MARIANA PRÓSPERI DE OLIVEIRA PAULA

CARACTERIZAÇÃO TAXONÔMICA E FUNCIONAL DE CONSÓRCIOS MICROBIANOS DEGRADADORES DE GLIFOSATO E SUA ATIVIDADE EM MICROCOSMOS DE SOLO

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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para obtenção do título de Doutor.

Prof. Dr. Victor Satler Pylro
Orientador

Prof. Dr. Luiz Fernando Wurdig Roesch
Prof. Dr. Marcos Rogério Tótola
Coorientadores

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Paula, Mariana Prósperi de Oliveira.
Caracterização Taxonômica e Funcional de Consórcios Microbianos Degradadores de Glifosato e sua Atividade em Microcosmos de Solo / Mariana Prósperi de Oliveira Paula. - 2024.
85 p.

Orientador(a): Victor Satler Pylro.
Coorientador(a): Luiz Fernando Wurdig Roesch, Marcos Rogério Tótola.
Tese (doutorado) - Universidade Federal de Lavras, 2024.
Bibliografia.

1. Glifosato. 2. Biodegradação. 3. Bactérias. I. Pylro, Victor Satler. II. Roesch, Luiz Fernando Wurdig. III. Tótola, Marcos Rogério. IV. Título.

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TAXONOMIC AND FUNCTIONAL CHARACTERIZATION OF GLYPHOSATE-DEGRADING MICROBIAL CONSORTIA AND THEIR ACTIVITY IN SOIL MICROCOSMS

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APROVADA em 12 de novembro de 2024.

Dr. Daniel Kumazawa Morais UiT
Dr. Jorge Teodoro de Souza UFLA
Dr. Leandro Nascimento Lemos CNPEM
Dr. Marcos Rogério Tótola UFV

Prof. Dr. Victor Satler Pylro
Orientador

Prof. Dr. Luiz Fernando Wurdig Roesch
Prof. Dr. Marcos Rogério Tótola
Coorientadores

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AGRADECIMENTOS

Agradeço ao meu orientador, professor Victor Satler Pylro, por seus ensinamentos, paciência e constante incentivo à ampliação da minha formação como jovem pesquisadora. Aos meus co-orientadores, professores Luiz Fernando Wurdig Roesch e Marcos Rogério Tótola, sou grata pela receptividade e pelo apoio que recebi durante meu período em seus laboratórios.

Minha gratidão se estende à equipe do Laboratório de Ecologia Microbiana e Bioinformática, com menção especial a Carlos e Cristiane, pela colaboração e amizade. Agradeço também à equipe dos laboratórios que integram o PPGMA e à secretaria integrada, pela parceria e prestatividade.

À Universidade Federal de Lavras (UFLA), ao Programa de Pós-Graduação em Microbiologia Agrícola, e à CAPES, agradeço a concessão da bolsa de estudos. Este trabalho foi realizado com o apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Aos meus amigos, especialmente aqueles que compartilharam ou ainda compartilham essa caminhada, tanto no Brasil quanto nos EUA — Amanda, Esteban, Jessica, Michelle, Sam, Brena, Carlos, Juliete, Luciano, Mirian e Cristiane — agradeço o apoio, incentivo e companheirismo. À minha família, pelo carinho e suporte incondicional, com especial gratidão à minha avó e professora Cida, por me ajudar a acreditar em minhas habilidades e por ser uma professora que sempre será uma referência para mim. Por fim, meu agradecimento sincero a todos que, direta ou indiretamente, contribuíram para a realização deste trabalho. Muito obrigada!

“A educação é a arma mais poderosa que você pode usar para mudar o mundo”

(Nelson Mandela)

RESUMO

O glifosato, herbicida não seletivo amplamente utilizado, pode afetar plantas não alvo e o solo, além de gerar preocupações ambientais e de toxicidade. Portanto, a remediação de áreas contaminadas é necessária, sendo a catálise enzimática microbiana a forma mais eficaz. O objetivo deste trabalho foi constituir um consórcio microbiano, por meio de enriquecimento utilizando glifosato como única fonte de carbono e sua caracterização por metataxonômica (gene codificador do rRNA 16S) e metagenômica (abordagem por *binning* genômico). Além disso, buscou-se avaliar o potencial desses consórcios na degradação do glifosato em microcosmos de solo, utilizando a atividade microbiana como indicador indireto do processo. A amostragem de solo ocorreu em lavouras de Café Conilon e Arábica do Estado do Espírito Santo, submetidas a manejos distintos de plantas daninhas. Foram retiradas alíquotas de solo e das unidades experimentais do enriquecimento para análise de sucessão das comunidades de microrganismos, por abordagens utilizando sequenciamento massivo de DNA. Além disso, foi utilizado protocolo de recuperação de genomas dos consórcios bacterianos estabelecidos, seguido pela anotação de genes que codificam enzimas-chave envolvidas na degradação do glifosato e do ácido aminometilfosfônico (AMPA). Nos microcosmos, a produção de CO_2 foi monitorada por 140 horas para avaliar a atividade catabólica em solos inoculados com os consórcios e controles não inoculados. Análises do gene 16S rRNA foram realizadas para investigar as alterações na estrutura da comunidade microbiana ao fim das 140h. O processo de enriquecimento revelou alterações dinâmicas na composição da comunidade microbiana ao longo do tempo, com *Achromobacter* sp. e *Serratia* sp. se destacando por possuírem o repertório metabólico necessário para a mineralização de glifosato e AMPA. Os consórcios bacterianos testados demonstraram alta atividade catabólica, especialmente os que foram cultivados em glifosato como fonte de fosfato antes da inoculação nos microcosmos de solo. A análise de abundância diferencial revelou aumentos significativos em *Achromobacter* e *Serratia* nos solos inoculados em comparação aos controles, juntamente com outros gêneros envolvidos na degradação do herbicida, como *Arthrobacter*, *Paenarthrobacter* e *Pseudarthrobacter*. Esses resultados sugerem o potencial do consórcio T6 para remediação de solos contaminados por glifosato, embora sejam necessários testes de segurança ambiental.

Palavras-chave: biodegradação; sequenciamento; 16S rRNA; microrganismos edáficos; herbicida.

ABSTRACT

Glyphosate, a widely used non-selective herbicide, can affect non-target plants and soil ecosystems, raising environmental and toxicity concerns. Therefore, remediation of contaminated areas is essential, with microbial enzymatic catalysis being the most effective approach. This study aimed to establish a microbial consortium through enrichment using glyphosate as the sole carbon source and to characterize it through metataxonomic (16S rRNA gene) and metagenomic (genomic binning approach) methods. Additionally, the study sought to evaluate the degradation potential of these consortia in soil microcosms, using microbial activity as an indirect indicator of the degradation process. Soil samples were collected from Conilon and Arabica coffee plantations in Espírito Santo, Brazil, which employed different weed management practices. Aliquots from soil samples and enrichment units were collected for analysis of microbial community succession, utilizing high-throughput DNA sequencing approaches. Furthermore, genome recovery protocols were applied to the established bacterial consortia, followed by annotation of genes encoding key enzymes involved in glyphosate and AMPA degradation. In the microcosms, CO_2 production was monitored over 140 hours to assess the catabolic activity in soils inoculated with the consortia and in non-inoculated controls. 16S rRNA gene analyses were conducted to investigate changes in microbial community structure after 140 hours. The enrichment process revealed dynamic alterations in the microbial community composition over time, with *Achromobacter* sp. and *Serratia* sp. standing out for possessing the metabolic repertoire required for glyphosate and aminomethylphosphonic acid (AMPA) mineralization. The tested bacterial consortia demonstrated high catabolic activity, especially those cultured with glyphosate as a phosphate source before inoculation in the soil microcosms. Differential abundance analysis showed significant increases in *Achromobacter* and *Serratia* in inoculated soils compared to controls, alongside other herbicide-degrading genera, such as *Arthrobacter*, *Paenarthrobacter*, and *Pseudarthrobacter*. These findings suggest the potential of the T6 consortium for the remediation of glyphosate-contaminated soils, though environmental safety tests remain necessary.

Keywords: biodegradation; sequencing; 16S rRNA; soil microorganisms; herbicide.

Impactos sociais, tecnológicos, econômicos e culturais

O crescimento populacional aumenta a demanda por solos produtivos, intensificando a necessidade de um gerenciamento sustentável da terra. Nesse cenário, o desenvolvimento de tecnologias verdes tem ganhado destaque, especialmente no que diz respeito à bioprospecção microbiana, que desempenha um papel crucial em práticas agrícolas sustentáveis. Este trabalho possui impacto tecnológico ao identificar consórcios bacterianos para a degradação do glifosato, que têm potencial para serem aplicados como bioinoculantes. Esses bioinoculantes não apenas favorecem o desenvolvimento vegetal, mas também contribuem para a remoção de resíduos de herbicidas do solo e das plantas, tornando a agricultura mais segura e ecologicamente responsável. Os impactos do estudo enquadram-se na área temática "Meio ambiente" da Política Nacional de Extensão, sendo diretamente alinhados às políticas sociais voltadas à preservação e recuperação ambiental. Além disso, o trabalho está em sintonia com os Objetivos de Desenvolvimento Sustentável (ODS) da Organização das Nações Unidas (ONU), especificamente com o objetivo "Fome zero e agricultura sustentável". Assim, este estudo apoia a Agenda 2030, ao contribuir para a restauração de ecossistemas e a mitigação dos impactos ambientais dos agroquímicos. Em resumo, esta pesquisa oferece soluções inovadoras para a agricultura no Brasil, incentivando práticas de manejo que atendem às demandas alimentares sem comprometer a saúde do solo e do meio ambiente.

Social, technological, economic, and cultural impacts

The growing population increases the demand for productive land, intensifying the need for sustainable land management. In this context, the development of green technologies has gained prominence, particularly in microbial bioprospecting, which plays a crucial role in sustainable agricultural practices. This study has a technological impact by identifying specific microbial consortia capable of degrading glyphosate, which can be applied as bioinoculants. These bioinoculants not only support plant development but also contribute to the removal of herbicide residues from soil and plants, making agriculture safer and more environmentally responsible. The impacts of this study fall under the “Environment” thematic area of the National Extension Policy, directly aligning with social policies focused on environmental preservation and recovery. Furthermore, this work aligns with the United Nations Sustainable Development Goals (SDGs), specifically with the goal “Zero Hunger and Sustainable Agriculture”. In doing so, this study supports the 2030 Agenda by aiding ecosystem restoration and mitigating the environmental impacts of agrochemicals. In summary, this research offers innovative solutions for agriculture in Brazil, promoting management practices that meet food demands without compromising soil and environmental health.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

O herbicida glifosato, amplamente utilizado em todo o mundo, foi lançado no mercado na década de 1970 e teve seu uso amplificado ao longo dos anos seguintes (Duke; Powles, 2008). Esse crescimento foi impulsionado, em grande parte, pelo uso de sintetases bacterianas insensíveis ao glifosato na engenharia genética de plantas tolerantes ao composto, permitindo sua aplicação em pós-emergência, o que aumentou significativamente sua comercialização (Benbrook, 2016). Embora o glifosato seja aplicado principalmente para eliminar ervas daninhas, ele pode expor outras plantas, invertebrados, microrganismos e até mesmo animais, por diversos meios e com diferentes graus de toxicidade. Essa ampla exposição gera preocupações sobre seus possíveis impactos ambientais e na saúde humana, tanto durante a fabricação, transporte e aplicação do herbicida (Gandhi *et al*., 2021). Após a aplicação, fatores como chuvas, vento, escoamento superficial e erosão podem transportar resíduos de glifosato para solo, água e alimentos, aumentando a necessidade de biorremediar ambientes contaminados (Schrübbers *et al*., 2016).

Diante desses desafios, a biorremediação com espécies microbianas que possuem potencial de degradação do glifosato surge como uma solução viável. Como exemplo, microrganismos como *Pseudomonas* sp. 4ASW e *Ochrobactrum intermedium* Sq20 são capazes de degradar o herbicida (Dick; Quinn, 1995; Kryuchkova *et al*., 2014; Firdous *et al*., 2017). Essas características destacam essas espécies como promissoras para o desenvolvimento de inoculantes. Para além de espécies isoladas, consórcios microbianos têm ganhado atenção em estudos de biofertilização e biorremediação, em decorrência da sua capacidade de promover resultados mais eficazes. Uma meta-análise comparou os efeitos de inoculantes bacterianos nessas aplicações, revelando que os consórcios foram significativamente melhores do que os tratamentos com espécies únicas. Enquanto cepas individuais aumentaram o crescimento das plantas em 29%, os consórcios alcançaram um aumento médio de 48%. Na biorremediação de metais pesados, os consórcios levaram a um aumento de 80% na eficácia, em comparação com um aumento de 48% com a inoculação de espécies únicas (Liu; Mei; Salles, 2023). De forma semelhante, a degradação de pesticidas pode ser ainda mais eficiente quando realizada por consórcios microbianos. Esses foram capazes de degradar até 80% de uma mistura dos

pesticidas agramina, atrazina, carbofuran, diazinon e glifosato, comparado a 50% quando os microrganismos foram isolados (Góngora-echeverría *et al*., 2020)

Com o avanço da bioinformática e a redução dos custos de sequenciamento de DNA, houve aumento nas pesquisas voltadas à investigação das características genômicas e funcionais de ecossistemas microbianos. Esses estudos, focados em genes e produtos-chave das vias metabólicas, têm impulsionado o desenvolvimento biotecnológico (Lemos *et al*., 2021). No entanto, as tecnologias de sequenciamento de primeira e segunda geração, embora revolucionárias na caracterização de microrganismos, apresentam limitações na montagem de genomas completos, processando apenas fragmentos curtos de DNA (<300 pb) (Ruppert; Kline; Rahman, 2019). Para superar essas limitações, são empregadas tecnologias de terceira geração como o sequenciamento de nanoporos, que permite a obtenção de genomas completos e a anotação de genes codificadores de enzimas-chave na degradação do glifosato (Kchouk *et al*., 2017).

O desenvolvimento de novos produtos e processos biotecnológicos tem se mostrado estratégia eficaz e sustentável para enfrentar os desafios crescentes relacionados à degradação do solo e ao impacto ambiental. Assim, este projeto teve como objetivo estabelecer o cultivo de consórcios microbianos degradadores de glifosato e caracterizá-los por meio de análises taxonômicas e funcionais, com foco na compreensão dos mecanismos envolvidos na degradação do herbicida. Além disso, buscou-se explorar o potencial desses consórcios para a biorremediação de solos contaminados, avaliando sua atividade em microcosmos de solo e sua capacidade de se estabelecer na comunidade microbiana nativa.

2 REFERENCIAL TEÓRICO

2.1 O solo como habitat microbiano

O solo é um importante recurso natural que hospeda biodiversidade dinâmica, distribuída ao longo de paisagens inteiras e também em micronichos. Microrganismos como bactérias, fungos, arqueias, algas e nematoides são a forma de vida mais abundante desse ambiente, podendo ser benéficos ou patogênicos. Tal diversidade microbiana é situada em diferentes tipos de solo, como os aluviais, argilosos, pretos, lateríticos e vermelhos, podendo também estar presente em habitats extremos, como solos salinos, ácidos, alcalinos, contaminados com agentes químicos, entre outros (Curtis; Sloan; Scannell, 2002; Yadav *et al*., 2021).

A distribuição e ocorrência desses microrganismos dependem de diversos fatores ambientais e intrínsecos. Assim, variáveis como a aeração, pH, umidade, temperatura, mineralogia, acúmulo de agentes químicos e presença de microrganismos antagonistas exercem influência sobre as comunidades microbianas do solo (Santoyo *et al*., 2017). Nesse sistema dinâmico, considerado a principal matéria-prima da agricultura, ocorrem interações entre fatores químicos, físicos e biológicos. O equilíbrio entre essas esferas rege a qualidade do solo, definida como a capacidade desse em sustentar a produtividade animal e vegetal, mantendo ou melhorando a qualidade da água, do ar e da saúde humana (Karlen *et al*., 1997; Vezzani; Mielniczuk, 2009; Andrade *et al*., 2021).

Dessa forma, além da avaliação de parâmetros físicos e químicos, há um grande potencial em avaliar a fração microbiológica do solo como fator de qualidade, pois microrganismos representam indicadores sensíveis às mudanças ocasionadas nesse sistema (Moreira; Siqueira, 2006). Alguns atributos microbiológicos comumente avaliados e relacionados à produtividade de plantas são a biomassa microbiana, o quociente metabólico, as atividades das enzimas urease, β-glucosidase e arilsulfatase e a hidrólise de diacetato de fluoresceína (Totola; Chaer, 2002; Silva Aragão *et al*., 2020). Ademais, a abundância e a diversidade genética microbianas presentes na rizosfera, endosfera e episfera das plantas são também avaliadas nos estudos de produtividade das plantações, pois muitos microrganismos são capazes de estabelecer relações simbióticas com as plantas (Chen *et al*., 2020).

É importante destacar que, para manutenção da sobrevivência, microrganismos do solo se organizam em comunidades microbianas que podem apresentar vantagens como a alta

diversidade metabólica. Essas comunidades consistem em grupos diferenciados de células que são altamente integrados e podem estabelecer interações sinérgicas que facilitam o surgimento de funções biossintéticas complexas. Assim, a utilização de diferentes substratos é otimizada em consórcios microbianos, pois há divisão de trabalho e maior resistência a perturbações do meio (Ibrahim; Raajaraam; Raman, 2021). A partir disso, tais consórcios podem ser aplicados para o bioprocessamento dos mais diversos substratos, como por exemplo, a decomposição dos componentes da biomassa lignocelulósica por diferentes organismos (Weiss *et al.*, 2021). Nessa perspectiva, deve-se explorar não somente o potencial biotecnológico de cepas únicas, como também de comunidades microbianas, voltadas para uma infinidade de funções na natureza (Borchert *et al*., 2021).

2.2 O potencial biotecnológico da microbiota dos solos agrícolas

O solo é um dos principais reservatórios da biodiversidade microbiana, principalmente em locais com considerável aporte de nutrientes, como a rizosfera de plantas ou ambientes que recebem carga de poluentes, fertilizantes ou pesticidas. Microrganismos do solo desempenham diversas funções no ecossistema, entre as quais destacam-se a ciclagem de nutrientes como nitrogênio, carbono, zinco, potássio e fósforo, além da produção de sideróforos para quelação de ferro e a liberação de fitormônios como citocinina, giberelina, auxina, ácido abscísico e etileno, importantes ao desenvolvimento vegetal (Kour *et al*., 2020; Yadav *et al*., 2021). Existem também papéis relacionados à proteção vegetal. Microrganismos da rizosfera podem controlar o crescimento de fitopatógenos por meio da síntese de metabólitos antimicrobianos, antibióticos, hormônios e enzimas (Ongena *et al*., 2007).

Práticas agrícolas inseguras e a rápida industrialização têm aumentado a poluição ambiental por agentes químicos como metais pesados, lixo nuclear, pesticidas e hidrocarbonetos. Consequentemente, a biodiversidade é exposta a esses agentes químicos, gerando efeitos deletérios à saúde humana e a outros organismos vivos. Como solução sustentável e de custo-benefício favorável, microrganismos do solo têm sido amplamente estudados por suas capacidades de metabolização de diversos contaminantes (Vasilyeva *et al*., 2020). Tais características fazem desses seres vivos a base de bioinoculantes, que podem combinar proteção vegetal e biorremediação de solos contaminados.

Microrganismos são base para insumos de interesse biotecnológico que, historicamente, têm sido explorados a partir de metodologias de cultivo e isolamento tradicionais. Porém, essas metodologias possuem limitações: dados da literatura afirmam que os microrganismos são o maior grupo da biodiversidade não descrita e que grande fração dessa diversidade atualmente só é acessível por meio de abordagens independentes de cultivo (Hug *et al*., 2016).

A partir dos anos 2000, as "ômicas" e o Sequenciamento de Alto Rendimento (HTS, em inglês) possibilitaram a bioprospecção molecular de espécies cultiváveis e não cultiváveis, gerando um volume significativo de dados moleculares anualmente. Para tornar essas informações acessíveis e promover a disseminação do conhecimento, as sequências genéticas recém-descobertas são inseridas em bancos de dados públicos e computadorizados, como o National Center for Biotechnology Information (NCBI). No Brasil, essa estrutura é complementada pela infraestrutura nacional de dados e informações em biodiversidade, representada pelo Sistema de Informação sobre a Biodiversidade Brasileira (SiBBr). Em paralelo, o projeto Microbioma Brasileiro busca compilar e disponibilizar a diversidade genética microbiana ainda em grande parte inexplorada no país (Pylro *et al*., 2014). Esse vasto potencial genético dos microrganismos, caracterizado por uma diversidade genética e metabólica, pode ser desvendado por métodos tradicionais e moleculares, oferecendo informações valiosas para diversas áreas científicas e contribuindo significativamente para a resolução de questões ambientais e econômicas globais.

2.3 O cultivo de café e o emprego de glifosato

No século XVIII, o português Francisco de Melo Palheta introduziu as primeiras mudas de café em solo brasileiro, inicialmente plantadas no estado do Pará. Em 1781, o plantio de café foi expandido para a região sudeste do país, dando início a um novo ciclo econômico na história do Brasil (Associação Brasileira Da Indústria De Café, 2020). O café é uma das principais *commodities* exportadas pelo Brasil. O país lidera o ranking de principal produtor, exportador e consumidor desse produto agrícola (ICO, 2016). Amplamente consumido no mundo, é estimado que mais de dois bilhões de xícaras dessa bebida são consumidas todos os dias e que o consumo de café aumentou em até 20 vezes entre 1952 e 2011.

O cafeeiro é uma planta perene que cresce em regiões de clima tropical, em áreas com temperaturas médias de 24 a 26 ◦C. É da família *Rubiacea* e pertence ao gênero *Coffea*, que

abrange mais de 70 espécies. Dentre essas, apenas duas são exploradas de forma comercial, no caso, *Coffea canephora var. conilon* e *Coffea arabica*, que correspondem a 23,6% e 76,4% da produção mundial, respectivamente (Mussatto *et al.*, 2011; Murthy; Madhava Naidu, 2012).

O manejo das plantações de café pode ocorrer a partir de dois modos: o convencional, como uma monocultura submetida à aplicação extensiva de pesticidas e fertilizantes sintéticos e por meio da agricultura orgânica, que busca desenvolver a produção a partir de métodos baseados na ecologia natural do sistema, não havendo uso de produtos sintéticos.

O controle de ervas daninhas no cafeeiro é realizado durante todo o ano, principalmente na estação chuvosa, que favorece o crescimento dessas plantas invasoras (Staver *et al.*, 2001). Esse controle é feito em sua maioria mediante herbicidas como o glifosato (N- (fosfonometil-glicina), agrotóxico mais comercializado atualmente no Brasil. Segundo Bombardi (2017), os estados de São Paulo, Minas Gerais, Bahia e Mato Grosso do Sul têm uma média de consumo de glifosato entre 5 e 9 kg por hectare, enquanto Mato Grosso, Paraná, Goiás e Rio Grande do Sul se enquadram entre 9 e 19 kg por hectare.

Segundo Schrübbers *et al*. (2016), plantas como o cafeeiro possuem metabolismo limitado de glifosato, além da possibilidade de ocorrência de translocação contínua desse composto das folhas diretamente expostas para as folhas mais novas. Isso pode causar uma redução no rendimento das plantas jovens. Além disso, a aplicação excessiva de defensivos agrícolas nas plantações e os efeitos da deriva da pulverização desses em outros ambientes contribuem para a contaminação do solo, água ou ar.

De acordo com Boesten (2016), resíduos de pesticidas ligados ao solo podem representar até um terço da massa total aplicada, o que representa cerca de 1 kg ha^{-1} ano^{-1} no campo agrícola. Em vista disso, é fundamental considerar os potenciais riscos que o uso desse herbicida representa para a saúde humana e para o meio ambiente, reforçando a necessidade de práticas agrícolas mais sustentáveis e de políticas de manejo que minimizem seus impactos.

2.3.1 Impactos ambientais em decorrência do uso de glifosato

Progressivamente, evidências mostram que os herbicidas à base de glifosato podem afetar a saúde humana e ambiental, apontando a necessidade de mais vigilância. Assim, países em todo mundo estão banindo ou restringindo o uso desse agroquímico (Gandhi *et al*., 2021).

Os herbicidas de glifosato são vendidos na forma de sais solúveis ou grânulos contendo aditivos e surfactantes. Apesar desses tipos de formulação aumentarem a solubilidade do

composto em água e a absorção desse nas plantas, essas tornam o herbicida mais tóxico (Gandhi *et al*., 2021). Por exemplo, o surfactante polioxietileno amina, presente na composição do herbicida Roundup®, foi apresentado como altamente tóxico a peixes e invertebrados de água doce (Giesy *et al*., 2000; Tsui; Chu, 2003).

A aplicação desses agroquímicos pode ocasionar mudanças na composição das comunidades bacterianas e fúngicas do solo, podendo resultar no crescimento de fitopatógenos resistentes ao composto como espécies de *Fusarium* spp. causadoras de podridão radicular (Rosenbaum *et al*., 2014). De forma semelhante *Staphylococcus aureus*, patógeno humano e animal, é também resistente ao composto, podendo tornar-se mais dominante em solos onde há acúmulo de glifosato (Funke *et al*., 2007).

Além disso, trabalhos recentes expuseram que a composição da microbiota intestinal das abelhas é modificada em razão da exposição ao glifosato puro e glifosato comercial, o que pode tornar esses insetos mais susceptíveis a infecções por patógenos (Motta *et al*., 2018; Motta *et al*., 2020; Motta & Moran, 2020). Ademais, também foi indicado que o glifosato prejudica as capacidades cognitivas necessárias para o inseto voar com sucesso de volta à colmeia, indicando um efeito negativo do herbicida no comportamento das abelhas (Balbuena *et al*., 2015).

Em relação à saúde humana, a alta exposição de pessoas ao glifosato ocorre majoritariamente nas populações que trabalham no campo ou vivem no entorno de áreas agrícolas e fábricas de manufatura e processamento desse herbicida. Nesse contexto, esses indivíduos possuem risco aumentado em desenvolver linfoma não-Hodgkin e outros tipos de câncer (Schinasi *et al*., 2014; Zhang *et al*., 2019). Além disso, estudos recentes indicam que a intensificação agrícola, como a expansão da produção de soja no Brasil, tem sido associada a um aumento na incidência de câncer infantil, como a leucemia pediátrica, possivelmente devido à exposição a pesticidas através da contaminação do abastecimento de água (Skidmore *et al*., 2023).Também foi relatado que, pelo fato de o glifosato ser um quelante de metal, inúmeros problemas de saúde poderiam surgir em uma população sujeita à exposição cumulativa desse herbicida, como infertilidade, defeitos congênitos, transtorno de ansiedade, disfunção da tireoide, doença de Alzheimer, entre outros (Samsel; Seneff, 2015).

Nessa perspectiva, cabe analisar o Limite Máximo de Resíduo (LMR), que representa a maior quantidade de resíduo de agrotóxico oficialmente aceita no alimento. No Brasil, o LMR permitido de glifosato no café é de 1 $mg.kg^{-1}$, dez vezes maior do que aquele permitido na

União Europeia (0,1 $mg.kg^{-1}$). Isso indica grande assimetria entre o uso de glifosato nas duas regiões e revela uma contradição ao constatar que parte desses resíduos do herbicida chegam aos países europeus por meio do alimento importado (Bombardi, 2017).

Dessa forma, em decorrência dos riscos atribuídos ao glifosato, é importante conhecer as implicações do uso desse herbicida a curto e longo prazo na saúde humana, nos organismos não-alvo e como são impactadas as comunidades microbianas dos mais diversos ecossistemas em que o herbicida pode ser encontrado.

2.3.2 Degradação microbiana de glifosato

O modo de ação do glifosato é baseado no bloqueio da síntese dos aminoácidos aromáticos triptofano, tirosina e fenilalanina a partir da inibição da 5-enolpiruvil-chiquimato-3-fosfato (EPSP) sintase, envolvida na via do chiquimato. Essa via não está presente apenas em plantas, mas também em bactérias e fungos, o que torna muitos táxons de microrganismos sensíveis ao herbicida. Deste modo, a aplicação desse composto pode afetar a composição microbiana e a atividade enzimática na endosfera da planta, na rizosfera e no solo circundante (Druille *et al.*, 2015; Van Bruggen *et al*., 2018).

A aplicação desse herbicida modifica as comunidades microbianas do solo após aplicações repetidas, de forma a ocasionar mudanças na composição de grupos bacterianos envolvidos em processos-chave para a ciclagem de C e N (Allegrini *et al*., 2017). São também reduzidas as atividades das enzimas beta-glicosidase e fosfatase com a aplicação do produto ao longo do tempo (Tejada, 2009).

À medida que os solos receberam glifosato, temporada após temporada e ano após ano, esses ambientes se tornaram locais de incidência de microrganismos degradadores desses agroquímicos (Ramakrishnan *et al.*, 2018). Isolados de locais contaminados, microrganismos como bactérias gram-positivas, negativas e fungos são capazes de degradar glifosato, assim como algumas espécies de plantas também o fazem (Duke *et al*., 2011; Benslama; Boulahrouf, 2013; Vemanna *et al*., 2017; Elarabi *et al*. 2020).

Acredita-se que as vias de degradação de glifosato nesses microrganismos estejam envolvidas na proteção celular contra fosfonatos de ocorrência natural, bem como no acesso ao fósforo, nitrogênio e carbono presentes nesses compostos (Metcalf; Van Der Donk, 2009; Hertel *et al*., 2021). Os principais mecanismos para a degradação de glifosato e dos seus

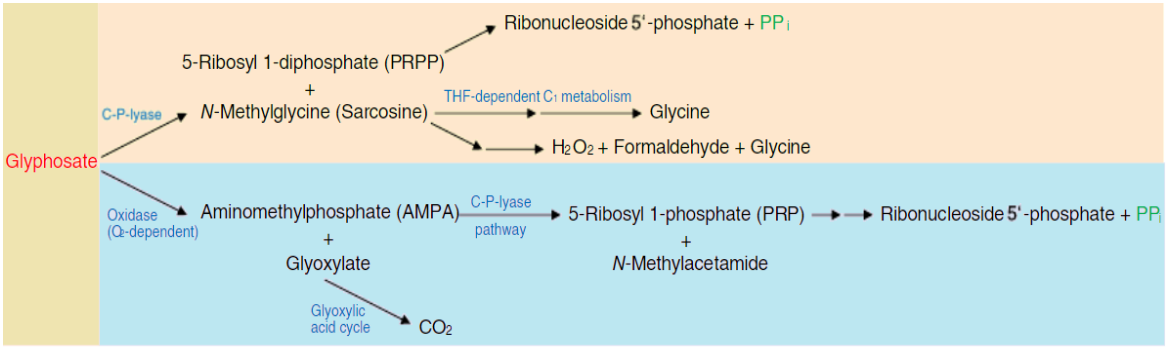
produtos secundários são a mineralização e o co-metabolismo por microrganismos como as bactérias (Ye *et al*., 2018; Ibrahim, Sevakumaran, Ariffin, 2023).

Como exemplos, *Alcaligenes* sp. GL, *Arthrobacter* sp. GLP-1, *Bacillus cereus* CB4, *Comamonas odontotermitis* P2, *Enterobacter* sp. Bisph2, *Flavobacterium* GD1, *Geobacillus caldoxylosilyticus* T20, *Pseudomonas pseudomallei* 22 foram documentadas como bactérias consumidoras de glifosato como única fonte de fósforo (Balthazor; Hallas, 1986; Penaloza-Vazquez *et al.*, 1995; Obojska *et al*., 2002; Benslama; Boulahrouf, 2016; Firdous *et al*., 2017). Há também bactérias que utilizam o glifosato como fonte de nitrogênio e carbono, tal como *Achromobacter* sp. LW9, *Agrobacterium radiobacter* SW9, *Ochrobactrum intermedium* Sq20 e *Ochratomyces* sp. (MCAuliffe; Hallas; Kulpa, 1990; Firdous *et al*., 2018; Firdous *et al*., 2020). A degradação bacteriana do glifosato é amplamente documentada, mas há relativamente poucos estudos sobre a degradação desse herbicida por fungos. No entanto, as pesquisas realizadas até o momento sugerem que esses microrganismos apresentam grande potencial para a biorremediação de ambientes contaminados com glifosato (Guo *et al*., 2022).

A taxa de degradação microbiana de compostos orgânicos no solo depende de fatores físico-químicos do ambiente, da natureza do substrato a ser catabolizado, bem como da acessibilidade da célula microbiana e de suas enzimas a esse (Moreira; Siqueira, 2006). Assim, o microrganismo capaz de degradar o glifosato deve apresentar um sistema de transporte para a absorção do composto, assim como de um sistema enzimático com afinidade para o mesmo (Hove-Jensen; Zechel; Joshimsen, 2014).

A biodegradação do glifosato geralmente ocorre por meio de duas vias principais. Na primeira, a enzima C-P liase degrada o glifosato em N-metilglicina (sarcosina), que é posteriormente convertida em formaldeído e glicina por meio da ação da sarcosina oxidase. O processo envolve o transporte e a clivagem do glifosato por um conjunto de 14 proteínas codificadas no operon *phn*, resultando na formação de 5-fosforibosil 1-difosfato (PRPP), que libera fósforo inorgânico e sarcosina, a qual pode ser oxidada pela sarcosina oxidase (Figura 1). A segunda via é iniciada por meio da clivagem C-N a partir da ação de oxidases com especificidade para o glifosato, como a glifosato oxidorredutase e a enzima glicina oxidase. O herbicida é então metabolizado em glioxilato e ácido aminometilfosfônico (AMPA). Esse metabólito pode ser posteriormente convertido pelas enzimas da via da C-P liase, liberando o fosfato de 5-ribosil 1-fosfato (PRPP) (Figura 1).

Figura 1 – A degradação microbiana de glifosato por meio de duas vias distintas



Fonte: Hertel *et al.* (2021)

Existem diferentes opiniões sobre qual via de degradação é predominante nos ambientes naturais. Hove-Jensen, Zechel e Jochimsen (2014), bem como Hertel *et al*. (2021), sugerem que a via da C-P liase é a mais comum, enquanto a outra via, iniciada pelas enzimas glifosato óxido-redutase e glicina oxidase, é ativada em ambientes com altas concentrações de glifosato ou ácidos fosfônicos, como uma resposta adaptativa para desintoxicação. Por outro lado, Sviridov *et al*. (2015) argumenta que, embora a via da sarcosina seja a única capaz de liberar fósforo inorgânico a partir do glifosato, a clivagem da ligação C-P depende das concentrações de Pi exógeno e endógeno. Isso sugere que a clivagem é induzida sob condições de deficiência de fósforo, o que pode não ser comum em muitos ambientes naturais.

Apesar dessas divergências, é amplamente reconhecido que uma variedade de microrganismos participa da degradação de glifosato, e ainda há muito a explorar sobre a diversidade de vias enzimáticas de degradação e seus mecanismos de regulação (Puglisi *et al*., 2007; Ermakova, Shushkova, Leont'evskii, 2008; Shushkova *et al*., 2012).

2.4 Microbiota dos solos em cultivos de café

Dois tipos de microbiota estão associados ao cafeeiro: a microbiota nativa, que vive em estreita associação com o cafeeiro na rizosfera, episfera e endosfera, e a microbiota proveniente da pós-colheita das cerejas de café, envolvida nos processos de fermentação. No que se refere à microbiota nativa, gêneros bacterianos como *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Serratia* e *Streptomyces*, além de fungos como *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium* e *Trichoderma*, são capazes de colonizar e dominar esses três compartimentos (Duong *et al*., 2020).

A composição da microbiota no cultivo do café pode ser influenciada por uma série de fatores bióticos e abióticos, incluindo as características físico-químicas do solo, alterações climáticas e microclimáticas, a topografia, o compartimento da planta analisado e o genótipo do cafeeiro (Fierer, 2017; Mina *et al*., 2020; Veloso *et al*., 2020).

Além disso, o manejo do solo tem papel fundamental na dinâmica dessa microbiota. Caldwell *et al*. (2015) investigaram como diferentes manejos de solo afetam a diversidade e composição das comunidades microbianas procarióticas na rizosfera do café. Usando sequenciamento massivo, os autores analisaram amostras de três fazendas com diferentes tipos de manejo (intensivo, de transição e orgânico). A análise revelou 48 filos bacterianos e 3 filos de arqueias, com bactérias predominando (98% das sequências) em relação às arqueias (2%). O filo *Proteobacteria* foi o mais dominante, seguido por *Acidobacteria* e *Actinobacteria*, enquanto o domínio *Thaumarchaeota* representou a maioria das sequências de arqueias. Embora houvesse semelhanças taxonômicas entre os locais, cada fazenda apresentou um perfil de estrutura da comunidade microbiana distinto. A hipótese de que fazendas orgânicas teriam maior diversidade microbiana não foi confirmada, já que essa fazenda apresentou os menores níveis de riqueza e diversidade. Os autores levantam a hipótese de que fatores como alta densidade de plantas ou o uso de fertilizantes em fazendas intensivas e de transição possam ter influenciado esses resultados. Esses resultados e outros similares devem ser avaliados com cautela, visto que o sequenciamento massivo de DNA baseado no gene 16S tem uma limitação importante: ele tende a acessar principalmente os grupos microbianos dominantes nas amostras analisadas. No entanto, em condições de distúrbio ou perturbação ambiental, essa dominância pode ser alterada, o que permite ao método detectar grupos microbianos previamente pouco abundantes.

Sternhagen *et al*. (2020) também exploraram o impacto ambiental nos sistemas de manejo convencional e orgânico, focando nas comunidades de fungos da rizosfera do café. Foi observado que os fungos saprotróficos e micorrízicos arbusculares foram os mais diversos, enquanto os saprotróficos e fitopatógenos foram os mais abundantes. A diversidade fúngica foi menor em campos com manejo convencional, possivelmente em razão do uso de fungicidas, menor sombreamento, baixa riqueza de árvores e alterações nos nutrientes do solo, como maior disponibilidade de nitrato e menor de magnésio. Além disso, os campos convencionais apresentaram menor diversidade de fungos saprotróficos, fitopatogênicos e micoparasitários, sugerindo uma possível alteração na estrutura trófica desses ambientes.

O manejo do solo e as práticas agrícolas têm impactos profundos na microbiota associada ao cafeeiro, influenciando a diversidade e a composição microbiana. A compreensão dessas dinâmicas é essencial para otimizar práticas de cultivo que promovam a sustentabilidade e a saúde do agroecossistema, além de melhorar o desempenho das plantas e a qualidade do produto.

2.4.1 Estratégias para o estudo da microbiota cafeeira

O primeiro estudo sobre microrganismos presentes na planta de café foi publicado em 1897 por Janse, que descreveu a colonização das raízes de *Coffea arabica* e *Coffea liberica* por fungos micorrízicos arbusculares (FMA). Desde então, surgiram diversos trabalhos focados no isolamento e purificação de microrganismos, utilizando métodos dependentes de cultivo, nos quais a cepa é caracterizada por meio de análises morfológicas e testes bioquímicos (Pederson; Breed, 1946; Teshome *et al*., 2017).

Com o passar dos anos, métodos bioquímicos mais sofisticados foram aplicados à caracterização de microrganismos, como a Eletroforese Enzimática Multilocus (MLEE) para estudos de bactérias fixadoras de nitrogênio, a cromatografia gasosa de ésteres metílicos de ácidos graxos (FAME-GC) para isolados bacterianos, e, mais recentemente, a ionização/dessorção de Matriz Assistida por Laser com Tempo de Vôo/Espectrômetro de Massa (MALDI-TOF-MS) para leveduras e bactérias (Jimenez-salgado *et al*., 1997; Miguel *et al*., 2013; Duong *et al*., 2020; Martins *et al*., 2020).

O desenvolvimento da biologia molecular, especialmente ao final do século XX, impulsionou pesquisas sobre a diversidade funcional de microrganismos, graças ao surgimento dos sequenciadores de DNA de primeira geração. Com a introdução de métodos de sequenciamento massivo de DNA (HTS, em inglês), a quantidade de leituras de alta qualidade a partir de amostras contendo misturas de espécies não identificadas aumentou significativamente. Essa abordagem, conhecida como metagenômica, combina sequenciamento de alto desempenho com identificação taxonômica e funcional baseada em DNA (Ruppert; Kline; Rahman, 2019).

Na caracterização molecular de microrganismos, são amplificados, sequenciados e depositados em bancos de dados sequências de genes que codificam o RNA ribossômico. Os genes que codificam o rRNA 16S, que são altamente conservados e contêm regiões muito variáveis, são marcadores amplamente utilizados para inferências filogenéticas de procariotos. No

entanto, sua principal limitação reside na baixa resolução taxonômica em nível de espécie para alguns grupos bacterianos (Santos *et al*., 2020).

Para identificar microrganismos eucariontes, o sequenciamento da região do espaço interno transcrito (ITS) é frequentemente empregado. Essa região, situada entre os genes 18S e 28S do rDNA, é altamente conservada intraespecificamente, mas variável entre diferentes espécies. A ocorrência de múltiplas cópias dessa região no genoma nuclear é uma limitação adicional para seu uso como marcador, em decorrência da potencial variação intragenômica (Coleman, 2007).

Os métodos moleculares ampliaram substancialmente a biodiversidade da árvore da vida, uma vez que superaram a necessidade de cultivar os microrganismos. Na abordagem independente de cultivo, o DNA é extraído, amplificado, preparado em bibliotecas e sequenciado por diferentes plataformas de sequenciamento massivo de DNA, como Illumina e Ion Torrent (Duong *et al*., 2020; Santos *et al*., 2020).

É importante ressaltar que existem pontos fortes e fracos associados às estratégias dependentes e independentes de cultivo. A abordagem dependente do cultivo permite o isolamento de microrganismos, o que viabiliza sua pronta aplicação em bioprocessos. No entanto, essa abordagem é mais laboriosa e apresenta uma capacidade limitada para abranger toda a diversidade microbiana (Duong *et al*., 2020). Em contrapartida, a abordagem independente de cultivo proporciona rapidez e eficiência ao investigar uma ampla biodiversidade que é difícil de cultivar ou que não pode ser cultivada em condições laboratoriais. Essa estratégia pode revelar a composição taxonômica de comunidades microbianas, seus potenciais funcionais, bem como todo o arcabouço metabólico (Addis *et al*., 2016).

Dessa maneira, observa-se que as abordagens mencionadas são complementares e, juntas, proporcionam melhor compreensão das interações estabelecidas entre os microrganismos e o café. Assim, é essencial fortalecer os conhecimentos teórico-práticos sobre esses aspectos, permitindo que a comunidade científica desenvolva estratégias de manipulação que explorem os recursos microbianos de forma eficaz e sustentável.

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SEGUNDA PARTE

Artigo 1

Glyphosate-degrading consortia: Microbial succession and key enzyme gene insights

Artigo elaborado conforme periódico mLife

Mariana Prósperi de Oliveira Paula[1], Alessandro Coutinho Ramos[2], Luiz Fernando Wurdig Roesch[3], Victor Satler Pylro[1,*]

[1,*]Laboratório de Ecologia Microbiana e Bioinformática, Departamento de Biologia, Universidade Federal de Lavras, Lavras, MG, Brasil. Laboratório de Microbiologia Ambiental e Biotecnologia, Universidade Vila Velha, Vila Velha, ES, Brasil[3]Institute of Food and Agricultural Sciences, Department of Microbiology and Cell Science, University of Florida, Gainesville, FL, US. *Correspondence: Victor Satler Pylro, victor.pylro@ufla.br

ORCID:

Mariana Prósperi de Oliveira Paula: 0000-0002-5917-8984
Victor Satler Pylro: 0000-0003-2154-9150
Luiz Fernando Wurdig Roesch: 0000-0003-1450-8828
Alessandro Coutinho Ramos: 0000-0002-4082-0076

Abstract

Glyphosate is a widely used non-selective herbicide with significant environmental and health concerns, highlighting the urgent need for effective remediation strategies. Among the available technologies, microbial enzyme-catalyzed degradation offers a more sustainable and practical solution. This study aimed to enrich and characterize microbial consortia from soils in coffee plantations in Espírito Santo, Brazil, exposed to various weed management practices. The enrichment process, followed by metataxonomic and metagenomics analysis, revealed dynamic shifts in the microbial community composition over time. Notably, *Achromobacter* and *Serratia* were identified as key genera, both harboring the complete metabolic repertoire necessary for glyphosate and AMPA mineralization. These results underscore the significant potential of these consortia for use in the bioremediation of glyphosate-contaminated environments.

Keywords: aminomethylphosphonic acid; biodegradation; bioprospection; coffee; C-P lyase; glyphosate oxidoreductase; herbicide; soil microbiota.

Impact Statement

This work uncovers microbial consortia from coffee plantation soils capable of degrading glyphosate, with *Achromobacter* and *Serratia* emerging as key genera. These genera possess complete metabolic pathways for glyphosate mineralization, offering potential for bioremediation applications in contaminated environments. Redundant metabolic capabilities suggest functional stability in diverse conditions, making these consortia promising candidates for field applications.

Keywords: Glyphosate degradation, microbial consortia, *Achromobacter*, *Serratia*, bioremediation.

1 Introduction

Widely used worldwide, the herbicide glyphosate began to be commercialized in the 1970s and followed an increasing number of uses in subsequent years. Bacterial synthetases insensitive to glyphosate in the genetic engineering of plants tolerant to the compound enabled their use post-emergence, significantly increasing their commercialization[1]. Although this herbicide is applied to eliminate weeds, other plants, invertebrates, microorganisms, and animals can be exposed to glyphosate. For these reasons, there is concern about the possible environmental and human health impacts caused during this product's manufacture, transportation, and application[2].

As soils accumulate pesticide residues, these environments have become hotspots for microorganisms that degrade these products[3]. Glyphosate degradation pathways in these microorganisms are believed to be involved in cellular protection against naturally occurring phosphonates and access to this compound's phosphorus, nitrogen, and carbon[4,5]. Bacterial genera such as *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Comamonas*, *Enterobacter*, *Flavobacterium*, *Geobacillus*, and *Pseudomonas* have been documented to degrade glyphosate as the sole source of phosphate [6,7,8,9,10]. Some genera use glyphosate as a nitrogen and carbon source, such as *Achromobacter*, *Agrobacterium*, *Ochrobactrum* and *Ochratomyces*[11,12,13].

Microbial glyphosate degradation happens by two distinct metabolic pathways: the sarcosine and AMPA (Aminomethylphosphonic acid). In the first one, the carbon-phosphorus bond of glyphosate is initially cleaved under the catalysis of C–P lyase, resulting in inorganic phosphate and sarcosine. The second one involves breaking the carbon-nitrogen bond by the

action of an oxidoreductase (glyphosate oxidoreductase or a glycine oxidase), yielding AMPA and glyoxylate as degradation products[14].

Soil microorganisms organize themselves into microbial communities with advantages such as high metabolic diversity. These communities consist of highly integrated groups of cells that frequently establish synergistic interactions, enabling the emergence of complex biosynthetic functions[15].

Furthermore, the population dynamics of microbial communities are strongly influenced by environmental context and the interactions among species, highlighting the intricate nature of microbial coexistence. This complexity enables multiple species to balance within a community, even though isolated pairs often exhibit competitive exclusion rather than coexistence. In a study of 12 stable bacterial communities, only 20.1% of isolated pairs coexisted under controlled conditions, whereas 71.5% displayed competitive exclusion, underscoring the importance of a broader community context for microbial balance[16]. Such interactions give rise to 'emergent properties' within ecosystems, representing functional patterns that cannot be deduced from individual components alone. These properties are vital for ecosystem functionality, supporting niche flexibility and the spatial organization that underpin resilience and resistance to environmental disturbances[17].

This complexity and interdependence within microbial communities provide a solid foundation for the superior efficiency of microbial consortia in processes such as the degradation of complex compounds. An example of this is a study where a microbial consortium achieved approximately 80% degradation of a mixture of pesticides, including agramine, atrazine, carbofuran, diazinon, and glyphosate, compared to the 50% degradation observed with isolated strains[18]

This work aimed to establish and characterize microbial consortia enriched from different soil sources, capable of degrading glyphosate. By profiling microbial communities and analyzing key genes involved in glyphosate degradation pathways, we sought to elucidate the ecological mechanisms driving this process. This comprehensive approach provides insights into the functional dynamics of these consortia and their potential role in mitigating glyphosate contamination in various soil environments.

2 Material and Methods

2.1 Soil sampling

Soil collections were carried out on coffee farms in Domingos Martins and Marechal Floriano, Espirito Santo, Brazil. In each collection area, three independent soil samples (0 to 20 cm deep) were collected along a transect, taking 30 meters of distance between the collection points. The soil samples were sieved (0.2 mm) and stored at 4 ^{o}C for microbial enrichment and at –20 ^{o}C for molecular analyses. According to each area where the soil samples were collected, we established seven treatments, as follows: Conventional Arabica Coffee, managed with glyphosate (AC), Organic Arabica Coffee (AO), Conventional Conilon Coffee (CC), Conilon Coffee without glyphosate application for 3 years (CC-G), Atlantic Forest adjacent to Conventional Arabica Coffee (MA), Weed present in the Conventional Arabica Coffee plantation (AC+W), and Weed present in the Conventional Conilon Coffee plantation (CC+W).

2.2 Enrichment culture

Firstly, 10 g of soil was added into Erlenmeyer flasks (250 mL) with 100 mL of culture media (pH 7) supplemented with glyphosate as a carbon source. The flasks were incubated on a shaker (150 rpm) at 25 °C for seven days. The culture media (GC) had the following composition (g.L^{-1}): 2 $(NH_4)2SO_4$, 0,2 $MgSO_2.7H_2O$, 0,01 $CaCl_2$, 1,5 KH_2PO_4, 1,5 $Na_2HPO_4.12\ H_2O$, 0,01 $FeSO_4.7H_2O$, and filtered (pore size 0,2 µm) commercial glyphosate (Roundup®) (8 g.L^{-1})[19]. The glyphosate concentration used falls within the range of 5 to 25 g.L^{-1}, reflecting typical field application rates for this herbicide[20]. After seven days, 10% (v/v) of the adaptation culture was transferred to Erlenmeyer flasks (500 ml) with 250 ml of GC media supplemented with commercial glyphosate (8 g.L^{-1}). These flasks were kept in the dark, shaking at 150 rpm, 27°C, for three weeks[10,21]. The enrichment experiment followed a completely randomized design, with plots divided by time. Seven soil treatments were assigned, each with three replicates. Sampling occurred at 192 and 480 hours, resulting in a total of 24 experimental units used to analyze the microbial community succession profile. After 480 hours of enrichment, the cultures were centrifuged at 9,000 rpm for 15 minutes to recover the consortium biomass. In addition to the GC medium, the recovered biomass was also inoculated in GP medium, where glyphosate served as the sole phosphorus source and glucose as the carbon source[22]. The composition of the GP medium was as follows (g.L^{-1}): 5 $C_6H_{12}O_6$, 2 $(NH_4)_2SO_4$, 0.2 $MgSO_4 \cdot 7H_2O$, 0.01 $CaCl_2$, 0.5 KCl, 0.5 NaCl, 0.001 $FeSO_4 \cdot 7H_2O$, and filtered (pore size

0,2 µm) commercial glyphosate (Roundup®) (8 $g.L^{-1}$). At the end of the incubation period, the biomass was recovered as above.

2.4 DNA extraction

First, genomic DNA from each microbial consortium sample was extracted and purified using the DNeasy PowerSoil Pro Kit (Qiagen), following the manufacturer's instructions. The purity of the extracted DNA was observed by spectrophotometry using the Nanodrop One equipment (Nanodrop Technologies, Wilmington, DE, USA) (260/280 nm), and quantification was performed with the Qubit® 4.0 fluorimeter, using Qubit® dsDNA HS Assay Kit (Invitrogen TM)[23].

2.5 Analysis of the taxonomic profile of the microbial community

The structure of the microbial communities in soil samples and microbial enrichment cultures were analyzed using metataxonomics. The 16S rRNA gene (hypervariable V4 region) and the ITS region were amplified using the primers 515F/806R and ITS1F/ITS2[24,25], respectively. According to the Ion Amplicon library preparation manual, PCR amplification was performed with barcodes attached to an A sequence from the Ion adapter and a trP1 sequence from the Ion adapter. PCR conditions were optimized for each primer set. The ITS region was amplified using the following protocol: 3 min at 96°C, followed by 30 cycles of 30 sec at 96°C, 45 sec at 45°C, and 1 min at 72°C, with a final extension of 7 min at 72°C[25]. For the 16S rDNA V4 region, PCR conditions were: 2 min at 95°C, followed by 30 cycles of 45 sec at 94°C, 45 sec at 56°C, and 1 min at 72°C, with a final extension of 10 min at 72°C[24].

Amplicons containing adapters and barcode sequences were purified using AMPureBeads (1.2×, Beckman Coulter). Emulsion PCR was performed using the Ion OneTouch 2™ system and the Ion Template PGM™ OT2 400 kit (Life Technologies). Sequencing of the amplicon libraries was conducted on an Ion 318™ Chip Kit v2 with the PGM Ion Torrent sequencer (Life Technologies). Post-sequencing, the PGM software filtered the Ion Torrent fastq files to remove polyclonal and low-quality sequences.

The operational taxonomic unit (OTU) table was generated using the UPARSE pipeline, following the Brazilian Microbiome Project recommendations[26]. Reads were truncated to 200 base pairs, and quality filtering allowed a maximum error rate of 1.0. Filtered reads were

dereplicated and clustered with a 97% similarity threshold, and representative sequences were obtained for each OTU[27]. Taxonomic classification was performed using QIIME with the UCLUST method, referencing the SILVA database (version 138). Alpha diversity was assessed using the Chao1 richness index, while beta diversity was calculated with the Bray-Curtis index [28, 29]. A Principal Coordinate Analysis (PCoA) was performed to visualize microbial community dissimilarity between samples, and a PERMANOVA test was applied to assess the statistical significance of the observed differences. Finally, the web-based tool MicrobiomeAnalyst was used to generate abundance and diversity plots, providing a comprehensive visual representation of microbial community composition and diversity dynamics[30].

2.6 Whole-genome sequencing

The DNA extracted from the consortia, which were enriched using glyphosate as a phosphorus source, underwent nanopore long-read sequencing. After quantification, we barcoded 200 ng of genomic DNA per sample using the Native Barcoding Kit 96 V14 (SQK-NBD 114.96). The final library was loaded onto an R10.4.1 (FLO-MIN106) flow cell and sequenced using a MinION device for 72 hours. The nanopore raw signal was processed into reads in real-time through Guppy. Barcodes were trimmed and reads with a q score <10 were filtered using Chopper[31]. Then, we followed the DIAMOND+MEGAN protocol[32], where the Unicycler pipeline was used for assembly and the Medaka tool for polishing. DIAMOND was employed to align the polished sequences against a protein database, using the frameshift-aware alignment mode. MEGAN used the interval-union LCA algorithm for the taxonomic binning of contigs by assigning reads to nodes in the NCBI taxonomy. These contigs were then retrieved from the MEGAN Community Edition 6.25.9 interface[33]. CheckM v1.0.18 was used to assess the completeness and contamination of the recovered metagenome-assembled genomes[34]. The nucleotide sequences were subsequently translated into amino acid sequences using the EMBOSS suite[35]. Protein annotations were conducted using both manual and online tools, including custom-built protein databases and KAAS (KEGG Automatic Annotation Server)[36]. The metagenomes were then blasted against these databases with an e-value threshold of 1E-8[37]. Finally, the recovered genomes were compared to reference genomes using ANI (Average Nucleotide Identity) through the JSpeciesWS tool[38], enabling precise prokaryotic species delineation via pairwise genome comparison.

3 Results

3.1 Diversity analysis of soil samples

Alpha diversity was measured using the Chao1 index, and the 16S rRNA alpha diversity analysis revealed significant differences in OTU richness among treatments (p-value: 7.3123e-07; [ANOVA] F-value: 26.356) (Figure 2). The CC-G treatment showed greater diversity compared to the CC treatment. This observed difference in taxon richness between these treatments is likely an indirect reflection of the disturbance caused by glyphosate management. The AC+W and CC+W treatments showed high diversity, indicating that there may be greater resistance stability of the weed microbiota, as higher microbial diversity is often associated with improved resilience and the ability of the community to withstand environmental disturbances. The Arabica coffee soil treatments, AC and AO, exhibited intermediate to high diversity, with both showing Chao1 indices ranging from 1000 to 1100. Lastly, the MA treatment presented the lowest diversity.

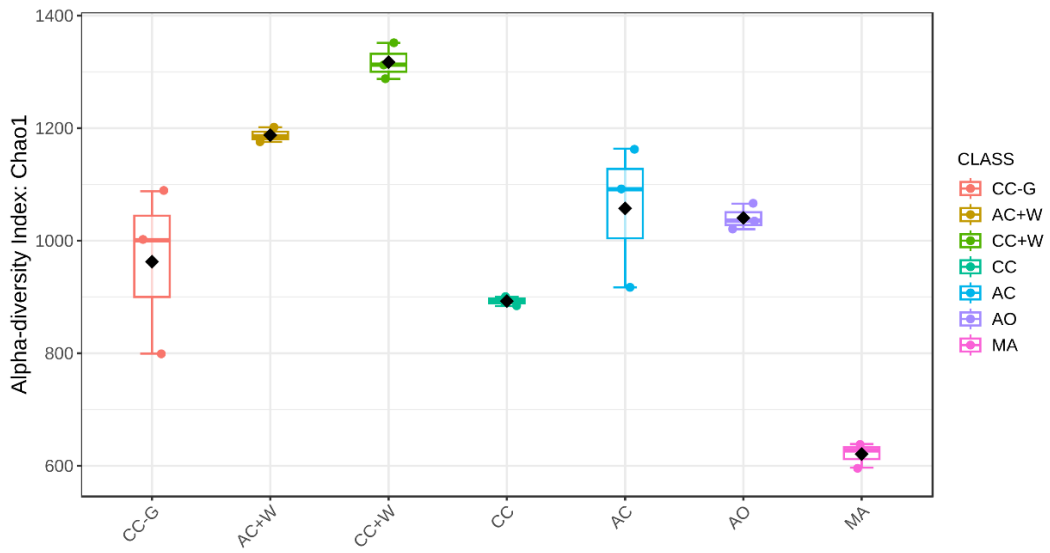

Figure 2 – Comparative Alpha diversity analysis (Chao1 index) based on 16s rRNA gene sequencing across different soil treatments. The treatments included: AC (Conventional Arabica Coffee, managed with glyphosate), AO (Organic Arabica Coffee), CC (Conventional Conilon Coffee), CC-G (Conilon Coffee without glyphosate application for 3 years), MA (Atlantic Forest adjacent to Conventional Arabica Coffee), AC+W (Weed present in the Conventional Arabica Coffee plantation), and CC+W (Weed present in the Conventional Conilon Coffee plantation).

Like bacterial alpha-diversity, the fungal alpha-diversity analysis showed significant differences between treatments (p-value: 0.00022032; [ANOVA] F-value: 9.9769)**.** All Arabica coffee treatments were more diverse than the Conilon coffee treatments, suggesting that the type of crop (Arabica vs. Conilon) may play an important role in fungal diversity. Additionally, MA presented diversity comparable to the AO treatment, indicating that organically managed systems can support diverse and potentially stable fungal communities (Figure 3).

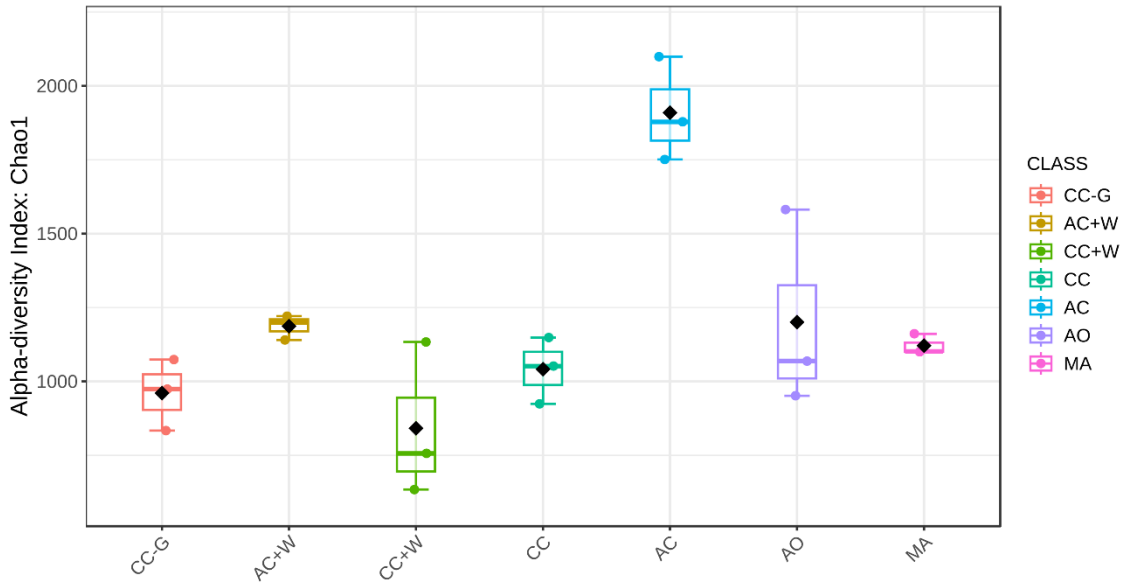

Figure 3 – Comparative Alpha diversity analysis (Chao1 index) based on ITS gene sequencing across different soil treatments. The treatments included: AC (Conventional Arabica Coffee, managed with glyphosate), AO (Organic Arabica Coffee), CC (Conventional Conilon Coffee), CC-G (Conilon Coffee without glyphosate application for 3 years), MA (Atlantic Forest adjacent to Conventional Arabica Coffee), AC+W (Weed present in the Conventional Arabica Coffee plantation), and CC+W (Weed present in the Conventional Conilon Coffee plantation).

The PCoA graph explores and visualizes data similarities or differences so that the distances between points are close to the original dissimilarities between the microbial communities. We conducted Principal Coordinate Analysis (PCoA) to assess Beta diversity based on a Bray-Curtis dissimilarity matrix, calculated in abundance of genus level. When evaluating the bacterial beta diversity of soils, the MA samples showed a clear separation from others along Axis 1, indicating significant differences in bacterial composition between forest and agricultural soils (Figure 4; PERMANOVA, $F = 4.7624$, $R^2 = 0.67116$, $p = 0.001$). This

finding suggests that agricultural land use markedly alters bacterial community structure. The AC and CC treatments clustered separately, demonstrating distinct bacterial compositions between conventional management of Arabica and Conilon coffee soils. This difference likely arises from crop type and specific management practices on the farms sampled. Weed treatments (AC+W and CC+W) also formed clusters distinct from cultivated coffee treatments, indicating that weed-associated bacterial communities differ from those in directly cultivated soils.

For fungal beta diversity, MA, AC, and AC+W treatments clustered together, reflecting their geographic proximity. Treatments without glyphosate (CC-G and AO) formed distinct, well-defined clusters separate from conventional management treatments (AC and CC), suggesting that glyphosate presence influences fungal community structure (Figure 5; PERMANOVA, $F = 5.1597$, $R^2 = 0.6886$, $p = 0.001$). Additionally, the separation of AC+W and CC+W clusters highlights distinct fungal communities associated with weeds in Arabica versus Conilon coffee systems.

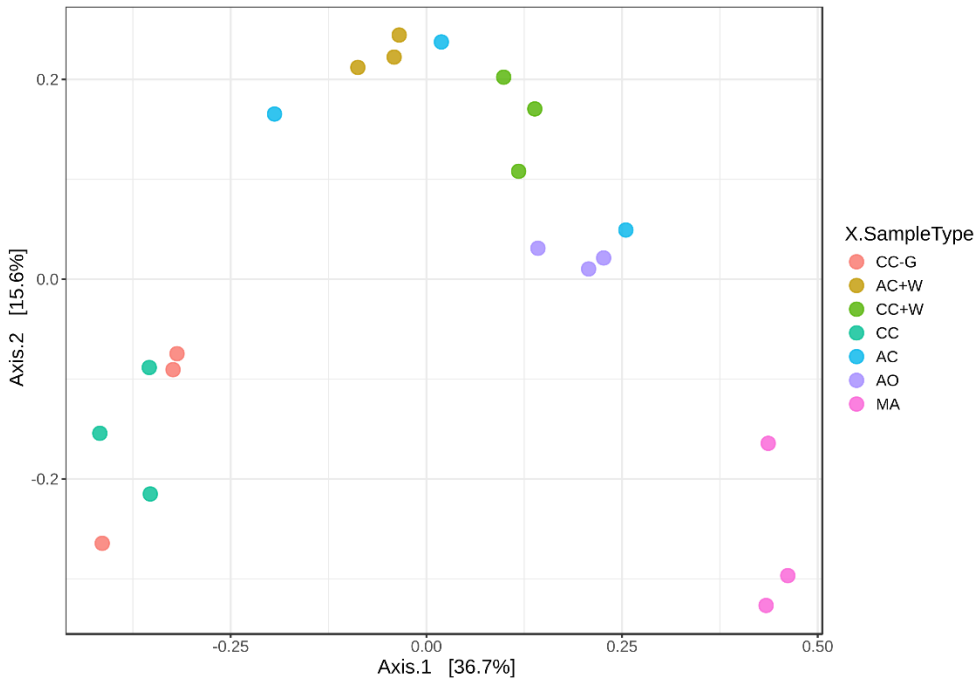

Figure 4 – Beta-diversity analysis of Bacteria in soil samples using Bray-Curtis index and PCoA based on rRNA 16S gene sequencing. The treatments included: AC (Conventional Arabica Coffee, managed with glyphosate), AO (Organic Arabica Coffee), CC (Conventional Conilon Coffee), CC-G (Conilon Coffee without glyphosate application for 3 years), MA (Atlantic Forest adjacent to Conventional

Arabica Coffee), AC+W (Weed present in the Conventional Arabica Coffee plantation), and CC+W (Weed present in the Conventional Conilon Coffee plantation).

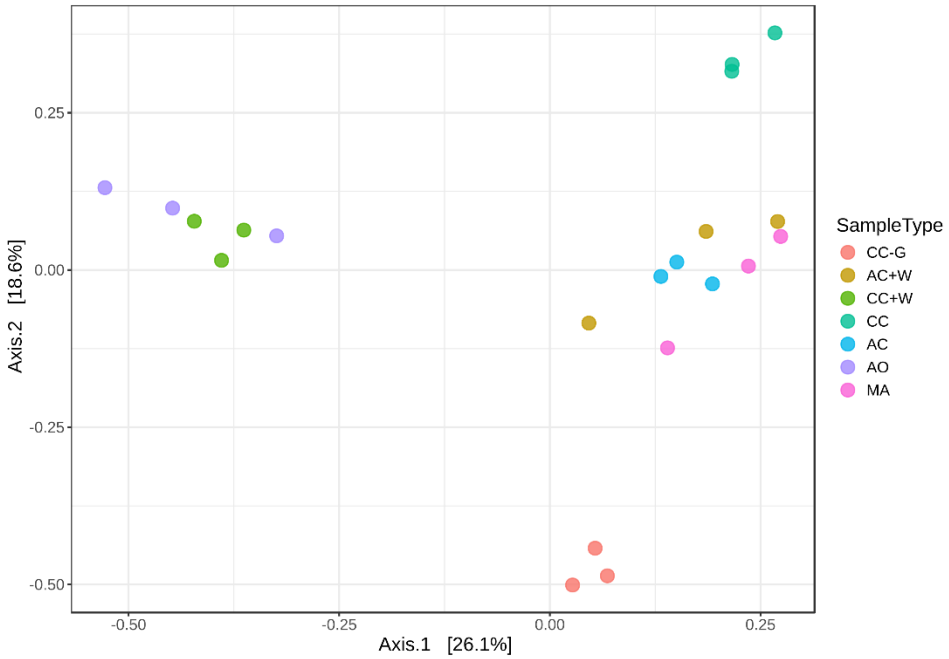

Figure 5 – Beta-diversity analysis of Fungi in soil samples using Bray-Curtis index and PCoA based on ITS rRNA sequencing. The treatments included: AC (Conventional Arabica Coffee, managed with glyphosate), AO (Organic Arabica Coffee), CC (Conventional Conilon Coffee), CC-G (Conilon Coffee without glyphosate application for 3 years), MA (Atlantic Forest adjacent to Conventional Arabica Coffee), AC+W (Weed present in the Conventional Arabica Coffee plantation), and CC+W (Weed present in the Conventional Conilon Coffee plantation).

3.2 Taxonomic classification of soil treatments

The Acidobacteria and Proteobacteria phyla were the most abundant across all treatments, particularly in the MA treatment. The Firmicutes phylum displayed relative abundances ranging from 9.89% to 43% in plantations that currently or previously employed conventional weed management. In contrast, this phylum represented only 6.12% in AO and 2.34% in MA. The Chloroflexi phylum was more abundant in the AO treatment compared to both conventional treatments and the Atlantic forest (Figure 6).

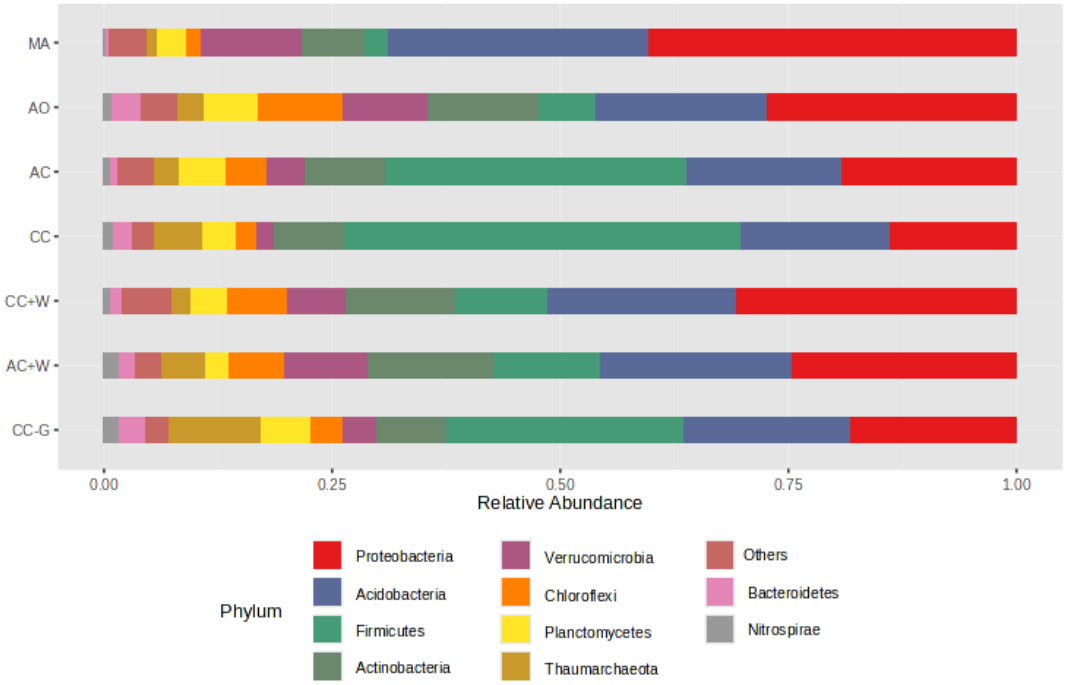

Figure 6 – Relative abundance of Bacterial phyla across different soil treatments.The treatments included: AC (Conventional Arabica Coffee, managed with glyphosate), AO (Organic Arabica Coffee), CC (Conventional Conilon Coffee), CC-G (Conilon Coffee without glyphosate application for 3 years), MA (Atlantic Forest adjacent to Conventional Arabica Coffee), AC+W (Weed present in the Conventional Arabica Coffee plantation), and CC+W (Weed present in the Conventional Conilon Coffee plantation).

For Fungi, Ascomycota phylum was the most abundant across all treatments, with the highest abundance in the AO treatment (79.36%) and a reduction in conventional treatments, such as CC+W (74.09%) and CC (37.33%). The Basidiomycota phylum showed a significant difference between treatments. In MA, its abundance was 13.92%, while in the AO treatment, it dropped to 8.08%. In conventional treatments, such as CC, Basidiomycota abundance was much higher (51.52%), suggesting that conventional management favors this fungal group. Additionally, the Calcarisporiellomycota phylum was nearly absent in MA and AO, but appeared in small amounts in conventional treatments, such as AC+W (0.41%) and CC+W (0.02%). Lastly, the Kickxellomycota phylum was more abundant in AO (0.66%) compared to conventional treatments and MA, where it showed very low or no abundance (Figure 7).

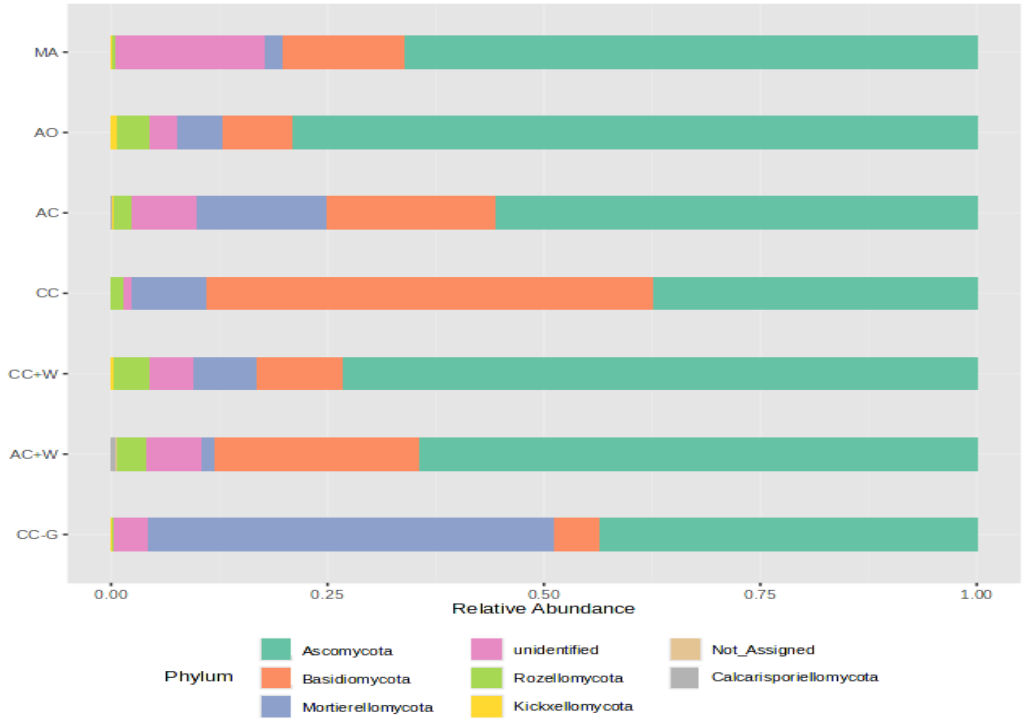

Figure 7 – Relative abundance of Fungi phyla across different soil treatments. The treatments included: AC (Conventional Arabica Coffee, managed with glyphosate), AO (Organic Arabica Coffee), CC (Conventional Conilon Coffee), CC-G (Conilon Coffee without glyphosate application for 3 years), MA (Atlantic Forest adjacent to Conventional Arabica Coffee), AC+W (Weed present in the Conventional Arabica Coffee plantation), and CC+W (Weed present in the Conventional Conilon Coffee plantation).

3.3 Taxonomic classification of microbial consortia

After the enrichment process, microbial biomass was generated from four soil treatments, and the resulting consortia were labeled as Con_AO (originating from organic Arabica coffee soil), Con_MA (from Atlantic Forest soil adjacent to a glyphosate-treated Arabica coffee plantation), Con_CC (from conventional glyphosate-treated Conilon coffee plantation soil), and Con_CC-G (from Conilon Coffee soil without glyphosate application for 3 years). The microbial consortia biomass was then processed to amplify the 16S and ITS rRNA genes, followed by DNA sequencing. The lack of ITS gene amplification indicates that the consortia were composed exclusively of bacteria, with fungi being selectively excluded during the enrichment process.

At the 192 h and 480 h points of enrichment with glyphosate as the sole carbon source, the Con_MA consortium is primarily characterized by the genera *Achromobacter*, *Labrys*, *Chitinophaga*, and *Ochrobactrum*. Notably, the abundance of *Labrys* increases over time (Figure 8). The Con_AO consortium also includes these genera, along with *Burkholderia*,

Comamonas, *Cupriavidus*, *Pseudomonas*, and uncultured bacteria. Similar to MR2, *Labrys* in Con_AO shows a rise in abundance over the time interval. These observations suggest that Con_AO and Con_MA share similar taxonomic profiles. The other two consortia, Con_CC and Con_CC-G, also display comparable taxonomic compositions, being dominated by the genera *Serratia*, *Ochrobactrum*, *Phenylobacterium*, *Chitinophaga*, and *Pseudomonas*, among others. In these consortia, *Serratia* becomes more abundant over time (Figure 9).

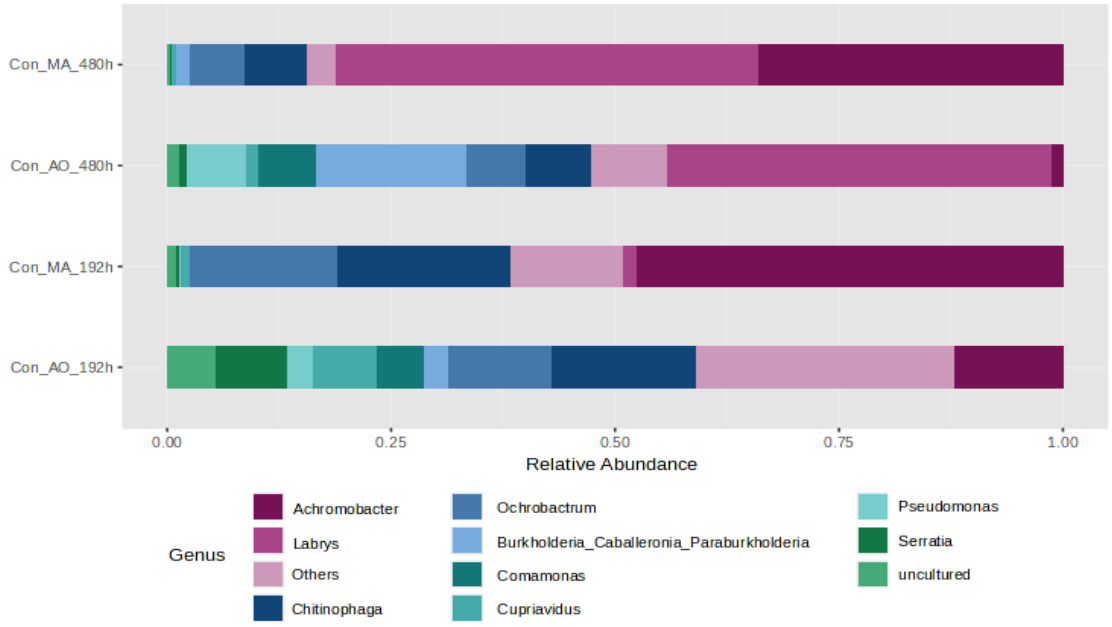

Figure 8 – Relative abundance of glyphosate-degrading bacteria over time for Con_AO and Con_MA consortia. The treatments included: Con_AO (originating from organic Arabica coffee soil), and Con_MA (from Atlantic Forest soil adjacent to a glyphosate-treated Arabica coffee plantation).

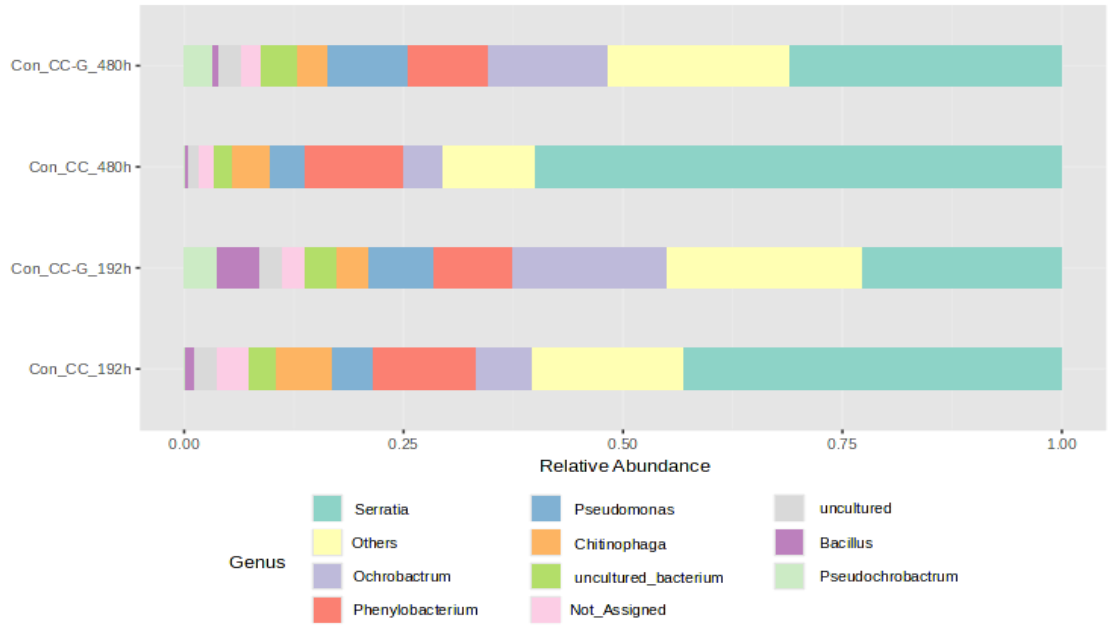

Figure 9 – Relative abundance of glyphosate-degrading bacteria over time for Con_CC and Con_CC-G consortia. The treatments included: Con_CC (from conventional glyphosate-treated Conilon coffee plantation soil), and Con_CC-G (from Conilon Coffee soil without glyphosate application for 3 years)

When analyzing the taxonomy of the consortia cultured post-enrichment, we found that the Con_AO and Con_MA consortia maintained similar taxonomic profiles, with *Labrys* emerging as the dominant genus in both. In contrast, the Con_CC and Con_CC-G consortia displayed a similar composition, where *Serratia* showed the highest abundance (Figure 10A).

Due to the poor growth of the consortia in glyphosate as the carbon source, they were inoculated in a medium with glyphosate as the phosphorus source (GP), where a substantial shift in consortium composition was observed (Figure 10B). In this second medium, the four consortia were composed of the genera *Achromobacter* and *Serratia*. With the change in culture medium, bacterial biomass accumulation was observed in a shorter time. After seven days of cultivation, the growth of the consortia using glyphosate as a carbon source indicated an Optical Density at 600 nanometers (OD600) of 0.2A, while in glyphosate as a phosphorus source, the OD reached 0.4A.

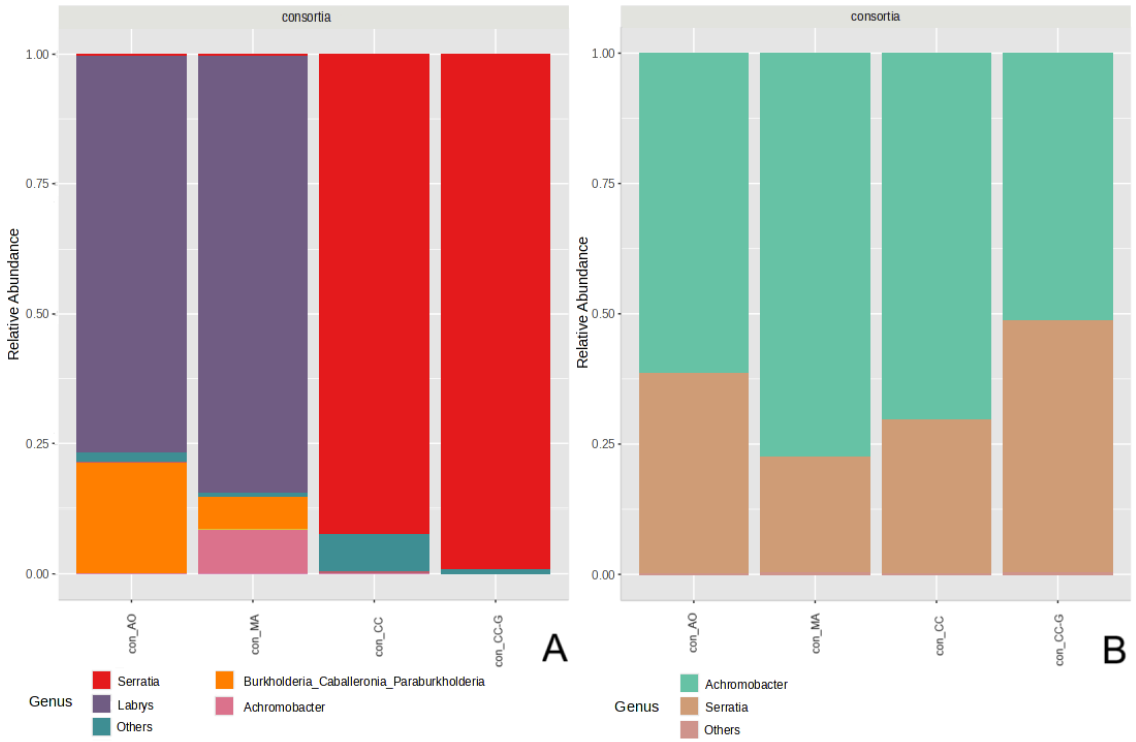

Figure 10 – Relative abundance of bacterial genera comprising the consortia in different culture media. 'A' indicates the bar charts related to the consortia with glyphosate as a carbon source, and 'B' with glyphosate as a phosphate source.

3.3 *Achromobacter* and *Serratia* genomes reconstructed from consortia

Two near-complete genomes were reconstructed (Table 1) from consortia DNA samples. The assembled genome of the *Achromobacter* genus resulted in a single contig of 6,622,132 bp, a size like other described *Achromobacter* genomes, namely *Achromobacter* sp. AONIH1 (GenBank assembly: GCA_018195695.1), *Achromobacter* sp. Marseille-Q0513 (GenBank assembly: GCA_018195695.1), *Achromobacter ruhlandii* (GenBank assembly: GCA_002082135.1), and *Achromobacter insuavis (*GenBank assembly: GCA_902859645.1). The pairwise ANI between the genome and the others ranged from 83.43 to 98.02%, indicating that the assembled genome is closely related to these *Achromobacter* species.

The most complete assembled genome of the *Serratia* genus consists of 11 contigs, with a total size of 4,828,039 bp (Table 1). Comparing this genome to those of *Serratia* sp. LS-1 (GCA_003719595.1), isolated from the host *Orthaga achatina*, and *Serratia marcescens* (GCA_030291735.1), isolated from soil, we found ANI percentages of 97.40% and 94.59%, respectively, indicating that the assembled genome is likely part of the *Serratia marcescens*

species complex, with high similarity to existing *Serratia* genomes and potential for classification as a strain within this species.

Table 1 – Genomic features of the two reconstructed genomes *Achromobacter* sp. and *Serratia* sp. from glyphosate-tolerant consortia

Genome	*Achromobacter* sp.	*Serratia* sp.
Phylum	*Proteobacteria*	*Proteobacteria*
Class	*Betaproteobacteria*	*Gammaproteobacteria*
Order	*Burkholderiales*	*Enterobacterales*
Family	*Alcaligenaceae*	*Yersiniaceae*
Genus	*Achromobacter*	*Serratia*
Estimated genome size (bp)	6,622,132	4,828,039
Number of contigs	1	11
Estimated completeness (%)	98.52	90.01
Estimated contamination (%)	1.32	0.24
G + C content (%)	67.65	59.45
N50 contig lenght	6,622,132	780,565

3.4 Genetic potential for glyphosate degradation in *Achromobacter* sp. and *Serratia* sp. genomes

Bacterial consortia metagenomes were examined to explore functions related to glyphosate breakdown. Key genes involved in its degradation, such as *gox* and *phnC-P*, were identified. The search for glyphosate degradation genes yielded positive results across all genome annotation methods. The best alignment results obtained through BlastP are provided in Supplementary material (Tables S1 and S2).

The genomes of *Achromobacter* sp. and *Serratia* sp. contained genes encoding proteins responsible for converting glyphosate and its intermediates into Pi, formaldehyde, and glycine, indicating the potential for glyphosate mineralization by these genera. In the C-P lyase pathway, all the 14 cistrons of the *phnCDEFGHIJKLMNOP* operon were identified and aligned with the reference genomes of *Achromobacter* sp. and *Serratia* sp., along with the gene for sarcosine oxidase. Genes encoding glyphosate oxidoreductase and 2-aminoethylphosphonate-

pyruvate transaminase were also found and aligned. In *Achromobacter* sp., genes for phosphonoacetaldehyde hydrolase and phosphonopyruvate hydrolase were located, while in *Serratia* sp., the genes for phosphonoacetaldehyde hydrolase and phosphonoacetate hydrolase were identified (Figure 11). Table S3 (Supplementary material) provides a detailed view of the genes, with the genes marked in figure 11 as 1 to 6.

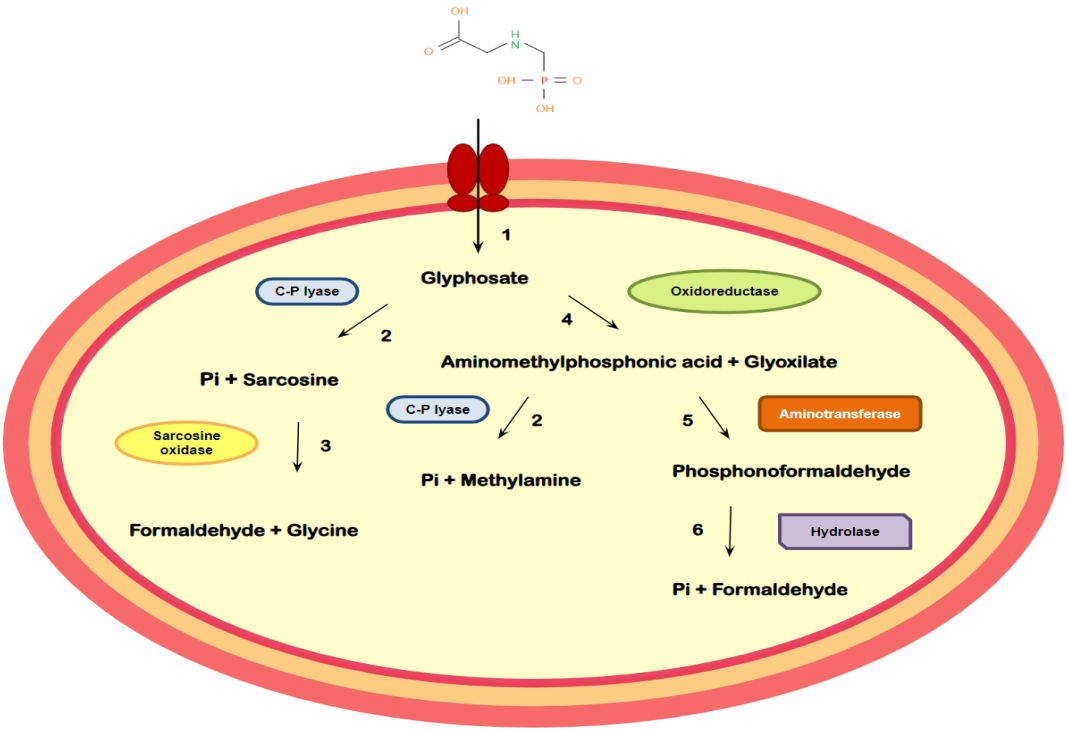

Figure 11 – Glyphosate degradation pathways in *Achromobacter* sp. and *Serratia* sp., adapted from the model proposed by Sviridov *et al*. (2011) for *Ochrobactrum anthropi* and *Achromobacter* sp.

4 Discussion

Here, we obtained microbial consortia from soils subjected to different weed management practices and from the Atlantic Forest. Upon analyzing the bacterial and fungi alpha diversity of the soil samples, we noticed that the alpha diversity of the forest soil, adjacent to the conventional Arabica coffee plantation, was lower than that of the soil of this plantation.

Alpha diversity can be lower in forest soils than in agricultural soils because agricultural practices, such as introducing fertilizers and other chemicals, tend to increase microbial richness and activity[39]. It is also noteworthy that, at low sampling levels, forests appear to have fewer taxa on average than pastures; however, as sampling effort increases, forests eventually surpass pastures in taxonomic richness[40].

16S rRNA gene analysis can have limitations in assessing alpha diversity, as it tends to predominantly capture dominant microbial groups, potentially underrepresenting less abundant communities. This limitation is particularly relevant in environments where microbiota composition is shaped by biotic and abiotic factors, such as soil characteristics and management practices, that influence microbial population dynamics. Under conditions of disturbance or environmental stress, 16S sequencing may temporarily reveal greater diversity, although this response may not accurately reflect the true species richness under normal conditions[41].

We also observed higher bacterial and fungal alpha diversity in the soil surrounding weeds present in the conventional Arabica and Conilon coffee plantations. Weeds can adopt various competitive strategies, often establishing beneficial interactions with diverse microbial groups, such as mycorrhizal fungi and nitrogen-fixing microorganisms. These interactions can promote a more diverse microbial community by creating microhabitats and enhancing nutrient availability in the rhizosphere[42]. Weeds may also release root exudates that attract a wide range of microbes, further increasing microbial diversity compared to the more homogeneous soil conditions typically found in monoculture plantations[43].

During the enrichment of bacterial consortia, we identified genera previously linked to glyphosate degradation in natural environments or as sources of key genes for developing herbicide-resistant transgenic plants. Dominant genera in the Con_AO and Con_MA consortia included *Achromobacter*, *Burkholderia*, *Chitinophaga*, *Comamonas*, *Labrys*, and *Ochrobactrum*[13, 44, 45, 46]. Notably, this is the first report of *Labrys*, known for degrading the neonicotinoid insecticide thiamethoxam, as a potential glyphosate degrader, utilizing it as a carbon source[47].

In the Con_CC and Con_CC-G consortia, the dominant genera included soil bacteria such as *Serratia*, *Ochrobactrum*, and *Pseudomonas*, alongside less common bacteria like *Phenylobacterium* and *Chitinophaga*. These genera have previously been reported as isolates or members of microbial consortia capable of degrading glyphosate in soil or aquatic environments [44,46,48,49,50].

Chitinophaga, a gliding, chitin-decomposing myxobacterium, was present in all consortia enriched with glyphosate as a carbon source. Although *Chitinophaga* is typically known for chitin degradation[51], its consistent presence in glyphosate-enriched consortia may indicate that it contributes to the degradation process, either directly or indirectly, possibly assisting in the initial breakdown of compounds or in stabilizing the microbial community[52].

When glyphosate was supplied as the sole phosphorus source and glucose as the carbon source, the microbial consortia experienced a shift in community composition, resulting in reduced bacterial diversity and a pronounced selection for *Achromobacter* and *Serratia*. This shift likely reflects competitive dynamics and niche specialization, with these genera better suited to the modified nutrient conditions. The additional energy from glucose facilitated the growth of organisms capable of utilizing phosphorus from glyphosate. *Achromobacter* and *Serratia* thus gained a competitive advantage within this specific nutrient niche due to their efficient mechanisms for exploiting both glyphosate-derived phosphorus and glucose as a carbon source. This outcome aligns with niche-based theory, suggesting that microbial composition is deterministically shaped by nutrient availability in the environment[53,54,55,56].

Achromobacter is known for its ability to degrade various pollutants, including pesticides, and it has been frequently found in soil environments where biodegradation of complex compounds occurs[57]. This genus has been extensively documented in glyphosate degradation experiments, with representatives capable of utilizing this herbicide as a source of carbon, nitrogen, and phosphorus[12,58]. Additionally, this genus is also known for its ability to promote plant growth. *Achromobacter sp.* 5B1 significantly enhanced the growth of the primary root in *Arabidopsis thaliana*, leading to an approximately fourfold increase in both the number and density of lateral roots[59].

The genus *Serratia* is frequently cited in microbial consortia for its plant growth-promoting properties and its role in degrading organic pollutants. For example, *Serratia marcescens* has been shown to degrade chlorpyrifos, fenitrothion, and parathion, utilizing these compounds as the sole carbon source at concentrations of 50 mg L^{-1}. While *Serratia* has also been involved in studies of environmental biodegradation, direct evidence of its ability to use glyphosate as a nutrient source remains limited[9,60].

Bacteria from the rhizosphere of corn and wheat were isolated from enrichment, in which glyphosate was used as a carbon source. Among the strains, *Serratia liquefaciens* demonstrated the ability to degrade 87.1% of 100 $mg.kg^{-1}$ of herbicide and promote corn growth

in vitro[50]. These results highlight the multifaceted potential of *Achromobacter* and *Serratia* consortia as both plant growth promoters and glyphosate degraders, underscoring their suitability for developing innovative biotechnological bioinputs with dual benefits for sustainable agriculture.

The presence of redundant metabolic capabilities across *Achromobacter* and *Serratia* genomes suggests that both can independently degrade glyphosate without requiring cooperation or cross-feeding between them. This can be seen as a form of functional redundancy, which might confer greater functional stability to the microbial community[61,62]. In an ecological sense, if one genus were diminished or lost due to environmental changes or selective pressures, the other could continue performing the necessary degradation activities, ensuring continued glyphosate breakdown.

These findings highlight the significant potential of *Achromobacter* and *Serratia* for bioremediation of glyphosate-contaminated soils. However, for successful application, microorganisms must meet specific criteria: they should exhibit low toxicity and be non-pathogenic, maintain high viability immediately after introduction, but with limited long-term survival, and effectively degrade glyphosate across varying environmental conditions. Additionally, their degradation pathways should prevent the accumulation of AMPA, ensuring minimal ecological impact[63]. Future studies should focus on optimizing the application strategies and assessing ecological impacts to harness the bioremediation potential of these genera fully.

Acknowledgments

The authors would like to thank the Fundação de Amparo à Pesquisa do Estado do Espírito Santo—FAPES (#2020QS1D) for the financial support in the development of the research/publication of the article and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) — Finance Code 001 — for their financial support. This work was supported by the Brazilian Microbiome Project (http://brmicrobiome.org).

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Supplemental Information for:

Glyphosate-Degrading Consortia: Microbial Succession and Key Enzyme Gene Insights

Mariana Prósperi de Oliveira Paula[1], Alessandro Coutinho Ramos[2],Luiz Fernando Wurdig Roesch[3],Victor Satler Pylro[1,*]

Table S1 – BlastP results for *Achromobacter* sp. against glyphosate-degrading enzyme databases.

Protein Name	Subject ID	Microorganism	% Identity	Alignment Length	E-value	Bit Score
Oxidoreductase	QEK94935.1	*Achromobacter insolitus*	93.836	438 bp	0.0	906
2-aminoethylphosphonate--pyruvate transaminase	WP_287144185.1	*Achromobacter* sp.	37.500	360 bp	7.68e-59	202
Phosphonoacetaldehyde hydrolase	WP_103273422.1	*Achromobacter* sp. AONIH1	98.901	273 bp	1.91e-178	547
Phosphonopyruvate hydrolase	WP_212319150.1	*Achromobacter* sp. Marseille-Q0513	100.000	295 bp	0.0	596
PhnC (phosphonate ABC transporter ATP-binding protein)	WP_063584983.1	*Achromobacter* sp.	94.118	255 bp	8.08e-160	492
PhnD (phosphate/phosphite/phosphonate ABC transporter substrate-binding protein)	WP_212314150.1	*Achromobacter* sp. Marseille-Q0513	99.699	332 bp	0.0	670
PnhE (phosphonate ABC transporter, permease protein)	WXR73837.1	*Achromobacter veterisilvae*	90.511	274	4.03e-163	503
PhnF (putative transcriptional regulator)	CAB3713684.1	*Achromobacter deleyi*	94.167	240	5.07e-154	475
PhnG (Alpha-D-ribose 1-methylphosphonate 5-triphosphate synthase)	CAB3713666.1	*Achromobacter deleyi*	81.290	155bp	2.01e-79	257
PhnH (phosphonate C-P lyase system protein)	WP_233772944.1	*Achromobacter* sp. AONIH1	95.631	206	2.29e-128	400
PhnI (Alpha-D-ribose 1-methylphosphonate 5-triphosphate synthase)	CAB3881543.1	*Achromobacter aegrifaciens*	90.296	371	0.0	691

PhnJ (alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase)	WP_212313042.1	*Achromobacter* sp. Marseille-Q0513	100	297	0.0	633
PhnK (phosphonate C-P lyase system protein)	WP_212313106.1	*Achromobacter* sp. Marseille-Q0513	99.612	258	1.54e-169	521
PhnL (phosphonate C-P lyase system protein)	WP_212313044.1	*Achromobacter* sp. Marseille-Q0513	98.361	244	5.09e-156	481
PhnM (phosphonate metabolism protein)	VEG66840.1	*Achromobacter insolitus*	89.487	390	0.0	706
PhnN (phosphonate metabolism protein/1,5-bisphosphokinase (PRPP-forming))	AUT49521.1	*Achromobacter sp. AONIH1*	97.500	200	2.58e-123	385
PhnO (Aminoalkylphosphonate N-acetyltransferase)	CAB3957350.1	*Achromobacter piechaudii*	79.221	154	9.04e-75	243
PhnP (phosphate/phosphite/phosphonate ABC transporter substrate-binding protein)	WP_103271052.1	*Achromobacter* sp. AONIH1	99.699	332	0.0	669

Table S2 – BlastP results for *Serratia sp.* against glyphosate-degrading enzyme databases.

Protein Name	Subject ID	Microorganism	% Identity	Alignment Length	E-value	Bit Score
Oxidoreductase	ALL19213.1	*Enterobacter* sp. E20	74.143	321	0.0	502
2-aminoethylphosphonate--pyruvate transaminase	ALL19851.1	*Enterobacter* sp. E20	92.568	296	0.0	610
Phosphonoacetaldehyde hydrolase	AIM21624.1	*Serratia* sp. SCBI	99.183	367	0.0	766
Phosphonopyruvate hydrolase	WP_197760021.1	*Serratia* sp.	97.407	270	4.59e-177	539

PhnC (phosphonate ABC transporter ATP-binding protein)	EZQ72255.1	*Serratia marcescens* BIDMC 80	100	110	2.83e-77	245
PhnD (phosphate/phosphite/phosphonate ABC transporter substrate-binding protein)	TXE52741.1	*Serratia bockelmannii*	99.639	277	0.0	576
PnhE (phosphonate ABC transporter, permease protein)	TXE40153.1	*Serratia marcescens*	99.042	313	0.0	638
PhnF (putative transcriptional regulator)	WXK86405.1	*Serratia marcescens*	98.734	237	3.43e-163	499
PhnG (Alpha-D-ribose 1-methylphosphonate 5-triphosphate synthase)	QSP86429.1	*Serratia marcescens* subsp. marcescens ATCC 13880	99.177	243	4.52e-162	494
PhnH (phosphonate C-P lyase system protein)	TXE52264.1	*Serratia bockelmannii*	100.000	147	1.08e-95	299
PhnI (Alpha-D-ribose 1-methylphosphonate 5-triphosphate synthase)	WYI59216.1	*Serratia nevei*	96.891	193	3.24e-122	377
PhnJ (alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase)	BEO88285.1	*Serratia nevei*	96.648	358	0.0	703
PhnK (phosphonate C-P lyase system protein)	BEM41129.1	*Serratia marcescens*	100.000	286	0.0	597
PhnL (phosphonate C-P lyase system protein)	WP_164049961.1	*Serratia* sp.	99.237	262	6.84e-175	532
PhnM (phosphonate metabolism protein)	WYI59212.1	*Serratia nevei*	99.573	234	7.72e-158	482
PhnN (phosphonate metabolism protein/1,5-bisphosphokinase (PRPP-forming)	AGE16265.1	*Serratia marcescens* WW4	88.095	378	0.0	671
PhnO (Aminoalkylphosphonate	KFD16262.1	*Serratia marcescens* subsp. marcescens	97.861	187	2.07e-119	369

N-acetyltransferase)		ATCC 13880				
PhnP (phosphate/phosphite/phosphonate ABC transporter substrate-binding protein)	OJH82416.1	*Serratia marcescens*	98.065	155	4.04e-101	314

Table S3 – Genes annotated in the *Achromobacter* sp. and *Serratia* sp. genomes

Gene number (Fig. 8)	KEGG number	Protein function	Gene name
1	K02044	Phosphonate transporter	Phosphonate transport system substrate-binding protein (phnD)
1	K02042	Phosphonate transporter	Phosphonate transport system permease protein (phnE)
1	K02041	Phosphonate transporter	Phosphonate transport system ATP-binding protein (phnC)
4	1.5.3.23	Oxidoreductase	Glyphosate oxidoreductase
3	1.5.3.24	Oxidoreductase	Sarcosine oxidase
2	4.7.1	Carbon-phosphorous Lyase	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase (phnJ)
5	2.6.1.37	Transferase	2-aminoethylphosphonate-pyruvate transaminase
6	3.11.1.1	Hydrolase	Phosphonoacetaldehyde hydrolase
6	3.11.1.2	Hydrolase	Phosphonoacetate hydrolase
6	3.11.1.3	Hydrolase	Phosphonopyruvate hydrolase

Artigo 2

Evaluating the effects of bacterial consortia inoculation on soil microbial activity and community dynamics in glyphosate-treated microcosms

Artigo elaborado conforme periódico Environmental Pollution

Mariana Prósperi de Oliveira Paula[1], Alessandro Coutinho Ramos[2], Marcos Rogério Tótola[3], Victor Satler Pylro[1,*]

[1,*]Laboratório de Ecologia Microbiana e Bioinformática, Departamento de Biologia, Universidade Federal de Lavras, Lavras, MG, Brasil. [2]Laboratório de Microbiologia Ambiental e Biotecnologia, Universidade Vila Velha, Vila Velha, ES, Brasil. [3]Laboratório de Biotecnologia e Biodiversidade para o Meio Ambiente, Departamento de Biologia, Universidade Federal de Viçosa, Viçosa, MG, Brasil. Correspondence: Victor Satler Pylro, victor.pylro@ufla.br

ORCID:

Mariana Prósperi de Oliveira Paula: 0000-0002-5917-8984
Alessandro Coutinho Ramos: 0000-0002-4082-0076
Marcos Rogério Tótola: 0000-0002-4235-2421
Victor Satler Pylro: 0000-0003-2154-9150

Abstract

This work assessed the performance of microbial consortia in soil microcosms contaminated with glyphosate, focusing on their catabolic activity and microbial diversity. Using respirometry, CO_2 production was monitored for 140 hours to evaluate microbial metabolic activity in inoculated soils and controls. Soil aliquots were collected for 16S rRNA marker gene analysis through metataxonomics to investigate changes in microbial community structure. Respiratory activity was stimulated in the treatments exposed to glyphosate. Differential abundance analysis revealed significant increases in *Achromobacter* and *Serratia* in inoculated soils compared to controls, alongside other genera involved in herbicide degradation, such as *Arthrobacter*, *Paenarthrobacter*, and *Pseudarthrobacter*. These results suggest the potential of the Con_CC-G consortium, obtained from Conilon Coffee soil without glyphosate application for 3 years, for large-scale bioremediation of glyphosate-contaminated soils, pending further toxicity and environmental safety assessments.

Keywords: Biodegradation. Bioremediation. Microbial activity. Herbicide. Soil microbiota.

1 Introduction

Glyphosate is an effective herbicide widely used worldwide; however, its extensive application has raised environmental concerns, particularly due to its accumulation in natural ecosystems and potential effects on biota. Although not specifically intended for soil application, substantial amounts of glyphosate can reach the soil surface during preplant broadcast or early-season treatments (Gandhi *et al*., 2021).

Bacterial consortia and isolates have emerged as a promising approach for the degradation of pollutants such as glyphosate. These microorganisms can utilize complementary metabolic pathways, which enhance the degradation of glyphosate and minimize the generation of unwanted by-products, such as aminomethylphosphonic acid (AMPA) (Firdous *et al*.,2020; Hertel *et al*., 2021). In the context of soil microbial activity, the inoculation of xenobiotic-degrading bacterial consortia can significantly increase the mineralization of the compound, accelerating its removal and promoting overall microbial activity, as evidenced by CO_2 production (de Lima *et al*., 2022).

The availability of herbicide to soil microorganisms is influenced by a range of soil factors, such as nutrient levels, pH, temperature, and moisture, although the significance of each factor can vary based on the pesticide in question (Haney et al., 2000). For glyphosate, a polar compound known for its strong adsorption to Fe and Al oxides and clay particles, availability in natural soils is further influenced by soil composition. This binding to soil particles and metal complexes reduces the pool of labile glyphosate, thereby limiting microbial uptake (Busse *et al*., 2021).

In living soil environments, introduced species frequently face competition and suppression from the native microbiome, which can diminish their survival and reduce the effectiveness of inoculation over time. Therefore, the interaction between microbial consortia and the native soil microbiota plays a crucial role in determining the success of these strategies, as the consortia must adapt and thrive within ecosystems characterized by complex microbial diversity (Liu, Mei & Salles, 2023). Thus, evaluating the degradation potential in soil microcosms offers a preliminary insight into the feasibility of applying these consortia on a larger scale, such as in agricultural areas contaminated with glyphosate.

This work aims to evaluate the potential of these bacterial consortia in glyphosate degradation in soils, using microbial activity analysis as an indirect indicator of this process. Additionally, we aimed to investigate the ability of these consortia to establish themselves in soils with complex native microbiota.

2 Methodology

2.1 Bacterial consortia

The glyphosate-degrading bacterial consortia used in this study are stored at the Laboratory of Microbial Ecology and Bioinformatics, Federal University of Lavras, Brazil. These microorganisms were previously obtained through microbial enrichment, where glyphosate was employed as the sole carbon source (GC medium). The composition of this medium consisted of ($g \cdot L^{-1}$): 2 $(NH_4)_2SO_4$, 0.2 $MgSO_4 \cdot 7H_2O$, 0.01 $CaCl_2$, 1.5 KH_2PO_4, 1.5 $Na_2HPO_4 \cdot 12\ H_2O$, 0.01 $FeSO_4 \cdot 7H_2O$, and filtered (pore size 0.2 μm) commercial glyphosate (Roundup®) (8 $g \cdot L^{-1}$) (Zhang *et al*., 2022). The glyphosate concentration used falls within the range of 5 to 25 $g.L^{-1}$, reflecting typical field application rates for this herbicide (Masotti *et al*., 2021). These consortia were labeled as Con_AO (originating from organic Arabica coffee soil), Con_MA (from Atlantic Forest soil adjacent to a glyphosate-treated Arabica coffee plantation), Con_CC (from conventional glyphosate-treated Conilon coffee plantation soil), and Con_CC-G (from Conilon Coffee soil without glyphosate application for 3 years).

After successive subcultures in glyphosate as the carbon source and observing limited microbial biomass growth, the consortia were subsequently inoculated into GP medium, where glyphosate was used as the phosphorus source. In this medium, satisfactory microbial biomass growth was achieved. The composition of this medium consisted of ($g \cdot L^{-1}$): 5 $C_6H_{12}O_6$, 2 $(NH_4)_2SO_4$, 0.2 $MgSO_4 \cdot 7H_2O$, 0.01 $CaCl_2$, 0.5 KCl, 0.5 NaCl, and 0.001 $FeSO_4 \cdot 7H_2O$, and filtered (pore size 0.2 μm) commercial glyphosate (Roundup®) (8 $g \cdot L^{-1}$) (Manogaran *et al*., 2018). For the respirometry analysis, the consortia inoculated in GP medium were used as the basis.

2.2 Respirometry analysis

This analysis was designed to examine how the metabolic activity (measured by CO_2 emission) of different bacterial consortia varied over time in an experiment, based on emission rate data (μMolHr) and the duration of the measurements (Dur). The experimental design consisted of 10 treatments: the first being soil (soil only), soil+gly (soil with glyphosate), Con_AO_C, Con_MA_C, Con_CC_C, Con_CC-G_C (soils inoculated with consortia previously cultured in GC medium), and Con_AO_P, Con_MA_P, Con_CC_P, Con_CC-G_P

(soils inoculated with consortia previously cultured in GP medium). All treatments had 3 replicates.

For the preparation of bacterial inoculants to be applied to the soil, these consortia, previously cultivated and established in GP medium, were divided into two sets: in the first set, the consortia were reinoculated in GC medium, and in the second, they continued to be inoculated in GP medium. Subsequently, after seven days of cultivation, the obtained biomass was centrifuged at 9,000 rpm for 15 minutes and prepared in a 0.8% saline solution for standardization. The inoculants were normalized based on optical density readings, adjusting all to the lowest concentration, which corresponded to an absorbance reading of approximately 0.2 at 600 nm. To set up the microcosms, 20 g of sieved Red Clay Oxisol were placed into 125 mL respirometric flasks. To standardize moisture levels across all treatments, the liquid inoculum containing 5 $g.L^{-1}$ of commercial glyphosate was adjusted to 60% of the soil's maximum water-holding capacity and thoroughly mixed into the soil (Haney *et al*., 2000). Control (soil only) received distilled water, while the control with glyphosate (soil + gly) received water containing glyphosate. After assembling each experimental unit, the respirometry flasks were kept at room temperature and connected to a respirometer coupled with an infrared gas analyzer with intermittent airflow (Sable System, NE, USA) (Figure 2). CO_2 production by the microbiota was measured over 140 hours, with readings taken every 4 hours. In the non-sterilized soil treatments, 0.5 mL of a solution containing 25 $g.L^{-1}$ of glyphosate was introduced into the flasks after 40 hours of the experiment to assess a potential increase in microbial respiration rate. At the end of the 140-h experiment, subsamples of soil were removed from the treatments for processing and sequencing of the 16S rRNA marker gene.

2.3 DNA extraction

First, genomic DNA from each soil sample was extracted and purified using the DNeasy PowerSoil Pro Kit (Qiagen), following the manufacturer's instructions. The purity of the extracted DNA was observed by spectrophotometry using the Nanodrop One equipment (Nanodrop Technologies, Wilmington, DE, USA) (260/280 nm), and quantification was performed with the Qubit® 4.0 fluorimeter, using Qubit® dsDNA HS Assay Kit (Invitrogen TM) (Pylro *et al*., 2014a).

2.4 Analysis of the taxonomic profile of the microbial community

The structure of the microbial communities in soil samples was analyzed using metataxonomics. The 16S rRNA gene (hypervariable V4 region) was amplified using the primers 515F/806R (Caporaso *et al*., 2012). According to the Ion Amplicon library preparation manual, PCR amplification was performed with barcodes attached to an A sequence from the Ion adapter and a trP1 sequence from the Ion adapter. For the V4 region, PCR conditions were: 2 min at 95°C, followed by 30 cycles of 45 sec at 94°C, 45 sec at 56°C, and 1 min at 72°C, with a final extension of 10 min at 72°C (Caporaso *et al*., 2012).

Amplicons containing adapters and barcode sequences were purified using AMPureBeads (1.2×, Beckman Coulter). Emulsion PCR was performed using the Ion OneTouch 2™ system and the Ion Template PGM™ OT2 400 kit (Life Technologies). Sequencing of the amplicon libraries was conducted on an Ion 318™ Chip Kit v2 with the PGM Ion Torrent sequencer (Life Technologies). Post-sequencing, the PGM software filtered the Ion Torrent fastq files to remove polyclonal and low-quality sequences.

2.5 Data analysis

The table of operational taxonomic units (OTUs) was generated using the UPARSE pipeline, following the Brazilian Microbiome Project recommendations (Pylro *et al*., 2014b). Sequences were truncated at 200 base pairs, and quality filtering was applied, allowing a maximum error rate of 1.0. The filtered reads were dereplicated, clustered with a 97% similarity threshold, and representative sequences were obtained for each phylum (Edgar, 2013). Taxonomic classification was performed using the Quantitative Insights Into Microbial Ecology (QIIME) software, based on the UCLUST method and the SILVA database (version 138).

The web-based tool MicrobiomeAnalyst (Dhariwal et al., 2017) was used to generate abundance and diversity plots, providing a comprehensive visual representation of microbial community composition and diversity dynamics. Soil treatments were compared through Single-factor Statistical Comparisons, employing a Two-group comparison approach. Log2FC (log fold change) values were used to define the direction of triangles in the visualization, where a positive Log2FC indicates an increase in the abundance of a taxon in a given treatment compared to the control or another condition. The EdgeR method was used to compute log2FC and p-values, with corrections applied using FDR (False Discovery Rate).

3 Results

3.1 Catabolic activity of bacterial consortia

In the sterilized soil, treatments that received inoculants and glyphosate showed a significant increase in catabolic activity compared to the control, which is represented by a low and constant line in the graph (Figure 12). Among the treatments, the consortia cultivated with glyphosate as a phosphate source exhibited higher metabolic activity overall, with the Con_CC and Con_CC-G consortia standing out.

In the non-sterilized soil, the consortia previously cultured with glyphosate as phosphate consistently exhibited higher CO_2 emission rates than those previously cultured with glyphosate as carbon, except the Con_CC-G_C consortium, which had rates as high as its counterpart cultured with glyphosate as phosphate, Con_CC-G_P (Figure 13). After the extra glyphosate was added to the soil at the 40-hour mark, there was an increase in CO_2 emission rates for all consortia, except for the control (soil only), suggesting a metabolic activation related to the increased availability of glyphosate (Figure 14).

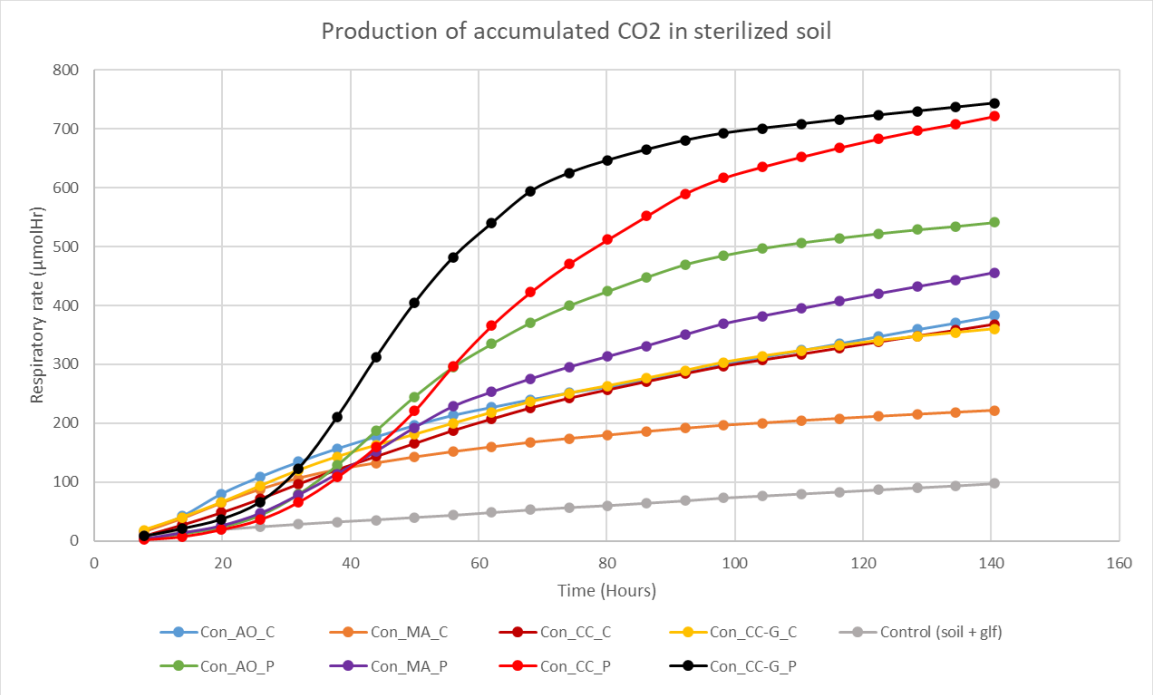

Figure 12 – Production of accumulated CO_2 in sterilized soil. Treatments: soil (only soil), soil+gly (soil+glyphosate), Con_AO_C (organic Arabica coffee soil, glyphosate as carbon source), Con_MA_C (Atlantic Forest soil, glyphosate as carbon source), Con_CC_C (conventional Conilon coffee soil, glyphosate-treated, glyphosate as carbon source), Con_CC-G_C (Conilon coffee soil, no glyphosate for 3 years, glyphosate as carbon source), Con_AO_P (organic Arabica coffee soil, glyphosate as phosphate source), Con_MA_P (Atlantic Forest soil, glyphosate as phosphate source), Con_CC_P (conventional

Conilon coffee soil, glyphosate as phosphate source), and Con_CC-G_P (Conilon coffee soil, no glyphosate for 3 years, glyphosate as phosphate source).

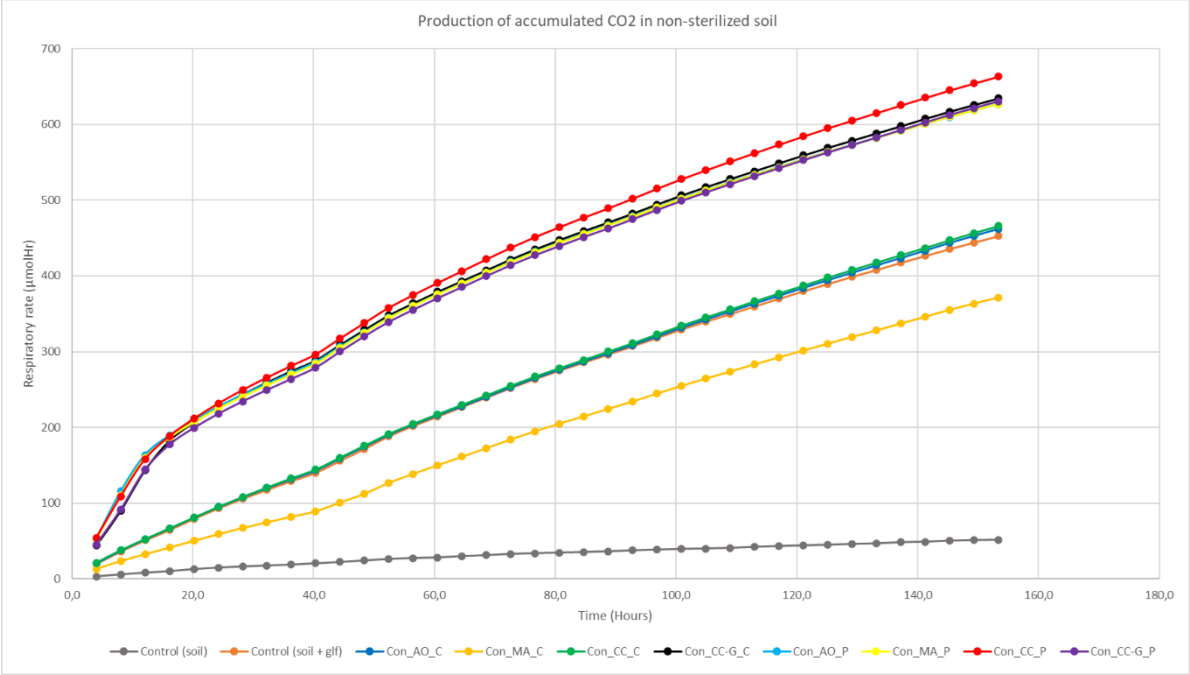

Figure 13 – Production of accumulated CO_2 in non-sterilized soil. Treatments: soil (only soil), soil+gly (soil+glyphosate), Con_AO_C (organic Arabica coffee soil, glyphosate as carbon source), Con_MA_C (Atlantic Forest soil, glyphosate as carbon source), Con_CC_C (conventional Conilon coffee soil, glyphosate-treated, glyphosate as carbon source), Con_CC-G_C (Conilon coffee soil, no glyphosate for 3 years, glyphosate as carbon source), Con_AO_P (organic Arabica coffee soil, glyphosate as phosphate source), Con_MA_P (Atlantic Forest soil, glyphosate as phosphate source), Con_CC_P (conventional Conilon coffee soil, glyphosate as phosphate source), and Con_CC-G_P (Conilon coffee soil, no glyphosate for 3 years, glyphosate as phosphate source).

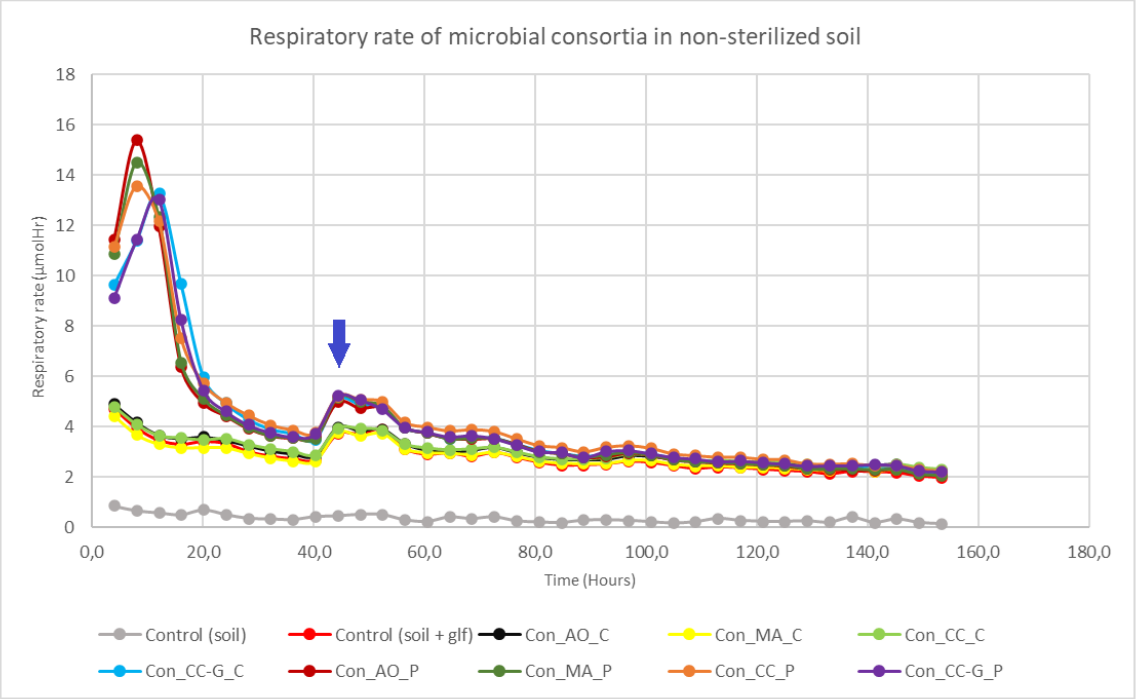

Figure 14 – Respiratory rate of microbial consortia in non-sterilized soil. The arrow indicates when an additional dose of glyphosate was added to the system. Treatments: soil (only soil), soil+gly (soil+glyphosate), Con_AO_C (organic Arabica coffee soil, glyphosate as carbon source), Con_MA_C (Atlantic Forest soil, glyphosate as carbon source), Con_CC_C (conventional Conilon coffee soil, glyphosate-treated, glyphosate as carbon source), Con_CC-G_C (Conilon coffee soil, no glyphosate for 3 years, glyphosate as carbon source), Con_AO_P (organic Arabica coffee soil, glyphosate as phosphate source), Con_MA_P (Atlantic Forest soil, glyphosate as phosphate source), Con_CC_P (conventional Conilon coffee soil, glyphosate as phosphate source), and Con_CC-G_P (Conilon coffee soil, no glyphosate for 3 years, glyphosate as phosphate source).

3.2 Diversity and Differential abundance analysis of bacterial genera in glyphosate-contaminated soils

The PCoA graph explores and visualizes data similarities or differences so that the distances between points are close to the original dissimilarities between the microbial communities. We conducted Principal Coordinate Analysis (PCoA) to assess Beta diversity based on a Bray-Curtis dissimilarity matrix. The beta-diversity analysis did not reveal statistically significant differences between the groups (F-value: 1.2049; R-squared: 0.35158; p-value: 0.158). (Supplementary figure S1). Despite the lack of statistically significant clustering observed in the beta-diversity analysis, the differential abundance analysis revealed that specific genera within the inoculated consortia were differentially abundant compared to the control treatments.

The bacterial consortia, initially composed of *Achromobacter* and *Serratia* as the most dominant genera (Supplementary figure S2), were prepared for soil inoculation. At the end of the experiment on catabolic activity, soil samples were collected to evaluate differences in the abundance of these genera and other microorganisms between the inoculated treatments and the control treatment (soil + glyphosate).

The results revealed differences in the abundances of *Achromobacter* and *Serratia* between the control treatment (soil+gly) and the treatments inoculated with consortia. In the Con_AO_C treatment compared to the control (soil+gly), a significant increase in the abundance of *Achromobacter* was observed (Log2FC 3.988, p-value 3.4354E-4) (Figure 15). This pattern was also observed in the T2 *glf* C treatment (Log2FC 4.0163, *p-value* 2.2483E-4).

In the Con_CC-G_C and Con_CC-G_P consortia, there was a significant increase in the abundance of *Serratia* compared to the control (Log2FC 5.7495, p-value 1.3098E-16), and (Log2FC 5.9276, p-value 1.6749E-17) (Figure 15). This increasing pattern was even more pronounced in the Con_AO_P treatment (Log2FC 6.7606, *p-value* 5.5338E-22) and the Con_MA_P treatment (Log2FC 7.1862, p-value 1.7558E-24). These results indicate that *Serratia* not only remained dominant in the cultured consortia, but also was one of the genera that contributed most to the significant difference compared to the control.

In addition to *Achromobacter* and *Serratia*, other genera such as *Arthrobacter*, *Paenarthrobacter*, and *Pseudarthrobacter* were also identified as differentially abundant compared to the soil+gly control, showing a significant increase in the Con_CC-G_C treatment (Log2FC 3.1577, p-value 1.1477E-5*;* Log2FC 3.114, p-value 4.011E-4*;* Log2FC *2.7867*, p-value 0.002004, respectively) (Supplementary figure S3).

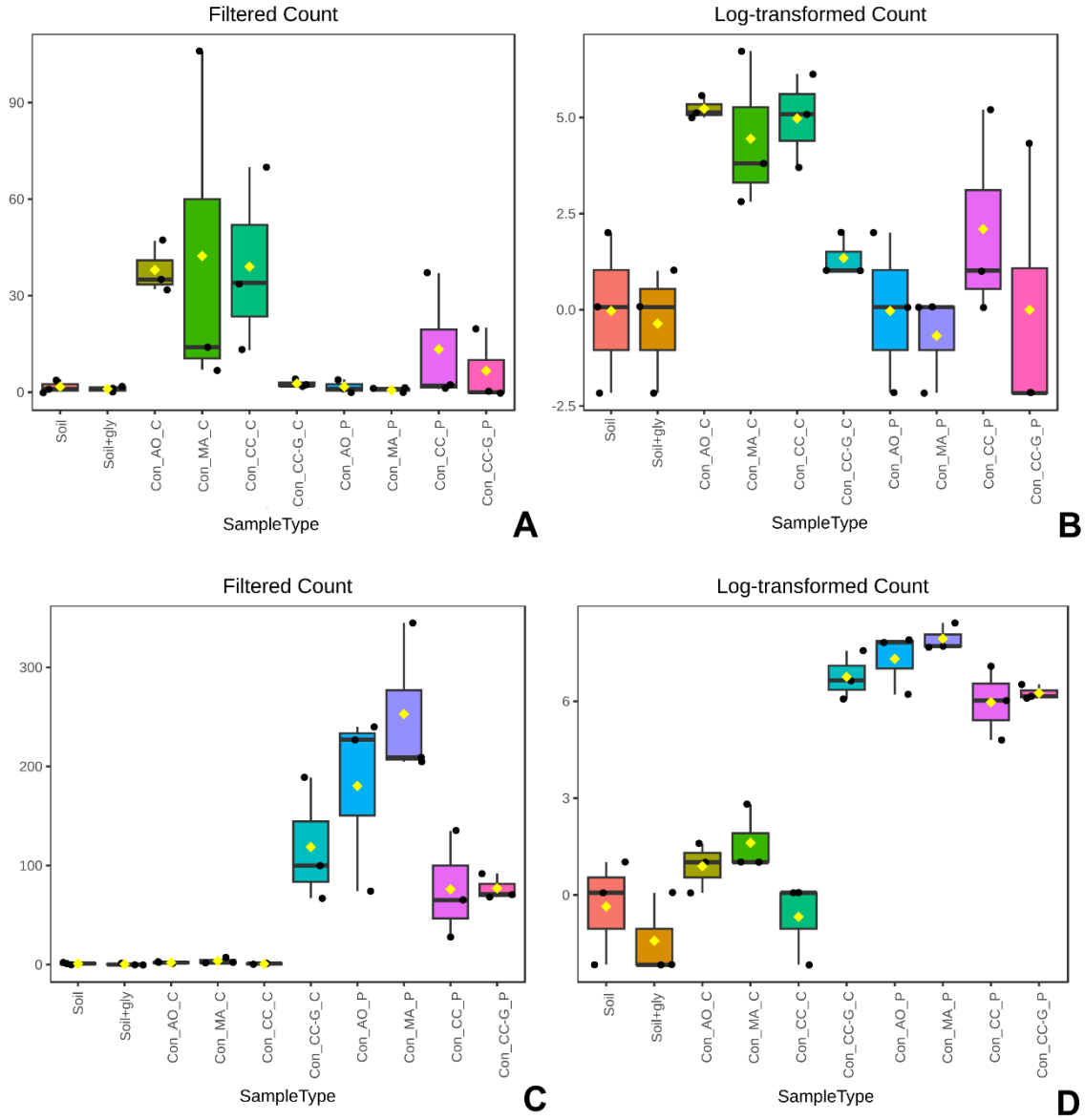

Figure 15 – Abundance of *Achromobacter* and *Serratia* across different treatments. *Achromobacter* is represented by A and B, and *Serratia* by C and D. A and C plots depict the filtered count data (raw abundance), and B and D plots represent the log-transformed count data, which normalizes the data to reduce skewness. Treatments: soil (only soil), soil+gly (soil+glyphosate), Con_AO_C (organic Arabica coffee soil, glyphosate as carbon source), Con_MA_C (Atlantic Forest soil, glyphosate as carbon source), Con_CC_C (conventional Conilon coffee soil, glyphosate-treated, glyphosate as carbon source), Con_CC-G_C (Conilon coffee soil, no glyphosate for 3 years, glyphosate as carbon source), Con_AO_P (organic Arabica coffee soil, glyphosate as phosphate source), Con_MA_P (Atlantic Forest soil, glyphosate as phosphate source), Con_CC_P (conventional Conilon coffee soil, glyphosate as phosphate source), and Con_CC-G_P (Conilon coffee soil, no glyphosate for 3 years, glyphosate as phosphate source).

Discussion

In this work, we evaluated the behavior of microbial consortia in soil microcosms contaminated with glyphosate, focusing on their respiratory activity and differential abundance. Respiratory activity is a widely recognized indicator for evaluating soil environmental status, as it highlights possible disruptions in carbon transformation processes triggered by the harmful effects of pesticides and various xenobiotics (Jezierska-Tys *et al*., 2020).

When analyzing non-sterilized soil, a comparison between the two controls, soil and soil+gly, revealed higher catabolic activity in the soil+gly control. This suggests that the native soil microbiota responded to the addition of glyphosate. This response aligns with findings that introducing pesticides into soil can initially enhance microbial respiration and enzymatic activity as an adaptation to chemical stress, resulting in increased CO_2 emissions (Floch *et al*., 2011).

During the respirometry assay in non-sterilized soil, glyphosate-sensitive microorganisms were impacted by the herbicide's inhibition of the shikimic acid pathway, which is essential for synthesizing aromatic amino acids. This disruption led to turnover within the microbial community, as susceptible organisms were eliminated, thereby providing a readily available carbon source for resistant microbes (Wardle & Parkinson, 1990). The presence of surfactants in the commercial glyphosate formulation may have further contributed to this effect by lysing microbial cells (Haney *et al*., 2000). Consequently, the increased carbon availability likely explains the elevated CO_2 emissions observed during the first 12 hours.

At the 40-hour mark, when the system showed signs of stabilization, an additional dose of glyphosate, five times higher than the initial one, was introduced, stimulating microbial activity and increasing CO_2 emissions. This increase aligns with observations by Zabaloy and Gómez (2008), where low application rates of glyphosate and 2,4-D produced only transient effects on CO_2 evolution, whereas higher doses of these herbicides stimulated microbial activity.

The Con_CC-G consortium, obtained from Conilon Coffee soil without glyphosate application for 3 years, stood out by showing the highest CO_2 emissions in both sterilized and non-sterilized soil, regardless of the cultivation medium used (glyphosate as a carbon source, GC, or as a phosphate source, GP). Its high microbial activity in soil after cultivation under different conditions suggests that this consortium does not rely on a single type of preparation, enhancing its large-scale applicability by allowing flexibility in the production phase of a

potential inoculant. Furthermore, the consortium was able to sustain its elevated respiratory activity even under the stress induced by glyphosate, including the presence of surfactants and other adjuvants.

The lower concentration at which the inocula were prepared may have contributed to the non-significant result in the beta-diversity analysis, as the inoculated consortia would need to comprise a larger proportion of the total microbial community to induce a detectable shift in community composition. However, despite the low concentration, differential abundance analysis revealed that certain genera were still notably abundant in inoculated treatments compared to the control.

The Con_CC-G_C consortium, for example, was associated with a greater diversity of differentially abundant genera relative to the control, with *Serratia* standing out, as it was originally part of the consortia before soil inoculation. In addition to Con_CC-G_C, *Serratia* was also differentially abundant in its counterpart, Con_CC-G_P, and in all other glyphosate as phosphate source consortia. This genus has previously been isolated from glyphosate-degrading consortia and is associated with the degradation of hydrocarbons and phenolic compounds (Cycoń *et al*., 2013; Mohy-ud-din *et al*., 2023). Other genera such as *Arthrobacter*, *Pseudarthrobacter*, and *Paenarthrobacter*, are known for their roles in herbicide and xenobiotic degradation (Cao *et al*., 2019; Chen *et al*., 2021; Harindintwali *et al*., 2024), also exhibited higher abundance compared to the control.

The genus *Achromobacter*, like *Serratia*, was part of the consortia before inoculation. It emerged as the only differentially abundant genus in the Con_AO_C and Con_CC_C consortia compared to the control. *Achromobacter* has been studied for its role in glyphosate degradation pathways, contributing to identifying and cloning genes involved in glyphosate resistance (Epiktetov *et al*., 2024).

Therefore, the respirometry and differential abundance analysis appear to be closely connected, suggesting that the higher metabolic activity observed in the Con_CC-G consortium may have driven an increase in microbial diversity, as reflected in the comparisons with the control.

The ability of Con_CC-G consortia to sustain high metabolic activity, as evidenced by its CO_2 emissions and its use of glyphosate as a dual-source nutrient, suggests that it could play a crucial role in the bioremediation of glyphosate-contaminated environments. However, before its application in field-scale bioremediation, it is crucial to assess the safety and potential toxicity of the Con_CC-G consortium to ensure it poses no risks to the surrounding ecosystem.

Ensuring the consortium's safety and ecological compatibility will be key to its successful application on a larger scale.

Acknowledgments

The authors would like to thank the Fundação de Amparo à Pesquisa do Estado do Espírito Santo—FAPES (#2020QS1D) for the financial support in the development of the research/publication of the article and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) — Finance Code 001 — for their financial support. This work was supported by the Brazilian Microbiome Project (http://brmicrobiome.org).

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Supplemental Information for:

Evaluating the effects of bacterial consortia inoculation on soil microbial activity and community dynamics in glyphosate-treated microcosms

Mariana Prósperi de Oliveira Paula[1], Alessandro Coutinho Ramos[2], Marcos Rogério Tótola[3], Victor Satler Pylro[1,*]

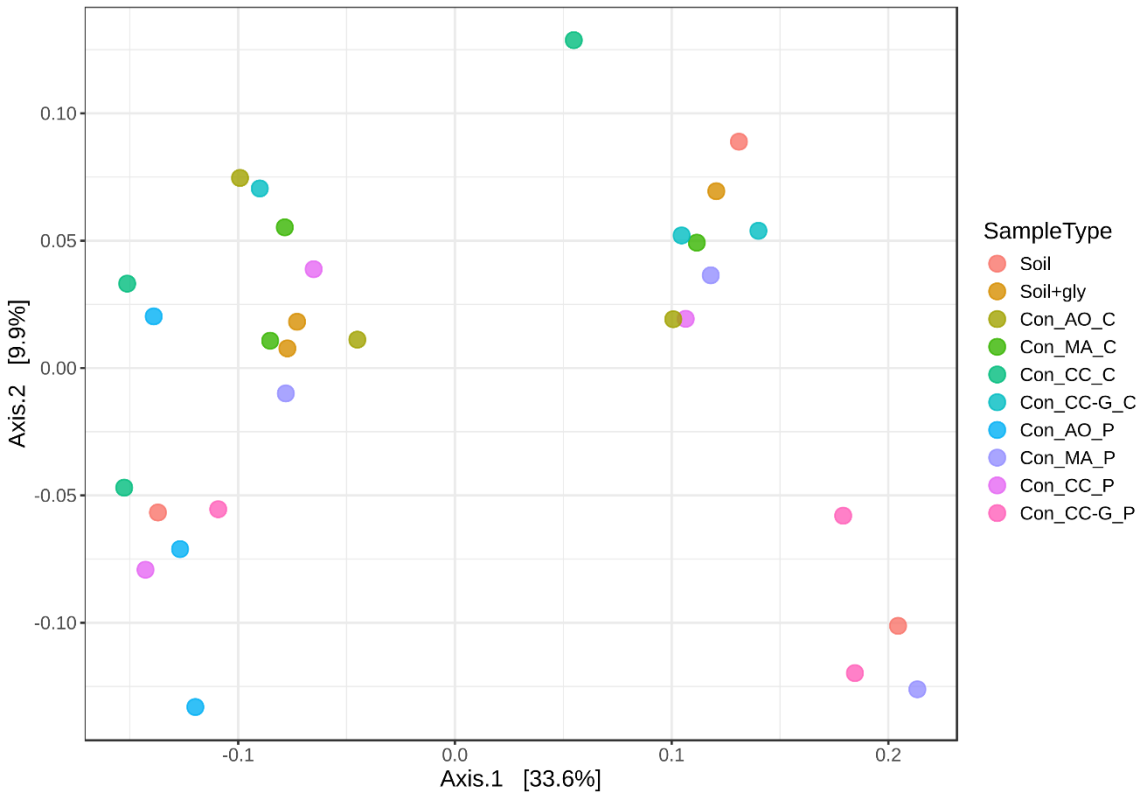

Figure S1 – Beta-diversity analysis of Bacteria in soil samples using Bray-Curtis index and PCoA based on rRNA 16S gene sequencing (F-value: 1.2049; R-squared: 0.35158; p-value: 0.158). Treatments: soil (only soil), soil+gly (soil+glyphosate), Con_AO_C (organic Arabica coffee soil, glyphosate as carbon source), Con_MA_C (Atlantic Forest soil, glyphosate as carbon source), Con_CC_C (conventional Conilon coffee soil, glyphosate-treated, glyphosate as carbon source), Con_CC-G_C (Conilon coffee soil, no glyphosate for 3 years, glyphosate as carbon source), Con_AO_P (organic Arabica coffee soil, glyphosate as phosphate source), Con_MA_P (Atlantic Forest soil, glyphosate as phosphate source), Con_CC_P (conventional Conilon coffee soil, glyphosate as phosphate source), and Con_CC-G_P (Conilon coffee soil, no glyphosate for 3 years, glyphosate as phosphate source).

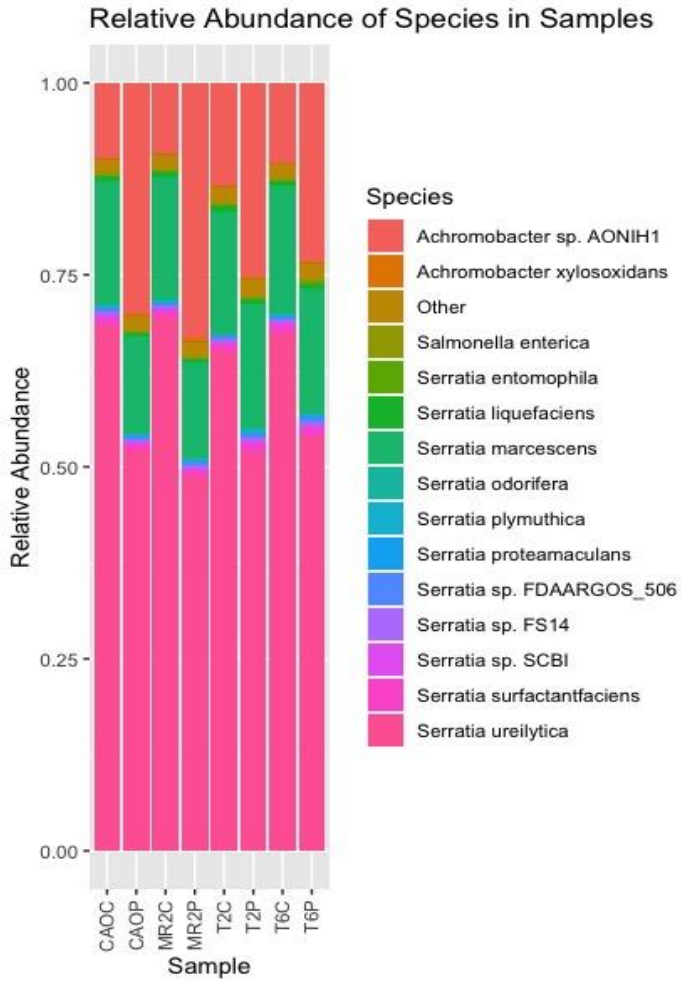

Figure S2 – Relative abundance, at the species level, of bacteria comprising the bacterial consortia when cultivated in different media. In the CAOC, MR2C, T2C, and T6C consortia, glyphosate was used as the sole carbon source, while in the CAOP, MR2P, T2P, and T6P consortia, it was used as the sole phosphate source. Treatments included: CAO (originating from organic Arabica coffee soil), MR2 (from Atlantic Forest soil adjacent to a glyphosate-treated Arabica coffee plantation), T2 (from conventional glyphosate-treated Conilon coffee plantation soil), and T6 (from Conilon Coffee soil without glyphosate application for 3 years).

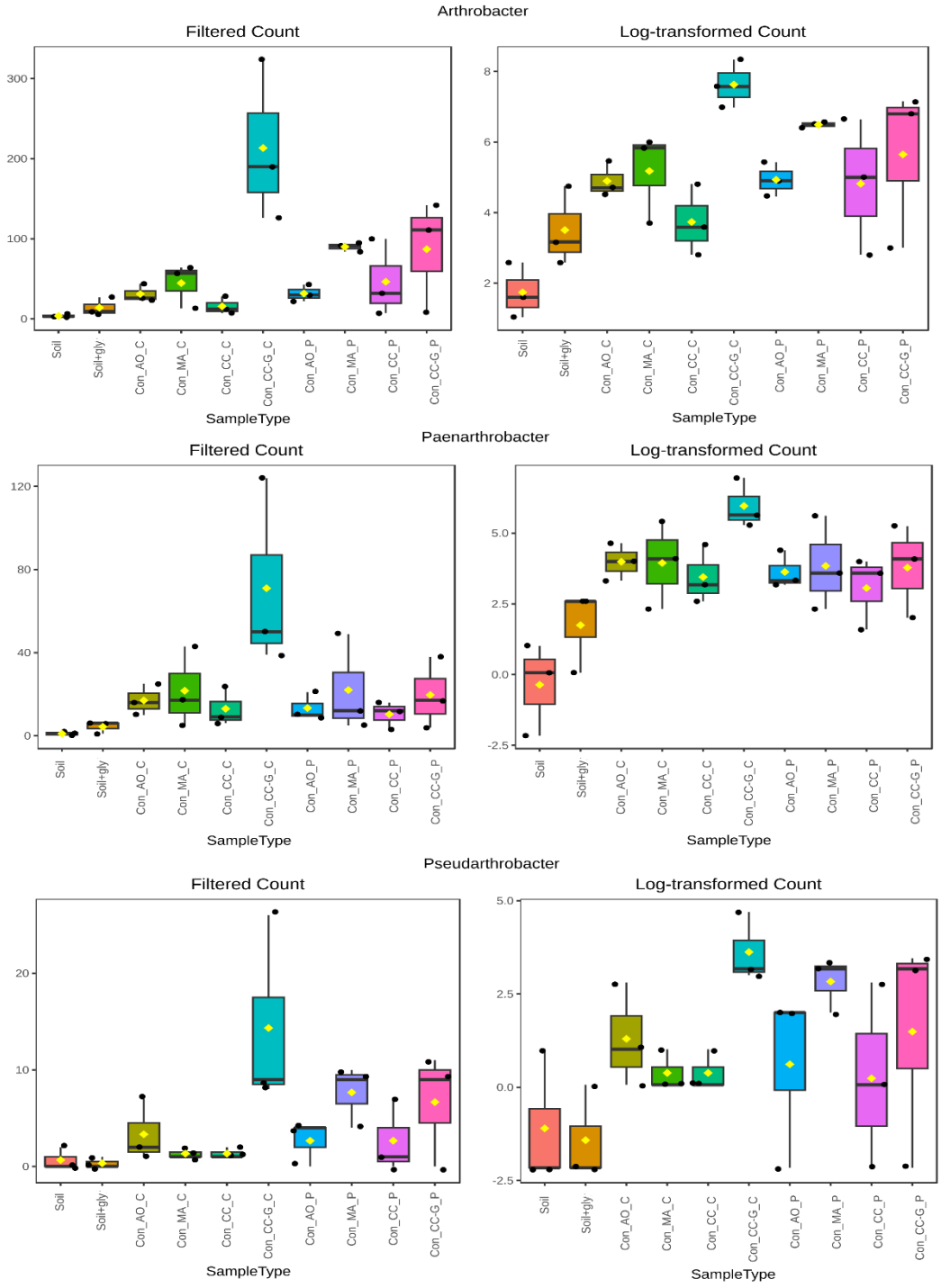

Figure S3 – Abundance of *Arthrobacter*, *Paenarthrobacter*, *and Pseudarthrobacter* across different treatments. The left plot depicts the filtered count data (raw abundance), and the right plot represents the log-transformed count data**,** which normalizes the data to reduce skewness. Treatments: soil (only soil), soil+gly (soil+glyphosate), Con_AO_C (organic Arabica coffee soil, glyphosate as carbon source), Con_MA_C (Atlantic Forest soil, glyphosate as carbon source), Con_CC_C (conventional Conilon coffee soil, glyphosate-treated, glyphosate as carbon source), Con_CC-G_C (Conilon coffee soil, no glyphosate for 3 years, glyphosate as carbon source), Con_AO_P (organic Arabica coffee soil, glyphosate as phosphate source), Con_MA_P (Atlantic Forest soil, glyphosate as phosphate source), Con_CC_P (conventional Conilon coffee soil, glyphosate as phosphate source), and Con_CC-G_P (Conilon coffee soil, no glyphosate for 3 years, glyphosate as phosphate source).

Considerações finais

Neste trabalho, realizamos a caracterização de consórcios bacterianos enriquecidos a partir de solos de plantações de café e da Mata Atlântica. Os gêneros *Achromobacter* e *Serratia* se destacaram nesses consórcios, uma vez que demonstraram elevada capacidade de adaptação metabólica, utilizando glifosato como fonte de carbono e fósforo. Ademais, a partir da anotação dos genomas recuperados desses gêneros, verificamos que *Achromobacter sp.* e *Serratia sp.* possuem genes codificadores de enzimas-chave na degradação de glifosato e AMPA, corroborando o potencial desses microrganismos na remediação de ambientes contaminados.

Além disso, os consórcios, quando aplicados em microcosmos de solo contaminado com glifosato, apresentaram, em geral, taxas respiratórias mais elevadas do que as observadas nos solos controle, indicando atividade catabólica em relação ao herbicida. Ao analisar a abundância diferencial entre solos inoculados com os consórcios e solos não inoculados, os gêneros *Achromobacter* e *Serratia* mostraram-se distintivos, indicando sua capacidade de persistir em solos com a microbiota nativa.

Esses resultados enfatizam a importância da interação entre a microbiota nativa e os consórcios introduzidos no solo, sugerindo que esses consórcios têm grande potencial para futuras aplicações em bioremediação em larga escala. No entanto, para uma aplicação eficaz em campo, é essencial realizar testes adicionais de segurança e impacto ambiental. Estudos futuros poderão explorar, de forma mais aprofundada, a estabilidade funcional e a longevidade desses consórcios em diferentes condições ambientais, além de otimizar suas aplicações em solos contaminados por glifosato.