UNIVERSIDADE FEDERAL DO PARANÁ

RENATA CAROLINI DE SOUZA

METAGENÔMICA TAXONÔMICA E FUNCIONAL EM SOLOS AGRÍCOLAS DO NORTE DO PARANÁ E DO DISTRITO FEDERAL

CURITIBA

2016

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Tese apresentada como requisito parcial à obtenção do grau de Doutor em Microbiologia, no Curso de Pós-Graduação em Microbiologia, Parasitologia e Patologia, Setor de Ciências Biológicas, da Universidade Federal do Paraná.

Orientadora: Profa. Dra. Vânia Aparecida Vicente

Co-Orientadora: Dra. Mariangela Hungria

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Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS BIOLÓGICAS
Departamento de Patologia Básica
Pós-graduação em Microbiologia, Parasitologia e Patologia.

TERMO DE APROVAÇÃO

"METAGENÔMICA TAXONÔMICA E FUNCIONAL EM SOLOS AGRÍCOLAS DO NORTE DO PARANÁ E DO DISTRITO FEDERAL"

por

RENATA CAROLINI DE SOUZA

Tese aprovada como requisito parcial para obtenção do grau de Doutor no Curso de Pós-Graduação em Microbiologia, Parasitologia e Patologia, pela Comissão formada pelos professores:

Profª. Drª. Vânia Aparecida Vicente – Presidente

Profª. Drª. Glaciela Kaschuk

Profª. Drª. Lygia Terasawa

Dr. Roberto Tadeu Raittz

Drª. Renata Rodrigues Gomes

Curitiba, 22 de março de 2016.

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
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ATA DE DEFESA DE TESE DE DOUTORADO

Aos vinte e dois dias do mês de março de dois mil e dezesseis, às oito horas e trinta minutos, na sala 129 do Departamento de Patologia Básica do Setor de Ciências Biológicas da Universidade Federal do Paraná, reuniu-se a Comissão Examinadora de defesa de tese de doutorado de autoria da Pós-Graduanda em Microbiologia, Parasitologia e Patologia, RENATA CAROLINI DE SOUZA sob o título "METAGENÔMICA TAXONÔMICA E FUNCIONAL EM SOLOS AGRÍCOLAS DO NORTE DO PARANÁ E DO DISTRITO FEDERAL" sob a orientação e Presidência da Professora Doutora Vânia Aparecida Vicente, do Departamento de Patologia Básica da UFPR. Banca Examinadora constituída pela Professora Doutora Glaciela Kaschuk, Departamento de Solos e Engenharia Agrícola, Setor de Ciências Agrárias, UFPR, Professor Doutor Roberto Tadeu Raittz, Programa de Pós-Graduação em Bioinformática da UFPR, Professora Doutora Lygia Terasawa, Programa de Pós-Graduação em Genética, UFPR e Doutora Renata Rodrigues Gomes, Pós-Doutoranda do Programa de Pós-Graduação em Microbiologia, Parasitologia e Patologia da UFPR. A Banca Examinadora iniciou os trabalhos com a candidata expondo oralmente seu trabalho por 60 (sessenta) minutos. Após, cada membro da banca examinadora fez uma arguição de durante 30 (trinta) minutos. No final a candidata foi aprovada, segundo a avaliação da Banca Examinadora. Para a devida publicação, o trabalho deverá sofrer as modificações sugeridas. Nada mais havendo a tratar, a Presidente encerrou a sessão, da qual foi lavrada a presente ata que será assinada pela Presidente e pelos demais Membros da Banca Examinadora, em Curitiba, 22 de março de 2016.

Profª. Drª. Vânia Aparecida Vicente – Presidente

Profª. Drª. Glaciela Kaschuk

Profª. Drª. Lygia Terasawa

Prof. Dr. Roberto Tadeu Raittz

Doutora Renata Rodrigues Gomes

AGRADECIMENTOS

Primeiramente à Deus por me conceder paciência e força nos momentos mais difíceis e, sobretudo, pela vontade de conquistar e passar por mais essa etapa da minha vida.

Aos meus pais e ao meu marido Fernando por todo apoio, amor e incentivo, por estarem sempre comigo.

Às minhas sobrinhas Maria Fernanda e Ana Clara pelas maiores alegrias da minha vida.

À Dra. Mariangela Hungria pela excelente orientação; por participar da minha formação acadêmica em todos esses anos; pela paciência, confiança, pelas inúmeras vibrações positivas e incentivo.

À Dra. Vânia pela orientação e oportunidade em fazer parte do programa.

Aos amigos do laboratório, os antigos e novos, pela ótima convivência e risadas na hora do almoço.

À todos os docentes que colaboraram para minha formação.

À Embrapa soja e UFPR pelo suporte e estrutura concedida.

À CAPES pelo apoio financeiro.

RESUMO

O Brasil é um país, em grande parte, sustentado pela agricultura, que ocupa milhões de hectares no território nacional, onde a região do Cerrado representa a principal cultivada e a região sul a mais antiga em cultivo. A introdução da agricultura causa perturbação do solo, resultando em diferenças em relação aos solos nativos; contudo, existem manejos com menor ou maior grau de alteração, como o plantio direto (PD) e o plantio convencional (PC), respectivamente. No entanto, ainda existem poucas informações sobre os impactos causados nas comunidades microbianas por diferentes manejos do solo e das culturas, que agora podem ser reveladas pelo uso de novas tecnologias, como a metagenômica, que detecta microrganismos independe do seu cultivo em meios de cultura. Neste trabalho, foram estudadas duas regiões agrícolas do Brasil: uma no norte do Paraná, Região Sul, e outra nos Cerrados, no Distrito Federal. A partir do solo amostrado em cada região, o DNA foi extraído e sequenciado, resultando em milhões de sequências em cada estudo. Para as análises taxonômicas e funcionais as sequências foram submetidas ao servidor online MG-RAST e para as análises estatísticas foram submetidas ao programa STAMP. Nos solos do Norte do Paraná, os dados metagenômicos entre diferentes manejos de solo (PD e PC), e das culturas (rotação e sucessão) foram comparados, e foram constatadas diferenças consideráveis na diversidade taxonômica, mas de menor magnitude na diversidade funcional, indicativo de redundância de vias metabólicas. Os solos sob PD apresentaram maior quantidade de sequências de metabolismo de compostos aromáticos, que podem auxiliar na degradação de pesticidas, enquanto que solos sob PC apresentaram grande quantidade de sequências de metabolismo de carboidratos, que podem estar relacionada à menor oferta de nutrientes nesse solo. No Distrito Federal, os dados dos solos agrícolas foram comparados com uma área nativa do Cerrado, identificando se a predominância de algumas ordens bacterianas, tais como, Rhizobiales e Burkholderiales, em solos agrícolas (PD e PC), em comparação com solos nativos do Cerrado, os quais apresentaram maior abundância dos gêneros *Rhizobium*, *Azospirillum*, *Xanthomonas*, *Pseudomonas* e *Acidobacterium*. O Cerrado também apresentou maior diversidade de subsistemas funcionais. Os resultados obtidos neste estudo indicam que a introdução da agricultura em uma área nativa, e o uso de manejos do solo menos conservacionistas provocam profundo impacto principalmente na diversidade taxonômica dos microrganismos do solo.

Palavras-chave: Metagenômica *Shotgun*, Biodiversidade funcional, Plantio direto, Cerrado, Biodiversidade microbiana.

ABSTRACT

Brazil is a country largely sustained by agriculture, which occupies millions of hectares, with the Cerrado representing the main cropping region and the southern region the most traditional. Agriculture introduction causes soil disturbance, resulting in differences in relation to the native soil; however, there are managements with greater or lesser degree of disturbance, such as no-tillage (NT) and conventional tillage (CT), respectively. However, there is still little information about impacts on soil microbial communities by different soil and agricultural managements, which can now be revealed with the use of new technologies, such as the metagenomics, which allows the detection of microorganism independently of their growth in culture media. In this thesis, we studied two agricultural regions in Brazil, one in the north of Paraná, southern Brazil and the other in the Federal District, in the Cerrado biome. The soil was collected from each region, DNA was extracted and sequenced, resulting in millions of sequences in each study. For taxonomic and functional analyzes sequences were submitted to the online server MG-RAST and for statistical analyzes the data were submitted to the STAMP program. In Paraná's soils, different soil (NT and CT) and crop (rotation and sucession) managements were compared and significant differences were observed in taxonomic diversity, but lower in magnitude on the functional analysis, indicating metabolic redundancy. The soils under NT were more abundant in sequences of the aromatic compounds metabolism, that could help in pesticide degradation, while soils under CT showed higher abundance of sequences of the carbohydrate metabolism, which might be related to the lower supply of nutrients. In the Cerrado, we compared the population of cropped soils with an undisturbed soil covered with native vegetation. We identified higher abundance of some bacterial orders, such as Rhizobiales and Burkholderiales in agricultural soils (NT and CT), while the native Cerrado showed greater abundance of *Rhizobium*, *Azospirillum*, *Xanthomonas*, *Pseudomonas* and *Acidobacterium* genus. The undisturbed Cerrado was also more abundant in the variety of functional subsystems. The results obtained in our study shown that the introduction of agriculture and the use of less conservationists practices deeply affect soil microbial community, especially the taxonomic diversity.

Key-words: Shotgun metagenome, Functional biodiversity, No-tillage, Cerrado, Microbial biodiversity.

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INTRODUÇÃO

Por ser um ambiente com grande riqueza e diversidade microbiana, o solo se destaca e torna-se um importante alvo de pesquisas científicas. Essa diversidade inclui microrganismos como bactérias, arqueias, fungos, protozoários e até mesmo vírus, constituindo o microbioma do solo, onde estão suscetíveis a constantes pressões de resistência, apesar de apresentarem grande capacidade de resiliência. No entanto, mesmo sabendo da enorme diversidade e riqueza microbiana no solo e sua grande capacidade em produzir substâncias com grande valor industrial, também é sabido que a grande maioria dos microrganismos não pode ser cultivada por metodologias microbianas clássicas (SIMON and DANIEL, 2011). Isso é devido a limitações nas técnicas de cultivo em laboratório, as quais não fornecem as mesmas condições e interações encontradas no ambiente. Com isso, além do baixo conhecimento sobre a diversidade microbiana do solo, deixa-se de contabilizar um grande potencial biotecnológico que pode estar contido nesses seres incultiváveis, que podem ser responsáveis pela síntese de moléculas de interesse biotecnológico ainda desconhecido, representando uma estratégia para encontrar novos antibióticos e enzimas, por exemplo (LEE and LEE, 2013).

A diversidade de microrganismos dos solos varia conforme o bioma, as condições climáticas, entre outros, por isso, cada local é único quanto à diversidade que abriga. Dessa forma, a utilização de técnicas independentes de cultivo, como a metagenômica, permite o acesso a uma maior diversidade microbiana de genes relacionados a processos metabólicos importantes. A metagenômica representa um conjunto de técnicas moleculares independentes de cultivo capaz de acessar a grande maioria dos microrganismos, inclusive os incultiváveis e, assim, facilitar o estudo da diversidade taxonômica e funcional, além de poder compreender melhor os processos e interações microbianas entre si e entre plantas, comparar o seu comportamento (diversidade e quantidade) sob diversas condições ambientais e perturbações, encontrar novos biocatalisadores, entre outros (HANDELSMAN *et al*., 1998).

A maioria dos estudos de bioprospecção de microrganismos e genes funcionais do solo não é direcionada às áreas agrícolas, as quais também podem representar um ambiente importante para o isolamento de microrganismos e substâncias de interesse biotecnológico. Ao contrário de solos nativos e nunca

cultivados, solos agrícolas são mais impactados, o que pode estimular a seleção de microrganismos com maior potencial para a realização de funções específicas, além de apresentarem ampla versatilidade nutricional. Além disso, determinados manejos de solo, por exemplo, o plantio direto e uso de adubos verdes, contribuem para o incremento no teor de matéria orgânica do solo, que é utilizada pelos microrganismos como fonte de carbono e energia, trazendo portanto mais benefícios do que técnicas menos conservacionistas (BALOTA *et al*., 2004). É possível, por exemplo, que em solos agrícolas os microrganismos produzam mais enzimas para a degradação de resíduos vegetais, inclusive com potencial tecnológico na indústria e em outros setores. Em contrapartida, solos nativos apresentam uma grande diversidade microbiana e esta também permanece desconhecida em sua maior parte (ROESCH *et al*., 2007).

Desse modo, o conhecimento da diversidade taxonômica e funcional de microrganismos em solos nativos ou submetidos a diferentes manejos agrícolas é de grande importância, pois além da possibilidade para explorar seu potencial biotecnológico, também permite aprofundar no conhecimento sobre esses microrganismos, visando a sustentabilidade agrícola, que representa um dos principais desafios da agricultura atual.

OBJETIVOS

OBJETIVO GERAL

Acessar a abundância relativa microbiana taxonômica e funcional em solos agrícolas situados em dois biomas distintos do Brasil.

OBJETIVOS ESPECÍFICOS

- Identificar a abundância metagenômica funcional de solos sob plantio direto e convencional com rotação e sucessão de culturas no Norte do Paraná;

- Conhecer a abundância metagenômica taxonômica e funcional em solos sob plantio direto, plantio convencional e mata nativa dos Cerrados no Distrito Federal;

CAPÍTULO 1 - REVISÃO DE LITERATURA

O Solo e sua Diversidade Microbiana

O solo é um ecossistema complexo que sofre intervenções das plantas, minerais e organismos. Apresenta uma grande biodiversidade microbiana, as quais compreendem bactérias, arqueias, fungos, além das partículas virais (FIERER *et al.*, 2007). Esses microrganismos são essenciais para a manutenção e a sustentabilidade do solo, atuando em uma gama de processos que incluem a ciclagem de nutrientes (SCHULZ *et al.*, 2013) com a decomposição da matéria orgânica (SCHMIDT *et al.*, 2011), além de relações mutualísticas e simbióticas com plantas auxiliando na supressão de doenças (MENDES *et al.*, 2011), na fixação biológica do nitrogênio (HUNGRIA *et al.*, 2005), na promoção do crescimento das plantas (BHATTACHARYYA and JHA, 2012) e, além disso, o solo ainda abriga grandes prováveis potenciais biotecnológicos (SIMON and DANIEL, 2011).

O solo sempre foi apontado como um dos principais reservatórios de microrganismos nos ecossistemas, com aproximadamente 1.000 Gb de sequências de genoma microbiano por grama de solo (VOGEL *et al.*, 2009). Embora haja o reconhecimento dessa grande diversidade e quantidade de microrganismos, apenas uma ínfima quantidade é conhecida. Isso é devido às limitações nas técnicas de cultivo em laboratório, que não podem oferecer as mesmas condições de desenvolvimento do seu hábitat natural, ou seja, existem inúmeros microrganismos incultiváveis que ainda não foram descritos ou descobertos. Tais microrganismos podem apresentar um grande potencial biotecnológico ainda desconhecido (LEE and LEE, 2013).

A microbiota do solo é naturalmente alterada por fatores como temperatura, pH, umidade, presença de animais e plantas (MENDES *et al.*, 2014; ZHALNINA *et al.*, 2015). No entanto, esta microbiota pode ser profundamente e irreversivelmente afetada por diferentes práticas de manejo agrícola (SOUZA *et al.*, 2013; SOUZA *et al.*, 2015), bem como a utilização de fertilizantes e agrotóxicos (IMFELD and VUILLEUMIER, 2012). Além disso, podem impactar as propriedades físicas e químicas do solo. Tudo isso pode resultar em desequilíbrios ecológicos com consequências imprevisíveis, mesmo porque, não conhecemos ainda a real diversidade microbiana do solo.

Na camada mais superficial do solo é onde ocorre a maior atividade biológica, porém é a mais sensível a processos de degradação. Dessa forma, em solos agrícolas, sistemas conservacionistas de manejo, como o plantio direto, ajudam a conservar essas atividades, uma vez que se caracterizam pela semeadura sobre os resíduos vegetais da cultura anterior, sem aração ou gradagem do solo. Os restos vegetais na superfície protegem o solo contra erosão, aumentam a retenção de água, mantêm a permeabilidade, permitem maior teor de matéria orgânica e melhores condições físicas do solo (CUNHA *et al.*, 2014), favorecendo e aumentando a atividade microbiana (HUNGRIA *et al.*, 2009). De modo contrário, o manejo conhecido como plantio convencional é realizado com a utilização intensiva de máquinas e implementos agrícolas para o revolvimento do solo, o que leva a sua desestruturação, compactação, suscetibilidade à erosão, flutuações térmicas e hídricas, favorecimento de patógenos, levando a alteração da qualidade biológica desse solo (AZIZ, MAHMOOD and ISLAM, 2013).

A monocultura ou sistema de sucessão de culturas contínuo (por exemplo, de soja no verão e trigo no inverno) também provoca degradação do solo, influenciando negativamente na microbiota e, até mesmo favorecendo o desenvolvimento de doenças. Já em um sistema de rotação de culturas ocorre a diminuição da incidência de patógenos, além de ser importante na manutenção da biodiversidade microbiana (DORR DE QUADROS *et al.*, 2012).

Desse modo, fica evidente que o manejo adequado do solo, assim como o manejo adequado das culturas são cruciais para a sobrevivência microbiana, caso contrário, podem causar danos ao agroecossistema. Nesse contexto, cresce ainda a necessidade de desenvolvimento de tecnologias mais avançadas que auxiliem a descrever a diversidade e as funções de comunidades microbianas no solo.

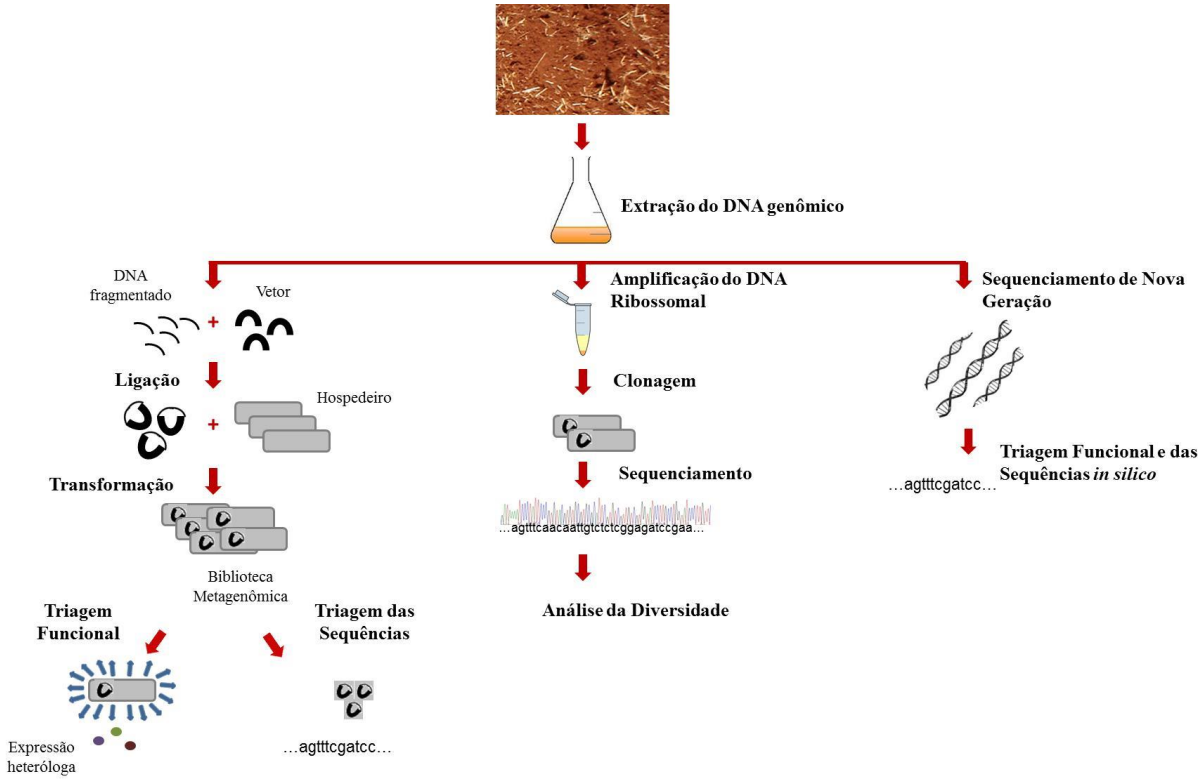
METAGENÔMICA

O termo metagenômica é relativamente recente, proposto por HANDELSMAN *et al.* (1998), quando trabalhavam com diversidade microbiana do solo. Esse termo foi definido como a análise funcional e das sequências nucleotídicas do metagenoma coletivo de uma microbiota encontrada em

determinada amostra ambiental, através da combinação de técnicas moleculares independentes de cultivo (HANDELSMAN *et al*., 1998).

A metagenômica permite o estudo de genes e funções microbianas anteriormente inacessíveis, possibilitando a descoberta de novos produtos para fins biotecnológicos e aplicação industrial (SATHYA and KHAN, 2014). Triagens das bibliotecas metagenômicas podem ser realizadas por estratégias baseadas na função e baseadas na sequência nucleotidica (HANDELSMAN *et al.*, 1998). Inicialmente, para sua realização é necessária a extração total do material genético de uma amostra e, a partir desse DNA, podem-se seguir diferentes formas de estudo. Uma delas consiste na clonagem desse material genético em um vetor apropriado (plasmídeo, fosmídeo, BAC) e, em seguida, a transformação em um hospedeiro cultivável (geralmente a bactéria *Escherichia coli*) produzindo, assim, as bibliotecas. Após a obtenção das bibliotecas metagenômicas, podem ser realizadas as triagens funcionais e triagens baseadas nas sequências (FIGURA 1).

FIGURA 1 - ESQUEMA COM AS ABORDAGENS QUE VÊM SENDO MAIS UTILIZADAS EM PESQUISAS DE METAGENÔMICA DE SOLOS.



FONTE: SOUZA, RC. 2013.

A triagem baseada na função deve ser capaz de detectar, isolar e caracterizar genes expressos na biblioteca metagenômica, sendo realizada com base na atividade metabólica dos clones, não necessitando de conhecimento prévio da sequência (EKKERS *et al.*, 2012).

Para identificar funções enzimáticas, clones individuais são incorporados aos meios de cultivo diferentes substratos e, depois, é realizada a detecção fenotípica, observando a presença de alguma “marca” facilmente visível nos clones positivos (EKKERS *et al.*, 2012), por exemplo, a formação de halo ao redor da colônia. Esse procedimento é trabalhoso, porém se for associado a tecnologias de alto desempenho, pode ser mais rápido e confiável. Por exemplo, as triagens para enzimas como proteases e amilases requerem a adição no meio de cultivo de leite e batata como substrato das respectivas enzimas, resultando na formação de halo ao redor da colônia (KENNEDY *et al.*, 2011). Contudo, é muito difícil conseguir isolar um produto funcional, pois a frequência de clones metagenômicos que expressam uma dada atividade é baixa. Couto e colaboradores (2010), pesquisando novas lipases a partir do sedimento do mangue na costa sul do Brasil, encontraram atividade em somente 1 a cada 2400 clones, por exemplo. Isso acontece porque existem alguns fatores limitantes, como a dificuldade de expressão heteróloga de genes em célula hospedeira e a baixa probabilidade de obtenção de fragmentos grandes de DNA contendo operons ou vias extensas (EKKERS *et al.*, 2012). Além disso, para o sucesso das triagens é necessária uma transcrição e tradução fiel do gene ou de genes de interesse e a secreção do produto gênico pelo hospedeiro (HANDELSMAN, 2004).

Outra forma de estudo, aplicada em estudos de diversidade, consiste na amplificação de uma região específica do DNA total de uma amostra, geralmente de regiões conservadas no genoma, como por exemplo, 16S e 18S de bactérias e fungos, respectivamente. Em seguida, essa região pode ser clonada em um vetor, que será transformado em um hospedeiro cultivável e, depois, sequenciado. Entretanto, atualmente o método de clonagem para esses genes não tem sido muito utilizado, pois geralmente eles são sequenciados diretamente (LOGARES *et al.*, 2014; WANG *et al.*, 2014). No entanto, alguns autores não consideram estudos metagenômicos verdadeiros aqueles que utilizam a PCR (*Polymerase Chain Reaction*) associada a bibliotecas de clones a partir de um único gene evolutivamente conservado, como os genes ribossomais, pois apenas um gene não

representaria toda a amostra metagenômica (KAKIRDE, PARSLEY and LILES, 2010).

Outra abordagem da metagenômica é a chamada metagenômica do tipo *shotgun*, na qual o material genético total extraído é fragmentado em sequências menores e diretamente submetido a sequenciadores de segunda ou terceira geração e, em seguida, são realizadas análises das sequências obtidas por meio de programas de bioinformática (FIGURA 1) (HODKINSON and GRICE, 2015). O surgimento das novas tecnologias de sequenciamento e o intenso desenvolvimento de *pipelines* de bioinformática forneceu maior precisão, rapidez e baixo custo ao estudo. Esse método permite o conhecimento taxonômico e metabólico da amostra, com triagens *in silico* dos genes, incluindo aqueles provenientes de microrganismos desconhecidos. No entanto, a eficiência dos métodos de extração de DNA e sequenciamento representam as principais fontes dos vieses desse tipo de protocolo.

Mesmo sendo recente, a metagenômica vem despertando o interesse em muitos pesquisadores frente às possíveis aplicações biotecnológicas dos microrganismos. Devido à importância da metagenômica, cada vez mais algoritmos, ferramentas de bioinformática, *pipelines* de análise de sequenciamento e banco de dados vêm sendo explorados, visando tornar possível a análise de grandes quantidades de dados metagenômicos.

SEQUENCIAMENTO E BIOINFORMÁTICA

Novas tecnologias de sequenciamento como as plataformas 454 GS FLX Life Sciences e Ion Torrent (PGM), possibilitam o sequenciamento de DNA com capacidade de gerar informações muitas vezes superiores à do sequenciamento de Sanger. Milhões de *reads* são gerados sem a necessidade de clonagem em célula hospedeira, evitando os vieses de clonagem, com uma grande economia de tempo e custo por nucleotídeo (KOBOLDT *et al.*, 2013). Em estudos metagenômicos, são obtidos milhões de sequências de DNA, e essa imensa quantidade de dados gerados necessita de ferramentas de bioinformática para a análise, processamento e armazenamento. Em projetos de metagenomas, são necessários programas de montagem, predição gênica, classificação taxonômica e análises funcionais.

Também é possível comparar o conjunto de dados gerados em diferentes amostras em um tipo de análise chamada de metagenoma comparativa, que estuda relações entre genes, segmentos de cromossomos e sequências proteicas, e é importante para estudos funcionais e evolutivos de comunidades microbianas coletadas em diferentes ambientes. Para este tipo de análise, programas foram desenvolvidos, sendo um deles o MG-RAST (*Metagenome Rapid Annotation using Subsystem Tecnology*) disponível para processar dados de sequências metagenômicas (MEYER, FOLKER *et al.*, 2008) comparando com banco de dados de proteínas e nucleotídeos, para atribuições funcionais e taxonômicas de sequências. As sequências podem ser categorizadas em subsistemas pelo SEED de forma a organizar as funções dos genes preditos de acordo com seus processos biológicos (OVERBEEK *et al.*, 2005).

Por meio de novas tecnologias, como a bioinformática e técnicas de sequenciamento de alto desempenho, tornou-se possível avaliar o potencial genético e funcional de um ambiente tão diverso como o solo, permitindo assim, entender melhor o funcionamento desse ecossistema e como o manejo pode influenciar a diversidade e o funcionamento microbiano no solo.

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CAPÍTULO 2 - Metagenomic analysis reveals microbial functional redundancies and specificities in a soil under different tillage and crop-management regimes

Applied Soil Ecology

Metagenomic analysis reveals microbial functional redundancies and specificities in a soil under different tillage and crop-management regimes

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http://dx.doi.org/10.1016/j.apsoil.2014.10.010

ABSTRACT

Information about microbial functionality in agricultural soils is still scarce, and in this study we used a shotgun metagenomic approach to compare different soil [conventional tillage (CT) with plowing and disking, and no-tillage (NT) with direct sowing into the residues of previous crops], and crop [crop succession (CS, soybean-summer/wheat-winter) or rotation (CR, soybean/maize-summer)/wheat/ lupine/oat-winter)] managements in a 13-year-old field experiment in southern Brazil. Differences were detected between NT and CT in some functional subsystems, e.g., NT had more sequences associated with the metabolism of aromatic compounds,

which might be related to higher capacity to degrade pesticides, more sequences of the adenylate cyclase (cAMP) pathway, which might confer stability to the microbial community, among others. On the other hand, CT showed more sequences related to carbohydrate metabolism, what could be related with a lower content of organic matter and need to metabolize a broader range of carbon sources. Also, we detected differences related to crop management, e.g., crop rotation showed more sequences in the metabolism of amino acids and derivatives and carbohydrate subsystems, what might result from higher diversity of crop residues added to the soil. However, it was notable that the differences in the diversity of taxa previously shown in the same experiment was far greater than the functional diversity reported now, emphasizing a high level of microbial functional redundancy.

Keywords: Environmental shotgun sequencing (ESS); Microbial biodiversity Soil metagenomics; Functional biodiversity Soil; management Crop management; No-tillage

1. INTRODUCTION

Brazil's economy is strongly based on agriculture and, in 2013/2014, 55.2 million hectares were cultivated for grain production under different soil and crop management systems (CONAB, 2014). The conventional tillage (CT) system consists of the traditional practices of plowing and disking before sowing, and degradation of large areas worldwide has been attributed to these mechanical practices. Contrarily, the no-tillage (NT) system consists of sowing directly into the residues of the previous crop, benefiting soil chemical, physical and biological properties, when compared with CT (BODDEY *et al*., 2010; CALEGARI *et al.,* 2008; CASTRO FILHO *et al*., 2002; DERPSCH, 1991; LAL *et al*., 2007; SILVA *et al*., 2014). The first trials with NT in Brazil date from the 1970s; it is now practiced on more than 32 million hectares (FEBRAPDP, 2014). NT has marked benefits on soil microorganisms, in terms of microbial biomass and metabolic quotient (BABUJIA *et al*., 2010; BALOTA *et al*., 1998, 2004; BERNARD and HABASH, 2009; FRANCHINI *et al*., 2007; HUNGRIA *et al*., 2009; SILVA *et al*., 2010, 2014), and also in terms of functional groups, such as nitrogen fixing rhizobia (FERREIRA *et al*., 2000;

HUNGRIA and VARGAS, 2000; KASCHUK *et al*., 2006; PEREIRA *et al*., 2007; SOUZA *et al*., 2013).

Another important practice for sustainability of productivity is crop rotation (CR). Although monoculture and crop succession (CS) with only two crops occupies most of the agricultural land globally, the adoption of CR with a minimum of three crops is a goal of agricultural conservationists (FAO, 2012). Benefits for soil chemical and physical properties have been reported in Brazil with the adoption of CR, in terms of increased carbon sequestration and organic matter (BABUJIA *et al*., 2010; BODDEY *et al*., 2010; CALEGARI *et al*., 2008; SILVA *et al*., 2014). However, it is noteworthy that detecting benefits in microbial biomass related parameters due to the adoption of CR in comparison with CS is more difficult than in the comparison of NT and CT (BALOTA *et al*., 1998; FRANCHINI *et al*., 2007; HUNGRIA *et al*., 2009; SILVA *et al*., 2010, 2014). It is also intriguing that in the studies performed in Brazil it has been difficult to detect changes in microbial diversity in the comparison of CS and CR, evaluated by the analysis of profiles of 16S rDNA-DGGE (SILVA *et al*., 2013) or by metagenomics (SOUZA *et al*., 2013). Worldwide, other studies have also reported benefits especially in the comparison of NT over CT, e.g., by using BIOLOGTM and other microbial parameters in areas cropped with wheat (LUPWAYI *et al*., 1998), maize (GOVAERTS *et al*., 2007) and barley (LUPWAYI *et al*., 2001), among others.

Studies of soil metagenomics are increasing worldwide (e.g., ACOSTA-MARTÍNEZ *et al*., 2010; DELMONT *et al*., 2012; FIERER *et al*., 2007, 2012a,b), but in agricultural tropical soils are still scarce. Estimates are that Brazil hosts approximately 20% of the world's biological diversity, but the degree to which this applies to microbial diversity is still largely unknown (PYLRO *et al*., 2014). Nevertheless, maintenance of ecosystem dynamics and land use sustainability demand knowledge about microbial diversity and functionality. Previously, by using a shotgun sequencing approach in soils of a long term field experiment 13 years under NT and CT with CS or CR, we revealed major differences in microbial diversity associated with tillage systems and, to a lesser extent, to crop management (SOUZA *et al*., 2013).

It is very important to establish linkages between soil microbial diversity and functionality, as our knowledge about this linkage, especially in tropical soils is still far from being clarified. Our hypothesis in this study is that soil microbial functionality

might have a buffering capacity aiming at minimizing the impact of agricultural practices. Investigating soil management systems with higher (conventional tillage, monocultures) or lower (no-tillage, crop rotation) impacts might help to indicate to which extend soil microbes may preserve soil functioning.

2. MATERIALS AND METHODS

2.1. DESCRIPTION OF THE FIELD TRIAL AND SOIL SAMPLING AND PROCESSING

The study was performed in a 13 year old field trial established at the experimental station of Embrapa Soja, in Londrina, state of Paraná, Brazil, latitude 23°110S, longitude 51°110W, and elevation of 620 m. The soil is classified as Latossolo Vermelho Eutroférrico (Brazilian system), rhodic eutrudox (US taxonomy). Information about climate conditions (SILVA *et al*., 2010) and chemical and physical properties (SOUZA *et al*., 2013) has been given before; however, to facilitate the discussion, information about chemical and physical properties is also given in Supplementary Table S1.

The treatments consisted of conventional tillage (CT, with the traditional practices of soil plowing and disking), and no-tillage (NT, with direct sowing into the residues of the previous crop), each under crop succession (CS) [soybean (*Glycine max* L. Merr.) in the summer and wheat (*Triticum aestivum* L.) in the winter], or crop rotation (CR) [soybean and maize (*Zea mays* L.) in the summer and wheat, lupine (*Lupinus angustifolius* L.) and oat (*Avena strigosa* Schreb.) in the winter]. Crops grown in succession and rotation for the last 7 years were listed before (SOUZA *et al*., 2013). The four treatments are designated as NTS, NTR, CTS and CTR. Each plot measured 8 m width x 15 m length; the trial had a completely randomized block design, with four replicates.

Sampling was performed in early November of 2010, when the experiment was 13 years old, immediately before sowing of soybean (summer crop), and three weeks after harvesting the winter crop, wheat. Wheat residues have been incorporated to the soil in the CT and left on soil surface in the NT system. Soil samples were taken from an area of 0.4 m^2 of the superficial layer (0–10 cm), with eight replicates per each of the four replicates of each treatment. A detailed

description of sampling procedure and processing was given before (SOUZA *et al*., 2013).

2.2. DNA EXTRACTION, SHOTGUN SEQUENCING AND SEQUENCES ANALYSES

In the laboratory, plant residues were removed, soil samples were homogenized and passed through a 2 mm sieve before analysis. Metagenomic DNA was extracted by using 10 g of each soil replicate and the PowerMax^{TM} Soil DNA Isolation Kit (Mo Bio Laboratories) and submitted to sequencing analysis in the 454 platform (GS-FLX Titanium Roche Applied Science) at the Labinfo of LNCC (Petrópolis, Rio de Janeiro, Brazil, http://www. labinfo.br). For the pyrosequencing, DNA was randomly fragmented by nebulization with compressed nitrogen gas. Fragments of 300–800 bp were selected and submitted to sequence analysis with the Titanium kit, and the support PicoTiterPlate (Roche Applied Science) (IMELFORT and EDWARDS, 2009). Replicates (GOMEZ-ALVAREZ *et al*., 2009) and Lucy Software (CHOU and HOLMES, 2001) were carried out to remove artificial duplicate reads (ADRs) and low quality sequences. The data set was deposited in the NCBI-SRA (Sequence Read Archive, http://www.ncbi.nlm.nih.gov/sra/?term=SRA050780) and at the DDBJ database (http://trace.ddbj.nig.ac.jp/DRASearch/submission?acc=SRA050780), with the submission accession number SRA050780. In this study functional data were analyzed and normalized by using the MG-RAST tools (MEYER *et al*., 2008).

2.3. FUNCTIONAL ANALYSES OF THE SEQUENCES WITH SEED AND KEGG

The sequences obtained were submitted to the MG-RAST (the Metagenomics RAST – http://metagenomics.anl.gov), and compared against SEED database for the functional classification in subsystems (OVERBEEK *et al*., 2005). According to the SEED website (http://theseed.org/wiki/Home_of_the_SEED), a subsystem represents a set of functional roles that make up a metabolic pathway, a complex, or a class of proteins. The levels of subsystems considered in SEED are: (1) highest level; (2) second highest level; (3) similar to a KEGG pathway; (4) actual functional assignment to the feature in question. Data were also compared against KEGG (Kyoto encyclopedia of genes and genomes) database (KANEHISA *et al*.,

2004); KEGG is a database resource that integrates genomic, chemical and systemic functional information (http://www. genome.jp/kegg/kegg1.html). For the BLAST (basic local alignment search tool, National Center for Biotechnology Information, http:// www.ncbi.nlm.nih.gov/) search, a cut-off of minimum identity of 60% and E-value of 1×10^{-5} were considered.

Due to the agricultural interest in C and N metabolism under NT and CT, the sequences were also analyzed for the metabolic profile towards the main processes related to C and N metabolism, based on MG-RAST with the KEGG map.

2.4. STATISTICAL ANALYSIS

Data from MG-RAST were submitted to statistical analysis with the STAMP (statistical analysis of metabolic profile) software (PARKS and BEIKO, 2010), to evaluate statistical differences in the metabolic profiles of the metagenomes considering all combinations in pairs. Statistical significance was estimated with the Fisher's test for $p \leq 0.05$, using the method of Newcombe-Wilson with the correction of Benjamini–Hochberg FDR. In addition, as STAMP only allows the comparison of pairs of treatments, replicated data (ten replicates of each set of about 1 million reads) were also analyzed considering the number of reads in each functional category (Tukey, $p \leq 0.05$, ANOVA).

3. RESULTS AND DISCUSSION

3.1. GENERAL SEQUENCING ANALYSIS AND FUNCTIONAL CLASSIFICATION

The four treatments, consisting of the combination of no-tillage (NT) and conventional tillage (CT) with crop rotation (CR) or crop succession (CS) resulted in about 1 million sequences per treatment (SOUZA *et al*., 2013). One might consider that metagenome represents a first step of study, to be followed by metatranscriptome and the metaproteome; therefore in this manuscript all assumptions will refer to "potential functionality". When the 4 million sequences were analyzed with MG-RAST, 0.8% was classified as unknown sequences, 44.7% as nucleic sequences related to unknown proteins (unknown proteins), 54.2% as nucleic sequences coding for known proteins and 0.3% as RNA ribosomes (Fig. S1).

The MG-RAST analyses indicate metabolic functions at four different levels; the subsystem 1 represents the highest level and specific genes the lowest (4). Subsystem classification of each soil in the 29 functional categories is shown in Table 1, and although in general the percentages of genes within each category seem similar, statistical differences were observed in some of the categories. If we consider that 1 g of soil might contain 10 billion microorganisms and thousands of different species, (ROSELLÓ-MORA and AMANN, 2001; TORSVIK and ØVREÅS, 2002), a difference of only 0.38%, e.g., as that observed in the category of protein metabolism between the NTS and the CTS, might represent a difference of more than 30 million microorganisms with that metabolic function. In general, the four soil metagenomes comprised sequences classified in pathways of constitutive genes, encoding proteins implicated in the basic metabolism needed for microbial survival in different environments, such as RNA and DNA synthesis, respiration, membrane transport, cell division and cell cycle (Table 1).

Among the 29 metabolic classes identified, the highest percentages of sequences fit into the clustering based and carbohydrates subsystems, followed by the amino acids and derivatives, while the subsystems of photosynthesis and dormancy and sporulation presented the lowest numbers of sequences (Table 1). Similar results have been reported in other soil studies, e.g., including natural conditions, as an untreated treatment with grassland in Rothamsted, UK (DELMONT *et al*., 2012), and soil micro ecosystems simulating soil with and without earthworms (FRISLI *et al*., 2013).

In the clustering-based subsystem, there is an evidence of coupling of functional genes, resulting in a group of hypothetical proteins explained by co-localization of conserved patterns in several genomes (GERDES *et al*., 2011), meaning that these genes have unknown specific functions. In general, this is the most abundant subsystem in functional metagenomic studies (DELMONT *et al*., 2012; LAVERY *et al.*, 2012), indicative that our current knowledge about the dynamics and structure of soil microorganisms in their habitats is still largely unknown, as pointed out a decade ago (BUCKLEY and SCHMIDT, 2003). This subsystem included several sequences coding for putative genes related to the metabolism of fatty acids, biosynthesis of galactoglucans and lipopolysaccharides, among other functions.

Table 1 - Functional classification of the genes detected in four metagenomes of an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop succession (S) or rotation (R).

Functional category	Percentage of sequences			
	CTR	NTR	NTS	CTS
Clustering-based subsystems	15.31 b[a]	15.30 b	15.50 a	15.49 a
Carbohydrates	10.94 a	10.81 b	10.82 b	10.80 b
Amino Acids and Derivatives	8.86 a	8.78 b	8.80 b	8.78 b
Miscellaneous	8.43 b	8.59 a	8.58 a	8.39 b
Protein Metabolism	7.57 a	7.32 b	7.20 c	7.58 a
Cofactors, Vitamins, Prosthetic Groups, Pigments	5.72	5.90	5.32	5.39
DNA Metabolism	3.75 c	3.87 b	3.63 d	3.99 a
RNA Metabolism	3.70	3.60	3.61	3.71
Respiration	3.53	3.51	3.48	3.52
Cell Wall and Capsule	3.77 a	3.62 b	3.63 b	3.78 a
Fatty Acids, Lipids, and Isoprenoids	3.11 c	3.20 b	3.25 a	3.20 b
Membrane Transport	3.82 b	3.82 b	3.99 a	3.83 b
Nucleosides and Nucleotides	3.01	3.02	3.02	3.03
Virulence, Disease and Defense	3.03 b	3.15 a	3.16 a	3.02 b
Stress Response	2.64	2.61	2.62	2.63
Metabolism of Aromatic Compounds	2.11 b	2.28 a	2.29 a	2.12 b
Regulation and Cell signaling	1.64 b	1.65 b	1.78 a	1.59 b
Cell Division and Cell Cycle	1.36 a	1.38 a	1.22 b	1.37 a
Sulfur Metabolism	1.33 b	1.35 b	1.48 a	1.34 b
Phages, Prophages, Transposable elements, Plasmids	1.33 b	1.32 b	1.48 a	1.34 b
Nitrogen Metabolism	1.08	1.12	1.13	1.09
Phosphorus Metabolism	0.94	0.92	0.89	0.91
Motility and Chemotaxis	1.07 a	0.92 b	0.93 b	1.08 a
Iron acquisition and metabolism	0.65 a	0.61 b	0.60 b	0.65 a
Secundary Metabolism	0.51 b	0.53 b	0.58 a	0.52 b
Potassium Metabolism	0.42 b	0.43 b	0.59 a	0.44 b
Dormancy and Sporulation	0.19	0.20	0.21	0.20
Photosynthesis	0.18	0.19	0.21	0.21

[a] Data obtained using MG-RAST with BLASTX against SEED database with a cut-off of 60% and E-value of 1 _10_5. As MG-RAST does not give statistical analysis, we used replicated data (ten replicates of each set) considering the number of reads in each functional category (Tukey, p _ 0.05) to indicate statistical differences. Categories without letters did not show statistical differences.

The comparative analysis of metabolic profiles using the level 1 of classification with STAMP revealed statistically significant differences in the comparison of CS with CR in the CT system. In the succession with soybean/wheat, there were more sequences related to DNA metabolism, clustering-based subsystems and fatty acids, lipids and isoprenoids (Fig. 2). In contrast, crop rotation resulted in a higher proportion of sequences related to amino acids and derivatives and to carbohydrates (Fig. 2). When comparing soil fertility properties between the CS and the CR (Table S1) we see that rotation resulted in higher pH, lower hydrogen + aluminum and higher content of bases, with an emphasis on calcium, all of them very important and favorable to the great majority of soil microorganisms. We may conclude that continuous cropping of soybean/wheat (CS) decreased soil fertility, and the more stressing conditions resulted in a need of increasing the genes related to DNA metabolism to improve survival. Contrarily, the inclusion of a higher diversity of plant species in the rotation, some of them very rich on N (as lupin), or with a good C:N ratio (as oat) increased the sequences related to amino acids derivatives and carbohydrates (Fig. 2).

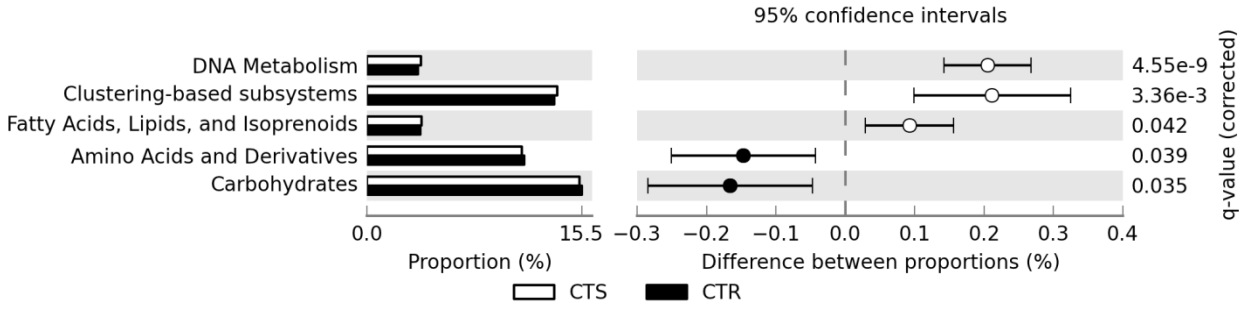

Figure 2 - Statistically significant differences in the functional subsystem in the level 1 of classification using the MG-RAST annotation platform and STAMP statistical software in the comparison of conventional tillage under crop succession (CTS) or crop rotation (CTR) in a 13-years field experiment. The graphic shows only the subsystems with statistical differences between the proportions of sequences in each treatment, with a confidence interval of 95%.

3.2. CARBON AND NITROGEN METABOLISM AND MOLECULAR SIGNALING: NOVELTIES RELATED TO TILLAGE AND CROPPING MANAGEMENT

At level 2 of the subsystem of metabolism of carbohydrates, the central carbohydrate metabolism presented the highest number of sequences (Fig S2), including genes related to the glycolytic, pentose-phosphate, tricarboxylic acid (TCA) and Entner–Doudoroff pathways. These genes are essential for the high energetic demands of microbial processes.

The quantity of carbohydrates in soils depends on the amount of plant and animal residues added, in addition to their decomposition rates. Sources of C are needed to provide energy for microbial decomposition to take place. It is well known that NT increases the reservoir of organic matter (BABUJIA *et al*., 2010; BALOTA *et al.*, 1998; BODDEY *et al*., 2010; CALEGARI *et al*., 2008; CASTRO FILHO *et al*., 2002; DERPSCH, 1991; FRANCHINI *et al*., 2007; LAL *et al*., 2007; SILVA *et al*., 2014), and also in our experiment the contents of C and N in soil were greater in the NT in comparison with the CT (Table S1). Therefore, initially one might be surprised by a slight difference towards a higher percentage of sequences of the carbohydrate metabolism in the CTR, when compared with NT (Table 1). However, we may consider that in one study with rhizobial microsymbionts of soybean, HUNGRIA *et al*. (2001) reported that isolates from the CT treatments had higher diversity in the use of C sources than the isolates from NT. According to WARDLE and GHANI (1995), although microbial metabolic quotient (qCO2) provides a useful measure of microbial efficiency, it has limitations, once it can be insensitive to disturbance and ecosystem development. However, observations from several of our studies are that metabolic efficiency of the microbial biomass is far greater under NT than in CT (BALOTA *et al*., 1998; FRANCHINI *et al*., 2007; PEREIRA *et al*., 2007; HUNGRIA *et al*., 2009). Considering both observations, we suggest that NT might favor microorganisms and C metabolic pathways of higher metabolic efficiency in the use of selected C sources, whereas with CT there is need for higher diversity of metabolic pathways aiming at utilizing a broader range of C sources. This would include the metabolism of recalcitrant C stocks in the CT, as the resistant fractions of the soil organic matter (including compounds such as lignines) (BENNER *et al*., 1987), would represent a higher proportion of the organic matter under the CT, in comparison to the NT, where the organic matter content was higher (Table S1) (FRANCHINI *et al*., 2007; SILVA *et al*., 2014). Apparently, to maintain activity in C limited soils, the microbial community selected with CT has to take advantage of every C source available, resulting in higher functional diversity. Other environmental factors might contribute to an

increase in the C metabolism under CT, e.g., soil temperatures are far greater than under NT and decomposition of C recalcitrant stocks are accelerated under higher temperatures (e.g., BENNER *et al*., 1987).

Although in several of our previous studies we have reported higher microbial biomass up to more than 100% in NT in comparison with CT, it has been difficult to detect differences associated with crop management regimes (crop succession x crop rotation) (BALOTA *et al*., 1998; FRANCHINI *et al*., 2007; PEREIRA *et al*., 2007; HUNGRIA *et al*., 2009; SILVA *et al*., 2010, 2014). It is noteworthy also that in our previous studies no differences have been detected in microbial diversity analyzed either by 16S rDNA DGGE (SILVA *et al*., 2013), or by metagenomics (SOUZA *et al*., 2013). In this study, the results shown in Table S2, of activity of acid phosphatase, dehydrogenase and cellulase also show differences between the soil managements, but not between the cropping systems. However, we now report more sequences in the carbohydrates' subsystem with CTR than with CTS treatment (Fig. 2). Similarly to what we reported for the sequences related to the DNA metabolism (Section 3.1), we may hypothesize that the higher diversity of crop residues with the crop rotation resulted in increased diversity of pathways in order to use different sources of carbohydrates. Again, although differences between rotation and succession may seem of minor magnitude, we might remember our observation of how many microorganisms are included in a small percentage of increased sequences. Second and very important, although rotation enriched the soil with a higher diversity of plant residues, it is still a very modest increase in the number of plant species five crops in the CR versus two in the CS, against hundreds of plant species in natural ecosystems and maybe not enough to result in more prominent changes.

We have also given attention to the subsystem of aromatic compounds, as agricultural soils usually receive heavy applications of pesticides and some microorganisms develop strategies to obtain energy from these compounds. Despite showing few sequences, we detected central and peripheral pathways of the catabolism of aromatic compounds (Fig. S3), e.g., the degradation of n-phenyl alkanoic acid described in *Pseudomonas putida* (OLIVERA *et al.,* 2001), several sequences attributed to isoquinoline 1- oxidoreductase (EC 1.3.99.16) and to the degradation of N-heterocyclic aromatics, which have herbicide activity and are degraded by soil microorganisms (SIMS, 2006). Bacteria in several genera, including *Alcaligenes*, *Acinetobacter*, *Rhodococcus*, *Nocardia* and *Pseudomonas*, are capable

of aerobic degradation of aromatic compounds, which may correlate with biodegradation of xenobiotics (CAO *et al*., 2009), and *Burkholderia* has an impressive set of genes for degradation of xenobiotics (ZULETA *et al*., 2014). *Bukholderia* and *Pseudomonas*, amongst the microorganisms detected in abundance in our previous study (SOUZA *et al*., 2013), may be chief contributors to the degradation of xenobiotics.

In the metabolism of aromatic compounds, the NT with crop succession (NTS) showed significantly higher number of sequences than the CT under the same crop management (CTS) (Fig. 3). The higher number of sequences with NT may be related to the degradation of pesticides favored by the higher soil organic matter content that strongly influences biodegradation (ANDRADE *et al*., 2010). Soils with higher organic matter content tend to adsorb pesticides but also show higher microbial activity, favoring microorganisms capable of using these C sources, such as those of the genera *Ralstonia*, *Burkholderia*, *Pseudoxanthomonas*, *Rhodococcus*, *Escherichia* and *Pseudomonas*, all detected before with NT (SOUZA *et al*., 2013). Other subsystems favored by the NTS in comparison to the CTS were those of virulence disease and defense, sulfur metabolism, regulation and cell signaling, potassium metabolism, membrane transport, phages and correlates, fatty acids, lipids and isoprenoids and secondary metabolism (Fig. 3). Instead, CTS showed more sequences associated with DNA and protein metabolism, cell wall and capsule synthesis, motility and chemotaxis, and iron acquisition and metabolism than the NTS (Fig. 3).

Based on matches with the KEGG database, it is noteworthy that we detected in both NT and CT genes related to the metabolism of methane (Fig. S4). Several hits were related to the transformation of methanol to formaldehyde, and one ferredoxin hydrogenase (EC 1.12.7.2) was found exclusively in the NT system. In our previous study we reported predominance of the Archaea domain in the NT when compared with the CT, including methanogenic Archaea (SOUZA *et al*., 2013), which may be involved in methane catabolism. Pathways involving methane may occur not only in anaerobic conditions (ANGEL *et al*., 2012), but also in aerobic soils containing anaerobic sites.

KEGG maps allow a more precise observation of the metabolic pathways. For example, in the metabolism of fructose and mannose, some key enzymes were found exclusively with NT (EC 2.4.1.217; EC 1.1.1.17) and others exclusively with

CT (EC 2.7.1.51; EC 3.1.3.70). Key enzymes for degradation of aminobenzoate, fluorobenzoate and dioxin were detected exclusively in the NT soil (EC 5.5.1.7 and EC 1.14.12.18), whereas, in the CT soil, there were enzymes for the degradation of naftalene (EC 1.13.11.38) and of benzoate (EC 1.14.12.1; EC 5.3.3.4). These observations might indicate higher facility to degrade some xenobiotics in CT or NT; however, it is possible that despite the absence of particular enzymes in some of the treatments, degradation of these compounds may take place by others.

Proportionally few sequences were attributed to the subsystem of nitrogen metabolism, and the majority was associated with ammonia assimilation, with higher abundance of the key enzymes glutamate synthase and glutamine synthetase (BERNARD and HABASH, 2009) (Figs. S4 and S5).Considering total N in soil, NT was higher than the CT and rotation slightly favored N accumulation (Table S1). Higher total N content in the soil might explain more sequences for glutamate synthase and ammonium transportation in the NTS. Other N related genes detected were associated with nitrate and nitrite reductases, and to the reduction of nitrite to nitric oxide, dinitrogen oxide, and gaseous nitrogen (Figs. S4 and S5). The results support the findings of a study of soil microbial communities across nitrogen gradients performed by FIERER *et al.* (2012a,b) that suggested shifts in the predominance of microbial communities, favoring the metabolism of proteins, related to a more active copiotrophic in comparison to oligotrophic bacteria under high N. In addition, the community favored by the N should be less likely to access more recalcitrant pools of C (FIERER *et al*., 2007, 2012a; MIKI *et al*., 2010), also in agreement with our results.

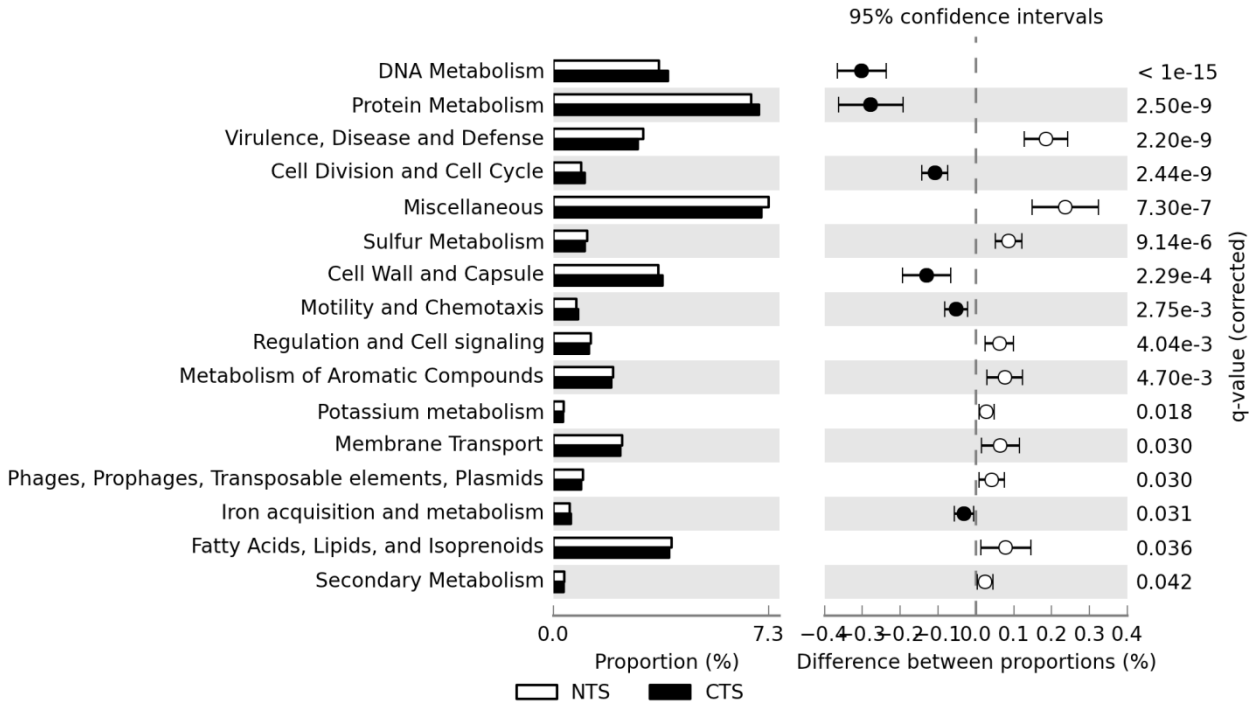

Figure 3 - Statistically significant differences in the functional subsystem in the level 1 of classification using the MG-RAST annotation platform and STAMP statistical software in the comparison of no-tillage under crop succession (NTS) and the conventional tillage with crop succession (CTS) in a 13-years field experiment. The graphic shows only the subsystems with statistical differences between the proportions of sequences in each treatment with a confidence interval of 95%.

Biological nitrogen fixation is a key process in agriculture, and we have shown that rates of biological fixation are higher in NT than in CT (e.g., HUNGRIA and VARGAS, 2000; PEREIRA *et al*., 2007); in addition, in our previous study the NT soils showed higher abundances of *Rhizobiales* (SOUZA *et al*., 2013). Now we confirmed more sequences related to the key gene nitrogenase transcriptional regulator nifA in the NT system in comparison to the CT, both under crop succession and rotation (Fig S6). Interestingly, the proportion of genes related to different components and types of nitrogenase varied with the treatments: CTR favored the Mo–Fe component, whereas the Fe–Fe component was lower in NTR and the Va–Fe nitrogenase was lower with NTS (Fig S6). However, we still do not know whether those differences have any impact in N2 fixation by free-living microorganisms, or when in symbiosis with legumes. Another interesting subsystem encountered with the MG-RAST analysis was that of regulation and signaling, with a higher number of sequences in the NTS (Table 1, Fig. 3). In this subsystem emphasis should be given

on genes of the enzyme adenylate cyclase (cAMP) signaling system in bacteria (Fig. S7). cAMP is known as an important secondary messenger used for intracellular signal transduction in prokaryotes and in eukaryotes; it participates in the regulation of important physiological processes, such as virulence factors in pathogenic bacteria (AGARWAL and BISHAI, 2009). It is also an internal cell “warning” of environmental and nutritional stresses. Soil microorganisms are frequently undergoing fluctuations at the substrate level and, thus, cAMP regulates energy and cell metabolism, as well as cell–cell signaling. The presence of this subsystem in greater proportion in soils under NTS (Fig. S7) might represent a more stable community, as cAMP represents an additional mechanism of regulation.

3.3 MICROBIAL FUNCTIONAL REDUNDANCIES AND SPECIFICITIES

In a previous study we reported several differences in microbial diversity in soils from long-term experiments under different soil and crop managements. Our main objective now was to investigate to which extend the differences detected in microbial diversity would reflect in potential soil functionality. The relevance of our study relies in that although it is well documented that microbial populations are sensitive to disturbance, microorganisms also have great potential for resilience in the long term, and disturbance may reduce some taxa while benefiting others, probably in an attempt to maintain functionality (e.g., KASCHUK *et al*., 2010; TORSVIK and ØVREÅS, 2002). However, in intensively cropped soils, time may not be sufficient for resiliency, and it is more likely that the resistance of certain species occurs, but there are still very few studies to confirm this hypothesis.

The CT system, especially with crop succession, is recognized as a land degrading practice and in a previous study we reported greater abundance of bacteria in CTS (SOUZA *et al*., 2013). That might be explained by the versatility of these microorganisms contributing to soil resilience, and attributable to higher resistance to stresses in these microorganisms. Contrarily, under NT, a system that is closer to the natural ecosystems (e.g., NOGUEIRA *et al*., 2006), the populations of Archaea and *Rhizobiales* were higher than in CT (SOUZA *et al*., 2013), indicating low resilience of these microrganisms. Interesting, it has been suggested that these two classes or microorganisms could well be used as bioindicators to monitor soil quality (SOUZA *et al*., 2013).

In this study we have confirmed some functional specificities related to no-tillage and crop rotation (e.g., higher percentages of sequences associated with carbohydrate metabolism). However, it is notable that differences in the diversity of taxa detected in our previous study (SOUZA *et al*., 2013) were far greater than the functional differences reported now, suggesting high microbial functional redundancy, one example being the metabolisms of C and N. As defined by ALLISON and MARTINY (2008), redundancy refers to the ability of one microbial taxon to carry out a process at the same rate as another under the same environmental conditions. And indeed, our study suggests that several taxa might be performing similar roles in microbial communities. Therefore, although microbial diversity can be drastically reduced with agriculture cultivation, with impacts depending on soil and crop management systems (e.g., BABUJIA *et al*., 2014; NOGUEIRA *et al*., 2006; SILVA *et al*., 2013; SOUZA *et al*., 2013), apparently the impact on microbial functional diversity is less marked, or it might be observed in due course in terms of composition of the microbial community.

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Anexos

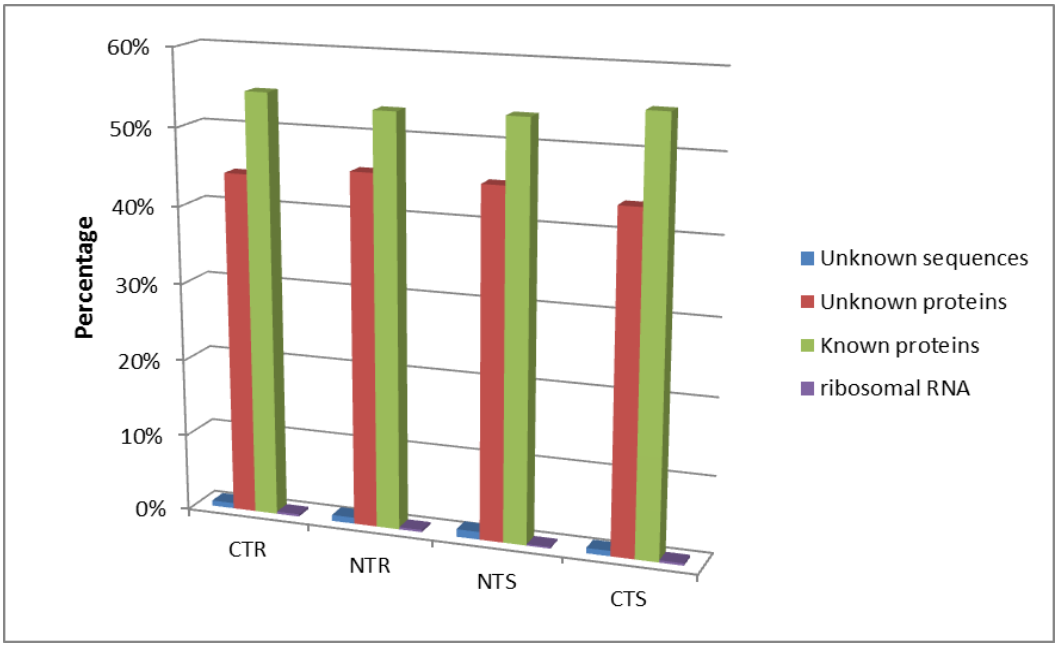

Figure S 1 - Classification of the sequences generated by MG-RAST annotation platform in each of the four metagenomes of an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop succession (S) or rotation (R).

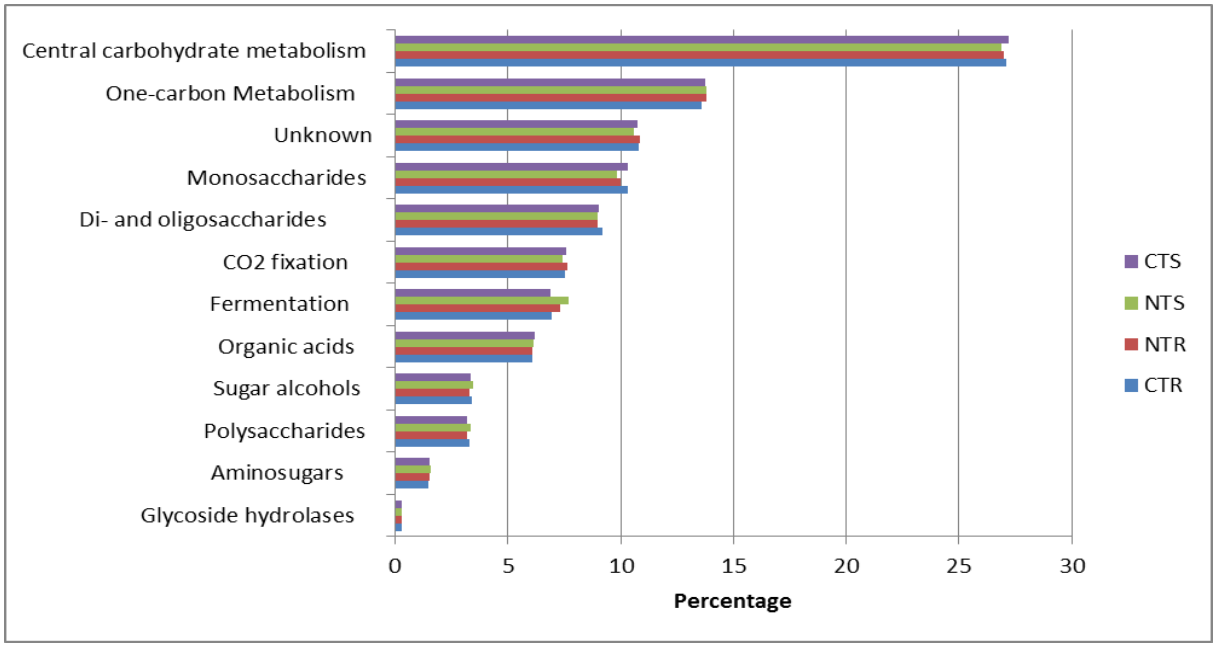

Figure S2 - Distribution of the level 2 of the carbohydrate subsystem generated by the MG-RAST in four metagenomes of an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop succession (S) or rotation (R).

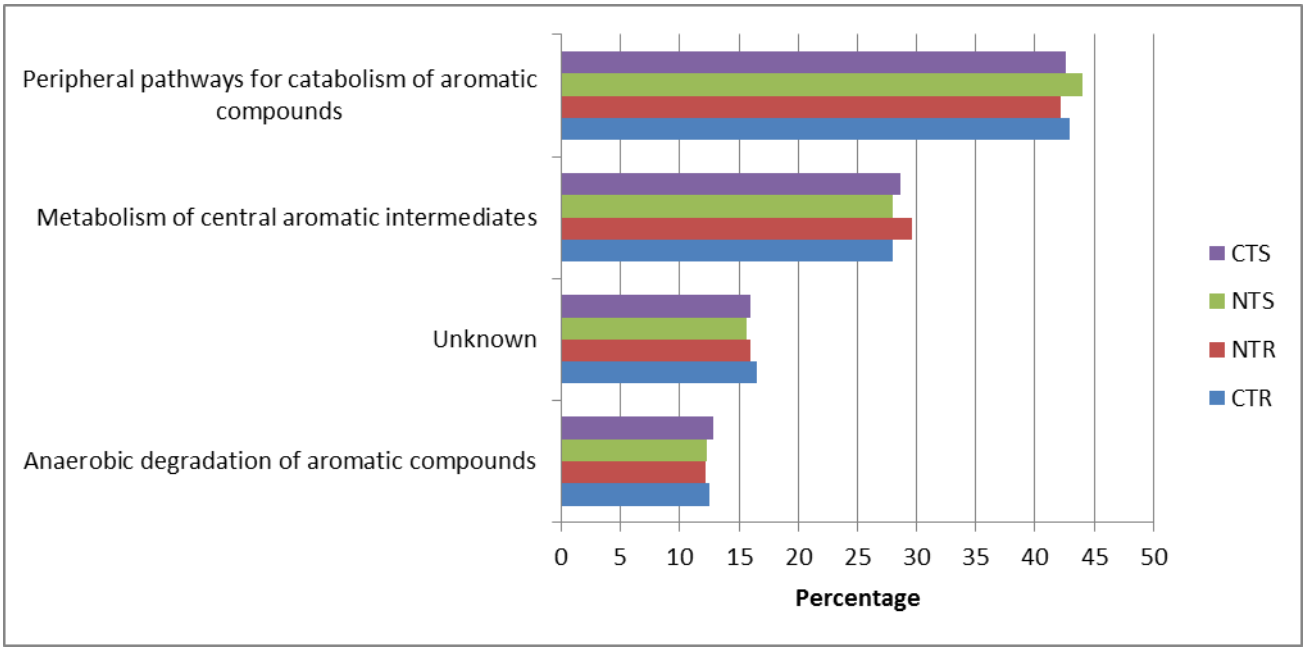

Figure S3 - Distribution of the level 2 of the metabolism of aromatic compounds generated by the MG-RAST in four metagenomes of an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop succession (S) or rotation (R).

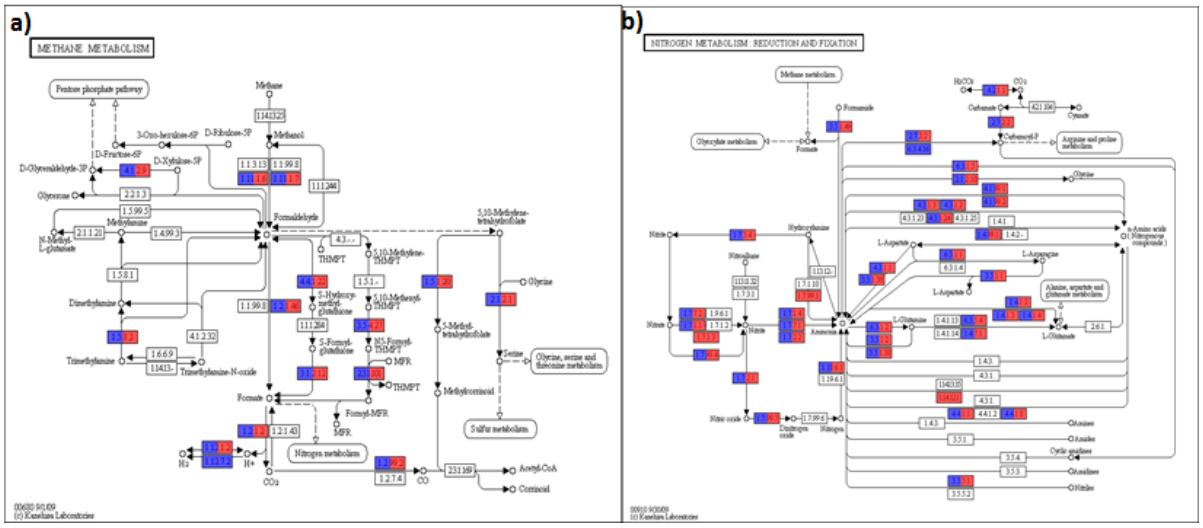

Figure S4 - Metabolic route of KEGG, for the (a) methane and (b) nitrogen; in blue is the no-tillage (NT) and in red the conventional tillage (CT).

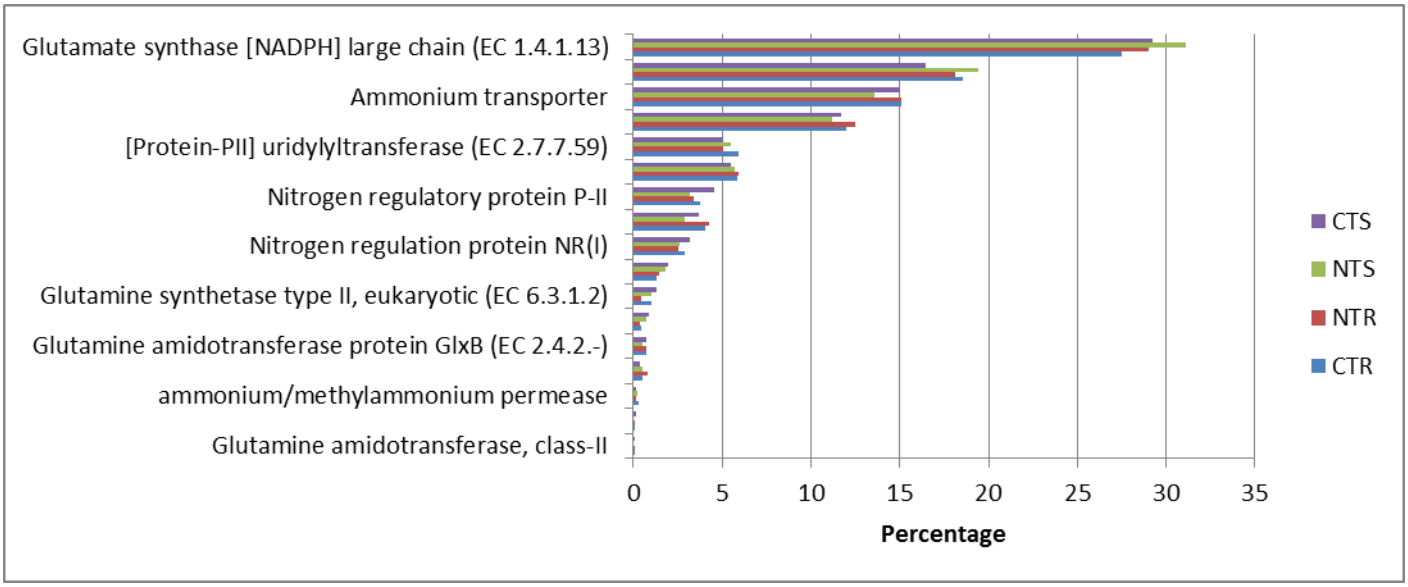

Figure S5 - Functional distribution of the assimilation of ammonia in the subsystem of metabolismo of N generated by MG-RAST in four metagenomes of an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop succession (S) or rotation (R).

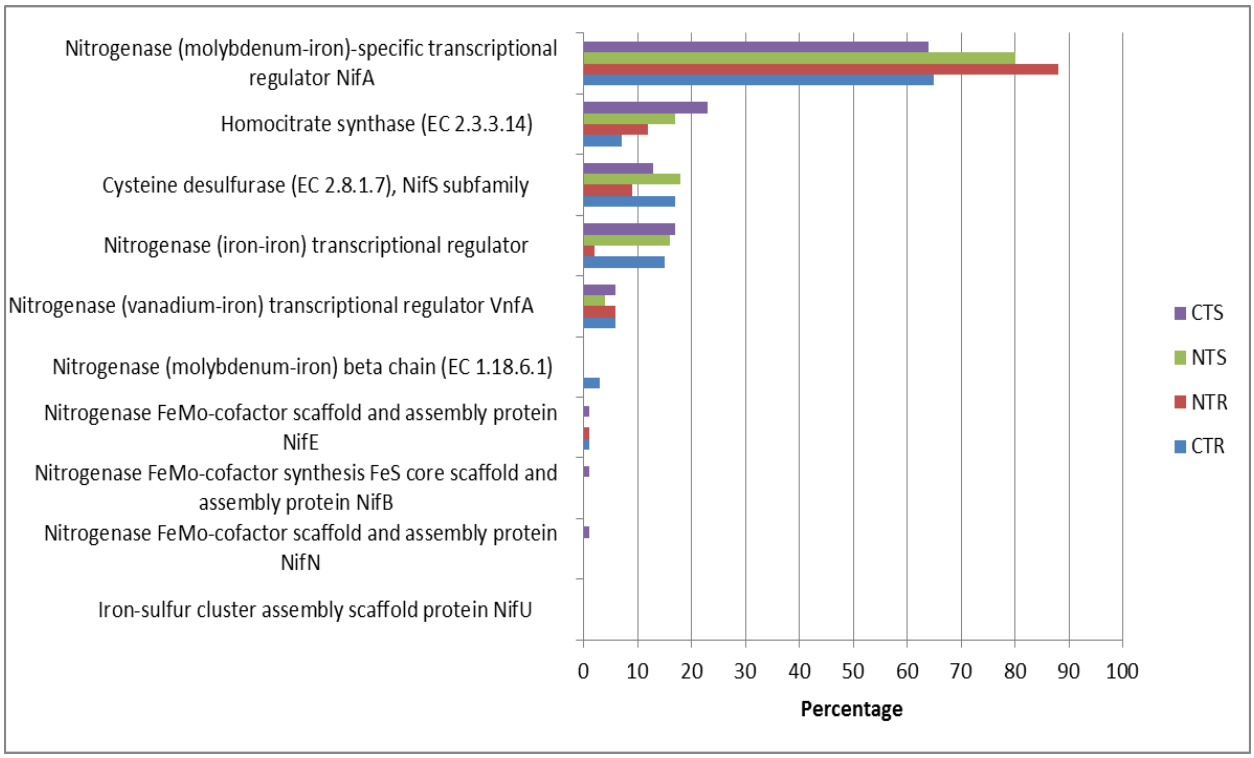

Figure S6 - Functional distribution of the nitrogen fixation-related genes in the subsystem of metabolismo of N generated by MG-RAST in four metagenomes of an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop succession (S) or rotation (R).

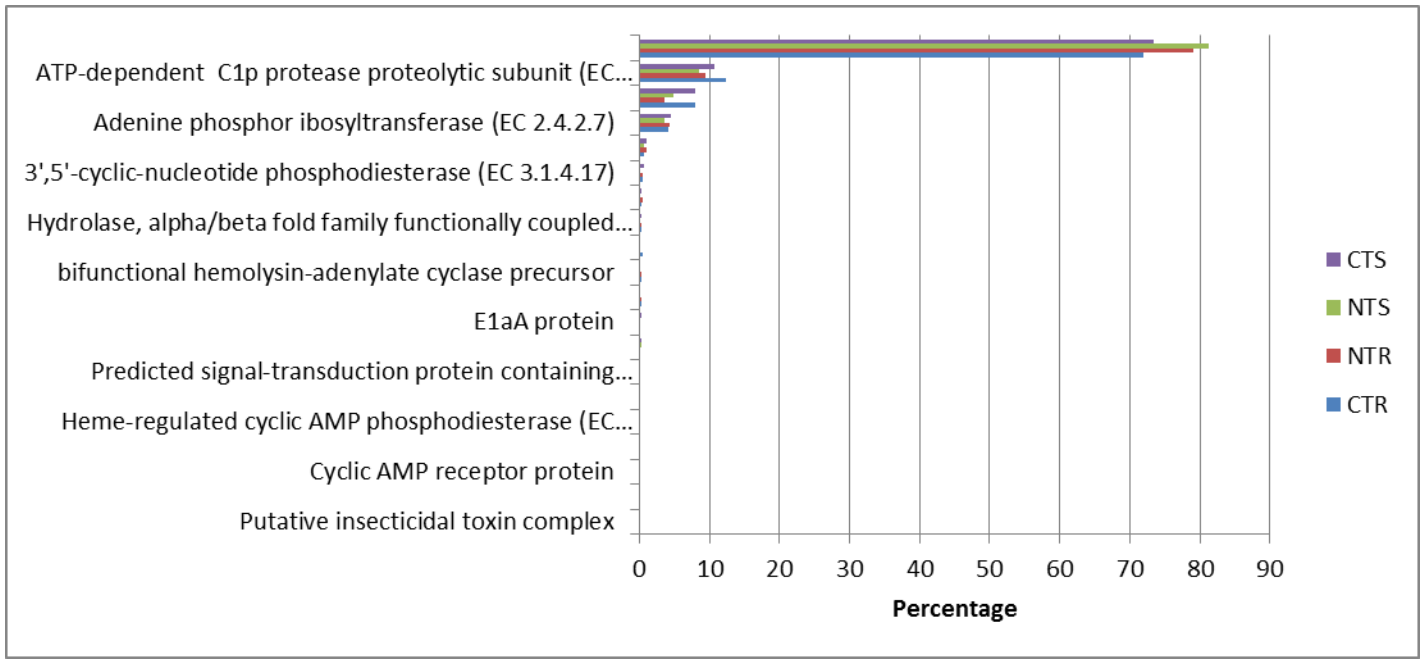

Figure S7 - Functional distribution of cAMP signalling in the subsystem of regulation and cell signaling generated by the MG-RAST in soils under no tillage (NT) or conventional tillage (CT) with crop rotation (R) or sucession (S) in a 13-year old field experimente.

Table S1 - Soil chemical and granulometric1 properties in the 0–10 cm layer.

Soil management[2]	Crop management[3]	pH	Al	H+Al	K	Ca	Mg	SB[4]	T_{CEC}[4]	P	C	N	BS[4]
		$CaCl_2$	----------	----------	----------	$cmol_c\ dm^{-3}$	----------	----------	----------	mg dm^{-3}	g dm^{-3}	g dm^{-3}	%
NT	CS	5.39[5]	0	4.67	0.53	4.03	1.24	5.79	10.47	66.0	24.0	2.6	55.00
NT	CR	5.68	0	3.95	0.58	4.50	1.20	6.28	10.22	55.5	25.8	2.8	61.24
CT	CS	5.23	0	4.77	0.46	3.73	1.01	5.20	9.97	14.8	19.2	1,6	52.11
CT	CR	5.32	0	3.91	0.47	4.06	1.15	5.68	9.60	20.6	19.4	1.8	59.22

[1] Granulometric composition: 710 g kg^{-1} of clay, 82 g kg^{-1} of silt and 208 g kg^{-1} of sand.
[2] Conventional tillage (CT), no-tillage (NT)
[3] Crop succession (CS) with soybean (summer) and wheat (winter) and crop rotation (CR) with soybean and maize (summer), and wheat, lupine, and oat in the winter, as described in Table 1S
[4] SB, sum of bases (Ca + Mg + K); T_{CEC}, Total Cation Exchange Capacity (H+Al + Ca + Mg + K); BS, Base saturation (SB/T_{CEC}) X 100.
[5] Data represent the means of four field replicates

Table S2 - Functional classification of the genes detected in an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT). Results obtained using MG-RAST with BLASTX against SEED database with a cut-off of 60% and E-value of 1 x 10-5.

Functional category	Percentage of sequences	
	NT	CT
Clustering-based subsystems	15.4%	15.4%
Carbohydrates	10.9%	10.9%
Amino Acids and Derivatives	8.8%	8.8%
Miscellaneous	8.5%	8.4%
Protein Metabolism	7.3%	7.5%
Cofactors, Vitamins, Prosthetic Groups, Pigments	6.6%	6.6%
DNA Metabolism	3.7%	3.8%
RNA Metabolism	3.7%	3.8%
Respiration	3.6%	3.6%
Cell Wall and Capsule	3.6%	3.7%
Fatty Acids, Lipids, and Isoprenoids	3.2%	3.2%
Membrane Transport	3.9%	3.8%
Nucleosides and Nucleotides	3.0%	3.0%
Virulence, Disease and Defense	3.1%	3.0%
Stress Response	2.6%	2.6%
Metabolism of Aromatic Compounds	2.2%	2.1%
Regulation and Cell signaling	1.7%	1.6%
Cell Division and Cell Cycle	1.3%	1.3%
Sulfur Metabolism	1.3%	1.3%
Phages, Prophages, Transposable elements, Plasmids	1.3%	1.3%
Nitrogen Metabolism	1.1%	1.0%
Phosphorus Metabolism	0.9%	0.9%
Motility and Chemotaxis	0.9%	1.0%
Iron acquisition and metabolism	0.6%	0.6%
Secundary Metabolism	0.5%	0.5%
Potassium Metabolism	0.5%	0.4%
Dormancy and Sporulation	0.2%	0.2%
Photosynthesis	0.2%	0.2%

Table S3 - Functional classification of the genes detected in an Oxisol under 13 years with crop rotation (CR) or crop succession (CS). Results obtained using MG-RAST with BLASTX against SEED database with a cut-off of 60% and E-value of 1 x 10-5.

Functional category	Perecentage of sequences	
	CS	CR
Clustering-based subsystems	15.5%	15.3%
Carbohydrates	10.9%	10.9%
Amino Acids and Derivatives	8.8%	8.8%
Miscellaneous	8.4%	8.5%
Protein Metabolism	7.4%	7.4%
Cofactors, Vitamins, Prosthetic Groups, Pigments	6.7%	6.6%
DNA Metabolism	3.8%	3.8%
RNA Metabolism	3.8%	3.8%
Respiration	3.6%	3.6%
Cell Wall and Capsule	3.7%	3.7%
Fatty Acids, Lipids, and Isoprenoids	3.2%	3.2%
Membrane Transport	3.9%	3.8%
Nucleosides and Nucleotides	3.0%	3.0%
Virulence, Disease and Defense	3.1%	3.1%
Stress Response	2.6%	2.6%
Metabolism of Aromatic Compounds	2.1%	2.2%
Regulation and Cell signaling	1.6%	1.6%
Cell Division and Cell Cycle	1.3%	1.3%
Sulfur Metabolism	1.3%	1.3%
Phages, Prophages, Transposable elements, Plasmids	1.3%	1.3%
Nitrogen Metabolism	1.1%	1.1%
Phosphorus Metabolism	0.9%	0.9%
Motility and Chemotaxis	1.0%	1.0%
Iron acquisition and metabolism	0.6%	0.6%
Secundary Metabolism	0.5%	0.5%
Potassium Metabolism	0.5%	0.4%
Dormancy and Sporulation	0.2%	0.2%
Photosynthesis	0.2%	0.1%

Table S4 - Activity of some soil microbial enzymes evaluated in the 0-10 cm layer, in the treatments under not tillage (NT) or conventional tillage (CT) with crop succession (S) of soybean/wheat or rotation (R) including soybean/wheat/maize/lupin/oat.

Treatment	Acid phosphatase (µg pNP/g/h)[1]	Dehydrogenase (mg TPF/g/h)[2]	Cellulase (µg glucose/g/day)
NTCR	128 a	25 a	134 b
NTCS	119 a	24 a	122 b
CTCR	82 b	15 b	198 a
CTCS	86 b	14 b	200 a

[1] *p*NP, p-nitrophenol.
[2] TPF, *reduction of* 2,3,5-triphenyltetrazolium chloride to triphenylformazan

CAPÍTULO 3 - Shifts in Taxonomic and Functional Microbial Diversity with Agriculture: How Fragile is the Brazilian Cerrado?

BMC Microbiology

Shifts in Taxonomic and Functional Microbial Diversity with Agriculture: How Fragile is the Brazilian Cerrado?

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Running title: Brazilian savannah metagenome

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ABSTRACT

Background

The Cerrado—an edaphic type of savannah— comprises the second largest biome of the Brazilian territory and is the main area for grain production in the country, but information about the impact of land conversion to agriculture on microbial diversity is still scarce. We used a shotgun metagenomic approach to compare undisturbed (native) soil and soils cropped for 23 years with soybean/maize under conservation tillage—"no-till" (NT)—and conventional tillage (CT) systems in the Cerrado biome.

Results

Soil management and fertilizer inputs with the introduction of agriculture improved chemical properties, but decreased soil macroporosity and microbial biomass of carbon and nitrogen. Principal coordinates analyses confirmed different taxonomic and functional profiles for each treatment. There was predominance of the Bacteria domain, especially the phylum Proteobacteria, with higher numbers of sequences in the NT and CT treatments; Archaea and Viruses also had lower numbers of sequences in the undisturbed soil. Within the Alphaproteobacteria, there was dominance of Rhizobiales and of the genus *Bradyrhizobium* in the NT and CT systems, attributed to massive inoculation of soybean, and also of Burkholderiales. In contrast, *Rhizobium*, *Azospirillum*, *Xanthomonas*, *Pseudomonas* and *Acidobacterium* predominated in the native Cerrado. More Eukaryota, especially of the phylum Ascomycota were detected in the NT. The functional analysis revealed lower numbers of sequences in the five dominant categories for the CT system, whereas the undisturbed Cerrado presented higher abundance.

Conclusion

High impact of agriculture in taxonomic and functional microbial diversity in the biome Cerrado was confirmed. Functional diversity was not necessarily associated with taxonomic diversity, as the less conservationist treatment (CT) presented increased taxonomic sequences and reduced functional profiles, indicating a strategy to try to maintain soil functioning by favoring taxa that are probably not the most efficient for some functions. Our results highlight that underneath the rustic appearance of the Cerrado vegetation there is a fragile soil microbial community.

Keywords: Shotgun metagenome, Soil microbiome, Functional biodiversity, Soil management, No-tillage, Cerrado

1. INTRODUCTION

The Cerrado region represents the second largest biome of the Brazilian territory, with an area of approximately 2 million km^2 (ARAUJO *et al.*, 2012). The soils are rich in aluminum, poor in nutrients and very acidic, supporting only an adapted vegetation typically composed of a gradient of grassland, savannah and forest, interspersed with riparian or gallery forests, patches of semi-deciduous forest, swamp and marshes (RUGGIERO *et al.*, 2002). However, with appropriate chemical correction, Cerrado soils can be very productive, and since the early 1960s large areas have been incorporated into agriculture (VIANA *et al.*, 2011), such that currently the region represents the main grain producing area in Brazil.

Soils are the more diverse environment in terms of microorganisms on Earth, with approximately 1,000 Gbp of microbial genome sequences per g of soil (VOGEL *et al.*, 2009). Microorganisms directly affect the environment and agricultural systems, by means of an array of mechanisms that include biological nitrogen fixation (HUNGRIA *et al.*, 2005), suppression of diseases (MENDES *et al.*, 2011), decomposition of organic matter (SCHMIDT *et al.*, 2011), plant growth promotion (BHATTACHARYYA and JHA, 2012), soil nutrient cycling (BRUSSAARD, 2012) and bioremediation (ALI *et al.*, 2012). However, soil microbial community structure and its associated biological processes can be readily affected by land use, as a result of changes in soil structure, water holding capacity, temperature fluctuations, organic matter and nutrients contents, pH, introduction of new plant species, and agrichemical inputs (e.g. VIANA *et al*., 2011; MENDES *et al*., 2011; BHATTACHAYYA and JHA, 2012).

For decades, several studies have measured the impact of agriculture on soil microorganisms diversity and function, but using limited methodologies that identified few microorganisms and/or detected only generalist activities or microbial biomass (e.g.(FERREIRA and HUNGRIA, 2002; KASCHUK, ALBERTON and HUNGRIA, 2010; 2011). Soil metagenome studies are finally revealing how deep the impacts of anthropogenic action may be. For example, (ROESCH *et al.*, 2007) confirmed that native forest soils had higher bacterial diversity than agricultural soils, while (SOUZA *et al.*, 2013) showed greater relative abundance of certain bacterial orders and

Archaea in a soil under conservation management, in comparison to another on which conventional practices had been adopted.

Brazilian economy greatly relies on agriculture, but the media frequently claims that the country adopts non-sustainable practices in agriculture. Considering soil microbial biomass, the Brazilian Cerrado is even more sensitive than the Amazon to the introduction of agriculture (KASCHUK *et al*., 2011), raising concerns about the impact on microbial community. Limitations of using specific methodologies or genes rely on bias of specific primers, detection of uncultivable microorganisms, among others, but great advances have been achieved with the metagenomic shotgun approach, opening opportunities for revealing genetic and metabolic diversity as well as new metabolic routes, genes and products (e.g. (SOUZA *et al.*, 2013; BENGTSSON-PALME *et al.*, 2014; SCHMIDT *et al.*, 2014; SINGH *et al.*, 2014; SOUZA *et al.*, 2015). Therefore, in order to better understand the impact of agriculture on the Cerrado soils, we used a shotgun metagenomic approach with taxonomic and functional analyses, comparing undisturbed and cropped areas.

2. MATERIALS AND METHODS

2.1 GENERAL DESCRIPTION OF THE AREAS AND SOIL SAMPLING

Soil samples were collected at the experimental station of Embrapa Cerrados in Planaltina, Federal District, Brazil (15º36'34'' S and 47º44'36'' W). The altitude of the sites is approximately 1170 meters, the climate is tropical seasonal (Aw, Köppen classification), with average rainfall of 1500 mm concentrated in the period from September to April, and a dry period lasting 5-6 months. The average annual temperature is 21°C, with an average high of 28°C in September and an average low of 17°C in June. The soil is classified as Latossolo Vermelho Amarelo argiloso (Brazilian system), clayey Typic Haplustox (US classification). The area relief is mostly plan.

The treatments were initially established in a very homogenous area. The area was transformed in two large experiments with two soil management systems. At the time of our study the experiments were 23-year-old under conventional tillage (CT) or no-

tillage (NT), both cropped with soybean (Glycine max (L.) Merr.) in one rainy season and maize (Zea mays L.) in the following one, and left as fallow in the winter (dry season). The CT area was annually prepared by ploughing and disking the soil before sowing and for incorporation of weeds after harvest, whereas the NT area was managed without ploughing or disking. Plots of CT measured 25 m width x 320 m length and plots of NT measured 50 m width x 320 m length. A treatment representing the undisturbed Cerrado stricto sensu (native) was included as a reference for the original soil conditions. The area has no history of anthropogenic activity and represents a typical area of native Cerrado stricto sensu.

Soils were sampled from each area in January of 2014 during the rainy season (summer), at 0-10 cm depth. Each sampling area under NT (8000 m2), CT (11200 m2), and native Cerrado (6700 m2) was split into three sub-areas in order to generate three replicates. Therefore, each biological replicated corresponded to 2267m2, 3733m2 and 2233 m2 for the NT, CT and native Cerrado, respectively. From each sub-area of each treatment ten subsamples spatially distributed to cover the whole area were taken to form a composite sample. Therefore, each of the three treatments ended up with three replicates, each composed by ten subsamples. At the sampling time the CT and NT area had maize at flowering stage.

Soil samples were placed in plastics bags and transported to the laboratory, plant residues and roots were removed and soil was sieved (<4 mm, 5 mesh). Subsamples were sent to chemical and physical analyses; others were stored at 4ºC for microbial biomass and soil enzymes analyses, the remaining being kept at -20º C for the metagenomic analysis.

For chemical and physical analyzes, samples were air-dried and sieved again through a 2-mm mesh for chemical analyses using routine methods (EMBRAPA, 1997). Soil pH was measured at a soil:water ratio of 1:2.5 by weight. Ca, Mg and Al were extracted with 1 N KCl and quantified through atomic absorption (Ca and Mg) and titration with NaOH 0.025 M (Al); P and K were extracted using the Mehlich 1 (H2SO4 0.0125 M + HCl 0.05 M) method, and quantified through flame spectrophotometry (K), or by using the blue-Mo method (P). Soil organic matter (SOM) was determined using the Walkley and Black method. Soil physical properties were analyzed using routine methods (EMBRAPA, 1997).

2.2 MICROBIAL BIOMASS AND ENZYMES ACTIVITY

To characterize and compare biological activity we evaluated microbial biomass-C and –N (MB-C, MB-N), and the soil enzymes β-glucosidase, arylsulfatase and acid phosphatase. Analyses were performed in each of the three replicates, each with three analytical replicates.

The soil MB-C and MB-N were determined using the chloroform-fumigation-extraction method (VANCE *et al*., 1987). C and N in the fumigated and non-fumigated samples were determined using a total organic C and N analyzer (Vario TOC Cube, Elementar Analysensysteme GmbH) with an infrared detector. For the calculation of MB-C and MB-N, kCE and kNE factors of 0.35 (JOERGENSEN, 1996) and 0.54 (BROOKES *et al*., 1985) were used.

The β-glucosidase (E.C. 3.2.1.21), acid phosphatase (E.C. 3.1.3.2), and arylsulfatase (E.C.3.1.6.1) activities were determined according to TABATABAI (1994). Due to their short incubation periods (1 h), toluene was omitted from the assays. These three soil enzymes were selected for their roles in the C cycle (□-glucosidase), P cycle (acid phosphatase), and S cycle (arylsulfatase), respectively.

2.3 METAGENOME

2.3.1 DNA extraction and shotgun sequencing

Metagenomic DNA was extracted using the PowerMaxTM Soil DNA Isolation Kit (MoBio Laboratories), following the manufacturer's procedure, and submitted to sequencing analysis in the Ion Torrent PGM sequencing platform (Life Technologies) at the Bioinformatics Laboratory of LNCC Petrópolis, Rio de Janeiro, Brazil, (http://www.lncc.br). Nine libraries of 400-base-pairs DNA fragments, using 100 ng of DNA from each sample were constructed. The libraries were prepared according to Ion Xpress™ Plus gDNA Fragment Library Preparation protocol. For DNA fragmentation the Ion Shear™ Plus Reagents were used. Emulsion PCR was carried out in the Ion OneTouch™ 2 System. Each library was unidirectionally sequenced in one Ion 318™ Chip v2 using an Ion PGM™ System. The metagenomic fragments were submitted to FastX-trimmer (http://hannonlab.cshl.edu/fastx_toolkit/) in order to remove low quality sequences (phred score<15) and short reads (<=50 bp). The

duplicated reads were filtered using the Replicates software (GOMEZ-ALVAREZ *et al.*, 2009). The retained sequences were submitted to MG-RAST v.3.3 server (MEYER *et al*., 2008).

2.3.2 Taxonomic and functional analysis

The sequenced fragments from the nine metagenomes were deposited on MG-RAST v.3.3 (the Metagenomics RAST – http://metagenomics.anl.gov) with the accession numbers 4577670.3 (CT_1), 4578926.3 (CT_2), 4578927.3 (CT_3), 7577671.3 (NT_1), 4578714.3 (NT_2), 4577672.3 (NT_3), 4577669.3 (NATIVE_1), 4578924.3 (NATIVE_2), and 4578925.3 (NATIVE_3). For the taxonomic classification, the sequences were compared against M5NR (M5 non-redundant) database based on the “best hit classification” method. The rarefaction curve and Principal Coordinates Analysis (PcoA) were derived from MG-RAST, estimated with the table of abundances for comparative analyses. The parameters used were: Max. e-value Cutoff: 1e−5; Min. % Identity Cutoff: 80%; Min. Alignment Length Cutoff: 50. These filters were used to avoid false positive sequences. It is worth mentioning that the classification in MG-RAST includes the categories of unclassified sequences (sequences that do not fit into the established parameters of size of the sequences), the unassigned category (unknown sequences showing no similarity with any known sequences) and the category of other sequences (including other sequences as small RNAs or regulation motifs). The PcoA was performed using the default parameters. For the functional analysis, the sequences were compared against SEED database and classified in subsystems (OVERBEEK et al., 2005) using the hierarchical classification method based on the distance method of Bray-Curtis.

2.3.3 Statistical analysis

For chemical properties, MB-C and MB–N and soil enzymes data were analyzed by one-way analysis of variance (ANOVA). Statistical differences between means were assessed by Tukey’s test ($p < 0.05$). All assumptions required by the analysis of variance were verified. These analyses were performed in MSTAT-C (Michigan State University)

To facilitate comparative analyses, visualization and statistical tests of abundance, the metagenome data were normalized with a log transformation, and this procedure is applied to each distribution in a group of distributions so that all distributions exhibit the same mean and the same standard deviation. Thereby all values are placed on a scale from 0 to 1, showing all abundance counts in a more intuitive scale (WILKE *et al*., 2013). For the metagenome data, the abundance profiles obtained from MG-RAST were submitted to STAMP (Statistical Analysis of Metabolic Profile) software (PARKS and BEIKO, 2010), to identify genus and functions statistically different among all treatments. As for each treatment three replicates were analyzed and data were not pooled, and several combinations of pairs were analyzed by STAMP.

For taxonomic data, the ANOVA test was used ($p<0.05$), Tukey-Kramer as post-hoc, and Storey's FDR (false discovery rate) for correction. First, the data were not grouped, but the Storey FDR test indicated that there were no statistical difference within each group. Then the samples from each treatment were pooled. For function analyses, the metagenomes were grouped according the treatments (CT1, CT2, CT3 - as group 1; NATIVE1, NATIVE2, NATIVE3 - as group 2, and NT1, NT2, NT3- as group3) and analyzed using the two groups approach, with Welch´s t-test, Welch´s inverted as CI method, and Storey FDR for correction. Each sample replicate was considered on the statistic test. Categories with biological relevance were obtained using a difference of proportions of 1 and ratio of proportions of 2 as filters.

3. RESULTS

3.1 SOIL PHYSICAL AND CHEMICAL PROPERTIES AND CLASSICAL MICROBIOLOGICAL PARAMETERS

When physical properties were addressed, we observed that 23 years of continuous cropping resulted in increased microporosity and decreased macroporosity, for either the NT and CT treatments. In addition, soil density was increased in the CT system (Table 1).

For the chemical properties, the results obtained in the undisturbed treatment highlight the typical properties of the Cerrado, with high Al content and acidity, low P and nutrients (Table 1). Soil liming and fertilizer inputs to the cropped area increased the level of nutrients, especially P, and increased pH. In comparison to the native undisturbed area, organic matter slightly decreased with cropping under the conservationist system of NT, with a further significant decrease in the CT (Table 1).

A three-fold decrease in soil MB-C and MB–N was observed with agriculture introduction in the Cerrado (Table 2). The arylsulfatase and acid phosphatase activities of the native area resembled those of the NT, but a decrease was verified in the CT treatment. β-glucosidase was also highest in the NT treatment, but it's activity in the CT was similar to that observed in the native Cerrado (Table 2).

Table 2 - Soil physical and chemical proprieties at the 0-10 cm layer in an Oxisol under native vegetation of Cerrado (Native) or cropped with soybean/corn under no-tillage (NT) or conventional tillage (CT) systems

Physical				
Treatment	*Total porosity* m^3/m^{-3}	*Microporosity* m^3/m^3	*Macroporosity* m^3/m^3	*Density* Mg/m^{-3}
NT	0.59	0.41	0.17	0.91
CT	0.59	0.44	0.15	0.95
Native	0.60	0.39	0.21	0.91

Chemical								
Treatment	*Al – exchangeable (titrimetry) me/100cc*	*Ca (atomic absorption) me/100cc*	*H+Al - Acidity (titrimetry) me/100cc*	*K (flame photometer) mg/L*	*Organic matter (OM) (Walkley & Black) %*	*Mg (atomic absorption) me/100cc*	*pH in water pH*	*P (Mehlich1 - spectrophotometry) mg/L*
NT	0.007 B	2.847 A	5.745 B	46.667 A	3.209 AB	0.978 A	5.670 A	26.563 A
CT	0.014 B	1.545 B	4.717 C	30.000 B	2.751 B	0.556 B	5.647 A	10.417 B
Native	0.682 A	0.093 C	8.432 A	44.667 A	3.667 A	0.142 C	4.687 B	0.180 C
p	0.0000	0.000	0.0003	0.0118	0.0262	0.0001	0.0001	0.0008
CV%	12.68	9.39	4.94	9.61	7.68	9.31	1.20	22.37

Values in columns sharing the same letter do not differ significantly ($p < 0.05$) as determined by the Tukey's test.

Table 3 - Microbial enzymes activities and microbial biomass of carbon and nitrogen evaluated in the soils samples at the 0-10 cm layer in an Oxisol under native vegetation of Cerrados (Native) or cropped with soybean/corn under no-tillage (NT) or conventional tillage (CT) systems

Treatment	**MB-N*	**MB-C*	*β-Glucosidase*	*Arylsulfatase*	*Acid Phosphatase*
	mg/kg soil	*mg/kg soil*	*mg p-nitrofenol/kg soil/h*		
NT	30.71 B	215.86 B	150.03 A	70.73 A	812.09 A
CT	23.85 B	150.65 C	92.51 B	41.75 B	621.13 B
Native	84.13 A	539.08 A	94.30 B	71.50 A	824.58 A
p	0.0017	0.0000	0.0124	0.0126	0.0061
CV%	18.19	3.34	12.63	12.04	5.41

Values in columns sharing the same letter do not differ significantly ($p < 0.05$) as determined by the Tukey's test.
*MB-N- Microbial Biomass-Nitrogen
*MB-C- Microbial Biomass-Carbon

3.2 Sequencing analysis

In the shotgun metagenomic approach, for each treatment about 5 million sequences were generated, resulting in 49.2 million reads and 1.31 x 10^{10} bp. When submitted to the MG-RAST server, an average of 2.23 million proteins were classified as known proteins and 2.98 million as predicted proteins but with unknown function (S1 Table).

3.3 Rarefaction curves and principal coordinates analysis

The rarefaction curves from samples generated in MG-RAST showed that even with almost 50 million sequences, the curves were not saturated, indicating high genetic diversity (S1 Fig.).

The results obtained in the PCoA anlaysis indicated that agricultural and undisturbed Cerrado soils had different taxonomic profiles (Fig. 4A), and similar results were observed for the functional profiles (Fig. 4B).

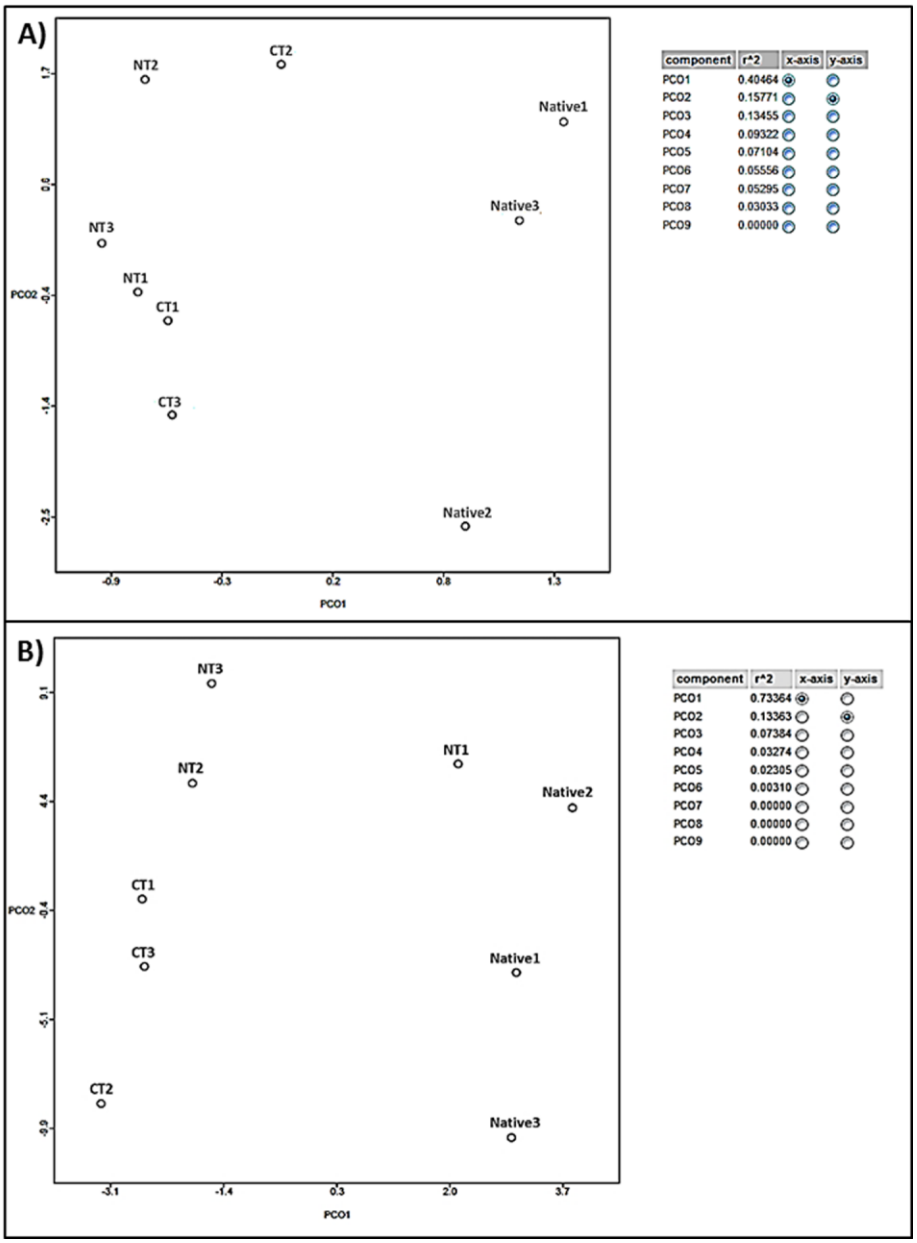

Figure 4 - PCoA analysis generated in (A) MG-RAST abundance compared to M5NR database and (B) of functional categories of subsystems generated in MG-RAST using normalized values (between 0 and 1) and Bray-Curtis distance for no-tillage (NT), conventional tillage (CT) and undisturbed Cerrado (Native) soil metagenomes.

3.4 MICROBIAL COMMUNITY COMPOSITION

The community structure analyses performed with the M5NR (M5 non-redundant protein) database available in the MG-RAST server (MEYER, F *et al.*, 2008) showed that in all treatments the majority of the sequences were attributed to the Bacteria domain, and the remaining were unclassified sequences (sequences that do not fit into the established parameters of size of sequences) of Archaea, Eukaryota, unassigned (unknown sequences showing no similarity with any known sequences), Viruses and other sequences (including other sequences as small RNAs or regulation motifs) (Fig. 5).

Differences in microbial composition at the domain level were detected among the treatments. The largest was observed in Bacteria domain, where the majority of the sequences were assigned to the NT and CT treatments, while the undisturbed soil had considerable lower numbers of sequences ($p < 0.05$ in the comparison of NT or CT with the Cerrado, but not between NT and CT). The second largest domain was of unclassified sequences equally distributed in all treatments. Interestingly, Archaea were very low in two replicates of the native Cerrado, but very abundant with the introduction of agriculture ($p < 0.05$). Eukarya was higher in two replicates of the NT treatment, but with no statistical difference between the treatments, and Viruses were lower in the native soil ($p < 0.05$) (Fig. 5).

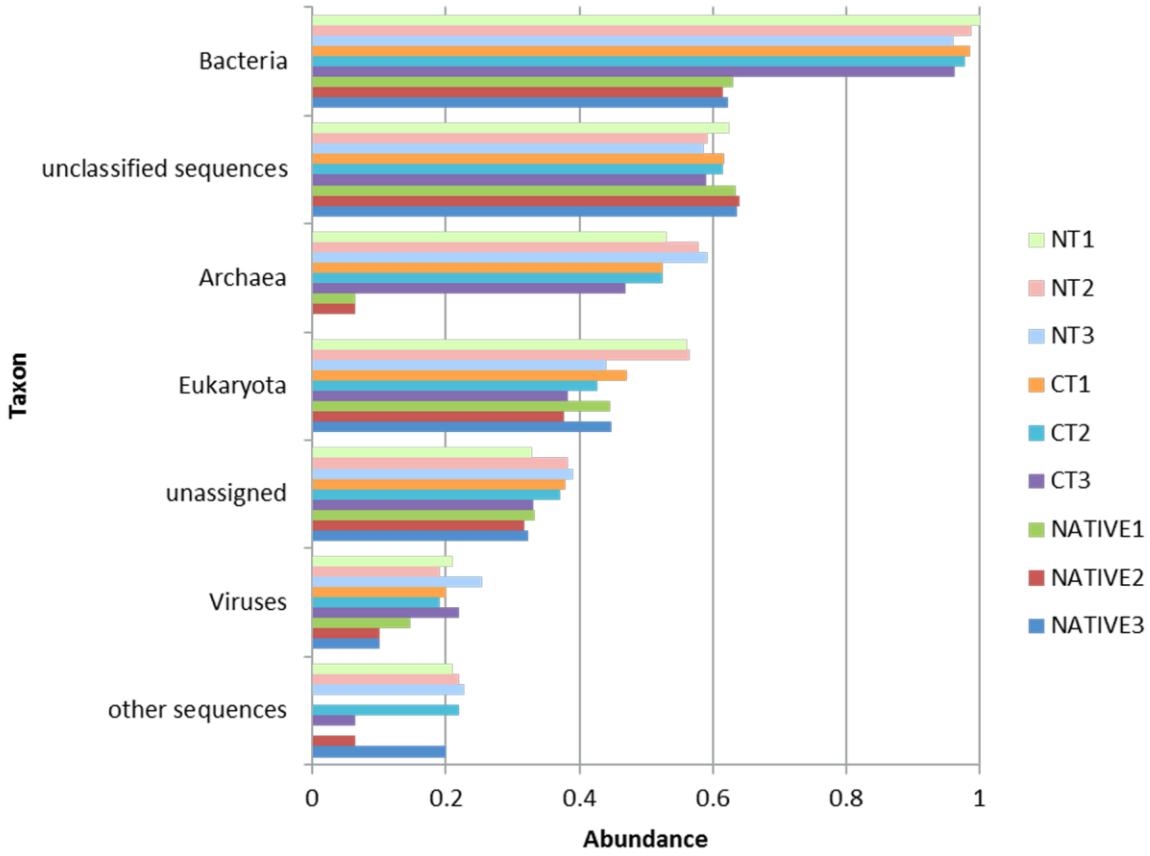

Figure 5 - Sequence abundance at the Domain level compared to M5NR database using normalized values between 0 and 1 for no-tillage (NT), conventional tillage (CT) and undisturbed Cerrado (Native) soil metagenomes.

Among the Bacteria, the most abundant phylum was Proteobacteria in both NT and CT, in comparison to the native area ($p < 0.05$) (Fig. 6). The two most abundant classes of Proteobacteria were Alphaproteobacteria and Betaproteobacteria (data not shown). Actinobacteria was the second most abundant phylum of the Bacteria domain, and in general was not very different among the treatments, except for one replicate of the CT. The Bacteroidetes, Firmicutes and unclassified sequences derived from Bacteria were more abundant in NT and CT treatments in comparison to the Cerrado, while the Acidobacteria phylum dominated in the native soil ($p < 0.05$) (Fig. 6).

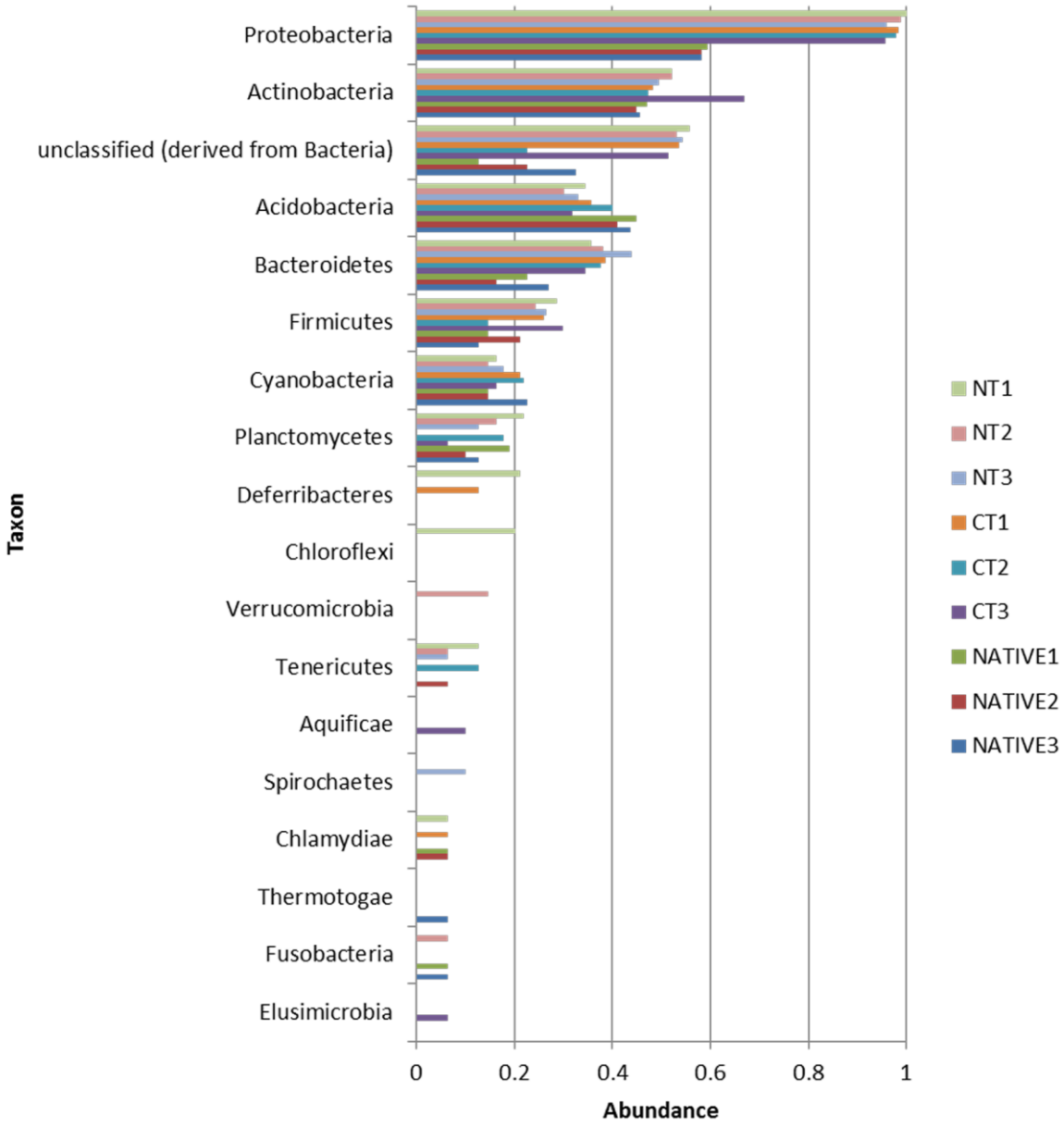

Figure 6 - Sequence abundance phylum of Bacteria Domain compared to M5NR database using normalized values between 0 and 1 for no-tillage (NT), conventional tillage (CT) and undisturbed Cerrado (Native) soil metagenomes.

We will focus on the results that have shown statistical differences between the treatments. The order Rhizobiales was the most dominant in Alphaproteobacteria in the CT and NT metagenomes ($p < 0.05$) (Fig. 7). Within the Rhizobiales, although the genus *Rhizobium* was significantly higher in native soils, *Bradyrhizobium* was higher in the soils under CT and NT (Fig. 8). Still in the Alphaproteobacteria, the genus

Azospirillum was more abundant in the undisturbed soil ($p < 0.05$) (Fig. 8). Within the Alphaproteobacteria, the Sphingomonadales was also higher in the CT and NT systems ($p < 0.05$) (Fig. 7).

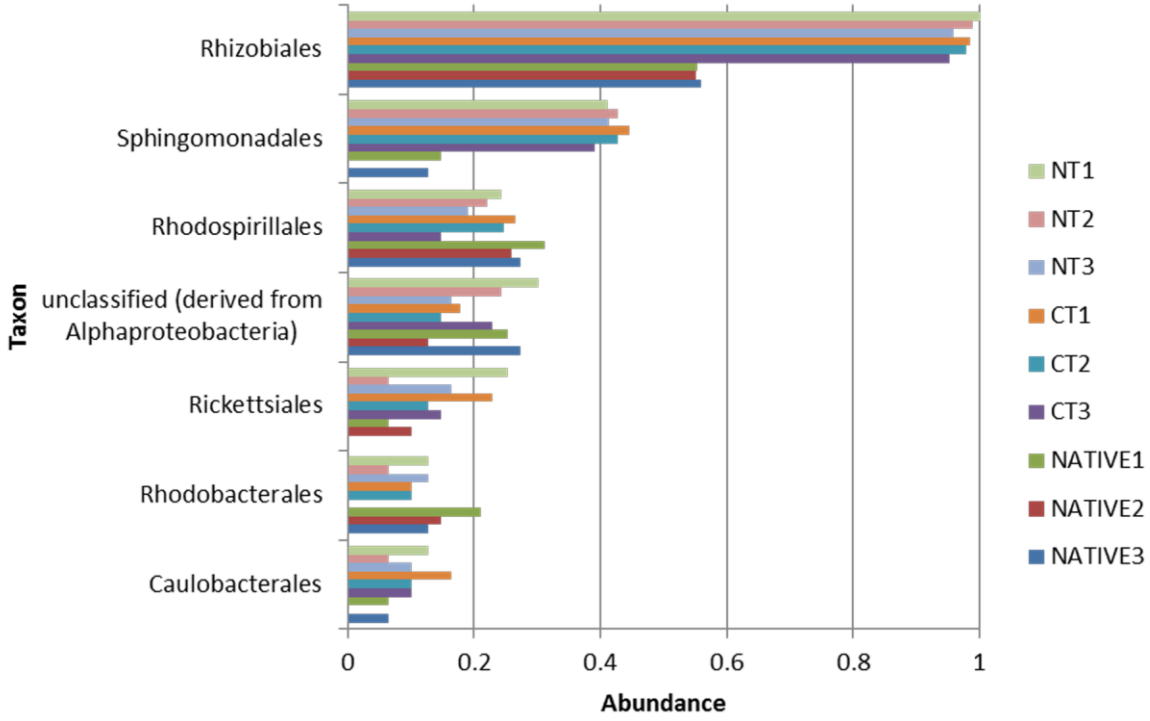

Figure 7 - Sequence abundance orders of Alphaproteobacteria compared to M5NR database using normalized values between 0 and 1 for no-tillage (NT), conventional tillage (CT) and undisturbed Cerrado (Native) soil metagenomes.

In the Betaproteobacteria class, the order Burkholderiales was the most abundant in the NT system, followed by Nitrosomonadales, both in the CT and NT systems ($p < 0.05$) (S2 Fig.). In the Betaproteobacteria, the genus *Nitrosomonas* was more abundant in the cropped areas, while in the Gammaproteobacteria the genus *Pseudomonas* and *Xanthomonas* were significantly more abundant in the undisturbed soil ($p < 0.05$) (Fig. 5). The genus *Acidobacterium* of the phylum Acidobacteria was also higher in undisturbed soil ($p < 0.05$) (Fig. 8).

Within the Archaea domain, the Crenarchaeota phylum was the most abundant in the NT soil, while the Thaumarchaeota phylum was the second most abundant and unclassified Archaea the third, in the NT and CT treatments, and all were practically not

detected in the native soil (p < 0.05) (S3 Fig.). Within this last phylum, the genus *Nitrosphaera* was more abundant in the NT and CT treatments (Fig. 8).

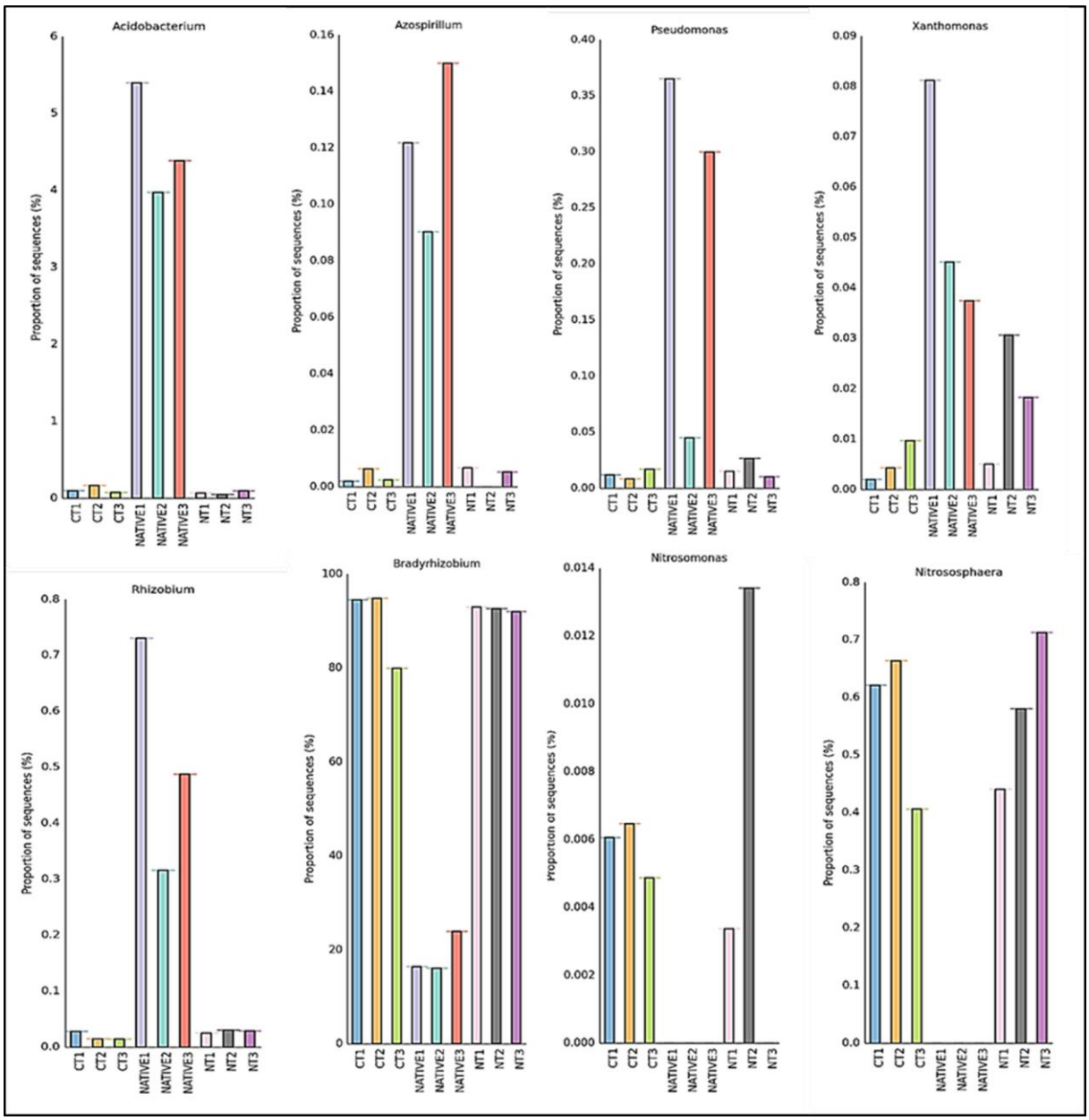

Figure 8 - Proportion of sequences of the main genera generated in STAMP software using MG-RAST genus abundance profiles (M5NR database) for no-tillage (NT), conventional tillage (CT) and undisturbed Cerrado (Native) soil metagenomes.

As pointed out before, the Eukaryota domain was more abundant in the NT treatment (Fig. 5), where there was dominance of the phylum Ascomycota (p < 0.05), followed by unclassified sequences and of Streptophyta, but these without statistical

difference (S4 Fig.). The low number of Viruses sequences, dominant in the CT and NT treatments (Fig. 5), was represented only by the Caudovirales order, higher in the CT and NT treatments in comparison with the Cerrado ($p < 0.05$) (S5 Fig.).

3.5 Functional metagenome profiles

Functional analysis generated by MG-RAST classified the sequences in 29 subsystems (Fig. 9), based on the relative abundance of the data normalized on a scale from 0 to 1. The five categories with more sequences were the RNA metabolism, protein metabolism, miscellaneous, clustering-based subsystems (functional coupling evidence but unknown function) and carbohydrates. The NT and the native soil showed similar numbers of sequences in all these subsystems, while the CT showed lower numbers of sequences. The CT had also lower numbers of sequences in other categories, including stress response, respiration, amino acids and derivatives, cell division and cell cycle. For the undisturbed area, we can mention higher numbers in the subsystems of cell division and cell cycling, motility and chemotaxis, dormancy and sporulation (Fig. 9).

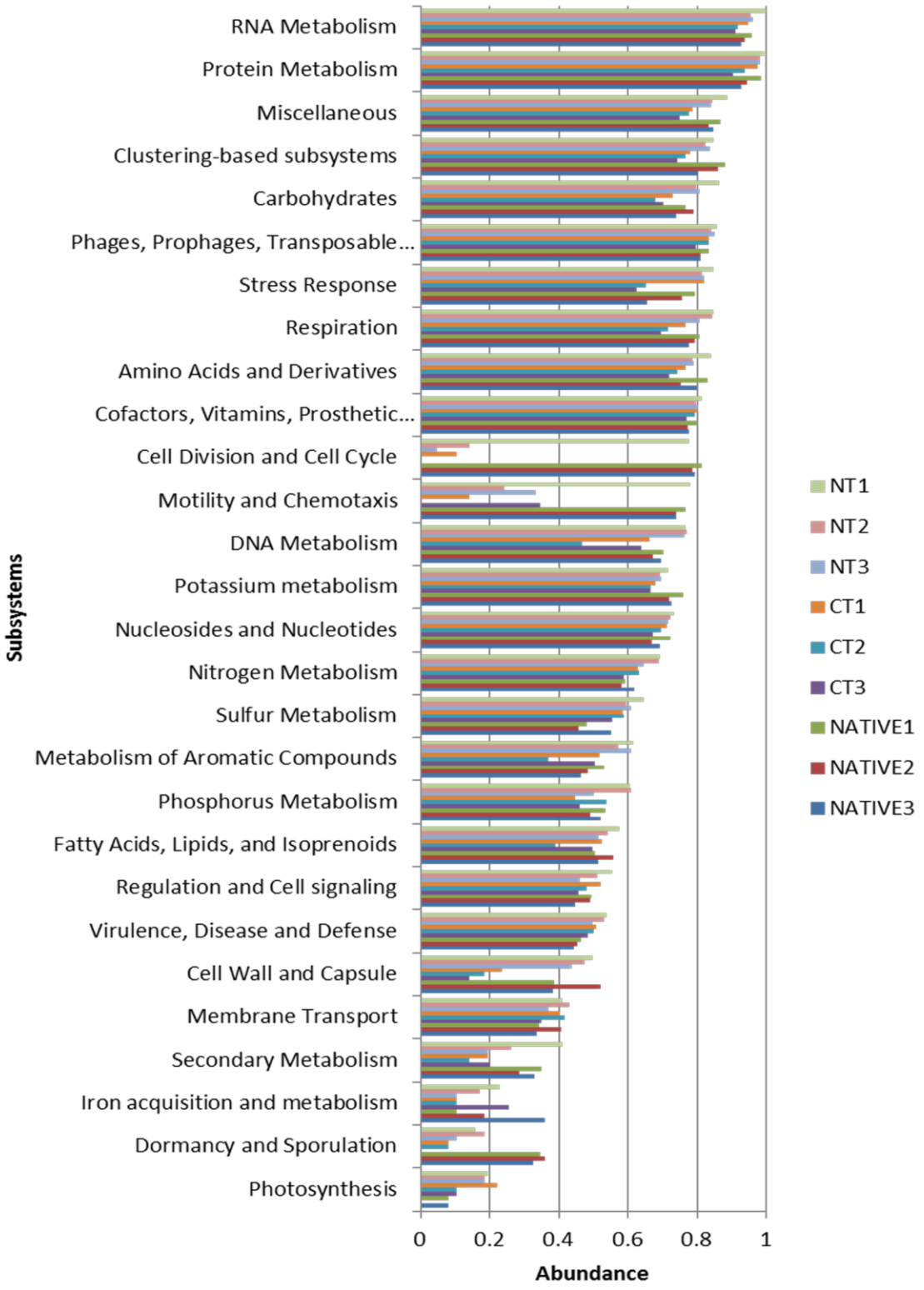

Figure 9 - Abundance of functional classification in subsystems categories using normalized values between 0 and 1 for no-tillage (NT), conventional tillage (CT) and undisturbed Cerrado (Native) soil metagenomes.

4. DISCUSSION

4.1 General Characterization of the Cerrado soils before and after the introduction of agricultural practices

The Brazilian Cerrado currently represents the most important grain producing area in the country, besides covering 24% of the Brazilian land (ARAUJO *et al.*, 2012). The edaphoclimatic conditions of the Cerrado find some parallel with the African savannahs, and in both cases, there are still few studies about microbial communities. The typical soil chemical properties of the Cerrado are of high Al content, low pH and low P, such that the use of lime and fertilizers is necessary to allow economic crop production and results in increased level of soil nutrients (Table 1). The no-tillage (NT) system is being increasingly adopted in the Cerrado over the conventional tillage (CT). Reduced soil disruption and soil cover by plant residues in the NT result in improved physical and chemical properties over the CT, including higher organic matter (OM) content, improved water retention capacity and lower oscillation of temperatures (e.g. (FRANCHINI *et al.*, 2007; BABUJIA *et al.*, 2010; SILVA *et al.*, 2010; SILVA *et al.*, 2014)). Our results confirmed higher OM and nutrient contents (Ca, K, Mg, P) in the NT in comparison to the CT after 23 years of cropping (Table 1).

As observed before (MENDES *et al.*, 2003; PEIXOTO *et al.*, 2010), significant reductions in microbial biomass (MB-C, MB-N), acid phosphatase and arysulfatase activities were observed in the CT areas in comparison to the NT and the native Cerrado (Table 3). Reduction in activity of both enzymes should be related to both the reduction in OM (Table 1) and the addition of chemical fertilizers when agriculture was established in the area. Contrarily, β-glucosidase (BG) activity was higher in the NT than in the Cerrado and in the CT, possibly due to the quality and quantity of plant residues, which are more complex in the undisturbed Cerrado and in the CT, since the β-glucosidase acts in less complex residues (MENDES *et al.*, 2003; PEIXOTO *et al.*, 2010; LOPES *et al.*, 2013)

4.2 Microbial taxonomic and functional diversity

The shotgun approach in metagenomic studies allows better understanding about soil microbial communities, indicating not only the taxonomic groups, but also

metabolic functions. The approach has already been successful in detecting differences in the composition and functionality of microbial communities in the comparison of NT and CT in a fertile oxisol of southern Brazil, subtropical climate (SOUZA *et al.*, 2013; SOUZA *et al.*, 2015). Now, in a different edaphoclimatic condition and having an undisturbed area for comparison, we confirmed that both soil managements caused profound changes in microbial structure and functioning (Fig. 4A, Fig. 4B).

In general higher taxonomic diversity was not associated to the native Cerrado, but rather to agricultural soils which showed higher abundances of Bacteria, the predominant domain in the soil, as well as of Archaea and Viruses (Fig. 5), in agreement with other studies carried out in Brazil (RAMPELOTTO *et al.*, 2013), North America (JANGID *et al.*, 2008) and Europe (NACKE *et al.*, 2011). The results indicate that the stresses imposed by agriculture modify soil microbiome by increasing its taxonomic diversity. For example, also using shotgun analyses MENDES *et al.* (2014) have shown that the soybean rhizosphere selected taxonomic and functional communities for its best development. Shifts may also be necessary to support the newly disrupted environment.

As in other metagenomic studies (SOUZA *et al.*, 2013; PACCHIONI *et al.*, 2014; XU *et al.*, 2014), Proteobacteria were dominant in all soils; however, one interesting observation of our study was the increase of this phylum in the NT and CT treatments (Fig. 6), and emphasis should be given to the genus *Bradyrhizobium* (Fig. 8). Brazilian soils are free of *Bradyrhizobium* compatible with soybean (FERREIRA *et al*., 2002), and massive inoculation is usually practiced every cropping season (HUNGRIA and MENDES, 2014). Genetic events such as high rates of horizontal transfer of symbiotic genes from the inoculant to indigenous rhizobia have been reported in the Cerrado as a result of massive inoculation (BARCELLOS *et al.*, 2007; BATISTA *et al.*, 2007), but no negative impacts on yield have ever been reported (HUNGRIA and MENDES, 2014). Now we show that massive inoculation can indeed affect soil microbial communities, and soil enrichment with inoculant strains might help to explain some failures in introducing new strains in soils with established populations (MENDES, HUNGRIA and VARGAS, 2004; HUNGRIA and MENDES, 2014). Interesting, *Bradyrhizobium* is related to the nitrogen metabolism subsystem, also more abundant in the agricultural soils (Fig. 9). In contrast, in the undisturbed Cerrado there was higher abundance of *Rhizobium*

(Fig. 8), although in a proportion 100-times lower than *Bradyrhizobium* in cropped soils (Fig. 8). Interestingly, studies with classical methods performed in undisturbed Cerrado areas have reported high abundance of *Rhizobium* species tolerant of acidity and stressful environmental conditions (e.g. (RIBEIRO *et al.*, 2012), indicating adaptation to the typical edaphoclimatic conditions of the region.

Other groups that are critical for soil functioning and more abundant in agricultural soils were Burkholderiales and Nitrosomonadales (S2 Fig.). The *Burkholderia* are highly versatile in their ecological niches, including agricultural soils (DRAGHI *et al.*, 2014), where they play important roles in soil bioremediation (ALI *et al.*, 2012), plant growth promotion and biological nitrogen fixation (BHATTACHARYYA and JHA, 2012; SUÁREZ-MORENO *et al.*, 2012; BASHAN *et al.*, 2014). Nitrogen-fixing *Burkholderia* are abundantly found in the Cerrado, especially in symbiosis with *Mimosa* spp., plants that have this biome as their major center of diversity (REIS JR *et al.*, 2010); in addition, *Burkholderia* can colonize diverse host plants (ESTRADA-DE LOS SANTOS, BUSTILLOS-CRISTALES and CABALLERO-MELLADO, 2001). Nitrosomonadales are related to nitrification processes (MONTEIRO, SÉNECA and MAGALHÃES, 2014), fitting into the nitrogen metabolism subsystem (Fig. 9), and their superiority in cropped soils (S2 Fig.) may reflect the use of N-fertilizer inputs to the maize crop, or N residues left by the soybean crop.

Acidobacteria plays several functions in soils, including the degradation of polymers and soil contaminants (YERGEAU *et al.*, 2012; VĚTROVSKÝ, STEFFEN and BALDRIAN, 2014), and the Cerrado is well known for the richness in these microorganisms (ARAUJO *et al.*, 2012; CASTRO *et al.*, 2013; RAMPELOTTO *et al.*, 2013; SILVA, A. P. *et al.*, 2013; SILVA, M. S. *et al.*, 2013; CATÃO *et al.*, 2014). This group of microorganisms was more abundant in the Cerrado (Fig. 6), in agreement with previous comparisons between undisturbed Cerrado and areas with agriculture and pastures. In general, Actinobacteria were found in similar abundances in all three treatments (Fig. 6). Reports about Actinobacteria vary with the biome; in Amazon the phylum was higher in undisturbed than in deforested soil (NAVARRETE *et al.*, 2015), while in Cerrado converted to pasture was higher than in the native area (QUIRINO *et al.*, 2009). *Streptomyces* is the most common Actinobacteria genus in nature, and predominantly found in soils (BONTEMPS *et al.*, 2013; SARIGULLU *et al.*, 2013). The

genus has high ability to synthesize metabolites such as antibiotics (JAURI *et al.*, 2013; SARAVANA KUMAR, DURAIPANDIYAN and IGNACIMUTHU, 2014), and several studies in the Cerrado biome have reported copious presence of these microorganisms (PEREIRA, NEVES and DROZDOWICZ, 1999; SILVA, M. S. *et al.*, 2013). Their biotechnological importance as promising sources of chitinases, proteases and xylanases (GOMES *et al.*, 2001; NASCIMENTO *et al.*, 2002; AZEREDO *et al.*, 2003) should also be mentioned. In addition, their antagonism against several microorganisms can represent a useful biological control tool (GOMES *et al.*, 2001; ANITHA and REBEETH, 2009), and they may also interfere with the introduction of beneficial microorganisms, as reported for inoculant strains of *Bradyrhizobium* (SCOTTI *et al.*, 1982).

Other microorganisms such as Bacteroidetes and Firmicutes were also abundant in our study, especially in agricultural soils (Fig. 6). The Firmicutes phylum includes the classes Bacilli and Clostridia that are well known spore-forming microorganisms, resulting in greater chance of survival in disturbed environments. There are also reports that these microorganisms are dominant in environments rich in P (KURAMAE *et al.*, 2012), and in our study the input of P-fertilizer to the cropped soils raised considerable their P content. In addition, this might explain the increase of sequences in the P metabolism subsystem in the NT treatment (Fig. 9). Bacteroidetes are usually very common in soils (VIERHEILIG *et al.*, 2012) and in one study were more abundant in agricultural ecosystems, in comparison to a forest soil (KURAMAE *et al.*, 2012). NACKE *et al.* (2011) observed that the relative abundances of Bacteroidetes increased with higher pH values, in agreement with the results from our study.

It has been suggested that soil pH (LAUBER *et al.*, 2009; ZHALNINA *et al.*, 2015) and plant residues (MIKI *et al.*, 2010; MITCHELL *et al.*, 2010; MENDES *et al.*, 2012) greatly affect the diversity and activity of soil microbial communities. Regarding soil pH, a good example is Acidobacteria, very abundant in the acidic native Cerrado soils (CASTRO *et al.*, 2011; ARAUJO *et al.*, 2012; CATÃO *et al.*, 2014) and also found in our study. However, every biome has different responses to soil disturbance. For example, in our study the abundant groups Acidobacteria and Alphaproteobacteria were affected by agriculture introduction, with decreases and increases in taxonomic diversity,

respectively. In contrast, NAVARRETE *et al.* (2015) found no differences in the same groups when compared an Amazon forest soil and a soil under slash-and-burn clearing.

As reported before in other metagenomic studies (FIERER *et al.*, 2012), there were few sequences of Archaea, and they were practically absent in the native Cerrado (Fig. 5). In a previous study, we detected more Archaea in the NT than in the CT system in southern Brazil (SOUZA *et al.*, 2013), and the result was attributed to a negative impact of tillage on this domain. Similar results were now confirmed in the NT vs. CT soils of the Cerrado, but they were— as also observed in another Cerrado soil (CASTRO *et al.*, 2011)—surprisingly low in the undisturbed soil. However, we must consider the observations of RODRIGUES *et al.* (2014), showing increase in richness and diversity of Archaea in the dry season, and our samples were obtained in the rainy season. Crenarchaeota, the most abundant phylum, was present only in the NT and Thaumarchaeota, the second most abundant, was present in both NT and CT systems (S3 Fig.); both phyla are relevant in agricultural soils due to their role in the nitrification process (NICOL *et al.*, 2008; XIA *et al.*, 2011; WU and CONRAD, 2014).

Eukaryota was the third most abundant domain, with higher number of sequences in the NT treatment (Fig. 5), and predominance of the Ascomycota phylum (S4 Fig.). These results are in agreement with (CASTRO *et al.*, 2008), that reported that human activity increased this phylum in comparison to the native soil, what could be related to a higher tolerance to environmental stresses (MA *et al.*, 2013; SOUZA *et al.*, 2013). The phylum includes a variety species that go from plant pathogens to decomposers of organic matter, but they are also important in undisturbed areas (BALDRIAN *et al.*, 2012) Disking in the CT system would favor hyphae disruption, decreasing fungi population (VANCE et al., 1987), but the subject needs more studies to be clarified, as in southern Brazil Eucaryota were more abundant in the CT, with a possible explanation relying on the higher tolerance of fungi to environmental stresses (SOUZA *et al.*, 2013).

Although Viruses sequences were low (Fig. 5), Caudovirales was higher in agricultural soils (S4 Fig.). The order consists of bacteriophages commonly found in soils, and their role in infecting Archaea and Bacteria may help in population control (WILLIAMSON, RADOSEVICH and WOMMACK, 2005); moreover, (ASHELFORD, DAY and FRY, 2003) showed that this predation is important for the control and growth promotion of bacterial population in soil.

In relation to microbial functions, the two most abundant subsystems, RNA and protein metabolism (transcription, translation, protein folding and degradation) are attributed to constitutive genes (Fig. 9). Miscellaneous (e.g. iron-sulfur cluster assembly and histidine degradation) was the third more abundant subsystem in the NT, in agreement with other studies, where they are usually positioned among the four most abundant subsystems (YU and ZHANG, 2012; UROZ *et al.*, 2013). Classification of sequences as “clustering-based” indicates an unknown function, and in general, this represents the most abundant subsystem in soil metagenomes (DELMONT *et al.*, 2012; UROZ *et al.*, 2013; SOUZA *et al.*, 2015). In our study, these subsystems included genes such as cytochrome biogenesis, proteases, cell-cycling and cell division and were higher in the undisturbed soil, followed by the NT. The carbohydrates subsystems, including central carbohydrate metabolism and fermentation, were more abundant in the NT, what could be related to the soil enrichment with crop residues (BABUJIA *et al.*, 2010; SILVA *et al.*, 2010; SILVA *et al.*, 2014). The increased β-glucosidase activity levels under NT, for instance, are closely associated with the composition of plant residues (LOPES *et al.*, 2013; LOPES *et al.*, 2015).

5. CONCLUSIONS

Our study highlights that the Brazilian Cerrado soils encompass high taxonomic and functional diversity of soil microorganisms; however, both are highly impacted by agriculture. Interestingly, as pointed out by (FIERER *et al.*, 2012), functional diversity was not necessarily associated with the taxonomic diversity, as the least conservation system, the CT treatment, presented increased taxonomic sequences and reduced functional metagenome profiles in comparison to the undisturbed Cerrado. That might indicate a strategy in the CT to try to maintain soil functioning by favoring taxa that are probably not the most efficient for some functions, leading to negative impacts in soil quality with time. In addition, in general agricultural soils changed to be more adapted to degrade accessible carbon and aromatic compounds substrates, as well as to be enriched in microorganisms related to the metabolism of N, P and S, as a response to the addition of fertilizers. We should also mention that native soils were rich in unknown functions, emphasizing the possibility of finding new functions and genes.

The typical vegetation of Cerrado, represented by stress-tolerant plant species, adapted to harsh conditions such as highly-weathered acidic soils, poor in nutrients, high temperatures, natural fire and long dry periods, might let us think that the biome could be less affected by anthropogenic activities. With the help of a metagenomic approach we have obtained new results that confirm previous observations using classical methods (KASCHUK *et al*., 2011), showing that the Cerrado is the most fragile biome in Brazil. Underneath the rustic appearance of the Cerrado vegetation there is a fragile soil microbial community.

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Anexos

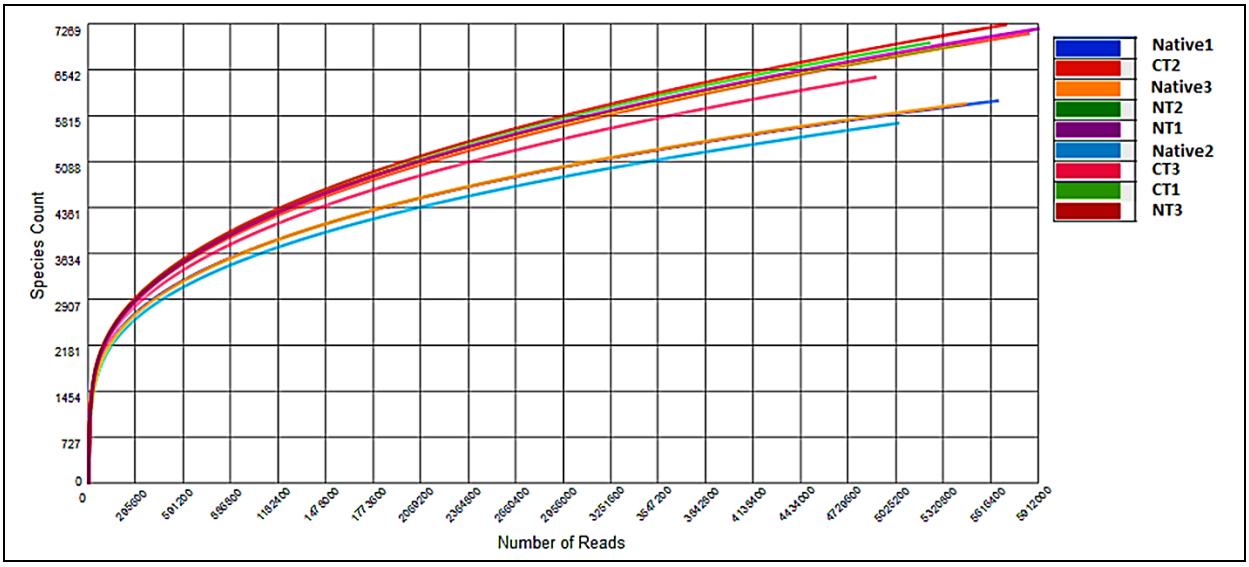

Figure S8 - Rarefaction curves generated with the MG-RAST software against M5NR database using normalized values between 0 and 1 for no-tillage (NT), conventional tillage (CT) and undisturbed Cerrado (Native) soil metagenomes.

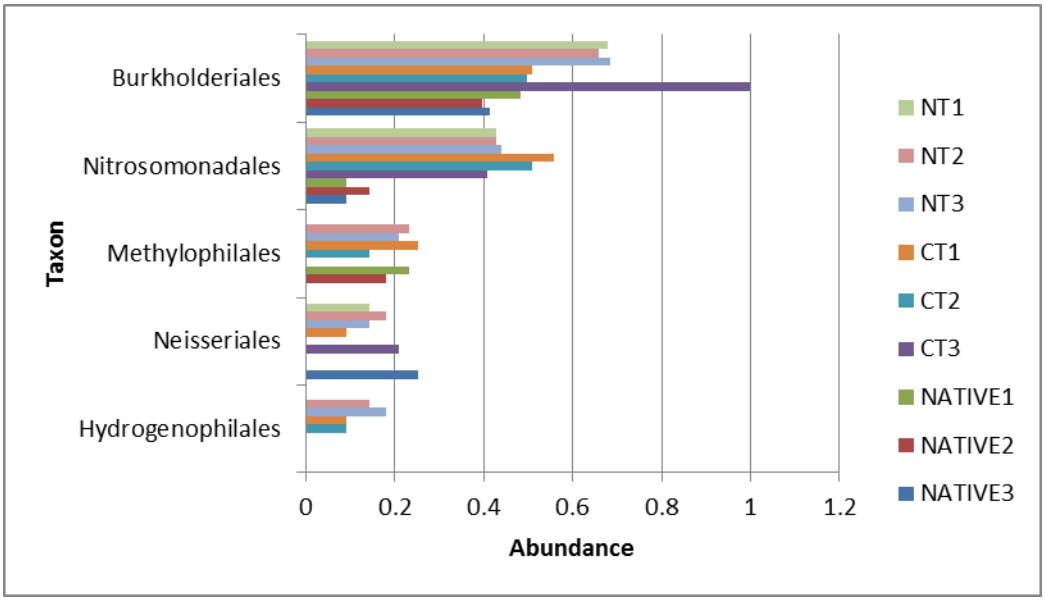

Figure S9 - Sequence abundance orders of Betaproteobacteria compared to M5NR database using normalized values between 0 and 1 for no-tillage (NT), conventional tillage (CT) and undisturbed Cerrado (Native) soil metagenomes.

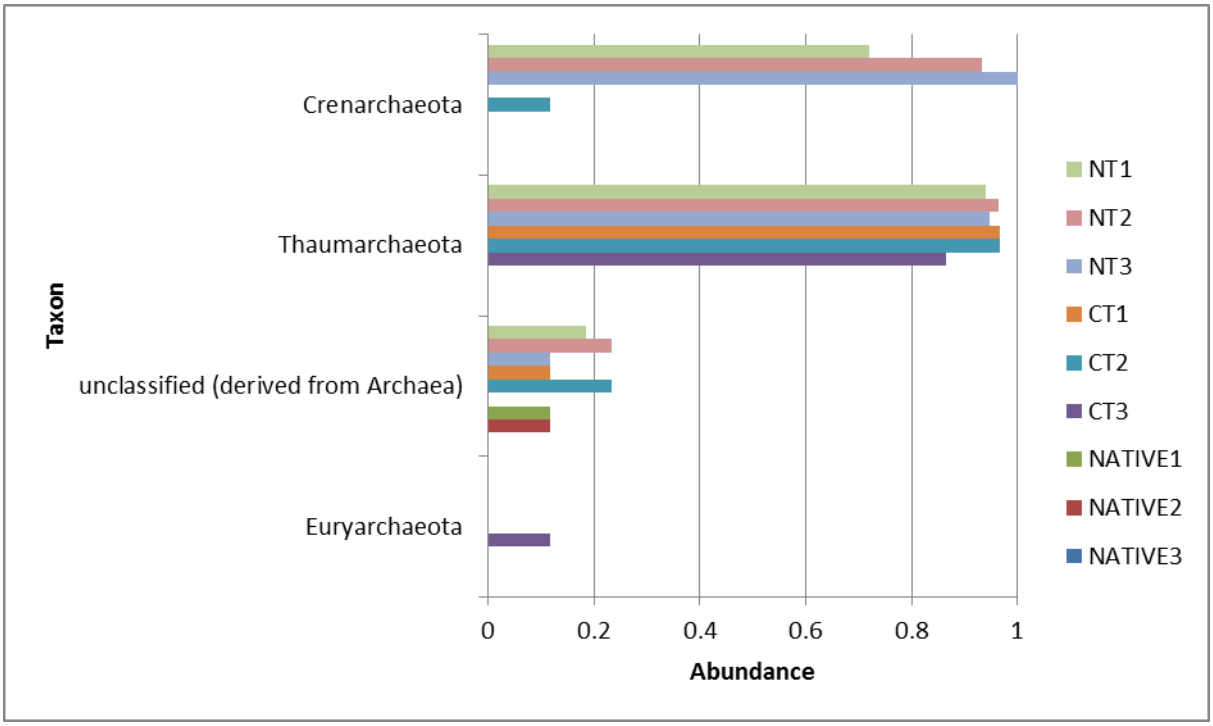

Figure S10 - Sequence abundance of phyla of Archaea Domain compared to M5NR database, and using normalized values between 0 and 1 for no-tillage (NT), conventional tillage (CT) and undisturbed Cerrado (Native) soil metagenomes.

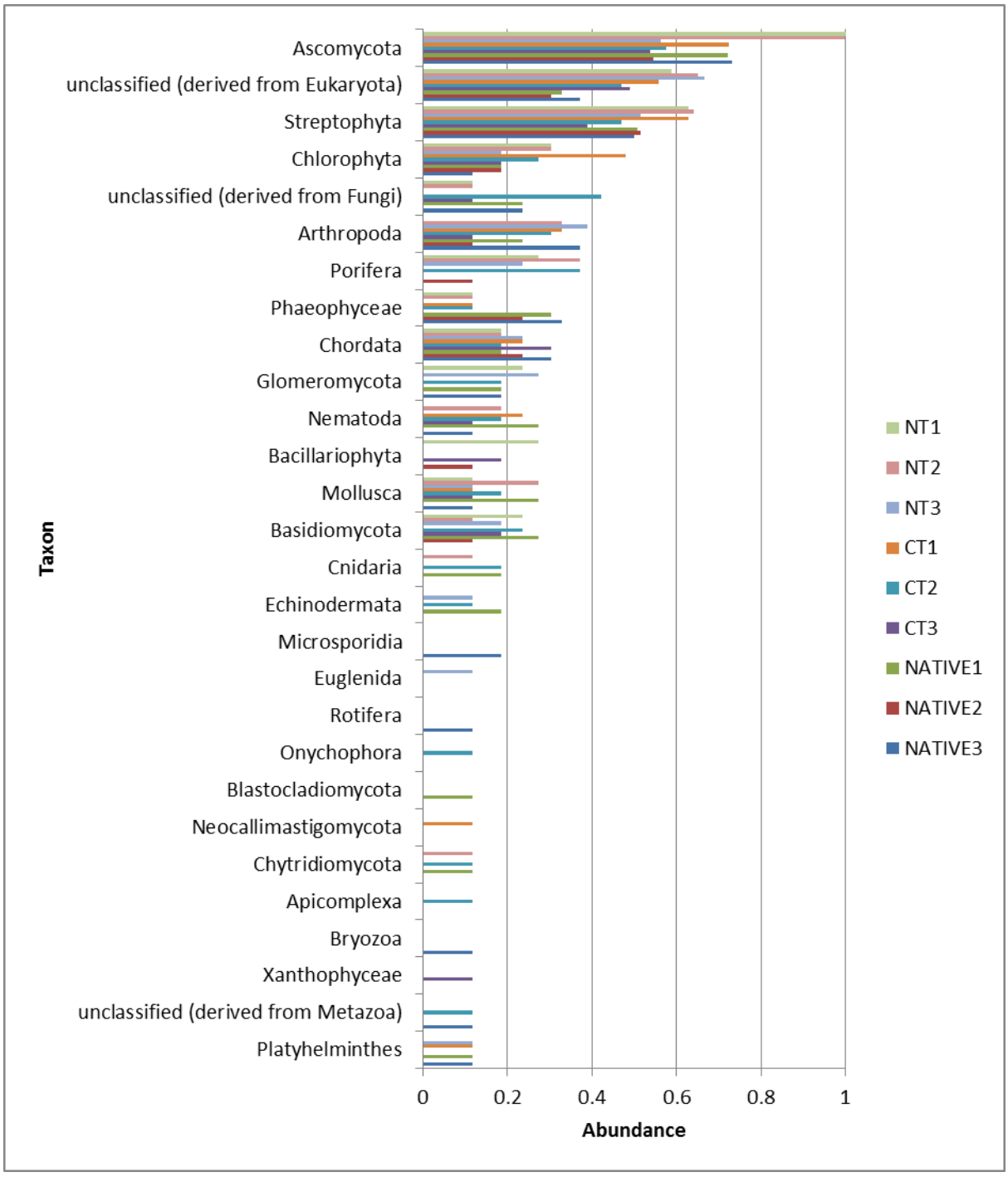

Figure S11 - Sequence abundance of the phyla of Eukaryota Domain compared to M5NR database and using normalized values between 0 and 1 for no-tillage (NT), conventional tillage (CT) and undisturbed Cerrado (Native) soil metagenomes.

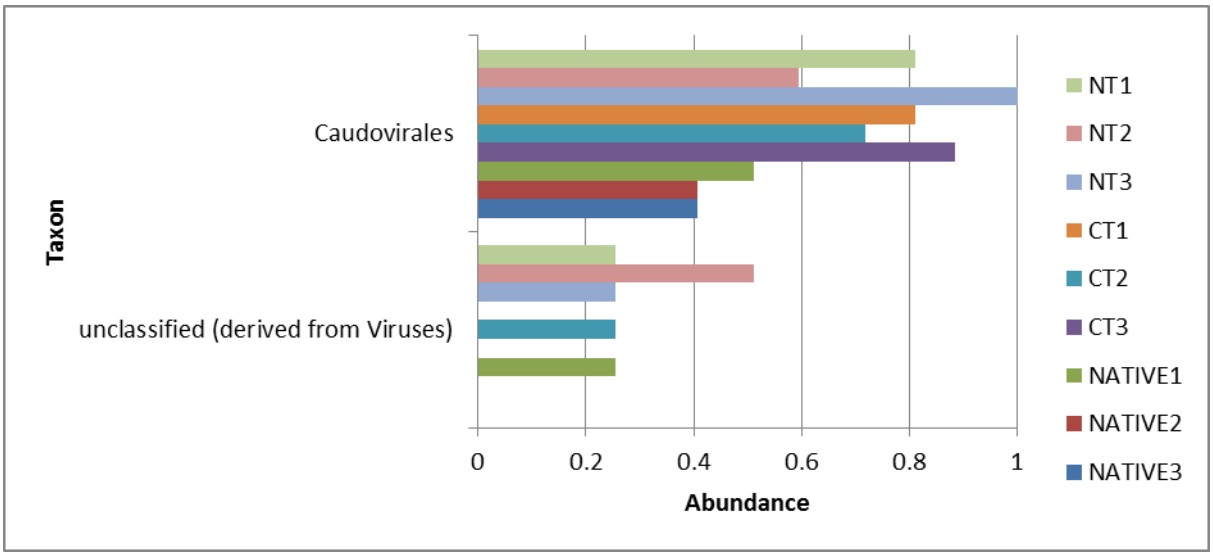

Figure S12 - Sequence abundance in the Viruses domain compared to M5NR database using normalized values between 0 and 1 for no-tillage (NT), conventional tillage (CT) and undisturbed (Native) soil metagenomes.

Table S5 - Main features of the compositing metagenomes based on the MG-RAST annotations. Treatments correspond to soils under native vegetation of Cerrado (Native) or cropped with soybean/corn under no-tillage (NT) or conventional tillage (CT) systems

Features	*Metagenome*								
	NT1	*NT2*	*NT3*	*CT1*	*CT2*	*CT3*	*Native1*	*Native2*	*Native3*
Total number of reads	5,912,295	5,234,816	5,708,651	5,420,302	5,850,205	4,898,256	5,657,434	5,040,252	5,460,208
Total number of base pairs	1,466,364,965	1,372,567,727	1,507,973,051	1,486,805,608	1,686,703,041	1,303,666,221	1,562,052,023	1,221,306,956	1,470,456,607
Mean sequence length	248 ± 58 bp	262 ± 79 bp	264 ± 68 bp	274 ± 78 bp	288 ± 82 bp	266 ± 92 bp	276 ± 78 bp	242 ± 84 bp	269 ± 80 bp
Mean GC contente (%)	63 ± 7%	63 ± 8%	63 ± 8%	63 ± 8%	63 ± 8%	63 ± 8%	60 ± 6%	61 ± 6%	61 ± 7%
Ribosomal RNA	30,702	23,298	27,374	25,119	4,441	3,710	42,110	2,524	2,850
Annotated proteins	2,484,162	2,221,408	2,419,021	2,344,760	2,515,825	1,918,496	2,362,338	1,888,382	2,179,884
*Unknown proteins	3,213,210	2,799,274	3,078,065	2,866,059	3,116,906	2,789,980	3,052,555	2,847,620	3,021,744
*Unknown	184,170	190,773	184,146	184,286	0	79,806	200,417	150,537	83,107
Failed QC*	50	62	44	77	213,033	106,181	13	151,110	172,529

*QC= Quality Control (base-call quality filtering, read-length filtering, and de-replication of reads).

*Know = match against the relevant database was significant.

*Unknown = no significant match was found in the database.

CAPÍTULO 4 – Considerações Finais

A população mundial vem mostrando um aumento progressivo que, consequentemente, demanda maior produção de alimentos. O grande desafio atual é manter essa produção sem degradar o solo, praticando uma agricultura sustentável. Solos de áreas nativas, como do Cerrado brasileiro, vêm sendo incorporados à agricultura, entretanto, são muitos os impactos causados com essa mudança. Um dos principais parâmetros para avaliar a qualidade do solo é a sua comunidade microbiana (indicador microbiológico), consequentemente, identificar alterações nos indivíduos dessa comunidade representa um grande indicativo para monitorar a “saúde” do solo.

Os solos apresentam uma grande biodiversidade de microrganismos, com enorme riqueza e abundância de espécies. A comunidade microbiana presente no solo, porém, pode ser alterada devido a mudanças nos ecossistemas. Assim, algumas espécies podem prevalecer e predominar à nova condição, já que apresentam maior especificidade com capacidade de utilizar as novas e mais complexas fontes de carbono. Ao contrário, outras espécies, sem tal capacidade, diminuem em abundância e podem até ser eliminadas, mas em alguns casos, ainda podem esporular e permanecer no ambiente até obter condições favoráveis novamente.

Neste estudo, foi constatado que mesmo os microrganismos possuindo grande capacidade de resiliência no solo, intensos manejos em solos agrícolas dificultam esse processo resultando em que algumas espécies tornem-se menos abundantes em relação a outras. Contudo, foi verificado que o impacto é de menor magnitude nos genes que codificam funções metabólicas, indicando redundância funcional microbiana, na qual diversas espécies desempenham funções semelhantes, visando à sobrevivência frente a alterações no solo. Dessa forma, este estudo mostrou que a diversidade funcional foi menos afetada com os impactos dos manejos do solo e das culturas do que a diversidade taxonômica. Contudo, quando comparado com uma área nativa do Cerrado, os resultados foram conclusivos em indicar a grande fragilidade dos ecossistemas frente a incorporação da agricultura mudanças para práticas agrícolas, com importantes alterações na riqueza e abundância taxonômica e funcional dos microrganismos.