UNIVERSIDADE FEDERAL DO PIAUÍ
CAMPUS PROFESSORA CINOBELINA ELVAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS AGRÁRIAS
DOUTORADO EM CIÊNCIAS AGRÁRIAS

ROMÁRIO MARTINS COSTA

MICROBIAL RESPONSES TO SOIL MANAGEMENT: BIOMASS, ENZYMATIC ACTIVITY, AND COMMUNITY STRUCTURE UNDER NO-TILLAGE AND CROP-LIVESTOCK INTEGRATION SYSTEMS

Bom Jesus – PI
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Thesis submitted to the Graduate Program in Agricultural Sciences - PPGCA, Campus Professora Cinobelina Elvas - CPCE, Federal University of Piauí - UFPI, in the field of Soil Science, as a requirement for obtaining the degree of Doctor in Agricultural Sciences.

Advisor: Prof. Dr. Ademir Sérgio Ferreira de Araújo.
Co-advisor: Prof. Dr. Henrique Antunes de Souza.

Bom Jesus – PI

2025

FICHA CATALOGRÁFICA
Universidade Federal do Piauí
Biblioteca Setorial Campus Professora Cinobelina Elvas
Setor de Processamento Técnico

C837m Costa, Romário Martins.
Microbial responses to soil management: biomass, enzimatyc, activity, and community estructure under no-tillage and crop-livestock integration systems / Romário Martins Costa. -- 2025.
67 f.

Tese (Doutorado) – Universidade Federal do Piauí. Programa de Pós-Graduação em Ciências Agrárias, Bom Jesus, Piauí, 2025.
"Orientador: Prof. Dr. Ademir Sérgio Ferreira de Araújo".
"Coorientador: Prof. Dr. Henrique Antunes de Souza".

1. Ciências agrárias. 2. Manejo do solo. 3. Sistemas de plantio. I. Araújo, Ademir Sérgio Ferreira de. II. Souza, Henrique Antunes de. III. Título.

CDD 630

Bibliotecária: Nayra Fontinele Feijão – CRB3/1249

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Approved on: July 30, 2025.

EXAMINATION COMMISSION

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Advisor

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Prof. Dr. Ricardo Silva de Sousa (UFPI)
External Member of the Program

ACKNOWLEDGMENTS

The completion of this thesis was only possible thanks to the support, encouragement, and contributions of many people, to whom I express my deepest gratitude.

First and foremost, I thank God for granting me strength, health, and wisdom to overcome the challenges of this journey in my life.

To my advisor, Dr. Ademir Sérgio Ferreira de Araujo, I am grateful for his guidance, trust in my work, and constant encouragement of critical thinking and academic independence. I also extend my sincere thanks to my co-advisor, Prof. Dr. Henrique Antunes de Souza, for his availability and technical-scientific support. To both, my admiration and gratitude.

To the Graduate Program in Agricultural Sciences – PPGCA/UFPI, I am thankful for the learning opportunities and the stimulating academic environment. I also extend my gratitude to the professors who contributed directly or indirectly to my training.

To the Research Support Foundation of the State of Piauí (FAPEPI), I am grateful for the financial support, without which this work would not have been possible.

To the Sugarcane Breeding Program (PMGCA – UFPI/RIDESA), I am thankful for providing the facilities necessary for the analyses.

To the members of the examining committee, I am grateful for their valuable contributions to improving this work.

I am especially grateful to the management of Fazenda Barbosa for authorizing the fieldwork, which was essential for the development of my thesis.

To all colleagues and friends from the research group, especially Dr. Sandra Mara Barbosa Rocha, I thank you for the enriching interactions, knowledge exchange, and assistance throughout different stages of the research.

To my friends Marcos Renan, Sâmia, and Maria Raimunda, I am grateful for your support, attentive listening during difficult moments, and words of encouragement along this journey.

To my family, I thank you for your unconditional love, patience, and support during the most challenging times. In particular, I thank my parents, Maria José and Raimundo, for always being by my side and cheering for me.

Finally, I thank all those who, in one way or another, contributed to making this journey possible. To each one of you, my sincere thanks.

Just as the soil harbors invisible life that sustains all that grows, it is in the silence of daily effort that true knowledge blooms.

Unknown author

ABSTRACT

The pursuit of sustainable agricultural practices has driven the adoption of management systems that promote soil health and the functioning of biological processes. In this study, we evaluated the impact of no-tillage (NT) and integrated crop-livestock (ICL) systems on soil microbiological attributes and the structure of prokaryotic communities in the soybean rhizosphere. Five sampling events conducted over the course of one year allowed us to assess the influence of seasonality on microbial attributes. During this period, we quantified microbial biomass carbon (MBC) and the activity of key enzymes, such as phosphatase, urease, dehydrogenase, and those involved in fluorescein diacetate hydrolysis. We also used 16S rRNA gene sequencing to assess the structure, composition, and potential nitrogen-cycle-related functions of bacterial and archaeal communities in the rhizosphere of crops grown under NT (soybean) and ICL (corn, Brachiaria grass, and soybean), as well as in bulk soil. Results indicated that management system, sampling time, and their interaction significantly influenced MBC and enzyme activity. The NT system showed higher phosphatase and urease activity in most periods, whereas the ICL system exhibited greater MBC, reflecting the effects of crop succession and grazing on microbial dynamics. The soybean rhizosphere microbiome composition was strongly shaped by management practices and preceding crops. Under NT, the soybean rhizosphere harbored a microbiome more similar to bulk soil, with greater microbial connectivity and strong associations with functional attributes such as MBC and enzyme activity. In contrast, the soybean rhizosphere under ICL formed a distinct microbiome influenced by grass and corn, with lower connectivity but enriched in specific taxa. Predictive functional analyses revealed a differentiated profile of nitrogen-cycle-related genes, with a higher abundance of genes linked to biological nitrogen fixation and assimilatory nitrate reduction in the soybean rhizosphere. These findings demonstrate that soil microbiological dynamics and rhizosphere microbiome assembly are strongly influenced by management practices and seasonality. Understanding these interactions is essential for developing more efficient and sustainable agricultural strategies capable of optimizing microbially mediated ecosystem services and enhancing the productivity and resilience of tropical agroecosystems.

Keywords: Conservation systems, Microbial stability, Prokaryotic communities, Soil management.

RESUMO

A busca por práticas agrícolas sustentáveis tem impulsionado o uso de sistemas de manejo que favoreçam a saúde do solo e o funcionamento dos processos biológicos. Neste estudo, avaliou-se o impacto dos sistemas de plantio direto (PD) e da integração lavoura-pecuária (ILP) sobre atributos microbiológicos do solo e a estrutura das comunidades procarióticas na rizosfera da soja. As cinco amostragens realizadas ao longo de um ano permitiram analisar a influência da sazonalidade sobre os atributos microbianos do solo. Nesse período, foram quantificados o carbono da biomassa microbiana (CBM) e a atividade de enzimas-chave, como fosfatase, urease, desidrogenase e as envolvidas na hidrólise do diacetato de fluoresceína. Também utilizamos o sequenciamento do gene 16S rRNA para avaliar a estrutura, a composição e as funções potenciais relacionadas ao ciclo do nitrogênio das comunidades bacterianas e arqueanas da rizosfera das culturas nos sistemas de PD (soja) e ILP (milho, capim Brachiaria e soja), além do solo não-rizosférico. Os resultados indicaram que o sistema de manejo, o tempo de amostragem e sua interação influenciaram significativamente o CBM e a atividade enzimática. O sistema PD apresentou maior atividade de fosfatase e urease na maioria dos períodos, enquanto o ILP exibiu maior CBM, refletindo os efeitos das sucessões de culturas e do pastejo sobre a dinâmica microbiana. A composição do microbioma rizosférico da soja foi fortemente modulada pelas práticas de manejo e pelas culturas antecessoras. A rizosfera da soja sob PD apresentou um microbioma mais semelhante ao solo não-rizosférico, com maior conectividade microbiana e forte associação com atributos funcionais como CBM e atividade enzimática. Em contraste, a rizosfera sob ILP formou um microbioma distinto, influenciado por capim e milho, com menor conectividade, porém enriquecido em táxons específicos. As análises funcionais preditivas revelaram um perfil diferenciado de genes associados ao ciclo do nitrogênio, com destaque para maior abundância de genes relacionados à fixação biológica e assimilação de nitrato na rizosfera da soja. Esses achados demonstram que a dinâmica microbiológica do solo e a montagem do microbioma rizosférico são fortemente influenciadas pelas práticas de manejo e pela sazonalidade. Compreender essas interações é essencial para o desenvolvimento de estratégias agrícolas mais eficientes e sustentáveis, capazes de otimizar os serviços ecossistêmicos mediados por microrganismos e promover a produtividade e resiliência dos agroecossistemas tropicais.

Palavras-chave: Comunidades procarióticas, Estabilidade microbiana, Manejo do solo, Sistemas conservacionistas.

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1 GENERAL INTRODUCTION

The agricultural sector has undergone significant transformations to meet the growing demand for food, driven by rapid population growth (Wang, 2022). This pressure is expected to intensify in the coming decades, with estimates projecting a global population of approximately 9.7 billion by 2050 and 10.9 billion by 2100 (UNITED NATIONS, 2019). Furthermore, the increasing scarcity of natural resources makes it essential to sustainably intensify agricultural production while enhancing productive efficiency (Rehman *et al.*, 2022; Sekaran *et al.*, 2021). In this context, conservation practices such as no-tillage (NT) and integrated systems, particularly crop-livestock integration (ICL), have gained prominence and are being widely adopted as strategies to reconcile productivity and sustainability.

Soil microbial biomass and the activity of enzymes such as phosphatase, urease, and dehydrogenase are recognized as sensitive indicators of soil quality and functionality, reflecting fundamental processes such as nutrient cycling and organic matter decomposition (Lopes *et al.*, 2018; Franco *et al.*, 2020; Bhaduri *et al.*, 2022). Seasonal factors have been reported to interact with management practices, influencing soil microbial stability and performance (Amaral *et al.*, 2023; Lopes *et al.*, 2018). However, information on the seasonal variation of soil microbial biomass and enzymatic activity remains limited, especially when simultaneously comparing NT and ICL systems. In this context, the second chapter of this thesis aimed to evaluate soil microbial biomass and the activity of nutrient-cycling enzymes in soils under NT and ICL systems over five sampling periods during one year.

Beyond processes occurring in bulk soil, the rhizosphere represents a key environment for the recruitment and selection of microbial communities that directly interact with plants, influencing their nutrition, growth, and stress resilience (Bhattacharyya; Furtak, 2022; Shi *et al.*, 2022). The composition and functionality of the rhizosphere microbiome are determined by factors such as soil type, cultivated plant species, and the management system adopted (Han *et al.*, 2020; Silva *et al.*, 2019). Conservation practices such as NT and ICL have the potential to positively modify soil microbial communities, stimulating beneficial taxa and contributing to soil health and crop performance. Despite the recognized agronomic benefits of these systems, studies directly comparing their impacts on soil biological properties remain limited, particularly in the soybean rhizosphere, the main crop cultivated under NT and ICL. In this context, the third chapter of this thesis aimed to understand how sustainable agricultural practices influence the recruitment of beneficial bacteria and archaea in the soybean rhizosphere, affecting the structure and functionality of these microbial communities.

2 CHAPTER 1

THEORETICAL FRAMEWORK

2.1 Conservation soil-use systems

Conventional agriculture, based on intensive soil tillage, monocultures, and the use of synthetic inputs, although it has boosted food production, has resulted in serious environmental impacts, such as soil degradation, loss of organic matter, erosion, compaction, reduction of microbial biodiversity, and increased greenhouse gas emissions (Hussain *et al.*, 2021). These effects compromise the sustainability of agricultural ecosystems and long-term food security (Mosier; Córdova; Robertson, 2021). In this context, conservation systems, such as no-tillage (NT) and crop-livestock integration (ICL), represent innovative approaches to agricultural management, aiming to optimize production and environmental sustainability (Fuentes-Llanillo *et al.*, 2021; Reis *et al.*, 2021).

The NT system is characterized by three basic principles: absence or minimal mechanical soil disturbance, permanent crop residue cover, and crop diversification (Peixoto *et al.*, 2020). This system offers advantages such as greater soil moisture retention, reduced costs for fuel, labor, and machinery maintenance, since the number of operations is lower compared to conventional systems (Fuentes-Llanillo *et al.*, 2021). The ICL system, in turn, is characterized by the rotation of crops and the production of forage for animal grazing during the off-season of the main crop (Costa *et al.*, 2021). In addition to agricultural intensification and production system diversification, ICL can offer higher economic returns due to reduced use of inorganic fertilizers, pesticides, and other inputs (Sekaran *et al.*, 2021).

In general, conservation systems can improve soil quality due to higher carbon inputs, reduce erosive processes (Soares *et al.*, 2019; Fuentes-Llanillo *et al.*, 2021; Sekaran *et al.*, 2021), and lower greenhouse gas emissions (Sato *et al.*, 2019) compared to conventional soil management. Furthermore, these systems can enhance soil biological properties, increasing microbial diversity and activity and benefiting functional groups essential for nutrient cycling and plant health (Li *et al.*, 2018; Bansal; Chakraborty; Kumar, 2022).

2.2 Biological indicators of soil quality

Soil microorganisms, such as bacteria, fungi, actinobacteria, and protozoa, represent the main living component of soil organic matter and account for 1 to 5% of total organic matter (Mendes *et al*., 2018). These microorganisms perform diverse functions, participating in the biogeochemical cycling of nutrients by mediating their availability to plants, as well as contributing to phosphate solubilization (Carneiro *et al*., 2004; Richardson; Simpson, 2011), biological nitrogen fixation (Hungria *et al*., 2009; Souza et al., 2016), protection against phytopathogens (Costa *et al*., 2023), phytohormone production (Majeed; Muhammad; Ahmad, 2018), soil aggregate stabilization (Mao *et al*., 2021), and pollutant degradation (Mishra *et al*., 2021), among other functions. Furthermore, microorganisms also release enzymes into the soil, which act in the decomposition of organic residues and nutrient cycling (Daunoras; Kačergius; Gudiukaitė, 2024).

Soil microbial biomass and enzymatic activity are strongly influenced by climatic factors, such as moisture and temperature, resulting in seasonal variations of these parameters (Lauber *et al*., 2013; Carvalho *et al*., 2018; Lopes *et al*., 2018). In cultivated systems, these parameters are also impacted by changes in land use and management (Lauber *et al*., 2013). Thus, due to their high sensitivity, these indicators have been widely used to assess soil quality (Amaral *et al*., 2023; Araújo *et al*., 2013; Peixoto *et al*., 2020; Zheng *et al*., 2023). Microbial indicators, such as basal respiration and microbial biomass, as well as biochemical indicators, such as enzymatic activity, are useful for monitoring the response of microorganisms to disturbances caused by management practices (Bhaduri *et al*., 2022). The effectiveness of these bioindicators is related to their ability to respond more rapidly to environmental changes than chemical soil attributes (Araújo *et al*., 2013; Mendes *et al*., 2018).

Studies indicate that conservation systems promote significant increases in soil microbial biomass compared to conventional management systems (Li *et al*., 2018; Chen *et al*., 2020). A global meta-analysis conducted by Li *et al*. (2018) revealed that no-tillage increased microbial biomass carbon (MBC) by 25% and microbial nitrogen by up to 64%. Similar results were reported by Chen *et al*. (2020), who observed higher fungal and bacterial biomass in no-tillage systems compared to conventional soil preparation. Additionally, a long-term study by Mbuthia *et al*. (2015) showed that no-tillage promoted significant increases in soil enzymatic activity.

Studies also show that ICL systems promote substantial increases in MBC and enzymatic activity compared to conventional systems. Bansal, Chakraborty and Kumar (2022),

when evaluating the combined effect of animal grazing and cover crops, observed marked increases in carbon and nitrogen associated with soil aggregates, boosting microbial biomass. Furthermore, in ICL systems, increased plant diversity enhances soil enzymatic activity (acid phosphatase, β-glucosidase, and urease) (Franco *et al*., 2020). Overall, most studies indicate increases in microbial biomass and enzymatic activity in NT and ICL systems compared to conventional systems (Li *et al*., 2018; Bansal; Chakraborty; Kumar, 2022). However, studies directly comparing NT and ICL systems remain scarce, particularly regarding the seasonal stability of microbial biomass and soil enzymatic activity.

The stability of soil microbial biomass and enzymatic activity over time is influenced by seasonal factors, vegetation type, plant developmental stage, and management practices (Araújo *et al*., 2013; Hsiao *et al*., 2019; Kostin *et al*., 2021). In native ecosystems, greater plant diversity, continuous cover, and litter deposition favor the stability of microbial biomass by providing higher carbon inputs, reduced temperature fluctuations, and more consistent moisture (Xi *et al*., 2023). In contrast, agricultural soils may exhibit lower temporal stability of microbial communities, which may be correlated with greater fluctuations in soil moisture and temperature (Lauber *et al*., 2013). However, Lopes *et al*. (2018) observed low temporal variation in microbial biomass and soil enzymatic activity after sixteen years of cultivation, indicating a state of equilibrium in agroecosystems.

Therefore, the integrated monitoring of soil microbial biomass and enzymatic activity, together with understanding their temporal stability under different management systems, constitutes an essential approach to more sensitively and comprehensively assess soil quality.

2.3 Soil microbial communities

Historically, knowledge about soil microbial communities was limited by the low efficiency of laboratory cultivation techniques, which access less than 1% of the microorganisms present in the soil (Garg *et al*., 2024). Advances in molecular biology, especially high-throughput sequencing platforms (e.g., Illumina MiSeq), combined with the use of bioinformatics, have enabled the study of microorganisms that have not yet been cultivated, significantly expanding our understanding of the soil microbiota (Schöler *et al*., 2017). In this context, amplicon-based approaches using markers such as the 16S rRNA gene (for bacteria and archaea) and 18S rRNA (for fungi) have been widely applied, allowing the assessment of structure, diversity (alpha and beta), and composition of microbial communities, which has

been essential for understanding the effects of soil use and management on microorganisms (Costa *et al*., 2022; Shi *et al*., 2022; Taerum *et al*., 2022).

Soil microbial communities, particularly those associated with the rhizosphere, perform essential functions in promoting plant growth and protecting plants against pathogens (Majeed; Muhammad; Ahmad, 2018; Shi *et al*., 2022). These communities are shaped by a variety of factors, including seasonal climatic conditions, plant type and developmental stage (Araujo *et al*., 2019; Silva *et al*., 2019; Sohn *et al*., 2021), edaphic characteristics such as soil type, pH, nutrients, and texture (Zheng *et al*., 2019; Pereira *et al*., 2022), as well as management practices and land-use changes (Souza *et al*., 2016).

Vegetation type acts as a primary determinant of microbial community structure, either through the release of root exudates — a source of energy for soil microbiota — or by contributing to the quantity and quality of litter, influencing the heterogeneity of microbial communities (Bresolin *et al*., 2010). In the rhizosphere, organic compounds (e.g., secondary metabolites, sugars, organic acids, vitamins, amino acids, and polysaccharides) are released, which can promote increased microbial diversity and functional potential associated with plant roots (Chukwuneme *et al*., 2021).

Systems with higher plant diversity tend to sustain more complex microbial communities due to the greater variety and quantity of exudates and carbon sources (Eisenhauer *et al*., 2017; Xi *et al*., 2023). For instance, Pires *et al*. (2021) observed that intercropping legumes with grasses favored soybean productivity, a result attributed to increased input of organic compounds and the consequent stimulation of soil microbiota. This positive effect can also be observed in the relationship between plant diversity and soil bacterial and fungal biomass, mediated by root biomass and exudation (Eisenhauer *et al*., 2017), indicating that more diversified systems, such as ICL, can benefit the soil microbial community. However, even in less diverse systems, such as monocultures, studies show that rhizosphere microbial communities maintain important functional traits, contributing to plant nutrition and growth (Mendes *et al*., 2014).

Microbial diversity plays a key role in maintaining ecosystem multifunctionality (Delgado-Baquerizo *et al*., 2016) and is a determinant factor for soil quality and fertility (Bhattacharyya; Furtak, 2022). However, land-use changes, such as converting native vegetation to pasture or agricultural areas, can cause significant losses in microbial diversity (Quirino *et al*., 2009; Souza *et al*., 2016; Sarto *et al*., 2020). Quirino *et al*. (2009) reported a tenfold reduction in bacterial richness when comparing Cerrado stricto sensu areas with pastures.

Long-term studies also highlight the impacts of intensive soil management. Zhou *et al*. (2015) observed that after 34 years of continuous inorganic fertilizer use in cultivated soils, bacterial diversity decreased, Proteobacteria and Actinobacteria increased in fertilized soils, and Acidobacteria were more prevalent in unfertilized soils. In general, the literature indicates the predominance of Proteobacteria, Bacteroidetes, and Actinobacteria in the maize rhizosphere (Li *et al*., 2014) and of Proteobacteria, Actinobacteria, and Acidobacteria in the soybean rhizosphere (Mendes *et al*., 2014).

In this context, understanding how NT and ICL modulate the soybean rhizosphere microbiota is fundamental to elucidating mechanisms that support productivity and sustainability. This conceptual framework underpins the investigation proposed in this study, focused on characterizing and comparing prokaryotic communities associated with soybean under different management systems, as well as assessing their relationships with soil chemical and biological attributes.

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3 CHAPTER 2

SEASONAL RESPONSES OF SOIL MICROBIAL BIOMASS C AND ENZYMATIC ACTIVITY COMPARING NO-TILLAGE AND INTEGRATED CROP-LIVESTOCK SYSTEMS

ABSTRACT

Sustainable agricultural systems, such as integrated crop-livestock (ICL) and no-tillage (NT), aim to sustainably produce crops and livestock while simultaneously conserving soil and its microbial properties, mainly in tropical regions. However, little is known about how microbial properties respond seasonally to management applied in NT and ICL. Thus, this study assessed the seasonal responses of soil microbial biomass C and enzymatic activity comparing both NT and ICL. The experimental area, under a block design with four replicates, with both NT and ICL management, was implemented in December 2022 on Yellow Argisol soil in Maranhao state, Brazil. Soil samples were collected (0–20 cm depth) in March, June, September, December, and March (2023). The results showed an effect size varying between 0.06 and 0.95 for agricultural systems, and 0.63 to 0.95 for sampling time. For the interaction between agricultural systems and sampling time, the effect size was superior to 0.86. NT showed initially higher microbial biomass C (~50%), leveling with ICL by the end of the sampling period. Phosphatase and dehydrogenase increased in ICL from March to June (~200% and ~700% for phosphatase and dehydrogenase, respectively), while fluorescein diacetate hydrolysis fluctuated in NT. Urease was higher (~100%) during all sampling times in NT. Linear discriminant analysis revealed distinct responses across sampling times, with a positive effect of pH on enzymatic activity in both systems and soil moisture and P impacting positively on microbial biomass in ICL. Our results revealed significant seasonal responses of soil microbial biomass and enzymatic activity comparing NT and ICL, but with distinct responses to agricultural systems. The study showed seasonal variation of soil microbial biomass and enzymatic activity dependent on the characteristics of NT and ICL. Therefore, understanding these differences helps farmers make better decisions for healthier soil and better crops.

Keywords: Sustainable agriculture, Soil biological properties, Soil enzymes.

3.1 Introduction

Integrated crop-livestock (ICL) and no-tillage (NT) are particularly important agricultural systems used worldwide in crop production. Despite both ICL and NT being considered sustainable systems, they differ in terms of soil and crop management. ICL focuses on integrating crop and livestock production within the same system, promoting the recycling of organic residues from crops and livestock (Reis *et al*., 2021). In contrast, NT involves directly seeding crops under the straw of the previous crop without disturbing the soil through tillage (Possamai *et al*., 2022). This maintains soil structure, reduces erosion, and conserves soil moisture (Bekele; Habtemariam; Gemi, 2022). Both systems contribute similarly to cycling nutrients, increasing soil organic matter, improving soil health, and decreasing greenhouse gas emissions (Bieluczyk *et al*., 2020; Alves *et al*., 2022). In Brazil, these systems are significantly important, occupying approximately 50 million hectares. Specifically, ICL is characterized by the annual rotation of pastures, used for meat or milk production (Moraes *et al*., 2014), while NT involves maintaining permanent soil cover with straw and crop rotation, primarily using soybean (Fuentes-Llanillo *et al*., 2021).

An important aspect observed with the use of both ICL and NT is their potential benefits to soil biological properties (Bansal; Chakraborty; Kumar, 2022; Bonetti *et al*., 2018; Laroca *et al*., 2018), which are essential for plant productivity. Particularly, soil microbial biomass (SMB) is one of the most important attributes of soil biological properties (Li *et al*., 2018), performing various functions in soils and showing high sensitivity to agricultural management (Yan *et al*., 2022). Additionally, SMB produces and releases enzymes that enhance biological activity in soils (Wang *et al*., 2021), influencing the cycling of C, N, and P (Moraes *et al*., 2018). For instance, phosphatase and urease are the primary enzymes involved in the cycling of P and N, respectively, contributing to increased availability of these elements in soils (Margalef *et al*., 2021; Piotrowska-Długosz *et al*., 2022). Therefore, agricultural systems that conserve and increase SMB have shown higher soil enzymatic activity, leading to increased cycling of nutrients and crop yield. Indeed, previous studies have demonstrated increased SMB content and enzymatic activity with the adoption of both ICL and NT compared to conventional systems (Franco *et al*., 2020; Sarto *et al*., 2020).

Although land use change, such as soil management, drives the responses of both SMB and enzymatic activity, some environmental factors, such as soil temperature and moisture, can also affect these biological properties (Deng *et al*., 2019). This is particularly important in tropical regions with high seasonal variation in environmental conditions, where both SMB and

enzymatic activity can exhibit significant annual variation (Amaral *et al*., 2023). Interestingly, previous studies have shown significant seasonal variation of SMB in NT (Lopes *et al*., 2021) and ICL (Maia *et al*., 2021) compared to conventional systems. For instance, Lopes *et al*. (2021) compared no-tillage and conventional systems and observed that the soil microbial properties, mainly microbial biomass C and N, changed significantly between seasons, being influenced by soil moisture and temperature. However, little is yet known about the seasonal variation in SMB and enzymatic activity comparing both NT and ICL, mainly in tropical regions, such as Brazil. Seasonal analyses of both SMB and enzymatic activity can provide key insights into the main drivers affecting these biological properties in both NT and ICL, contributing to a better understanding of potential soil biological activity, particularly related to nutrient cycling. In this study, we hypothesize that there will be significant differences in the seasonal variations of SMB and enzymatic activity under NT and ICL across a year due to their specific management practices. To address this hypothesis, we assessed both SMB and enzymatic activity in soils under ICL and NT during five sampling times across a year.

3.2 Material and methods

3.2.1 Study area

The study was conducted at Farm Barbosa, Brejo, Maranhao state (3◦42′66″S; 42◦56′25″W, 102 m). The soil is classified as Yellow Argisol containing 76% sand, 8% silt, and 16% clay. The annual precipitation and temperature are shown in Figure 1A. This farm has agricultural areas under NT and ICL systems that were implemented after the deforestation of native Cerrado in 2003. This was followed by applying 2.0 tons ha^{-1} of calcitic limestone + 0.5 ton ha^{-1} of gypsum. In 2004, NT was implemented by applying reduced tillage. This area has been annually cultivated with soybean and millet, used as cover, under crop succession. Every year, soil fertilization consists of 100 kg ha^{-1} of ammonium sulfate, 150 kg ha^{-1} of monoammonium phosphate, and 170 kg ha^{-1} of potassium chloride. ICL was implemented in 2009 with soybean being cultivated followed by pasture (*Urochloa brizantha* cv. Marandu) and intercropped with maize. Annually at the sowing of soybean, the soil is fertilized with 340 kg ha^{-1} (formulation NPK 12-30-00). After 30 days, there is the application of 200 kg ha^{-1}

(formulation NPK 10-00-30). After the crop harvest (soybean and maize), the pasture is grazed by cattle (three units of animals per hectare).

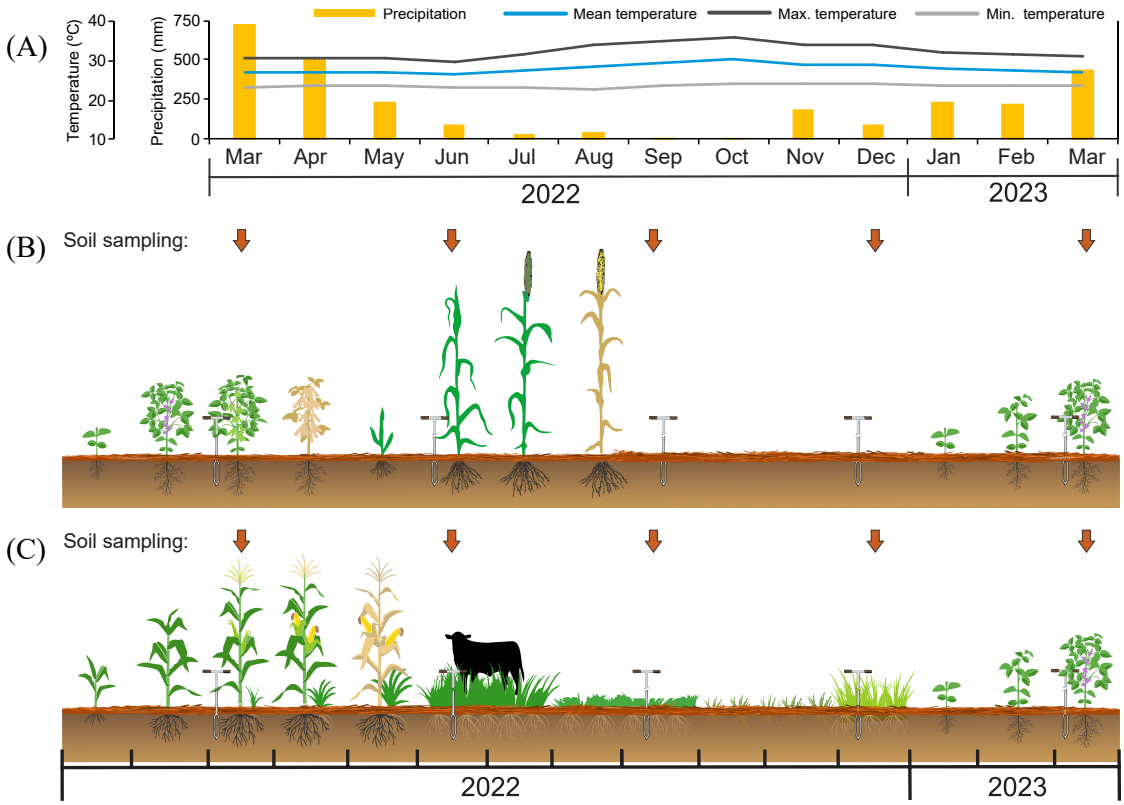


Figure 1 - Climatic characteristics during the 2022/2023 crop season (A) and the agricultural management applied in no-tillage (B) and integrated crop-livestock (C) systems.

To evaluate the effect of these systems on soil properties, experimental plots (30 m × 40 m) were established in December 2021 under a block design with four replicates. The agricultural practices of both NT and ICL were applied in these plots. In NT, soybean was sown in January and harvested in April, followed by millet sown in May and left as soil cover until September (Figure 1B). In ICL, maize was sown in January and harvested in May, followed by pastures sown and cattle grazing from June to December (Figure 1C). In 2023, soybean was sown in both NT and ICL in January.

3.2.2 Soil sampling and analysis

To assess the seasonal variation of SMB and enzymatic activity, soil samples were collected at a depth of 0–20 cm in 2022 (March, June, September, and December) and 2023 (March) (Fig. 1B and C). In each period, five randomized points were sampled in each plot, and the samples were combined to form four composite samples in each system (NT and ICL),

totaling eight composite soil samples per period. Since the experiment had five sampling times, a total of 40 soil composite samples were obtained (8 composite samples × 5 sampling times). All composite samples were placed in labeled plastic bags and stored in an icebox for transportation. In the laboratory, the samples were sieved (2 mm) and stored at 4 ◦C before soil biological analysis. Soil properties (Table 1) were analyzed according to Embrapa (1999). Soil pH was determined in a 1:2.5 soil/water extract. Available phosphorus (P) was estimated by photometry. Soil moisture content was estimated using the gravimetric method, expressed as the mass of water per mass of dry soil.

Table 1 - Soil chemical properties in no-tillage (NT) and integrated crop-livestock (ICL) systems.

Agricultural system	Sample time	pH	TOC	P	Al^{3+}	Ca^{2+}	Mg^{2+}	K^{+}	CEC
		H_2O	g kg^{-1}	mg dm^{-3}			$cmol_c$ dm^{-3}		
NT	1	5.69	14.24	59.43	0.13	3.50	1.25	0.05	9.93
	2	5.35	11.34	44.71	0.50	2.23	0.70	0.02	8.97
	3	5.70	13.60	37.69	0.00	2.98	1.08	0.09	9.38
	4	5.94	9.93	44.50	0.06	2.73	1.00	0.07	9.06
	5	6.04	9.96	40.82	0.00	2.35	1.23	0.04	8.93
ICL	1	5.72	9.88	54.51	0.13	2.33	0.60	0.03	7.89
	2	5.84	10.27	42.57	0.06	2.70	1.25	0.03	8.84
	3	5.97	11.57	31.21	0.06	2.65	1.18	0.13	9.73
	4	6.15	10.94	39.15	0.00	2.63	1.25	0.16	9.74
	5	6.49	13.42	17.54	0.00	2.10	1.25	0.12	8.30

Sampling times (1- March 2022; 2- June 2022; 3- September 2022; 4- December 2022; 5- March 2023). TOC – total organic C; CEC – cation exchange capacity.

The microbial biomass carbon (MBC) was assessed according to Vance, Brookes and Jenkinson (1987), through the extraction of C from fumigated and unfumigated soils by potassium dichromate. An extraction efficiency coefficient of 0.41 was applied to convert the difference in C between fumigated and unfumigated soil into microbial biomass C. Soil respiration (SRP) was determined according to Alef and Nannipieri (1995) by monitoring CO_2 evolution after 7 days. Total organic C (TOC) was determined by the wet combustion method using a mixture of potassium dichromate and sulfuric acid under heating aquecimento (Yeomans; Bremner, 1988). The microbial quotient (qMic) was defined as the ratio between MBC and TOC.

The hydrolysis of fluorescein diacetate (FDA) and the dehydrogenase activity (DHA) were estimated according to Schnürer and Rosswall (1982) and Casida *et al*. (1964), respectively. The evaluation of FDA, which measures the activities of esterases, proteases, and lipases (Adam; Duncan, 2001), was conducted by incubating soil samples with and without fluorescein diacetate, followed by the measurement of fluorescein content using spectrophotometry. The evaluation of DHA was based on the spectrophotometric determination of triphenyl tetrazolium formazan (TTF). The urease activity was determined according to Kandeler and Gerber (1988), using urea as a substrate and the amount of ammonia produced being measured by spectrophotometry. The activity of acid phosphatase was evaluated through colorimetric estimation of p-nitrophenol (Alef; Nannipieri, 1995).

3.2.3 Statistical analysis

The significance tests of the treatment effects (soil management [no-tillage (NT) and integrated crop-livestock (ICL)], and collection time [five sample times] and their interactions) were performed using analysis of variance with repeated measures (ANOVAR) in RStudio software. Shapiro-Wilk, Levene's, and Mauchly's tests for normality, homogeneity of variances, and sphericity, respectively, were used to ensure non-violation of the ANOVAR assumptions. Effect size was calculated as generalized eta squared (η^2G) (Cohen, 1988). All data showed sphericity and Greenhouse-Geisser correction was not applied. The normality and homogeneity of variances were observed in our dataset. Bonferroni's test was used to assess significant pair-wise differences. Linear discriminant analysis (LDA) was performed in Past 4.13 software. Permutational multivariate analysis of variance (PERMANOVA), based on Bray-Curtis's index, was used to confirm the groups formed in LDA. Redundancy analysis (RDA) was carried out to evaluate the relationships between soil properties (explanatory variables) and microbial properties (response variables) using the vegan package in RStudio software. The temporal stability of soil microbial biomass and enzymatic activity was assessed according to Tilman, Reich and Knops (2006). Temporal stability was calculated as the ratio between the mean and its temporal standard deviation for each variable evaluated in the experiment, subsequently tested using the non-parametric Wilcoxon test ($p < 0.05$).

3.3 Results

3.3.1 Soil microbial biomass C

Soil microbial biomass C, microbial quotient, phosphatase, and urease were significantly affected by agricultural system, sampling time, and their interaction (Table 2). The results showed an effect size varying between 0.06 and 0.95 for agricultural systems, and between 0.63 and 0.95 for sampling time. The effect size for the interaction between agricultural systems and sampling time was superior to 0.86. Soil microbial biomass C was significantly higher in NT (March 2022), with a temporary decrease observed in June and a consistent increase from September to March (2023) (Figure 2A). In contrast, microbial biomass C was initially lower in ICL (March 2022), temporarily increased in June, and then stabilized from September to March (2023). By the end of the sampling period (March 2023), the levels of soil microbial biomass C were similar in both NT and ICL. The microbial quotient initially showed similar values in both NT and ICL, while it increased in ICL and decreased in NT by June (Figure 2B). From September (2022) to March (2023), the microbial quotient increased in NT, while ICL showed a decrease in this soil parameter.

Table 2 - ANOVA with repeated measures for the effects of soil management (SM), sampling time (ST) and their interaction (SM x ST) on all measured variables.

Propriedades do solo	SM		ST		SM x ST		Sphericity‡
	p-value	η^2_G	*p*-value	η^2_G	*p*-value	η^2_G	
Total organic C	0.41^{NS}	0.06	< 0.05	0.63	< 0.05	0.90	0.15
Microbial biomass C	< 0.05	0.78	< 0.05	0.63	< 0.05	0.94	0.60
Microbial quotient	< 0.05	0.84	< 0.05	0.58	< 0.05	0.94	0.42
Acid phosphatase	< 0.05	0.90	< 0.05	0.94	< 0.05	0.93	0.92
Urease	< 0.05	0.95	< 0.05	0.95	< 0.05	0.86	0.26
Dehydrogenase	0.18^{NS}	0.07	< 0.05	0.94	< 0.05	0.87	0.24
Fluorescein diacetate	0.31^{NS}	0.07	< 0.05	0.97	< 0.05	0.91	0.50

‡Sphericity was examined with the Mauchly's Test. Effect size was calculated as generalized eta squared (η^2G). NS = not significant. NS = non-significant.

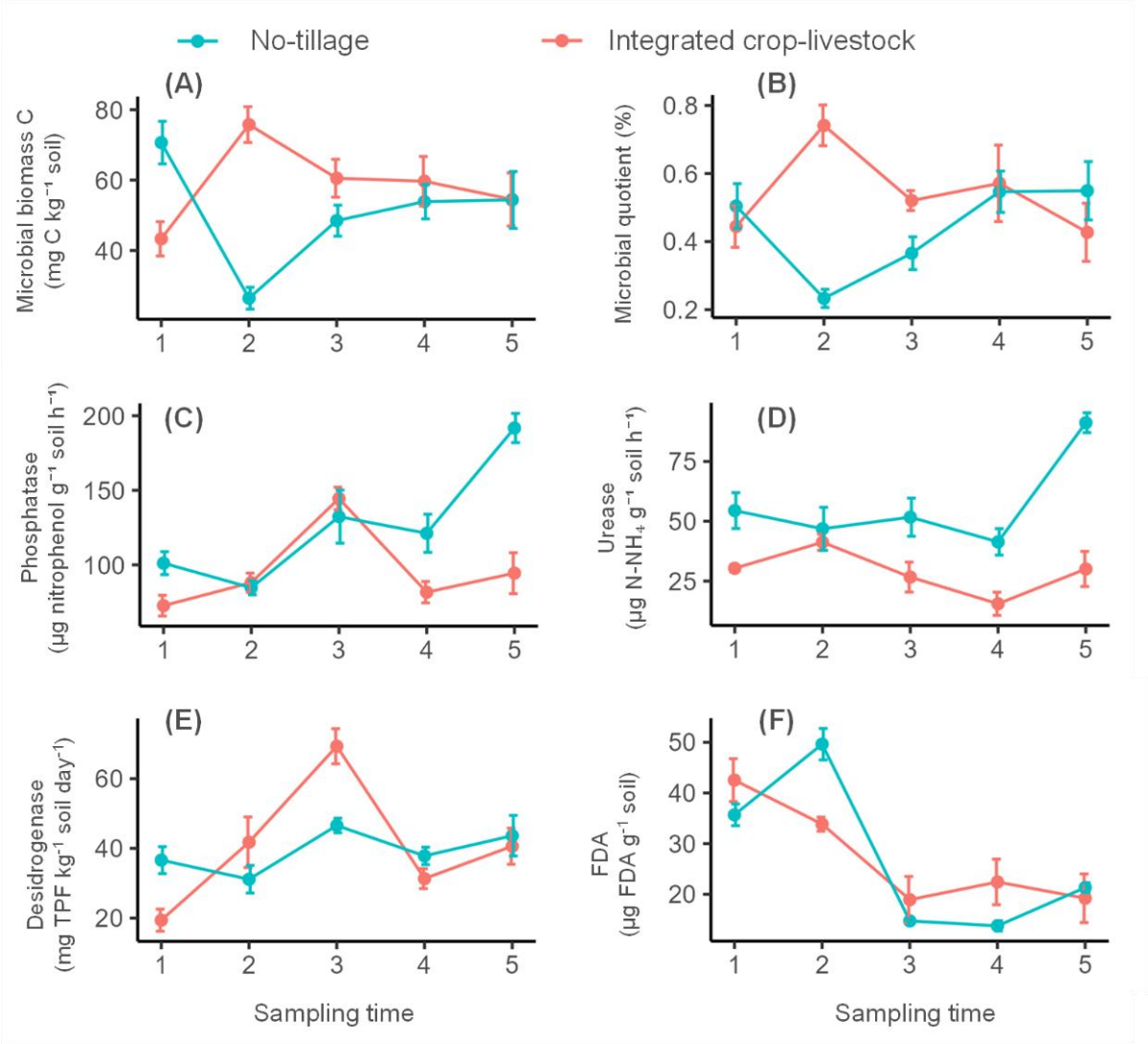


Figure 2 - Soil microbial attributes comparing five soil sampling periods under no-tillage and integrated crop-livestock systems. FDA = fluorescein diacetate hydrolysis; 1 – March 2022; 2 – June 2022; 3 – September 2022; 4 – December 2022; 5 – March 2023.

3.3.2 Soil enzymatic activity

From March to June (2022), both phosphatase and dehydrogenase increased in ICL, followed by a subsequent decrease from September (2022) to March (2023) (Figure 2C and E). In NT, both phosphatase and dehydrogenase consistently increased over time. Urease activity was consistently higher in NT and increased by the end of the sampling period (March 2023) (Figure 2D). Regarding fluorescein diacetate hydrolysis, NT showed a temporary increase in June followed by a decrease in September. Afterwards, fluorescein diacetate hydrolysis stabilized from December to March (2023). In ICL, fluorescein diacetate hydrolysis consistently decreased over time (Figure 2F).

3.3.3 Multivariate responses and temporal stability of microbial biomass and enzymatic activity

The linear discriminant analysis showed distinct responses of soil microbial biomass and enzymatic activity at different sampling times (Figure 3). In March 2022, we observed that both NT (with soybean) and ICL (with maize) exhibited a clear separation from other sampling times. From June (2022) through March (2023), the responses of each system differed. In NT, soil microbial properties clustered separately in June (with millet) compared to September and December (with straw), and March (2023; with soybean) which were closer. In ICL, soil microbial properties were closer in June and September (with pasture), while they showed separation in December (dried pasture) and March (2023; with soybean).

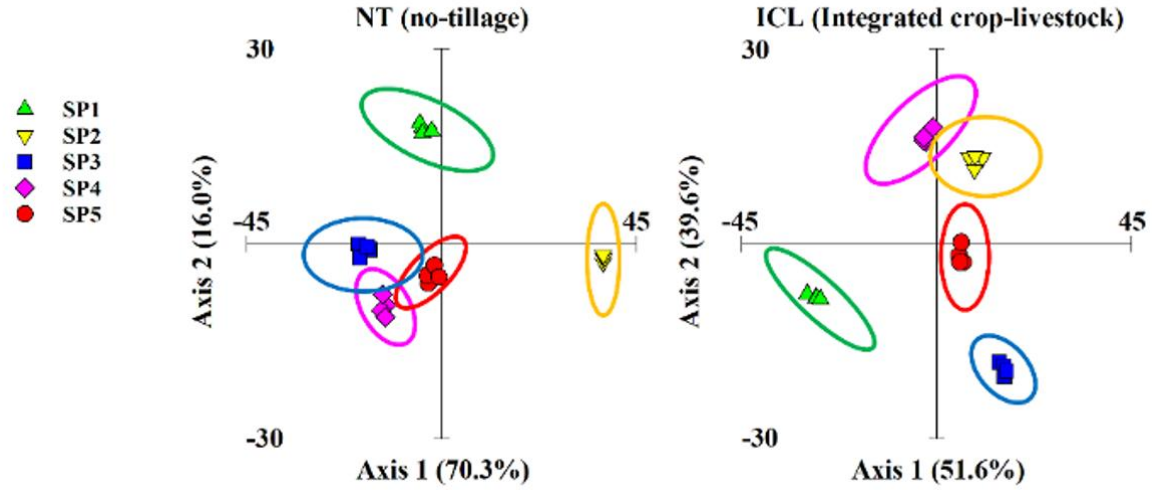


Figure 3 - Linear discriminant analysis (LDA) showing differences between the five collection times in soil under no-tillage (NT) or integrated crop-livestock (ICL). Sampling times: SP1 (March 2022); SP2 (June 2022); SP3 (September 2022); SP4 (December 2022); SP5 (March 2023).

On the other hand, when comparing the main drivers of soil biological properties (Figure 4), we observed soil pH positively influencing both dehydrogenase and phosphatase in NT (September and December) and ICL (September). Conversely, soil moisture and P positively drove the responses of microbial biomass C, microbial quotient, and TOC, mainly in ICL. The temporal stability of microbial biomass C and enzymatic activity did not differ when comparing both NT and ICL (Figure 5). The exception was dehydrogenase activity, where temporal stability was higher in NT ($P = 0.029$; Figure 5E).

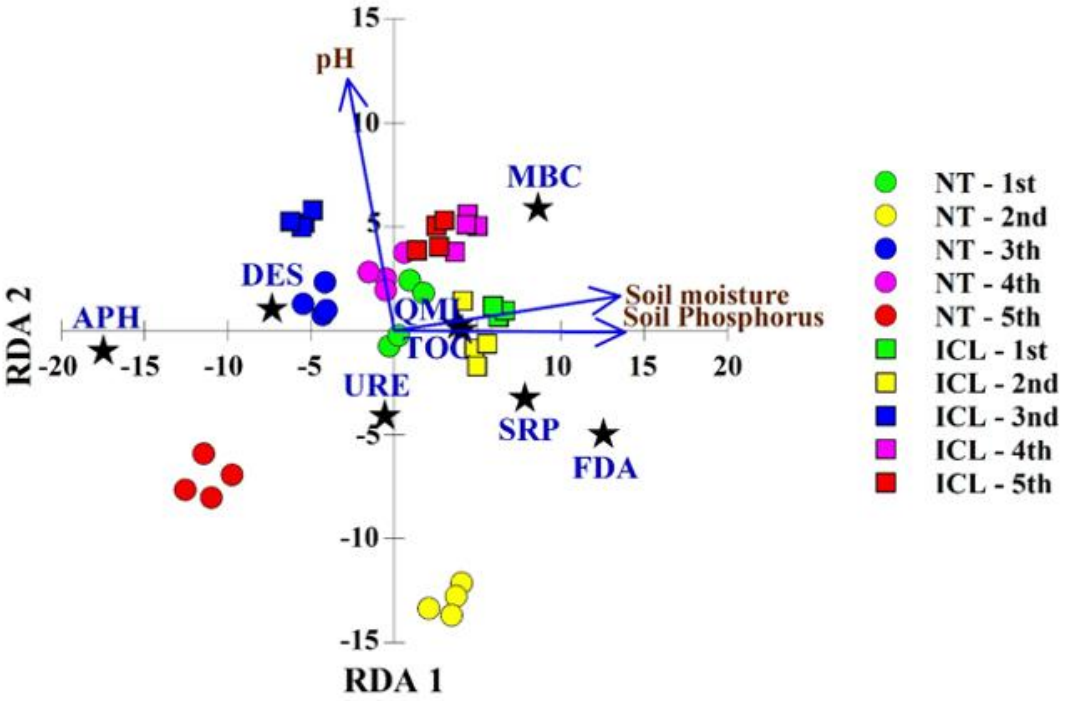


Figure 4 - Redundance analysis showing the relationships between chemical soil proprieties and biological soil parameters. RDA model was significant by ANOVA (F: 3.18; p: 0.012). TOC: total organic C; MBC: microbial biomass C; SRP: soil respiration; QMI: microbial quotient; APH: acid phosphatase; URE: urease; DES: dehydrogenase; FDA: fluorescein diacetate.

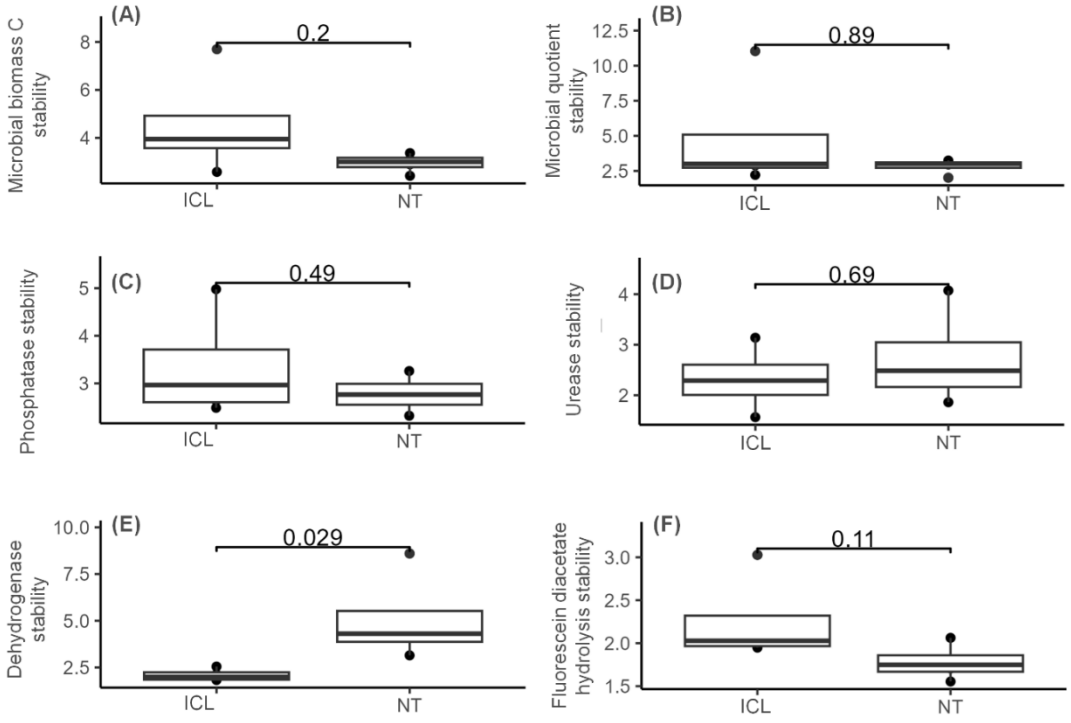


Figure 5 - Temporal stability of soil microbiological attributes under no-tillage (NT) and integrated crop-livestock (ICL) systems. The values at the top of each figure were tested using the non-parametric Wilcoxon test ($p < 0.05$).

3.4 Discussion

In this study, we compared two well-known sustainable agricultural systems: no-tillage versus integrated crop-livestock, to assess their impact on soil microbial biomass and enzymatic activity across seasons. In tropical regions, soil moisture is the main factor influenced by seasonal variation. Typically, higher levels of soil moisture are observed during the rainy season than the dry season, and soil moisture is recognized as a main driver of soil microbial biomass and enzymatic activity (Amaral *et al*., 2023; Araújo *et al*., 2013). Therefore, it is expected that the responses of soil microbial properties will be primarily influenced by soil moisture in tropical regions.

However, our results demonstrated that, although seasonal variation is a well-recognized determining factor in tropical regions, the effects of management on soil microbial attributes were distinct and, in some cases, modulated by this seasonality. These results confirm our hypothesis, indicating varied responses of soil microbial biomass C and enzymatic activity to seasonal variation when comparing no-tillage and integrated crop-livestock. Our results also showed different effect sizes when comparing agricultural systems, sampling time, and interactions, where the effect size was significant for all variables when analyzing the interaction between agricultural systems and sampling time (Cohen, 1988). Regardless of seasonal variation, the results showed higher values of microbial biomass C and microbial quotient under integrated crop-livestock, while urease was more prominent under no-tillage.

Specifically, higher microbial biomass C and microbial quotient were found from June to December in integrated crop-livestock. These results can be attributed to the permanent presence of pasture species, which provide abundant living roots (Laroca *et al*., 2018), associated with the contribution of cattle excreta (Bonetti *et al*., 2018). These characteristics support higher microbial biomass content and microbial quotient due to the input of more decomposable organic residues (Wang *et al*., 2006; Egan *et al*., 2018;). Indeed, a high microbial quotient may indicate the presence of less stable organic matter (Rui *et al*., 2016). Previous studies in Brazilian cerrado observed high microbial biomass C and microbial quotient in soil under integrated crop-livestock due to the presence of pastures and the contribution of animal excreta (Bonetti *et al*., 2018; Laroca *et al*., 2018). On the other hand, no-tillage is influenced by the addition of more stabilized C from long-term straw cover, which contributes less available C to microbial biomass (Zheng *et al*., 2023). Thus, this factor promotes a lower microbial quotient (Rui *et al*., 2016).

At the beginning of the experiment, we observed crop-specific effects on soil microbial biomass C, with soybean in no-tillage increasing microbial biomass C compared to maize in integrated crop-livestock. This difference might be attributed to distinct root traits, such as root exudates, probably providing distinct C sources for microbial biomass (Ma *et al*., 2022). For instance, soybean releases root exudates that provide more energy sources to soil microorganisms (Sugiyama, 2019), leading to increased microbial biomass. Indeed, a previous study identified several C sources in the form of sugars released by soybean roots, such as glucose, arabinose, galactose, sucrose, kojibiose, and oligosaccharides (Timotiwu; Sakurai, 2002). During soybean cultivation (March 2023), both systems exhibited similar values of microbial biomass carbon, highlighting the effect of the crop on this attribute regardless of the management history.

The activity of enzymes such as phosphatase and urease directly reflects nutrient mineralization and biogeochemical cycling in the soil (Sobucki *et al*., 2021). At the beginning and end of the experiment, phosphatase activity increased in no-tillage, suggesting a potential contribution of soybean associated with straw cover (Tyler, 2020). For instance, Tyler (2020) reported cover crops enhancing phosphatase activity under no-tillage, due to increased substrate availability. In integrated crop-livestock, lower phosphatase activity may be due to higher P-fertilizer addition in maize (2022) and soybean (2023) (Mori, 2022).

Regarding urease activity, this remained consistently higher in no-tillage and increased by the end of the sampling time (under soybean). Similar to phosphatase activity, this higher urease activity can be attributed to straw presence (Yan *et al*., 2023). In addition, high input of organic C (Garcia; Sampaio; Nahas, 2011) and the N-fixing ability by soybean contribute to increased urease activity (Nakei; Venkataramana; Ndakidemi, 2022). Recently, Wen et al. (2023) showed through a global meta-analysis that no-tillage associated with straw input effectively increases soil enzyme activity.

We found no significant effect of both no-tillage and integrated crop-livestock on dehydrogenase and fluorescein diacetate hydrolysis. Since dehydrogenase and fluorescein diacetate hydrolysis are indicators of microbial activity (Melo *et al*., 2020), these results suggest that the practices applied in both systems were not enough to distinctly influence soil microbial activity. Particularly, both no-tillage and integrated crop-livestock maintain permanent soil cover, by organic residues and plants, respectively, and presence of roots (Francaviglia; Almagro; Vicente-Vicente, 2023). These factors contribute to the maintenance of soil microbial activity (Breil *et al.*, 2023). When comparing the effect of sampling time, the transition from soybean to pastures, in integrated crop-livestock, increased dehydrogenase activity, indicating

a positive effect of pastures stimulating this enzyme due to high plant biomass (Santos *et al.*, 2015). In no-tillage, dehydrogenase showed lower variation, slightly increasing with straw presence, as also reported by Yan *et al*. (2023a; 2023b). In no-tillage, fluorescein diacetate hydrolysis temporarily increased in June (under millet) and decreased in September (under straw). In integrated crop-livestock, fluorescein diacetate hydrolysis consistently decreased over time. This result could be due to high substrate availability, potentially saturating esterase, which is an extracellular enzyme that degrades fluorescein, thus reducing fluorescein diacetate hydrolysis (Dzionek *et al.*, 2018).

The linear discriminant analysis showed that soil microbial biomass and enzymatic activity responded to different plant species and soil management practices. When comparing soybean and maize grown under no-tillage and integrated crop-livestock, respectively, distinct responses in soil microbial properties were observed. For instance, soil microbial biomass C temporarily decreased in no-tillage when millet was cultivated and increased in integrated crop-livestock when pasture and cattle were present. This means that changing plant species within these systems (e.g., switching soybean to millet, and maize to pastures, in no-tillage and integrated crop-livestock, respectively) led to different microbial responses, particularly when straw cover was present on the soil surface in no-tillage. The presence of millet adds a more recalcitrant C in no-tillage, while pastures and cattle manure, in integrated livestock, contribute to a more labile C and N. This main difference can contribute to distinct responses of soil microbial biomass and enzymatic activity. Our results confirm that the distinct plant species (Sun *et al.*, 2023) grown and the way the soil is managed significantly impact soil microbial biomass and activity (Hartman *et al*., 2018; Tian *et al.*, 2020). In addition, the presence of organic residues, in no-tillage, modulates the response of microbial biomass and enzymatic activity, and this occurs due to the chemical composition of the organic residues returned to the soil, which influences microbial responses (Almagro *et al.*, 2021). In our study, millet straw has a more recalcitrant C, which temporarily reduces soil microbial biomass in the no-tillage system when this residue is present.

Soil pH was the main driver of dehydrogenase in NT (September and December) and ICL (September). During these periods, there is the presence of straw cover in no-tillage (millet straw) and integrated crop-livestock (dried pastures), confirming the effect of straw associated with pH on soil dehydrogenase activity (Sharma *et al*., 2023). Soil moisture, as an important driver in tropical regions, was found to be related to microbial biomass and organic C in integrated crop-livestock, suggesting the effect of living plants and roots (Cheng; Coleman, 1990). Additionally, available P was shown to be an important driver of microbial biomass in

integrated crop-livestock. This means that higher availability of P can increase soil microbial biomass C (Camargo *et al.*, 2024). However, greater microbial biomass could increase the availability of P from organic matter in integrated crop-livestock through enzymatic processes (Deiss *et al.*, 2016).

Our results showed that the temporal stability of microbial biomass and enzymatic activity does not differ when comparing integrated crop-livestock and no-tillage. This may be due to the continuous input of organic residues associated with less disturbance by zero or reduced tillage in both systems (Page; Dang; Dalal, 2020). Indeed, Kostin *et al*. (2021) reported that agricultural systems with fewer perturbations have higher stabilization of microbial properties. Thus, the adoption of agricultural practices with lower soil disturbance associated with the addition of organic residues will contribute to maintaining more stable and resilient microbial properties over time (Kostin *et al*., 2021), promoting long-term soil health and ecosystem sustainability.

Although different temporal dynamics were observed comparing both agricultural systems, the no-tillage promoted in general higher phosphatase and urease activities. These findings support the idea of continuous input of straw into the soil to increase enzymatic activity related to P and N cycling (Xie *et al.*, 2021). This increases the availability of plant nutrients and crop productivity (Li *et al*., 2018). These findings reinforce the importance of agricultural management practices, such as the no-tillage system, in stimulating enzymatic activity. These microbial indicators are critical for maintaining soil health and long-term agricultural productivity (Daunoras; Kačergius; Gudiukaitė, 2024). Future studies should elucidate the mechanisms driving these seasonal variations to direct agricultural management strategies for enhancing soil health and sustainability.

3.5 Conclusion

This study demonstrated that the responses of soil microbial biomass and enzymatic activity differ between no-tillage and integrated crop-livestock systems. Furthermore, the impact of seasonal variations in tropical soils on soil microbial biomass and enzymatic activity depends on the characteristics of each agricultural system. Our findings showed the effects of crop succession and millet straw cover in integrated crop-livestock and no-tillage, respectively, on microbial biomass C, urease, and phosphatase activity. Additionally, the distinct responses of soil microbial properties observed at different sampling times suggest a direct effect of agricultural systems, which can have implications for system-specific management practices.

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4 CHAPTER 3

SOIL MANAGEMENT SHAPES BACTERIAL AND ARCHAEAL COMMUNITIES IN SOYBEAN RHIZOSPHERE: COMPARISON OF NO-TILLAGE AND INTEGRATED CROP-LIVESTOCK SYSTEMS

ABSTRACT

Sustainable agricultural systems play a crucial role in improving soil properties and enhancing crop yields. Particularly for soybean, a vital agricultural commodity, no-tillage (NT) and integrated crop-livestock (ICL) systems have been employed in tropical regions. Despite the recognized benefits of using NT and ICL, there is a significant knowledge gap regarding their impact on the rhizosphere microbiome of soybean. Therefore, this field study aimed to explore and compare the responses of the bacterial and archaeal communities within the soybean rhizosphere in both NT and ICL systems. To address this objective, in addition to sampling the soybean rhizosphere, we collected samples from the bulk soil in the NT area and the rhizospheres of grass (*Urochloa brizantha*) and corn (*Zea mays* L.) in the ICL system, covering the typical land use in this region. The results revealed distinct bacterial and archaeal communities in the soybean rhizosphere under NT and ICL. Specifically, the ICL system enriched the soybean rhizosphere with KD4_96 (score 3), Vicinamibacteraceae (score 3), *Candidatus Nitrocosmicus* (score 2.5), and *Methylobacterium* (score 2.5). In contrast, NT led to an enrichment of *Solirubrobacter* (score 3), *Amycolatopsis* (score 2.8), *Sphingomonas* (score 2.8), and Nitrososphaeraceae (score 2.5). Microbial community interactions exhibited greater complexity in the soybean rhizosphere under NT (676 nodes and 7095 edges). Notably, both bacterial and archaeal communities in the soybean rhizosphere under NT and ICL demonstrated potential functionality in nitrogen fixation. Thus, this study showed that NT and ICL promoted different responses of bacterial and archaeal communities within the soybean rhizosphere, which can influence the plant's performance.

Keywords: Archaea, Bacteria, Conservation systems, Microbial communities.

4.1 Introduction

During the last decade, agricultural systems have placed a strong emphasis on sustainability, particularly from an environmental perspective. This emphasis includes integrated agricultural systems that introduce different production methods within the same area and employ distinct strategies (Bieluczyk *et al*., 2020), such as no-tillage (NT) and intercropping systems. In Brazil, the total area under both NT and intercropping agricultural systems encompasses approximately 50 million hectares. These agricultural systems yield various benefits for both agriculture and the environment, such as increased productivity (Chai *et al*., 2021) and reduced greenhouse gas emissions (Tran *et al*., 2020). Concerning the positive impact on the soil environment, these systems contribute to an increase in soil organic matter and nutrients (Bieluczyk *et al*., 2020), preserve soil moisture (Glatzle *et al*., 2021), and create favorable conditions for soil microbial communities (Bertola; Ferrarini; Visioli, 2021).

The soil microbial communities play a crucial role in both agricultural production and environmental sustainability. Notably, bacterial and archaeal communities are among the most abundant and diverse microbial communities found in soils (Singh *et al*., 2009), playing essential roles in soil and plant productivity, particularly within integrated agricultural systems (Pratibha *et al*., 2023). Indeed, both bacterial and archaeal communities contribute to nutrient cycling through the decomposition of organic residues (Raza *et al*., 2023), being involved in various steps of nitrogen cycling (Gubry-Rangin; Nicol; Prosser, 2010), such as ammonification and nitrification (Diao *et al*., 2023). In addition, these communities facilitate processes such as nitrogen fixation promotion (Sepp *et al*., 2023) and phosphate solubilization (Tian *et al*., 2021). These processes, orchestrated by both bacterial and archaeal communities, make nutrients more accessible to plants, thereby promoting higher productivity (Kebede, 2021). Particularly, both bacterial and archaeal communities can help reduce gas emissions into the atmosphere, thereby mitigating the greenhouse effect (Bardgett; Freeman; Ostle, 2008).

Therefore, the knowledge of structure, diversity, and composition of both bacterial and archaeal communities in agricultural systems is important to increase their sustainability. NT is a well-known agricultural system that requires the maintenance of plant coverage on soil and has been recognized for its ability to enhance soil properties and increase crop yields (Busari *et al*., 2015; Calonego *et al*., 2017; Galdos *et al*., 2019). Over the last decade, integrated crop-livestock (ICL) has emerged as a noteworthy system that promotes plant productivity through the enhancement of nutrient cycling (Farias *et al*., 2020). Several studies have shown the positive effect of ICL on soil properties, increasing the soil organic matter content (Bieluczyk

et al., 2020) and improving the biological properties (Sekaran; Kumar; Gonzalez-Hernandez, 2021). Regarding the effect on soil bacterial community, ICL has positively contributed to changing the community composition and diversity (Bansal; Chakraborty; Kumar, 2022; Chen *et al*., 2018; Sarto *et al*., 2020). For instance, Bansal, Chakraborty and Kumar (2022) observed enhanced phospholipid fatty acid (PLFA), total bacterial biomass, gram (+), gram (–) bacteria in ICL as compared to conventional agriculture.

Despite its beneficial effects on agriculture, there are few studies specifically comparing NT and ICL systems regarding their impact on soil properties and crop yield (Assmann *et al*., 2014; Souza *et al*., 2016). Concerning soybean, the primary crop cultivated under both NT and ICL, it is not known how each system affects its rhizosphere and the associated microbial communities. This is a critical question as the rhizosphere of plants is influenced by soil management, and the rhizospheric microbial communities are in turn affected by the rhizosphere (Leite *et al*., 2024). For instance, a recent study by Pires *et al*. (2021) observed higher mycorrhiza diversity and richness in the rhizosphere of soybean intercropped with *Urochloa* under ICL.

Therefore, we hypothesized that the rhizosphere of soybean under the ICL system could show distinct diversity and composition of bacterial and archaeal communities, differing from those observed in soybean under NT. Thus, this study is designed to evaluate the bacterial and archaeal communities in the rhizosphere of soybean under the ICL system, particularly when intercropped with grasses and compare them with the rhizosphere of soybean under the NT system. For this, we assessed the microbial communities through the sequencing of the 16S rRNA gene and correlated them with chemical and biological soil parameters. With this, we aimed to gain insights into how more sustainable agricultural practices benefit the recruitment of beneficial microbes in the rhizosphere of soybean, potentially influencing plant growth and health.

4.2 Material and methods

4.2.1 Field location

The study was carried out from March 2022 to April 2023 at Fazenda Barbosa, Brejo, MA, Brazil (3◦42’1.66"S; 42◦56’25.08"W; 102 m). The soil is classified as Yellow Argisol Typical Dystrocohesive (PAdx), with a predominance of kaolinite (Dantas *et al*., 2014), and presents 76% sand, 8% silt, and 16% clay. The climate is tropical (Aw) according to the

Köppen-Gerger (Brito *et al*., 2023). The average annual precipitation and temperature are 1,613 mm and 27 ◦C, respectively (Maranhão, 2022).

4.2.2 Agricultural systems

We selected two main agricultural crop systems: no-tillage (NT) and integrated crop-livestock (ICL) (Figure 1). The NT area was deforested in 2003, with the application of 2 tons ha^{-1} of calcitic limestone, and it has been annually cultivated with soybean and millet under crop succession. Each year, soil fertilization consists of 100 kg ha^{-1} of ammonium sulfate, 150 kg ha^{-1} of monoammonium phosphate, and 170 kg ha^{-1} of potassium chloride. The ICL area was deforested in 2009, with the application of 1 ton ha^{-1} of dolomitic limestone + 0.5 ton ha^{-1} of gypsum. The no-tillage system was implemented with soybean and millet under crop succession until 2020. Starting from 2021, soybean has been cultivated followed by pasture (*Urochloa brizantha* cv. Marandu) and intercropped with corn. In both systems, soybean is sown at a population of 300,000 plants per hectare. Annually, soil fertilization consists of 340 kg ha^{-1} (formulation NPK 12-30-00) plus 200 kg ha^{-1} (formulation NPK 10-00-30) after 30 days. After the crop harvest (soybean and corn), the pasture is grazed by cattle (three units of animals per hectare).

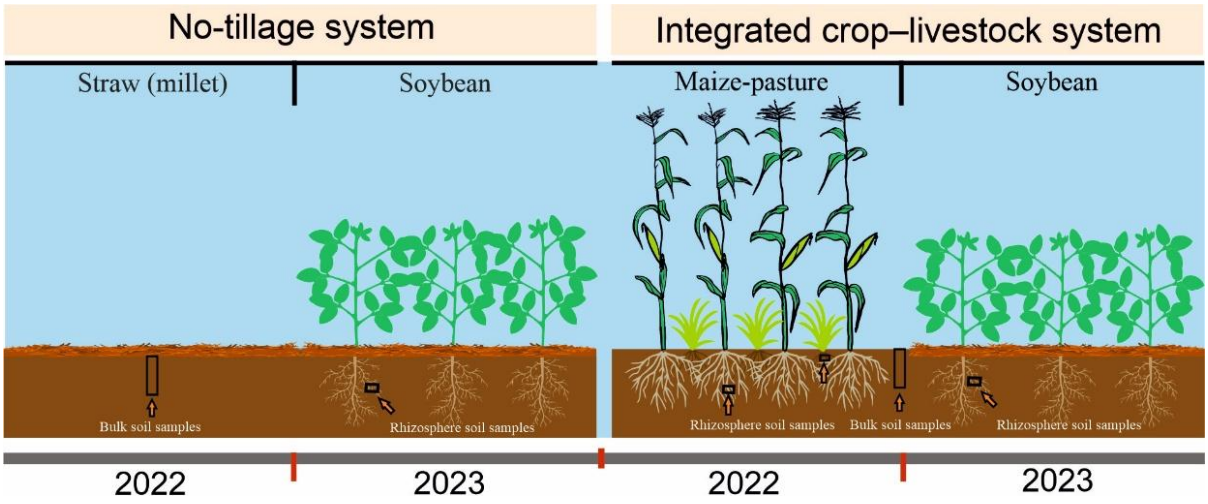


Figure 6 - Illustration depicting the variances in agricultural management systems.

4.2.3 Rhizosphere sampling

In both NT and ICL systems, the sampling of soybean rhizosphere was conducted in March 2023 (Figure 6). However, to evaluate the effect of both NT and ICL systems on bacterial

communities in the rhizosphere of soybean, we collected samples from bulk soil, and the rhizosphere of grass (*Urochloa brizantha*) and corn (*Zea mays* L.) in ICL. To collect the rhizosphere samples, the soil adhered to roots was carefully collected and put into sterile Eppendorf tubes, which were immediately frozen at a temperature of −80 °C. The bulk soil was collected at 0–20 cm depth and stored in sterile bags at −80 °C. Both bulk soil samples from NT and ICL were chemically and biologically. Briefly, total organic carbon (TOC) was estimated according to Yeomans and Bremner (1988). Microbial biomass carbon (MBC) was determined following Vance, Brookes and Jenkinson (1987). The activities of urease, dehydrogenase, acid phosphatase, and fluorescein diacetate (FDA) hydrolysis were assessed according to Tabatabai (1994).

4.2.4 DNA extraction and sequencing

To extract DNA, 0.5 g of soil samples were used with a DNA Isolation Kit (DNeasy PowerSoil Pro Kit; Qiagen, CA, USA). The quality and concentration of DNA were measured using a spectrophotometer. The primers 515F (5' -GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVHHHTWTCTAAT-3'), were used to amplify the V4 region of the 16S rRNA by polymerase chain reaction (PCR) (Caporaso *et al*., 2011). PCR program was: 95 °C for 3 min, followed by 35 cycles at 98 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. This procedure includes sequencing adapters and dual-index barcodes to differentiate each sample. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) and quantified by Qubit fluorometer with dsDNA BR Assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The libraries were paired-end sequenced using the Illumina MiSeq Reagent Kit v2 (300-cycles, 2 × 150 bp) (Illumina) at the Genomic and Bioinformatic Facility Centre (CeGenBio) of the Federal University of Ceará, Brazil.

The 16S rRNA sequences were preprocessed using the QIIME 2 v2021.11 software. Demultiplexing and quality control were conducted using DADA2 (Callahan; McMurdie; Holmes, 2017), employing the consensus method to eliminate any remaining chimeric and low-quality sequences (<q20). The 16S rRNA sequencing approach yielded approximately 11,409,000 reads, averaging 475,370 sequences per sample. These sequences were then rarefied to 252,750 sequences, based on the lowest sample count, and singleton and doubleton sequences were removed. Taxonomic classification for the 16S rRNA region was done using the SILVA

database version 138 at 97% similarity (Quast *et al*., 2013). The 16S rRNA data are available at NCBI SRA under the identification PRJNA1069081.

4.2.5 Data analysis

To compare the structure of bacterial and archaeal communities and its correlation with environmental parameters, Principal Component Analysis (PCA) and Redundancy Analysis were employed using Canoco 5 (Biometrics, Wageningen, The Netherlands). Furthermore, a permutational multivariate analysis of variance (Permanova; Anderson, 2001) was performed to identify significant differences in bacterial community structures among the samples. Subsequently, microbial composition was compared using the LDA Effect Size (LEfSe) analysis, as outlined by Segata *et al.* (2011). For this analysis, the OTU table at the phylum, genus, and OTU levels was used to assess community composition. A functional prediction annotation was carried out to evaluate the potential functions exhibited by both bacterial and archaeal communities using PICRUSt2 (Douglas *et al*., 2020), with a focus on the nitrogen cycle. Finally, we assessed the complexity of community interactions through network analysis. SparCC correlations (Friedman; Alm, 2012) were calculated, and only significant ($p < 0.01$) and strong (>0.9 or < -0.9) correlations were selected. For network visualization and the calculation of topological properties, we utilized the interactive platform Gephi (Bastian; Heymann; Jacomy, 2009).

4.3 Results

4.3.1 Structure of bacterial and archaeal communities

The results of PCA explained 18.38% of total variation and revealed a distinct clustering of bacterial and archaeal communities between NT and ICL (Permanova $F = 2.809$, $P = 0.0001$; Figure 7A). Notably, the bacterial and archaeal communities in the rhizosphere of soybean under NT clustered with the communities found in bulk soil. In the ICL, the bacterial and archaeal communities in the soybean rhizosphere were distinctly separated from those found in rhizospheres of grass and corn, which clustered together. The RDA analysis comparing only rhizospheres and its correlation with environmental parameters explained 20.36% of the total variation and showed that bacterial and archaeal communities in soybean from both NT and ICL were clustered apart from the rhizosphere of corn and grass, as shown in the first axis of

the RDA (Permanova F = 2.51, P = 0.0001; Figure 7B). Also, the samples from the rhizosphere of soybean were different between NT and ICL. The community of the soybean rhizosphere under no-tillage correlated with soil microbial biomass and enzymes, while the samples from grass and corn correlated with fluorescein diacetate and microbial quotient.

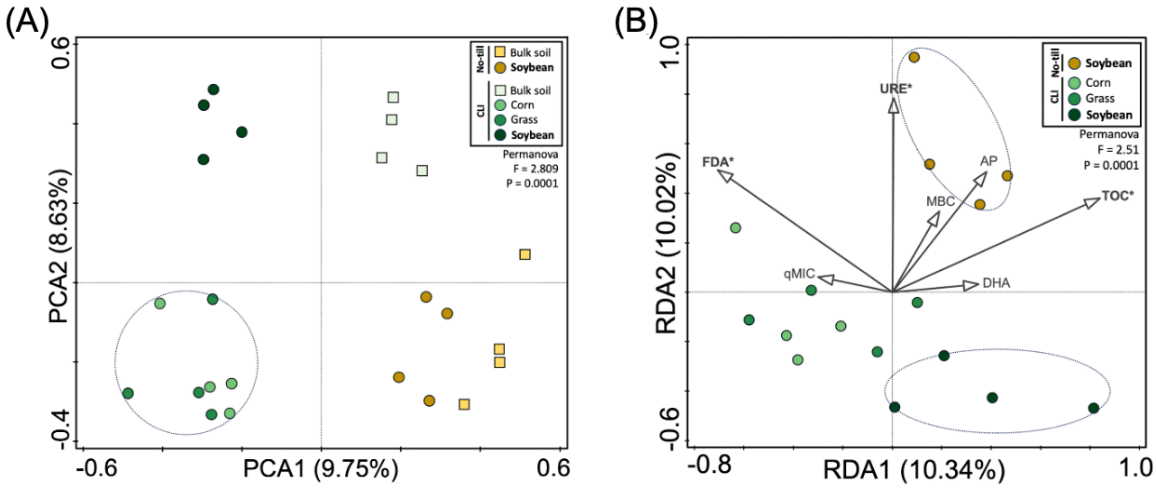


Figure 7 - Structure of the bacterial and archaeal community in bulk soil and rhizosphere associated with crops in no-tillage (No-till) and integrated crop-livestock (ICL) systems based on the 16S rRNA gene. (A) Principal component analysis (PCA) including samples from bulk soil and rhizosphere. (B) Redundancy analysis (RDA) of the rhizosphere in the two evaluated systems. Arrows indicate the correlation between microbial profile, organic carbon, and soil microbial properties. The analysis of permutation (Permanova) is indicated in the top right corner of the graphs. TOC: total organic carbon; MBC: microbial biomass carbon; qMic: microbial quotient; DHA: dehydrogenase; FDA: fluorescein diacetate; URE: urease; AP: acid phosphatase.

4.3.2 Composition of bacterial and archaeal communities

The three most abundant microbial phyla identified in bulk soil and rhizosphere across both agricultural systems were Actinobacteriota (30% of total sequences), followed by Proteobacteria (19%) and Firmicutes (10%) (Figure 8A). Comparing soybean bulk soil and rhizosphere, LDA analysis showed that the rhizosphere increased the abundance of Proteobacteria and Bacteroidota, whereas Acidobacteriota, Chloroflexi, and Crenarchaeota were more abundant in bulk soil ($P < 0.05$, Figure 8B). The rhizosphere of maize and grass showed higher abundances of Actinobacteriota and Myxococcota, respectively ($P < 0.05$, Figure 8C).

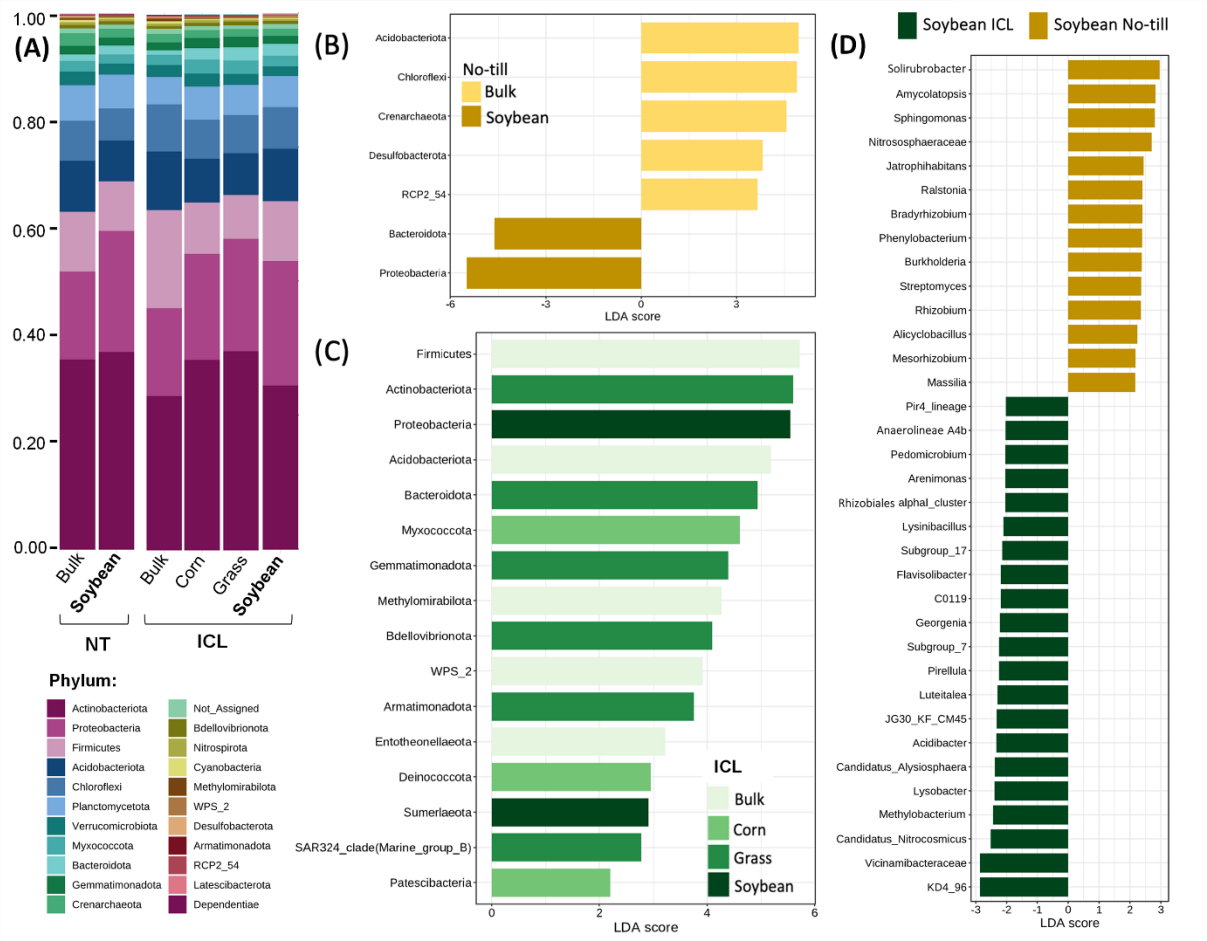


Figure 8 - Microbial community composition in bulk soil and rhizosphere of crops under no-tillage (NT) and integrated crop-livestock (ICL) systems. (A) Most abundant bacterial phyla. Linear discriminant analysis (LDA) effect size (LEfSe) of the main microbial phyla enriched in (B) no-tillage and (C) integrated crop-livestock, and of the main microbial genera associated with (D) soybean cultivated in both systems.

We also examined the enrichment of bacterial and archaeal groups at the genus level in the soybean rhizosphere (Figure 8D). This analysis revealed that the soybean rhizosphere under ICL exhibited an increase in 21 taxa, whereas the soybean rhizosphere under NT showed an increase in 14 taxa. More specifically, the comparison of the soybean rhizosphere communities in both systems highlighted an enrichment of KD4_96, Vicinamibacteraceae, *Candidatus Nitrocosmicus*, and *Methylobacterium* in ICL, while under NT the soybean rhizosphere showed enrichment of *Solirubrobacter*, *Amycolatopsis*, *Sphingomonas*, and Nitrososphaeraceae.

4.3.3 Dynamics of bacterial and archaeal communities

The co-occurrence network analysis showed a distinct pattern of bacterial and archaeal interactions in bulk soil and rhizosphere comparing both NT and ICL (Figure 9; Table 3). In

NT, both bulk soil and the rhizosphere of soybean showed a similar number of nodes (671 and 676 nodes, respectively, in bulk soil and rhizosphere). However, the bacterial and archaeal communities in the rhizosphere of soybean under no-tillage presented a higher number of edges (4442 and 7095 edges, respectively, in bulk soil and rhizosphere). In the integrated crop-livestock system, bulk soil (568 nodes and 2896 edges) and the rhizosphere of corn (569 nodes and 2637 edges) showed lower complexity compared to the rhizosphere of grass (676 nodes and 4493 edges) and soybean (628 nodes and 4028 edges). Comparing soybean under NT and ICL, we observed higher complexity in the rhizosphere of soybean in NT (676 nodes and 7095 edges). In addition, the bacterial and archaeal communities in the rhizosphere of soybean under NT showed higher values of average degree (Table 3).

Table 3 - Topological properties of soil and rhizosphere microbiome networks in no-tillage (NT) and integrated crop-livestock (ICL) systems, as inferred by SparCC and calculated using Gephi.

	NT		ICL			
Network properties	Bulk	Soybean	Bulk	Corn	Grass	Soybean
Number of nodes[a]	671	676	568	569	676	628
Number of edges[b]	4442	7095	2896	2637	4493	4028
Positive edges[c]	73%	69%	63%	58%	75%	78%
Negative edges[d]	27%	31%	37%	42%	25%	22%
Modularity[e]	1.33	1.407	2.057	3.179	1.133	1.089
Number of communities[f]	95	66	73	57	68	90
Network diameter[g]	20	20	19	16	16	25
Average path length[h]	6.48	5.93	6.485	5.589	6.056	8.060
Average degree[i]	13.24	20.99	10.197	9.269	13.293	12.828
Average clustering coeffic.[j]	0.413	0.417	0.350	0.342	0.364	0.422

[a]Microbial taxon (at genus level) with at least one significant ($P < 0.01$) and strong (SparCC > 0.9 or < -0.9) correlation;
[b]Number of connections/correlations obtained by SparCC analysis;
[c]Positive correlation (> 0.9 with $P < 0.01$);
[d]Negative correlation (< -0.9 with $P < 0.01$);
[e]The capability of the nodes to form highly connected communities, that is, a structure with high density of between nodes connections (inferred by Gephi);
[f]A community is defined as a group of nodes densely connected internally (Gephi);
[g]The longest distance between nodes in the network, measured in number of edges (Gephi);
[h]Average network distance between all pair of nodes or the average length off all edges in the network (Gephi);
[i]The average number of connections per node in the network, that is, the node connectivity (Gephi);
[j]How nodes are embedded in their neighborhood and the degree to which they tend to cluster together (Gephi).

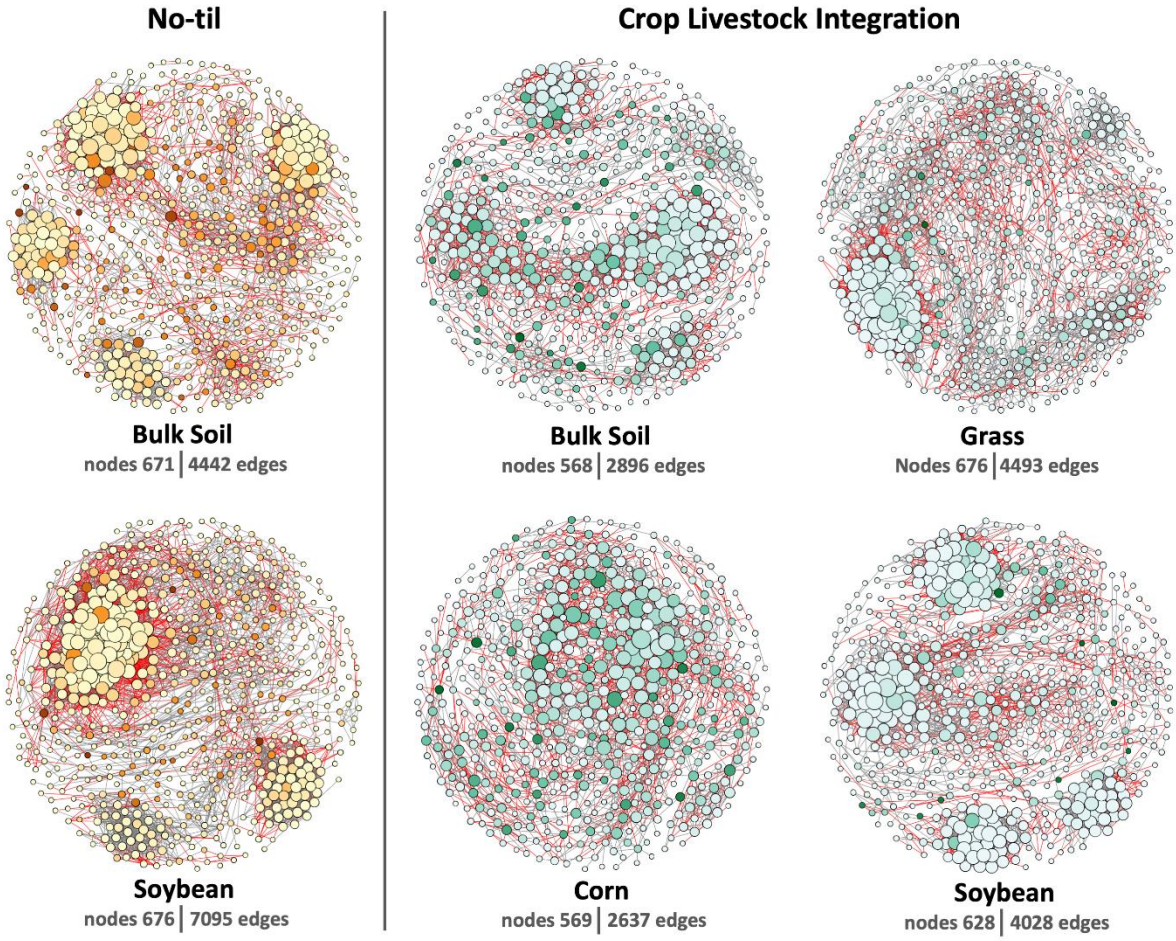


Figure 9 - Network co-occurrence analysis of the bacterial communities in soils under no-tillage and integrated crop-livestock systems. A connection represents a SparCC correlation with magnitude > 0.9 (positive correlation – gray edges) or < –0.9 (negative correlation – red edges) and is statistically significant ($P < 0.01$). Each node represents taxa at the OTU level, and the node size is proportional to the number of connections (i.e., degree). Node color is based on betweenness centrality, where darker colors indicate higher values.

4.3.4 Functional prediction profiling

Since both NT and ICL aim to increase and maintain soil sustainability through organic matter, we predicted potential functions associated with nitrogen cycling (Figure 10). We observed distinct responses of potential functions when comparing soybean under NT and ICL. Potential functions related to organic degradation and nitrification were observed in both bulk soils. However, the bacterial and archaeal communities in the rhizosphere of soybean under NT showed potential functions related to nitrogen organic degradation. In addition, both bacterial and archaeal communities in the rhizosphere of soybean under NT and ICL showed potential functions related to nitrogen fixation. Interestingly, a higher abundance of genes related to

assimilatory nitrate reduction was observed in the rhizosphere compared to bulk soils in both systems.

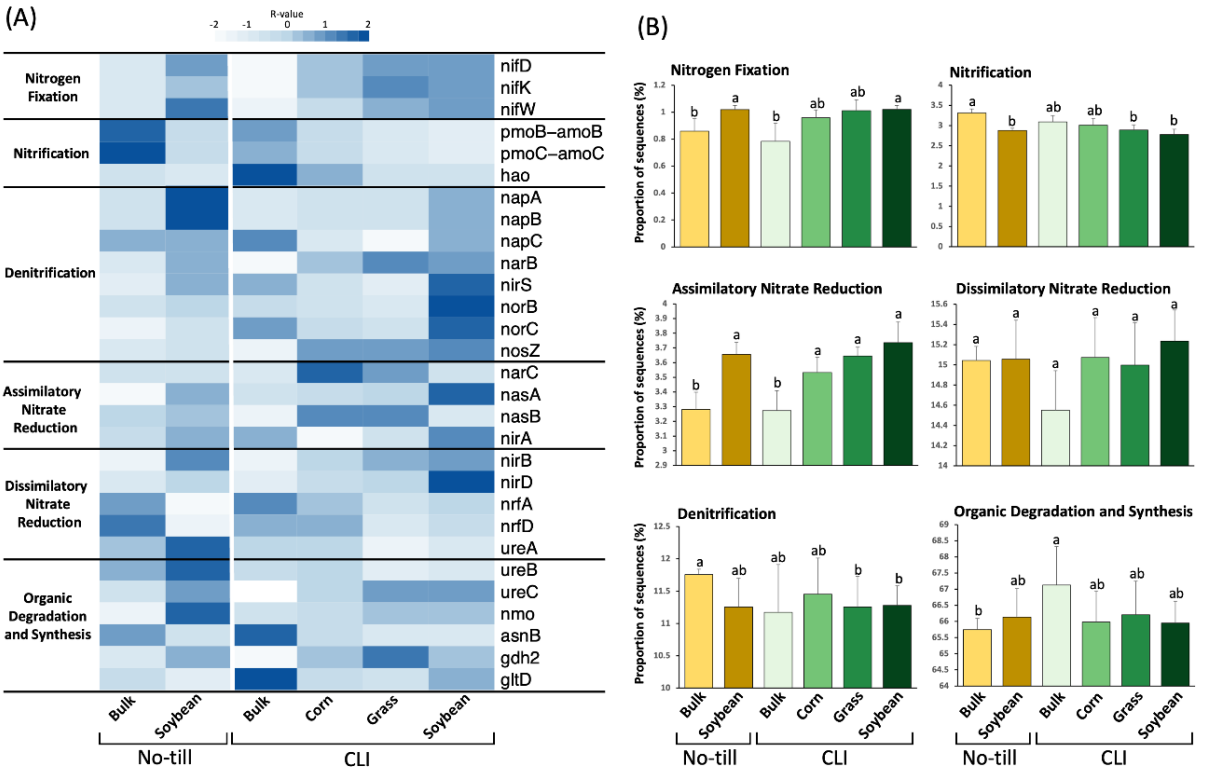


Figure 10 - (A) Heatmap and (B) bar graph showing the differential abundance of predicted functions related to the nitrogen cycle in no-tillage (No-till) and integrated crop-livestock (ICL) systems. The classification is based on functional predictions using the FAPROTAX database. Different letters indicate significant differences ($P < 0.05$) based on a post-hoc Tukey-Kramer test followed by Benjamini-Hochberg FDR correction.

4.4 Discussion

4.4.1 Structure of bacterial and archaeal communities

The results of this study confirmed the hypothesis that the rhizosphere of soybean in both no-tillage and integrated crop-livestock systems exhibited distinct bacterial and archaeal communities and their interactions. This suggests that, despite being the same crop species, the rhizosphere composition is influenced by the specific agricultural system (Chen, *et al*., 2022a). The bacterial and archaeal communities in the rhizosphere of soybean differed between no-tillage and integrated crop-livestock systems. In the no-tillage system, the bacterial and archaeal communities in the soybean rhizosphere were closely related to those in the bulk soil, indicating an influence of millet straw added to the soil surface. This aligns with previous studies that have

reported the impact of straw returning on microbial communities in bulk soil, which are then recruited by the rhizosphere (Bu *et al*., 2020). Additionally, Ridder-Duine *et al*., (2005) reported that bacterial communities in the rhizosphere are generally more similar to those in the bulk soil than to other rhizosphere communities. This may explain why the rhizosphere of soybean under integrated crop-livestock systems was not influenced by the microbial communities associated with both grass and corn rhizospheres.

The distinct characteristics of plant species within integrated crop-livestock systems, particularly grass and corn (Poaceae family), may contribute to differences in the bacterial and archaeal communities present in the soybean rhizosphere (Leguminosae family). Regarding the effect of microbial properties, the presence of grass stimulates microbial biomass due to a higher proportion of carbon (C) than nitrogen (N), thus increasing the microbial population (Amorin *et al*., 2020). When the bacterial community was considered, a previous study observed that the rhizosphere of corn (Poaceae) and cowpea (Leguminosae) in subsequent cultivation were distinct, suggesting an effect of plant species and demonstrating that the microbiome from distinct rhizospheres was not shared (Araujo *et al*., 2019). This effect is attributed to differences in root exudation between plant species, where Poaceae family plants typically release carbohydrates, while leguminous plant species release amino acids (Alvey *et al*., 2003; Merbach *et al*., 1999). In addition, a previous study has shown that leguminous plant species present a higher amount of rhizosphere carboxylates than grasses (Kidd *et al*., 2016). Interestingly, when comparing rhizospheres, the prokaryotic community in soybean under no-tillage was associated with soil organic carbon, microbial biomass, and enzymes, suggesting a direct effect of millet straw as the main carbon source for soil microorganisms (Zhang *et al*., 2019).

4.4.2 Composition of bacterial and archaeal communities

In general, we observed a high relative abundance of Actinobacteria, Proteobacteria, and Firmicutes, as expected in both agricultural systems. This prevalence can be attributed to their role in facilitating the maintenance of organic carbon, primarily through the input of straw and root debris, consequently fostering the proliferation of these microbial groups (Chen *et al*., 2022b). The high abundance of these three phyla in both agricultural systems is interesting, as members of these microbial groups play essential roles in the soil-plant continuum. They act as decomposers of organic residues, releasing nutrients, and function as plant-growth promoters, thereby enhancing plant performance (Spain; Krumholz; Elshahed, 2009; Bao *et al*., 2021).

Although Actinobacteria, Proteobacteria, and Firmicutes have dominated the microbial community, specific bacterial and archaeal groups were enriched in the rhizospheres of plants, particularly in soybean under no-tillage and integrated crop-livestock systems, confirming the observed pattern in the structure of bacterial and archaeal communities. Specifically, in the rhizosphere of soybean under integrated crop-livestock, there was an enrichment of *KD4_96*, *Vicinamibacteraceae*, *Candidatus Nitrocosmicus*, and *Methylobacterium*. Notably, the enrichment of *Methylobacterium* in the soybean rhizosphere is significant, given its recognized role as an important nitrogen fixer and hormone producer (Grossi *et al*., 2020). Previous studies have observed *Methylobacterium* associated with the rhizosphere of soybean under sustainable farming systems (Bender *et al*., 2022; Agyekum *et al*., 2023). Additionally, we can highlight the enrichment of *Candidatus Nitrocosmicus*, a recognized aerobic ammonia oxidizer (Alves *et al*., 2019), which can contribute to nitrogen cycling in integrated crop-livestock.

The rhizosphere of soybean under no-tillage enriched bacterial groups involved in carbon and nitrogen cycling, such as *Sphingomonas* and members of the family Nitrososphaeraceae (Dong *et al*., 2017; Behnke *et al*., 2021). The presence of straw in the no-tillage system could explain the enrichment of these specific groups, such as *Sphingomonas*, known as plant growth promoters through nitrogen fixation, contributing to soybean growth (Luo *et al*., 2020; Kafi *et al*., 2021). Regarding Nitrososphaeraceae, previous studies have reported this nitrifying microorganism impacting nitrogen cycling (Tourna *et al*., 2011), especially during soybean growth (He *et al*., 2014), contributing to an increase in nitrogen availability to plants.

4.4.3 Bacterial and archaeal community dynamics

In general, we observed a more complex network of interactions among bacterial and archaeal communities in both bulk soil and the rhizosphere of soybean in the no-tillage system. This high number of microbial interactions in the bulk soil suggests greater soil microbial stability in no-tillage systems, which may contribute to the assembly of the soybean rhizosphere microbiome (Gu *et al*., 2022). In addition, the higher average degree in the co-occurrence network observed in the rhizosphere of soybean under no-tillage suggests greater interdependence among bacteria and archaea and less divergent functional groups (Gu *et al*., 2022). Indeed, the rhizosphere of soybean under no-tillage showed increased bacterial and archaeal interactions, which contribute to improving soybean growth through biological control of pathogens, production of plant regulators, and biological nitrogen fixation (Volpiano *et al*.,

2022). The heightened complexity of the microbial community may mitigate pathogen invasion by enhancing competition for resources and niche occupancy, thereby promoting greater efficiency (Mendes *et al*., 2023).

In contrast, the integrated crop-livestock system exhibited variations in microbial interactions according to distinct plant rhizospheres. This variability suggests plant-specific microbial dynamics within integrated crop-livestock systems, potentially affecting the network of interactions among bacterial and archaeal communities. For instance, Schmidt *et al*. (2019) assessed microbial communities in various plant species and agricultural management practices and observed that plant selection drives rhizosphere microbial communities more significantly than agricultural management practices. This means that, despite integrated crop-livestock being a sustainable system, distinct rhizosphere microbial communities from different plant species, such as grass and corn, did not necessarily contribute to an increase in microbial interactions within the soybean rhizosphere.

4.4.4 Functional prediction profiling

It is known that both no-tillage and integrated crop-livestock systems aim to increase and maintain soil organic matter (Bieluczyk *et al*., 2020). Since organic matter is a primary source of nitrogen (N), we predicted potential functions related to N cycling to compare no-tillage and integrated crop-livestock systems. The results revealed contrasting responses of predicted functions related to N in the rhizosphere of soybean under no-tillage and integrated crop-livestock, indicating that distinct agricultural management practices potentially promote different effects on soil and rhizosphere functionality. In bulk soil, the potential functions related to organic degradation and nitrification indicate that the presence of organic matter in the soil stimulates biological processes related to N mineralization, thereby increasing N availability to plants (Hoffland *et al*., 2020).

Comparing samples from the rhizosphere across different systems, soybean under no-tillage exhibited potential functions related to organic degradation, suggesting that microbial communities play a role in organic matter decomposition (Khan *et al*., 2023). This could result from a similar composition of microbial communities and their interactions observed in the bulk soil and rhizosphere of soybean under no-tillage. On the other hand, both bacterial and archaeal communities in the rhizosphere of soybean under no-tillage and integrated crop-livestock systems showed potential functions related to nitrogen (N) fixation, which is important as this process makes N available from the atmosphere to plants, particularly soybean

(Ciampitti *et al*., 2021). Interestingly, a higher potential for nitrate reduction was observed in the rhizosphere compared to bulk soils in both systems, suggesting that plant-microbe interactions in the rhizosphere can contribute to increasing the assimilation of nitrate, potentially influencing N availability for the plants (Moreau *et al*., 2019).

4.5 Conclusions

This study presents novel findings regarding the rhizosphere microbiome in sustainable agricultural management systems, demonstrating that NT and ICL practices elicit distinct responses from bacterial and archaeal communities in the soybean rhizosphere. These results underscore the significant influence of the microbiome present in the soil, such as bulk soil in NT and the rhizospheres of pasture and corn in ICL, on the rhizosphere of soybean. Consequently, the study reveals that the assembly of the rhizosphere microbiome is strongly associated with the soil management system, regardless of whether the main crop belongs to the same plant species.

In addition to highlighting the intricate interplay between soil management practices and rhizosphere microbiome composition, our study underscores the importance of considering broader ecosystem dynamics in sustainable agriculture. Understanding how different agricultural practices shape microbial communities can lead to more effective management strategies that optimize crop productivity while promoting environmental sustainability.

Further studies could elucidate the specific mechanisms by which different soil management practices influence the rhizosphere microbiome and how these microbial communities, in turn, impact plant health and productivity. By gaining a more comprehensive understanding of these dynamics, we can continue to refine agricultural practices to ensure long-term sustainability and resilience in food production systems.

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5 FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

The study revealed significant differences between no-tillage and crop-livestock integration systems, underscoring the importance of considering temporal variation when analyzing soil biological attributes. Both the type of agricultural management and the sampling time exerted strong influence on microbial biomass and enzymatic activity (Appendix 1). However, the interaction between management and sampling time was even more pronounced, showing that soil biological responses are dynamic and modulated by seasonal variations. This finding highlights the complexity of microbial processes in soils and the need for continuous monitoring over time to more accurately assess the sustainability and efficiency of tropical agricultural systems.

The characterization of the soybean rhizosphere microbiome demonstrated that bacterial and archaeal communities are strongly influenced by the management system, reflecting microbial legacies left by previous vegetation and adopted practices (Appendix 2). Under no-tillage, greater similarity with bulk soil was observed, along with higher network complexity and stronger correlations with functional soil attributes such as microbial biomass and enzymatic activity. In contrast, in crop-livestock integration, the soybean rhizosphere displayed a distinct functional profile, indicating that management directly influences the composition and functionality of the microbiome.

Overall, the results reinforce that sustainable agricultural systems, such as no-tillage and crop-livestock integration, have the potential to positively modulate soil and rhizosphere microbiota, with direct implications for soil health, the functioning of biogeochemical cycles, and crop productivity.

As future perspectives, it is important to deepen functional analyses through omics approaches such as metagenomics and metatranscriptomics, as well as to evaluate, in the long term, the impact of management systems on the functional stability of the soil microbiome. Additionally, studies that integrate different soil types and climatic conditions may broaden the understanding of the applicability of microbiological indicators as tools for more sustainable agricultural management.

APPENDICES

European Journal of Soil Biology 121 (2024) 103628

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: www.elsevier.com/locate/ejsobi

Original article

Seasonal responses of soil microbial biomass C and enzymatic activity comparing no-tillage and integrated crop-livestock systems

Romario Martins Costa [a], Erica Maria Batista Araujo [a], Davila Esmelinda Oliveira Silva [a], Sandra Mara Barbosa Rocha [a], Aurenivia Bonifacio [b], Ricardo Silva Sousa [c], Arthur Prudencio de Araujo Pereira [d], Erika Valente de Medeiros [e], Edvaldo Sagrilo [f], José Oscar Lustosa de Oliveira Junior [f], Henrique Antunes de Souza [f], Ademir Sergio Ferreira Araujo [a,*]

[a] Soil Microbial Ecology Group, Universidade Federal Do Piauí, Teresina, PI, Brazil
[b] Departamento de Biologia, Universidade Federal Do Piauí, Teresina, PI, Brazil
[c] Departamento de Engenharia Agrícola e Solos, Universidade Federal Do Piauí, Teresina, PI, Brazil
[d] Departamento de Ciencias Do Solo, Universidade Federal Do Ceará, Fortaleza, Ceará, Brazil
[e] Universidade Federal Do Agreste Pernambucano, Garanhuns, Pernambuco, Brazil
[f] Embrapa Meio Norte, Teresina, PI, Brazil

ARTICLE INFO

Keywords:
Sustainable agriculture
Soil biological properties
Soil enzymes

ABSTRACT

Sustainable agricultural systems, such as integrated crop-livestock (ICL) and no-tillage (NT), aim to sustainably produce crops and livestock while simultaneously conserving soil and its microbial properties, mainly in tropical regions. However, little is known about how microbial properties respond seasonally to management applied in NT and ICL. Thus, this study assessed the seasonal responses of soil microbial biomass C and enzymatic activity comparing both NT and ICL. The experimental area, under a block design with four replicates, with both NT and ICL management, was implemented in December 2022 on Yellow Argisol soil in Maranhao state, Brazil. Soil samples were collected (0–20 cm depth) in March, June, September, December, and March (2023). The results showed an effect size varying between 0.06 and 0.95 for agricultural systems, and 0.63 to 0.95 for sampling time. For the interaction between agricultural systems and sampling time, the effect size was superior to 0.86. NT showed initially higher microbial biomass C (~50 %), leveling with ICL by the end of the sampling period. Phosphatase and dehydrogenase increased in ICL from March to June (~200 % and ~700 % for phosphatase and dehydrogenase, respectively), while fluorescein diacetate hydrolysis fluctuated in NT. Urease was higher (~100 %) during all sampling times in NT. Linear discriminant analysis revealed distinct responses across sampling times, with a positive effect of pH on enzymatic activity in both systems and soil moisture and P impacting positively on microbial biomass in ICL. Our results revealed significant seasonal responses of soil microbial biomass and enzymatic activity comparing NT and ICL, but with distinct responses to agricultural systems. The study showed seasonal variation of soil microbial biomass and enzymatic activity dependent on the characteristics of NT and ICL. Therefore, understanding these differences helps farmers make better decisions for healthier soil and better crops.

1. Introduction

Sustainable agricultural systems are recognized as farming techniques that produce crops or livestock while conserving the environment, including the protection of soil and biodiversity, and being economically and socially viable [1]. Integrated crop-livestock (ICL) and no-tillage (NT) are particularly important agricultural systems used worldwide in crop production. Despite both ICL and NT being considered sustainable systems, they differ in terms of soil and crop management. ICL focuses on integrating crop and livestock production within the same system, promoting the recycling of organic residues from crops and livestock [2]. In contrast, NT involves directly seeding crops under the straw of the previous crop without disturbing the soil through tillage [3]. This maintains soil structure, reduces erosion, and conserves soil

* Corresponding author.
E-mail address: ademir@ufpi.edu.br (A.S.F. Araujo).

https://doi.org/10.1016/j.ejsobi.2024.103628
Received 12 February 2024; Received in revised form 13 May 2024; Accepted 21 May 2024
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Appendix 1 - Published article: Seasonal responses of soil microbial biomass C and enzymatic activity comparing no-tillage and integrated crop-livestock systems.

Rhizosphere 30 (2024) 100886

Contents lists available at ScienceDirect

Rhizosphere

journal homepage: www.elsevier.com/locate/rhisph

Soil management shapes bacterial and archaeal communities in soybean rhizosphere: Comparison of no-tillage and integrated crop-livestock systems

Romario Martins Costa [a,*], Mayanna Karlla Lima Costa [a], Sandra Mara Barbosa Rocha [a], Marcos Renan Lima Leite [a], Francisco de Alcantara Neto [b], Henrique Antunes de Souza [c], Arthur Prudencio de Araujo Pereira [d], Vania Maria Maciel Melo [e], Erika Valente de Medeiros [f], Lucas William Mendes [g], Ademir Sergio Ferreira Araujo [a]

[a] Soil Microbial Ecology Group, Universidade Federal do Piauí, Teresina, PI, Brazil
[b] Departamento de Fitotecnia, Universidade Federal do Piauí, Teresina, PI, Brazil
[c] Embrapa Meio Norte, Teresina, PI, Brazil
[d] Federal University of Ceará (Department of Soil Science), Fortaleza, Ceará, Brazil
[e] Federal University of Ceará (Department of Biological Sciences), Fortaleza, Ceará, Brazil
[f] Federal University of the Agreste of Pernambuco, Garanhuns, Pernambuco, Brazil
[g] Center for Nuclear Energy in Agriculture, University of Sao Paulo, Piracicaba, São Paulo, Brazil

ARTICLE INFO

Keywords:
No-tillage
Integrated crop-livestock
Microbial communities
Bacteria
Archaea

ABSTRACT

Sustainable agricultural systems play a crucial role in improving soil properties and enhancing crop yields. Particularly for soybean, a vital agricultural commodity, no-tillage (NT) and integrated crop-livestock (ICL) systems have been employed in tropical regions. Despite the recognized benefits of using NT and ICL, there is a significant knowledge gap regarding their impact on the rhizosphere microbiome of soybean. Therefore, this field study aimed to explore and compare the responses of the bacterial and archaeal communities within the soybean rhizosphere in both NT and ICL systems. To address this objective, in addition to sampling the soybean rhizosphere, we collected samples from the bulk soil in the NT area and the rhizospheres of grass (*Urochloa brizantha*) and corn (*Zea mays* L.) in the ICL system, covering the typical land use in this region. The results revealed distinct bacterial and archaeal communities in the soybean rhizosphere under NT and ICL. Specifically, the ICL system enriched the soybean rhizosphere with KD4_96 (score 3), Vicinamibacteraceae (score 3), *Candidatus Nitrocosmicus* (score 2.5), and *Methylobacterium* (score 2.5). In contrast, NT led to an enrichment of Solirubrobacter (score 3), Amycolatopsis (score 2.8), Sphingomonas (score 2.8), and Nitrososphaeraceae (score 2.5). Microbial community interactions exhibited greater complexity in the soybean rhizosphere under NT (676 nodes and 7095 edges). Notably, both bacterial and archaeal communities in the soybean rhizosphere under NT and ICL demonstrated potential functionality in nitrogen fixation. Thus, this study showed that NT and ICL promoted different responses of bacterial and archaeal communities within the soybean rhizosphere which, can influence the plant's performance.

1. Introduction

During the last decade, agricultural systems have placed a strong emphasis on sustainability, particularly from an environmental perspective. This emphasis includes integrated agricultural systems that introduce different production methods within the same area and employ distinct strategies (Bieluczyk et al., 2020), such as no-tillage (NT) and intercropping systems. In Brazil, the total area under both NT and intercropping agricultural systems encompasses approximately 50 million hectares (Embrapa, 2023). These agricultural systems yield various benefits for both agriculture and the environment, such as increased productivity (Chai et al., 2021) and reduced greenhouse gas

* Corresponding author.
E-mail address: asfaruaj@yahoo.com.br (R.M. Costa).

https://doi.org/10.1016/j.rhisph.2024.100886
Received 10 March 2024; Received in revised form 1 April 2024; Accepted 2 April 2024
Available online 7 April 2024
2452-2198/© 2024 Elsevier B.V. All rights reserved.

Appendix 2 - Published article: Soil management shapes bacterial and archaeal communities in soybean rhizosphere: comparison of no-tillage and integrated crop-livestock systems.