UNIVERSIDADE ESTADUAL PAULISTA - UNESP
CÂMPUS DE JABOTICABAL

TEMPORAL DYNAMICS OF MICROBIOME COMPOSITION AND GENE EXPRESSION IN A LIGNOCELLULOSIC BIOMASS-DEGRADING CONSORTIUM AT DIFFERENT ADAPTATION STAGES

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Biólogo e Mestre em Microbiologia Agropecuária

2025

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – UNESP, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Microbiologia Agropecuária

2025

C331t

Carvalho, Lucas Amoroso Lopes de

Temporal dynamics of microbiome composition and gene expression in a lignocellulosic biomass-degrading consortium at different adaptation stages / Lucas Amoroso Lopes de Carvalho. -- Jaboticabal, 2025

160 p.

Tese (doutorado) - Universidade Estadual Paulista (UNESP), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal

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1. Degradação biológica. 2. Lignocelulose. 3. Microbiologia ambiental. 4. Sequenciamento de nucleotídeo. 5. Enzimas microbianas. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Dados fornecidos pelo autor(a).

Impacto potencial desta pesquisa

Esta pesquisa oferece potencial para o avanço da energia renovável e da gestão sustentável de resíduos. Ao identificar bactérias-chave e vias metabólicas ativas na degradação da lignocelulose, ela ajuda a aprofundar a compreensão da adaptação microbiana e destaca estratégias para aumentar os rendimentos de biocombustíveis. A eficiência aprimorada dos processos de degradação da biomassa pode reduzir os custos operacionais, estimular novos mercados para resíduos agroindustriais e minimizar os danos ambientais ao reduzir a dependência de pré-tratamentos mais intensivos. Além disso, consórcios microbianos otimizados podem ajudar a impulsionar uma bioeconomia circular ao converter resíduos agroindustriais em produtos de valor agregado e mitigar os impactos climáticos. Tomadas em conjunto, as descobertas desta pesquisa conectam a ecologia microbiana e as aplicações práticas, auxiliando no desenvolvimento de soluções de energia mais limpas e sustentáveis.

Potential impact of this research

This research offers potential for advancing renewable energy and sustainable waste management. Identifying key bacteria and metabolic pathways active in lignocellulose degradation helps deepen understanding of microbial adaptation and highlights strategies for increasing biofuel yields. Improved efficiency of biomass degradation processes can reduce operational costs, stimulate new markets for agro-industrial waste, and minimize environmental damage by reducing reliance on more intensive pretreatments. Furthermore, optimized microbial consortia can help drive a circular bioeconomy by converting agro-industrial waste into value-added products and mitigating climate impacts. Taken together, the findings of this research connect microbial ecology and practical applications, aiding the development of cleaner and more sustainable energy solutions.

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: TEMPORAL DYNAMICS OF MICROBIOME COMPOSITION AND GENE EXPRESSION IN A LIGNOCELLULOSIC BIOMASS-DEGRADING CONSORTIUM AT DIFFERENT ADAPTATION STAGES

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Aprovado como parte das exigências para obtenção do Título de Doutor em Microbiologia Agropecuária, pela Comissão Examinadora:

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Jaboticabal, 11 de abril de 2025

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Lucas Amoroso Lopes de Carvalho was born on 6 of July 1992 in the municipality of Jaboticabal, São Paulo/Brazil, son of Paula Regina Amoroso Lopes de Carvalho and Gilberto Lopes de Carvalho. He earned a bachelor's degree in Biological Sciences (2015–2019) from the Faculty of Agricultural and Veterinary Sciences (FCAV) at São Paulo State University "Júlio de Mesquita Filho" (UNESP) – Jaboticabal, São Paulo, where he conducted undergraduate research and completed his final work entitled "Eritrocitograma de suínos em diferentes fases de criação no estado de São Paulo" under the supervision of Prof. Dr. Aureo Evangelista Santana (*in memoriam*). From March 2019 to March 2021, he completed his master's degree in the Postgraduate Program in Agricultural Microbiology at FCAV/UNESP under the supervision of Prof. Daniel Guariz Pinheiro. His research, "Investigação sobre o efeito do sistema de cultivo na composição da microbiota da cana-de-açúcar", culminated in the work published at https://doi.org/10.1016/j.micres.2021.126866. In March 2021, he began his Ph.D. studies in the same program, under the supervision of Prof. Dr. Daniel and co-supervision of Prof. Dr. Lúcia Maria Carareto Alves, developing the current research project titled "Temporal Dynamics of Microbiome Composition and Gene Expression in a Lignocellulosic Biomass-Degrading Consortium at Different Adaptation Stages". Throughout his postgraduate studies, he co-authored more than 20 articles indexed in international journals, mainly focusing on the application of omics approaches — such as metabarcoding, (meta)genomics, and (meta)transcriptomics — across various themes related to agricultural microbiology. His complete academic curriculum is available at: http://lattes.cnpq.br/9100748298129844.

ACKNOWLEDGMENTS

To my parents, Paula and Gilberto, my sister Julia, and my partner Michelle, who have always believed in my capabilities and supported me throughout this journey.

To my friends Felipe Rodrigues and Bruno Mangili for tolerating my academic frustrations and for the moments of entertainment and laughter.

To the Faculty of Agricultural and Veterinary Sciences, São Paulo State University “Júlio de Mesquita Filho” (FCAV/Unesp) for the academic support and infrastructure provided during my Ph.D. studies.

To Prof. Dr. Daniel Guariz Pinheiro and Prof. Dr. Lúcia Maria Carareto Alves for their guidance, invaluable lessons, and, most importantly, for the trust and freedom they granted me throughout this process.

To my past and present colleagues at the Bioinformatics Laboratory — Luis Guillermo, Michelli, Rafael, Maria Fernanda, André Aquino, Ana Paula, Carlos, Felipe, Gabriella, and Andrea — for their companionship and support.

To Prof. Dr. Juan “Nacho” Ignacio Vílchez and all the colleagues at the iPlantMicro laboratory at the Institute of Chemical and Biological Technology (IQTB), Universidade Nova de Lisboa, for warmly welcoming me and providing an incredible period of learning and growth in Portugal.

To all collaborators, professors, and colleagues who believed in my work, placed their trust in me, and contributed to meaningful and enriching partnerships throughout my postgraduate studies.

To the faculty and staff of the Department of Agricultural and Environmental Biotechnology, who were always willing to help whenever work-related demands arose.

To the Graduate Program Council in Agricultural Microbiology and the Technical Postgraduate Section staff for their dedication and efficiency in addressing any issues that arose during my master's studies.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.”.

Therefore, I am grateful for their financial assistance.

I also extend my gratitude to the São Paulo Research Foundation (FAPESP) for funding research projects No. 2016/16624-1 and No. 2019/20825-0, which enabled the execution of this study.

Finally, to anyone who contributed — directly or indirectly — to the development of this work, my sincerest thanks!

SUMMARY *p.*

TEMPORAL DYNAMICS OF MICROBIOME COMPOSITION AND GENE EXPRESSION IN A LIGNOCELLULOSIC BIOMASS-DEGRADING CONSORTIUM AT DIFFERENT ADAPTATION STAGES

RESUMO – A biomassa lignocelulósica (BLC) representa um recurso renovável com grande potencial para produção de energia sustentável, mas é estruturalmente recalcitrante e difícil de usar. Nesse sentido, os microrganismos do solo podem ser soluções promissoras devido às suas diversas capacidades metabólicas e enzimáticas para lidar com esse tipo de material. Para entender a capacidade de adaptação dos microbiomas naturais ao uso da BLC como fonte exclusiva de carbono, dois estudos complementares foram conduzidos. No primeiro estudo, a metataxonômica do gene *16S rRNA* (região V4) foi usada para explorar a dinâmica temporal de um consórcio bacteriano em três pontos de tempo (dia 0, dia 5 e dia 10) nos estágios inicial (duas semanas) e final (vinte semanas) de adaptação. Apesar das mudanças substanciais na composição da comunidade — exemplificadas por abundâncias reduzidas de *Caulobacter* e *Chitinophaga* no estágio final — as previsões funcionais indicaram que os traços funcionais eram altamente compartilhados entre os estágios. Enquanto o estágio inicial foi caracterizado por maior instabilidade, mas uma prevalência notável de táxons potencialmente lignocelulolíticos, o estágio tardio revelou maior diversidade taxonômica e funcional, e uma prevalência maior de genes previstos associados ao metabolismo de carboidratos, sugerindo uma comunidade microbiana funcionalmente versátil especializada para a quebra de BLC. Da mesma forma, o segundo estudo empregou uma abordagem metatranscriptômica para capturar a expressão gênica ativa deste consórcio microbiano exposto ao substrato lignocelulósico nos dias 5 e 10 dos estágios inicial e tardio. Uma transição na contribuição relativa no nível do filo foi notada, com Bacteroidota declinando de aproximadamente 18% para menos de 1% do volume total de transcritos, enquanto Bacillota aumentou de aproximadamente 3% para mais de 27% no estágio tardio. Esta mudança foi acompanhada por um aumento proporcional em enzimas ativas em carboidratos, particularmente aquelas que visam a quebra da celulose e hemicelulose. Mais de 30% dos transcritos de lignocelulase detectados foram afiliados ao gênero *Paenibacillus* (Bacillota), destacando o papel fundamental dessas bactérias na degradação da biomassa. No geral, esses resultados revelam como a exposição prolongada a substratos lignocelulósicos enriquece seletivamente os táxons microbianos e mecanismos enzimáticos que impulsionam a conversão eficiente de biomassa. Esses resultados destacam a plasticidade taxonômica e funcional inerente dos microbiomas do solo, evidenciando a capacidade de reestruturação da comunidade e o aumento da expressão de enzimas degradativas quando expostos a essa pressão seletiva. Assim, essas descobertas podem servir como base para o design de consórcios microbianos otimizados, bioprospecção de novas enzimas e desenvolvimento de estratégias de melhoramento direcionadas para aprimorar o uso industrial do BLC.

Palavras-chave: Sequenciamento de RNA, metataxonômica, enzimas ativas em carboidratos, desconstrução de lignocelulose, bioprospecção

TEMPORAL DYNAMICS OF MICROBIOME COMPOSITION AND GENE EXPRESSION IN A LIGNOCELLULOSIC BIOMASS-DEGRADING CONSORTIUM AT DIFFERENT ADAPTATION STAGES

ABSTRACT – Lignocellulosic biomass (LCB) represents a renewable resource with great potential for sustainable energy production, but it is structurally recalcitrant and difficult to use. In this sense, soil microorganisms can be promising solutions due to their diverse metabolic and enzymatic capabilities to deal with this type of material. To understand the adaptation capacity of natural microbiomes to the use of LCB as an exclusive carbon source, two complementary studies were conducted. In the first study, metabarcoding of the *16S rRNA* gene (V4 region) was used to explore the temporal dynamics of a bacterial consortium at three time points (day 0, day 5 and day 10) in the early (two weeks) and late (twenty weeks) stages of adaptation. Despite substantial changes in community composition — exemplified by reduced abundances of *Caulobacter* and *Chitinophaga* in the final stage — functional predictions indicated that functional traits were highly shared between stages. While the early stage was characterized by greater instability but a notable prevalence of potentially lignocellulolytic taxa, the late stage revealed greater taxonomic and functional diversity, and a higher prevalence of predicted genes associated with carbohydrate metabolism, suggesting a functionally versatile microbial community specialized for LCB breakdown. Similarly, the second study employed a metatranscriptomic approach to capture active gene expression from this same microbial consortium exposed to lignocellulosic substrate on days 5 and 10 of the early and late stages. A transition in relative contribution at the phylum level was noted, with Bacteroidota declining from approximately 18% to less than 1% of the total transcript volume, while Bacillota increased from approximately 3% to over 27% in the late stage. This change was accompanied by a proportional increase in carbohydrate-active enzymes, particularly those targeting cellulose and hemicellulose. More than 30% of the detected lignocellulase transcripts were affiliated with the genus *Paenibacillus* (Bacillota), highlighting the key role of these bacteria in biomass degradation. Overall, these results reveal how prolonged exposure to lignocellulosic substrates selectively enriches microbial taxa and enzymatic mechanisms that drive efficient biomass conversion. These results highlight the inherent taxonomic and functional plasticity of soil microbiomes, illustrating the capacity for community restructuring and increased expression of essential degradative enzymes when exposed to this selective pressure. Thus, these findings can serve as a basis for the design of optimized microbial consortia, bioprospecting for potent enzymes, and developing targeted breeding strategies to enhance the industrial use of LCB.

Keywords: RNA-Seq analysis, *16S rRNA* gene sequencing, carbohydrate-active enzymes, lignocellulose deconstruction, bioprospection

CHAPTER 1 – GENERAL CONSIDERATIONS

1. INTRODUCTION

Lignocellulosic biomass (LCB) represents a vast and renewable source of carbon, including agricultural residues and forestry by-products (Andrade et al., 2022; Zhao et al., 2022). The main components of LCB — cellulose, hemicellulose, and lignin — form a rigid matrix that provides structural support to plants. This matrix, however, also confers high recalcitrance, making the efficient conversion of LCB into bioenergy and other value-added products a significant challenge (Rezania et al., 2020; Broda et al., 2022). Despite these obstacles, the abundance and renewability of LCB continue to attract attention for its potential, especially related to its application as a feedstock for second-generation (2G) biofuels (Faria et al., 2024).

To utilize residual LCB, pretreatment processes are required that facilitate the release of fermentable sugars (Mankar et al., 2021). These pretreatment processes generally require substantial energy input, specialized equipment, and can sometimes result in inhibitory products for the subsequent steps of obtaining 2G fuels, in addition to being environmentally harmful. Thus, the importance of developing innovative strategies to overcome the structural challenges of LCB nature is emerging (Haldar and Purkait, 2021).

Numerous studies highlight the key role of microbial communities in the deconstruction of lignocellulosic materials (López-Mondéjar et al., 2019; Thapa et al., 2020; Gurovic et al., 2023). Unlike conventional chemical or physical pretreatment approaches, microorganisms can employ an arsenal of adapted enzymes to degrade the complex network of lignin, hemicellulose, and cellulose under diverse environmental conditions (Benatti and Polizeli, 2023). These enzymatic processes not only reduce energy demands but can also produce fewer inhibitory byproducts when compared to more aggressive methods (Kumar et al., 2020). Thus, microbial degradation strategies are increasingly seen as an environmentally friendly and sustainable alternatives that capitalize on the metabolic diversity found in nature.

Different microbial species can specialize in the breakdown of distinct components of the lignocellulosic substrate. This division of labor is especially valuable for soil-derived consortia, where an extensive reservoir of lignocellulolytic organisms coexists, shaped by local environmental conditions (Champreda et al., 2019; Puentes-

Téllez and Salles, 2020; Weiss et al., 2021). Understanding how these complex microbial communities adapt, and function is essential to harness their full potential in biotechnological applications.

To elucidate the composition and functional capability of lignocellulose-degrading communities, modern -omics approaches have gained prominence. Metabarcoding, often relying on marker genes such as *16S rRNA* for prokaryotes and ITS for fungi, provides rapid and high-throughput insight into the assemblage and has proven effective in detecting community composition cultivated under different LCB sources (Ventorino et al., 2015; Houfani et al., 2019; Puentes-Téllez and Salles, 2020). Additionally, metatranscriptomics, which focuses on gene expression, is emerging to understand the active metabolic contributions of these organisms, thus offering insights into how microbial communities dynamically respond to factors such as LCB availability throughout degradation (Peng et al., 2018).

Foundational studies using the same soil-derived microbial consortium used here set the stage for the current work. The first of these, by Constancio et al. (2020), characterized the degradative capabilities of the consortium by quantifying glucose production following exposure to different lignocellulosic biomass sources. This study also included preliminary metagenomic sequencing, identifying several enzymes of biotechnological relevance, such as glucanases, amylases, proteases, and lipases. Taxonomic profiling highlighted the dominance of genera including *Burkholderia*, *Pandoraea*, and *Dyella*.

Building on this, Weiss et al. (2021) conducted a 20-week experiment in which the same consortium was cultivated with sugarcane bagasse. Metagenomic sequencing at weeks 2 and 20 enabled the recovery of 52 metagenome-assembled genomes (MAGs) from eight bacterial classes: Actinobacteria, Alphaproteobacteria, Bacilli, Bacteroidia, Cytophagia, Gammaproteobacteria, Oligoflexia, and Thermoleophilia. Many of these MAGs contained genes associated with various lignocellulose degradation mechanisms, further supporting the metabolic potential of the consortium.

In this context, the present study investigates how this lignocellulolytic consortium adapts over time to a lignocellulosic substrate. Taxonomic composition was evaluated by sequencing the *16S rRNA* gene (V4 region), while RNA sequencing

revealed the expression profiles of biomass-degrading genes and how their patterns shifted throughout the temporal dynamics of LCB breakdown. These analyses were designed to complement the previous genomic efforts by providing a deeper resolution of microbial taxonomy and directly linking community structure to active functions under biomass exposure.

5. CONCLUSION

This study provided crucial insights into the temporal dynamics of microbial consortia during lignocellulose degradation, revealing a shift from a diverse early-stage community to a specialized late-stage composition dominated by taxa with optimized lignocellulolytic functions, such as *Paenibacillus*. These findings highlight the role of microbial succession in shaping biomass degradation efficiency, supporting the hypothesis that functional redundancy found in natural environments allows a gradual transition towards a simpler but more efficient system through induced selective pressures. In addition to advancing the understanding of functional adaptation of the microbial community, this work also highlights the potential of natural consortia for biotechnological applications in biofuel production and waste management. Future studies should explore individual capabilities and optimization processes for those industrial applications.

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