights of the water-saturated and dried samples. The macro-porosity was obtained by the difference between the water content of the water-saturated samples from Richard's pressure chamber. The micro-porosity was based on the difference between the total porosity and macro-porosity.

2.2.4. Soil DNA extraction

The total DNA from soil samples was extracted using the Power Soil DNA Isolation Kit (Qiagen, Hilden, German) based on 0.25 g and following the manufacturer's instructions. Briefly, the soil sample was homogenized, and the microbial cells were lysed by mechanical and chemical methods. After that, the total genomic DNA was captured on a silica membrane in a spin column format, washed, and eluted from the membrane to obtain the extract. In addition, the DNA concentration was measured using the Qubit fluorometer (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Finally, the DNA quality was checked through 1% sodium boric acid agarose gel electrophoresis analysis (Brody and Kern 2004).

2.2.5. PCR in real-time (qPCR) of marker-genes

The abundance of microbial communities was quantified through StepOnePlus™ Real-Time PCR System (qPCR) with 96-well plates (Applied Biosystems, Foster City, CA, USA). The qPCR was carried out for the bacterial (based on 16S rRNA from Bacteria), archaeal (also based on 16S rRNA gene), and fungal communities (based on ITS gene). Also, the quantification of microbes related to the N-cycle was performed, including the N-fixers (based on the nifH gene) and the bacterial ammonia-oxidizers (AOB, based on the amoA gene from Bacteria) and archaeal ammonia-oxidizers (AOA, based on the amoA gene from Archaea). First, a PCR was performed using DNA extracted from strains that harbor the gene of interest for each gene. After the PCR product was quantified, standard curves were created after serial dilutions. The 16S rRNA gene quantification (from Bacteria and Archaea) was based on 10μL that contained: 5μL of qPCR SYBR Green Master Mix, 1μL of each primer (5pmol), 1.5μL of ultrapure water, 1μL template DNA, and 0.5μL bovine serum albumin (BSA; 10 mg ml⁻¹). For the ITS gene, the qPCR analysis was based on 25μl that contained: 12.5μl qPCR SYBR Green Master Mix, 1.25μl of each primer (10pmol), 2.5μL BSA (10 mg ml⁻¹), and 1μL template DNA. The analyses of melting curves were performed from 68 to 95°C, and all standard curves had R² greater than 0.98. The results were analyzed using the StepOnePlus™ Real-Time software version 2.2.2 (Applied Biosystems, Foster City, CA, USA). Strains used to construct the standard curves, primers, and reaction conditions for the amplification of the genes are described in Supplementary Table 1.4.

2.2.6. DNA sequencing and bioinformatic analysis

The amplicon sequencing analyzes were performed for the 16S rRNA and ITS genes at the Center for Functional Genomic Research (ESALQ/USP), located in the municipally of Piracicaba, São Paulo state, Brazil. A modified version of the pair primers 515F-806R (v4 region of the 16S SSU rRNA) was chosen to characterize
bacterial and archaeal communities (or prokaryotic; Parada et al. 2016; Caporaso et al. 2012; Apprill et al. 2015). The primers pair were modified to remove known biases against Crenarchaeota/Thaumarchaeota and recommended by the Earth Microbiome Project (www.earthmicrobiome.org) to represent bacterial and archaeal communities. The primers ITS1F-ITS2 (Gardes and Bruns 1993; Smith and Peavy 2015) were chosen for sequencing fungal communities. Details about these primers and reaction conditions used for amplification are described in Supplementary Table 1.4. In total, 20 libraries for each barcode were prepared using the MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA), following the manufacturer’s instructions for the Illumina MiSeq platform (2 × 150 bp paired-end). The sequences for both prokaryotic (based on 16S rRNA) and fungal communities (based on the ITS) were processed using the DADA2 pipeline (Callahan 2017) in the R environment (R Core Team, 2021). First, the primers from the demultiplexed data were removed. After, the data were trimmed and filtered to remove low-quality sequences and, the denoising inference step was performed (based on the learn error step). After that, the forward and reverse sequences were merged, and the chimeric sequences were removed based on the “consensus” method. Next, the taxonomic inference was performed using the SILVA database (v. 138; Quast et al. 2012) to the 16S rRNA data and the UNITE (v. 8.2; Köljalg et al. 2013) to the ITS. A compositional matrix was generated for each amplicon. Finally, the prokaryotic matrix was filtered to remove sequences classified as mitochondria and chloroplast and rarefied to 27535 sequences per sample, and the fungal matrix to 22687 sequences per sample (Supplementary Figure 1.2 and Supplementary Table 1.5). Sequences were submitted to the NCBI Sequence Read Archive under the identification BioProject ID PRJNA947540.

2.2.7. Statistical analysis

The statistical analysis was performed using the varieties of Brachiaria (4 varieties + Control) as a factor and with 4 biological replicates, totaling 20 samples. The effect of the blocks was only considered for statistical analysis. The data were analyzed using the R platform (R Core Team, 2021), graphs were created with the “ggplot2” package (v. 3.4.0; Wickham 2016), and statistical analysis was performed with the packages “vegan” (v.2.6-4; Oksanen et al. 2013) and “agricolae” (v. 1.3-5; De Mendiburu and Simon 2015). Distance-based redundancy analysis (db-RDA) was performed to check the soil prokaryotic and fungal communities' structure and its correlation with the soil characteristics. For the analysis, the ASVs compositional table from the 16S rRNA and ITS sequencing were used as biological matrices to calculate the Bray-Curtis distance. After that, the soil characteristics were used as an explanatory matrix. The significance of each soil characteristic in the distribution of the biological data was tested by the PERMANOVA test (based on 999 permutations). The significance of the obtained clusters was calculated by the ADONIS permutational test followed by the pairwise Adonis test (both based on 999 permutations and adjusted by Bonferroni correction). The soil microbial diversity (Shannon-Index), richness (Taxa S index), and Simper dissimilarity (in %) were calculated using the “vegan” package. The gene abundance obtained by the qPCR analysis, the soil microbial diversity, and richness were represented as a box-plot. The Simper results were based on the comparison between the Control and each of the varieties of Brachiaria. The Simper index and abundance of the 5 most important taxa for the dissimilarity analysis were plotted using Excel (Microsoft) and STAMP program (Parks and Donavan, 2014). Besides, random forest analysis was performed to rank the 20 most important taxa groups (at the genus level, for Prokaryotic and Fungal communities) across the different varieties of Brachiaria (and without the Control treatment). The analyses were carried out in the "microeco" package (Liu et al. 2021) and considered the Mean Decrease Gini index as the indicator value, followed by the abundance plotting of the significant groups. The
statistical differences between groups for the soil physical-chemical and plant characteristics, microbial diversity, richness, and gene abundance datasets were based on the One-Way ANOVA test, followed by the least significant difference (LSD) test to compare groups \( (P<0.05) \) with p-value adjusted by Bonferroni correction. A functional prediction annotation was performed to access the potential functions displayed by the soil microbes. The prokaryotic community matrix at the genus level was used as input in the FAPROTAX database \( \text{v. 1.2.4; Louca et al. 2016} \), which maps prokaryotic taxa to putative functions using information based on functional annotations of cultivated representatives. For fungal communities, their compositional matrices at the specie level were used as input in the FUNGuild database \( \text{v.1.1; Nguyen et al. 2016} \). It annotated these soil communities in trophic modes, and only confidence scores of ‘Probable’ and ‘Highly Probable’ were used. The group comparison for microbial composition and potential functions datasets was based on the One-Way ANOVA, followed by the post-hoc Tukey-Kramer test \( (P < 0.05) \) and after Benjamin-Hochberg correction for false discovery rate (FDR). To test the correlation between gene abundance, soil, and plant characteristics, a Spearman rank analysis was carried out and represented as a correlogram using the “corrplot” package \( \text{v. 0.92; Wei and Simko, 2021} \).

2.3. Results

2.3.1. The soil and plant characteristics

The soil physics and chemical characteristics are represented in Table 1. Our results showed that the varieties of Brachiaria changed the soil pH and nutrient availability. The BR presented the lowest soil pH compared to the other treatments, including the Control. Consequently, the availability of soil nutrients dependent on the pH also changed. In general, soil K, Ca\(^{2+}\), Mg\(^{2+}\), SB, V\(^{\%}\), and S under the influence of the BR variety presented the lowest values compared to the Control and the other varieties. On the other hand, the BR showed the highest potential acidity \( (H^+:Al) \). The varieties changed the SOC concentration and P availability in the soil. Soils cultivated with Brachiaria varieties presented more SOC than the Control; in addition, BI showed the highest SOC value. Also, the varieties presented more abundance of P compared to the Control. Interestingly, soil N also changed according to the varieties. The BR variety presented the lowest value for N-total and N-Organic, whereas the varieties BP and BI presented the highest ones. The varieties showed the lowest N-Inorganic values compared to the Control. For NH\(_4^+\), the Control and the varieties BM and BI showed the lowest values, while the varieties BR and BP presented the highest ones. However, the Control showed the highest values for NO\(_3^-\) compared to the varieties. Lastly, the soil-physical characteristics also changed according to the presence of the varieties of Brachiaria. The varieties BR and BI decreased the soil density compared to the other treatments. However, the presence of plants increased the soil Total-Porosity and Macro-Porosity, compared to the Control. Regarding the N content in the plants, the N-Leaf concentration and SPAD index were 30\% lower in the BR variety compared to the BI. However, the Shoot-Biomass was 30\% higher in the BI variety compared to the average of the other varieties. Lastly, the BI variety showed a Root-Biomass 61\% higher than the BP variety.
Tabela 1. Soil and Plant characteristics from soil samples of varieties of Brachiaria. The average and standard deviation values are based on 4 replicates. Lower-case letters indicate statistical differences based on the One-Way ANOVA test, followed by the LSD test to compare groups with p-value adjusted by Bonferroni correction. Ctrl – Control; BR - Brachiaria ruziensis; BP - Brachiaria brizantha cv. BRS Paiaguás; BM - Brachiaria brizantha cv. Marandu; BI - Brachiaria spp. cv. BRS Ipyporá.

<table>
<thead>
<tr>
<th>Soil chemical characteristics</th>
<th>Control</th>
<th>BR</th>
<th>BP</th>
<th>BM</th>
<th>BI</th>
<th>P- Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (CaCl₂)</td>
<td>5.98± 0.10 a</td>
<td>5.50± 0.00 c</td>
<td>5.93± 0.13 a</td>
<td>5.88±0.10 ab</td>
<td>5.70± 0.08 bc</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>S.O.C. (g kg⁻¹)</td>
<td>8.90± 0.50 b</td>
<td>9.3± 2.1 ab</td>
<td>11.4±1.9 ab</td>
<td>9.6±2.1 ab</td>
<td>12.7±0.5 a</td>
<td>0.02</td>
</tr>
<tr>
<td>P (mg kg⁻¹)</td>
<td>26.75± 1.71 b</td>
<td>34.25± 4.35 a</td>
<td>41.25± 1.89 a</td>
<td>38.50±3.70 a</td>
<td>38.75± 2.99 a</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>K (mmol kg⁻¹)</td>
<td>4.0± 0.23 c</td>
<td>3.80± 0.00 c</td>
<td>5.50± 0.24 a</td>
<td>4.58±0.15 b</td>
<td>3.15± 0.10 d</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ca²⁺ (mmol kg⁻¹)</td>
<td>27.0± 1.14 a</td>
<td>18.25± 0.50 b</td>
<td>26.50± 2.08 a</td>
<td>25.50± 1.0 a</td>
<td>25.75± 0.96 a</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mg²⁺ (mmol kg⁻¹)</td>
<td>19.0± 0.82 a</td>
<td>13.0± 1.15 c</td>
<td>18.25± 1.26 a</td>
<td>15.75± 0.5 b</td>
<td>16.0± 0.0 b</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>H₃Al (mmol kg⁻¹)</td>
<td>18.0± 0.0 d</td>
<td>28.0± 0.0 a</td>
<td>16.0± 0.0 c</td>
<td>19.50±1.0 c</td>
<td>21.50± 1.0 b</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Al (mmol kg⁻¹)</td>
<td>0.0± 0.0</td>
<td>0.75± 0.50</td>
<td>0.0± 0.0</td>
<td>0.50± 0.58</td>
<td>0.50± 0.58</td>
<td>0.09</td>
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<tr>
<td>S.B. (mmol kg⁻¹)</td>
<td>50.0± 0.0 a</td>
<td>35.25± 1.50 c</td>
<td>50.25± 3.40 a</td>
<td>46.0±1.41 a</td>
<td>45.74± 0.96 b</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C.E.C. (mmol kg⁻¹)</td>
<td>68.0± 2.16</td>
<td>65.25± 1.50</td>
<td>66.25± 3.40</td>
<td>66.50± 1.75</td>
<td>66.25± 0.50</td>
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<td>V (%)</td>
<td>73.50± 10 a</td>
<td>55.75± 0.96 c</td>
<td>76.0± 0.82 a</td>
<td>70.25±1.26 b</td>
<td>67.75± 1.50 b</td>
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<td>m (%)</td>
<td>0.0± 0.0</td>
<td>2.25± 1.50</td>
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<td>1.0± 1.15</td>
<td>1.0± 1.15</td>
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<td>S (mg/kg)</td>
<td>11.50± 1.41 bc</td>
<td>8.25± 2.22 c</td>
<td>10.75±1.71 bc</td>
<td>18.0±2.0 a</td>
<td>13.0±1.63 b</td>
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<tr>
<td>N-Total (mg kg⁻¹)</td>
<td>1015± 40 ab</td>
<td>785± 101 c</td>
<td>1137±105 a</td>
<td>840± 99 bc</td>
<td>1050± 128 ab</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>N-Organic (mg kg⁻¹)</td>
<td>922± 31 ab</td>
<td>752± 100 c</td>
<td>1124±103 a</td>
<td>831.9±103 b</td>
<td>1047± 128 ab</td>
<td>&lt;0.01</td>
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<td>N-Inorganic (mg kg⁻¹)</td>
<td>93.14± 11.24 a</td>
<td>32.68± 5.53 b</td>
<td>13.32±3.38 c</td>
<td>8.17± 8.27 c</td>
<td>2.60± 1.57 c</td>
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<tr>
<td>NH₄⁺ (mg kg⁻¹)</td>
<td>0.0± 0.0</td>
<td>23.04± 4.64 a</td>
<td>12.16±2.76 b</td>
<td>3.34±2.98 c</td>
<td>0.0± 0.0 c</td>
<td>&lt;0.01</td>
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<tr>
<td>NO₃⁻ (mg kg⁻¹)</td>
<td>93.35± 11.24 a</td>
<td>9.65± 4.43 b</td>
<td>11.6±0.72 b</td>
<td>4.82± 7.54 b</td>
<td>2.60± 1.57 b</td>
<td>0.01</td>
</tr>
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</table>

Soil physics characteristics

<table>
<thead>
<tr>
<th>Soil Physics characteristics</th>
<th>Control</th>
<th>BR</th>
<th>BP</th>
<th>BM</th>
<th>BI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g cm⁻³)</td>
<td>1.50±0.01 a</td>
<td>1.30±0.06 b</td>
<td>1.46±0.03 a</td>
<td>1.47±0.03 a</td>
<td>1.25±0.07 b</td>
</tr>
<tr>
<td>Total Porosity (cm² cm⁻³)</td>
<td>0.40±0.01 c</td>
<td>0.51±0.02 ab</td>
<td>0.45±0.01 abc</td>
<td>0.44±0.01 bc</td>
<td>0.53±0.02 a</td>
</tr>
<tr>
<td>Micro-porosity (cm² cm⁻³)</td>
<td>0.34±0.02</td>
<td>0.28±0.01</td>
<td>0.30±0.01</td>
<td>0.30±0.01</td>
<td>0.30±0.02</td>
</tr>
<tr>
<td>Macro-porosity (cm³ cm⁻³)</td>
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<td>0.23±0.03 a</td>
<td>0.15±0.02 ab</td>
<td>0.15±0.02 ab</td>
<td>0.23±0.04 a</td>
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</table>

Plant characteristics

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<th>Plant characteristics</th>
<th>Control</th>
<th>BR</th>
<th>BP</th>
<th>BM</th>
<th>BI</th>
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<tr>
<td>N-Leaf (unit)</td>
<td>-</td>
<td>14.88±1.75 b</td>
<td>21.34±1.44 a</td>
<td>18.04±2.58 ab</td>
<td>21.54±1.45 a</td>
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<tr>
<td>N-SPAD</td>
<td>-</td>
<td>32.05±2.23 c</td>
<td>43.25±2.88 ab</td>
<td>35.92±4.75 bc</td>
<td>44.77±2.22 a</td>
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<tr>
<td>Shoot-Biomass (T ha⁻¹)</td>
<td>-</td>
<td>7.65± 0.7 b</td>
<td>9.17± 1.8 b</td>
<td>8.6± 1.6 b</td>
<td>12± 0.9 a</td>
</tr>
<tr>
<td>Root-Biomass (T ha⁻¹)</td>
<td>-</td>
<td>0.96± 0.1 b</td>
<td>0.49± 0.3 b</td>
<td>0.98± 0.2 a</td>
<td>1.28± 0.2 a</td>
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</tbody>
</table>

### 2.3.2. The structure and diversity of microbial communities

The db-RDA analysis was performed to investigate the prokaryotic and fungal communities' structure and their correlation to the soil characteristics, represented in Figure 1. The results indicated that the varieties of *Brachiaria* performed as a driver for the soil microbial community structure and that soil characteristics played an important role in these changes. For the prokaryotic communities, the analysis indicated 58% of the data inertia was explained by the varieties of *Brachiaria* (Supplementary Table 1.6) and 44% considering the sum of axis 1 and 2, as represented in Figure 1A. The samples clustered according to the varieties, and the multivariate Adonis test statically validated these segregations (Supplementary Table 1.7 and dashed lines represented in Figure 1A). Regarding the soil characteristics, the results pointed out that almost all soil chemical characteristics (except K, S, and Micro-Porosity) contributed significantly to the found segregations of the samples (based on the PERMANOVA test; 999 permutations; P<0.05). Similar results were found for the fungal communities (Figure 1B). The data inertia explained by the varieties was 63% (Supplementary Table 1.6) and 47% considering the sum of the axis 1 and 2. Also, the varieties significantly segregate the samples (Supplementary Table 1.7 and dashed lines represented in Figure 1B).
The only soil characteristics that did not correlate significantly with the samples were K, S, soil density, and Total-Porosity (based on the PERMANOVA test; 999 permutations; P<0.05). The prokaryotic diversity was lower in the BI variety compared to the other treatments (Supplementary Figure 1.3A, P=0.02). However, the fungal diversity was higher in the BI and BP varieties compared to the BR (Supplementary Figure 1.3B, P<0.01). Also, even though we did not observe statistical differences in the microbial richness (Supplementary Figure 1.3C and 3D, P>0.05), the values in the soils under the influence of varieties of *Brachiaria* were higher compared to the Control.

**Figure 1.** Distance-based redundancy analysis (db-RDA) of prokaryotic (A) and fungi (B) communities and their correlation with soil characteristics. The analysis was based on the Bray-Curtis distance. The arrows indicate soil characteristics significantly correlating with microbial community composition (based on 999 permutations and P<0.05). The dashed lines indicated significant sample clusters based on the multivariate Adonis test (999 permutations).

### 2.3.3. The composition of microbial communities

The 16S rRNA and ITS amplicon sequencing generated 1 004 440 sequences after the quality control, filtering, and rarefaction steps (Supplementary Table 1.5). The 16S rRNA sequences were classified into 34 phyla, including 3 from Archaea and 35 from Bacteria (Figure 2A). The five most abundant phyla were Actinobacteriota (28% of all sequences), followed by Proteobacteriota (26%), Acidobacteriota (11%), Chloroflexi (7%), Firmicutes (5%), Bacteroidota (5%) and Crenarchaeota (4%). Together they represented 86% of all sequences. Furthermore, we found that the ITS sequences were classified into 13 fungal phyla (Figure 2B). The most 5 abundant ones were Ascomycota (72%), followed by Basidiomycota (13%), Chytridiomycota (3%), Mortierellomycota (3%), and Glomeromycota (2%). Together, these groups represented 93% of all sequences. A Simper analysis was performed to check the percentage of the soil microbial dissimilarity carried out by the varieties of *Brachiaria* compared to the Control (Figure 2C). For the prokaryotic community, the variety BR showed the most dissimilar community with 32%, followed by BI (23%), BP (22%), and BM (21%). For the fungal communities, it was found a similar pattern. The BR showed the most dissimilar community with 68%, followed by BM (64%), BI (62%), and BP (58%). The 5 main taxa that contributed to Simper's dissimilarity analysis for each variety compared to the Control treatment are represented in Supplementary Figure 1.4, and its % abundance in Supplementary Figure 1.5. The biomarker indicator analysis (Supplementary Figure 1.6) was performed to check the difference between microbial taxa among varieties.
of Brachiaria (and without the Control treatment). Based on the random forest algorithm, the analysis ranked the 20 most important prokaryotic (A) and fungal genera (B). For prokaryotic genera, *Pseudarthrobacter*, *Pseudomonas*, *Paenarthrobacter*, *Paenibacillus*, and *Puia* were the biomarker genera for the BR variety. *Bacillus*, *Steroidobacter*, and *Dactylosporangium* were identified as biomarker genera for the BP variety. *Angustibacter*, *Microvirga*, *Luteolibacter*, *Nitrocosmicus* candidatus, and *Fimbriiglobus* were the biomarker genera for the BM variety. Lastly, *Gaiella*, *Nitrospira*, and *Parafilimonas* were identified as marker genera for the BI variety. Considering the fungal genera, the analysis highlighted *Trimorphomyces*, *Papilio trema*, *Leptospora*, and *Humicola* as the biomarker genera for the BR variety. *Gibberella*, *Pseudorobillarda*, *Volutella*, *Lipomyces*, and *Pyrenochaetopsis* were identified as biomarker genera for the BP variety. *Nectriopsis* and *Cyphellophora* were found to be biomarker genera for the BM variety. Finally, the biomarker genera for the BI variety were *Trichoderma*, *Saitozyma*, *Glomus*, *Talaromyces*, *Entrophospora*, *Meteobizium*, *Mortierella*, and *Phallus*. 
2.3.4. The functional profile of microbial communities

The prokaryotic functional profile was obtained using the FAPROTAX database, which assigned putative functions according to the microbial compositional profile (Figure 3A and Supplementary Table 1.8). The Control showed lower values than the varieties for microbial metabolism functions such as chemoheterotrophy and aerobic chemoheterotrophy. However, the phototrophy, photoheterotrophy, photoautotrophy, and anoxygenic photoautotroph functions were lower in the BR variety compared to the other treatments. Also, the BR variety...
showed a lower % for functions related to N-metabolism. The nitrification, aerobic ammonia oxidation, nitrate respiration, nitrite respiration, nitrogen respiration, nitrate denitrification, nitrite denitrification, nitrous oxide denitrification and anoxygenic photoautotrophy S oxidizing functions were lower in the BR variety compared to the other treatments. However, the BR variety showed a higher percentage for ureolysis function. The cellulolysis function was higher in the BP variety compared to the BI variety. The fungi functions were signed as guilds by the FUNGuild database. Considering the “Trophic-level” classification (Figure 3B), we found that the Control showed a higher % of Saprotroph but lower values for Pathotroph-Saprotroph. The Saprotroph-Symbiotroph and Symbiotroph were higher in the BI variety. Finally, according to the “Guild” classification, the BI variety has a higher % for Endophyte, Ectomycorrhizal, and Arbuscular Mycorrhizal Fungi (Supplementary Figure 1.7).

**Figure 3.** (A) Bubble plot representing the differences in the abundance (%) of potential prokaryotic functions according to the FAPROTAX database (based on 16S rRNA sequencing). The “Rare” function is based on the sum of functions that represents less than 1% (Supplementary Table 1.6). (B) Bubble-plot representing the differences in the abundance (%) of fungal functional groups (trophic mode) according to the FUNGuild database (based on ITS sequencing). The lower-case letters represent functions with a statistical difference based on the One-Way ANOVA, followed by the post-hoc Tukey-Kramer test (P < 0.05) and after the Benjamini-Hochberg FDR correction. Ctl – Control; BR - Brachiaria ruiziiensis; BP - Brachiaria brizantha cv. BRS Paiaguás; BM - Brachiaria brizantha cv. Marandu; BI - Brachiaria spp. cv. BRS Ipyporã.

### 2.3.5. Microbial marker-genes abundance and their correlations with soil and plant characteristics

The quantification of marker genes indicated that the abundance of microbial communities changed according to the treatments. The bacterial abundance (Figure 4B) was lower in the BI and BP varieties compared to the other treatments. However, the BI and BM varieties presented the highest archaeal abundance (Figure 4C). The size of the fungi communities also changed. The BP showed the highest abundance compared to the other treatments (Figure 4A). The microbial N-fixers were lowest in the Control and highest in the BR variety (Figure 4D). We found that the BI variety showed the lowest value for bacterial ammonia-oxidizers (Figure 4E). However, the BI and BP varieties showed the highest values for archaeal ammonia-oxidizers (Figure 4F).

A correlogram was carried out to assess the correlation between the gene’s abundance to soil and plant characteristics (Supplementary Figure 1.8). The bacterial abundance correlated positively with AOB and nifH genes. However, it correlated negatively with the AOA gene, SOC, N-total, and N-Organic from the soil, and N-Leaf and
N-SPAD from plants. The archaeal abundance correlated positively with the soil characteristics P and S and the plant Root-Biomass, Leaf-Biomass, N-Leaf, and N-SPAD. Nonetheless, the gene correlated negatively with the soil characteristics Mg\textsuperscript{2+}, SB, NO\textsubscript{3}\textsuperscript{-}, N-Inorganic, Density, and Micro-Porosity. The fungal abundance correlated positively with the gene \(nifH\) and the soil P, K, and NH\textsubscript{4}\textsuperscript{+}. However, the gene correlated negatively with NO\textsubscript{3}\textsuperscript{-} and Micro-Porosity. The bacterial ammonia-oxidizers correlated positively with the \(nifH\) gene and soil NH\textsubscript{4}\textsuperscript{+}. However, the gene correlated negatively with the gene AOA and the soil characteristics SOC, Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, CEC, N-Total, and N-Inorganic. The archaeal ammonia-oxidizers correlated positively with SOC, Mg\textsuperscript{2+}, N-Total, N-Organic, Leaf-Biomass, N-Leaf, and N-SPAD but negatively with the gene \(nifH\) and the NH\textsubscript{4}\textsuperscript{+} from the soil. Lastly, the \(nifH\) gene correlated positively with the H-Al, NH\textsubscript{4}\textsuperscript{+}, Macro-porosity, and Root-Biomass. However, the gene correlated negatively with the pH, Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, SB, CEC, V\textsubscript{\%}, N-Total, N-Organic, and Micro-Porosity.

![Box plot representing the abundance of the marker genes ITS (A), 16S rRNA of Bacteria (B), and Archaea (C), nifH (D), amoA of Bacteria (E), and Archaea (F).](image)

**Figura 4.** Box plot representing the abundance of the marker genes ITS (A), 16S rRNA of Bacteria (B), and Archaea (C), \(nifH\) (D), \(amoA\) of Bacteria (E), and Archaea (F). Lower case letters indicate statistical differences based on the One-Way ANOVA test, followed by the LSD test to compare groups with p-value adjusted by Bonferroni correction. All P-Values were smaller than 0.01.

### 2.4. Discussion

#### 2.4.1. Soil Brachiaria varieties growth, nitrogen acquisition, and influence on soil physicochemical properties

In our study, the soil physicochemical characteristics changed between the varieties of *Brachiaria*. We found that the presence of the plants increased the soil P and SOC, and both positively correlated with the plant biomass. *Brachiaria* varieties are characterized by a high biomass production that, after decomposing, can increase SOC and soil fertility (Brandan et al. 2017). Also, the *Brachiaria* roots can exude organic acids, OH\textsuperscript{-} and H\textsuperscript{+}, to increase the soil P availability (Merlin et al. 2016), which explains the increase of nutrients in the treatments with the plant in our study. In general, the utilization of *Brachiaria* sp. can provide a wide range of beneficial services to
tropical agroecosystems (Baptistella et al. 2020). Its versatility allows it to be introduced as an isolated crop (pasture), used as a cover crop (predecessor crop), combined with annual or perennial crops (e.g., coffee and orange), or incorporated into intercropping systems (e.g., maize). The soil quality legacy left by *Brachiaria* cultivation can be decisive in increasing crop productivity in tropical soil-plant systems (Baptistella et al. 2020).

Interestingly, the BR variety increased the soil acidity and decreased many nutrients dependent on pH, such as K, Ca\(^{2+}\), and Mg\(^{2+}\). The soil pH is considered one of the main factors responsible for regulating the availability of nutrients in the soil and, consequently, making them available for plant and microbes’ absorption (Fernández and Hoeft 2009). Also, the BR variety can exudate high rates of organic acids, C-based compounds, and H\(^+\) to make P available in conditions of scarcity of this element (Chigira and Oyama 2000; Louw-Gaume et al. 2017; Almeida et al. 2020). In our study, this root exudation ability linked with cation-based nutrient uptake may explain why the acidity increased in the BR variety compared to the others. The soil nitrogen dynamics also changed according to the varieties of *Brachiaria* and revealed different strategies of N acquisition, microbial abundance, and plant growth. Plants and microbes can absorb N in their soil-available forms (NH\(_4^+\) and NO\(_3^-\)), which commonly enter into agricultural lands through N-based fertilizers, biological N fixation (BNF), and organic matter mineralization (Robertson and Vitousek 2009). Therefore, the last two were the only N sources in this study, considering there was no N-fertilizer application in the field during the experiment. The highest NO\(_3^-\) amount in the Control treatment indicated the influence of the plants on the availability of this nutrient in the soil. The element is the final product of soil nitrification and has high mobility in the soil-plant system. It can be uptake by plants, immobilized by microbes and it is highly susceptible to denitrification and leaching (Powlson 1993; Momesso et al. 2022). Considering the difference between varieties, the BR and BI showed the most contrasting results for soil N characteristics. The BR variety showed an accumulation of soil NH\(_4^+\). Interestingly, this variety also showed a higher abundance of microbial N-fixers and accumulated more SOC than the Control, suggesting the plant uses multiple strategies to acquire the nutrient. Rocha et al. (2020) and Bossolani et al. (2020) also found an increase in the soil N-fixers in the BR variety. They highlighted the plant’s potential to improve biological N fixation in the rotational agriculture system. Also, the highest amount of N-inorganic found in soil and the lower amount of N accumulation in the leaves of the BR variety indicated a less pronounced need for N to its growth.

The BR variety did not show the highest values of shoot-biomass in this study. However, this variety decreased soil density and increased porosity, indicating high root growth, leading to better soil physical structuration. These soil changes provided by BR root development are a management tool to make the soil suitable for crop rotation systems (Favilla et al. 2021). This legacy from the previous cultivation of forage grasses is essential for reducing soil compaction and increasing soil porosity (Silva et al. 2021). On the other hand, the BI variety presented a contrasting result regarding the soil N-cycle. This variety presented the lowest values for the soil N-Inorganic, NH\(_4^+\), and NO\(_3^-\). However, these nutrients accumulated in the plant contributed to its growth, confirmed by the highest values of N-Leaf concentration and plant biomass. In addition, we also found an increase in soil porosity and a decrease in soil bulk density. Forage grasses, in general, have a high potential to accumulate leaf nutrients and produce high plant biomass (below- and aboveground), but some varieties require soils with high fertility and N amendments to maintain their growth (Valle et al. 2017; Camargo et al. 2022). In addition, the BI variety had more microbial N-fixers than the Control but less than the BR variety, as well as, had a smaller influence on the accumulation of SOC compared to other treatments. Together, these results suggest that this variety can extract more of the required N from soil organic matter than through the association with N-fixing microbes.
2.4.2. The selection of the soil microbial community by Brachiaria varieties

The prokaryotic communities changed according to the varieties of Brachiaria, which explained almost 60% of the data variation (based on the db-RDA analysis). Also, the soil's chemical and physical characteristics strongly influenced this selection. It was found that the Control samples were segregated from Brachiaria samples. The SOC, P, NH$_4^+$, and Macro- and Total-Porosity indicated a strong correlation with the Control samples. Interestingly, all these parameters increased with the presence of the plants in this study (except the NH$_4^+$). One of the reasons that explain these changes is that Brachiaria plants can have profound root growth in the soil, increasing their contact area with soil particles and enabling a high uptake of water and nutrients (Rao et al. 1996; Santos et al. 2013). The aggressive Brachiaria growth allows the plants to produce a high amount of biomass and root-soil colonization (Oliveira et al. 2019; Silva et al. 2021). Consequently, we can find an increase in SOC metabolization and soil porosity, favoring C decomposers and aerobic microbes (Uribe et al. 2022; Abán et al. 2022). Besides improving soil properties, Brachiaria varieties can improve their nutrition by modulating specific soil microbial groups. For example, the Brachiaria plants can extract P retained in the soil colloids by exudation of organic acids and associating with P-solubilizing bacterial groups (Merlin et al. 2016; Oliveira et al. 2021) and mycorrhizal fungi (Clark and Zeto 2000). Also, the plants can inhibit microbial ammonia-oxidizers (a process known as biological nitrification inhibition or BNI), increasing their advantage in the competition for soil N uptake against microbes (Moreta et al. 2014; Nakamura et al. 2020). We found that Brachiaria samples were strongly associated with soil nutrients (Ca$^{2+}$, Mg$^{2+}$, and N) and indicators of soil acidity (pH and H-Al). In our study, most of these soil characteristics increased in the presence of the plants (except for the BR variety). Soil pH is considered one of the main drivers of soil microbiome due to its indirect effects on the availability of nutrients and toxic elements that can change microbial growth (Lammel et al. 2018). The biomarker indicator analysis revealed the ability of the Brachiaria varieties to increase the abundance of specific groups, with many of them described in the literature for improving plant nutrition and resistance to stresses. The bacterial groups Pseudarthrobacter, Pseudomonas, and Paenibacillus were considered biomarkers for the BR variety, encompassing BNF agents, siderophore producers, and plant growth promoters (Mutai et al. 2017; Tshishonga and Serepa-Dlamini 2020; Langendries et al. 2021). For the BP variety, the Bacillus group was highlighted, including plant growth promoters that protect the plant host against pathogens and abiotic stressors through the production of phytohormones (Saxena et al., 2020). As for the BM variety, Microvirga, Nitrocosmicus candidatus, and Fimbriiglobus were identified as responsible for harboring nodulating groups, displaying higher tolerance to nitrite and ammonia, and acting as degraders of complex heteropolysaccharides (Msaddak et al. 2017; Lehtovirta-Morley et al. 2016; Ravin et al. 2028). When considering the BI variety biomarkers, Gaiella and Nitrospira were identified, including lignocellulose decomposers and nitrifiers (Zhang et al. 2019; Xu et al. 2020).

The Brachiaria varieties also changed the soil fungal communities and explained 42% of their variation (based on the db-RDA analysis). Soil acidity characteristics (pH and V%), many pH-dependent nutrients (N-Total, Ca$^{2+}$, Mg$^{2+}$), and the soil components SB and CEC contributed to the observed segregation from the Control samples. While the pH can indirectly and directly modulate the bacterial communities (Lammel et al. 2018), some studies suggested that soil fungal communities are less responsible for variations in soil pH and more correlated to soil nutrients (Lauber et al. 2008; Rousk et al. 2010). The soil porosity (Micro- and Macro-Porosity), nutrients (N-Inorganic, P), and SOC contributed to the segregation of Brachiaria varieties samples. The increase in soil porosity can contribute to aerobic activity from bacterial and, especially, fungal communities and consequently increase SOC.
metabolization (Yang et al. 2019). The positive correlation between Brachiaria species and the P availability in the soil is not only due to the association with bacterial communities’ groups (Merlin et al. 2016; Oliveira et al. 2021) but also with AMF. These fungi groups supply their C needs by associating with plant roots and, in exchange, provide P and other nutrients to their hosts (Clark and Zeto 2000). The AMF also benefits from the BNI process performed by a wide variety of Brachiaria (Nakamura et al. 2020). The increase in the NH$_4^+$ availability can supply the plant needs and the AMF, mainly when N and P supplies are scarce in the soil (Teutschnerova et al. 2019). The BR variety showed the most dissimilar fungal community compared to the Control (as found for the prokaryotic communities). As fungal biomarker indicators for the BR variety, we highlighted Papiliotrema genus, which harbors groups described for metabolizing complex root secretions (Sarkar et al. 209). In the BP variety, the Humicola genus taxa have members with the potential for biological control against plant diseases (Ko et al. 2001). Additionally, this variety increased the abundance of Gibberella and Volutella, which are known to harbor plant pathogen groups (Cannon et al. 2012; Dalla Lana et al. 2021). Finally, the BI variety showed many biomarkers considered as AMFs (arbuscular mycorrhizal fungi), such as Trichoderma, Glomus, and Entrophospora (Amer et al. 2008; Karasawa et al. 2012; Palenzuela et al. 2010).

2.4.3. Brachiaria varieties influence microbial soil potential functions

The presence of the plants increased the prokaryotic activity metabolism, indicated by the higher percentage of chemoheterotroph and aerobic-chemoheterotroph in the Brachiaria soil samples. The findings converged with prokaryotic structure and composition results that showed a strong correlation of microbial aerobic groups with the improvements in soil structure (porosity and density) performed by plants’ presence. Besides, the improvements in soil nutrient availability by Brachiaria plants can contribute to soil microbial activity (Brandan et al. 2017; Cardozo Junior et al. 2018). Interestingly, we found a decrease in phototroph metabolism, nitrification, and denitrification in the BR variety. The increased soil acidity and toxic elements in the BR soil can explain those changes. These elements can directly affect the growth of the microbial community responsible for those functions (Sauze et al. 2017; Bossolani et al. 2020; Naz et al. 2022).

The presence of plants also decreased the percentage of Saprotroph fungi but increased the Pathotroph-Saprotroph ones. The results indicate that the presence of plants can make other functional groups more competitive in the soil than saprotrophs (Schmidt et al. 2019). The BI variety showed a higher percentage for the Saprotroph-Symbiotroph and Symbiotroph trophic levels. Also, the guild classification showed a higher percentage for Endophyte, Ectomycorrhizal, and Arbuscular Mycorrhizal. Interestingly, all of them are related to bringing nutritional benefits to their hosts (Clark and Zeto 2000; Nakamura et al. 2020). However, most BI variety studies focused on plant productivity and their potential for animal nutrition (Valle et al. 2017; Camargo et al. 2022). Our results indicated that the BI variety also presented a higher association with the fungal community, highlighting its potential to select the soil microbiome to help its nutrition.

The size of the bacterial communities decreased in the BP and BI varieties. The positive correlation of the bacterial abundance with ammonia-oxidizer bacteria and N-fixers abundance indicated these functional groups’ contribution to the soil bacterial communities’ size. The archaeal abundance increased in the BM and BI varieties. The gene correlated positively with P and S, highlighting the importance of these elements for archaeal growth. For example, the S is important for archaeal groups due to their need for the element as a cofactor for soil methanogenesis (Liu et al. 2012). And more recently, the archaea were characterized as drivers of soil stoichiometry in phosphorus-deficient habitats (Wang et al. 2022). The size of the fungal communities increased in the BP variety.
It correlated positively with N-fixers, P, K, and NH$_4^+$, reinforcing their role in N and P soil cycle on the fungal community (Teutschroeva et al. 2019). Also, the negative correlation with micro-porosity indicates that soil structure can play a role in the fungal community (Yang et al. 2019). Our results showed that the Brachiaria varieties could select N-fixing microorganisms in different quantities and highlighted the higher abundance in the BR and BP varieties, which may decrease the dependency of these plants on N fertilizers (Reis et al. 2001). However, the negative correlation of the gene with soil acidity characteristics and the positive with soil porosity reinforce the preferences of these groups for non-acidic and aerobic soils (Bossolani et al. 2020). Interestingly, the N-fixers and NH$_4^+$ availability influenced our study’s soil ammonia oxidizers abundance. The bacterial (AOB) and archaeal ammonia-oxidizers (AOA) display an important role in nitrification by converting NH$_4^+$ to NO$_3^-$ and increasing the availability of nutrients in the soil (Prosser et al. 2020). Both microbial groups compete for the same N substrate. Still, the archaeal group has limited growth in soils rich in NH$_4^+$ due to their low tolerance to the element (French et al. 2012) and has a growth advantage in acid and oligotrophic soils (Sims et al. 2012). Also, the bacterial group can have an advantage in agricultural lands due to the liming and nutrient availability found in these soils (Banning et al. 2015). Interestingly, the higher abundance of N-fixers in the BR and BP, and consequently the increase in NH$_4^+$ availability, favored the AOB abundance and inhibited the AOA in our results. Also, we found the contrary pattern on BP and BI varieties. Both had less N-fixer abundance, which may have given a competitive advantage to archaeal ammonia oxidizers over the bacterial group. The explanation was reinforced by decreased AOB gene abundance and increased AOA in the soils from BP and BI varieties.

2.5. Conclusion

In this study, we investigated the impact of four Brachiaria varieties on soil properties and microbial communities. All treatments resulted in improved soil P, SOC, and soil porosity. However, the BR and BI varieties presented the most contrasting results. The BR variety led to a decrease in soil pH and nutrients, along with lower plant biomass, while the BI variety exhibited higher biomass but depleted nitrogen from the soil. Changes in soil acidity, nutrient availability, and soil porosity were identified as the main drivers of microbial community structure. Each of the Brachiaria varieties increased the abundance of specific microbial groups, which were considered biomarkers in this study. These groups included N-fixers, siderophores, decomposers, nitrifiers, and AMFs, as described in the literature. The presence of the Brachiaria plants increased microbial functions such as chemoheterotroph, aerobic-chemoheterotroph, and Pathotroph-Saprotroph potential functions. The BR variety decreased potential prokaryotic phototroph metabolism, nitrification, and denitrification, while the BI variety showed a higher proportion of symbiotrophic fungi. The abundance of bacteria, archaea, and fungi varied based on the Brachiaria varieties and positively correlated with functional bacterial genes, P, N, and soil porosity. In our study, the BI variety exhibited a greater potential to enhance soil quality through increased soil porosity, potential AMFs, and higher biomass production. Additionally, all varieties demonstrated promising benefits for intercropping and crop rotation systems. Future studies should address the empirical relation between these identified biomarker microbes and the studied varieties. Finally, choosing an efficient Brachiaria variety with superior nutrient acquisition capabilities and the ability to leave a positive legacy in the soil, can be a highly effective strategy for improving agricultural systems and increasing food production.
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3. N FERTILIZERS IN Brachiaria GRASSLANDS: EFFECTS ON SOIL MICROBIOME DYNAMICS AND NITRIFICATION

Resumo

**Fertilizantes Nitrogenados em Pastagens de Brachiaria: Efeitos na Dinâmica do Microbioma do Solo e na Nitrificação**

Aproximadamente 60% das pastagens brasileiras enfrentam algum grau de degradação devido à manutenção inadequada e ao pastoreio. Dentro deste contexto, a fertilização com nitrogênio (N) é reconhecida como uma abordagem eficaz para melhorar recuperar pastagens degradadas. A fertilização com N tem o potencial de alterar não apenas o microbioma do solo como um todo, mas também comunidades específicas envolvidas na nitrificação do solo, um processo crítico que governa a disponibilidade de N. Um entendimento dos impactos de diferentes fontes de N nas comunidades microbianas do solo, e suas implicações para o ciclo do N e o crescimento das plantas, pode auxiliar na otimização dos manejos existentes. Dado este cenário, este estudo teve como objetivo explorar como diferentes fontes de fertilizantes nitrogenados afetam o microbioma do solo, especialmente aqueles envolvidos no processo de nitrificação, e seu subsequente impacto no crescimento das plantas de Brachiaria. Para este propósito, aplicamos técnicas moleculares, incluindo PCR quantitativo e sequenciamento em larga escala, para analisar comunidades procarióticas e fúngicas e suas funções potenciais, juntamente com análises químicas tradicionais do solo e medições de biomassa vegetal. Os tratamentos incluíram sulfato de amônio, ureia e torta de mamona como fontes de N, além de um controle sem N em gramíneas de Brachiaria (Sin. Urochloa). Nossas descobertas revelaram que todos os fertilizantes aumentaram a disponibilidade de N e alteraram as propriedades químicas do solo como pH e enxofre. Sulfato de amônio, o mais eficaz em liberar N, promoveu o crescimento de procarióteis em detrimento dos fungos, além de promover funções relacionadas ao ciclo do N. A ureia e torta de mamona favoreceram o crescimento fúngico, especialmente aqueles ligados a doenças de plantas, além de reduzirem funções relacionadas a ciclagem de N. O sulfato de amônio afetou significativamente a nitrificação, provavelmente através de seu impacto nas bactérias oxidantes de amônia, e todos os fertilizantes promoveram o desenvolvimento radicular de plantas sobre a biomassa aérea. Dessa maneira, concluímos que diferentes fertilizantes nitrogenados modificaram as comunidades procarióticas e fúngicas, com implicações para a ecologia do solo e funções relacionadas ao N. Estudos futuros devem se concentrar em identificar práticas agrícolas sustentáveis que promovam a produtividade agropecuária, enquanto minimizam os seus potenciais impactos ambientais.

**Palavras-chave:** Fertilizantes nitrogenados; Comunidades microbianas; Ciclo do nitrogênio 16S rRNA gene; ITS gene; Oxidantes de amônia
Abstract

N FERTILIZERS IN Brachiaria GRASSLANDS: EFFECTS ON SOIL MICROBIOME DYNAMICS AND NITRIFICATION

Approximately 60% of Brazilian pastures are facing some degree of degradation due to inadequate maintenance and overgrazing. Within this framework, N fertilization is recognized as an effective approach to enhance tropical forage for the rehabilitation of these degraded pastures. N fertilization has the potential to alter not just the overall soil microbiome but also specific communities involved in soil nitrification, a critical process governing the availability of N. An understanding of the impacts of different N sources on the soil microbial communities, and their implications for the N cycle and plant growth, can inform the optimization of current fertilization practices. Given this scenario, this study aimed to explore how different N fertilizer sources affect the soil microbiome, especially those involved in the nitrification process, and their subsequent impact on plant growth in Brachiaria grass. For this purpose, we applied molecular techniques, including quantitative PCR and amplicon sequencing, to analyze prokaryotic and fungal communities and their potential functions, alongside traditional soil chemical analyses and plant biomass measurements. Treatments included ammonium sulfate, urea, and castor meal as nitrogen sources, plus a control without nitrogen on Brachiaria grass (Syn. Urochloa). Our findings revealed that all N fertilizers increased soil N availability and altered soil properties like pH and sulfur content. Ammonium sulfate, the most effective at releasing N, promoted prokaryotic over fungal growth, maintaining nitrification and denitrification functions. Urea and castor meal favored fungal growth, especially those linked to plant diseases, while reducing N-cycling functions. Ammonium sulfate significantly affected nitrification, likely through its impact on bacterial ammonia oxidizers, and all fertilizers improved root development more than aboveground biomass. We concluded that different N fertilizers modified the soil prokaryotic and fungal communities, with implications for soil ecology and N functions. Future studies should focus on identifying sustainable agricultural practices that foster crop growth while minimizing the potential negative environmental impacts associated with specific fertilizers.

Keywords: N fertilizers; Microbial communities; N-cycle; 16S rRNA gene; ITS gene; Ammonia-oxidizers

3.1. Introduction

The genus Brachiaria (also known as Urochloa) is a notable tropical grass in South America, particularly in Brazil, where it occupies extensive areas to support livestock production (Mutimura and Ghimire 2021; Gonçalves et al. 2023). Furthermore, Brachiaria is used in intercropping systems and crop rotations, contributing to soil structure improvement as well as increasing carbon and phosphorus content, which positively affects soil health for subsequent crops (Baptistella et al. 2020; Almeida et al. 2020; Gonçalves et al. 2023). Still, approximately 60% of Brazilian pastures, or 109.7 million hectares, are affected by degradation due to poor management practices and the challenges posed by weathered, acidic soils (Rao et al. 1995; Eigenolf et al. 2022; Bolfe et al. 2024). This degradation leads to reduced yield and grazing capacity, the proliferation of invasive species, soil erosion, and increasing land use change through deforestation (Feltran-Barbieri and Fére 2021). To mitigate these issues, nitrogen (N) fertilization is identified as an important tool for the recovery of degraded Brachiaria grasslands (Sales et al. 2020; Pereira et al. 2022). The application of N, particularly during the establishment of new pastures or in the recovery phase following intensive grazing, is an effective management strategy for improving plant growth and ecosystem resilience (Delevatti et al. 2019).
The N sources are also important strategies to meet the nutritional demands of pastures, with options ranging from synthetic sources, such as urea and ammonium sulfate, to organic ones, such as manure and castor meal (de Matos et al. 2018; Pereira et al. 2022). The selection of N sources for pasture fertilization is influenced by several factors, such as the nutritional demand of the target species, economic viability, and potential environmental impacts (Pereira et al. 2022; Souza et al. 2023). Furthermore, the implications of N fertilization extend beyond plant development by affecting soil properties, such as pH and the availability of other soil nutrients as well (Batista et al. 2015). These alterations can significantly influence the composition of bacterial and fungal populations within the soil, thereby affecting nutrient dynamics within the soil-plant system (Momesso et al. 2022; Merloti et al. 2023).

Bacterial and fungal communities are integral to the soil ecosystem, facilitating energy transfer between aboveground and belowground systems and significantly contributing to the formation and decomposition of organic matter (Cui et al. 2018; Wang et al. 2022). Bacteria typically metabolize less complex substrates, while fungi are capable of decomposing more recalcitrant materials (Koranda et al. 2014; Cui et al. 2018). The application of different N fertilizers can shift the competitive balance between these microbial groups, directly impacting the ecological services provided by soil organisms, such as carbon sequestration and plant productivity (Zhang et al. 2016; Ai et al. 2018; Cui et al. 2018). Recent studies underscore the complex relationship between Brachiaria grass species and N cycling, underscoring the necessity of further research into how N fertilizers impact soil microbial communities (Momesso et al. 2022; Merloti et al. 2023). Understanding these interactions is crucial for optimizing N uptake by plants, enhancing soil fertility, and reducing negative environmental outcomes, such as nutrient leaching and greenhouse gas emissions (Schulz et al. 2017; Yue et al. 2022).

The process of soil nitrification plays a pivotal role in making N available for plant uptake. Bacterial and archaeal ammonia oxidizers (AOB and AOA, respectively) are the main agents in converting ammonia (NH4+) to nitrite (NO2−), which is then quickly transformed into nitrate (NO3−) by nitrite-oxidizing bacteria (Pajares and Bohannan 2016). Both AOA and AOB are widely distributed and abundant in various soil environments, and their prevalence and activity levels often surpass those of nitrite-oxidizing organisms, making them more influential in the overall soil nitrification process (Prosser and Nicol 2008). The activity and population dynamics of AOA and AOB are sensitive to a wide range of ecological and environmental factors, including soil pH, temperature, moisture content, and the presence of ammonia and organic matter (Levičnik-Höfferle et al. 2012). This sensitivity makes them important indicators of soil health and fertility, and their study can provide insights into the effects of agricultural practices and climate change on N cycling (Ayiti and Babalola 2022; Zhang et al. 2024). Thus, this study aims to explore how different N fertilizer sources affect the soil microbiome, especially those involved in the nitrification process, and their subsequent impact on plant growth in Brachiaria grasslands. We hypothesize that variations in N fertilizer sources will change soil nutrient availability, influencing the competition dynamics for resources among prokaryotic and fungal communities, as well as among ammonia-oxidizing organisms. These changes are anticipated to affect plant growth and development in Brachiaria grasslands. Throughout the investigation, we aimed to address the following key questions: (i) How do different N fertilizers affect soil characteristics? (ii) How do prokaryotic and fungal communities, and their potential functions, vary with different N fertilizers? (iii) To what extent do N fertilizers affect nitrification and its microbial agents in the soil? (iv) What are the implications for Brachiaria development? Through addressing these questions, we aim to enhance understanding of the complex interplay between N fertilizers and the soil microbiome, contributing to sustainable Brachiaria grassland management in Brazil.
3.2. Methodology

3.2.1. Site characteristics and fieldwork experiment

The field experiment was conducted at the Experimental Farm Station of São Paulo State University (UNESP) in Botucatu, São Paulo State, Brazil (22° 83′ 3″ S, 48° 42′ 64″ W, 765 m). The soil site is classified as sandy clay loam, kaolinitic, and thermic Typic Haplorthox (USDA, 2022). The region’s climate classification, according to the Köppen-Geiger system, is Cwa, with an average annual rainfall of 1360 mm. The region experiences an annual temperature of 20.7°C (based on measurements from 1956 to 2020), with a maximum of 26.1°C and a minimum of 15.3°C. The predominant soil texture in the field experiment was clayish. Supplementary Table 1 provides the land-use history of the experimental field for the previous 10 years.

The experiment, conducted in 2019 within a no-till site, involved the sowing of Brachiaria grass in plots measuring 15 m × 20 m with a 2 m space between each plot. Initial phosphorus (P) and potassium (K) fertilization were applied in the seedling line based on prior soil fertility analysis and recommended values for São Paulo state (Cantarella et al. 1998). Four varieties of Brachiaria grasses were sown (B. ruiziiensis, B. brizantha cv. BRS Paiaguás, B. brizantha cv. Marandu, B. spp. cv. BRS Ipyporã), but they were not considered as a main factor in the study and were included as a random factor in the subsequent statistical analysis. Additional information about the Brachiaria grass varieties chosen in this study is available in Merloti et al. (2023).

After fifteen months post-sowing, mature forage grasses underwent mechanical mowing and were removed from the site to simulate intensive grazing. Subsequently, N sources were applied in Brachiaria grass. These included ammonium sulfate ((NH₄)₂SO₄; treatment SA; 20% of N) as an inorganic N source, urea as an amidic source (NH₂; treatment U; 45% of N), castor meal as an organic source (treatment CM; 5% of N). The last one by-product with high N content remaining after extracting oil from the seeds of the castor bean plant (Ricinus communis). In addition, a control without N fertilizers was added (treatment C). All N source fertilizers were applied in a single dose of 60 kg/ha, following recommended guidelines for grass recovery after intense grazing (Costa et al. 2006). The experimental design comprised 16 plots per level of fertilizer (control without N, ammonium sulfate, castor meal, and urea), totaling 64 plots in 4 randomized blocks (schematic figure is available in Supplementary Figure 2.1). Four soil samplings were conducted over one month following the application of N sources (1st, 7th, 15th, and 30th day). Based on ammonium and nitrate results, the 7th day was identified as the most suitable for representing the soil microbiome response to the proposed treatments (Supplementary Figure 2.2). Following this selection, soil samples were characterized for their physico-chemical properties, and total DNA was extracted for further analysis.

3.2.2. Soil and plant sampling

In each plot, a total of five points were sampled, including one central point and four in each corner, within the 0-10 cm soil layer. A meticulous filtering process involved shaking the sampled roots to eliminate non-adhered soil. Afterwards, each of the 5 points were mixed into a composite sample. Approximately 600 g of these samples were collected for detailed analysis of soil chemical and texture characteristics. Undisturbed soil samples were gathered using an auger and metal rings for subsequent analysis of density and porosity. Furthermore, 50 mg samples were immediately frozen in liquid nitrogen and stored at -20°C for follow DNA extraction. In each plot, the
biomass of the shoot plants was gathered in an area measuring 1 × 1 m, while the roots of the *Brachiaria* were sampled five times, matching the location of soil sampling using an auger and metal rings (100 cm³).

### 3.2.3. Plant biomass and soil characteristics

The shoot biomass and roots of the *Brachiaria* were then dried for three days at 60°C in an oven with forced ventilation. The samples were then weighed and converted to kg/ha. The soil nutrients were measured based on the methodology proposed for Brazilian tropical soils (Cantarella et al. 1998). Details about each method were described by Merloti et al. (2023). Briefly, soil pH and organic matter were measured using 0.01M CaCl₂ and Walkley–Black method, respectively. Phosphate (P), potassium (K⁺), calcium (Ca²⁺), and magnesium (Mg²⁺) were determined using anion exchange resin and calorimetric method. Sulfate (S) was quantified using BaSO₄ turbidimetric method after extraction with 0.01 M calcium phosphate solution. Potential acidity (H⁺Al) was determined by the Shoemaker-McLean-Pratt method. Based on the results, the sum of bases (SB) was calculated by the sum of the cations K⁺, Ca²⁺, and Mg²⁺; cation exchange capacity (CEC) by the sum of H⁺Al and cations; soil available nutrients (Vₐ) was determined by SB/CEC. Ammonium (NH₄⁺) and nitrate (NO₃⁻) were extracted using KCl and quantified by the calorimetric method. The sum of NH₄⁺ with NO₃⁻ resulted in the N-available. The soil porosity analysis was based on saturation, weighing, and the use of Richard’s pressure chamber. The total porosity was calculated by determining the difference in weight between the water-saturated and dried samples (Klute and Dirksen 1986; Smith and Mullins 2001).

### 3.2.4. Soil DNA extraction and PCR in real-time (qPCR)

The total DNA from soil samples was extracted using the Power Soil DNA Isolation Kit (Qiagen, Hilden, German) based on 0.25 g and following the manufacturer’s instructions. The DNA concentration was measured using the Qubit fluorometer (Invitrogen, Carlsbad, USA) and its quality was checked through agarose gel in electrophoresis analysis (Brody and Kern 2004). The abundance of microbial communities was quantified through StepOnePlus™ Real-Time PCR System (qPCR) with 96-well plates (Applied Biosystems, Foster City, CA, USA). The qPCR was carried out for the bacterial, archaeal and fungal communities. Also, the quantification of microbes related to the N-cycle was performed, including the bacterial ammonia-oxidizers and archaeal ammonia-oxidizers. Details about the qPCR mix components for each gene are described by Merloti et al. (2023). Strains used to construct the standard curves, primers, and reaction conditions for the amplification of the genes are described in Supplementary Table 1.4.

### 3.2.5. DNA sequencing and bioinformatic analysis

The DNA sequencing and bioinformatic analysis were conducted at the Center for Functional Genomic Research (ESALQ/USP) in Piracicaba, São Paulo, Brazil. Amplicon sequencing was performed using the primers 515F-806R for V4 region of 16S rRNA for prokaryotic communities (Caporaso et al. 2011; Apprill et al. 2015; Parada et al. 2016) and ITS1F-ITS2 for fungi communities (Gardes and Bruns 1993; Smith and Peay 2014). A total
of 60 libraries for each barcode were prepared using the Illumina MiSeq platform, generating paired-end sequences (2 × 150 bp). The DADA2 pipeline (Callahan 2017) in the R environment was employed for data processing, involving primer removal, trimming, filtering for quality, denoising inference, merging of forward and reverse sequences, and removal of chimeric sequences. Taxonomic inference utilized the SILVA database for 16S rRNA data (Quast et al. 2012) and UNITE for ITS (Kõljalg et al. 2013). Finally, the prokaryotic matrix was filtered to remove sequences classified as mitochondria and chloroplast and rarefied to 27535 sequences per sample, and the fungal matrix to 22687 sequences per sample (Supplementary Figure 2.3). Sequences were submitted to the NCBI Sequence Read Archive under the identification BioProject ID PRJNA947540.

3.2.6. Statistical analysis

The data were analyzed using the R platform, graphs were created with the “ggplot2” package (Wickham 2016), and statistical analysis was performed with the packages “vegan” (Oksanen et al. 2013), “agricolae” (De Mendiburu and Simon 2015) and “lme4” for mixture models (Bates et al. 2014). Distance-based redundancy analysis (db-RDA) assessed the structure of soil prokaryotic and fungal communities and their correlation with soil characteristics. The significance of soil characteristics in the distribution of biological communities was determined through the PERMANOVA test. Fertilizer effects on microbial communities were tested using the Adonis permutational test, followed by pairwise Adonis tests. Soil microbial diversity and richness were calculated using the "vegan" package. Utilizing the "microeco" package (Liu et al. 2021), random forest analysis and Mean Decrease Gini index identified the 20 most important taxa groups for prokaryotic and fungal communities across different fertilizers, considered as biomarkers. Functional prediction annotation was performed using the FAPROTAX database for prokaryotic communities (Louca et al. 2016) and the FUNGuild database for fungal communities (Nguyen et al. 2016). Mixture models tested variables from soil characteristics, microbial gene abundance, diversity, richness, and potential functions, with fertilizer as a fixed factor and blocks and varieties of Brachiaria grass as random factors.

3.3. Results

3.3.1. Soil characteristics and plant biomass

The N-fertilizer ammonium sulfate decreased the soil pH (5.4) and H+Al (28 mmolc/dm3) compared to the other treatments (Figure 5A and 5B). The urea fertilizer statistically increased soil available nutrients (72.4 mmolc/dm³; Figure 5C). The S increased with ammonium sulfate (51 mg/kg of dry soil) and castor meal fertilizer application (16.5; Figure 5F). Soil N-available increased with all treatments, with ammonium sulfate showing the highest value (32 g/kg of dry soil), followed by urea (24) and castor meal (10; Figure 5G). A decrease in shoot biomass of 1003, 220, and 330 kg/ha was observed with the application of ammonium sulfate, castor meal, and urea, respectively, compared to the control treatment (Figure 5H). In contrast, there was an increase in root biomass of 110, 130, and 240 kg/ha with ammonium sulfate, castor meal, and urea, respectively (Figure 5I). There were no statistical differences for soil organic matter and P-Total (Figure 5D and 5E), as well as soil clay content, density, porosity, and N-total (Supplementary Figure 2.4).
Figura 5. Box plots representing soil and plant characteristics of Brachiaria grass under N-fertilizers. Values represent averages and standard deviations from 20 replicates. Lowercase letters denote differences determined by a linear mixed-effect model. Group comparisons utilized the Tukey test, with p-values adjusted using FDR correction. Abbreviations for fertilizers: C = control without N, AS = ammonium sulfate, CM = castor meal, U = urea.

3.3.2. Soil microbiome structure, composition, and biomarkers

The db-RDA analysis illustrated the structure of the prokaryotic community (Figure 6A) and revealed that pH, soil available nutrients (V), pH, and H+Al were the three primary environmental variables influencing the assemblage of the prokaryotic community (Figure 6B, PERMANOVA test, all with P<0.01). Moreover, the application of fertilizers statistically altered the soil prokaryotic communities (PERMANOVA test, P<0.01, Supplementary Table 2.1), indicating that all treatments were significantly different from each other (Supplementary Table 2.2). Additionally, the fertilizers showed no statistical difference in prokaryotic diversity and richness (Supplementary Figure 2.5A and B).

The prokaryotic composition is represented in Supplementary Figure 2.6. The most abundant phyla were Proteobacteria (29%), Actinobacteria (28%), Acidobacteria (10%), Bacteroidetes (6%), Chloroflexi (6%), Crenarchaeota (4%), and Firmicutes (4%), collectively constituting 87% of all prokaryotic communities. Biomarker analysis (Gini index) demonstrated that the genera Nocardioides, Chthoniobacter, Flavitalea, Solirubrobacter, and
Sphingomonas were associated with the ammonium sulfate fertilizer. For castor meal fertilizer, the analysis identified Chryseobacterium, Exiguobacterium, Stenotrophomonas, Staphylococcus, Flavobacterium, Taihuella, Dyadobacter, Pseudomonas, and Sphingobacterium as biomarkers. In the case of the urea fertilizer, the genera Sphingobium, Janibacter, and Flaviaesturariibacter were considered as biomarkers (Figure 7A).

The structure of fungal communities is represented through the db-RDA analysis (Figure 6C), highlighting soil available nutrients (V%), pH, and H+Al as the primary drivers of these communities (Figure 6D, PERMANOVA test, all with P<0.01). The application of fertilizers induced statistically significant modifications in soil fungal communities (PERMANOVA test, P<0.01, Supplementary Table 2.1), with all treatments showing statistical differences from each other, exceptionally urea fertilizer that showed no statistical difference with the control (Supplementary Table 2.2). Furthermore, the fertilizer exhibited no statistical difference in fungal diversity and richness (Supplementary Figure 2.5C and D, respectively, and P>0.05).

The composition of soil fungal communities is presented in Supplementary Figure 7. The phyla Ascomycota (77%), Basidiomycota (10%), Mortierellomycota (4%), Chytridiomycota (2%), and Glomeromycota (1%) collectively constitute 94% of all identified phyla. Biomarker analysis identified Vishnyacoziina, Isaria, Phaeosphaeria, and Spiromyces genera as biomarkers associated with the ammonium sulfate fertilizer. Wallemia, Nectriopsis, Gibberella, Mortirella, and Microdochium genera were designated as biomarkers for the castor meal fertilizer. In the case of the urea fertilizer, the genera Volutella and Humicola were considered biomarkers (Figure 7B).

**Figura 6.** Distance-based redundancy analysis (db-RDA) of prokaryotic (A) and fungal (C) communities correlated with soil characteristics, based on Bray-Curtis distance. Arrows indicate significant correlations between soil properties and microbial community composition (999 permutations, P < 0.05). Ranked bar plots for significant soil correlations are presented for prokaryotic (B) and fungal (D) communities. Abbreviations for fertilizers: C = control without N, AS = ammonium sulfate, CM = castor meal, U = urea.
Figura 7. Biomarkers analysis using the random forest algorithm for prokaryotic (A) and fungal (B) communities in soil under Brachiaria grass with N-fertilizers. The ranking showcases the 20 most important genera based on the Gini index and their respective relative abundances. Abbreviations for fertilizers: C = control without N, SA = ammonium sulfate, TM = castor meal, U = urea.

### 3.3.3. Soil microbiome potential functions and abundance of marker genes

The potential prokaryotic functions, as assigned by the FAPROTAX database, are illustrated in Figure 8A. All the fertilizers increased the abundance of chemoheterotrophy and aerobic chemoheterotrophy functions, but only urea and castor meal were significantly higher compared to the control. Nitrogen and Nitrate respiration functions were statistically higher in ammonium sulfate compared to castor meal. Also, photoautotroph and denitrification related functions statistically decreased with the fertilizers input compared to the control, exceptionally ammonium sulfate. Aromatic compound degradation was statistically higher in the castor meal while cellulolysis function in the control. The fungal communities were categorized into trophic levels using the FUNGuild database, and the results are presented in Figure 8B. The urea fertilizer notably increased the fungal groups classified with multiple trophic levels as Pathotroph.Saprotroph.Symbiotroph compared to the castor meal and ammonium sulfate fertilizers. The castor meal significantly increased the fungal groups identified as Saprotroph.Symbiotroph compared to the other treatments.
Figura 8. (A) Bubble-plot representing the differences in the abundance (%) of potential prokaryotic functions according to the FAPROTAX database. The “rare” function is based on the sum of functions that represents less than 1%. (B) Bubble-plot representing the differences in the abundance (%) of fungal functional groups (trophic mode) according to the FUNGuild database. Lowercase letters denote statistically differences determined by a linear mixed-effect model. Group comparisons utilized the Tukey test, with p-values adjusted using FDR correction. Abbreviations for fertilizers: C = control without N, SA = ammonium sulfate, TM = castor meal, U = urea.

The abundance of bacterial communities (represented by the 16S rRNA gene) exhibited a significant decrease with the application of castor meal (1.80e+05) and urea (1.40e+05) fertilizers, in comparison to ammonium sulfate (2.72e+05; Figure 9A). All the fertilizers decreased the total archaeal abundance (16S rRNA), but mainly urea (4.26e+03) and ammonium sulfate (4.37e+03) were significant compared to the control (5.73e+03; Figure 9B). Fungal abundance (ITS gene) increased with castor meal (1.83e+05) and urea (2.15e+05) fertilizers compared to ammonium sulfate (4.68e+04) and the control (1.11e+05; Figure 9C). Regarding bacterial ammonia oxidizers (AOB gene), only urea fertilizer (6.21e+01) significantly increased its abundance, while ammonium sulfate (4.47e+01) also showed a slight increase, compared to the control (3.26e+01; Figure 9D). Lastly, the abundance of archaeal ammonia oxidizers (AOA gene) significantly decreased with the application of ammonium sulfate fertilizer (4.22e+02) compared to U (1.77e+03; Figure 9E).
3.4. Discussion

*Brachiaria* grass plays a crucial role in Brazil, covering a significant portion of the territory and supporting the cattle industry (Nehring 2023). The application of N is essential for promoting plant growth, particularly in grassland establishment or recovery after intensive grazing (Boddey et al. 2004). N utilization efficiency is linked to the N source applied to the soil and its subsequent decomposition into assimilable forms through the microbial nitrification process (Norton and Ouyang 2019). Furthermore, N sources can alter other soil characteristics, such as pH, thereby changing the availability of soil nutrients and potentially leading to consequences for the soil microbiome (Lammel et al. 2018). In this study, we investigated N fertilization in grasses using various N sources (organic, amidic, and inorganic) and examined its consequences on the soil microbiome, including bacterial and fungal communities, as well as nitrifying organisms. These changes may have significant repercussions on N cycling and the development of grasses.

All sources increased the amount of N available in the soil, with ammonium sulfate showing the highest concentration, followed by urea and castor meal. This sequence of N release into the soil solution is likely related to the chemical characteristics of each fertilizer. When added to the soil, the ammonium sulfate molecule \((\text{NH}_4)_2\text{SO}_4\)
undergoes chemical dissociation into 2 moles of NH$_4^+$ and 1 of SO$_4^{2-}$ (Chien et al. 2011). The dissociation is dependent on soil pH, temperature, moisture and is less dependent of soil microbial communities, which emerge as important factors in the subsequent nitrification process, converting NH$_4^+$ molecules into NO$_3^-$ (Chien et al. 2011; Norton and Ouyang 2019). This makes the N from ammonium sulfate quickly available for absorption by plants and/or immobilized by microbes but also susceptible to leaching, causing water contamination due to the high soil mobility of NO$_3^-$ molecules, or denitrification with potential consequences for climate change through N$_2$O emission (Chien et al. 2011; Norton and Ouyang 2019). In addition, ammonium sulfate can decrease soil pH due its high H$^+$ dissociation in the soil (Chien et al. 2011), leading to a reduction in soil nutrient availability (Lammel et al. 2018), as confirmed by the higher H+Al found in our results. Furthermore, we observed a significant increase in S as part of its chemical dissociation.

In contrast to ammonium sulfate, the transformation of urea fertilizer depends on the soil microbial enzyme called urease. Together with two moles of water, urease hydrolyzes the CO(NH$_2$)$_2$ molecule into two moles of NH$_4^+$ and one of HCO$_3^-$ (bicarbonate) (Chien et al. 2011). Urease can be produced by a range of microbial groups and plants, and its activity can be influenced by soil texture, pH, temperature and moisture (Kumari et al. 2016; Matczuk and Siczek 2021). Although urea increases soil N availability to an intermediate level compared to other sources, it also presents the highest amount of available soil nutrients, likely due to its non-soil acidification. Compared to ammonium sulfate and due the quick activity of urease enzymes, urea can undergo to a high volatilization, diminishing its efficiency to supply the plant N needs and potentially contaminating water and atmosphere (Powlson and Dawson 2022).

Castor meal showed the lowest increase in available N compared to the other treatments. This by-product of Ricinus communis seeds has a high dependency on microbial diversity for mineralizing the available N in its components (Lima et al. 2011). On contrary of other N organic sources, castor meal can be quicker mineralized by soil microbiome and surpassing the crop needs in many cases due its high N concentration (Lima et al. 2011; de Almeida et al. 2021). However, the castor meal C/N ratio, N concentration, soil moisture, and pH can affect its decomposition and N-releasing rate (Lima et al. 2011; de Almeida et al. 2021).

Our results indicated a pronounced focus on root development in Brachiaria grass, as evidenced by the increased root biomass rather than shoot biomass. The experiment, involving N application, was conducted during the dry season in Brazil, which may explain the grasses’ preference to invest in root growth for enhanced water capture (Santos et al. 2013).

Interestingly, we observed that all N fertilizers promoted prokaryotic chemoheterotroph and aerobic chemoheterotroph metabolism. These microbial groups extract energy and carbon sources from organic material (Balkwill 1989). The promotion of root development is often associated with increased aeration and the plant’s exudation of organic compounds that can increase bacterial and fungal abundance (Momesso et al. 2022; Merloti et al. 2023). Both factors likely occurred in our study, contributing to the promotion of microbial chemoheterotroph groups. The ammonium sulfate fertilizer specifically promoted Novosphingobium and Sphingomonas bacterial genera as biomarkers. These genera harbor species known to promote plant growth during stress conditions such as drought, salinity, and heavy metals in agricultural soil (Asaf et al. 2020; Ma et al. 2023). Castor meal revealed several biomarker genera with the potential for bioremediation and plant growth promotion, including Exiguobacterium, Staphylococcus, and Flavobacterium, known for cellulose decomposition (Máté et al. 2022). Urea highlighted biomarker genera with members applied for bioremediation and degradation, such as Sphingobium and Janibacter (Kalsi et al. 2020; Osaro-Matthew et al. 2022; Liu et al. 2024).
The application of fertilizers influenced fungal community guilds, with an increase in pathotrophic groups and a decrease in saprotrophic and symbiotrophic ones, although not statistically significant. The promotion of root growth by N fertilizers may contribute to increased resources available for pathogens, outcompeting symbiotrophic and saprotrophic groups due to their rapid growth and resilient structures that persist in the soil (Lekberg et al. 2021). Interestingly, urea and castor meal fertilizers promoted fungal growth over bacterial communities, while the opposite was observed for ammonium sulfate, which maintained bacterial communities outnumbering fungi. As a consequence, urea and castor meal fertilizers led to a decrease in prokaryotic functions related to photoautotroph and denitrification, whereas no significant changes were found for ammonium sulfate, maintaining these functions at control levels. The higher NH$_4^+$ release rate by ammonium sulfate may have favored bacterial abundance and competition for resources against fungal communities. The NH$_4^+$ is considered an important energy source for microbial groups, promoting their growth and carbon fixing (Shanmugam and Valentine 1975; Dividson et al. 1990; Kim et al. 2022). This explanation is further supported by the higher organic matter content found in soils receiving ammonium sulfate in our study compared to other N sources. Conversely, the lower and constant rate of NH$_4^+$ release in soils treated with urea and castor meal favored fungal growth over total bacterial communities, nurturing fast-growing groups that included many plant pathogens. This is supported by urea promoting the *Volatella* genus as a biomarker, known to harbor various crop disease groups (Cannon et al. 2012). Castor meal, in turn, presented *Gibberella* and *Microdochium* genera as biomarkers, both recognized for harboring phytopathogenic fungi (Hsuan et al. 2011; Gao et al. 2022). Furthermore, urea stimulated the *Humicola* genus, encompassing decomposer fungi (Ibrahim et al. 2021), while castor meal favored the *Mortierella* genus, known for hosting decomposers and phosphorus-solubilizing groups (Sang et al. 2022; Li et al. 2023).

The abundance of bacterial and archaeal ammonia-oxidizers revealed that ammonium sulfate was the fertilizer that most promoted bacterial ammonia-oxidizers, while simultaneously decreasing the archaeal ones. These findings align with previous studies highlighting the preference of bacterial ammonia oxidizers for N available rich environments (Norton and Ouyang 2019; Prosser et al. 2020), as observed in our results and promoted by ammonium sulfate. The continuous and slow release of N from organic sources can benefits AOA over AOB groups in soil due the AOA higher affinity with environments with less available NH$_4^+$ (Prosser et al. 2020), which may explain its decrease with ammonium sulfate and increase with castor meal. However, urea promoted the abundance of both bacterial and archaeal ammonia-oxidizer groups, suggesting that the type of N fertilizer can selectively influence the agents responsible for soil nitrification, and urea benefiting both groups.

### 3.5. Conclusion

Our study assessed the impact of various N fertilizers on soil prokaryotic and fungal communities, with a specific focus on the nitrification process. Considering changes in soil characteristics, we found a soil acidification by ammonium sulphate and an increased in S content by castor meal and ammonium sulphate. Besides, the fertilizers released N in different rates and consequently affecting the soil microbiome. Ammonium sulphate, recognized as the most efficient N releaser, promoted the growth of bacterial communities over fungal communities, maintaining N functions related to nitrification and denitrification at the same level of the control. Conversely, urea and castor meal stimulated fungal growth over bacterial communities, particularly favoring fungal genera associated with plant diseases, while diminishing N functions related to N cycling. Ammonium sulphate, being the most effective N releaser, also demonstrated a pronounced impact on nitrification, likely associated with bacterial ammonia-oxidizers.
All fertilizers enhanced root development over shoot biomass. Future research should explore sustainable agricultural practices that promote crop development while mitigating potential adverse environmental effects linked to specific fertilizers.

References


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Matczuk D, Siczek A (2021) Effectiveness of the use of urease inhibitors in agriculture: a review. International Agrophysics 35:


Powlsen DS, Dawson CJ (2022) Use of ammonium sulphate as a sulphur fertilizer: Implications for ammonia volatilization. Soil use and Management 38:622–634


Smith DP, Peay KG (2014) Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. PloS one 9:e90234
4. THESIS CONCLUSION

This thesis has comprehensively explored the interactions between soil, plant varieties, and fertilizers with regard to their impact on soil properties and microbial communities, emphasizing agricultural productivity and environmental sustainability.

The first study provided detailed insights into how different *Brachiaria* varieties influence soil chemistry, structure, and microbial dynamics. Particularly, the *Brachiaria Ruziziensis* and *Brachiaria spp.* cv. Ipyporã varieties illustrated distinct impacts on soil pH, nutrient levels, and microbial communities, with the BI variety enhancing soil porosity, biomass production, and promoting beneficial microbial functions like those associated with arbuscular mycorrhizal fungi (AMFs). These findings suggest that selecting *Brachiaria* varieties based on their specific impacts on soil and microbial health can significantly enhance soil quality and sustainability in agricultural systems.

The second study focused on the effects of various nitrogenous fertilizers on soil microbial communities and highlighted the dual role of fertilizers in enhancing plant growth and altering microbial dynamics. Ammonium sulphate emerged as a potent N releaser, promoting bacterial communities that support crucial nitrogen cycling processes such as nitrification and denitrification. In contrast, urea and castor meal promoted fungal growth, including genera associated with plant diseases.

An important limitation of the studies is that none included animal interactions as a variable, which could have introduced significant modifications to the outcomes. The presence of grazing animals could alter physical soil parameters, such as density and porosity, and influence soil chemistry through the addition of manure and urine. These changes might have further interacted with the consumption patterns of the pastures, thus impacting both soil and plant characteristics, as well as microbial community dynamics.

Despite these limitations, the findings contribute valuable knowledge for the application of *Brachiaria* in intercropping and rotational cropping systems. Integrating the results from both studies highlights the critical roles of plant variety selection and fertilizer application in shaping soil health and microbial ecosystems. These insights are pivotal for the development of sustainable agricultural strategies that enhance crop productivity while minimizing adverse environmental impacts.
APPENDICES

APÉNDICE A. Appendix A - Supplementary material from chapter 1.

**Supplementary Table 1.1.** Land use history, including the crops grown and liming timeline from 2002 to 2019.

<table>
<thead>
<tr>
<th>Growing season</th>
<th>Crops</th>
<th>Liming</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002/2003</td>
<td>Oriza sativa</td>
<td>2000 kg ha⁻¹ of dolomitic lime (71% ECCE)†</td>
</tr>
<tr>
<td></td>
<td>Apr. Avena strigosa</td>
<td></td>
</tr>
<tr>
<td>2003/2004</td>
<td>Jan. Phaseolus vulgaris</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Apr. Avena strigosa</td>
<td>-</td>
</tr>
<tr>
<td>2004/2005</td>
<td>Nov. Arachis hypogaea</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Apr. Avena sativa</td>
<td>-</td>
</tr>
<tr>
<td>2005/2006</td>
<td>May Avena sativa</td>
<td>-</td>
</tr>
<tr>
<td>2006/2007</td>
<td>Feb. Zea mays</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Jun. Avena strigosa</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dec. Zea mays</td>
<td>2500 kg ha⁻¹ of dolomitic lime (76% ECCE)†</td>
</tr>
<tr>
<td>2007/2008</td>
<td>Jun. Avena sativa</td>
<td>-</td>
</tr>
<tr>
<td>2008/2009</td>
<td>Dec. Glycine max</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mar. Zea mays</td>
<td>-</td>
</tr>
<tr>
<td>2009/2010</td>
<td>Oct. Glycine max</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mar. Sorgum vulgare</td>
<td>-</td>
</tr>
<tr>
<td>2010/2011</td>
<td>Oct. Glycine max</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mar. Zea mays</td>
<td>-</td>
</tr>
<tr>
<td>2011/2012</td>
<td>Oct. Glycine max</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mar. Zea mays</td>
<td>-</td>
</tr>
<tr>
<td>2012/2013</td>
<td>Oct. Glycine max</td>
<td>3000 kg ha⁻¹ of dolomitic lime (88% ECCE)†</td>
</tr>
<tr>
<td></td>
<td>Mar. Zea mays</td>
<td>-</td>
</tr>
<tr>
<td>2013/2014</td>
<td>Oct. Glycine max</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mar. Zea mays</td>
<td>-</td>
</tr>
<tr>
<td>2014/2015</td>
<td>Oct. Glycine max</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mar. Zea mays</td>
<td>-</td>
</tr>
<tr>
<td>2015/2016</td>
<td>Oct. Glycine max</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mar. Zea mays</td>
<td>-</td>
</tr>
<tr>
<td>2016/2017</td>
<td>Oct. Glycine max</td>
<td>5000 kg ha⁻¹ of dolomitic lime (69% ECCE)†</td>
</tr>
<tr>
<td></td>
<td>Mar. Zea mays</td>
<td>-</td>
</tr>
<tr>
<td>2017/2018</td>
<td>Oct. Glycine max</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mar. Zea mays</td>
<td>-</td>
</tr>
<tr>
<td>2018/2019</td>
<td>Ago. Fallow</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Jan. Brachiaria varieties *</td>
<td>-</td>
</tr>
</tbody>
</table>

Lime reapplication was performed when base saturation reached ≤ 50%.

†ECCE: effective calcium carbonate equivalents

*Beginning of the experiment

**Supplementary Table 1.2.** Soil texture characteristics before the experiment implementation.

<table>
<thead>
<tr>
<th>Soil Texture</th>
<th>Clay (g/ Kg)</th>
<th>Silt</th>
<th>Total Sand</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>309</td>
<td>35</td>
<td>656</td>
</tr>
</tbody>
</table>
Supplementary Table 1.3. Characteristics of the varieties of *Brachiaria* selected in this study.

<table>
<thead>
<tr>
<th>Varieties/Characteristics</th>
<th>BR Brachiaria ruziziensis</th>
<th>BP Brachiaria brizantha cv. BRS Paiaguás</th>
<th>BM Brachiaria brizantha cv. Marandu</th>
<th>BI Brachiaria spp. cv. Ipyporã - BRS RB331</th>
</tr>
</thead>
<tbody>
<tr>
<td>Usage</td>
<td>Grazing and hay</td>
<td>Grazing and hay</td>
<td>Grazing and hay</td>
<td>Grazing and hay</td>
</tr>
<tr>
<td>Intercropping</td>
<td>All legumes</td>
<td>All legumes</td>
<td>All legumes</td>
<td>All legumes</td>
</tr>
<tr>
<td>Life cycle</td>
<td>Perennial</td>
<td>Perennial</td>
<td>Perennial</td>
<td>Perennial</td>
</tr>
<tr>
<td>Drought tolerance</td>
<td>Good</td>
<td>Tolerant</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Leafhopper tolerance</td>
<td>Medium</td>
<td>Medium</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Waterlogging tolerance</td>
<td>Medium</td>
<td>Medium</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Tolerance to cold</td>
<td>Medium</td>
<td>Medium</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Digestibility</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Super High</td>
</tr>
<tr>
<td>Palatability</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Altitude</td>
<td>Up to 2000m</td>
<td>Up to 2000m</td>
<td>Up to 2000m</td>
<td>Up to 2000m</td>
</tr>
<tr>
<td>Annual precipitation</td>
<td>Over 1000m</td>
<td>Over 800m</td>
<td>Over 1000m</td>
<td>Over 1000m</td>
</tr>
<tr>
<td>Growth habit</td>
<td>Clumping</td>
<td>Tufted</td>
<td>Tufted</td>
<td>Tufted</td>
</tr>
<tr>
<td>Germination</td>
<td>7 to 21 days</td>
<td>7 to 21 days</td>
<td>7 to 21 days</td>
<td>7 to 21 days</td>
</tr>
<tr>
<td>Planting depth</td>
<td>2 to 3 cm</td>
<td>2 to 3 cm</td>
<td>2 to 3 cm</td>
<td>2 to 6 cm</td>
</tr>
<tr>
<td>Breeding goals</td>
<td>Resistance to pasture plague insects (<em>Notozulia entreiriana</em> and <em>Deois flavopicta</em>)</td>
<td>High productivity and resistance to drought and pests</td>
<td>High productivity and adaptation to different soils</td>
<td>Resistance to pasture plague insects (<em>Notozulia entreiriana</em> and <em>Deois flavopicta</em>)</td>
</tr>
</tbody>
</table>


Supplementary Figure 1.1. Schematic graphic representing the experimental study design, size, and distribution of each plot. Black dots represent the points sampled within each plot and mixed to form a composite sample per plot.
**Supplementary Table 1.4.** Description of primers used in qPCR and amplicon sequencing analysis.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>*DSMZ code</th>
<th>Primers</th>
<th>Sequences (5'→3')</th>
<th>Fragment length (pb)</th>
<th>Reference(s)</th>
<th>Amplification condition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>16S rRNA</strong> of Bacteria</td>
<td>11192 (Gordonia sp.)</td>
<td>Eub 338f Eub 518r</td>
<td>ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG</td>
<td>180</td>
<td>Bakke et al. (2011)</td>
<td>qPCR: 95°C-10 min, 40 cycles, 95°C-40s, 54°C-40s, 72°C-40s; Melting: 95°C-15s, 55°C-1 min, 95°C-15s</td>
</tr>
<tr>
<td><strong>16S rRNA</strong> of Archaea</td>
<td>23604 (Methanothermoana mesophile)</td>
<td>519F 915R</td>
<td>CAGCCGCCGCGGTAA GTGCTCCCCCGCCAATTCCT</td>
<td>397</td>
<td>Coolen et al. (2004); Stahl, Amann (1991)</td>
<td>qPCR: 95°C-10 min; 40 cycles, 95°C-30s, 57°C-30s, 72°C-30s; Melting: 95°C-15s, 57°C-1 min, 95°C-15s</td>
</tr>
<tr>
<td><strong>ITS</strong></td>
<td>Fragment isolated from Rhizoctonia solani</td>
<td>ITS1f 5.8S</td>
<td>CTGGTCATTTAGAGGAAGTA A CGGTGCGGTTCATTCG</td>
<td>300</td>
<td>Fierer et al. (2005)</td>
<td>qPCR: 94°C-15 min; 40 cycles, 94°C-1 min, 53°C-30s, 72°C-1 min; Melting: 95°C-15s, 72°C-1 min, 96°C-15s</td>
</tr>
<tr>
<td><strong>amoA</strong> of Bacteria</td>
<td>28437 (Nitrosomonas europaea)</td>
<td>amoB1F amoB2R</td>
<td>GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC</td>
<td>491</td>
<td>Rotthauwe et al. (1997)</td>
<td>qPCR: 95°C-10 min; 40 cycles, 95°C-45s, 60°C-45s, 72°C-45s; Melting: 95°C-15s, 60°C-1 min, 95°C-15s</td>
</tr>
<tr>
<td><strong>amoA</strong> of Archaea</td>
<td>Environmenta 1DNA</td>
<td>amoA1F amoA2R</td>
<td>STA ATG GTC TGG CIT AGA CG GCG GCC ATC CAT CTG TAT GT</td>
<td>635</td>
<td>Francis et al. (2005)</td>
<td>qPCR: 95°C-5 min; 40 cycles, 95°C-40s, 56°C-30s, 72°C-1 min; Melting: 95°C-15s, 57°C-1 min, 95°C-15s</td>
</tr>
</tbody>
</table>

| 16S rRNA (v4 region) | - | 1132r | CCGTCAATTHCTTYAART | 300 | Parada et al. (2016) and Apprill et al. (2015) – updated primers | PCR: 25ul, Roche FastStart High Taq, 58°C, 26 cycles |
| **ITS** | - | ITS1F | CTGGTCATTTAGGAAGTA A | 300 | Gardes, Bruns (1993); Smith, Peavy (2014) | PCR: 25ul, Qiagen HotStar Taq, 52°C, 33 cycles |

| **ITS** | - | ITS2 | GCTGCGTTCCTCATCGATGC | Variable | |

*Reference code of the cell catalog of the Leibniz Institute DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), used in the construction of the standard curves for qPCR analysis*
### Supplementary Table 1.5. Count sequences from 16S rRNA and ITS after processing step (which includes filtering, denoise, merging, and removal of chimerical sequences) and after rarefaction.

<table>
<thead>
<tr>
<th>Sample_ID</th>
<th>ITS</th>
<th>16S</th>
<th>After filtering mitochondria and chloroplast/rarefaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>After processing</td>
<td>After rarefaction</td>
<td>After processing</td>
<td></td>
</tr>
<tr>
<td>BR_1</td>
<td>38640</td>
<td>22687</td>
<td>41388</td>
</tr>
<tr>
<td>BR_2</td>
<td>49775</td>
<td>22687</td>
<td>42734</td>
</tr>
<tr>
<td>BR_3</td>
<td>53181</td>
<td>22687</td>
<td>44098</td>
</tr>
<tr>
<td>BR_4</td>
<td>28702</td>
<td>22687</td>
<td>42407</td>
</tr>
<tr>
<td>BP_1</td>
<td>44935</td>
<td>22687</td>
<td>38990</td>
</tr>
<tr>
<td>BP_2</td>
<td>28808</td>
<td>22687</td>
<td>47004</td>
</tr>
<tr>
<td>BP_3</td>
<td>22798</td>
<td>22687</td>
<td>38414</td>
</tr>
<tr>
<td>BP_4</td>
<td>26671</td>
<td>22687</td>
<td>37135</td>
</tr>
<tr>
<td>BM_1</td>
<td>38299</td>
<td>22687</td>
<td>41576</td>
</tr>
<tr>
<td>BM_2</td>
<td>37814</td>
<td>22687</td>
<td>44197</td>
</tr>
<tr>
<td>BM_3</td>
<td>22687</td>
<td>22687</td>
<td>36267</td>
</tr>
<tr>
<td>BM_4</td>
<td>52041</td>
<td>22687</td>
<td>46263</td>
</tr>
<tr>
<td>BI_1</td>
<td>28869</td>
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<td>40776</td>
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<td>BI_2</td>
<td>27214</td>
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<td>49721</td>
</tr>
<tr>
<td>BI_3</td>
<td>35133</td>
<td>22687</td>
<td>45691</td>
</tr>
<tr>
<td>BI_4</td>
<td>28593</td>
<td>22687</td>
<td>60923</td>
</tr>
<tr>
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<td>22687</td>
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<tr>
<td>Ctrl_3</td>
<td>38460</td>
<td>22687</td>
<td>40114</td>
</tr>
<tr>
<td>Ctrl_4</td>
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<td>57308</td>
</tr>
<tr>
<td>Total</td>
<td>727282</td>
<td>453740</td>
<td>885241</td>
</tr>
</tbody>
</table>

Ctrl = Control; BR = *Brachiaria ruziziensis*; BP = *Brachiaria brizantha* cv. BRS Paiaguás; BM = *Brachiaria brizantha* cv. Marandu; BI = *Brachiaria* spp. cv. BRS Ipyporã.

### Supplementary Figure 1.2. Rarefaction curve from 16S rRNA (A) and ITS (B) after processing step (which includes filtering, denoise, merge and, removal of chimerical sequences).
Supplementary Table 1.6. Inertia values from the distance-based redundancy analysis (db-RDA).

<table>
<thead>
<tr>
<th></th>
<th>Prokaryotes</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inertia</td>
<td>Proportion (%)</td>
</tr>
<tr>
<td>Total</td>
<td>0.6086</td>
<td>100</td>
</tr>
<tr>
<td>Constrained</td>
<td>0.3508</td>
<td>57.64</td>
</tr>
<tr>
<td>Unconstrained</td>
<td>0.2578</td>
<td>42.36</td>
</tr>
</tbody>
</table>

Supplementary Table 1.7. Adonis test result for the bacterial and fungal soil communities. The analysis was based on the Bray-Curtis distance with 999 permutations. The analysis model considered the block as an aleatory factor and the varieties of Brachiaria as a fixed factor.

<table>
<thead>
<tr>
<th></th>
<th>Prokaryotes</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Model R² (%)</td>
<td></td>
</tr>
<tr>
<td>F-Value</td>
<td>5.10</td>
<td>6.35</td>
</tr>
<tr>
<td>P-Value</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Supplementary Figure 1.3. Box-plot representing the prokaryotic diversity (A) and richness (C) and fungal diversity (B) and richness (D). Prokaryotic data was based on 16S rRNA sequencing classification at the genera level, and fungal data was based on ITS sequencing classification at the species level. Lower case letters indicate statistical differences based on the One-Way ANOVA test, followed by the LSD test to compare groups (P<0.05) with p-value adjusted by Bonferroni correction.

- **A**: Bacteria Diversity
- **B**: Fungi Diversity
- **C**: Bacteria Richness
- **D**: Fungi Richness

Legend:
- **Ctrl**: Control
- **CR**: Brachiaria ruziensis
- **BP**: Brachiaria brizantha cv. BR5 Palagás
- **BM**: Brachiaria brizantha cv. Marandu
- **BR**: Brachiaria spp. cv. BR5 ljuven3
Supplementary Figure 1.4. Top 5 Fungal and Prokaryotic taxa that contributed (in %) to Simper dissimilarity analysis between the Control and each variety of *Brachiaria*. **Ctrl** – Control; **BR** - *Brachiaria ruziziensis*; **BP** - *Brachiaria brizantha* cv. BRS Paiaguás; **BM** - *Brachiaria brizantha* cv. Marandu; **BI** - *Brachiaria* spp. cv. BRS Ipyporã.

**Simper Dissimilarity Contribution**

**Legend:**
- **Fungi taxa**
- **Prokaryotic taxa**

Supplementary Figure 1.5. Top 5 Fungal and Prokaryotic taxa abundance that contributed to Simper dissimilarity analysis between the Control and each variety of *Brachiaria*. **Ctrl** – Control; **BR** - *Brachiaria ruziziensis*; **BP** - *Brachiaria brizantha* cv. BRS Paiaguás; **BM** - *Brachiaria brizantha* cv. Marandu; **BI** - *Brachiaria* spp. cv. BRS Ipyporã.
Supplementary Figure 1.6. Random forests analysis for biomarkers of each variety of Brachiaria. Ranking of the 20 most important genera (Gini index) and their relative abundance for Prokaryotic (A) and Fungal taxa (B). Ctrl – Control; BR - Brachiaria ruiziiensis; BP - Brachiaria brizantha cv. BRS Paiaguás; BM - Brachiaria brizantha cv. Marandu; BI - Brachiaria spp. cv. BRS Ipypora.
### Supplementary Table 1.8. Potential predicted functions results (%) according to the FAPROTAX database (for prokaryotic communities).

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<th>Potential Functions</th>
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<th>BP</th>
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<th>BI</th>
<th>Average</th>
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<td>5.83</td>
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<td>1.69</td>
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</table>

*The “Rare” function is based on the sum of functions that represents less than 1%.

### Supplementary Figure 1.7. Box-plot representing differences in the abundance (%) of fungal functional groups (Guild classification) according to the FUNGuild database (based on ITS sequencing). Low case letters in the box-plot represent functional groups with a statistical difference based on the One-Way ANOVA, followed by the post-hoc Tukey-Kramer test (P < 0.05) and after Benjamini-Hochberg FDR correction. Ctrl – Control; BR - Brachiaria ruziziensis; BP - Brachiaria brizantha cv. BRS Paiaguás; BM - Brachiaria brizantha cv. Marandu; BI - Brachiaria spp. cv. BRS Ipyporã.
Supplementary Figure 1.8. Spearman rank correlogram between genes abundance with plant and soil characteristics. Only the significant correlations were plotted (P< 0.05 and after Bonferroni correction).
APÊNDICE B. Appendix B - Supplementary material from chapter 2.

**Supplementary Figure 2.1.** Schematic plot representing the experimental study design, size, and distribution of each plot. Black dots represent the points sampled within each plot and mixed to form a composite sample per plot.

**Supplementary Figure 2.2.** Average N-Available (NH$_4$+NO$_3$) content in soils under *Brachiaria* grass and N-fertilizers.
Supplementary Figure 2.3. Rarefaction curve from 16S rRNA (A) and ITS (B) after-processing step (which includes filtering, denoise, merge and, removal of chimerical sequences).

Supplementary Figure 2.4. Box plots illustrating in soil characteristics under Brachiaria grass and N-fertilizers. Values represent averages and standard deviations from 20 replicates. Lowercase letters denote differences determined by a linear mixed-effect model. Group comparisons utilized the Tukey test, with p-values adjusted using FDR correction. Abbreviations for fertilizers: C = control without N, AS = ammonium sulfate, CM = castor meal, U = urea.

Supplementary Table 2.1. Multivariate Permutational Adonis Test from Prokaryotic and Fungi communities, based on Bray-Curtis distance and 999 permutations. Varieties were added as random factor.

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<th>Fungi</th>
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<td>R² (%)</td>
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<td>11.39</td>
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<tr>
<td>F-Value</td>
<td>2.63</td>
<td>2.57</td>
</tr>
<tr>
<td>P-Value</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Supplementary Table 2.2. Multivariate Permutational Pairwise Adonis Test, based on Bray-Curtis distance and 999 permutations.

<table>
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<tr>
<th>Pairs</th>
<th>Prok. Community</th>
<th>Fungi Community</th>
</tr>
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<tr>
<td></td>
<td>P-Value (after “FDR” correction)</td>
<td></td>
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<tr>
<td>C vs TM</td>
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<tr>
<td>C vs U</td>
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<tr>
<td>C vs SA</td>
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<td>0.03</td>
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<tr>
<td>TM vs U</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TM vs SA</td>
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<tr>
<td>U vs SA</td>
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Supplementary Table 2.3. Inertia values from the distance-based redundancy analysis (db-RDA).

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<th>Fungi Composition</th>
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</thead>
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<td></td>
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<td>Proportion (%)</td>
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<td>Total</td>
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<td>100</td>
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<tr>
<td>Unconstrained</td>
<td>0.95</td>
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Supplementary Figure 2.5. Box-plot representing prokaryotic diversity (A) and richness (B), and fungal diversity (C) and richness (D) in soils under Brachiaria grass and N-fertilizers. Values represent averages and standard deviations from 20 replicates. Lowercase letters denote differences determined by a linear mixed-effect model. Group comparisons utilized the Tukey test, with p-values adjusted using FDR correction. Abbreviations for fertilizers: C = control without N, SA = ammonium sulfate, TM = castor meal, U = urea.
Supplementary Figure 2.6. Pizza plot showing the relative differential abundance of prokaryotic phyla in soils under *Brachiaria* grass and N-fertilizers. Abbreviations for fertilizers: C = control without N, SA = ammonium sulfate, TM = castor meal, U = urea.

Supplementary Figure 2.7. Pizza plot showing the relative differential abundance of fungi phyla in soils under *Brachiaria* grass and N-fertilizers. Abbreviations for fertilizers: C = control without N, SA = ammonium sulfate, TM = castor meal, U = urea.