UNIVERSIDADE FEDERAL DO PARANÁ

DANIEL VASCONCELOS RISSI

Análise metagenômica de comunidades microbianas de solos do Paraná baseada no gene 16S rRNA

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ANÁLISE METAGENÔMICA DE COMUNIDADES MICROBIANAS DE SOLOS DO PARANÁ BASEADA NO GENE 16S rRNA

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Orientador: Prof. Dr. Leonardo Magalhães Cruz
Coorientador: Prof. Dr. Luciano Fernandes Huergo

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Pós-Graduação em Bioinformática WWW.BIOINFO.UFPR.BR
E-mail: bioinfo@ufpr.br **Tel: 41 33614906**

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em BIOINFORMÁTICA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **DANIEL VASCONCELOS RISSI** intitulada: **Análise metagenômica de comunidades microbianas de solos do Paraná baseada no gene 16S rRNA**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua **APROVAÇÃO** no rito de defesa. A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 05 de fevereiro de 2018.

Leonardo Magalhães Cruz
Presidente
Programa de Pós-graduação em Bioinformática – UFPR

Dr. Helisson Faoro
Avaliador Interno
Programa de Pós-graduação em Bioinformática – UFPR
Instituto Carlos Chagas-FIOCRUZ/PR

Dr. Rafael Mazer Etto
Avaliador Externo
Universidade Estadual de Ponta Grossa - UEPG

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"Não importa o quanto você bate, mas sim o quanto aguenta apanhar e continuar. O quanto pode suportar e seguir em frente. É assim que se ganha."

Rocky Balboa – Personagem Fictício

RESUMO

Metagenômica é o estudo das comunidades microbiológicas encontradas em um ambiente, combinando técnicas de extração de material genético, genômica e bioinformática. Através deste estudo, podemos acessar um microbioma que não pode ser encontrado e analisado normalmente através de técnicas tradicionais de cultivo, devido à impossibilidade de cultivar muitos microrganismos em condições de laboratório. O acesso mais amplo à biodiversidade que esta tecnologia pode fornecer, nos permite identificar novos microrganismos, sua quantidade e seu papel no ecossistema, através da classificação taxonômica e predição funcional. O solo é considerado um dos ecossistemas biológicos mais complexos. Eles são responsáveis pelos ciclos biogeoquímicos mais fundamentais na Terra, mantendo relações vitais entre si e com organismos superiores. Os seus papéis principais estão relacionados com a decomposição da matéria orgânica, fornecimento de nutrientes para as plantas e degradação de substâncias químicas. A inoculação de microrganismos que podem realizar uma relação simbiótica benéfica com plantas, pode ajudar no crescimento vegetal e aumentar a produção agrícola. Vários microrganismos já foram identificados como bactérias promotoras de crescimento vegetal, como por exemplo o *Azopirillum brasilense*, uma bactéria fixadora de nitrogênio, que é usada como bio-fertilizante na agricultura em vários compostos comerciais. Este microrganismo não é aplicado apenas para promover o crescimento das plantas, mas também para moldar a região da rizosfera com outros microrganismos benéficos, que também podem atuar diretamente como bactérias promotoras de crescimento vegetal ou indiretamente na solubilização de minerais, controle biológico patógenos ou contribuindo para a estrutura e agregação de solo.

Palavras-chave: Bioinformática, Fixação Biológica de Nitrogênio, Bactérias Promotoras de Crescimento Vegetal

ABSTRACT

Metagenomics is the study of the microbiological communities recovered from a environment by combining techniques of genetic material extraction, genomics and bioinformatics. Through this study we are able to access a microbiome that can't be found normally with traditional techniques of cultivation and analysis due to the impossibility to cultivate many microorganisms in laboratory conditions. The broader access to biodiversity that this technology can provides allows us to identify new microorganisms, their abundance and their roles in the ecosystems through taxonomic classification and functional predictions. The soil is considered one of the most complex biological ecosystems. They are responsible to the most fundamentals biogeochemical cycles in earth, maintaining vital relationships between each other and with higher organisms. Their main roles are related with the decomposition of the organic matter, nutrients privation to the plants and the degradation of chemical substances.

The inoculation of microorganisms that can performed a beneficial symbiotic relationship with plants, can help in the plant growth and increase the agricultural production. Several microorganisms were already identified as plant growth promoting bacterias such as *Azopirillum brasilense*, a nitrogen-fixation bacteria, that has been used as bio-fertilizer in agriculture in several commercial compounds. This microorganism is not only applied to promote plant growth, but also to shape the rhizosphere region with other beneficial microorganisms that can also acts directly as plant promoting bacterias or indirectly in the solubilization of minerals, biological control of plant pathogens or contributing to the structure and aggregation of soil.

Keywords: Bioinformatics, Biological Nitrogen Fixation, Plant Growth Promoting Bacterias

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1. INTRODUÇÃO

A metagenômica expandiu amplamente nosso conhecimento de microbiologia (SANTOS et al., 2017). A tecnologia é baseada no isolamento direto do DNA genômico de amostras ambientais e se tornou uma poderosa ferramenta para explorar a diversidade genética e metabólica de ecossistemas complexos (BIDDLE et al., 2008). O fato de não mais ser necessário o cultivo de microrganismos para estudo de amostras biológicas, revolucionou as pesquisas em microbiologia abrindo uma nova gama de possibilidades a partir dessa ciência (MADHAVAN et al., 2017).

Estes avanços no conhecimento microbiano, se deve ao fato de a tecnologia utilizar técnicas de sequenciamento de DNA, que agora são capazes de uma grande quantidade de dados sequenciados de DNA (WATSON, 2014). A alta profundidade de sequenciamento oferecida por tais avanços significa que até mesmo os microrganismos menos abundantes em um ambiente são possíveis de serem representados (ROUMPEKA et al., 2017).

Tais tecnologias de sequenciamento aliadas com desenvolvimento em bioinformática, fizeram analises metagenômicas acessíveis em muitos laboratórios (ROUMPEKA et al., 2017) e permitiram uma análise mais ampla e ágil para a identificação da diversidade microbiana de diversos ambientes e proporcionar acesso à informação genética da biota independentemente de ser cultivável ou não (RONDON et al., 2000).

Dentre os ambientes com maior diversidade, o solo é o lar de 25% de todas as espécies vivas na terra (TURBÉ et al., 2010) e contêm uma grande diversidade genética, principalmente derivados de microrganismos, mas também fauna e flora (TORSVIK et al., 1990; TORSVIK & OVREAS, 2002; VEREECKEN et al., 2016). Diversas vias metabólicas codificadas por microrganismos são fundamentais em diversos ciclos biogeoquímicos e são a base para a sustentação de um ecossistema (DANIEL 2004; FERRER et al., 2009; CHAN et al., 2013; VEREECKEN et al., 2016).

Ambientes distintos, podem diferir consideravelmente nas suas características abióticas, abundâncias microbianas, taxas de atividade microbiana e composição da comunidade microbiana (FIERER 2017). Até em um determinado perfil do solo, as condições ambientais podem variar consideravelmente nos diferentes ambientes microbianos nele encontrados no solo, incluindo a rizosfera (FIERER 2017).

Em escala global, as condições ambientais do solo são altamente variáveis pois estão sujeitas à diversas alterações climáticas. Décadas de pesquisa mostraram

que as propriedades dos solos superficiais - incluindo pH, concentração de carbono, salinidade, textura e concentração de nitrogênio disponível - apresentam uma enorme variedade. Esta variação é um produto dos principais fatores que afetam a formação do solo: nomeadamente, clima, organismos (incluindo microrganismos e microrganismos) (FIERER 2017).

O primeiro estudo está relacionado a solos do ambiente de Mangue e a Sambaqui. O trabalho buscou diferenciar as comunidades microbianas em ambos os solos e prever os ciclos metabólicos envolvidos em cada ambiente, já que as características físicas e químicas dos ambientes estudados podem influenciar diretamente a diversidade do ecossistema.

O segundo estudo está relacionado a melhor compreensão do impacto causado na biodiversidade microbiana da rizosfera após inoculação de *Azospirillum brasilense,* uma bactéria promotora de crescimento vegetal, em sementes de milho anteriormente ao plantio. O estudo busca identificar se a adição desta bactéria pode reduzir o uso de aditivos nitrogenados ao solo, reduzindo os custos de produção e ainda aumentar a produtividade, avaliando-se também as alterações causadas na microbiologia da rizosfera para microrganismos promotores de crescimento vegetal.

O terceiro estudo apresentado também está relacionado a inoculação de *Azospirillum brasilense* em sementes de milho previamente ao plantio, buscando-se estudar as alterações no solo e na rizosfera. Em adição, também foi realizado adubação verde, proveniente dos resíduos do milho. Essa ação pode alterar a microbiota por fornecer matéria orgânica adicional ao solo e auxiliar no desenvolvimento de microrganismos benéficos a planta podendo vir a substituir a adição de fertilizantes.

2. FUNDAMENTAÇAO TEÓRICA

2.1. Ecossistema do Solo

A maioria dos ecossistemas na Terra são dependentes ou substancialmente influenciadas por interações e processos que ocorrem dentro e entre os solos do planeta (EISENHAUER, et al., 2017), que provem serviços essenciais do ecossistema e foram o fundamento da vida terrestre (JACKSON, et al 2017).

A interação entre solo e matéria orgânica é fundamental para aumentar a produção de alimentos e fibras e proteger espécies e ecossistemas (JACKSON, et al 2017), gerando e mantendo a biodiversidade, como a especiação, a extinção e a interação das espécies (MITTELBACH, et al., 2007)

Um dos grandes responsáveis pela manutenção de um ecossistema são as complexas interações entre microrganismos que coexistem em complexas matrizes (FUHRMAN, 2009; HALLAM & MCCUTCHEON 2015). Portanto, identificar e definir as interações que ocorrem entre os microrganismos do solo é fundamental para a compreensão da diversidade e função microbiana (HALLAM & MCCUTCHEON 2015; REN et al., 2015).

Os microrganismos dos solos associados com a fauna, em interações próximas com plantas, suportam ciclos de nutrientes eficientes, resistência a patógenos e saúde geral da cultura (BENDER, WAGG & van der HEIJDEN 2016). Desde então, as plantas vêm co-evoluindo beneficamente com determinados grupos de microrganismos (VRIES et al., 2017), principalmente com as denominadas bactérias promotoras de crescimento vegetal através da interação entre os exsudados da planta com os microrganismos que se localizam aos arredores da raiz da planta (rizosfera) (BAIS et al., 2006). Esta interação promove uma seleção entre os microrganismos presentes no solo, trazendo os organismos mais benéficos para mais próximo da raiz da planta e afastando outros que podem a vir ser patogênicos (VRIES et al., 2017).

As interações planta-microrganismos são fundamentais componentes no desenvolvimento de um solo saudável e um fator crítico para o desenvolvimento agrícola e produção de alimentos (VIRES et al., 2017) e pode ser amplamente melhorada com a aplicação de fertilizantes (KAISER et al., 2007). Quando associados com a presença de nutrientes, geralmente a produtividade da planta aumenta em

ecossistemas naturais e agrícolas (MARSCHNER & MARSCHNER, 1995) por isso nutrientes como Nitrogênio, Fósforo e Potássio (NPK) vem sendo adicionada ao solo com objetivo de aumentar a produção agrícola (LIN et al., 2012). Entretanto, a adição de fertilizantes pode encarecer a produção agrícola. Como exemplo, a adubação nitrogenada é uma das práticas de gestão mais caras para os produtores (Clemente et al., 2016) e outras tecnologias podem ser aplicadas como adubação de origem animal (Almeida et al., 2016) e vegetal (GAO et al., 2016) e inoculação de microrganismos promotores de crescimento vegetal (BHATTACHARYYA & JHA 2012), dentre outras.

2.2. Mangue

Os mangues são ambientes que possuem um ecossistema de transição entre ambiente terrestre e o marinho (ALONGI 2009). Essa característica torna o mangue uma importante fonte de biodiversidade já que, apresenta uma série de comportamentos ecológicos próprios (LIMA & TOGNELLA, 2012). Alguns mangues se desenvolvem em litorais onde estão sujeitos à uma alta atividade de maré, outros estão localizados em ambientes mais protegidos, como em baías. Entretanto, ambos são frequentemente inundados pela água marinha.

Muitos manguezais são as planícies costeiras, que incluem estuários. Os manguezais são geralmente limitados a uma faixa de elevação estreita dentro do quadro de maré (MCKEE et al., 2012). A distribuição de manguezais em uma linha costeira muda com o tempo, envolvendo saldos sutis entre e subsidência, erosão e estabilização vegetativa, produtividade e decomposição e eficiência de drenagem das marés (FITZGERALD et al., 2008).

As florestas de mangue, contêm alto nível de matéria orgânica viva acima do solo, e grandes estoques subterrâneos de matéria orgânica em decomposição e em forma de minerais al (TWILLEY et al. 1992, DONATO et al. 2011, MCLEOD et al. 2011, LOVELOCK et al. 2014). Atualmente o mangue vem sendo avaliado como o principal ambiente armazenador de carbono (HAMILTON & CASEY, 2016) contribuindo, na redução de gases de efeito estufa, tornando uma opção para mitigar as mudanças climáticas (SIIKAMÄKI et al., 2012; MURDIYARSO et al., 2002), mesmo ocupando apenas cerca de 0,2% da superfície terrestre.

Diversos fatores relacionados por exemplo a concentração salina, atividades aeróbicas e anaeróbicas são importantes no acolhimento de diversas comunidades microbianas em seu ambiente. Essas comunidades desempenham papéis críticos no funcionamento e na manutenção desses sistemas sensíveis e complexos (KATHIRESAN & BINGHAM, 2001; SAHOO & DHAL, 2009) e podem ser interessantes do ponto de vista biotecnológico com a busca por novos genes e enzimas de interesse comercial (Couto et al., 2010). Estes biomas atualmente se encontram em risco devido ao aumento do nível dos oceanos (GILMAN et al. 2007; ELLISON 2014). O que causa prejuízos não so do ponto de vista biológico quanto no equilíbrio de gases atmosféricos.

2.3. Comunidade Microbiana da Rizosfera e Endofíticas

A rizosfera é uma limitada região que cerca o sistema radicular de plantas vasculares e se constitui em um ambiente complexo e dinâmico onde a raiz interage com componentes orgânicos, minerais, solutos, gases, e diversas bactérias que conduzem os ciclos biogeoquímicos dos elementos (OBURGER & SCHMIDT, 2015). As raízes das plantas podem secretar uma variedade de metabolitos primários (ácidos orgânicos, hidratos de carbono e aminoácidos) e metabolitos secundários (alcaloides, terpenos e compostos fenólicos) que de algum modo, afetam a microflora da rizosfera (VENTURI & KEEL, 2016). Sendo, portanto, importante fonte de nutrientes e de chamariz químico para alguns microrganismos, e função repelente para outros, regulando assim a comunidade bacteriana presente na rizosfera (GONZALEZ-PASTOR, 2015).

A associação planta-microrganismo é muitas vezes mutuamente benéfica. Os microrganismos obtêm nutrientes dos exsudatos de plantas, enquanto a planta pode adquirir hormônios, nutrientes, precursores, bem como a proteção contra os agentes patogénicos dos microrganismos. (HIGHLANDER *et al.*, 2015). A microbiota colonizadora da rizosfera (imediatamente em torno da raiz) e o compartimento endofíticos (dentro da raiz), contribuem para o crescimento da planta, produtividade, sequestro de carbono e fitorremediação e é determinada pelas características do sistema e pela interação com as bactérias endofíticas (LUNDBERG *et al.*, 2014).

Muitas das transformações no ciclo de nutrientes comumente ocorrem na rizosfera através da atividade microbiana da sua comunidade. Como por exemplo a da desmineralização do nitrogênio e a (CANBOLAT *et al*., 2006) e a solubilização de

fósforo e carboidratos (KOHLER *et al*., 2007). Quando presentes em terras agricultáveis, possuem um profundo efeito na sustentabilidade biológica da produção. rizobactérias podem estabelecer uma interação positiva com raízes de plantas promovendo um importante papel no ambiente agrícola e na agricultura sustentável. O acesso a comunidade microbiana pode ser realizado utilizando métodos dependentes de cultivo quanto independentes de cultivo (GARBEVA *et al*., 2004)).

Entretanto, estimasse que a população microbiana associada com as raízes varia de 10^8 a 10^9 células por grama de solo da rizosfera (Gonzalez-Pastor, 2015), entretanto, a partir de técnicas dependentes de cultivo, se consegue acessar apenas 1 a 10% dessa comunidade microbiana. Devido a isso, técnicas que utilizam a biologia molecular foram empregadas para maior acessibilidade a essa microbiota (LÓPEZ *et al*., 2015). A técnica conhecida como Metagenômica, se tornou promissora e comumente utilizada para acessar a composição filogenética e o potencial funcional das comunidades microbianas já que, pela técnica consegue-se acessar grande parte da amostra devido ao fato de todo microrganismo possuir ácidos nucleicos, principalmente o DNA (RINCON *et al*., 2013; LI *et al*., 2014).

2.4. Milho

A domesticação do milho começou entre 9000 a 11000 anos atrás a partir do seu progenitor selvagem Teosinto no México (DOEBLEY, 2004). Desde sua domesticação, diferentes variedades surgiram, resultando em diversas variações morfológicas, fisiológicas e genéticas para diferentes usos e condições de crescimento (AGUIRRE-LIGUORI *et al*., 2016). Hoje, o milho possui um importante papel na economia brasileira e mundial - mais de 70 países produzem milho (DAS, *et al*., 2016) - devido a sua posição de destaque entre as espécies exploradas agricuturalmente (MÔRO & FRITSCHE, 2015). Sendo aplicado como principal componente na alimentação de aves, gado e porcos (PURWANTO *et al*., 2015). A produção de milho para a safra 2016/2017 no Brasil foi de 93 mil toneladas de milho (CONAB: Companhia Nacional de Abastecimento, 2017) e a produção mundial está acima de 1 bilhão de toneladas, sendo o Estados Unidos o maior produtor (35% do total da produção mundial), seguido por China, Brasil, México, Indonésia, Índia, França e Argentina (DAS, *et al*., 2016).

A busca por maiores produtividades do milho, tem feito pesquisadores e produtores buscarem cada dia mais inovações tecnológicas que facilitem o incremento da produção. Melhoramento genético, seleção de híbridos, tratamento de sementes com produtos fitossanitários, diferentes espaçamentos e inoculação de microrganismos fixadores de nitrogênio - como o *Azospirillum spp.* - são técnicas usadas visando maiores produções (EMBRAPA, 2004).

A inoculação de plantas com bactérias benéficas para aumentar o crescimento e proteger plantas de doenças, é uma estratégia promissora nos sistemas de plantio modernos (VAN LOON, 2007; VESSEY, 2003). O uso de inoculantes a base de *Azospirillum spp*., proporciona um aumento na produtividade de grãos de milho (HUNGRIA *et al*., 2010). Além disso, pode ser considerada uma alternativa para aumentar a produção de maneira sustentável reduzindo o uso de agroquímicos, que poluem o ambiente (MYRESIOTIS, *et al*., 2015).

2.5. Biodisponibilidade de Nitrogênio

O nitrogênio é um dos mais importantes nutrientes para todos os seres vivos e serve como um importante componente estrutural de proteínas, ácidos nucleicos e celulares. A atmosfera é composta por cerca de 78% de nitrogênio livre, entretanto ele não se encontra disponível para a planta devido a sua incapacidade de metaboliza-lo. Portanto, apesar de ser um nutriente essencial, sua disponibilidade para o desenvolvimento vegetal é limitada. Por isso, o ciclo do nitrogênio é um componente vital nos ciclos biogeoquímicos. Para que o nitrogênio seja metabolizado, é necessário que os microrganismos presentes no solo, transformem o nitrogênio livre do ambiente em outros compostos para que ele possa ser assimilado pela planta (SHARMA, *et al*., 2016). A maioria da fixação de nitrogênio atmosféricoacontece pela ação de diazotróficos como *Azospirilium*, *Herbaspirilium* e *Burlkoderia* dentre outros (SESSITSCH, *et al*., 2012) (Figura 1).

No plantio da maioria das culturas, o nitrogênio é aplicado em diversas formulações químicas, para suprimento do crescimento e desenvolvimento vegetal. Estas aplicações representam aumento de custo significativo para a produção agrícola, seja pela compra do produto a ser aplicado ou pela mão de obra e maquinário para sua aplicação (VERCHOT, *et al*., 2006). Entretanto, o custo de

fertilização nitrogenada é exacerbado porque cerca de 50% do nitrogênio aplicado nas culturas é perdido (VAN NOORDWIJK, 1987).

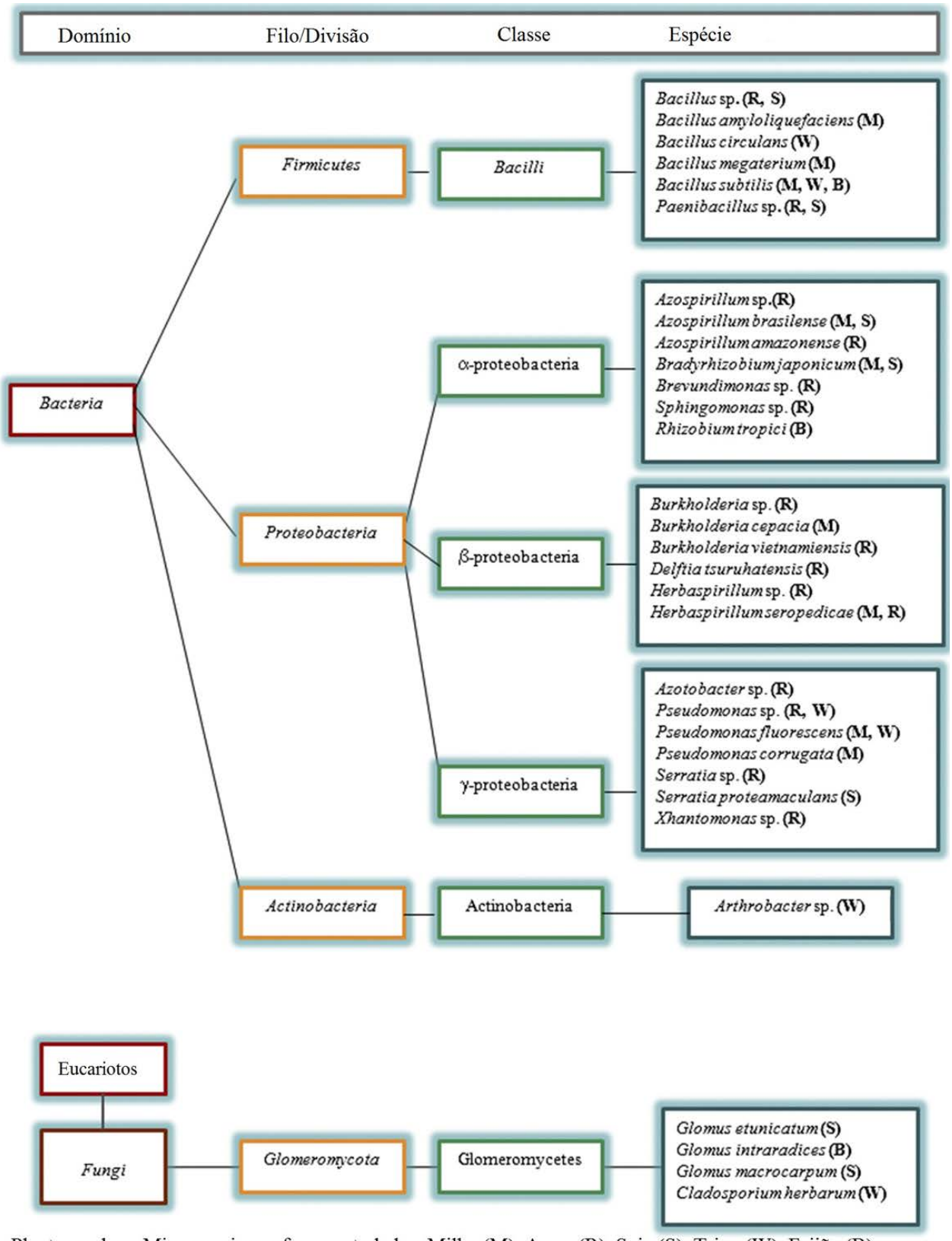
A inoculação de microrganismos vem sendo considerada uma alternativa amigável para reduzir o uso de fertilizantes sintéticos de nitrogênio adicionados sem comprometer o rendimento da lavoura (HUNGRIA *et al*., 2010). Entretanto, no Brasil esta prática ainda é muito precária devido ao tipo de tecnologia de aplicação e a inconsistência de resultados científicos que pode depender de vários fatores bióticos e abióticos (MORAIS, *et al*., 2016). Além disso, o uso de inoculantes microbianos pode ajudar o Brasil a atingir os objetivos em reduzir a emissão de gases poluente, assim como manter posição de liderança no *ranking* de países que podem ajudar a prevenir a fome no mundo (HUNGRIA, *et al*., 2010).

Os benefícios no crescimento das plantas, devido à adição de bactérias promotoras de crescimento vegetal, incluem aumentos nas taxas de germinação, crescimento da raiz, e de rendimento - incluindo grãos-, área foliar, teor de clorofila, teor de magnésio, teor de nitrogênio, teor de proteína, atividade hídrica, tolerância à seca, tamanho e pesos das raízes, e senescência (LUCY *et al*., 2004).

A colonização bacteriana na rizosfera e endosfera das raízes do milho, é influenciada pela inoculação de bacterias promotoras de crescimento vegetal, o que pode influenciar fortemente a biodisponibilidade de nitrogênio para a planta (KUAN, et al., 2016). No milho, os principais microrganismos encontrados na rizosfera, são transmitidos através da semente (JOHNSTON-MONJE, et al., 2016).

As bactérias do gênero *Azospirillum,* veem sendo consideradas uma das mais importantes rizobactérias para crescimento da planta, pela produção dos hormônios vegetais promotoras de crescimento como, ácido indol acético, ácido abscísico e giberelina (BASHAN *et al*., 2004; PERRIG *et al*., 2007) e por realizarem atividade simbiótica com a planta, promovendo a disponibilidade de nitrogênio para os cultivares (BASHAN & BASHAN, 2005). A produção desse hormônio vegetal pelas espécies do gênero, alteram o metabolismo e a morfologia de raízes de plantas, resultando em uma melhor absorção de minerais e água e produzindo raízes maiores e mais saudáveis (BASHAN & DE BAHSAN, 2010). Além, de promover o rendimento da cultura, como foi observado no estudo de Hungria (2010), utilizando cepas de *Azospirillum brasilense*, onde houve um aumento na produção de grãos de milho de até 30%, em relação aos tratamentos não inoculados.

Figura 1: Microrganismos promotores de crescimento de planta mais frequentemente usados em estudos



Plantas onde os Microrganismos foram estudados: Milho (M), Arroz (R), Soja (S), Trigo (W), Feijão (B)

FONTE: Pérez-Montano, et al., 2014 (modificado)

2.6. Bactérias do Gênero *Azospirillum.*

O gênero *Azospirillum* possui atualmente cerca de 12 espécies (LAVRINENKO *et al*. 2010) e veem atraindo a atenção de pesquisadores ao redor do mundo devido seu alto potencial agro-biotecnológico e fitoestimulante (WISNIEWSKI-DYÉ et al., 2013; VERESOGLOU & MENEXES, 2008). Os estudos Iniciais dessas bactérias foram realizados em cereais e posteriormente em outros tipos de plantas, tornando-se claro que, muitas cepas de *Azospirillum*, poderiam colonizar rizosferas em geral, aumentar o crescimento e influenciar o metabolismo de muitas espécies de plantas, incluindo plantas anuais, perenes, arbóreas, ornamentais, selvagens e até mesmo células únicas de microalgas (PEREG *et al*., 2016) devido a suas inúmeras propriedades que permitem que ele possa prosperar em um meio rico em nutrientes como a da rizosfera de plantas (STEENHOUDT & VANDERLEYDEN, 2000).

Azospirillum induz o crescimento vegetal e controle de doenças através inúmeros mecanismos, como o aumento da biomassa radicular, fixação de nitrogênio, solubilização do fosfato, síntese de fito hormônios, sideróforos, enzimas, vitaminas, e a possibilidade de que mais de um mecanismo esteja envolvido ao mesmo tempo (BASHAN & DE-BASHAN 2010; BASHAN *et al*., 2014)

A espécie *Azospirillum brasilense*, veem atraindo um interesse particular dos pesquisadores, por possuir cepas epífitas (colonizadoras da superfície de raízes) e endofíticas facultativas (capazes de penetrar dentro do tecido das raízes da planta) (ROTHBALLER *et al.*, 2003), possuindo diferentes comportamentos adaptativos (KOVÁCS *et al*., 2016) e sendo capaz de alterar a arquitetura das raízes da planta, aumentando a formação lateral e adventícios e também pelos radiculares (BASHAN *et al*., 2014 ; BASHAN & DE-BASHAN, 2015) promovendo um aumento da superfícies de absorção das raízes e consequentemente aumentando o volume de substrato do solo explorado, podendo assim, aumentar também a absorção de outros nutrientes e água (OKON & VANDERLEYDEN, 1997). Sua associação com plantas superiores, auxilia no crescimento e desenvolvimento vegetal, através de diferentes mecanismos, como fito-hormônios e fixação biológica de nitrogênio atmosférico (WISNIEWSKI-DYÉ *et al*., 2013; BASHAN & DE-BASHAN, 2010) promovendo aumento de produção em importantes cultivares como milho, trigo e arroz sob várias condições de clima e solo (OKON & VANDERLEYDEN, 1997) e induzir resistência a doenças em plantas

por ativação de rotas metabólicas (VLEESSCHAUWER *et al*., 2006; TORTORA *et al*., 2012).

2.7. Metagenômica

A metagenômica garante acesso a uma grande diversidade microbiana não é cultivável (COUGHLAN *et al*., 2015), permitindo uma melhor caracterização da microbiologia em um determinado ambiente. Também provem informações das atividades metabólicas e papel funcional dos microrganismos presentes em determinada população (LANGILLE *et al.*, 2013) fornecendo uma valiosa ferramenta para a descoberta de novos genes, vias metabólicas e de extrema importância biotecnológica (CULLIGAN *et al*., 2014)).

Análises metagenômicas são mais frequentemente realizadas por sequenciamento do16S do RNA ribossomal (rRNA) ou por sequenciamento total (*shotgun*), normalmente em uma plataforma de sequenciamento de próxima geração.

O gene 16S rRNA compreende-se na subunidade menor de um ribossomo bacteriano. O estudo metagenômico que utiliza do gene 16S rRNA como marcador filogenético, se beneficia do fato desse gene ser extremamente conservado mas, que possui regiões em seu gene hipervariadas que são espécie-específicas (TIKHONOV et al., 2015). Portanto, a partir da análise dessa região hipervariada, é possível se identificar a nível taxonômico as espécies relativas a cada sequência, e por consequência, acessar a diversidade biológica de um ambiente de interesse. A metodologia padrão desse tipo de análise busca formar *clusters* com as sequencias de 16S rRNA semelhantes, esses clusters são denominados de “Unidade taxonômica operacional” (OTU’s). A partir desses OTU’s, apenas uma sequência é selecionada como representante desse grupo, e será definido a qual organismo ele pertence (TIKHONOV et al., 2015).

O estudo conhecido como *shotgun* ou metagenômica de DNA total, busca estudar todo o material genético encontrado na amostra. Esse material é sequenciado em pequenos fragmentos que, após disso são juntados em fragmentos maiores utilizando sobreposição de sequencias em uma técnica denominada *assembly* (WANG, 2014). Após isso, esses fragmentos maiores, são classificados taxonomicamente (*binning*) baseados em bancos de dados de referências ou através da metodologia *de novo* (CARR, 2013*)*. Este estudo permite uma abordagem mais ampla, onde se pode ser realizado estudos relacionados a genes de interesse e enzimas, proteínas

e demais produtos de origem transcricional. Essa abordagem é se tornam mais fácil para estes estudos, pois já possuímos acesso direto aos genes apresentados no ecossistema estudado (SHARPTON, 2014).

Estas tecnologias exploram comunidades que independem de métodos microbiológicos tradicionais de cultivo e têm melhorado nossa compreensão das ricas comunidades microbianas que estão presentes em determinados habitats (PREIDIS & HOTEZ, 2015) e também têm sido aplicadas na busca de genes e produtos gênicos com interesses biotecnológicos, que possam ser utilizados e incorporados em processos industriais, agrícolas ou medicinais, visando melhorias e redução de custos nesses processos (SCHMEISSER *et al*. 2007).

Analises de comunidades metagenômicas requerem o uso de ferramentas de bioinformática para processar de maneira eficiente e reprodutível a grande quantidade de dados gerados pelo sequenciamento, e assim obter uma visão geral da taxonomia (PLUMMER *et al*., 2015).

3. ARTIGOS CIENTÍFICOS

Esta seção apresenta os artigos científicos desenvolvidos ao longo do mestrado e que foram ou serão ainda submetidos a revistas científicas.

O primeiro artigo apresentado nesta dissertação, busca avaliar as diferenças na microbiologia de solo de um Manguezal localizado na costa paranaense. Tal estudo avaliou solos de mangue com a presença de Sambaqui comparado com o solo sem Sambaqui. Neste estudo identificamos uma microbiologia semelhante entre os solos e identificamos um ecossistema importante em diversos ciclos geoquímicos e de armazenamento de carbono.

No segundo artigo, esta apresentado um estudo que busca entender as mudanças na microbiologia da rizosfera do milho com a adição de fertilizantes de nitrogênio e inoculação de diferentes cepas comerciais de *Azospirillum brasilense* na semente de milho. Neste estudo verificamos que a presente de nitrogênio no solo altera significativamente a microbiota da rizosfera, aumentando principalmente bactérias promotoras de crescimento vegetal. Tais bactérias ainda tiveram sua presença aumentada quando as sementes foram inoculadas com *A. brasilense*.

3.1 INFLUENCE OF ANCIENT ANTHROPOGENIC ACTIVITIES ON THE MANGROVE SOIL FROM MATA ATLÂNTICA (BRAZIL) BIOME

Luciano F. Huergo[1]*, Daniel V. Rissi[2], Andressa S. Elias[1], Maria V. Gonçalves[1], Marco V. Gernet[1], Flávio Barreto[1], Gilson W. Dahmer[1], Rodrigo A. Reis[1], Fábio O. Pedrosa[3], Emanuel M. Souza[3], Rose A. Monteiro[3], Valter A. Baura[3], Eduardo Balsanelli[3] and Leonardo M. Cruz[3].

1- Setor Litoral, UFPR, Matinhos, PR, Brazil
2- Programa de pós-graduação em Bioinformática, SEPTI, UFPR, Curitiba, PR, Brazil
3- Departamento de Bioquímica e Biologia Molecular, UFPR, Curitiba, PR, Brazil.

*Correspondence to: Luciano F. Huergo, Tel: +55 41 33611570, E-mail: huergo@ufpr.br Tel. +55 41 996765856. Fax. +55 41 33161570.

Running title: Shell midden and mangrove microbiome

Key words: biodiversity, mangrove, 16S sequencing

Summary

Mangroves are highly productive ecosystems located at the transition between the terrestrial and marine environments usually found in estuarine regions in tropical climate. Mangroves play an important role in carbon storage, nutrient cycling and support for the marine food web. Mangrove soils are formed by fine particles rich in organic carbon and are subject to constant fluctuations in oxygen, salinity and nutrients availability due to fresh water flux and tidal variations. Microbes play an important role in nutrient cycling in mangrove soils; however, studies on mangrove soil microbiome are scarce. Here we describe the microbiome profile of pristine mangrove soil located on an environmental protected area in Guaratuba, south of Brazil, and compared with mangrove soil affect by the presence of carbonaceaous debris eroding from an archeological site known as Sambaqui. We show that the while the Sambaqui site has a major effect on the mangrove soil active acidity, increasing the soil pH in 2.6 units, just minor changes in the soil microbiome profile were observed. The high alpha diversity indexes and predicted metabolic potential suggest that the mangrove soil microbiome not only provides important ecological services but also may host a broad range of microbes and genes of biotechnological interest.

1. Introduction

The Atlantic forest biome hosts a significant portion of the Brazilian biological diversity and is considered as a “hot spot” of biodiversity by UNESCO. This biome is one of the most threatened on the planet with less than 8% of the original forest remaining (CARNAVAL, *et al.*, 2009). The major remnants of continuous Atlantic forest are located on the cost of the Paraná state due to the presence of several Public and Private Conservation Units within this region (TIEPOLO, 2015).

A particular important ecosystem within the Atlantic forest is the mangrove, waterlogged areas located at the transition between the terrestrial and marine environments in estuarine regions (ALONGI, 2014). Due to its typical characteristics such as salinity, oxygen and pH variations there are just few vegetal species adapted to survive those conditions (ALONGI, 2014). Mangrove ecosystems are important environments

for the protection and reproduction of many animal species, including arthropods, fish, amphibians, reptiles, birds and mammals (Hossain and Nurudin, 2016).

Mangroves occupy 0.5% of the global coastal area, approximately 138,000 km^2, Brazilian mangroves represents 7% of the global mangrove area (GIRI et al. 2011; ALONGI, 2014). Mangroves are highly productive ecosystems, providing support to marine and terrestrial food web and contributing to 10-15% to coastal sediment carbon storage and export of 10-11% of the particulate terrestrial carbon to the ocean (Alongi, 2014). It has been estimated that the carbon biomass in mangrove soil is about three times the biomass that makes up the mangrove vegetation (Hossain & Nurudin, 2016). The mangrove ecosystem is claimed to play an important role in climate regulation, contributing to carbon sequestration and helping counterbalance anthropogenic CO_2 emissions (ALONGI, 2014), but are disappearing at an alarming rate and loss of this ecosystem will contribute to climate change (ATWOOD, et al., 2017). Anthropogenic activities such as city expansion, ports, contamination by oil splits, domestic sewage, agriculture and others are the main threat to this ecosystem (ALONGI, 2014).

Mangrove sediments in Brazil are typically formed by river and marine alluvium deposits, with its texture mainly composed by silt and clay, in the fine fractions, combined with high concentrations of organic matter and salts, with a dark gray color commonly defined as mud (HOSSAIN & NURUDIN, 2016). The water tidal cover along with fine texture and organic matter observed in mangrove sediments produce a gradient from aerobic conditions (in the surface), where degradation of organic matter occur mainly through aerobic respiration, to anaerobic process, creating conditions for anaerobic decomposition including sulfate reduction that produces H_2S giving the mangrove soil a typical strong odor (GHIZELINI et al., 2012; HOSSAIN & NURUDIN, 2016).

Given the unique physicochemical characteristics of mangrove soil such as oxygen, salinity and pH variations, this ecosystem is very diverse in microbial life forms (MENDES $ TSAI, 2017) and hence it is a promising repository of microbes and genes of biotechnological interest (THATOI et al., 2013). There are just few studies describing the composition, ecology and dynamics of the mangrove soil microbiome in Brazil (GHIZELINI et al., 2012).

Shell middens are archeological sites found in coastal zones all over the globe. These sites are spread all over the Brazilian coast line and are known as Sambaquis (OKUMURA & EGGERS, 2005; GERNET *et al*., 2014). Sambaquis consist mostly of mollusk shells that are of waste products of meals eaten by groups of hunter-fishers-gatherer indigenous people how lived in the area between 2,000 – 10,000 years ago (SCHEEL-YBERT, 2001). These sites can reach up to several meters high and sites of up to 300,000 m^3 have been described (PARMALEE, et al., 1974; SIMPSON et al., 1996; ESTÉVEZ et al., 2001).

The structure of Sambaqui are typically arranged in layers with varying composition that are likely to reflect the occupation of these sites by different groups (Fig. 1), the bottom layer represents the debris of the most ancient groups. It is common to find stone artifacts, bonfire and human remains inside the Sambaqui suggesting that these sites were general waste deposits. Some Sambaqui sites have been characterized at the social-cultural anthropological perspective (PARMALEE et al., 1974; SIMPSON et al., 1996). However, there is no report on pedology or microbiome analysis of Sambaqui soils.

Several Sambaqui sites have been described in the cost of Paraná state in Brazil (BIGARELA, 2011), in this study we focused on Sambaqui built over a mangrove area in the Boguaçú River which is part of the Guaratuba bay estuary (Fig. 1 and 2). We analyzed how the presence of this Sambaqui site influences the physicochemical and microbial composition of the nearby mangrove soil.

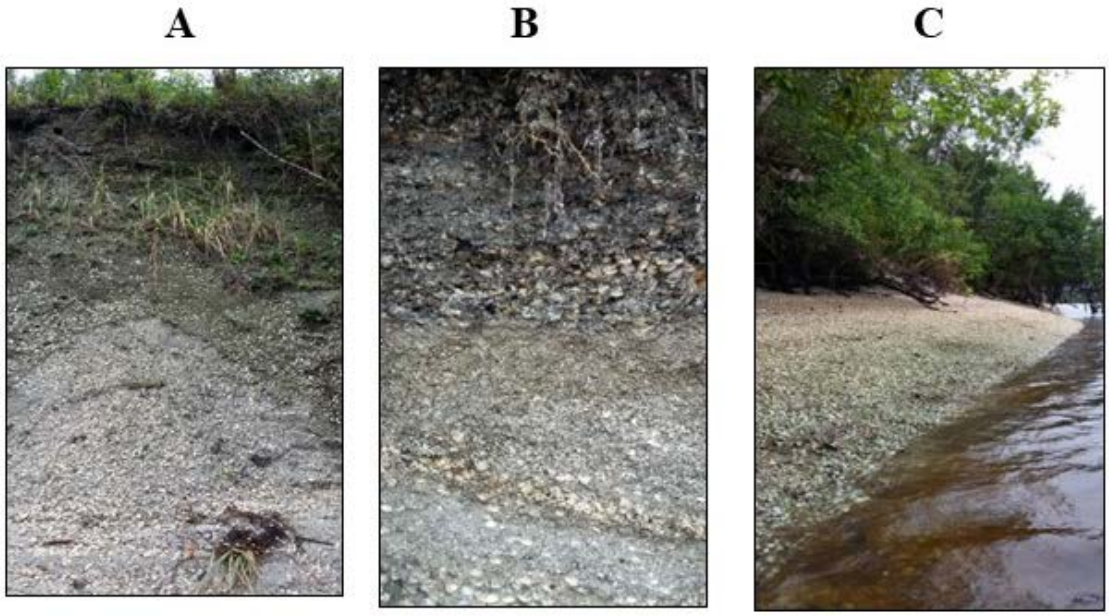
2. Material and Methods

2.1 Sampling and experimental design

The Sambaqui Boguaçu is located at the margin of Boguaçú river in a place called Ilha do Casqueiro, Guaratuba Bay estuary, Paraná, Brazil (25 ° 55'11 "S / 48 ° 37'39" W). This Sambaqui is 7 m height and are likely to date between 10,000 and 2,000 year from present (BIGARELA, 2011). Two sampling areas were defined; one consisting of pristine mangrove soil located 30 m from the Sambaqui site (Figure 1). The other area is where the mangrove soil is covered and naturally mixed with the

shells that are eroding from the Sambaqui Mountain (Figure 1). Three samples were collected from each site for statistical comparison. In order to reduce local variations, each of these three samples was made up by mixing three sub-samples 2 m distant from each other in the same tide line.

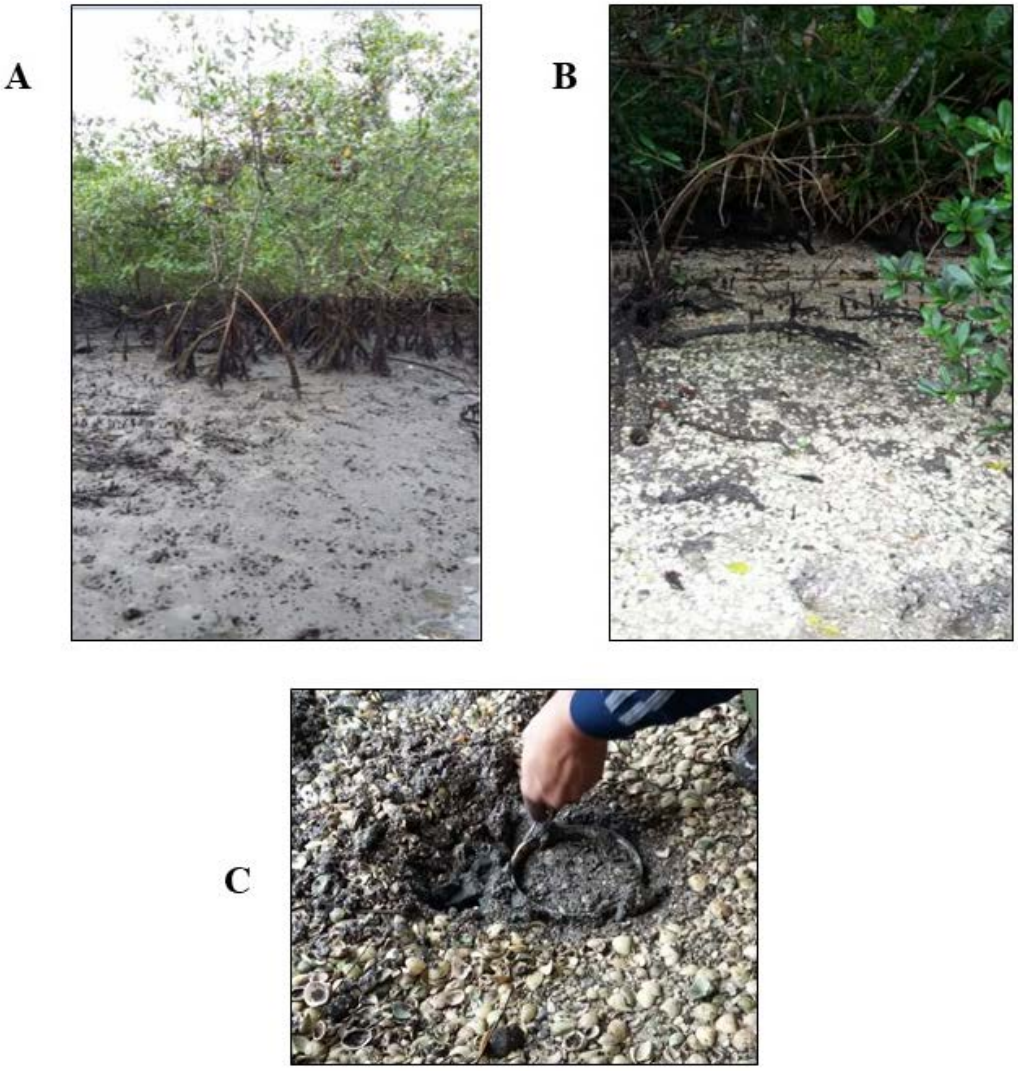
Figure 1: The Sambaqui Boguaçú – Guaratuba, PR, Brazil.



(A) View of the Sambaqui from the left margin of the Boguaçú river. The top is 7 m high from the river level. (B) Different layers of shell debris that represent the chronological history of the site. (C) View from the base of the Sambaqui at the left margin of the Boguaçú river.

Samples were collected on low tide on 25/10/2016. PVC pipes with 10 cm diameter and 15 cm in length were used to collect blocks of soil (Figure 2), three blocks of sub-samples were collected in a plastic bag and homogenized to make up each sample. Loose shells were manually removed from the top of the soil before collection. Samples had their total DNA extracted and were diluted for bacteria cultivation two hours after collection at the Setor Litoral, Universidade Federal do Paraná, Matinhos, Paraná.

Figure 2: The area of sample collection



(A) The natural mangrove. (B) The mangrove area where the Sambaqui shells are mixed with the mangrove soil by natural processes. (C) Sampling of the Sambaqui affected area, loose shells on the top were removed before soil extraction.

2.2. Heterotrophic bacteria counting

A 1g sample of each replicate of the pristine and Sambaqui affected soils were diluted with 9 ml of sterile saline (10^{-1} dilution), 100 µl of serial dilutions 10^{-2}, 10^{-3} and 10^{-4} were spread on LB agar containing cyclohexamide 50 $\mu g.ml^{-1}$ to inhibit the growth of fungi. Plates were incubated aerobically at 30ºC for 48 hours for Colony Forming Units (CFU) counting.

2.3. Soil analysis

The physicochemical characterization of the soil was performed at the Laboratory of Soil Chemistry and Fertility, Universidade Federal do Paraná, Curitiba, Brazil. All the routine analysis was performed according to Manual de Métodos de Análise de Solo – EMBRAPA (Donagema *et al*., 2011). pH was determined in the presence of $CaCl_2$ 10 mM as described (Abreu Jr et al., 2003).

2.3. Total DNA extraction and 16S Ilumina sequencing

Total DNA extraction from the soil was performed using the MOBIO Laboratories, Inc. "Power Soil DNA Kit", DNA quality was determined by agarose gel electrophoresis and quantity was determined by Qubit HS dsDNA kit (Thermo). PCR amplification, barcoding and Ilumina sequencing was performed as described (Caporaso *et al.*, 2012). Briefly, the V4 region of the 16S gene was PCR amplified in 10 µl reactions using: 20 ng of template DNA; 1 µM primer 515F; 1 µM primer 806R (which includes the barcode for each sample); and 5 µl KlenTaq DV ReadyMix (Sigma-Aldrich). The PCR cycles were 94ºC 3 min, 18x (94ºC 45 s, 50ºC 30 s, 68ºC 1 min), 72ºC 10 min. Three PCR technical replicates were performed for each sample. Amplicons were quantified by qPCR with Kapa NGS quantification kit (Roche) and quality assessed using a 2100 Bioanalyzer (Agilent), and equimolar amounts of the technical replicates were mixed. Two technical replicate sequencing reactions were performed for each sample using a MiSeq platform and MiSeq 500v2 Reagent Kit (Illumina) pair-end reads (2x of 250 pb) at Department of Biochemistry, Universidade Federal do Paraná, Curitiba, Brazil.

2.4. DNA sequence data processing and analysis

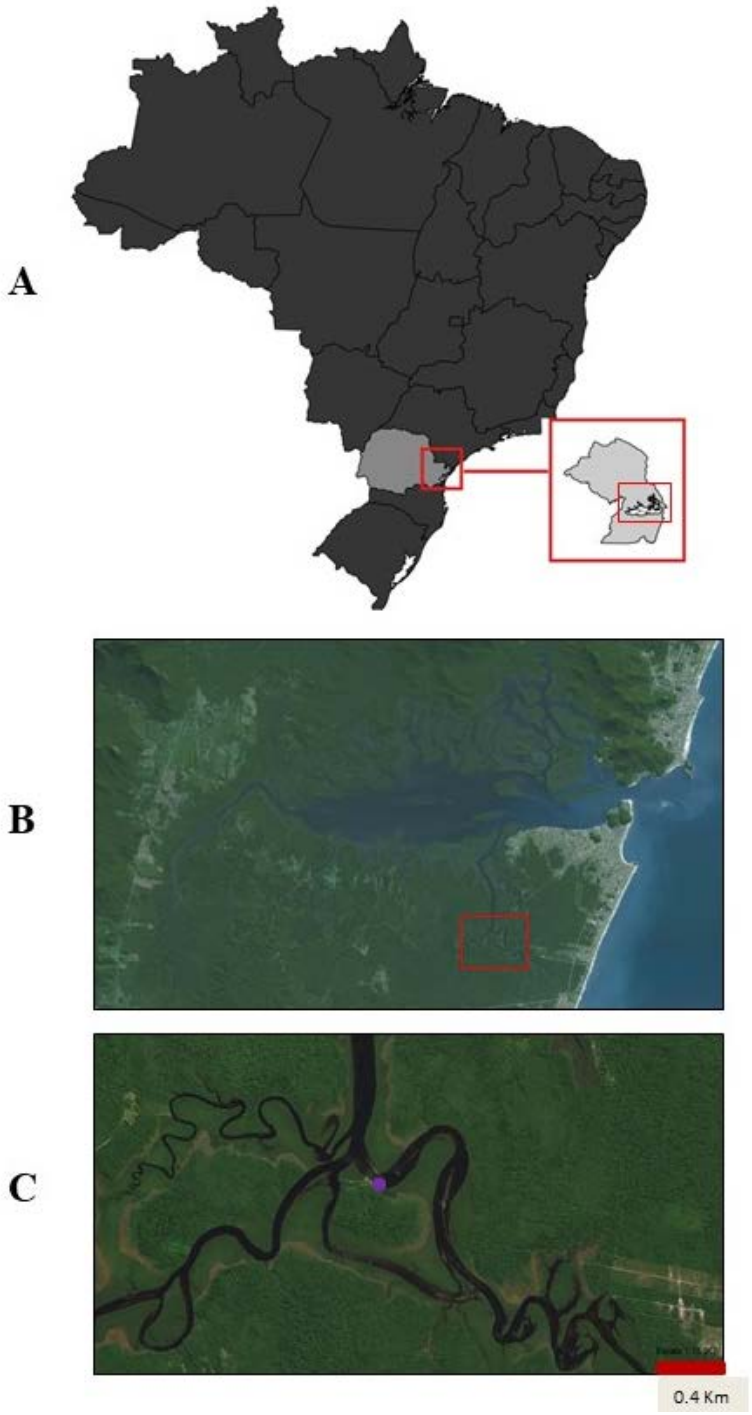
The sequences were quality analyzed using FASTQC tool. The forward sequences of the two technical DNA sequencing replicates were joined and processed using QUIIME v1.8.0 (KUCZYNSKI *et al.*, 2012). The reads were clustered into Operating Taxonomic Units (OTUs) and taxonomy assigned using the 16S SILVA v128 (QUAST *et al.*, 2013). Mitochondrial and chloroplast sequences were removed. Statistical analyses were performed using samples normalized by the number of reads.

Quantitative and statistical analyses were performed using MetaCoMET (WANG *et al.*, 2016), STAMP (PARKS & BEIKO, 2010) and R (R Development Core Team) using the Vegan pack. Functional prediction based on 16S sequences was performed using Tax4Fun (ASSHAUER *et al.*, 2015), Parallel META-3 (JING *et al.*, 2017) and KEEG (KANEHISA & GOTO, 2000)

3. Results

In this study we focused on Sambaqui present over a mangrove area in the Boguaçú River which is part of the Guaratuba bay estuary (Figure. 3), an environmental protected area. The Sambaqui Boguaçú is 7 m high and the Boguaçú river flow, tidal variations and wind erosion spreads the Sambaqui shells all over the nearby mangrove area (Fig. 1). We hypothesized that the shell deposition over the mangrove soil would create a unique alkaline environment due to carbonate release from the shells and this would affect the soil microbial community. Hence, we have set a study to compare the physicochemical and microbial composition between pristine and Sambaqui affected mangrove soil on the Boguaçú river, Guaratuba, Paraná, Brazil.

Figure 3: The site of study



A) Map of Brazil showing the Paraná state in Grey. Guaratuba is shown in the insert and the estuary of Guaratuba Bay is indicated in a red mark. B) Satellite image of the Guaratuba Bay the study area is marked in red square and amplified in the image C) with the precise location of the collection site indicated with a purple dot (the red bar indicates the scale = 0.4 Km). Maps were generated using Qgis http://qgis.org/en/site/about/index.html. Satellite images were acquired from http://www.paranainterativo.pr.gov.br/.

Samples from the Sambaqui affected soil and nearby pristine mangrove soil (from here on simply named S and M, respectively) were collected (Figure 2) and

compared physicochemical analysis and microbial composition. In order to determine the number of heretrophic bacteria, soil samples were serial diluted and plated on LB agar. After 48 hours at 30ºC, 3.2 10^4 CFU/g and 7.6 10^3 CFU/g, were counted for M and S samples, respectively. This suggests a lower number of heterotrophic bacteria in the Sambaqui area. The CFU counts are in agreement with lower amounts of total DNA extracted /mg of sample in the Sambaqui area (Figure. 3) and reduced levels of carbon detected in the soil (Table 1). These data suggest that the Sambaqui soil is less dense in microbial life forms; this may be caused either by its unique chemical composition and/or due to the presence of visible high mass of inorganic shell pieces.

Table 1 – Physicochemical analysis of soil

Sample	*Sand	Silt	Clay	*pH	Al^{3+}	Ca^{2+}	Mg^{2+}	*K^{+}	*P	*C
M1	725	163	113	4.7	0.3	5.1	4.3	0.75	43	52
M2	713	125	163	3.6	2.8	5.6	4.9	0.77	60	34
M3	725	113	163	3.6	1.9	5.8	4.8	0.72	86	39
S1	788	88	125	6.5	0.0	5.3	4.1	0.69	119	19
S2	788	113	100	6.6	0.0	5.6	4.0	0.55	125	19
S3	788	100	113	6.6	0.0	7.0	4.3	0.56	119	22

*Significant differences M vs S ($p<0.05$). Sand, Silt and Clay are given in $g.kg^{-1}$. pH was determined in the presence of $CaCl_2$ 10 mM. Al^{3+}, Ca^{2+}, Mg^{2+} and K^{+} units are $cmol/dm^3$. P and C are given in mg/dm^3 and g/dm^3, respectively.

3.1. Soil physicochemical properties

The physicochemical properties of the pristine and Sambaqui affected mangrove soil were determined. The silt and clay contents were similar for both M and S types of soils. The sand fraction was significant higher in S samples (Table 1) and this is probably caused by the shell pieces that accumulate in the sand fraction of these samples (Fig. 1). The pH values in the Sambaqui samples were significant higher (up to 2.6 units in average) than those values measured in the natural mangrove area (Table 1). This difference in the pH confirmed our initial hypothesis that the carbonate

release from the shell material would neutralize H^+ ions rendering the Sambaqui soil more alkaline.

The Ca^{2+} levels were not significant different between the M and S samples, thought higher trends were observed in the S samples (Table 1). The detected Ca^{2+} levels in both M and S are high in comparison to most soil types in Brazil (Abreu Jr *et al.,* 2003). We hypothesize that the Ca^{2+}released from the decomposing shell material may became available to bind to the clay particles not only in the S soil but also in to the nearby M soil, and this could explain the similarities and high levels of Ca^{2+} detected in both S and M samples

Lower amounts of K^+ and higher P were detected in the Sambaqui soil. Given that this type of soil exhibited lower carbon (Table 1), CFU counts (see above) and DNA yields than pristine soil, the most likely explanation for the increased levels of these elements would be the presence of ancient anthropogenic waste materials such as small pieces of shells and/or bones.

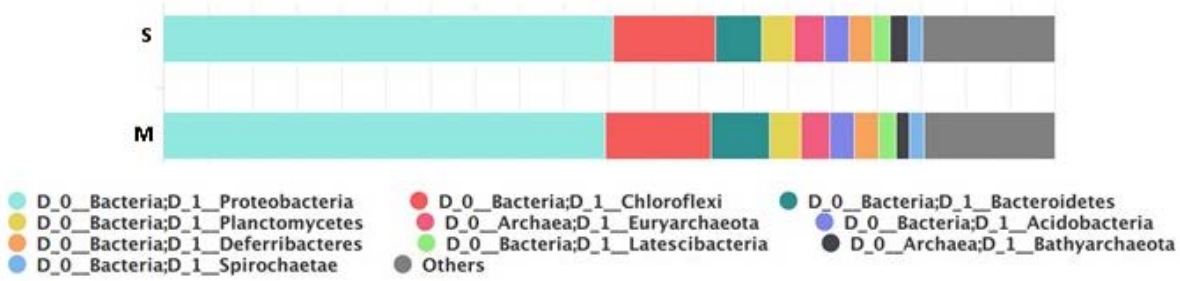
3.2. Prokaryotic biodiversity

The total DNA extracted from triplicate M and S samples were subjected to PCR amplification of V4 region of 16S rRNA gene and next generation Ilumina sequencing using primers and methodology described in (CAPORASO *et al.*, 2012). A total of 73,981 reads of 250 bp in length from 16S rRNA were analyzed. Average reads per sample were 12,330 with counts ranging from 9,222 to 16,077. Rarefaction plots are shown in Figure. S1 (Apendice).

In all subsequent analysis, samples were normalized by the number of reads, leaving a total of 55,332 reads, 9,222 reads per sample, comprising a total of 7,816 OTUs. The most abundant phylum observed were Proteobacteria 49%, Chloroflexi 11.6%, Bacteroidetes 6.4%, Planctomycetes 3.6%, Euryarchaeota 3.3%, Acidobacteria 2.6%, Deferribacteres 2.6%, Latescibacteria 2%, Bathyarcheota 1.5% and Spirochetes 1.5% (Figure 4). Venn diagram indicate that 3,933 OTUs were shared between M and S samples with 1,972 and 1,911 unique OTUs for the M and S samples, respectively (Figure S2 Apendice). Alpha diversity indexes were similar between M and

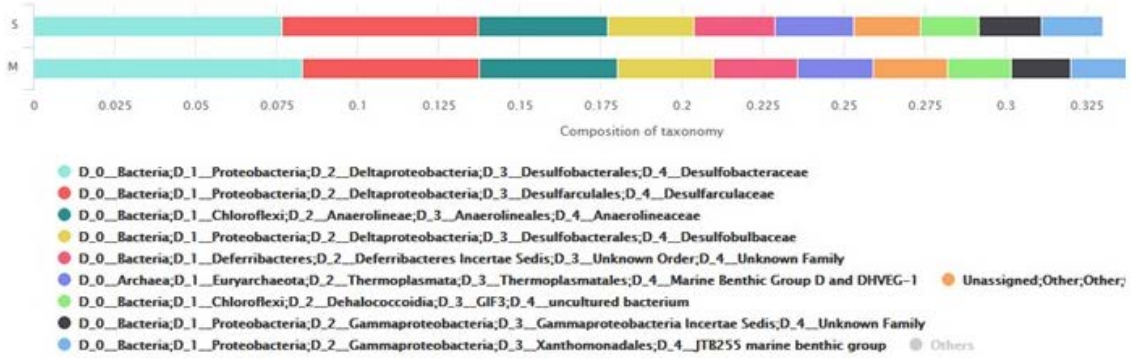
S samples (Table 2). Despite the significant differences in the soil chemical composition, the general microbial community profile was strikingly similar between M and S samples when compared at the phylum level (Fig. 4). Among the 10 most representative families were Desulfobacteraceae, Desulfarculaceae and Desulfobulbaceae, these groups comprise anaerobic sulfate reducing bacteria typically found in marine sediments.

Figure 4: Main Phylums from Sambaqui and Mangrove Samples



In this figure, all subsamples from Mangrove and Sambaqui were joined together in one compose samples that represents each environment. We can notice a high similarity at phylum level between environments, by which made the Sambaqui and Mangrove quite similar in microbiology repository.

Figure 5: Main families from Sambaqui and Mangrove Samples



In this figure, all subsamples from Mangrove and Sambaqui were joined together in one compose samples that represents each environment. We can notice a high similarity at genus level between environments, by which made the Sambaqui and Mangrove quite similar in microbiology repository.

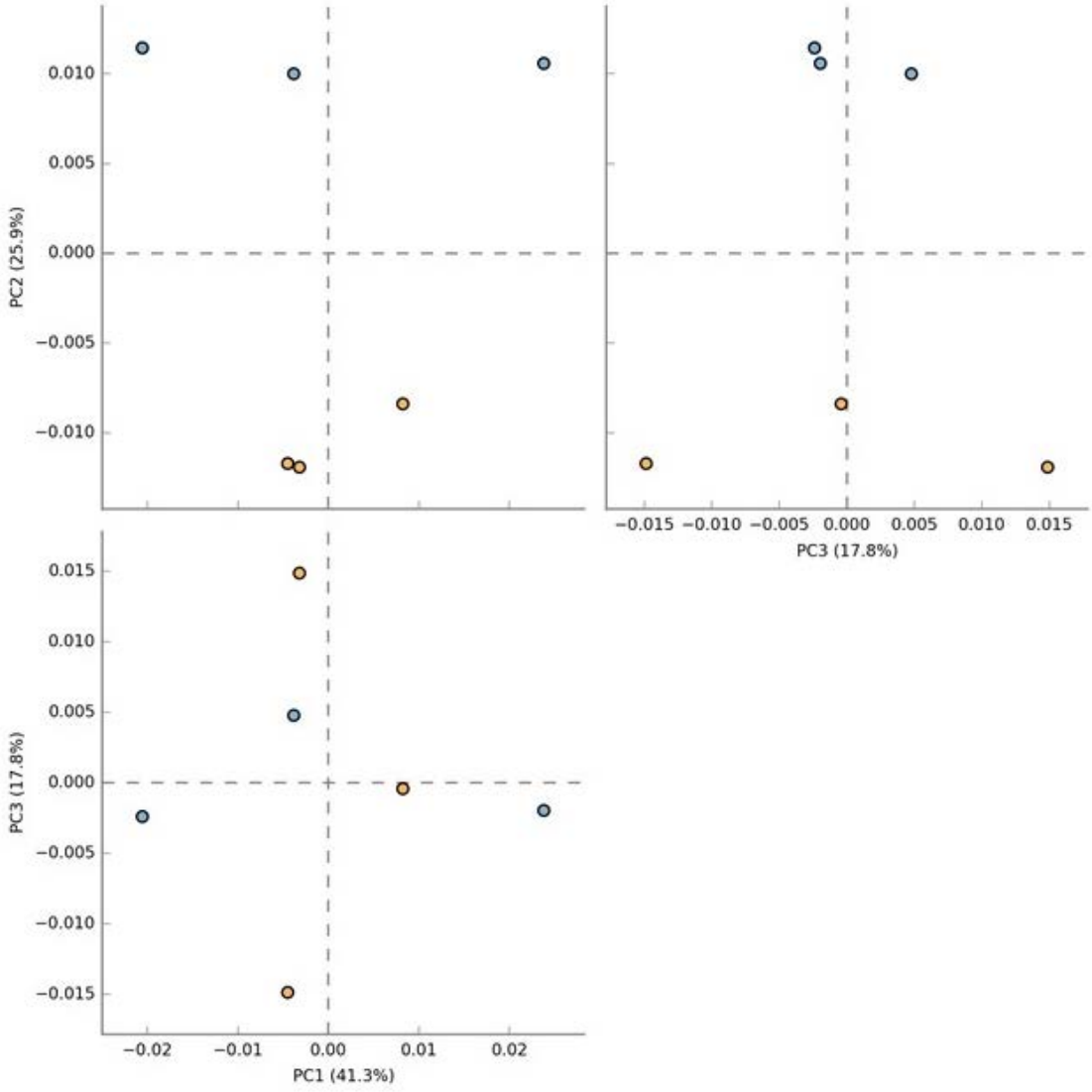
Table 2 – Alpha diversity analysis

Sample	Chao1	Shannon	OTUs	PD Tree
M1	5,743	10.68	3,272	187
M2	5,931	10.66	3,265	187
M3	5,584	10.58	3,139	180
S1	5,350	10.53	3,132	177
S2	5,407	10.65	3,217	183
S3	5,576	10.66	3,251	183

The alpha diversity analysis represented in this table showed a similarity in biological diversity between Sambaqui and Mangrove, using different methods of analysis.

Even though the two environments had similar Alpha diversity indexes, Beta diversity analysis support differences in abundances of OTUs in each type of soil. This was revealed by the clear separation of the M and S samples in Principal Component Analysis plots when analyzed at lower taxonomic level (Figure 6, see PCA plots, PC1 vs PC2 and PC2 vs PC3). Hierarchical clustering heat map also separated the M and S samples in different groups and clear differences in the relative abundance of few OTUs were readily detected (Figure S3 Apendice). The distinction of the microbiome composition between the two environments was also detected using Jackknifed UP-GMA clustering (Figure S4 Apendice).

Figure 6: Principal component analysis of OTUs between M and S samples

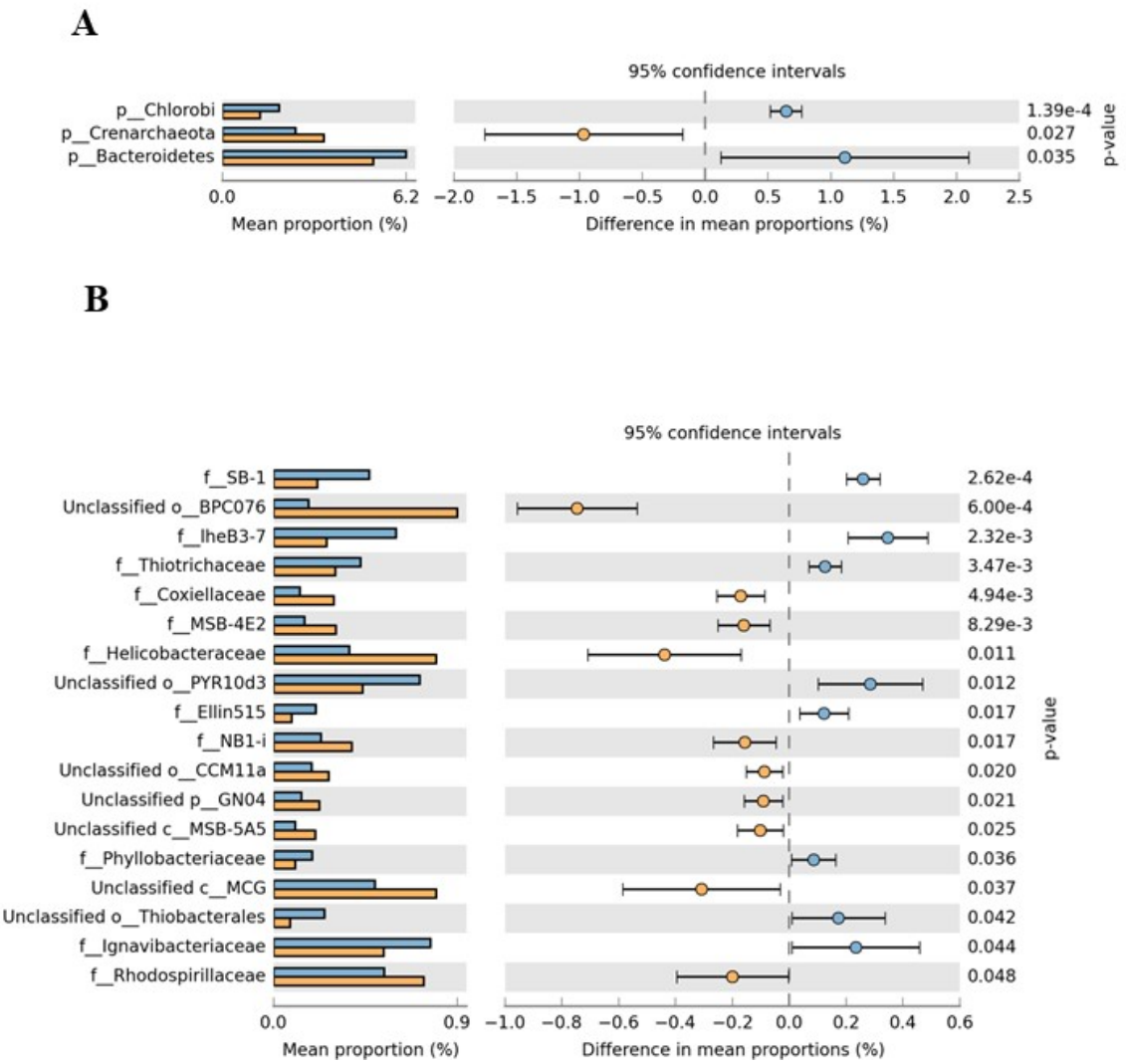


PCA plots were generated in STAMP using welch´s t-test two sided, 0.95. OTUs were analyzed at genus level . M and S samples are indicated using blue and orange dots, respectively and were clustered separated mainly in the PC2 vs PC3 and PC2 vs PC1, that represents a better sample variation in Beta diversity graph.

To identify differences in the abundance of OTUs between the S and M samples statistical analysis were performed using normalized data. OTUs representing less that 0.2% of each sample were removed to reduce bias occurring in less representative groups, results were filtered to $p<0.05$. *Chlorobi* and *Bacterioidetes* were decreased whereas *Crenarchaeota* was increased in the Sambaqui affected soil (Figure 7.A). At family level, *Thiotrichaceae*, *Phyllobacteriaceae* and *Ignavibacteriaceae* were decreased whereas *Coxiellacea*, *Helicobacteriaceae* and *Rhodospirillaceae* were in-

creased in the Sambaqui affected soil (Fig. 7.B). Furthermore, few groups of unclassified bacteria were also significant different when comparing S vs M samples (Fig. 7.B).

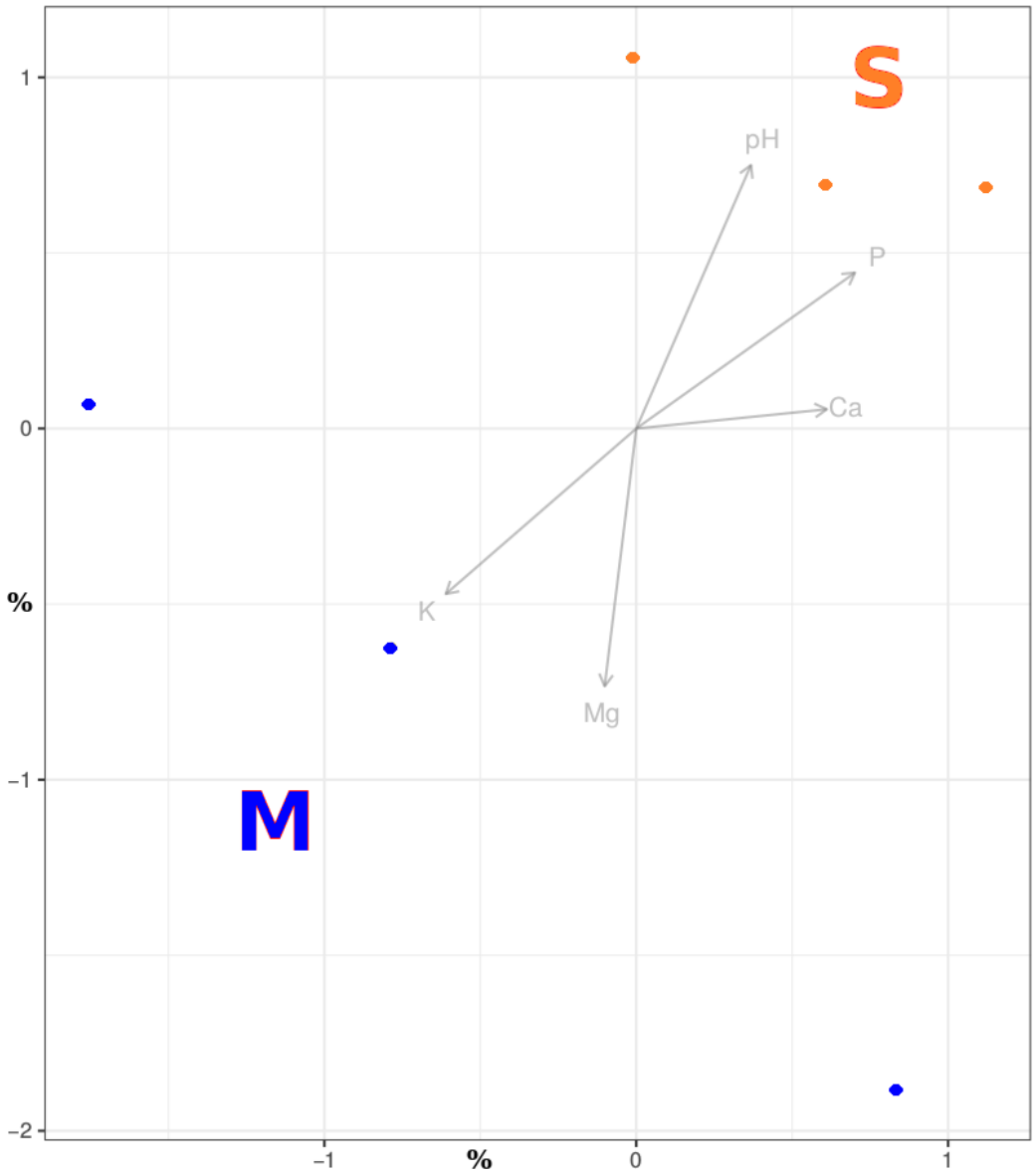
Figure 7: Statistical significant abundance in OTUs from native vs Sambaqui affected soil



Mean proportion bars of significant groups at Phylum (A) and Family (B) levels were generated in STAMP using T-test. Data were filtered for sequences with at least 0.2% representation. M and S samples are indicated using blue and orange dots, respectively.

Canonical correspondence analysis (CCA) were performed using distance matrices and permutation test with Pseudo F-ratios to explore which set of environmental variables most influence the community composition. The CCA plot confirmed the separation between S and M and revealed that pH, Ca, K, Al and Mg are the major factors influencing the M community composition (Figure 8).

Figure 8: Canonical correspondence analysis (CCA plots) of OTUs abundance and significant physic-chemical soil variables



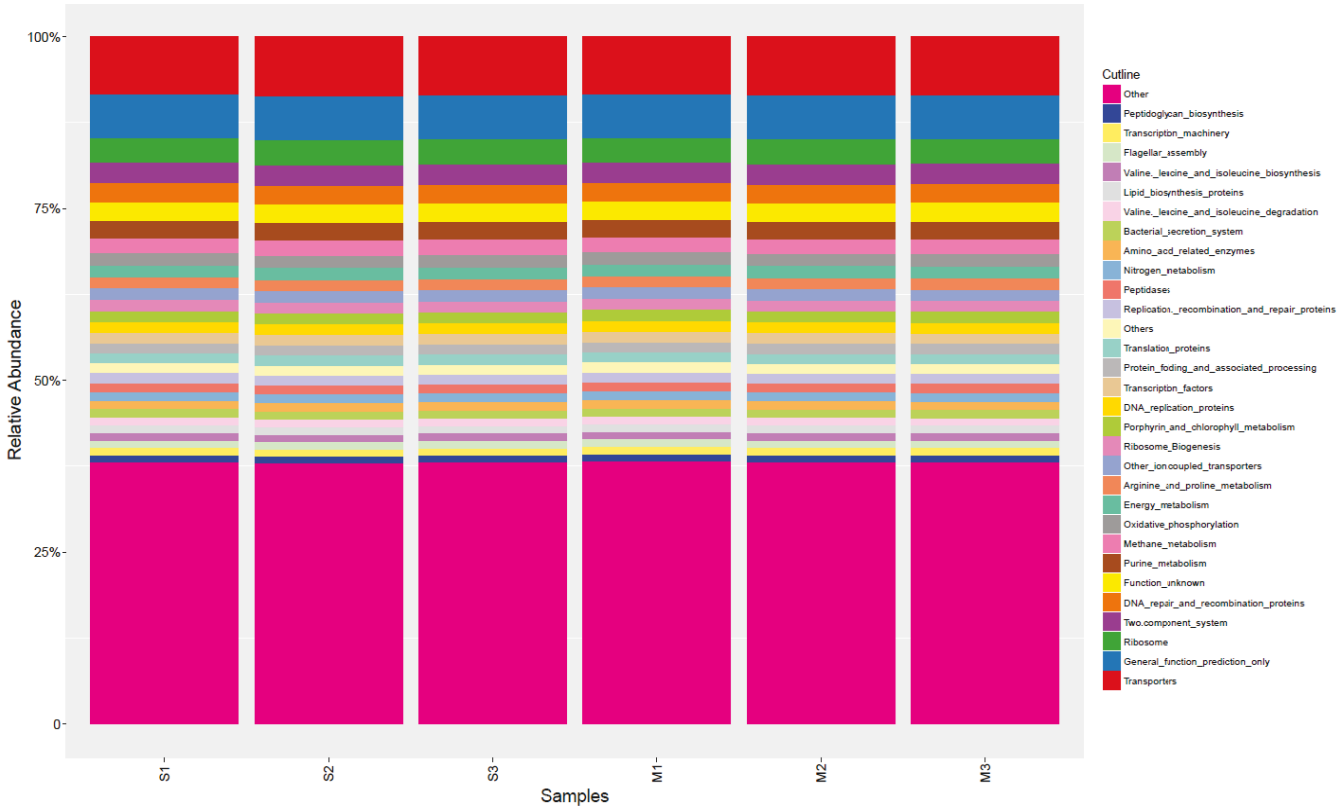
The vegan library was used to create the CCA plots using the microbial biodiversity with the environmental variables that are found to be significant affect the community composition show as vectors. M and S samples are indicated using blue and orange dots, respectively. p-value=0.095

3.3. Functional prediction of the microbial community based on 16S sequences

In order to explore the metabolic potential of the microbial communities, a functional prediction based on the rRNA 16S sequences was performed using

Tax4Fun and Parallel META-3. These computational approaches have a strong correlation with overall shotgun metagenomic sequencing (ASSHAUER *et al.*, 2015; JING *et al.*, 2017). A significant difference between KEGG orthology occurrence between M and S samples was observed (Wilcoxon test p=0.00184), this was expected given the small differences in microbial community composition (Fig. 5). However, the general functional profile was very similar between all samples (Figure 9). Furthermore, from a total of 6,661 KEGGs identified, only 70 had p <0.01 between M and S samples (Table S1). Particularly, 15 KEGGs involved in pathogenesis were more represented in the S samples (Table S1) and this could be explained by the increased counts of potential pathogenic organisms from Helicobacteriaceae and Coxiellaceae families in the S samples (Figure 7.B).

Figure 9: Functional profile prediction of the microbial community based on 16S sequences

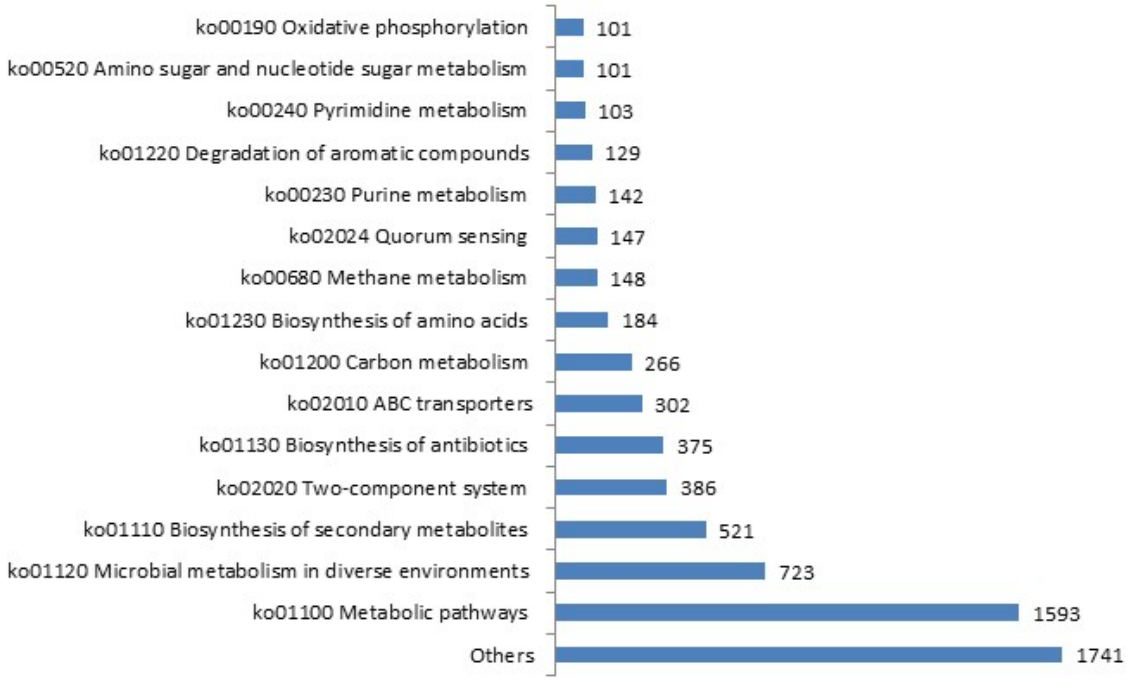


The samples were subjected to functional classification using Parallel META-3.

Most of the KEGG hits were mapped to central metabolic pathways (Figure 10). Several hits for KEGGs involved in the biosynthesis of secondary metabolites and antibiotics were found (Figure 9). The functional prediction identified KEGGs related to carbon, sulphur and nitrogen cycling. Particularly, methane metabolism was well

represented (Figure 9 and Figure 10). All steps of the nitrogen cycle were represented including nitrification, denitrification and nitrogen fixation.

Figure 10: Major predicted KEGG pathways in the soil samples



All the ko entries identified by Tax4fun (using data from M and S samples combined) were submitted to a KEGG pathway search. The graphic shows only pathways with more than 100 hits, the number of hits are indicated

4. Discussion

In this study we focused on an archeological Sambaqui site that is eroding over a mangrove soil in order to determine the effects of ancient anthropogenic activities on soil physicochemical properties and microbiome composition. The comparative analysis between pristine vs Sambaqui affected mangrove soil revealed that the sea shells deposits in the soil resulted in a pH increase of 2.6 units in average (Table 1). Despite this ≈400x difference in H^+ molar concentration, the general microbiome profile of M and S samples were statically similar (Figure 4 and Figure 5). This result was unexpected as several studies indicate that pH has a major influence of microbial communities (FIERER &JACKSON, 2006; PAN, *et al.*, 2014; MENDES &TSAI, 2017; LAUBER, *et al.*, 2009). Nevertheless, small changes in the microbiome composition were observed between M and S samples (Figure 7) and CCA analysis support that pH is an important factor influencing these difference in the microbial community (Figure 8).

The microbiome profile observed in the current study correlates well with previous microbiome studies performed in mangrove soils in other regions of Brazil with Proteobacteria being the most represented Phylum and followed by Deferribacteres, Chloroflexi, Acidobacteria, Bacteroidetes and Planctomycetes (NOGUEIRA et al., 2015; PEIXOTO *et al.*, 2011; DOS SANTOS *et al.*, 2011). Our data indicate that mangrove soil hosts a broad diversity of microbial life forms as suggested by the high number of OTUs and high Shannon diversity indexes (Table 2). Given that mangrove is located in the transition of freshwater, marine and terrestrial environments, the mangrove soil high microbial diversity may be explained by the coexistence of microorganisms from all these three different environments (MENDES & TSAI, 2017). Furthermore, mangroves are very diverse and dynamics environments considering the oxygen level stratification and constant fluctuation in salinity and abundance of nutrients due to fresh water flux and tidal variations. Such diversity and dynamics may contribute to the formation of diverse microbial niches thereby explaining its high microbial diversity.

The microbial diversity correlated with a notable diverse range of metabolic pathways operating in the mangrove soil microbiome, as suggested by *in silico* metabolic reconstruction (Fig. 9 and Figure 10). Genes involved in cycling of major nutrients and in the production of secondary metabolites and antibiotics were vary abundant (Figure 9). As a significant part of the rDNA 16S sequences where assigned to uncultured and/or unclassified microorganisms, mangrove soils are promising sites for the prospection of novel microbes and/or genes of biotechnological interest.

5. Conclusions

We conclude that the archeological Sambaqui site on the Boguaçu River, on the Guaratuba bay estuary (with a estimate data between 2,000 – 10,000 years ago), has a major effect on the nearby mangrove soil pH, however, it has caused minor impacts on the composition nearby mangrove microbiome. This work highlights the high degree of microbial diversity associated with the mangrove soil, suggesting that these sites are very important microbial and genetic repositories that could be further explored by human kind to identify novel enzymes and/or biological active compounds.

The data also supports that the mangrove microbiome provides a vast range of ecological services including carbon storage and cycling of major nutrients, thereby helping to support other forms of life. Urgent conservation actions are needed to protect mangroves all over the world.

6. Acknowledgments

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3.2. ROOT-MAIZE ASSOCIATED MICROBIOME CHANGES INFLUENCED BY *AZOSPIRILLUM BRASILENSE* INOCULATION

Daniel Vasconcelos Rissi[1]; Maria Isabel Stets[2]; Emanuel Maltempi de Souza[2]; Fábio de Oliveira Pedrosa[2]; Vandeir Francisco Guimarães[3]; Leonardo Magalhães Cruz[12*]

[1]Sector of Professional and Technological Education, Federal University of Paraná (UFPR), Curitiba, Paraná, Brazil

[2]Department of Biochemistry and Molecular Biology, Federal University of Paraná (UFPR), Curitiba, Paraná, Brazil

[3]Centro de Ciências Agrárias, Western Paraná State University (UNIOESTE), PR, Brazil

*Corresponding author (leonardo@ufpr.br)

Abstract

The plant growth promoting bacteria *Azospirillum brasilense*, is a nitrogen-fixating bacteria and has been applied in several crops to improve agricultural productions in several cultivars. When this bacteria is associated with the rhizosphere, it is possible to verify an increase in the roots with helps the plant to increase their production. Although, it is still unknown the effects of this microorganism in the microbiota of Maize rhizosphere. Through metagenomics analysis from 16S rRNA gene using Qiime tool, it is possible to access the microbiology that surrounding the Maize rhizosphere. Were inoculated 3 strains of *Azospirillum brasilense* Ab-V5, Ab-V6 and Ab-V7 with half potion of Nitrogen fertilizer. In the total 17 rhizosphere, samples were analyzed and we observed an increase in the presence of plant growth promoting bacteria that can helped to improve agronomics characters in plant and improve profits in agriculture. Despite that all strains increase plant development, the strains that provided a better development of the plant promoting bacterias were inoculated with the Ab-V5 and Ab-V7 strains associated ($N_{50}I^{+56}$). This study proved that inoculation with *Azospirillum brasilense* could change the rhizosphere and raise the plant production and became a good alternative to reduce the application of NPK fertilizers, reducing coasts of production.

1. Introduction

Since the maize (*Zea mays*) domestication, many varieties emerged, resulting in several variations in morphology, physiology, and genetics that make it to adapt to different crop conditions (CASAS *et al*., 2016). Besides that, maize is one of the most extensively grown cereal crop in the world, ranking second in production (HASSAN et al., 2015), being highly applied in animal feed (MINARDI *et al*., 2015). In this way, the

search for greater yields of maize has made researchers and producers seek for more technological innovations that facilitate the increase of production and decrease production costs.

One promising biotechnological strategy in modern systems of production has being the crop inoculation with bacteria that promote plant growth and protect it from diseases (VAN LOON 2007; VESSEY 2003), such as *Azospirillum* spp. based inoculants, that provides an increase in grain production in maize (HUNGRIA *et al*., 2010). This technology has been considered a friendly alternative to reduce the use of Nitrogen synthetic fertilizers and to decrease the use of agrochemicals without compromising the yield (HUNGRIA *et al*., 2010; MYRESIOTIS *et al*., 2015).

The plant-microorganism association in the rhizosphere is often mutually beneficial. The rhizosphere is a limited narrow region that surrounds the root system of vascular plants, and constitutes as a complex and dynamic environment where the root interacts with organic components, minerals, solutes, gases, and bacterias that drive the biogeochemical cycles (OBURGER & SCHMIDT, 2016). The microorganisms associated with the rhizosphere, can obtain nutrients from plant exudate, while the plant can acquire hormones, nutrients, metabolite precursors, and protection against pathogenic microorganisms, as well (MARTIN et al., 2016), playing an important role in plant growth and productivity (LUNDBERG et al., 2012).

The bacterial community colonization in the rhizosphere and endosphere of maize roots are influenced by the inoculation of growth-promoting bacteria (KUAN et al., 2016). The *Azospirillum* genus is considered to be one of the most important rhizobacteria for plant growth, benefiting the plant with phytohormones production, such as indole acetic acid, abscisic acid, and gibberellin (BASHAN et al., 2005; PERRIG et al., 2007), and biological nitrogen fixation (DOS SANTOS et al., 2017). The production of this plant hormones by species of *Azospirillum* genus, alter the metabolism and morphology of plant roots, resulting in a better absorption of minerals and water and stimulate the increase in the size of the roots (BASHAN & DE BAHSAN, 2010). In addition, strains of *Azospirillum brasilense* were reported to increase the production of corn grains up to 30% against to the non-inoculated treatments (HUNGRIA *et al*., 2010). In this work our goal was to identify changes in rhizosphere

microbiology, mainly plant-growth promoting bacterias, with the Inoculation of different strains of A. brasilense (Ab-V5, Ab-V6, Ab-V7) in Maize Seeds and Nitrogen Adiction.

2. Material and Methods

2.1. Field experiment and Inoculant Preparation

The field experiments were conducted at the UNIOESTE experimental station locate in the city of Marechal Cândido Rondon, Paraná, Brazil (54°22' W and 24°46' S, 420 m altitude). The climate, in the Koppen system, is subtropical humid mesothermal (Cfa) (MARINI et. al. 2015). The soil was classified as clayey Oxisol (Lvef).

The *Azospirillum brasilense* strains Ab-V5, Ab-V6, Ab-V7 were develop in the National Institute of Science and Technology of Biological Nitrogen Fixation at Federal University of Paraná, Curitiba, Paraná, Brazil, in cooperation with Embrapa Soja, Londrina, Paraná, Brazil (HUNGRIA *et al*., 2010). These strains are authorized for the production of commercial inoculants in Brazil (HUNGRIA *et al*., 2010), to be used in wheat and maize crops. The strains were cultivated in medium NFbHP, supplemented with lactate as carbon source and Glutammate 5 mmol L^{-1} as nitrogen source (MACHADO et al., 1991), and inoculated at 10^8 bacterias per seed (Table 1). The inoculation with the different strains of *A. brasilense*, both individually and in association was performed 2 h before sowing. For the individual inoculation of the strains, 6 mL of inoculant were mixed to 1,000 maize seeds. Co-inoculation treatments of two or three strains used mixed inoculant in proportion of 1:1 or 1:1:1, respectively.

Table 1. Sample identification and treatments detail.

SAMPLES	Treatment - Nitrogen (N) fertilization	Inoculation with *A. brasilense* strains
N_0I^-	0kg/ha of N (N_0)	non-inoculated (I^-)
$N_{50}I^-$	60kg/ha of N (N_{50})[1]	non-inoculated (I^-)
$N_{50}I^{+7}$	60kg/ha of N (N_{50})	inoculated with Ab-V7 strain (I^{+7})
$N_{50}I^{+56}$	60kg/ha of N (N_{50})	inoculated with Ab-V5 e Ab-V6 strains (I^{+56})
$N_{50}I^{+567}$	60kg/ha of N (N_{50})	inoculated with Ab-V5, Ab-V6 e Ab-V7 strains (I^{+567})
[1]subscript 50 means 50% of total (120kg/ha) dosage of recommended nitrogen fertilization.		

In the first column, "N" represents do dosage of nitrogen fertilizer and "I" the bacterial strains of *A. brasilense* inoculated in each sample. Those strains were selected in these combinations due to provide a statistical increase in Maize production.

Soil samples were collected along the experimental plots at 20 cm depth. 17 subsamples were collected and homogenized in a composite sample, which was packed in plastic bags and immediately placed on ice and transported to the laboratory.

Five specimens were collected during harvesting of their grains in each experimental plot and joined to form a composite sample. For each treatment, two to eight subsamples were obtained as replicates. Once harvested, the plants were packed in plastic bags, labeled and placed immediately on ice and transported to the laboratory where they were processed as quickly as possible. The excess soil adhered to the plants was removed and the plants were washed in tap water and dried on paper towel. The roots and aerial parts were separated and weighed.

2.2. DNA Extraction, PCR amplification, and 16S rRNA gene sequencing

Metagenomic DNA was extracted with two different techniques. The Soil DNA extraction was performed using 1g of soil with "UltraClean Soil DNA Kit" (MOBIO laboratories). Roots were sterilized and then crushed in liquid nitrogen and DNA were

extracted using “Power Plant DNA Kit” (MOBIO Laboratories). All samples were amplified in triplicate.

The 16S rRNA gene were amplified with the primer pair 27F/1492R (LANE 1991). The products of PCR were subjected to electrophoresis in agarose gel of low melting point, where two DNA bands of about 2.0 kb and 1.5 kb were observed. The primer pair used in PCR is able to amplify mitochondrial DNA, corresponding to the 2,000 pb DNA band on the gel (MAGNANI et al., 2013). The 1.5 kb band, corresponding to 16S rRNA gene in prokaryotes, was extracted and purified from the gel and subjected to a nested PCR with Illumina primer pair, including primer sequences for V4 region of 16S rRNA gene 515f/806r, well suited for accurate diversity studies at the domain and bacterial phylum levels (LIU et al., 2007; PEIFFER et al., 2013) generating an amplicon of about 250bp. The forward strand of the amplicons were sequenced using MiSeq Illumina platform NGS.

2.3. Quality and diversity community analyses

Reads quality (e.g., length, GC content, etc.) was checked using FastQC tool (Babraham Bioinformatics. FastQC: a quality control tool for high throughput sequence data https://www.bioinformatics.babraham.ac.uk/projects/fastqc).

The raw fastq sequence files were uploaded into Quantitative Insight into Microbial Ecology tool (QIIME, version 1.9.1) and processed through the pyrosequencing pipeline (CAPORASO et al., 2010). Sequences were trimmed at 250 bp to eliminate small reads and end-bases with low quality. Trimmed reads were used to determine operational taxonomic units (OTUs) with QIIME open-reference strategy using Uclust at a similarity threshold of 97% (EDGAR, 2010). This strategy is composed by two rounds of analysis. In the first round, the sequences are align against the reference database to taxonomic classification. Those sequences that failed to OTU assignment using this first strategy, were subjected to a second round of clusterization using QIIME *de novo* strategy. The OTU’s ware taxonomic assigned based on the Greengenes (version 13_8) database. The taxonomic assignment frequencies and metadata of soil samples was exported as Biological observation matrix (biom format) (MCDONALD et al., 2012) and filtered for mitochondria and

Chloroplast sequences. The counts of each OTU were normalized by the total number of aligned reads per sample.

Alpha and Beta diversity, hierarchical clustering and principal coordinate analysis (PCoA) were applied using QIIME and MetaCoMET (WANG et al., 2016), and STAMP (PARKS et al., 2014) in order to compare microorganisms abundance. For those analysis, BIOM file was used as input and methodological specifications are presented in text and figures.

2.4. Functional Prediction Analsysis

OTU representative sequences were used for functional metagenomic prediction analyses using PICRUSt (LANGILLE et al., 2013). The database includes 1,756,783 prokaryotic and 1,262,986 eukaryotic sequences (KOO, et al., 2017). The taxonomical assignment using for PICRUSt analysis was based on Greengenes reference database (DESANTIS et al., 2006) release 13_5 of 2013, containing 99,322 16S rRNA sequences.

The PICRUSt/Greengenes approach use the KEGG database as reference for metabolic predictions and include normalization for 16S rRNA gene copy number. The predictive functions of the microbial communities were determined by linearly combining the normalized taxonomic abundances into the precomputed association matrix of KEGG Orthology reference profiles version 13.5.

2.5. Statistical Analysis

Statistical analysis were made using STAMP (PARKS et al., 2014), Statistical Analysis of Metagenomic Profiles, using two sided Welch’s t-test with 0.95 of confidence interval. Samples with p-value lower than 0.05 were considered statistically significant.

3. Results

3.1. Agronomics Characteristics

Through the analysis of variance, a significant effect was detected for all sources of variation used in the present study. However, in relation to the effect of the treatments used, a significant difference was observed for the biometric variables and for the components of the production, as well as for the productivity obtained, there are no significant difference between the treatments in relation to the P and K contents of the treatments leaves and grains of maize.

The inoculation of the Ab-V5, Ab-V6 and Ab-V7 strains of *Azospirillum brasilense*, showed statistical significance in biometric parameters: stem diameter (SD), shoot dry matter (SDM) and leaf area (LA) (Table 2).

Table 2: Biometric Data for Maize Inoculation Experiment with *Azospirillum braliense* Strains Ab-V5, Ab-V6 and Ab-V7 in Field, Led in 2012

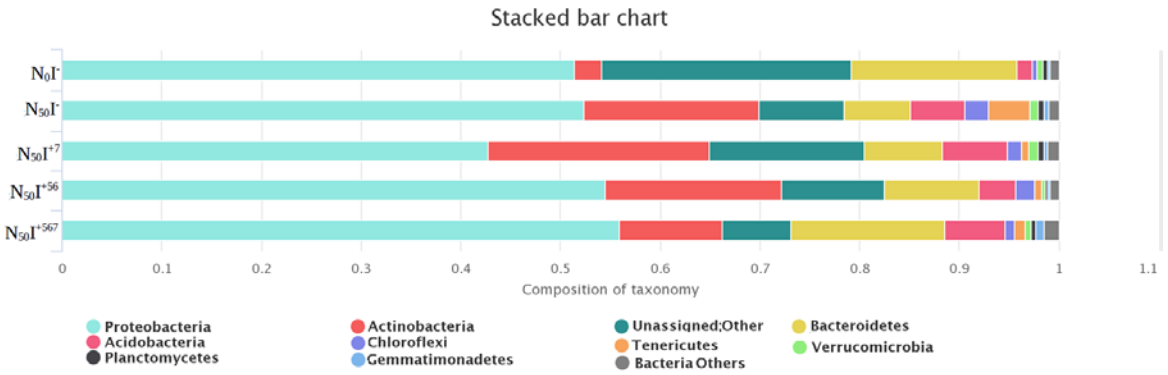
Treatment	SD (cm)	SDM (g per plant)	LA (dm^2)
N_0I^-	24,19 cA*	33,00 cB*	45,50
$N_{50}I^-$	24,68 cA*	40,00 cB*	57,00
$N_{50}I^{+7}$	29,10 aA	57,25 aB*	71,75
$N_{50}I^{+56}$	29,21 aA	59,00 aA	74,50
$N_{50}I^{+567}$	26,72 bB*	46,50 aB*	59,25

Legend: Stem Diameter (SD), Shoot Dry Matter (SDM) and Leaf Area (LA) using Scott-Knott's Test At 5%. Subsamples from experimental samples were jointed in one sample. Same letter in lower and upper case represents agronomics characters without statistical significance. (*) character with statistical significance

3.2. Taxonomic Classification

The normalized data had 3.696 sequences per sample totalizing 62.832 sequences in 17 samples, one subsamples were removed due to the short number of sequences. The taxonomic analysis showed that 13.4% of the reads belongs to Unassigned reads. The most representative taxonomic groups are Proteobacteria (50.5%), Actinobacteria (15.3%), Bacteroidetes (10.4%) and Acidobacteria (4.8%) (Figure 1).

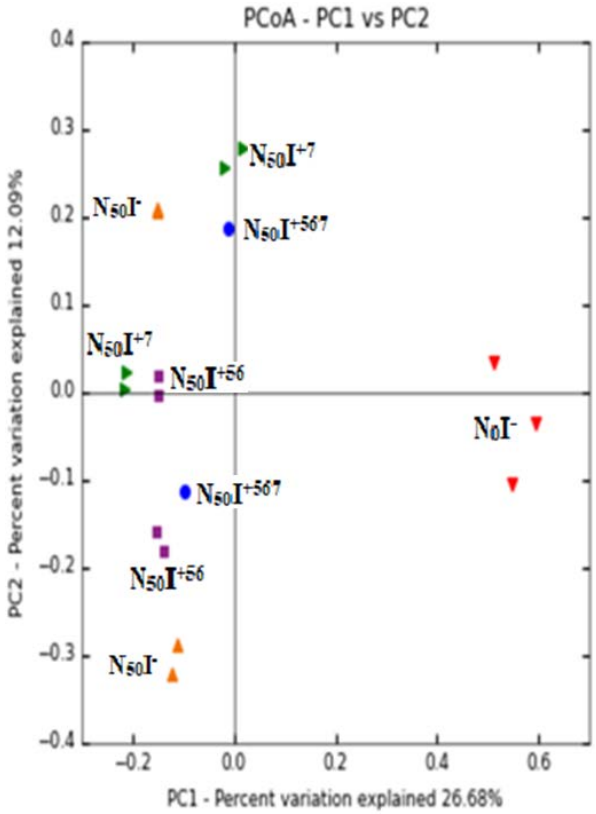
Figure 1: Bar Chart of Phylum Distribution.



Distribution of the ten most common phylum in rhizosphere soil of maize plants subjected to different treatments of N fertilization and inoculation with *Azospirillum brasilense* strains. The samples ID are according to table 1, where N_0 and N_{50} indicate percentage of N fertilization from a total of 120 kg/ha (100%). Through this figure, we can observe that the main phylum groups kept their abundance in the study.

Through analyzes of Beta diversity, (Figure 2) it is possible to verify that samples without Nitrogen Fertilization (N_0I^-) are grouped separated of all other samples. The graphs PC1-PC2 and PC2-PC3, shows how different is the microbial composition between control samples and all other samples.

Figure 2: PCOA graph from Beta Diversity analysis



Beta Diversity principal coordinate analysis (PCoA) of microbial community in rhizosphere soil of maize plants subjected to different treatments of N fertilization and inoculated with *Azospirillum brasilense* strains using all taxonomic levels evaluated from Bray-Curtis calculated dissimilarities. evaluated from Bray-Curtis calculated dissimilarities. We can observe that the nitrogen addition, promote a change in microbial communities causing a displacement in nitrogen fertilized groups.

With the Nitrogen Fertilizer addition ($N_{50}I^-$ samples), we can observe a large dislocation of the groups in the Beta Diversity plot (Figure 2). Analyzing ate Phylum level, a higher change can be noticed in Actinobacteria phylum (Figure 3). Also, at genus level we could identity changes in microbiological communities, it is possible to observe mainly a strong increase in *Streptomyces* (Bhattacharyya & Jha 2012;, GOUDJAL et al., 2014), *Burkholderia* (WAKELIN et al., 2017), *Amycolatopsis* (Xu et al., 2016) among other genus that belongs to families of *Bradyrhizobiaceae* (KONISHI et al., 2017), *Xanthomonadaceae* (AFZAL et al., 2017), *Acidobacteriaceae* (KIELAK et al., 2016), all well know groups of plant-growth promoting bacterias (Figure 4).

Figure 3: Statistical Difference between $N_{50}I^-$ samples and N_0I^- samples at Phylum level.

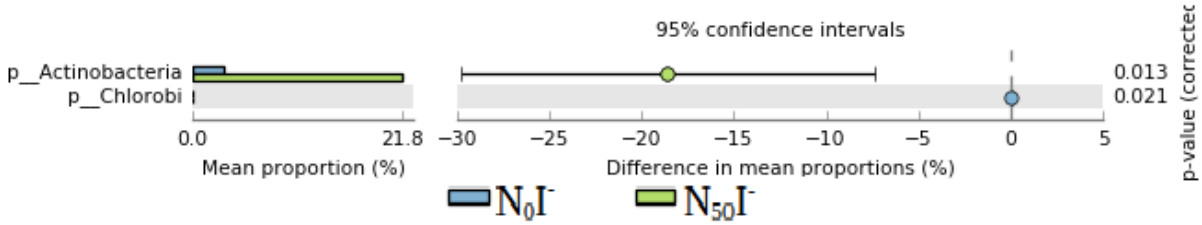
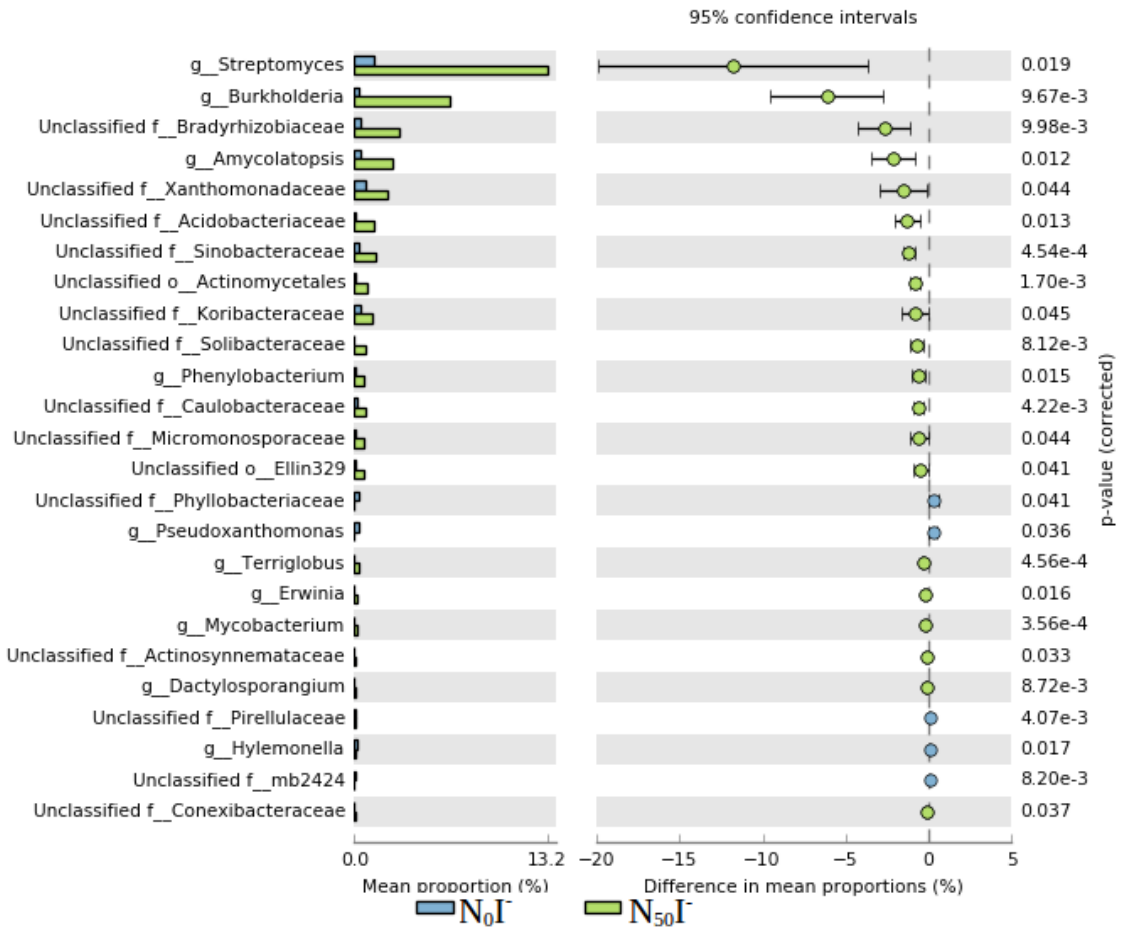


Figure 4: Statistical Difference Between N_0I^- samples and $N_{50}I^-$ samples at Genus Level



Samples inoculated with strain Ab-V7 had a increase in genus from *Sinobacteriaceae* and *Micromonosporaceae* families and *Kutzneria* genus among others with representativity. A decrease in Caulobacteraceae family and *Devosia* genus is also noticed with the Ab-V7 strain inoculation when compared with $N_{50}I^-$ samples (Figure 5.a).

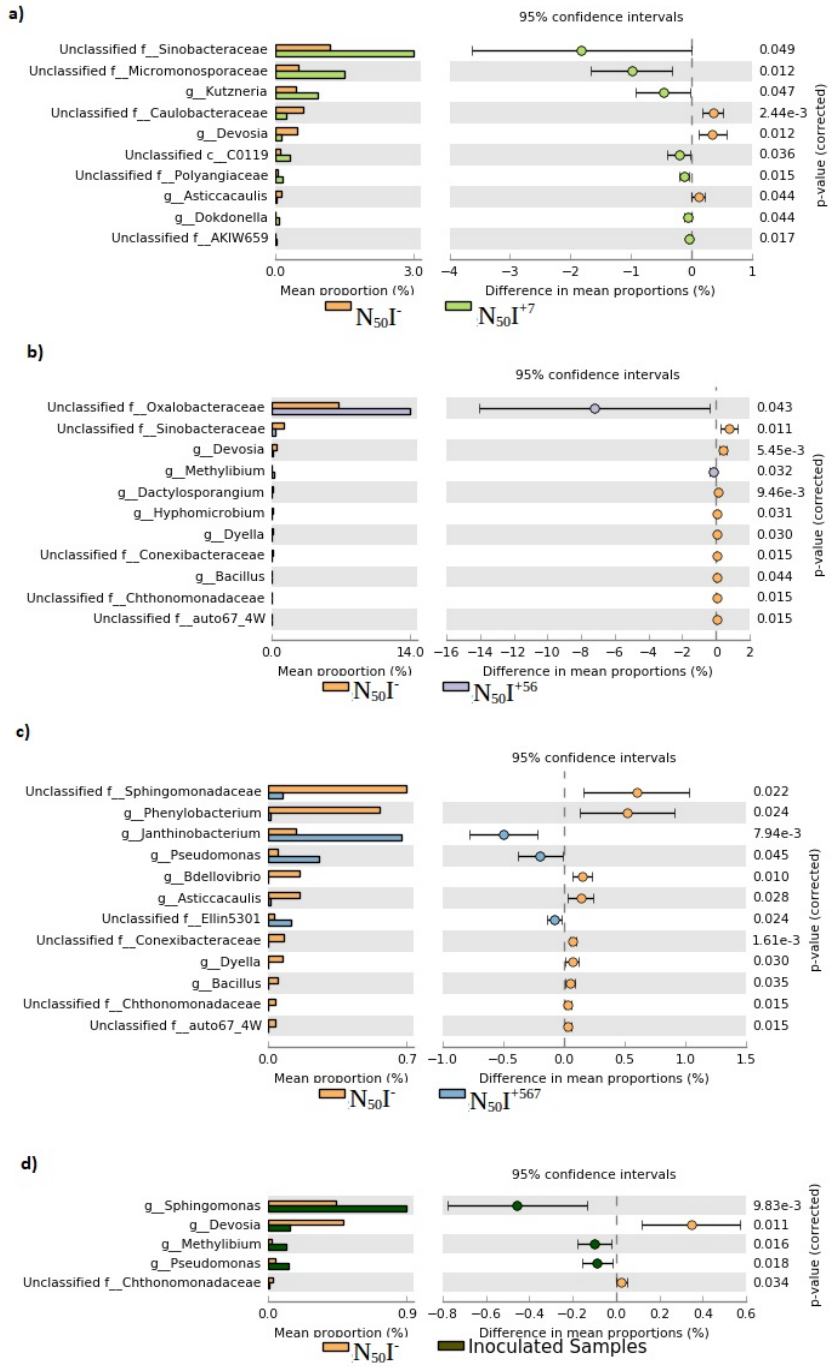
Sample with inoculation of Ab-V5 and Ab-V6 strains of *A. brasilense* ($N_{50}I^{+56}$) had a increase in some unknown genus of *Oxalobacteraceae* family when compared with samples with nitrogen fertilization ($N_{50}I^-$)(Figure 5.b).

The sample inoculated with all the strains in this study Ab-V5, Ab-V6 and Ab-V7, had increase in the *Janthinobacterium* and Pseudomonas genus. Although, a unknown genus of *Sphingomonadaceae*, *Phenylobacterium* and *Conexibacteraceae* family together with *Bdellovibrio*, *Asticcacaulis*, *Dyella* and Bacillus genus were

reduced with the inoculation of *A. brasilense* strains and were more representative in $N_{50}I^-$ (Figure 5.c).

By joining all inoculated samples as one group, against $N_{50}I^-$ samples, a increment a rise in *Sphingomonas*, *Methylibium* and *Pseudomonas* genus were identified. Although, a decrease in *Devosia* genus in all inoculated samples were detected with statistical significance (Figure 5.d).

Figure 5: Statistical Significance Between $N_{50}I^-$ Samples and Inoculated Samples

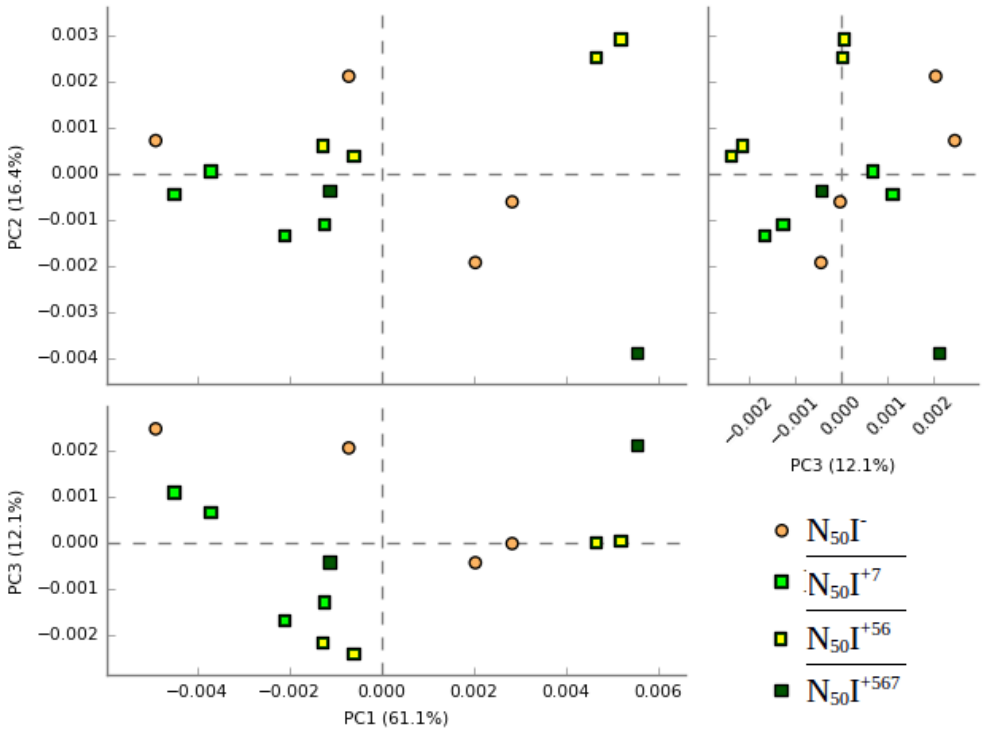


a) Statistical Significance Between non inoculated samples with nitrogen fertilization ($N_{50}I^-$) and samples inoculated with A.brasilense strain Ab-V7. b) Statistical Significance Between non inoculated samples with nitrogen fertilization ($N_{50}I^-$) and samples inoculated with A.brasilense strain Ab-V5 and Ab-V6. c) Statistical Significance Between non inoculated samples with nitrogen fertilization ($N_{50}I^-$) and samples inoculated with A.brasilense strain Ab-V5, Ab-V6 and Ab-V7. d) Statistical Significance Between $N_{50}I^-$ Samples and All Inoculated Samples together. It is possible to observe a increase in plant-growth promoting bacterias.

3.3. Functional Predictions

Analyzing the PCOA of the functional profiles (Figure 6), It is possible to observe a difference in the functional predictions profile between samples. Samples N_0I^- and $N_{50}I^-$ are distant from each other and group in different places in the plot showing a higher change in microbiological communities with the Nitrogen addition in soil.

Figure 6: PCOA Plot of Functional Predictions



Comparing $N_{50}I^-$ and $N_{50}I^{+7}$ samples had 356 features with significant difference. The features with most effect size with significant difference were antibiotic transport system permease protein, antibiotic transport system ATP-binding protein, 3-demethylubiquinone-9 3-methyltransferase, long-chain acyl-CoA synthetase and DNA ligase (ATP) (Figure 7.a).

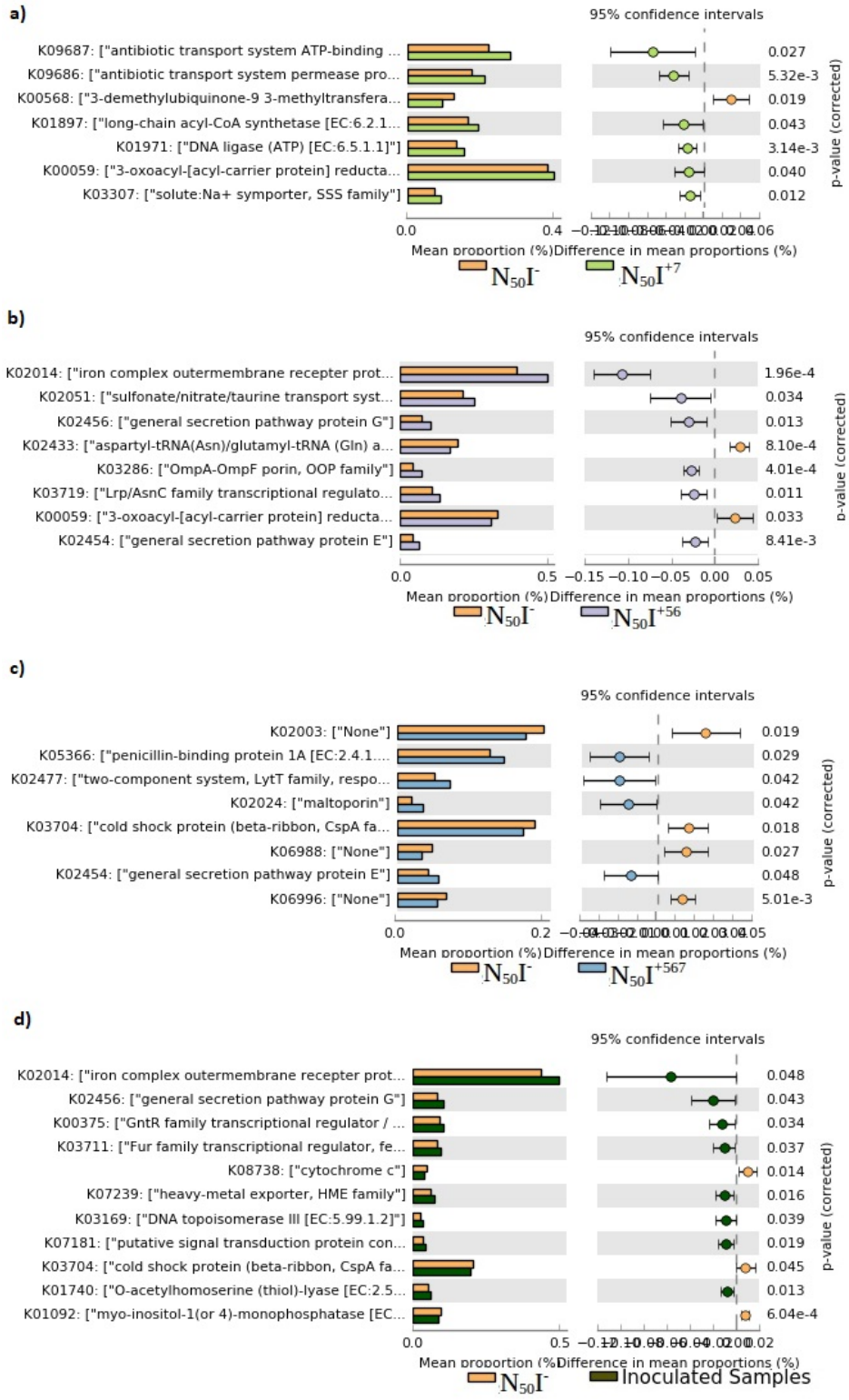
Comparing $N_{50}I^-$ and $N_{50}I^{+56}$ samples, 697 features had a significant difference in functional predictions. The features with most effect size with significant difference were iron complex outermembrane receptor protein, sulfonate/nitrate/taurine transport system substrate-binding protein, general secretion pathway protein G, aspartyl-

tRNA(Asn)/glutamyl-tRNA (Gln) amidotransferase subunit A, OmpA-OmpF porin, OOP family (Figure 7.b)

Comparing $N_{50}I^{-}$ and $N_{50}I^{+567}$ samples, 244 features had a significant difference in functional predictions. The features with most effect size with significant difference were penicillin-binding protein 1A, two-component system, LytT family, response regulator, maltoporin (Figure 7.c).

$N_{50}I^{-}$ samples in comparison with all inoculated samples, had 268 features with statistical significant difference. The features with most effect size with significant difference were iron complex outermembrane receptor protein, general secretion pathway protein G, GntR family transcriptional regulator / MocR family aminotransferase, Fur family transcriptional regulator, ferric uptake regulator Figure 7.d).

Figure 7: Statistical Difference in Functional Prediction Features between samples



a) $N_{50}I^{-}$- $N_{50}I^{+7}$. b) $N_{50}I^{-}$ – $N_{50}I^{+56}$ c) $N_{50}I^{-}$- $N_{50}I^{+567}$ d) $N_{50}I^{-}$ - All Inoculated Samples

4. Discussion

A. brasilense are one of the most study plant-growth promoting bacteria, with positive effects proved in studies with in Cereal, Maize, Soy, Pastures and several crops in distinct soils types and climatic changes (OKON & LABANDERA-GONZALEZ, 1994; HERSCHKOVITZ et al., 2005; HUNGRIA, et al., 2016)

Previous studies reported that inoculation with *Azospirillum*, result in significant changes in the secondary metabolites in rhizosphere, suggesting an interaction mechanism between the rhizosphere microbiome with *Azospirillum* bacterium (WALKER, 2011). These secondary metabolites may play a key role in the vegetative development mainly by the production of phytohormones (BASHAN & DE-BASHAN, 2010; D'ANGIOLI, et al., 2017) and and in the interaction of rhizosphere-microorganisms (D'ANGIOLI et al., 2017).

With the soil nitrogen fertilization, we could noticed a increase in *Actionobacteria* phylum (Figure 4) and at genus level, in many plant-growth bacterias such as Streptomyces (BHATTACHARYYA & JHA, 2012), *Burkholderia* (WAKELIN et al., 2017), *Amycolatopsis* (XU et al., 2016) among other genus that belongs to families of *Bradyrhizobiaceae* (KONISHI et al., 2017), *Xanthomonadaceae* (AFZAL et al., 2017), *Acidobacteriaceae* (KIELAK et al., 2016), all well know groups of plant-growth promoting bacterias. This shows that nitrogen fertilization has a positive contribution in the development of microbiological communities in rhizosphere with plant-growth promoting bacterias. (Figure 5) when compared with soil samples without nitrogen fertilization.

Comparing each inoculated samples with the samples without inoculation and fertilizer with nitrogen ($N_{50}I^{-}$), the samples $N_{50}I^{+7}$ inoculated with Ab-V7 strain, contribute to increase in *Sinobacteraceae* a plant-growth promoting bacteria family (SITEPU et al., 2007) and reported most recently degrading organic compounds (WANG et al., 2017), *Micromonosparaceae* family, reported to produce IAA, a phytohormone (El-TARABILYA et al., 1997; GOMES et al. 2000; SOUSA et al. 2008; GANGWAR, et al., 2012) and *Caulobacteraceae* a plant growth promoting bacteria families (BHATTACHARYYA & JHA, 2012). At genus level, we could confirm a

increase in *Kutzneria* genus which play a important role in synthesize novel antibiotics (DINESH, et al., 2017) and anti-fungal activity against common root fungi (BROBERG et al., 2006), as most representative genus with statistical difference between samples (Figure 6.a).

$N_{50}I^{+56}$ samples in comparison with N_0I^- samples, the only microbiological community that shows a higher statistical significance was the Oxalobacteraceae family. The *Oxalobacteraceae* family has some root colonizing bacteria (OFEK et al., 2012) and have important groups of plant-growth promoting bacterias such as *Herbaspirillum* (PEDROSA, et al., 2011) and *Massilia* (KUFFNER et al., 2010) that produced IAA phytohormones. This family is also reported to have bacterias with antifungal activity such as *Janthinobacterium* and *Duganella* genus (HAACK et al., 2016), induced most likely to degradation products, and secondary metabolites being suppressive toward fungi plant pathogens (HAAK, et al., 2016; SCHLEMPER, et al., 2017) (Figure 6.b).

The sample inoculated with all strains ($N_{50}I^{+567}$) had a positive effect in the development of *Janthinobacterium* and *Pseudomonas* genus that promote plant-growth (KUFFNER et al., 2010; MA et al., 2010) and prevent plant infection (KONG et al., 2016). Although, many bacterial groups decrease their proportion. Those groups have biological activity related with plant-growth with *Sphingomonadacea* family produce IAA increasing plant growth (KHAN et al., 2014) and *Phenylobacterium* genus also known as nitrogen fixation bacteria (YANG et al., 2017), obligate parasites such as *Bdellovibrio* (JURKEVITCH, 2008), and *Asticcacaulis* (ISHIZAWA et al., 2017) (Figure 6.c)

Putting together all inoculated samples, we could identify a statistical increase in plant-growth promoting bacteria in *Sphingomonas* (SADEGHI et al., 2012), *Methylibium* (SANTIAGO et al., 2017), Pseudomonas (WAKELIN et al., 2017) (Figure 6.d**)**

In study developed in Mayze using DGGE analysis (HERSCHKOVITZ et al., 2005), inoculated with two strains of *A. brasilense* did not alter bacterial community structures. In our study using 16S rRNA gene to access the microbiological community in the rhizosphere, the inoculated samples ($N_{50}I^{+7}$, $N_{50}I^{+56}$, $N_{50}I^{+567}$), had a statistical

increase in proportion of plant-growth promoting bacterias and bacterias that contribute to the plant health with antibiotic or anti-fungi activity showing that the seed inoculation with A. brasiliense strains can change the soil microbiology and helped to build a microbiome that can improve plant development and possible improve the production (Figure 6).

The absence of *Azospirillum* genus might be associated with the stage of the soil sampling. It is rather to detect more metabolites from *Azospirillum brasilense* in early stages of cultive (WALKER et al., 2011) and with phosphorus supply (D'ANGIOLI et al., 2017) so it is possible that their absence in the result be related to the stage of analysis or physico-chemical quality of the soil. Although, their inoculation showed to be determinant to increase of some plant growth promoter bacterias (Figure 5.d), which could be explained by the interaction of *A. brasilense* metabolites with other microorganisms in early stages of cropping.

The observed difference between the microbiological communities inoculated with different *A. brasilense* strains, could be explained by a strain-dependent mechanism (WALKER et al., 2011), that could be able to change the microbiology in the rhizosphere by their own way depending on the strain inoculated in the seed

The inoculation with this bacteria, may help the plant growth even without the addition of any chemical nitrogen fertilization which decrease the production cost in agriculture (HUNGRIA et al., 2015)

In conclusion the inoculation of *A. brasilense* in Maize seed, promote an increase in agronomics characters, which might improve profits in agricultural systems. By all means, the metagenomics analysis was able to determinate that the inoculation of Ab-V5 and Ab-V7 ($N_{50}I^{+56}$), has better interaction with the environment witch than other inoculated samples, which contribute more in the changes of the rhizosphere microbiome, plant growth and increase Maize production the production.

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4 CONSIDERAÇÕES FINAIS

A partir das analises metagenômicas desenvolvidas, foi possível identificar melhor a microbiana dos ambientes analisados e perceber ligeiras mudanças ocorridas nos ambientes.

Entre os ambientes de Mangue e Sambaqui, percebemos que apesar de serem biomas diferentes com condições físico-químicas diferentes, houve pouco impacto na alteração da microbiota, podendo ser explorado futuramente para descoberta de novos produtos de interesse biotecnológico. Os dados também suportam que o microbioma de mangue oferece uma vasta gama de serviços ecológicos, incluindo armazenamento de carbono e ciclagem de nutrientes importantes.

Os demais estudos sugerem que a inoculação de *Azospirillum brasilense* pode alterar no desenvolvimento da microbiota ao redor da rizosfera. Essa microbiota é benéfica para a planta e possui uma rede de interações melhores entre elas do que as apresentadas em solo. Essa microbiota é formada em uma combinação da atividade metabólica do microrganismo, provavelmente ainda nos estágios iniciais de cultivo, combinados com uma “atividade de seleção” promovida pelo exsudado da planta.

5. REFERÊNCIAS COMPLEMENTARES

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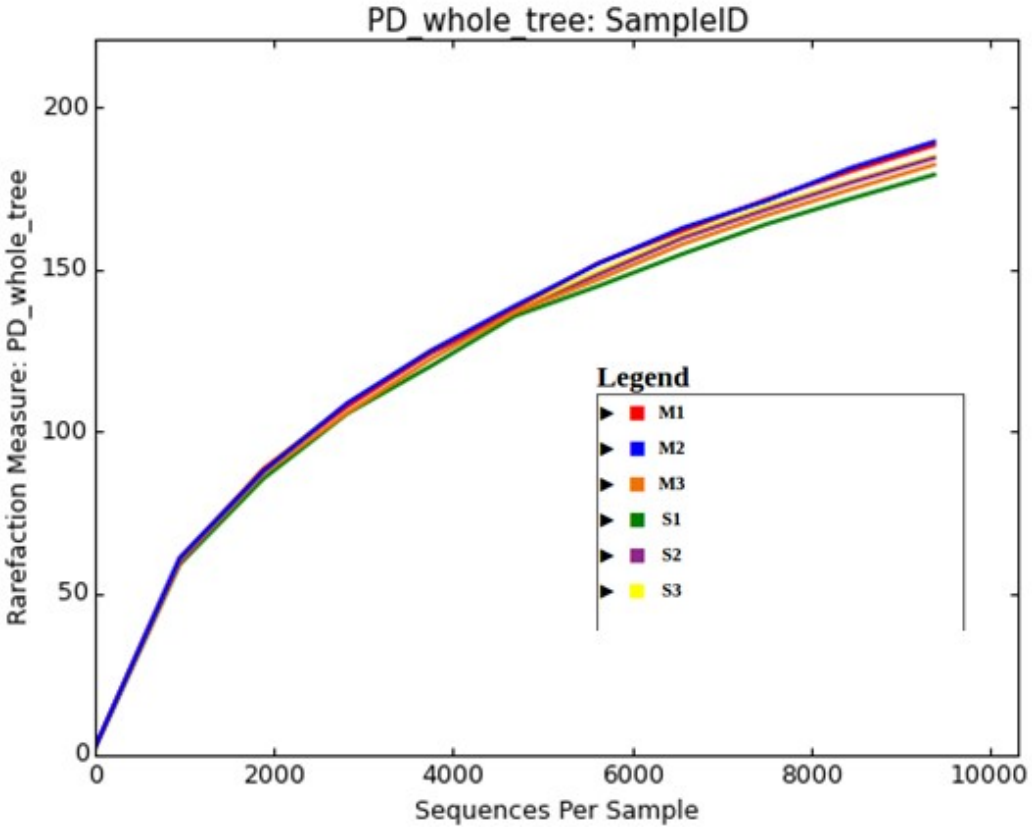
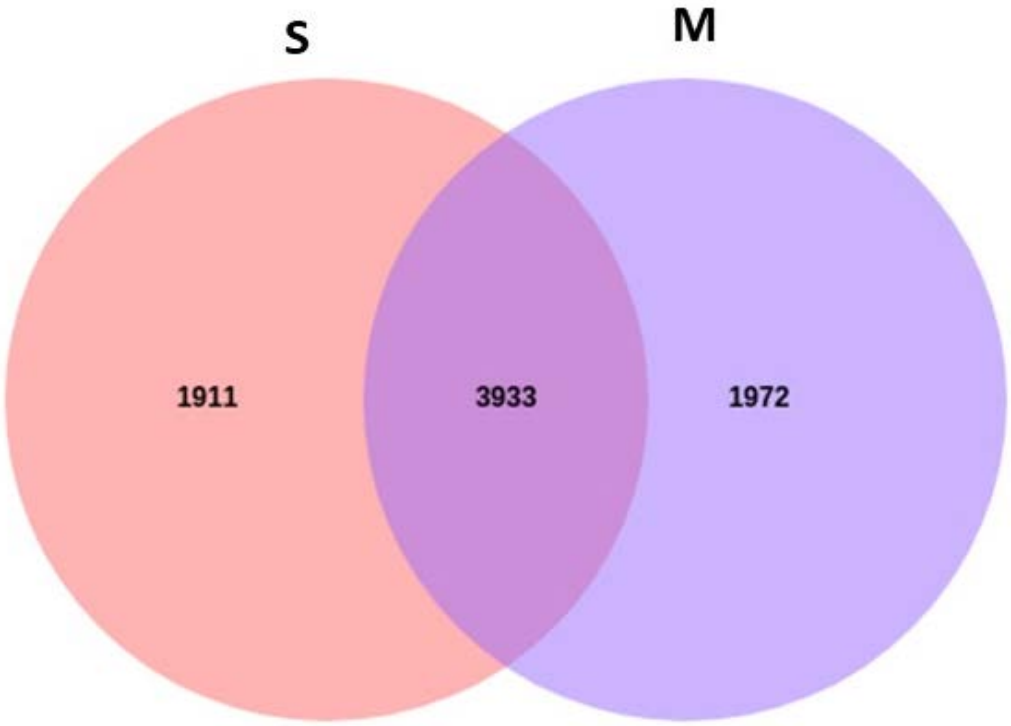
APÊNDICE

Figure S1: Rarefaction Plots



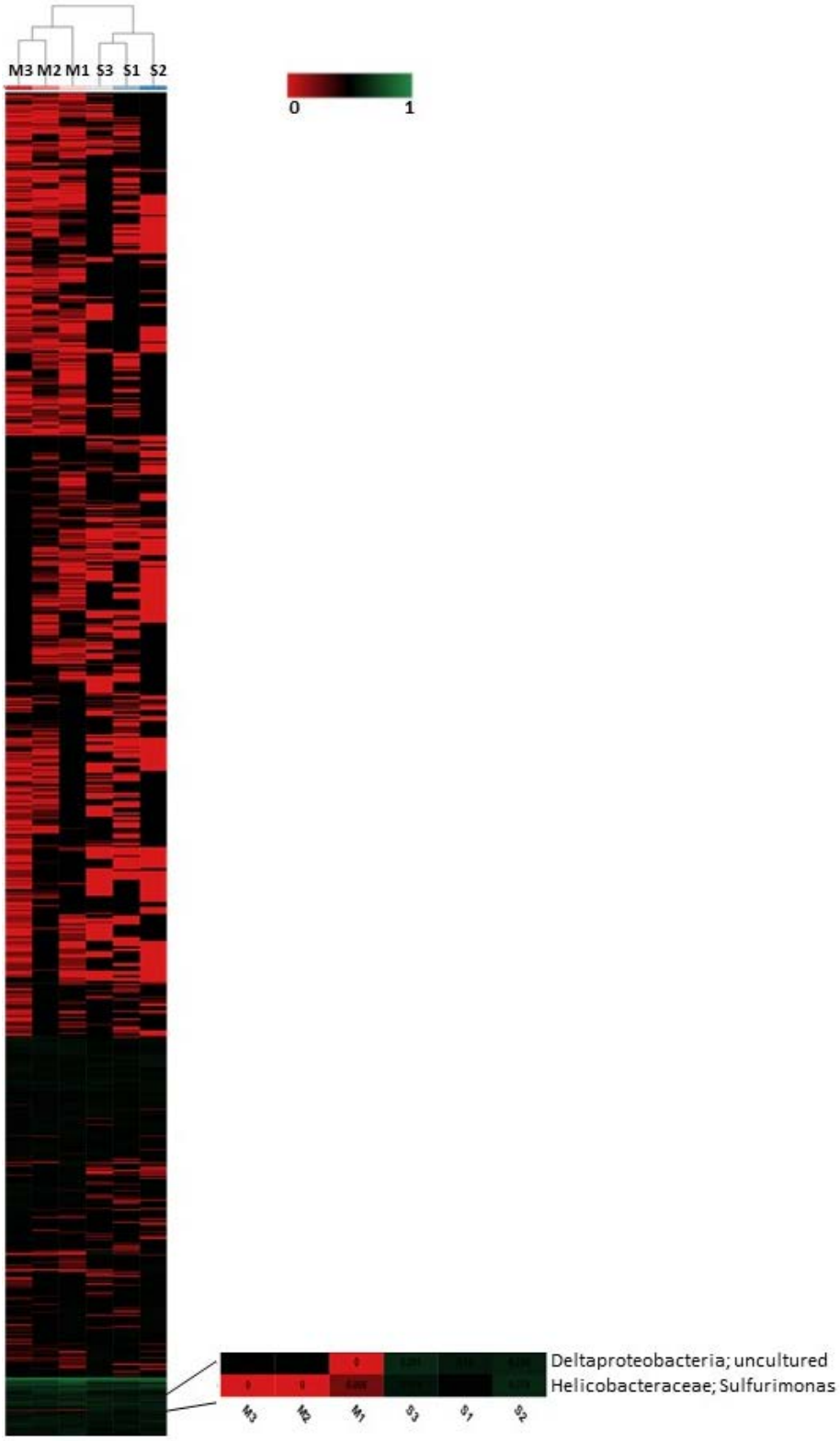
. The 16S DNA sequences of each sample were used to create rarefraction plots using Phylogenetic diversity tree (PD) using QUIIME

Figure S2: Veen Diagrame



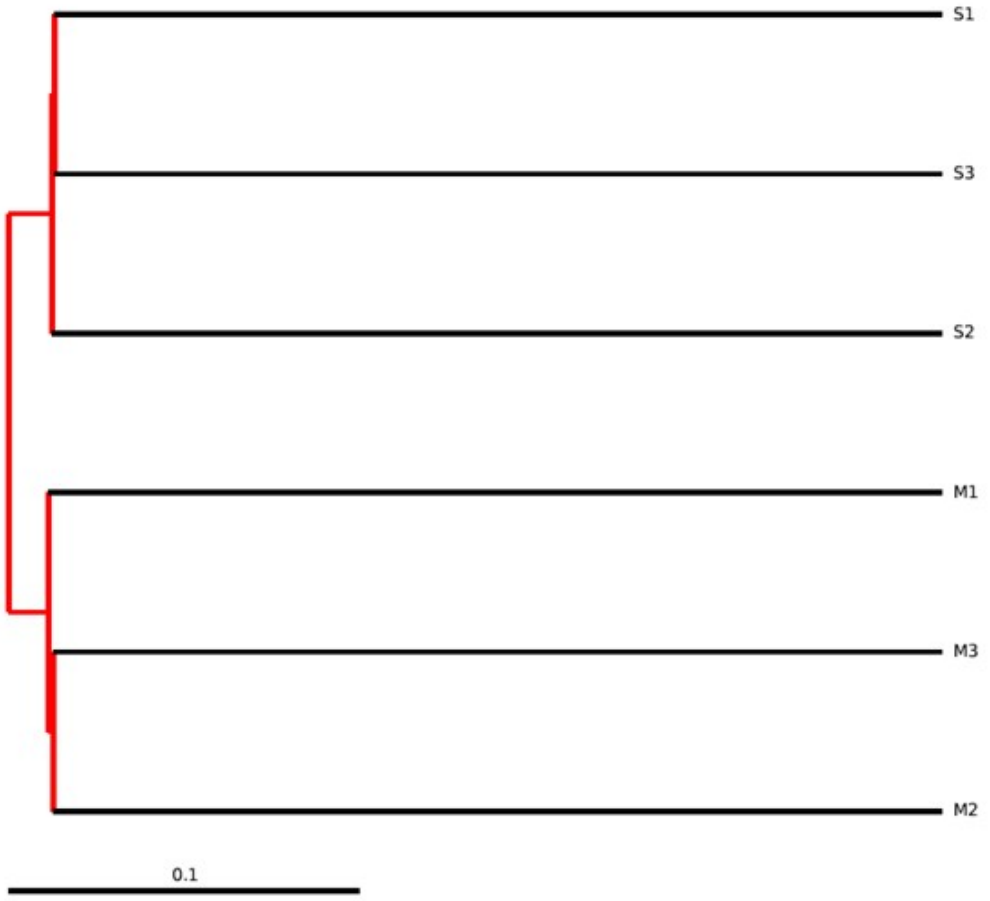
Venn diagram using all the OTUs identified in all S and M samples, showing that 3933 OUT's are shared between S and M samples

Figure S3: Hierarchical clustering heat map analysis of samples



OTUs were analyzed at the lowest taxonomic level (Genus) using MetaCoMET. Each line represents a OTU and its abundance in each sample M1, M2, M3, S1, S2, S3 is represented as a heat map. The expanded part of the heat map indicates two OTUs that are highly divergent in abundance between the M and S samples

Figure S4: Jackknifed UPGMA Tree.



The tree shows the phylogenetic similarity of bacterial communities based on 16S rRNA genes. The data was obtained using QUIIME.