MINERAÇÃO GENÔMICA DE ISOLADOS BACTERIANOS DO GÊNERO *BACILLUS* PARA BIOCONTROLE DE FUNGOS FITOPATOGÊNICOS

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CAMPOS DOS GOYTACAZES - RJ

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Dissertação apresentada ao Centro de Biociências e Biotecnologia da Universidade Estadual do Norte Fluminense Darcy Ribeiro, como parte das exigências para obtenção do título de Mestre em Biotecnologia Vegetal.

Orientador: Dr. Thiago Motta Venancio

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Dr. Thiago Motta Venâncio (Orientador) – UENF

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ACHO-VASQUEZ, Hector Julio, M.Sc., Universidade Estadual do Norte Fluminense - RJ, Fevereiro de 2026. **Mineração genômica de isolados bacterianos do gênero *Bacillus* para biocontrole de fungos fitopatogênicos**. Orientador: Thiago Motta Venancio.

Até 2050, a agricultura precisará ampliar a produção global em 70% para abastecer uma população estimada em 9,8 bilhões. Atualmente, entre 20% e 40% das perdas no campo decorrem de pragas e doenças fúngicas. Embora eficazes, o uso intensivo de fungicidas sintéticos eleva a toxicidade residual, acelera o surgimento de resistência e amplia os riscos ambientais e à saúde humana. Nesse cenário, biofungicidas microbianos especialmente os derivados de *Bacillus* têm se destacado como alternativa sustentável, atuando por antibiose e pela produção de enzimas hidrolíticas. Este estudo descreve a mineração genômica em larga escala para identificar espécies de *Bacillus* com potencial biofungicida, classificá-las filogeneticamente e mapear genes associados à atividade antifúngica, resistência a antibióticos, virulência e biofertilização. Foram analisados 10.839 genomas do GenBank, com controle de qualidade via CheckM e BUSCO. A identidade genômica foi estimada com Mash, resultando em 10.276 genomas distribuídos em 78 comunidades; nove das quais exibiram conexões fortes e formaram complexos. A delimitação com fastANI auxiliou no refinamento da estrutura para 103 comunidades. Através de reconstrução filogenética, conseguimos organizar os genomas em cinco grupos: *B. cereus* sensu lato, *B. pumilus*, *B. amyloliquefaciens*, *B. licheniformis* e *B. subtilis*. Através de revisão da literatura, identificamos 135 genes candidatos (enzimas hidrolíticas e metabólitos secundários), por buscas com USEARCH e antiSMASH, com destaque para *B. spizizenii*, *B. velezensis* e *B. subtilis*. Genes de resistência e virulência concentraram-se principalmente em *B. cereus* sensu lato, enquanto a maioria das espécies apresentou potencial de biofertilização (ferro, nitrogênio e fosfato). Ao indicar cepas candidatas ao biocontrole, o presente trabalho contribui para o desenvolvimento de bioinsumos sustentáveis.

Palavras-chave: *Bacillus*, biofungicida, resistência a antibióticos, virulência e biofertilização.

ABSTRACT

ACHO-VASQUEZ, Hector Julio, M.Sc., Universidade Estadual do Norte Fluminense - RJ, January 2026. **Genomic mining of bacterial isolates of the genus *Bacillus* for biocontrol of phytopathogenic fungi**. Advisor: Thiago Motta Venancio.

By 2050, global agricultural production will need to increase by approximately 70% to sustain an estimated population of 9.8 billion people. Currently, between 20% and 40% of crop losses are due to pests and fungal diseases. Although effective, the intensive use of synthetic fungicides increases residual toxicity, accelerates the emergence of resistance, and heightens environmental and human health risks. In this context, microbial biofungicides particularly those derived from *Bacillus* have emerged as sustainable alternatives, acting through antibiosis and the production of hydrolytic enzymes. This study conducted large-scale genomic mining to identify *Bacillus* species with biofungicidal potential, classify them phylogenetically, and map genes associated with antifungal activity, antibiotic resistance, virulence, and biofertilization. A total of 10,839 genomes from GenBank were analyzed, with quality control via CheckM and BUSCO. Genomic identity was estimated with Mash, resulting in 10,276 genomes distributed across 78 communities, nine of which exhibited strong connections and formed complexes. Delimitation with fastANI helped refine the structure to 103 communities. Through phylogenetic reconstruction, we were able to organize the genomes into five groups: *B. cereus sensu lato, B. pumilus, B. amyloliquefaciens, B. licheniformis,* and *B. subtilis.* Through a literature review, we identified 135 candidate genes (hydrolytic enzymes and secondary metabolites) using USEARCH and antiSMASH searches, with emphasis on *B. spizizenii, B. velezensis,* and *B. subtilis*. Resistance and virulence genes were mainly concentrated in *B. cereus* sensu lato, while most species showed biofertilization potential (iron, nitrogen, and phosphate). By indicating candidate strains for biocontrol, this study contributes to the development of sustainable bio-inputs.

Keywords: *Bacillus*, biofungicide, biofertilization, antibiotic resistance, virulence.

1. CAPÍTULO 1

1.1. INTRODUÇÃO

A agricultura tem sido historicamente a base para o crescimento e sobrevivência da civilização humana. Estima-se que até 2061 será necessário um aumento mínimo de 70% na produção de alimentos para garantir a segurança alimentar de uma população que deverá atingir 10,0 bilhões de pessoas (Fusar Poli & Fonte Francesco, 2024; Roser et al., 2023; Syed Ab Rahman et al., 2018). Nesse cenário, destacam-se também perdas de 20% a 40% por pragas e doenças, relacionadas a fungos fitopatogênicos (Oerke & Dehne, 2004; Savary et al., 2012), especialmente de algumas espécies dos gêneros *Fusarium* (*Fusarium graminearum*), *Aspergillus* (*Aspergillus flavus*), *Alternaria* (*Alternaria solani*), *Geotrichum* (*Geotrichum candidum*), *Penicillium* (*Penicillium expansum*) e *Sclerotinia* (*Sclerotinia sclerotiorum*) são responsáveis por perdas agrícolas significativas em cultivos essenciais como trigo, milho, arroz e soja (Azeem et al., 2022; Crovo & Clemente, 2015; Nazarena Torres Álvarez & Susana Aquino Jara, 2010; Rivero González et al., 2012).

Diferentes estratégias podem ser aplicadas para prevenir, mitigar ou controlar doenças causadas por fungos fitopatogênicos (Azeem et al., 2022). Os fungicidas sintéticos têm desempenhado um papel crucial na mitigação dos danos causados por fungos fitopatogênicos nas últimas décadas (Fenta & Mekonnen, 2024; Mir et al., 2023). No entanto, o uso prolongado e intensivo de pesticidas químicos levanta series preocupações como a toxicidade residual e riscos ao meio ambiente e à saúde humana (Ahmad et al., 2024; Nicolopoulou-Stamati et al., 2016). Além disso, a aplicação excessiva desses compostos acelera o desenvolvimento de resistência, o que gradualmente compromete a eficácia dos fungicidas. Esse fenômeno força os agricultores a recorrerem a tratamentos cada vez mais agressivos e potencialmente prejudiciais, elevando o risco de contaminação do solo, da água e das plantações, além de aumentar a exposição de trabalhadores rurais e consumidores a substâncias nocivas (Yoon et al., 2013; Zhang et al., 2023).

Nesse contexto, os biofungicidas fungicidas biológicos derivados de microrganismos, plantas ou substâncias naturais emergem como alternativa

promissora. Globalmente, o mercado global de biopesticidas, que foi avaliado em US$2,4 bilhões no início de 2014, tem previsões de atingir US$100 bilhões até 2063, impulsionado por regulamentações ambientais e demanda por agricultura sustentável (Rodrigues et al., 2023). No Brasil, líder na América Latina, o setor gerou aproximadamente USD 58 milhões em 2017, alcançou USD 690 milhões na safra 2023/24, refletindo uma elevada taxa de crescimento anual composta (CAGR) de 49.38%. Esses avanços evidenciam a transição do agronegócio brasileiro para práticas mais sustentáveis, embora desafios relacionados à estabilidade dos produtos e à escala de produção ainda persistam (Felipe De Lima Andreata et al., 2025).

Nesse sentido, o uso de microrganismos com potencial de biocontrole surge como uma alternativa promissora, economicamente viável e ecologicamente sustentável para reduzir o uso de fungicidas químicos. Esses microrganismos, ao não deixarem resíduos tóxicos e serem geralmente específicos para o seu alvo biológico, constituem uma solução viável para práticas agrícolas mais seguras e eficazes (Bautista et al., 2018; Dame et al., 2021). Além disso, sua utilização como biopesticidas está alinhada aos Objetivos de Desenvolvimento Sustentável (ODS) das Nações Unidas, também conhecidos como Agenda 2030. Essa abordagem promove a agricultura sustentável como uma estratégia integrada para alcançar metas globais, como a erradicação da fome e a redução da pobreza. Entre os dezessete ODS, oito estão diretamente relacionados ao desenvolvimento sustentável (ODS 6, ODS 7, ODS 9, ODS 12, ODS 14, ODS 15), sendo os mais impactantes o ODS 2 (fome zero) e o ODS 1 (erradicação da pobreza) (Fenibo, Ijoma e Matambo, 2021; Fusar Poli & Fonte Francesco, 2024)

No entanto, microrganismos benéficos, como as espécies do gênero *Bacillus*, surge como uma solução promissora (Lahlali et al., 2022; Zhang et al., 2023). Estes microrganismos são conhecidos por suas múltiplas capacidades benéficas, não apenas pela habilidade de solubilizar fosfatos e produzir fitohormônios, mas também por suas propriedades antagonistas contra patógenos (Khan et al., 2022; Nihorimbere et al., 2011; C. Tran et al., 2022; Velho et al., 2011; Zhu et al., 2020). Principalmente por meio de mecanismos como competição por nutrientes e espaço, produção de sideróforos, lipoppeptídeos

como iturina (Tsuge et al., 2001; Yaraguppi et al., 2023), surfactina (Dong et al., 2022; Souto et al., 2004), bacillomicina (Ramarathnam et al., 2007), fengicina (Dong et al., 2022; Loeffler et al., 1986), micosubtilina (Tsuge et al., 2001), bacillaeno (W. Y. Wang et al., 2022; Zhu et al., 2020) e difficidina (Wu et al., 2015), assim como de enzimas líticas como quitinasas (Chernin et al., 1995; D. M. Tran et al., 2022), proteases (Ayaz et al., 2023; Hegedüs & Marx, 2013; Theis & Stahl, 2004) e chitosanasas (Pang et al., 2021). Tais moléculas comprometem a estrutura das paredes celulares fúngicas (Tiwari et al., 2019; Zhang et al., 2023). Portanto, as cepas antifúngicas de *Bacillus* compreendem uma promissora estratégia de biocontrole na agricultura sustentável (Chen et al., 2020; Khan et al., 2022; Zhang et al., 2023).

A necessidade de identificar e anotar novas espécies de *Bacillus* com potencial de biocontrole é, portanto, uma estratégia crucial para o desenvolvimento de soluções alternativas eficazes. Espécies amplamente utilizadas, como *Bacillus amyloliquefaciens, B. subtilis, B. pumilus, B. velezensis, B. licheniformis e B. thuringiensis*, já foram comprovadas e estão no mercado (Lens, 2024). Além disso, a descoberta de novas espécies com propriedades de biocontrole pode ajudar a reduzir a dependência de pesticidas químicos, promovendo práticas agrícolas mais verdes e sustentáveis (Fenibo et al., 2021; Zhang et al., 2023). A integração desses microrganismos nas práticas de manejo agrícola pode melhorar a saúde do solo, aumentar a resiliência das culturas e contribuir para a conservação do meio ambiente.

1.2. REVISÃO BIBLIOGRÁFICA

1.2.1. Gênero *Bacillus*

Bacillus é um gênero pertencente à família Bacillaceae, ordem Bacillales, classe Bacilli, filo Firmicutes e domínio Bacteria. Foi descrito pela primeira vez em 1872 por Cohn (Borriss, 2020; Cohn, 1872; Göker & Oren, 2024). As espécies do gênero *Bacillus* apresentam morfologia bacilar, são tipicamente Gram-positivas, com tamanhos variando de 0,5 a 2,5 µm x 1,2-10 µm. Muitas espécies apresentam mobilidade por meio de flagelos e podem crescer em condições aeróbicas ou anaeróbicas facultativas. Seu crescimento ideal requer pH neutro

e um intervalo de temperatura que varia de 20 a 30 °C, sendo algumas encontradas até 45 °C (bactérias mesófilas) (Drobniewski, 1993; Le Marc et al., 2021; Niall A Logan & Vos, 2015; Vos et al., 2009).

No entanto, essas características não são homogêneas em todo o gênero, pois existe uma marcada diversidade fenotípica entre as espécies como resultado de processos evolutivos e adaptativos, o que permite a presença de espécies acidófilas, psicrofílicas, alcalófilas e termofílicas (Fajardo-Cavazos et al., 2014; Niall A. Logan & Vos, 2015). Uma de suas características mais distintivas é a capacidade de formar endósporos, o que contribui decisivamente para sua resistência a condições ambientais adversas, favorecendo a ampla dispersão no ambiente. Além disso, a esporulação faz parte do seu ciclo de vida e é ativada diante de limitações nutricionais ou mudanças no ambiente, dando origem a estruturas altamente resistentes à escassez de nutrientes, radiação, calor e dessecação (Christie & Setlow, 2020; Setlow, 2014). Essa capacidade explica a ampla distribuição do gênero em diversos ambientes, incluindo ar, água, solo, planta, trato gastrointestinal de animais e alimento para consumo (Dérozier et al., 2023).

Desde sua identificação inicial, o gênero *Bacillus* passou por profundas modificações taxonômicas impulsionadas pelo avanço da ciência e pela incorporação de novas ferramentas de identificação, que vão desde métodos bioquímicos clássicos até técnicas moleculares e genômicas de alta resolução. Como consequência, numerosas cepas anteriormente atribuídas ao *Bacillus* foram reclassificadas em gêneros distintos, enquanto outras foram incorporadas como novas espécies dentro do gênero (Blanco Crivelli et al., 2024; Borriss, 2020; Milase et al., 2024; Patel & Gupta, 2020). Atualmente, *Bacillus* é considerado um dos gêneros com maior diversidade dentro da família Bacillaceae. Até janeiro de 2026, foram registradas 663 espécies associadas ao gênero, das quais 118 têm nomes validamente publicados e aceitos de acordo com a Lista de nomes procarióticos com status na nomenclatura (LPSN - List of Prokaryotic names with Standing in Nomenclature) (Meier-Kolthoff et al., 2022).

1.2.1.1. Grupos operacionais

No gênero *Bacillus*, o uso de grupos operacionais atende a uma necessidade metodológica, uma vez que muitas espécies formam complexos filogeneticamente muito próximos, apresentam elevada similaridade genômica, compartilham características morfológicas e são difíceis de discriminar por meio de testes fenotípicos de rotina ou pela análise do gene 16S rRNA (Liu et al., 2015; Niall A. Logan & Vos, 2015; Xu & Kovács, 2024). Por isso, recorre-se a esquemas de maior resolução, como *Multi-locus Sequence Analysis* (MLSA) e *Average Nucleotide Identity* (ANI), para delimitar e discutir conjuntos coerentes de espécies (Glaeser & Kämpfer, 2015; Jain et al., 2018; Nu Dieu Hong et al., 2022; Rodriguez-R et al., 2024). Nesse contexto, grupos *como B. subtilis, B. amyloliquefaciens, B. pumilus* e *B. licheniformis*, bem como o complexo *B. cereus* sensu lato, representam unidades biológicas distintas, com histórias evolutivas, nichos ecológicos e aplicações diferenciadas (Carroll, Cheng, et al., 2022; Caulier et al., 2019; Fu et al., 2021; Ngalimat et al., 2021).

A. Grupo *B. subtilis*

Dentro do gênero *Bacillus*, o grupo *Bacillus subtilis* é o mais estudado, tendo como principal modelo a cepa *B. subtilis* subsp. *subtilis* 168. Essa linhagem apresenta células vegetativas de pequeno tamanho (< 1 µm de largura), metabolismo predominantemente mesófilo e preferência por condições neutrofílicas, embora algumas linhagens demonstrem tolerância a valores elevados de pH (Barbe et al., 2009). Uma das características mais distintivas do grupo *B. subtilis* é seu amplo potencial biossintético. Estudos genômicos indicam que aproximadamente 4-5% de seu genoma é dedicado à produção de compostos antimicrobianos, principalmente peptídeos antimicrobianos (*Antimicrobial peptides*, AMPs) (Stein, 2005). Esses metabólitos geralmente apresentam estruturas cíclicas e hidrofóbicas, com modificações químicas como aminoácidos na configuração D ou ligações tioéter intramoleculares (Jack et al., 1995; Wang et al., 2015). Além disso, análises genômicas comparativas demonstraram que a presença, organização e conservação dos clusters de

genes biossintéticos (*Biosynthetic gene clusters*, BGCs) são fortemente estruturadas pela filogenia. Mesmo a perda parcial ou inativação de BGCs pode ser mantida entre clados relacionados, o que sugere uma evolução adaptativa do potencial antimicrobiano em função da linhagem e do nicho ecológico (Kiesewalter et al., 2021; Steinke et al., 2021). As espécies do grupo são: *B. subtilis, B. cabrialesii, B. tequilensis, B. vallismortis, B. spizizenii, B. inaquosorum, B. stercoris, B. halotolerans* e *B. mojavensis* (Caulier et al., 2019; Ngalimat et al., 2021).

Devido a essas propriedades, diversas cepas do grupo são utilizadas comercialmente como agentes de biocontrole. Por exemplo, produtos formulados com *B. subtilis* incluem Biobac biofungicide e Tacap biofungicide, desenvolvidos pela UPL, utilizados no controle de patógenos como *Rhizoctonia solani*, *Botrytis cinerea* e *Alternaria porri*. Bio-imune; Multi-Attack; Multi-Guard; FungiOuro; Biologic Aerum; Brandt Clearfol; Power BAC Inductor; SKDefense; Solution Pro Evolution, desenvolvidos pela Vittia. Outros produtos comerciais, como Milarum biofungicide da Tradecorp, exploram a capacidade dessa espécie de suprimir fitopatógenos por meio da produção de metabólitos antifúngicos e da indução de resistência sistêmica em plantas (Felipe De Lima Andreata et al., 2025).

B. Grupo *B. amyloliquefaciens*

A espécie-tipo do grupo *B. amyloliquefaciens* foi originalmente descrita por sua capacidade de produzir α-amilase extracelular por Juichiro Fukumoto em 1943 (Fukumoto, 1943). Embora intimamente relacionada com *B. subtilis*, este grupo apresenta características morfológicas, genômicas e funcionais próprias. Morfologicamente, exibem morfologia bacilar altamente conservada, com dimensões celulares que variam ligeiramente entre as espécies, mas permanecem dentro de uma faixa estreita, aproximadamente de 0,5 a 0,9 µm de largura e 1,5 a 3,5 µm de comprimento, bem como a formação de endósporos elipsoidais em posição central ou subterminal (Ngalimat et al., 2021). Estudos genômicos permitiram distinguir inicialmente duas linhagens: *B.*

amyloliquefaciens subsp*. amyloliquefacien*s (cepa tipo DSM7^{T}) e *B. amyloliquefaciens* subsp*. plantarum*, representada pela cepa FZB42, amplamente utilizada em aplicações agrícolas (Fan et al., 2017). No entanto, análises filogenômicas posteriores demonstraram uma elevada similaridade genômica entre *B. amyloliquefaciens* subsp. plantarum e os táxons *B. velezensis, B. methylotrophicus, B. oryzicola* e *B. vanillea*, o que levou à sua reclassificação como sinônimos heterotípicos tardios de *B. velezensis*. Apesar dessa complexidade taxonômica, essas linhagens formam um conjunto filogeneticamente coerente (Fan et al., 2017; Ngalimat et al., 2021). Para refletir essa estreita relação, foi proposto o termo grupo operacional *Bacillus amyloliquefaciens* (operational group *B. amyloliquefaciens*, OGBa), que agrupa *B. amyloliquefaciens, B. velezensis, B. siamensis* e *B. nakamurai* (Ngalimat et al., 2021).

Este grupo foi validado por meio de análises de genomas e genes marcadores, sendo caracterizado por uma ampla distribuição ecológica e elevada capacidade de sobrevivência associada à formação de endósporos. De uma perspectiva funcional, os membros do OG*Ba* se destacam por seu potencial biotecnológico (Ngalimat et al., 2021; S.-Y. Wang et al., 2022). Diversas cepas atuam como bactérias promotoras do crescimento vegetal (*Plant growth promoting bacteria*, PGPB), melhorando a nutrição e o desenvolvimento radicular, suprimindo patógenos fitopatogênicos por meio da competição ecológica e da produção de metabólitos antimicrobianos, e induzindo respostas de defesa nas plantas. Da mesma forma, os genomas de cepas representativas mostram uma alta densidade de clusters biossintéticos responsáveis pela produção de lipopeptídeos, policetídeos e compostos voláteis, o que posiciona como um eixo funcional central no potencial de biocontrole desse grupo (Chun et al., 2019).

Diversas cepas desse grupo são amplamente utilizadas em biofungicidas comerciais. Um exemplo clássico é o produto Sonata biofungicide da Bayer, formulado com Bacillus amyloliquefaciens, utilizado contra patógenos como *Botrytis cinerea*, *Alternaria solani* e *Uncinula necator*. De forma semelhante, biofungicidas baseados em Bacillus velezensis, como Certano biofungicide e

Arvatico biofungicide da Syngenta, são empregados no controle de patógenos de solo como *Rhizoctonia solani*, *Fusarium solani* e *Macrophomina phaseolina* (Felipe De Lima Andreata et al., 2025).

C. Grupo *B. licheniformis*

O grupo *B. licheniformis* inclui a espécie *B. licheniformis* e espécies filogeneticamente muito próximas, como *B. paralicheniformis*, *B. sonorensis*, *B. haynesii*, *B. swezeyi* e *B. glycinifermentans* (Ngalimat et al., 2021). Essas espécies apresentam identidades extremamente altas no gene 16S rRNA, o que dificulta sua discriminação por meio de marcadores clássicos; por exemplo, na descrição de *B. paralicheniformis* sp. nov., a análise filogenética revelou identidade de 99,5% com *B. sonorensis* KCTC-13918T e de 99,4% com *B. licheniformis* DSM 13T (Dunlap et al., 2015). Essa proximidade filogenética tem gerado uma confusão taxonômica persistente, com implicações práticas relevantes (Du et al., 2019; Dunlap et al., 2015). Sob a perspectiva regulatória, a European Food Safety Authority (EFSA) destacou que *B. paralicheniformis* pode ser erroneamente identificado como *B. licheniformis*, dependendo do método empregado, e concluiu que apenas o uso de genomas completos permite uma identificação inequívoca de determinadas cepas, superando as limitações da caracterização através do gene do 16S rRNA (Barat Baviera et al., 2024).

B. licheniformis destaca-se por sua elevada relevância industrial, sendo um produtor frequente de enzimas extracelulares de interesse biotecnológico (Gudiña & Teixeira, 2022). Entre essas enzimas, sobressaem as proteases alcalinas, amplamente utilizadas em processos industriais devido à sua atividade ótima em condições alcalinas e em temperaturas moderadas a elevadas, especialmente após a otimização das condições de cultivo e fermentação. Além disso, α-amilases com perfis de atividade dependentes de pH e temperatura têm sido caracterizadas, com suporte de estudos experimentais em sistemas fermentativos (Divakaran et al., 2011; Mabrouk et al., 1999; Sarker et al., 2013). A comparação com espécies próximas revela diferenças funcionais relevantes. *B. paralicheniformis*, por exemplo, pode portar operons de metabólitos

secundários, como aqueles associados ao sistema de fengicina, ausentes até o momento em genomas de *B. licheniformis*. Com base nisso, foram propostos marcadores gênicos específicos (por exemplo, *fenC* e *fenD*) para a discriminação rápida dessas espécies (Du et al., 2019; Olajide et al., 2021). Por fim, sob a perspectiva de inocuidade alimentar, algumas cepas de *B. licheniformis* têm sido associadas a surtos de intoxicação alimentar em decorrência da produção de lichenisina, um biossurfactante com atividade citotóxica. Esse aspecto reforça a importância de uma identificação taxonômica e genômica precisa em contextos industriais e alimentares (Barat Baviera et al., 2024; Salkinoja-Salonen et al., 1999; Yeak et al., 2022).

Do ponto de vista aplicado, espécies deste grupo também têm sido incluídas em formulações comerciais de biocontrole agrícola. No Brasil, *B. licheniformis* aparece em alguns biofungicidas registrados. Entre os exemplos encontram-se o produto Nacillus Max, desenvolvido pela empresa Bio Insumos, utilizado no controle de patógenos como *Alternaria solani*, *Fusarium solani* e *Rhizoctonia solani*. Formulações multiespécies também incluem Biolucro e BTP 167-21A, desenvolvidas pela Total Biotecnologia, que combinam Bacillus licheniformis com outras bactérias benéficas para o manejo de patógenos de solo, como *Macrophomina phaseolina* e *Rhizoctonia solani* (Felipe De Lima Andreata et al., 2025).

D. Grupo *B. pumilus*

O grupo *B. pumilus* exemplifica claramente a utilidade de grupos operacionais dentro do gênero *Bacillus*, dada a dificuldade de sua delimitação taxonômica. Diversas espécies intimamente relacionadas compartilham mais de 99,5% de identidade na sequência do gene 16S rRNA e exibem diferenciação fenotípica e bioquímica limitada, o que favorece identificações errôneas quando esse marcador é usado exclusivamente (Espariz et al., 2016; Fu et al., 2021). Estudos baseados em MLSA e outras abordagens genômicas demonstraram que numerosos isolados historicamente atribuídos a *B. pumilus* correspondem, na verdade, a espécies distintas, como *B. safensis* ou *B. altitudinis*, tornando

necessário o uso de ferramentas de maior resolução para resolver as relações filogenéticas dentro do grupo. O chamado “grupo *B. pumilus*” abrange um amplo conjunto de espécies filogeneticamente relacionadas, incluindo *B. pumilus, B. safensis, B. altitudinis, B. xiamenensis, B. zhangzhouensis* e *B. australimaris* (Liu et al., 2016). As espécies deste grupo são descritas como bactérias Gram-positivas, móveis, em forma de bastonete, que formam endósporos elipsoidais, características associadas à sua persistência ambiental e ampla distribuição em ambientes terrestres e marinhos (Fu et al., 2021; Liu et al., 2013).

De uma perspectiva aplicada, membros do grupo *B. pumilus* foram relatados em aplicações biotecnológicas e agrícolas, incluindo cepas com potencial PGPR e inibição fúngica (Dobrzyński et al., 2022, 2023; Munimbazi & Bullerman, 1998), bem como no contexto de patogenicidade vegetal; um exemplo relevante é a descrição de *B. pumilus* como o agente causal da doença do abaulamento do tronco em seringueiras (*Hevea brasiliensis*) (Husni et al., 2021). Em conjunto, essas evidências confirmam que o gene 16S rRNA é insuficiente para discriminar espécies dentro do grupo *B. pumilus* e que abordagens como MLSA e genômica comparativa são essenciais para a identificação confiável e a correta interpretação ecológica.

Na agricultura, biofungicidas formulados com *B. pumilus* são utilizados para o controle de doenças foliares e de solo. Entre eles destaca-se o produto Serenade biofungicide da Bayer, bem como formulações comerciais como Caravan biofungicide e BTP 005-19 biofungicide desenvolvidas pela Koppert, utilizadas contra patógenos como *Cercospora kikuchii* e *Septoria glycines* (Felipe De Lima Andreata et al., 2025).

E. Grupo *B. cereus* sensu lato

O grupo *B. cereus* sensu lato, também conhecido como complexo de espécies *B. cereus* sensu lato, constitui um macrogrupo operacional distinto dentro do gênero *Bacillus*, caracterizado por uma alta identidade genômica, combinado com uma marcante diversidade fenotípica, ecológica e de impacto na saúde e no setor agroalimentar (Carroll, Pierneef, et al., 2022). Inclui espécies

classicamente reconhecidas como *B. cereus, B. thuringiensis, B. anthracis, B. paranthracis, B. mycoides* e *B. pseudomycoides*, cuja delimitação taxonômica é particularmente complexa (Carroll, Cheng, et al., 2022; Carroll et al., 2020; Carroll, Pierneef, et al., 2022). Estudos filogenéticos e genômicos demonstraram que características fenotípicas marcantes do grupo, como toxicidade inseticida ou virulência associada ao antraz, dependem em grande parte da aquisição de plasmídeos e outros elementos genéticos móveis, enquanto o genoma cromossomal exibe uma estrutura predominantemente clonal que não separa estritamente *B. cereus* e *B. thuringiensis* em clados exclusivos (Bower et al., 2022; Carroll, Pierneef, et al., 2022; Magome et al., 2025). Consequentemente, o pangenoma do grupo é considerado aberto, refletindo alta plasticidade genética, aspecto que está alinhado à amplitude de estilos de vida que variam de saprófitos oportunistas a patógenos de humanos, animais ou insetos (Bazinet, 2017; Liu et al., 2015; Priest et al., 2004).

A diversidade interna do grupo pode ser interpretada sob um modelo ecotípico, com pelo menos sete grandes grupos filogenéticos (I–VII), posteriormente ampliados para oito grupos (I–VIII), essa classificação foi inicialmente proposta com base na análise do gene *panC*, um marcador com maior capacidade de resolução do que o gene *16S rRNA* para discriminar linhagens próximas dentro do complexo (Guinebretière et al., 2008). Os grupos filogenéticos definidos por meio de *panC* foram posteriormente validados por estudos genômicos, particularmente por análises de Average Nucleotide Identity (ANI) e filogenias baseadas em genomas completos (Carroll, Cheng, et al., 2022; Carroll, Marston, et al., 2022). Esses enfoques demonstraram que cada grupo corresponde a clados genômicos bem delimitados, geralmente com valores de identidade superiores a 92,5–95% (Carroll, Marston, et al., 2022). Além disso, os grupos apresentam padrões ecológicos e fisiológicos distintos, especialmente em relação à tolerância térmica. De modo geral, os grupos I–V incluem cepas mesófilas, com temperaturas ótimas de crescimento entre 30 e 37 °C; o grupo VI reúne bactérias psicrotolerantes, capazes de crescer abaixo de 7 °C, o que explica sua presença frequente em alimentos refrigerados; enquanto os grupos VII–VIII apresentam características intermediárias ou adaptações particulares a determinados nichos ambientais (Carroll, Cheng, et al., 2022).

1.2.2. Enzimas e metabólitos antifúngicos

Os fitopatógenos fúngicos são particularmente difíceis de controlar devido à sua ampla gama de hospedeiros. Consequentemente, seu manejo frequentemente requer o uso de altas doses de fungicidas, o que aumenta significativamente o impacto ambiental (Anckaert et al., 2021; Etesami et al., 2023). Nesse contexto, o gênero *Bacillus* se destaca por sua notável capacidade de produzir uma ampla variedade de compostos antimicrobianos, incluindo agentes com atividade antifúngica (Shahid et al., 2021). *Bacillus* é capaz de sintetizar compostos de baixo peso molecular com atividade antibiótica, bem como enzimas líticas direcionadas contra fitopatógenos fúngicos (Figura 1). Essas enzimas incluem proteases, quitinases, quitosanases e β-glucanases, que participam da degradação das paredes celulares de diversos microrganismos. Diversos estudos demonstraram que espécies do gênero *Bacillus* alocam até 8,5% de seu repertório genético para a biossíntese de compostos antimicrobianos (Chen et al., 2009).

1.2.2.1. Enzimas hidrolíticas

As enzimas líticas produzidas por espécies do gênero *Bacillus* constituem um dos mecanismos centrais de sua atividade antifúngica e de biocontrole contra fitopatógenos (Shahid et al., 2021). Esse sistema enzimático é composto principalmente por proteases, quitinases, quitosanases e β-glucanases, que atuam de forma coordenada sobre os componentes estruturais da parede celular de fungos e oomicetos, promovendo a degradação das hifas e inibindo a germinação dos esporos (Abdelmoteleb et al., 2017; Huang et al., 2005; Pang et al., 2021; Xu et al., 2016). Diversas espécies de *Bacillus*, particularmente *B. subtilis, B. velezensis* e *B. amyloliquefaciens*, consolidaram-se como agentes de biocontrole amplamente utilizados na agricultura (Anckaert et al., 2021; Legein et al., 2020). A produção dessas enzimas geralmente é induzida na presença de polímeros da parede celular fúngica, como quitina, quitosana ou β-glucanas, ou durante a fase de crescimento pós-exponencial, coincidindo com o aumento da competição microbiana na rizosfera (Ruiz-Herrera & Ortiz-Castellanos, 2019). O gênero *Bacillus* é responsável por aproximadamente 50% do mercado global de

enzimas industriais, gerando US$1,6 bilhão. Essas aplicações incluem, por exemplo, proteases para detergentes e amilases usadas em processos industriais associados a amido, além de usos no setor têxtil (Schallmey et al., 2004).

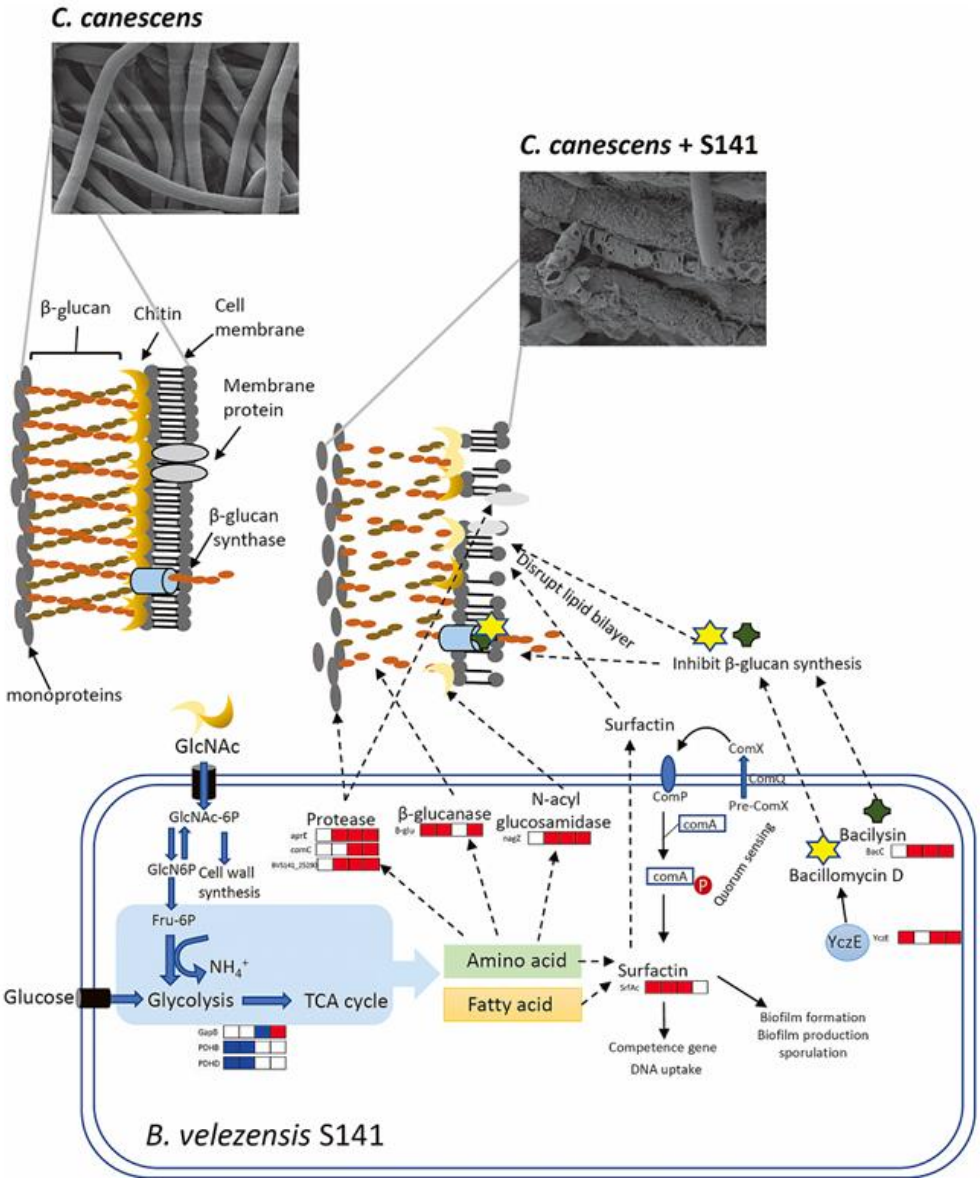

Figura 1. Mecanismos da atividade antifúngica de *B. velezensis* S141 contra *Cercospora canescens.* A S141 inibe o crescimento fúngico por meio (i) da degradação enzimática da parede celular (proteases, β-glucanases e N-acetilglucosaminidases) e (ii) produção de metabólitos secundários (surfactina, bacilisina e bacilomicina D), que alteram a integridade da membrana e bloqueiam a biossíntese da parede celular (Songwattana et al.,2023).

A. Proteases

As proteases são um grupo de enzimas altamente complexas que atuam na hidrólise de ligações peptídicas em uma reação essencialmente irreversível (Fink & Jerala, 2022). Dentre essas enzimas, as proteases extracelulares desempenham um papel central na atividade antifúngica de *Bacillus*. Estas são, em sua maioria, serina proteases alcalinas, dentre as quais a subtilisina, codificada pelo gene *aprE*, destaca-se como responsável por mais de 70% da atividade proteolítica total em *B. subtilis*. Sua expressão aumenta durante a transição para a fase estacionária e é regulada por promotores reconhecidos pela RNA polimerase associada a $\sigma^{(A)}$ (Jan et al., 2000; Zolfaghari Emameh et al., 2022). Essas proteases degradam proteínas estruturais da parede celular fúngica, bem como enzimas secretadas pelos próprios patógenos, rompendo a matriz proteica que sustenta a arquitetura celular. Esse processo facilita o acesso de outras enzimas líticas e aumenta a susceptibilidade dos fungos a compostos antimicrobianos (Hu et al., 2022). Variantes como *sprE*, associadas à esporulação (Wong et al., 1984), e a ação sinérgica com lipopeptídeos como a surfactina produzida pelo operon *srfA* intensificam o efeito antifúngico. Esse efeito é particularmente evidente contra patógenos como *Fusarium oxysporum, Cercospora spp*. e *Botrytis cinerea* (Hussain et al., 2024; Rodríguez-Pedroso et al., 2008; Songwattana et al., 2023).

B. Quitinases

As quitinases são enzimas, classificadas como glicosidases (EC 3.2.1.14), que catalisam a hidrólise das ligações β-1,4 da quitina, um polímero insolúvel de N-acetilglicosamina que constitui entre 10 e 20% da parede celular fúngica (Martínez-Zavala et al., 2020; Monzingo et al., 1996; Ruiz-Herrera & Ortiz-Castellanos, 2019). As quitinases bacterianas pertencem principalmente às famílias GH18 e GH19 e possuem uma arquitetura multidomínio que frequentemente inclui domínios de ligação à quitina (CBDs, *chitin binding domains*), os quais aumentam significativamente a eficiência catalítica (Funkhouser & Aronson, 2007; Martínez-Zavala et al., 2020; Otsuka et al., 2022). Em *B. thuringiensis*, genes como *chiA, chiB* e *chiC* foram extensivamente

caracterizados, com destaque para quitinases como ChiA74, possui um domínio CBD C-terminal e apresenta alta eficácia contra *F. oxysporum* (Martínez-Zavala et al., 2024). Essas enzimas também demonstram sinergia marcante com proteínas Cry e lipopeptídeos, otimizando seu uso em estratégias de biocontrole (Martínez-Zavala et al., 2020, 2024). A atividade quitinolítica de diferentes subespécies de *B. thuringiensis*, bem como de *B. amyloliquefaciens, B. velezensis* e *B. subtilis*, tem sido associada à inibição do crescimento micelial e à germinação de esporos de fungos como *Fusarium spp., Sclerotium rolfsii* e *Penicillium digitatum* (Gomaa, 2012; Liu et al., 2024; María et al., 2017; Mian et al., 2024).

C. Quitosanases

As quitosanases ampliam o espectro da atividade antifúngica ao atuarem sobre a quitosana, um polímero parcialmente desacetilado derivado da quitina. Essas enzimas (EC 3.2.1.132) hidrolisam ligações β-1,4 e geram quito-oligossacarídeos (ChOS) de baixa polimerização, que possuem atividade antimicrobiana intrínseca (Cui et al., 2021; Luo et al., 2020; Monzingo et al., 1996). As quitosanases de *Bacillus* pertencem a múltiplas famílias GH, sendo a GH46 a mais representativa dentro do gênero (Cui et al., 2021; Pang et al., 2021; Tomita et al., 2013). Essas enzimas exibem características biotecnológicas favoráveis, como atividade ótima em pH ligeiramente ácido, estabilidade térmica moderada e produção eficiente de ChOS com efeitos antifúngicos comprovados. Genes como csn-SH em *B. atrophaeus* ou BaCsn46B em *B. amyloliquefaciens* codificam quitosanases altamente ativas contra patógenos agrícolas relevantes, incluindo *M. oryzae*, *C. higginsianum*, *F. oxysporum* e *B. cinerea,* induzindo alterações citológicas como vacuolização, espessamento da parede celular e perda da integridade estrutural (Cui et al., 2021; Luo et al., 2020).

D. β-glucanases

As β-glucanases são um componente chave na degradação da matriz de glucano da parede celular fúngica, que pode representar entre 30 e 60% de sua

composição. Essas enzimas hidrolisam ligações β-1,3 e β-1,6 e pertencem a diversas famílias GH, incluindo GH16, GH17, GH55 e GH81 (Otsuka et al., 2022; Vargas-Arispuro et al., 2017; Vela-Corcia et al., 2025). No contexto do biocontrole, as β-glucanases bacterianas não apenas degradam diretamente a parede celular, mas também expõem a quitina subjacente, potencializando a ação das quitinases e desencadeando respostas de resistência sistêmica em plantas hospedeiras. Exemplos notáveis incluem a β-glucanase BvlzGluc de *B. velezensis*, com potente atividade contra *B. cinerea* (Vela-Corcia et al., 2025), e enzimas produzidas por *B. subtilis* e *B. amyloliquefaciens* que inibem a germinação de esporos e causam graves malformações hifais. A ação dessas enzimas se estende a uma ampla gama de patógenos, incluindo *Fusarium spp*. e *Phytophthora meadii* (Abraham et al., 2013; Khan et al., 2018), cujas paredes celulares contêm β-glucanos como um alvo vulnerável.

Em conjunto, a produção coordenada de proteases, quitinases, quitosanases e β-glucanases posiciona o gênero *Bacillus* como uma plataforma biotecnológica com enorme potencial para o desenvolvimento de estratégias sustentáveis de controle biológico, capazes de reduzir a dependência de fungicidas químicos e antecipar os desafios fitossanitários da agricultura moderna.

1.2.2.2. Metabólitos secundários

Espécies do gênero *Bacillus* produzem um repertório diversificado de metabólitos secundários antifúngicos cuja eficácia é diretamente determinada pela presença, organização e regulação de complexos biossintéticos específicos (Salazar et al., 2023). Esses compostos podem ser classificados em grandes categorias de acordo com suas vias de biossíntese, incluindo peptídeos não ribossomais (NRPs) e policetídeos sintetizados por sistemas do tipo *polyketide synthase* (PKS) (Munimbazi & Bullerman, 1998; Shahid et al., 2021; Steinke et al., 2021). Sob uma perspectiva funcional, a atividade antifúngica real e relevante do gênero concentra-se principalmente em lipopeptídeos e, em menor grau, em certos policetídeos, que atuam predominantemente rompendo membranas e

alterando a permeabilidade celular ou interferindo em processos essenciais para o crescimento fúngico.

A. Peptídeos não ribossomais (NRPs)

Os NRPs constituem um dos principais e mais eficazes arsenais antifúngicos do gênero *Bacillus*. Esses metabólitos anfipáticos incluem compostos formados por um peptídeo cíclico ligado covalentemente a uma cadeia lipídica, o que lhes confere alta afinidade por membranas biológicas e permite a interação direta com a membrana plasmática de fungos (Meena & Kanwar, 2015; Velho et al., 2011). Dentro desse grupo, três famílias apresentam maior relevância funcional: surfactinas, iturinas e fengicinas. A família das surfactinas é codificada pelo operon *srfA* (*srfAA–D*), responsável pela produção de um dos biossurfactantes mais potentes conhecidos. Embora sua atividade antifúngica direta seja limitada, a surfactina exerce um papel estratégico como metabólito facilitador, promovendo alterações subletais na membrana fúngica que aumentam a susceptibilidade a outros lipopeptídeos antifúngicos (Danevčič et al., 2021; Ramesh et al., 2024; Vollenbroich et al., 1994; Zhi et al., 2017). Esse efeito sinérgico é crucial para a eficácia geral do biocontrole exercido por cepas que produzem múltiplos NRPs. Em contraste, a família das iturinas constitui o núcleo da atividade antifúngica direta. Codificada por operons como *itu* (iturina A), *myc* (micosubtilina) e *bam/bmy* (bacilomicina D), essa família compreende lipoheptapeptídeos cíclicos de elevada atividade biológica. Esses compostos atuam predominantemente por meio da formação de poros iônicos na membrana plasmática fúngica, promovendo a perda do equilíbrio osmótico, o colapso do potencial de membrana e, consequentemente, a morte celular (Danevčič et al., 2021; Duitman et al., 1999; Leclère et al., 2005; Tsuge et al., 2005; Vollenbroich et al., 1994; Yu et al., 2021; Zhi et al., 2017). Cepas de *B. subtilis* e *B. amyloliquefaciens* portadoras desses clusters exibem inibição eficaz de fitopatógenos de importância agronômica, como *Fusarium spp., Rhizoctonia solani, B. cinerea* e *Colletotrichum spp*. (Mejía-Bautista et al., 2016; Pedraza et al., 2020; Souto et al., 2004; Yu et al., 2021).

A família das fengicinas, é sintetizada pelo operon *fen/pps* (*ppsA-E* ou *fenA-E*) e caracteriza-se pela alta seletividade para fungos filamentosos e oomicetos (Ramarathnam et al., 2007; Yin et al., 2024; Zeng et al., 2021).

Esses lipodecapeptídeos cíclicos exibem um mecanismo de ação dependente da concentração: em baixas doses, induzem a formação de poros transitórios, enquanto em concentrações mais elevadas causam a solubilização direta da membrana celular (Manetsberger et al., 2025; Stellerl et al., 1999). Essa dupla funcionalidade explica sua alta eficácia contra patógenos como *Magnaporthe oryzae*, *B. cinerea* e várias espécies de *Fusarium,* posicionando a fengicina como um dos metabólitos mais relevantes no controle de doenças radiculares e de solo (Ajuna et al., 2024).

B. Poliquetídeo sintases (PKS)

Os PKS, cujos clusters biossintéticos contêm enzimas multimodulares análogas às NRPS que catalisam a condensação de unidades de acetil-CoA e malonil-CoA. Em *Bacillus*, predominam os sistemas PKS do tipo I trans-AT (modular ou iterativo) e do tipo II (aromático), produzindo antibióticos poliénicos e macrolídeos com atividade antimicrobiana (Aleti et al., 2015; Hegedüs & Marx, 2013; C. Tran et al., 2022). O operon da dificidina (*dfnA-I*) destaca-se; trata-se de um antibiótico poliénico da classe dos policetídeos sintetizado por PKS do tipo I trans-AT e produzido por espécies como *B. amyloliquefaciens* e *B. velezensis*. Embora sua atividade primária seja antibacteriana, a dificidina pode interagir com membranas fúngicas e processos de tradução, exibindo atividade antifúngica contra *B. cinerea e Fusarium spp.* (Li et al., 2022; Tian et al., 2021; Wu et al., 2015). O operon do bacillaeno (*baeA–D*) produz um metabólito com atividade antifúngica limitada. No entanto, quando atua em sinergia com outros compostos, sua eficácia aumenta, embora sua função principal seja antibacteriana (Aleti et al., 2015; Yang et al., 2025). Em contraste, o operon *mlnA–H* codifica a biossíntese de macrolactinas, macrolídeos de cadeia longa que interferem em membranas celulares e sistemas enzimáticos. Esses compostos apresentam atividade antifúngica comprovada contra *Aspergillus niger* e *Candida albicans* (Ajuna et al., 2024; Karpiński, 2019).

C. Outros metabólitos relacionados

Além dos lipopeptídeos e policetídeos, *Bacillus* produz outros metabólitos secundários que, embora não constituam o núcleo antifúngico, podem contribuir de forma complementar para a supressão de fungos por meio de mecanismos diretos ou indiretos. Entre eles, destacam-se a bacilisina e a bacillibactina, cujos clusters biossintéticos e modos de ação explicam seu papel ecológico na competição microbiana.

A bacilisina é um dipeptídeo não ribossomal de baixo peso molecular, composto por L-alanina e pelo aminoácido não proteinogênico L-anticapsina, produzido por espécies como *B. subtilis*, *B. amyloliquefaciens* e *B. pumilus*. Sua biossíntese é codificada pelo operon *bac* (*bacABCDE*), que coordena a conversão de prefenato em L-anticapsina (*bacA–C*), a formação final do dipeptídeo (*bacD*) e sua exportação (*bacE*) (Islam et al., 2022; Rajavel et al., 2009). Funcionalmente, a bacilisina atua como um pró-fármaco que, após hidrólise intracelular, libera L-anticapsina, um inibidor competitivo da glucosamina sintase. Considerando que essa enzima é essencial para a síntese de quitina, sua inibição leva à desestabilização da parede celular fúngica (Islam et al., 2022; Loeffler et al., 1986; Parker & Walsh, 2012; Wu et al., 2015). A bacillibactina é um sideróforo do tipo catecolato produzido por *B. subtilis*, *B. anthracis* e *B. amyloliquefaciens*, cuja estrutura corresponde a um trímero cíclico de 2,3-di-hidroxibenzoil-glicina-treonina. Sua biossíntese é codificada pelo operon *dhb* (*dhbACEBF*), no qual *dhbE* ativa o di-hidroxibenzoato*, dhbB* atua como proteína carreadora e *dhbF* catalisa a montagem sequencial de glicina e treonina para formar a molécula final (Chakraborty et al., 2022; Manetsberger et al., 2025). A bacillibactina não apresenta atividade antifúngica direta, mas exerce um efeito antimicrobiano indireto por meio da quelação de ferro férrico (Fe^{3+}), um micronutriente essencial para o crescimento fúngico. Ao sequestrar ferro do ambiente, limita a disponibilidade desse elemento para microrganismos competidores, incluindo fungos do solo e da rizosfera. Esse mecanismo de exclusão nutricional contribui para a eficiência competitiva de *Bacillus* em ambientes naturais e pode potencializar indiretamente o efeito de outros metabólitos antifúngicos produzidos pela mesma cepa (Dimopoulou et al., 2021).

1.2.3. Compostos orgânicos voláteis (COVs)

São moléculas orgânicas de baixo peso molecular (100–500 Dá), produzidas por diversos organismos, incluindo microrganismos do solo, plantas e algumas fontes antropogênicas. Entre os microrganismos, as espécies do gênero *Bacillus* destacam-se por sua capacidade de sintetizar uma ampla diversidade de VOCs com funções antifúngicas e promotoras do crescimento vegetal por meio da resistência sistêmica induzida (ISR) (Poulaki & Tjamos, 2023; Grahovac et al., 2023). VOCs compreendem diversos grupos químicos, entre eles álcoois (2,3-butanodiol, 3-pentanol), cetonas (acetoin, 2-nonanona), aldeídos (benzaldeído, nonanal), ácidos carboxílicos (ácido acético, ácido 3-metilbutanoico), fenóis (2,4-di-tert-butilfenol), pirazinas e compostos sulfurados como dissulfeto de dimetila (He et al., 2020). Esses compostos se originam principalmente em rotas de fermentação primária, no catabolismo de aminoácidos e no metabolismo lipídico. Seu perfil varia conforme a cepa bacteriana e as condições de cultivo, como meio de cultura, pH, disponibilidade de oxigênio e fase de crescimento (Chaves-López et al., 2015; He et al., 2020; Ling et al., 2023).

Os VOCs produzidos por *Bacillus* apresentam elevada atividade antifúngica. Compostos como 2-nonanona, benzotiazol e 2-undecanona demonstram altos níveis de inibição contra patógenos como *F. oxysporum*, *B. cinerea* e *C. gloeosporioides* (Tian et al., 2025). Outros metabólitos, como acetoin e 2,3-butanodiol, atuam principalmente como indutores de ISR em plantas (Ryu et al., 2003). Da mesma forma, ácidos voláteis (ácido acético e ácido 3-metilbutanoico) e compostos aromáticos como pirazinas e fenóis inibem patógenos de solo e de pós-colheita, incluindo *R. solani*, *Sclerotinia sclerotiorum* e *P. expansum* (Chaves-López et al., 2015; He et al., 2020). A atividade antifúngica dos VOCs é explicada por múltiplos mecanismos complementares. Entre eles destacam-se a alteração da integridade da membrana celular, que leva à perda de permeabilidade, vacuolização e lise celular; a modulação da expressão gênica em patógenos, afetando genes associados à virulência e ao estresse oxidativo; e a indução de defesas na planta, incluindo acúmulo de espécies reativas de oxigênio, ativação de enzimas relacionadas à defesa e deposição de calose (Figura 2). Além disso, alguns VOCs inibem a síntese de

pigmentos ou micotoxinas em fungos (Grahovac et al., 2023; Ryu et al., 2003; Surovy et al., 2023).

1.2.4. *Bacillus* como biofungicida

Na agricultura, diversas espécies de *Bacillus*, incluindo *B. subtilis, B. amyloliquefaciens, B. velezensis, B. pumilus* e cepas não patogênicas de *B. cereus* e *B. thuringiensis*, são consideradas rizobactérias promotoras do crescimento vegetal (PGPR, *plant growth-promoting rhizobacteria*) e agentes de controle biológico eficazes contra fungos fitopatogênicos, oomicetos, bactérias e nematoides (Beneduzi et al., 2012; Choudhary & Johri, 2009; Prasad et al., 2023; Zhang et al., 2023). Sua eficácia baseia-se na ação integrada de múltiplos mecanismos, tanto diretos quanto indiretos, o que posicionou esse gênero como uma plataforma central em programas de manejo integrado de doenças. Os mecanismos diretos incluem antibiose mediada por metabólitos secundários, lise de hifas fúngicas e competição por nutrientes e nicho ecológico (Isela Parra-Cota et al., 2024; Prasad et al., 2023; Raaijmakers et al., 2010; Zhang et al., 2023). Além disso, *Bacillus* pode induzir resistência sistêmica na planta, melhorar seu vigor e modular a microbiota da rizosfera. Essa multifuncionalidade explica seu amplo espectro de ação e seu uso contínuo em biofungicidas comerciais.

O sucesso de *Bacillus* como biofungicida reside em três principais características biológicas. Primeiro, a formação de endósporos permite o desenvolvimento de formulações estáveis pós molháveis, grânulos ou suspensões concentradas com prazos de validade prolongados, como documentado para produtos à base *de B. subtilis QST713* (Serenade®) (Lahlali et al., 2013) e *B. amyloliquefaciens* D747 (Double Nickel®) (Pethybridge et al., 2019). Segundo, muitas cepas exibem alta capacidade de colonização rizosférica e, em alguns casos, endofítica, facilitada pela formação de biofilme e pelo uso eficiente de exsudatos radiculares. Isso possibilita a ocupação precoce do nicho e limita o estabelecimento do patógeno. Finalmente, *Bacillus* se destaca por seus diversos metabólitos, que incluem lipopeptídeos (iturinas, fengicinas e surfactinas), policetídeos, sideróforos, enzimas degradadoras da parede celular fúngica, compostos orgânicos voláteis e fitormônios. A coexistência de vários

desses metabólitos na mesma cepa gera efeitos sinérgicos e reduz o risco de seleção de resistência (Beneduzi et al., 2012; Huang et al., 2023; Raaijmakers et al., 2010; Teixeira et al., 2021).

De uma perspectiva comparativa, os biofungicidas à base de *Bacillus* oferecem vantagens claras sobre os fungicidas químicos, incluindo múltiplos modos de ação, baixo risco de resistência e perfis favoráveis de segurança ambiental e alimentar (Anckaert et al., 2021; Figueiredo et al., 2025). No entanto, sua eficácia no campo pode ser variável e dependente de fatores ambientais, tipo de solo, manejo agronômico e época de aplicação, apresentando melhores resultados com aplicações preventivas ou precoces. De modo geral, as evidências científicas e a experiência comercial acumulada posicionam o gênero *Bacillus* como um dos pilares dos biofungicidas modernos (C. Tran et al., 2022; Zhang et al., 2023). Sua capacidade de combinar potente antibiose, indução de defesas vegetais, promoção do crescimento e estabilidade da formulação o torna uma ferramenta fundamental para uma agricultura mais sustentável, com menor dependência de fungicidas sintéticos e melhor alinhada às demandas regulatórias e dos consumidores por sistemas de produção de baixo impacto ambiental (Etesami et al., 2023).

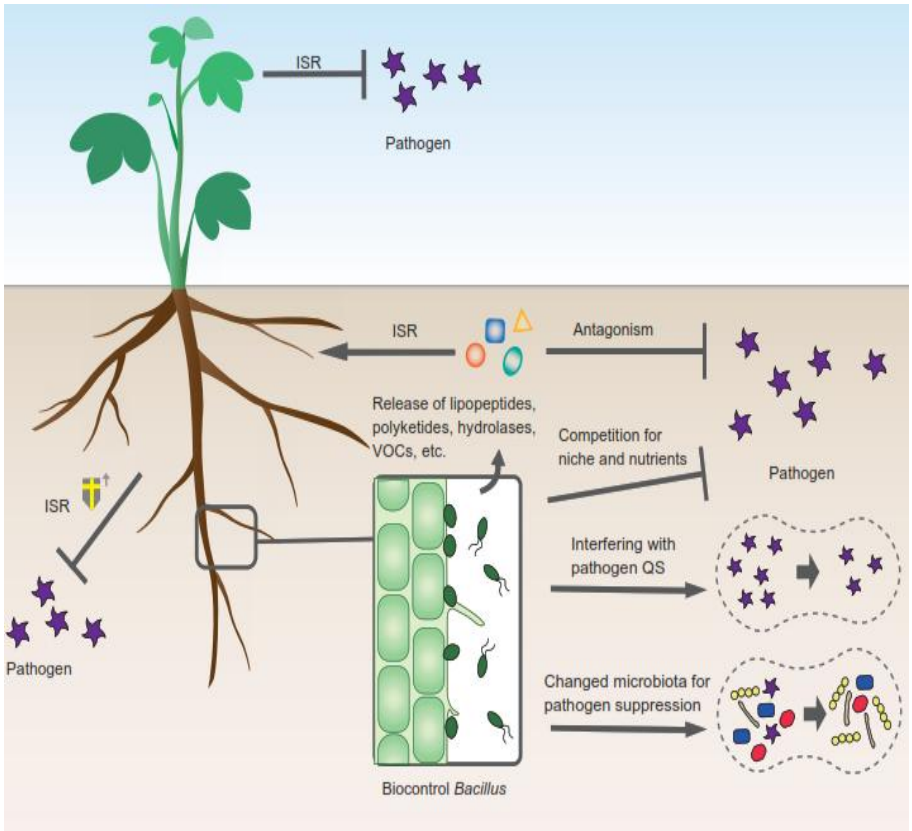

Figura 2. Representação esquemática dos múltiplos mecanismos biológicos de proteção em espécies do gênero *Bacillus*, tanto diretos e indiretos. De forma direta, atua por meio da produção de metabólitos antagonistas, incluindo peptídeos antimicrobianos (como bacteriocinas e lipopeptídeos), polipeptídeos, enzimas líticas e compostos orgânicos voláteis (COVs). De forma indireta, essas bactérias competem por nichos ecológicos e nutrientes, além de induzirem a resistência sistêmica nas plantas (ISR). Além disso, as espécies de *Bacillus* podem secretar enzimas específicas, como AHL-lactonases e AHL-oxidoredutases, que interferem nos sinais de *quorum sensing (QS)* produzidos pelos patógenos, afetando sua comunicação, agregação e patogenicidade (Zhang et al., 2023).

1.2.5. Promoção do crescimento vegetal (PGPR)

As rizobactérias promotoras do crescimento vegetal (Plant Growth-Promoting Rhizobacteria, PGPR) constituem um grupo funcional de microrganismos capazes de colonizar a rizosfera e modular o desenvolvimento vegetal por meio de interações bioquímicas e ecológicas (Kaymak, 2010; Sivasakthi et al., 2014). Esses efeitos são geralmente classificados em mecanismos diretos, associados à nutrição e à regulação fisiológica da planta, e indiretos, relacionados à supressão de patógenos e à ativação de respostas de defesa (Arora et al., 2013; Etesami et al., 2023; Nazir et al., 2018). Dentro desse grupo, o gênero *Bacillus* tem se destacado como um dos mais relevantes devido à sua plasticidade metabólica, à sua capacidade de formar endósporos altamente resistentes e à sua aptidão para estabelecer biofilmes persistentes na interface solo-raiz, características que sustentam tanto sua eficácia ecológica quanto sua viabilidade em aplicações agrícolas comerciais (Hayat et al., 2012; Kashyap et al., 2019; Kumar et al., 2011; Sivasakthi et al., 2014).

Em termos de promoção direta do crescimento, *Bacillus* modula a fisiologia vegetal principalmente por meio da produção de fitormônios e do aumento da disponibilidade de nutrientes (Poveda & González-Andrés, 2021). A biossíntese de compostos como o ácido indolacético (AIA), giberelinas (GA) e ácido abscísico (ABA) permite influenciar processos-chave como a arquitetura radicular, a divisão celular e a adaptação ao estresse abiótico (Ali et al., 2022; Kang et al., 2019; Sarti et al., 2023). Em particular, a produção de AIA tem sido amplamente associada ao aumento na formação de raízes laterais e pelos

radiculares, o que se traduz em maior capacidade de absorção de água e nutrientes (Poveda & González-Andrés, 2021; Sun & Shahrajabian, 2025). De forma complementar, as giberelinas contribuem para a regulação do crescimento da parte aérea por meio da ativação de vias biossintéticas específicas na planta, incluindo a expressão de genes como *GA20ox* e *GA3ox* (Adjei et al., 2025; Hu et al., 2024; Yuan et al., 2024). No nível nutricional, diversas espécies de *Bacillus* facilitam a aquisição de nutrientes limitantes. A solubilização de fosfatos inorgânicos, mediada pela secreção de ácidos orgânicos e enzimas como a glicose desidrogenase (*gcd*) (Boonkumkrong et al., 2024; Rasul et al., 2019), aumenta a fração biodisponível de fósforo no solo e promove a ativação de transportadores vegetais como *PHT1* (Huang et al., 2024). Além disso, a produção de sideróforos, como a bacilibactina, otimiza a captação de ferro em condições de baixa disponibilidade, ao mesmo tempo em que restringe seu acesso a microrganismos competidores (Chakraborty et al., 2022; Dimopoulou et al., 2021). Outro mecanismo relevante é a atividade da enzima ACC desaminase, que modula os níveis de etileno vegetal sob condições de estresse, atenuando seu efeito inibitório sobre o crescimento radicular (Orozco-Mosqueda et al., 2020).

Por outro lado, os mecanismos indiretos de *Bacillus* concentram-se na proteção da planta contra patógenos, por meio de uma combinação de antagonismo microbiano e indução de resistência sistêmica (ISR) (Choudhary & Johri, 2009). A produção de lipopeptídeos cíclicos, como surfactina, iturina e fengicina, constitui um dos principais determinantes de sua atividade antifúngica, ao alterar a integridade das membranas celulares dos patógenos e inibir processos como a germinação de esporos e o crescimento hifal (Bai et al., 2025; Bakker et al., 2024; Ramesh et al., 2024; Zhu et al., 2023). Esse efeito é reforçado pela secreção de enzimas hidrolíticas, incluindo quitinases, β-glucanases e proteases, capazes de degradar componentes estruturais das paredes celulares fúngicas (Ajuna et al., 2024; Mardanova et al., 2017). Paralelamente, algumas cepas interferem nos sistemas de comunicação bacteriana (quorum sensing) por meio de enzimas lactonases, como *aiiA*, que degradam moléculas sinal do tipo AHL, reduzindo a virulência de fitopatógenos (Domnin et al., 2025; Lopes et al., 2017).

Em nível de diversidade funcional, diferentes espécies do gênero exemplificam a amplitude desses mecanismos (Figura 3). Por exemplo, *B. subtilis* constitui um modelo amplamente estudado, caracterizado por sua capacidade de formar biofilmes regulados por redes genéticas complexas (incluindo *Spo0A*, *DegU* e *ComA*) e por sua eficiência na colonização radicular (Sharipova et al., 2023; Vasilchenko et al., 2022). Por sua vez, *B. amyloliquefaciens* e *B. velezensis* destacam-se por seu potencial como agentes de biocontrole e por sua contribuição para a nutrição vegetal (Rabbee et al., 2023; Wang et al., 2022). Em conjunto, as evidências disponíveis sugerem que o sucesso de *Bacillus* como PGPR reside em sua capacidade de integrar múltiplas funções metabólicas, ecológicas e regulatórias em resposta a sinais do ambiente rizosférico, incluindo exsudatos radiculares (Kashyap et al., 2019; Kumar et al., 2011). Essa integração funcional não apenas otimiza o crescimento vegetal, mas também posiciona esse gênero como um componente estratégico no desenvolvimento de sistemas agrícolas sustentáveis baseados em bioinsumos.

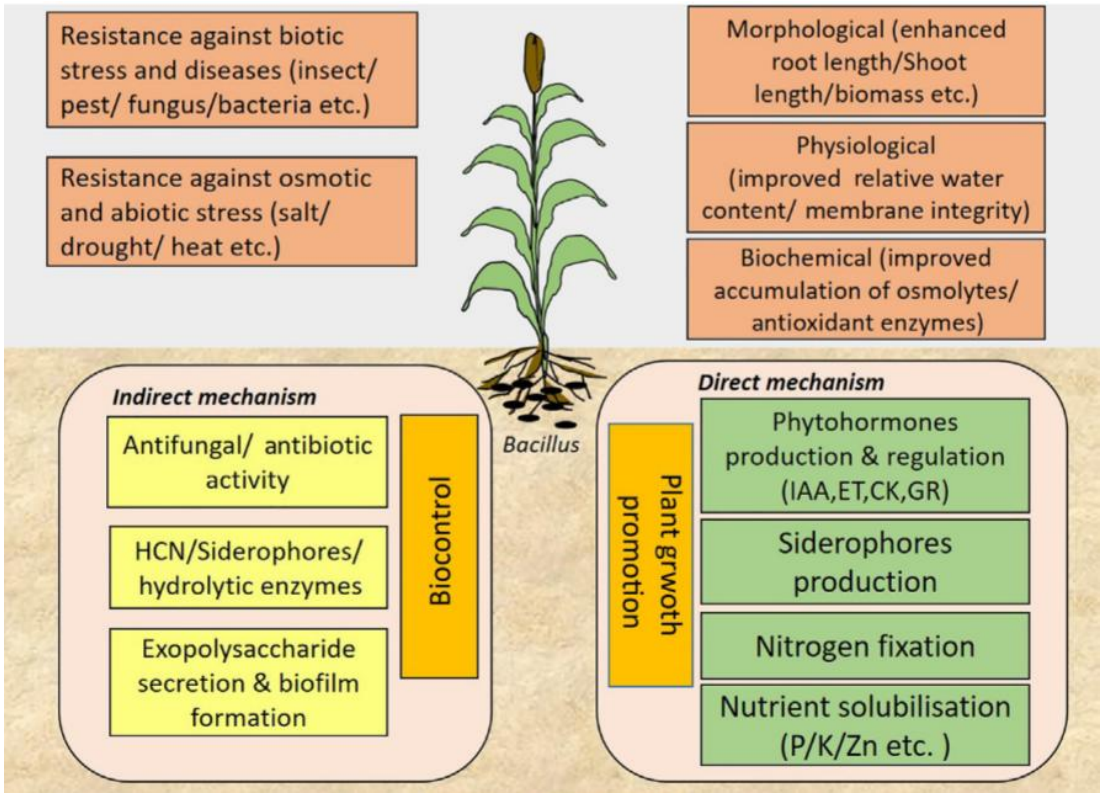

Figura 3. Mecanismo de promoção do crescimento vegetal por *Bacillus sp.* (Tiwari et al., 2019).

1.3. OBJETIVOS

1.3.1. Objetivo Geral

- Realizar a prospecção genômica de cepas do gênero *Bacillus* com potencial de biocontrole de fungos fitopatógenos.

1.3.2. Objetivos específico

- Compilar todos os genomas de *Bacillus* disponíveis publicamente.
- Reconstruir a filogenia do gênero.
- Realizar uma curadoria de genes envolvidos com biocontrole de fungos.
- Identificar cepas candidatas a partir da presença de genes previamente associados ao biocontrole de fungos.
- Classificar genes de acordo com seu modo de ação contra fitopatógenos.
- Verificar presença de outros genes relevantes nestas espécies, como genes envolvidos com biofertilização, resistência a antibióticos e virulência.

2. CAPÍTULO 2. Artigo publicado: World Journal of Microbiology and Biotechnology

Integrative phylogenomic and pangenome landscape of *Bacillus*: insights from 10,000 genomes into taxonomy, functional potential, and biotechnological applications

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2.1. ABSTRACT

The genus *Bacillus* comprises Gram-positive, endospore-forming rods with a ubiquitous distribution across terrestrial, aquatic, and aerial environments, as well as associations with plants, animals, and food. To explore its diversity, we conducted an integrated phylogenomic and pangenome analysis using 10,839 publicly available RefSeq genomes (67 type strain genomes). Taxonomic delimitation combining genomic-distance metrics (Mash/ANI), network analyses, and a label-propagation algorithm assigned 10,276 genomes to operational communities, revealing novel complexes within *B. cereus* sensu lato and other clades. A robust phylogeny reconstructed from 103 representative genomes corroborated the ANI-based groupings. Forty-eight communities (≥10 genomes) were further analyzed for pangenome openness, showing a strong negative correlation between the saturation coefficient (α) and genomic fluidity (φ) ($\rho = -0.636$), indicating that open pangenomes exhibit high gene-content variability. Functional profiling revealed 135 antifungal genes and 15 secondary metabolite clusters, highlighting *B. velezensis*, *B. amyloliquefaciens*, and *B. subtilis* as rich reservoirs of hydrolytic enzymes, NRPS/PKS systems, and nutrient-competition traits. Additionally, 135 resistance determinants and 115 virulence factors were identified, mainly within *B. cereus* sensu lato. Biofertilization genes related to phosphorus, nitrogen, siderophore, potassium, and sulfur metabolism were broadly conserved, underscoring the genus biotechnological potential.

Keywords: comparative genomics; pangenome dynamics; *Bacillus cereus*; functional genomics; microbial biotechnology.

2.2. INTRODUCTION

The genus Bacillus comprises Gram-positive, rod-shaped bacteria belonging to the family Bacillaceae (order Bacillales, class Bacilli, and phylum Bacillota). These microorganisms are distinguished by their ability to form endospores and by their high metabolic plasticity, which enables growth under both aerobic and facultatively anaerobic conditions (Borriss, 2020; Göker & Oren, 2024; Oren & Garrity, 2021; Maughan & Van der Auwera, 2011; Vos et al., 2009). The genus was first described by Cohn (1872) and currently includes more than 656 entries in the List of Prokaryotic names with Standing in Nomenclature Permanence (LPSN), of which 115 species have validly published names (Parte et al., 2020) (accessed July 2025).

Bacillus species are ubiquitously distributed and have been isolated from diverse habitats, including freshwater and marine environments, soils of various characteristics, air, animals, plants, and even food (Dérozier et al., 2023). Among the best characterized members are B. cereus, the etiological agent of foodborne illnesses such as emetic and diarrheal syndromes (Griffiths & Schraft, 2017; Yang et al., 2023); B. anthracis, the causative agent of anthrax (Bower et al., 2022); and B. thuringiensis, extensively used as a bioinsecticide in agricultural pest management (Sanahuja et al., 2011). These species belong to the B. cereus sensu lato group (Carroll et al., 2022). In contrast, members of the B. subtilis group (e.g., B. subtilis and B. stercoris) and the B. amyloliquefaciens group (e.g., B. velezensis and B. amyloliquefaciens) (Ngalimat et al., 2021) are recognized for their agricultural applications as plant growth-promoting bacteria (PGPB) and biocontrol agents with antifungal activity (Figueiredo et al., 2025; Li et al., 2023; Pengproh et al., 2023; Soliman et al., 2023; Teixeira et al., 2021). Ecological preferences within the genus appear to be shaped, at least in part, by genomic architecture, as organisms with similar lifestyles often share a higher proportion of genes (Henaut-Jacobs et al., 2023). However, accurately delineating these ecologically and functionally distinct groups remains challenging when relying solely on 16S rRNA gene-based taxonomy, whose limited resolution often obscures fine-scale relationships and misrepresents closely related taxa such as those within the B. cereus sensu lato complex (Chung et al., 2024; Maughan & Van der Auwera, 2011; Poretsky et al., 2014).

The rapid expansion of genome sequences in public repositories has enabled the adoption of whole-genome approaches such as average nucleotide identity (ANI), genomic distance metrics, and pan-genome analysis—methods that offer superior taxonomic resolution and allow inference of key aspects of strain lifestyle (Passarelli-Araujo et al., 2022, 2025). Recent studies demonstrate Bacillus diversity, particularly

within well-defined species groups such as the B. subtilis, B. amyloliquefaciens, and B. cereus sensu lato complexes. These studies have provided important insights into pangenome openness, genomic fluidity, and the functional diversification associated with ecological adaptation and pathogenicity (Bazinet, 2017; Chun et al., 2019; Wang et al., 2024; Wu et al., 2021). In parallel, efforts to curate high-quality pangenomes have emphasized the importance of controlling for confounding genomes, including misclassified strains, engineered or genome-reduced isolates, and highly redundant clonal lineages, which can inflate accessory gene content and bias estimates of pangenome openness (Table S1) (Wu et al., 2021; Yang & Gao, 2022).

While these approaches are essential for fine-scale species-level analyses, a critical challenge remains integrating large-scale genome collections into a coherent taxonomic and functional framework that preserves biological signals while enabling comparative analyses across the entire genus. In this study, we analyzed 10,839 genomes, of which 10,276 high-quality genomes clustered were resolved into 103 distinct communities, representing the most comprehensive genus-wide dataset analyzed to date. By combining genome-distance metrics, network-based community detection, and representative-genome phylogenetics, we systematically characterized pangenome architecture, antifungal potential, resistance determinants, virulence factors, and biofertilization-related traits. This large-scale framework enables direct comparison with previous species-focused studies while providing a unified view of how genomic diversity, functional potential, and taxonomic structure are interlinked across the genus Bacillus, thereby paving the way for the development of safe and sustainable agricultural bioinputs.

2.3. METHODS

2.3.1. Dataset collection

A total of 10,839 publicly available *Bacillus* genomes from the NCBI RefSeq database in October 2024 using Datasets v.16.25.0 (https://www.ncbi.nlm.nih.gov/datasets/). Genome quality was assessed with CheckM v.1.2.3 (Parks et al., 2015), applying a minimum completeness threshold of 90% and a maximum contamination of 5%. BUSCO v5.8.0 (Simão et al., 2015) was additionally used with the Bacillales dataset as a reference, discarding genomes with completeness ≤90% or duplication ≥5 %.

2.3.2. Type strain validation and network analysis

Type strains of the genus *Bacillus* were retrieved from the LPSN (Parte et al., 2020) to validate species names and to obtain the corresponding Genomic Taxonomy Database

Designated Representative Genome (GTDB) representative genomes (Parks et al., 2018). Genomic distances for 1000-, 3000-, and 5000-bp sketches were computed using Mash v2.3.0 (Ondov et al., 2016). Reciprocal Mash distances were converted into genomic identity values (1 - Mash) and genome pairs with identities <0.95 were excluded, as they did not clearly resolve community structure.

Communities were identified using the label propagation algorithm (LPA) (Raghavan et al., 2007), a graph-based clustering approach for detecting groups of highly interconnected nodes in large networks. In this framework, nodes represent genomes and edges reflect genomic similarity (e.g., Mash). Through iterative label propagation among neighboring nodes, genomes sharing high similarity converge into stable communities without requiring predefined cluster numbers. (Passarelli-Araujo et al., 2022).

Communities containing type genomes were named according to their corresponding validated type species (67 in total), whereas those lacking type genomes are assigned arbitrary labels (e.g. *Bacillus* sppN, where N corresponds to the number assigned to the community) (Henaut-Jacobs et al., 2023). In such cases, a representative genome was selected according to GTDB reference genome designation. To resolve highly connected or complex clusters, fastANI v1.34 (Jain et al., 2018) was applied for each complex separately. The resulting communities were compared with NCBI RefSeq database and GTDB classifications to identify potential misclassifications, which were manually inspected. For type strain comparisons, ANI was computed using pyANI v0.3.0.0-alpha using MUMmer-based alignment (Pritchard et al., 2016), ensuring that all genome pairs exhibited ≥95% identity. Community networks were visualized with the igraph v.2.1.4 package (Han et al., 2010).

2.3.3. Phylogenetic reconstruction and pangenome analysis

All *Bacillus* genomes were re-annotated using Prokka v.1.14.6 (Seemann, 2014). Phylogenetic inference was performed using representative genomes from each community. Orthologous genes were identified with Orthofinder v. 3.0.1 (Emms & Kelly, 2015), and their sequences were aligned with MAFFT v7.526 (Rozewicki et al., 2019). The resulting alignments were concatenated and filtered using BMGE v1.12 (Criscuolo & Gribaldo, 2010). Phylogenetic inference was performed with IQ-TREE v.2.4.0 (Nguyen et al., 2015), employing the *TEST* option for automatic model selection. Node support values were computed from 1,000 nonparametric bootstrap replicates. *Metabacillus fastidiosus*, *M. litoralis*, and *M. niabensis* (accession number: GCF_001591625.1,

GCF_007994985.1, GCF_030813165.1, respectively) were used as the outgroup. Phylogenies were visualized using iTOL v7 (Letunic & Bork, 2024).

Pangenome analysis was carried out for communities containing at least 10 genomes using Panaroo v.1.5.2 (Tonkin-Hill et al., 2020). The selected parameters were -c 0.95, -f 0.85, --clean-mode moderate, --refind_prop_match 0.5, --search_radius 1000, and --remove-invalid-genes, aiming to conservatively define orthologous gene families, control the proportion of core and accessory genes, and minimize the impact of assembly artifacts, annotation errors, and fragmented genes on the pangenome analysis (Passarelli-Araujo et al., 2025).

Genomic openness was estimated by fitting a power law (α) model, and pangenome accumulation curves were generated with pagoo v0.3.18 (Ferrés & Iraola, 2021). Genomic fluidity, representing pairwise gene content diversity, was calculated with micropan v2 (Snipen & Liland, 2015).

2.3.4. Identification and classification of genes involved in antifungal activity

A comprehensive literature review was performed to identify genes with experimentally validated antifungal activity in *Bacillus* strains. The corresponding coding sequences were retrieved from UniProt (Bateman et al., 2021) and NCBI Protein database (https://www.ncbi.nlm.nih.gov/protein/). Genes sharing the same name across different species were renamed by appending the initials of the respective species at the end (e.g., *chiA-Bv* for *B. velezensis*). When multiple genes with the same name were present in one species, a consecutive number was added (e.g., *chiA-Bv1*). Gene presence was determined with USEARCH v11.0.667 (Edgar, 2010), with thresholds of ≥60% identity and ≥50% coverage. Presence-absence matrices were generated and annotated in R with the tidyverse v.2.0.0 package (Wickham et al., 2019). To further evaluate the potential of these strains to produce antifungal secondary metabolites, antiSMASH v8.0.2 (Blin et al., 2025) was employed, considering similarity confidence values ≥50% (classified as Medium, 50–75%, and High, 75–100%).

We also analyzed 103 representative genomes previously annotated with Prokka v.1.14.6 (Seemann, 2014), to confirm the presence of Carbohydrate-active enzymes (CAZymes), identified using dbCAN v4.1.4 (Zheng et al., 2023), employing the parameters --dia_cpu 26 --hmm_cpu, --dbcan_thread 26 and --gram p. Signal peptide prediction and secretion probability were assessed using SignalP v6.0 (Teufel et al., 2022), using the parameters -m signalp.predict, --organism other, and --mode slow-sequential. Two matrices were subsequently integrated: (i) CAZy family

presence/absence and (ii) signal peptide probability for secreted CAZymes, denoting families containing at least one signal peptide-bearing protein with the suffix "_SP".

2.3.5. Identification of genes for antibiotic resistance, virulence, and biofertilization.

Genes linked to antibiotic resistance, virulence, and biofertilization were identified using three specialized databases: the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2023) for resistance genes, the Virulence Factor Database (VFDB) (B. Liu et al., 2022) for virulence factors, and PlaBAse (Patz et al., 2021) for biofertilization-related genes. All databases were downloaded on June 6, 2025. Gene detection was carried out with USEARCH v11.0.667 (Edgar, 2010) using thresholds of ≥60% identity and ≥50% coverage. Presence–absence matrices for all *Bacillus* genomes were generated in R with the tidyverse v2.0.0 package (Wickham et al., 2019). Scripts used in the genomic characterization of *Bacillus:* https://github.com/Grrt53/Bacillus_total.git

2.4. RESULTS AND DISCUSSION

2.4.1. Genome curation and taxonomic delimitation

We retrieved 10,839 *Bacillus* genomes from the NCBI RefSeq database (October 2024; Table S2). After applying stringent quality criteria (completeness ≥ 90% and contamination/duplication ≤ 5%), 10,625 genomes were retained, ranging from 2.7 to 8.0 Mb in size. Sixty-seven type strains with validly published names, as listed in LSPN (April 2025), were identified, each having a corresponding reference genome in GTDB. For *B. paramobilis*, which lacks GTDB species assignment, a representative genome was manually selected (Table S3). Classification with LPA assigned 10,276 genomes into 78 *Bacillus* communities, within which nine preliminary species complexes were identified (Table S4, Table S6; see Methods for details). Genomes excluded at this stage primarily belonged to closely related genera within Bacillaceae, such as *Niallia, Domibacillus, Pseudobacillus*, and *Priestia*. Each community was linked to a GTDB reference genome; those without a reference were labeled with the suffix (_XX).

Highly interconnected communities were grouped into species complexes, encompassing 76.5% of the dataset (Table S6). These complexes were further refined with FastANI , yielding in 103 communities, including 34 communities associated with the previously identified species complexes (Table 1; Table S5). One representative genome from each of the 103 communities was subsequently analyzed using PyANI to

achieve a higher resolution view of genomic relationships (ANI ≥ 95%; Table S7). This analysis confirmed the genomic coherence of the nine species complexes previously defined (Table S6), while also revealing the relationships among 25 representative species belonging to those complexes—three of which were unchanged after refinement (Figure 1).

Within the *B. anthracis* complex, the *B.* spp35 community was closely related to *B. anthracis* (ANI ≥ 95.77%), whereas *B.* spp36 exhibited multiple linkages to *B. paranthracis, B. tropicus*, and *B. pacificus* (ANI ≥ 95.53%), with *B. paranthracis* occupying a central, highly connected position. Likewise, *B. basilensis* and *B.* spp34 formed a distinct complex (ANI ≥ 95.4%), clearly separated from the initial cluster. In the *B. cereus* complex (including *B. cereus* and *B. thuringiensis*), *B.* spp32 displayed strong connectivity with both species (ANI ≥ 96%).

In the *B. mobilis* complex, *B.* spp28 was linked to *B. wiedmannii* (ANI = 95.07%), while *B.* spp30 and *B.* spp31 were associated with to *B. paramobilis*, itself connected to *B. mobilis* and *B. wiedmannii* (ANI = 95.07%). Notably, *B.* spp30 is also connected to *B. sanguinis* (≥ 95.25%), which initially belonged to another complex (*B. anthracis*). Conversely, the complexes formed by *B. haynesii*, *B. licheniformis*, and *B. paralicheniformis* (ANI ≥ 95.27%), as well as those involving *B. halotolerans* with *B. mojavensis* (ANI ≥ 95.75%) and *B. subtilis* with *B. stercoris* (ANI ≥ 95.47%), remained stable after PyANI refinement (Table 1). Overall, the pyANI network analysis corroborated the *Bacillus* delineation obtained with FastANI while providing higher resolution within species complexes (Table 1, Table S5, Table S6, and Table S7).

Table 1. Species complexes in *Bacillus* resolved by community detection and ANI-based delimitation.

Species complex	No. of genomes	ANI (%)
B. anthracis, B. basilensis, B. dicomae, B. pacificus, B. paranthracis, B. sanguinis, B. tropicus, B. spp34, B. spp35, B. spp36	1461	96.8
B. cereus, B. thuringiensis, B. spp32, B. spp33	2501	97.2
B. halotolerans, B. mojavensis	124	96.8
B. haynesii, B. licheniformis, B. paralicheniformis	776	95.8
B. hominis, B. mycoides	262	95.0
B. mobilis, B. paramobilis, B. wiedmannii, B. spp28, B. spp29, B. spp30, B. spp31	416	96.2
B. nitratireducens, B. proteolyticus	101	95.0
B. siamensis, B. velezensis	1172	95.0
B. stercoris, B. subtilis	1055	96.8

2.4.2. Species assignment correlation

To compare species assignments across classification frameworks, we analyzed genome counts derived from NCBI RefSeq, GTDB, and our graph-based LPA (Table S8). For species with validly published names listed in LPSN (as curated by GTDB, April 2025), Spearman's rank correlation revealed strong and highly significant concordance among schemes. Genome counts assigned by RefSeq and GTDB were strongly correlated ($\rho = 0.932$, $p < 2.35 \times 10^{-30}$), as were those between RefSeq and LPA ($\rho = 0.961$, $p < 7.64 \times 10^{-38}$) and between GTDB and LPA ($\rho = 0.969$, $p < 2.54 \times 10^{-41}$) (Figure 2). These results indicate that, despite methodological differences, genome assignments are broadly consistent across reference-based (RefSeq, GTDB) and graph-based (LPA) taxonomic frameworks. Notably, LPA showed the highest concordance with GTDB, underscoring that the network-based approach effectively captures taxonomic relationships in agreement with genome-based species definitions.

Nevertheless, marked discrepancies were observed for several taxa. For example, NCBI RefSeq classified 2,059 genomes as *B. cereus*, whereas GTDB and LPA assigned 1,303 and 1,571 genomes, respectively (Table S8, Figure 2). In addition *B. amyloliquefaciens* included 234 genomes in RefSeq, compared with only 74 in both GTDB and LPA. In contrast, *B. paranthracis* accounted for 265 genomes in RefSeq versus 563 in GTDB and 508 in LPA. Likewise, *B. subtilis* comprised 990 genomes in RefSeq compared with 1,045 in GTDB and 1,019 in LPA, whereas *B. velezensis* included 983 genomes in RefSeq versus 1,198 in GTDB and 1,147 in LPA. These discrepancies highlight persistent challenges in *Bacillus* species delimitation and reflect the divergent criteria applied across classification systems. Importantly, these discrepancies are not merely taxonomic artifacts but have direct consequences for downstream pangenome inference, as misclassified or phylogenetically divergent "confounding" genomes can artificially inflate gene pools.

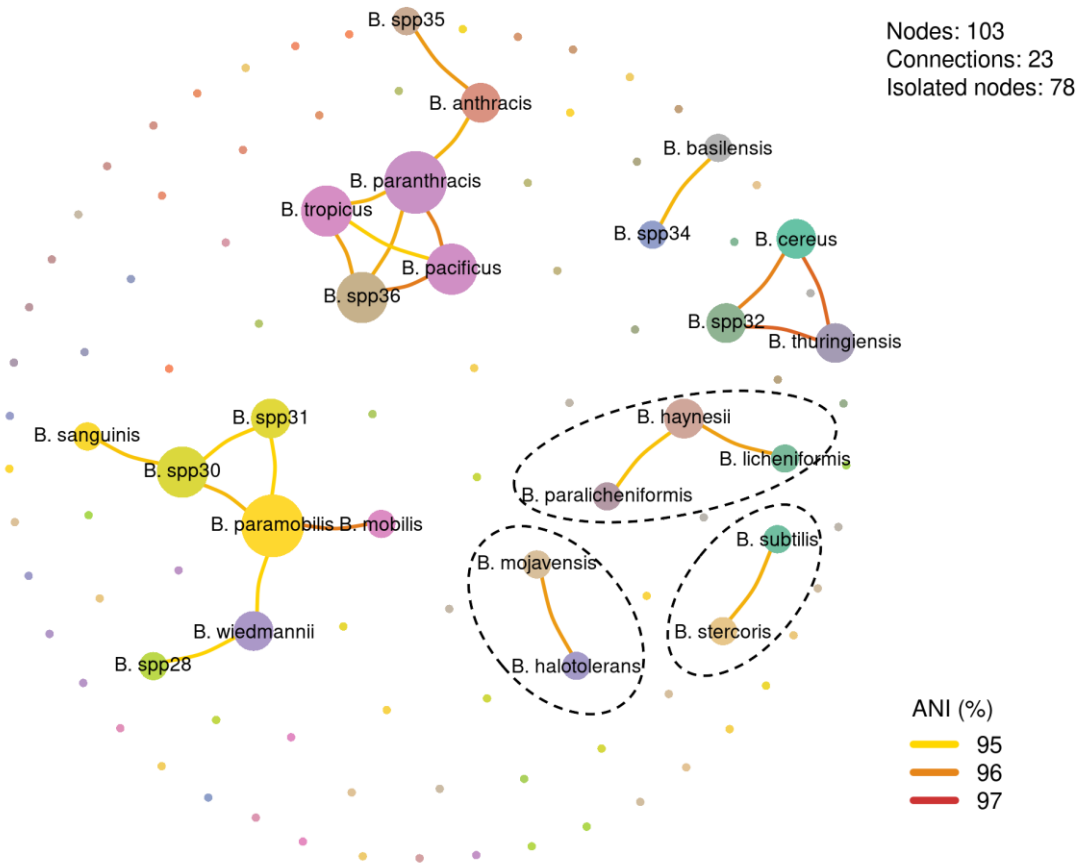

Figure 1. Network of genomic communities in the genus *Bacillus* based on average nucleotide identity (ANI). The network includes 103 representative genomes, of which 25 form 23 connections, while 78 remain isolated. Each node represents a genome, with node size proportional to its degree of centrality (number of connections). Edge colors denote ANI values: yellow (≥ 95%), orange (≥ 96%), and red (97%). Newly identified communities (e.g., *Bacillus* spp32, spp35, and spp36) show strong connectivity with reference species such as *B. cereus, B. thuringiensis, B. paranthracis*, and *B. paramobilis*, supporting their inclusion in the *B. cereus* sensu lato group or close phylogenetic proximity to recognized species (see also Figure 3). The communities delimited by dashed lines correspond to complexes that remained unchanged after refinement using pyANI. The remaining complexes were refined, including the resolution of 9 species (*i.e.*, separated communities)

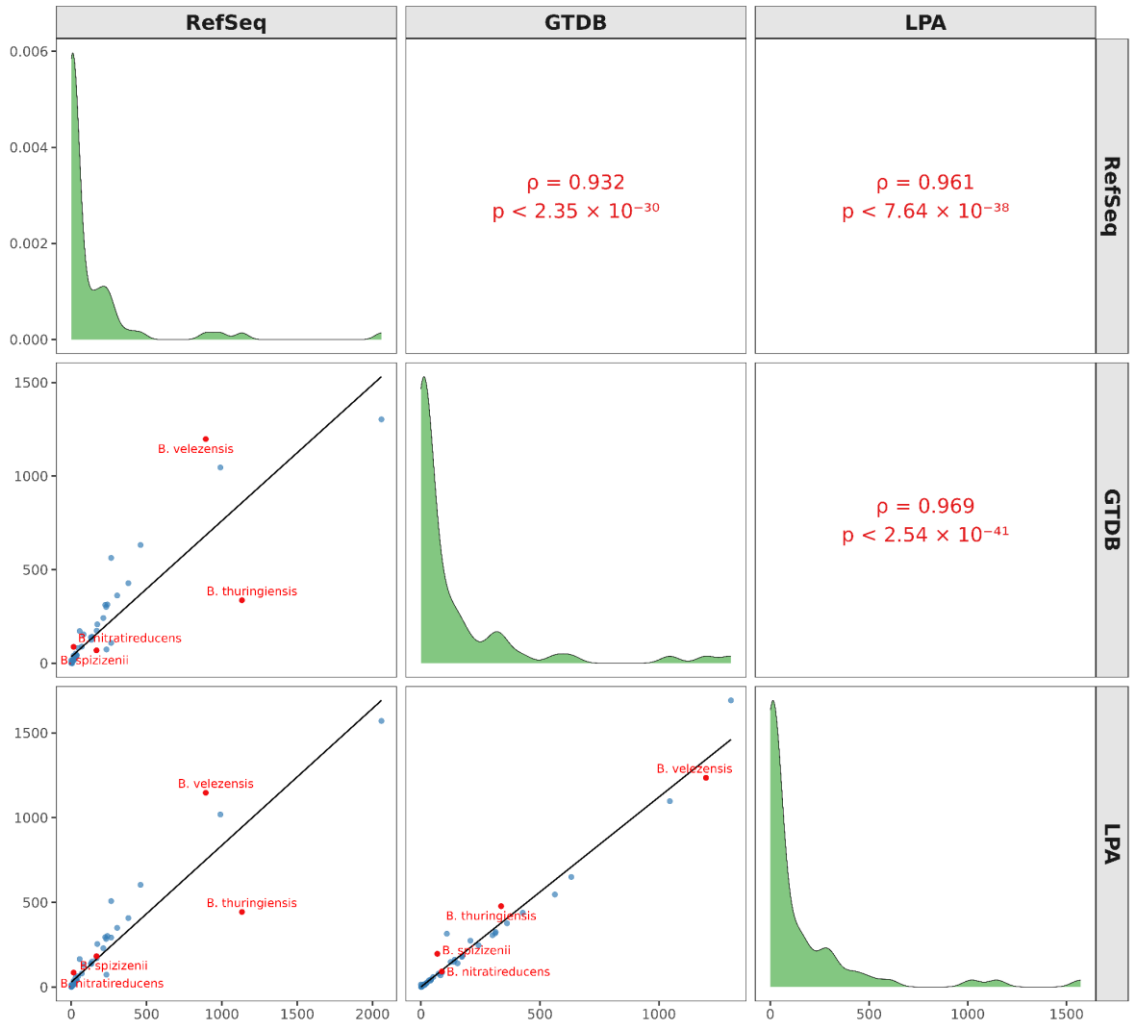

Figure 2. Taxonomic assignments among classification systems for selected *Bacillus* species. *B. thuringiensis* shows substantial overallocation in NCBI RefSeq (1,133 genomes) compared with GTDB (337) and LPA (444). *B. velezensis* is more inclusively defined by GTDB (1,198) and LPA (1,147) relative to RefSeq (893). In contrast, *B. spizizenii* is assigned significantly fewer genomes in GTDB (69) than in RefSeq (168) and LPA (183), suggesting a narrower taxonomic definition. *B. nitratireducens* appears underrepresented in RefSeq (16) compared to GTDB (88) and LPA (86), indicating potential misclassification or incomplete representation.

2.4.3. Phylogenomic resolution of *Bacillus* communities

Phylogenetic reconstruction was conducted using one representative genome from each of the 103 communities (Table S3). ModelFinder, implemented in IQ-TREE, selected LG+F+I+G4 as the best-fit evolutionary model. Genomes of *Metabacillus* were used as outgroup taxa (see Methods). The resulting phylogeny showed strong branch support across all nodes (Figure 3). Species clustered according to their major operational

groups, including the *B. cereus* sensu lato group (Carroll et al., 2022), *B. pumilus* (Fu et al., 2021), *B. amyloliquefaciens* (Ngalimat et al., 2021)*, B. licheniformis*, and *B. subtilis* (Caulier et al., 2019) groups. Within the genus *Bacillus*, these operational groups represent phylogenetically cohesive complexes of closely related species that frequently exhibit high genomic similarity and overlapping phenotypic traits, which can hinder discrimination using conventional phenotypic tests or limitations of 16S rRNA-based classification, which lacks the resolution needed to discriminate among closely related species (Liu et al., 2015; Xu & Kovács, 2024).

This resolution corroborates the network-based analysis (Figure 1), which also revealed complex communities. Overall, ANI-based species delimitation provided a robust framework for taxonomic resolution, while phylogenetic and functional group analyses offered complementary insights into species relationships and the biological basis of complex community formation

2.4.4. Pangenome architecture and genomic fluidity in *Bacillus*

Pangenome analysis was performed for 48 communities containing at least 10 genomes, enabling exploration of the relationship between genomic fluidity and the saturation coefficient (α) as indicators of genomic architecture and species dynamics. The pangenome, defined as the complete set of genes across all strains of a species or closely related group (Golchha et al., 2024; Rouli et al., 2015), is typically partitioned into three fractions: (i) the core genome, comprising genes present in all strains and linked to essential cellular functions and taxonomic identity; (ii) the accessory genome, consisting of genes shared by some but not all strains, often associated with niche adaptation, host interaction, or stress responses and resistance; and (iii) private or unique genes, found in a single strain, frequently reflecting specific adaptations or recent horizontal gene transfer (Golchha et al., 2024; Tettelin et al., 2008; Tettelin & Medini, 2020; Vernikos et al., 2015). Within this framework, genomic fluidity (φ) was calculated to quantify pairwise gene content dissimilarity, while α was used to assess pangenome openness. Lower α values denote open pangenomes with continuous gene acquisition, whereas higher values reflect closed, conserved pangenomes (Figure S1, Table S9). Spearman's rank correlation revealed a significant negative association between α and genomic fluidity ($\rho = -0.636$; $p = 1.19 \times 10^{-6}$). A linear regression confirmed this trend ($F_{1,46} = 41.28$; $p = 6.66 \times 10^{-8}$), explaining 46.1% of the variance (adjusted $R^2 = 0.461$). The resulting equation, $\varphi = 0.4499 - 0.4242\alpha$, suggests that genomic fluidity decreases by approximately 0.42 units for every unit increase in α (Figure 4).

Interestingly, species such as *B.* spp11*, B. sonorensis, B.* spp34*, B. basilensis*, and *B. cytotoxicus*—all members of the *B. cereus* sensu lato group (Carroll et al., 2022)—displayed relatively high α values and a larger proportion of core genes, a pattern that may reflect the limited number of genomes analyzed and/or genuine ecological specialization. By contrast, *B. licheniformis* and *B. paralicheniformis* exhibited the most convincingly "closed" pangenomes, supported by extensive sampling (408 and 230, respectively) and consistently high α values.

Conversely, species with low α values, including *B. mycoides* (n = 255), *B. cereus* (n = 1,592), *B. thuringiensis* (n = 444), and *B. wiedmannii* (n = 285), showed highly open pangenomes and elevated genomic fluidity (Table S7). This pattern is consistent with a generalist lifestyle, extensive genomic plasticity, and higher rates of HGT, enabling rapid adaptation to diverse environments. Notably, this group includes opportunistic pathogens (e.g., *B. cereus* group), where genetic versatility likely contributes to ecological success and pathogenic potential (Walker-York-Moore et al., 2017). Similarly, the presence of open pangenomes and high genomic fluidity has been described in non-pathogenic species such as *B. subtilis, B. spizizenii, B. halotolerans,* and *B. atrophaeus*, belonging to the *B. subtilis* group (Chun et al., 2019; Wu et al., 2021; Wang et al., 2024), reflecting intense HGT and marked ecological generalism.

Collectively, these results indicate that pangenome openness and genomic fluidity in *Bacillus* are shaped by a combination of sampling depth and species-specific ecological strategies. It is important to note that, although clonal or nearly identical genomes were not explicitly removed, the analytical framework implemented incorporates multiple strategies to mitigate the impact of redundancy on pangenome inference. These include strict genome-quality filtering and the pre-grouping of isolates into ANI-defined communities prior to pangenome analysis. This approach, which is consistent with recent recommendations for the construction of high-quality pangenomes in *B. subtilis* and *Escherichia coli* (Wu et al., 2021; Yang & Gao, 2022), prioritizes large-scale biological interpretability and complements studies focused on fine-grained comparisons among highly related strains.

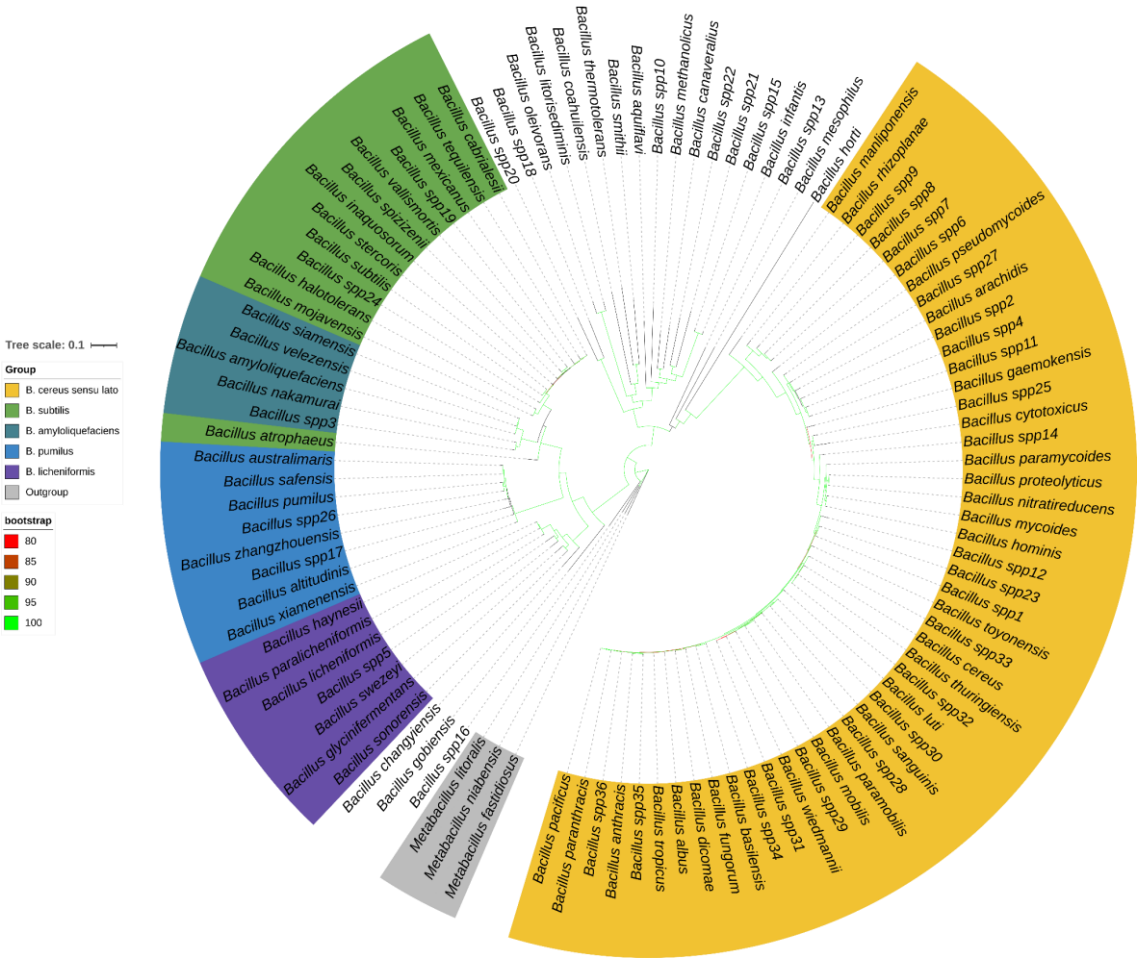

Figure 3. Maximum-likelihood phylogeny of 103 representative *Bacillus* genomes. Node support values ≥80% are indicated by green lines. Genome identifiers corresponding to each species are listed in Table S3. Major species groups are resolved and cluster according to their operational groups (e.g., *B. cereus* sensu lato, *B. pumilus*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. subtilis*), in agreement with ANI-based community delimitation (see Figure 1).

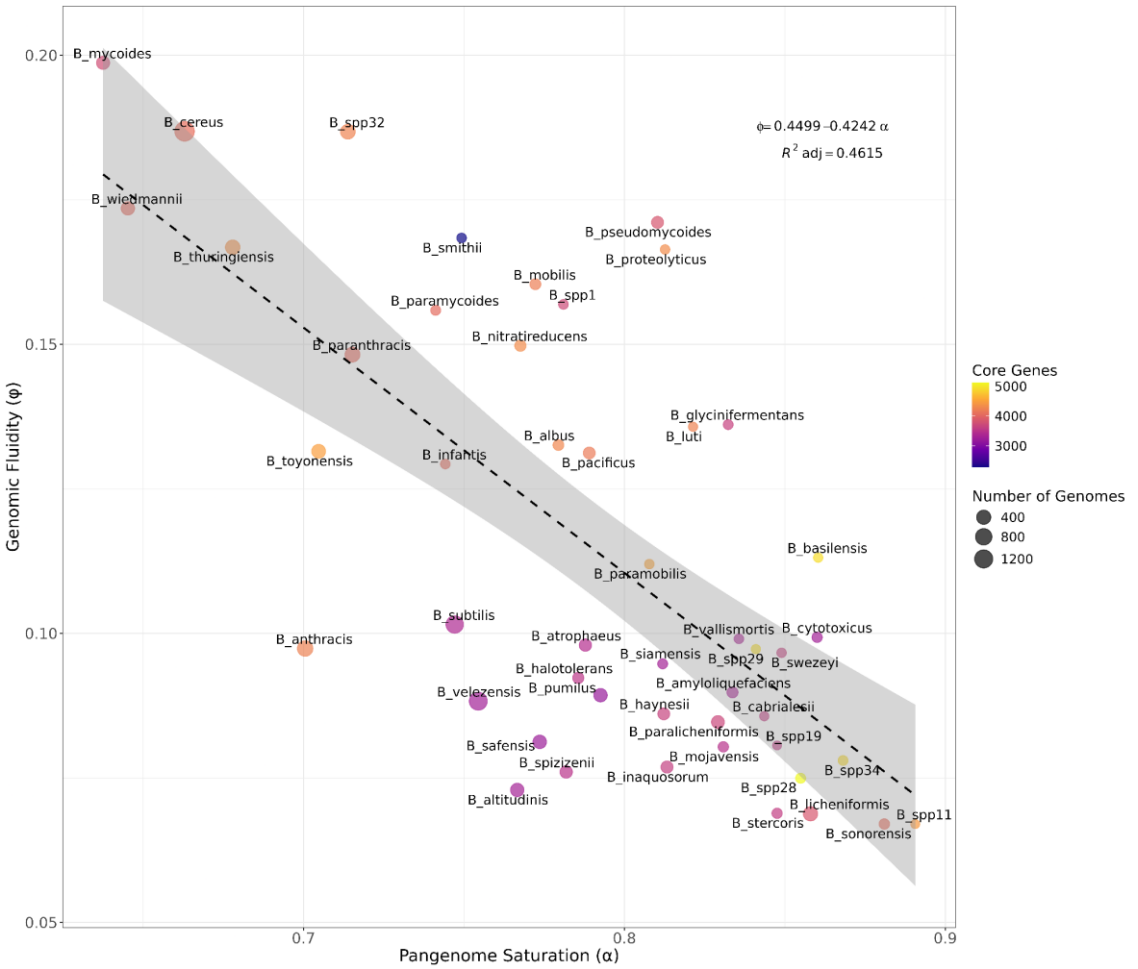

Figure 4. Pangenome openness and genomic fluidity in *Bacillus* species.. Relationship between pangenome openness (saturation curve coefficient, α) and genomic fluidity (φ). The linear regression model (φ = 0.4499 − 0.4242α) revealed a significant negative association ($F_{1,46}$ = 41.28, p = 6.66 × 10^{-8}), showing that species with more open pangenomes tend to exhibit higher genomic fluidity.

2.4.5. Secondary metabolites and competitive antifungal strategies in *Bacillus*

Across the 103 communities analysed (10,276 genomes in total), three major antifungal mechanisms were identified: (i) cell wall degradation, via hydrolytic enzymes, (ii) growth inhibition mediated by peptides and secondary metabolites, (iii) competitive inhibition through nutrient sequestration. In total, 135 genes involved in these mechanisms were evaluated (Figure S2, Tables S10, S11, and S12).

The first mechanism, cell wall degradation, targets fungal structural polysaccharides. Chitinases and glucanases play central roles in breaking down chitin and glucans, which are essential components of fungal cell walls (Ruiz-Herrera & Ortiz-Castellanos, 2019; Synowiecki & Al-Khateeb, 2003). Additionally, exposed chitin can be

converted into chitosan, a pathogenicity strategy employed by fungi infecting both plants and humans (e.g. *Colletotrichum graminicola* and *Cryptococcus neoformans*) (Baker et al., 2011; El Gueddari et al., 2002; Nampally et al., 2012). Genes encoding chitinases (e.g., *chiA*, *chiB*, *chiD*), glucanases (e.g., *bglA, bglS*), and chitosanase (e.g., csn), were widely distributed and often present as multiple homologs across *Bacillus* species. These enzymes act complementarily, degrading chitin, β-glucans, and chitosan. When evaluated individually, however, their activity is limited (Mauch et al., 1988), for instance, synergistic activity markedly enhances antifungal effects, as demonstrated in *B. subtilis* and *B. methylotropicus* (syn. *B. velezensis*), which suppress the growth of *Alternaria triticina* and *Bipolaris sorokiniana* through combined chitinase-glucanase production (Saini et al., 2024). Likewise, chitosanase in *B. subtilis* has been shown to inhibit *Botrytis cinerea, Fusarium oxysporum*, and *F. solani* (Pang et al., 2021; Park et al., 2008).

The highest potential for chitinase-mediated degradation was observed in the *B. cereus* sensu lato clade, with nine genes identified, particularly in *B. toyonensis, B. thuringiensis, B.* spp32*, B. cereus, B. wiedmannii*, and *B. tropicus*. In contrast, smaller chitinase repertoires (seven genes) were detected in the *B. pumilus, B. amyloliquefaciens, B. licheniformis*, and *B. subtilis* groups, where the presence of the *chiA-Bv* gene was notable (Tran et al., 2022). Interestingly, *B. paranthracis*, a member of the *B. cereus* sensu lato clade, also harbored this gene despite its divergence from more distant phylogenetic lineages. By comparison, *B. velezensis, B. siamensis*, and *B. amyloliquefaciens* (*B. amyloliquefaciens* group), together with *B. atrophaeus, B. mojavensis*, and *B. halotolerans* (*B. subtilis* group), exhibited greater enzymatic potential associated with chitosanases and glucanases. Notably*, B. atrophaeus, B. mojavensis*, *B. halotolerans, Bacillus* spp3*, B. velezensis, B. siamensis*, and *B. amyloliquefaciens* harbored at least two genes from each of the three hydrolytic categories, indicating broad antifungal potential.

The second antifungal mechanism involves the production of antifungal peptides and secondary metabolites. Non-ribosomal peptide synthetases (NRPSs) encode compounds such as surfactin (*srfA*), bacillomycin (*bmy*), fengycin (*fen*), and plipastatin (*pps*), which bind to fungal membranes and disrupt their integrity (Cawoy et al., 2014; Jourdan et al., 2009; Khatoon et al., 2022). Polyketide synthase (PKS) clusters, responsible for difficidin (*dfn*) and macrolactin (*mln*) production, were also detected; these metabolites interfere with cell envelope biosynthesis and nucleic acid or protein synthesis (Khatoon et al., 2022; Kumariya et al., 2019; Papagianni, 2003). Hybrid NRPS/PKS pathways, including *pks* (bacillaene), *myc* (mycosubtilin), iturin (*itu*), and *zma* (zwittermicin), combine polyketide and peptide modules to yield structurally complex

compounds with dual activity against fungal membranes (Abdelmoteleb et al., 2017; Emmert et al., 2004; Kevany et al., 2009, Markelova, N., & Chumak, A., 2025). Ribosomally synthesized and post-translationally modified peptides (RiPPs), such as subtilin (*spa*) and subtilosin (*alb* + *sbo*), were also identified; both act by inducing membrane permeabilization and apoptosis (Klein et al., 1992; Marx et al., 2001). In addition, rhizocticin (*rhi*), a phosphonopeptide that acts synergistically with the protease subtilisin (*aprE*), was observed. Together, they inhibit filamentous and budding fungi by interfering with intracellular metabolism (Abdelmoteleb et al., 2017; Borisova et al., 2010; J. Hu et al., 2022; Kugler et al., 1990).

Analysis with antiSMASH v8.0.2 confirmed the presence of 15 secondary metabolite clusters associated with antifungal activity (Figure 5, Table S13). Fengycin was the most widespread metabolite, detected in 33 species including *B. altitudinis*, *B. amyloliquefaciens*, and *B. atrophaeus*. Surfactin was present in 17 species, while mycosubtilin was restricted to five (*B. atrophaeus*, *B. inaquosorum*, *B. nakamurai*, *B. spizizenii*, and *B. velezensis*). Bacillomycin D occurred only in *B. mexicanus* and *B. velezensis*, whereas plipastatin was identified in nine species. Subtilosin A was found in 12 species, and zwittermicin A was limited to *B. cereus*, *B.* spp32, *B. thuringiensis*, and *B. wiedmannii*. Difficidin was exceptionally rare, occurring only in *B. siamensis* and *B. velezensis*. Macrolactin H and rhizocticin A were present in six species, while subtilin was identified in seven species, including *B. amyloliquefaciens* and *B. subtilis*.

Notably, the Iturin biosynthetic gene cluster (BGC) was not detected by antiSMASH, likely due to its low similarity (<50%) to MIBiG reference clusters and the complex architecture of NRPS/PKS systems. Previous studies in *B. velezensis* have reported that the fengycin and iturin BGCs may share overlapping genomic regions, which can hinder the accurate identification of the iturin cluster by automated genome mining tools. Consequently, antiSMASH may fail to resolve this cluster when its modular organization diverges from canonical references (Bach et al., 2025).

In contrast, the USEARCH analysis identified the complete cluster in species such as *B. amyloliquefaciens*, *B. siamensis*, and *B. velezensis* (Figure S2). This discrepancy arises from fundamental methodological differences: antiSMASH integrates multiple layers of information through ClusterCompare and KnownClusterBlast, including the presence of biosynthetic domains, NRPS/PKS module architecture, gene functions, sequence identity, and synteny to produce a composite similarity score. By contrast, USEARCH relies solely on direct protein-level alignments, reporting identities above 60% with coverage >50%. As a result, even when individual genes show strong similarity, the

aggregate score calculated by antiSMASH may remain low, potentially underestimating the functional presence of this biosynthetic gene cluster.

The third antifungal mechanism involved nutrient competition, such as bacillibactin (*dhb*), a siderophore responsible for iron acquisition (Dimopoulou et al., 2021; Manetsberger et al., 2025), was present in 71 species, although partial operon losses (e.g., *dhbB*, *dhbC* by USEARCH) were occasionally observed. Thirty-two species lacked the operon entirely, including *B. aquiflavi*, *B. arachidis*, and *B. coahuilensis*. Together, these results show that antifungal potential is unevenly distributed across the genus. Notably, *B. velezensis, B. siamensis, B. amyloliquefaciens, B. spizizenii*, *B. subtilis*, and *B. atrophaeus* exhibited the broadest repertoire of antifungal genes, encompassing all three major mechanisms.

However, the marked phylogenetic structure observed between species and their operational groups, together with the presence of well-defined antifungal repertoires within the different groups, contrasts with what described in the *B. amyloliquefaciens* and *B. subtilis* operational groups, suggesting that vertical inheritance plays a central role in the organization of these traits, while horizontal gene transfer likely contributes to lineage-specific expansions and the emergence of particular functional combinations (Chun et al., 2019; Neal et al., 2024).

The CAZy profiles (Figure S3, Table S14) are dominated by the glycoside hydrolase family GH18 (Monzingo et al., 1996), which shows two contrasting patterns: a highly conserved, non-secreted variant present in nearly all genomes (102/103; 99.0%) and a secreted GH18_SP variant detected in 57/103 genomes (55.3%) with strong secretion support (median = 0.974). The latter likely corresponds to extracellular chitinases targeting insoluble substrates such as fungal cell walls, hyphae, and spores, consistent with the associated genes shown in Figure 5. In this context, the presence of the AA10 family, encoding copper-dependent oxidative enzymes that disrupt crystalline chitin and enhance chitinase efficiency (Busk & Lange, 2015; Yao et al., 2023), was identified in 52/103 genomes (50.5%), also with strong secretion support (median = 0.972). In addition, the CBM50/LysM module, which binds N-acetylglucosamine-rich polymers and improves catalytic efficiency (Takashima et al., 2020), was detected in specific taxa (*B. litorisediminis* and *B. canaveralius*). This modular architecture mirrors experimentally validated systems, such as *B. velezensis* RB.IBE29, where secreted GH18, AA10, and CBM50/LysM domains contribute to chitin degradation and inhibition of fungal spore germination (Tran et al., 2022). Collectively, these results support that clades enriched in lytic families particularly their signal peptide bearing variants

constitute plausible candidates for biocontrol, and that such repertoires may vary even among genomes of the same species, reflecting environmental adaptation.

Additionally, the GH46 family (GH46_SP) was detected in 15/103 genomes (14.6%), with very strong secretion support (median = 0.975), consistent with activity against chitosanized fungal cell walls (Monzingo et al., 1996; Viens et al., 2015), while the β-glucanase/lichenase-type GH16_21_SP subfamily was present in 27/103 genomes (26.2%; median = 0.977), where it may weaken the glucan matrix and act synergistically with chitinolytic enzymes (Otsuka et al., 2022; Zalila-Kolsi et al., 2018). Notably, these patterns further support the conclusion that lineages enriched in secreted lytic CAZymes constitute priority candidates for biocontrol applications and targeted experimental validation.

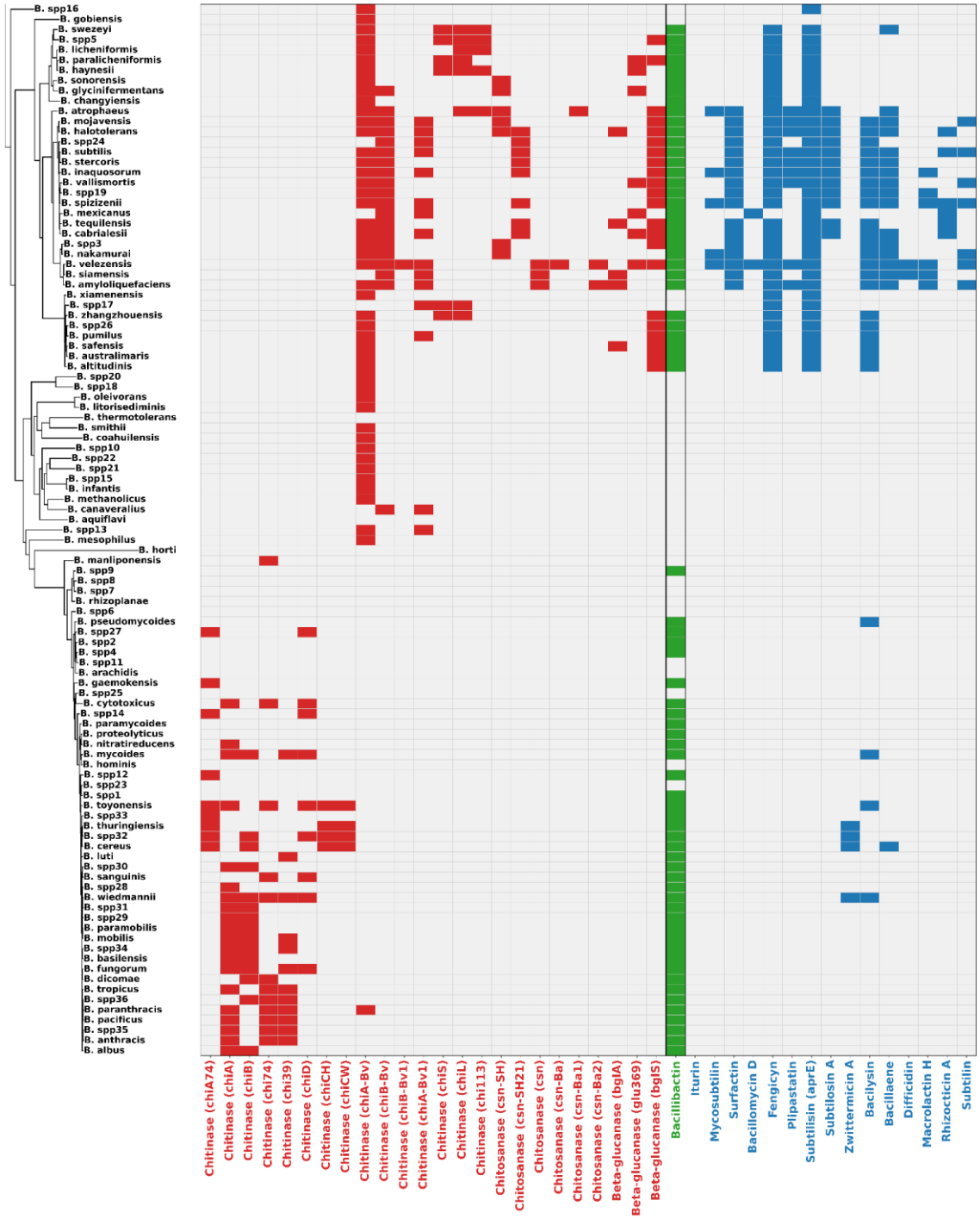

Figure 5. Presence–absence profiles of chitinases, chitosanases, glucanases, and antifungal peptide biosynthetic clusters across *Bacillus* species. Genes listed with locus identifiers (e.g., chitinase *chi74*, subtilisin *aprE*) were detected using USEARCH v11.0.667. Metabolite names without gene identifiers (e.g., bacillibactin, surfactin, fengicyn) correspond to biosynthetic clusters predicted with antiSMASH v8.0.2 (Table S10). Categories: Cell wall degradation (red), Competitive inhibition (green) and Growth inhibition (blue).

2.4.6. Antibiotic resistance landscape across *Bacillus*

Analysis of antibiotic resistance determinants across 10,276 genomes identified 135 unique genes (Figure 6, Table S15). Genes involved in antibiotic inactivation were of particular clinical relevance. Among these, TEM β-lactamase variants (e.g., *TEM-1, TEM-116, TEM-181, TEM-229*) stood out, as they are widely reported in enterobacteria such as *Enterobacter, Citrobacter, Klebsiella*, and *Proteus*, where they act as major drivers of β-lactam resistance (Naidoo et al., 2020; Oduro-Mensah et al., 2016; Salverda et al., 2010). In addition, intrinsic β-lactamases characteristic of *Bacillus* were detected, including *BcII*, *Bla1*, and *Bla2*, were detected and are known to confer basal resistance to penicillin and ampicillin (Zheng et al., 2024). Collectively, these genes provide resistance to penicillins, cephalosporins, and carbapenems, and, in the case of TEM variants, also to monobactams. They were identified in several species, including *B. safensis, B. thuringiensis, B. altitudinis, B. cereus*, and *B. pacificus*. Additional inactivation determinants included FosBx1 and FosB, linked to fosfomycin resistance, which were exclusive to the *B. cereus* sensu lato group (Kowalska et al., 2024; Roberts et al., 2013).

Genes associated with antibiotic target alteration were also observed, most notably the *vanA* and *vanB* operons and their regulatory components (*vanR*, *vanS*, *vanY*, *vanZ*), which mediate glycopeptide resistance by replacing the terminal D-Ala-D-Ala dipeptide with D-Ala-D-Lac, thereby reducing vancomycin binding affinity (Hill et al., 2010; Moosavian et al., 2018; McInnes et al., 2024). In our dataset, these operons were detected in species such as *B. albus, B. anthracis, B. mycoides, B. basilensis*, and *B. cereus*.

The antibiotic efflux mechanism was represented by tetracycline resistance genes, including *tet(M)*, *tet(L)*, *tet(45)*, *tetA(58)*, and *tetB(58)*, which were identified in members of the *B. cereus* group as well as in streptococci (Agersö et al., 2002; Hedayatianfard et al., 2014; X. Hu et al., 2022; Jeong & Lee, 2021). In the *B. amyloliquefaciens* group, *tet(L)* conferred only low-level resistance to tetracycline, as it was not associated with mobile genetic elements and is therefore considered an intrinsic determinant with very limited risk of horizontal transfer (Nøhr-Meldgaard et al., 2022). These genes were detected in multiple species, including *B. cereus, B. infantis, B. anthracis, B. cabrialesii, B. atrophaeus*, and *B. amyloliquefaciens*, highlighting the potential for widespread efflux-based resistance. Additional efflux-related determinants included *bcrA*, *bcrB* and *bcrC* belonging to Antibiotic target alteration mechanism, which form an ABC transporter system that provides self-protection against bacitracin by actively exporting the antibiotic. First characterized in *B. licheniformis*, a natural

bacitracin producer (Neumüller et al., 2001; Podlesek et al., 1995), this system was also present in *B. paralicheniformis, B. haynesii*, and *B. sonorensis* (all members of the *B. licheniformis* group). Furthermore, *ykkC* and *ykkD,* genes implicated in chloramphenicol resistance in *B. subtilis* (Jin et al., 2025), were identified in the *B. subtilis, B. amyloliquefaciens*, and B. *pumilus* groups.

In addition to efflux systems, genes associated with antibiotic target alteration were also observed. Notably, *vanZ_in_vanF_cl* genes were identified, conferring resistance to glycopeptides such as vancomycin (Hill et al., 2010; Moosavian et al., 2018).

Finally, antibiotic inactivation mechanisms were represented by the *BcII* β-lactamase, which provides resistance to penicillins and cephalosporins (Zheng et al., 2024). Two additional genes, *FosBx1* and *FosB,* confer resistance to fosfomycin (Kowalska et al., 2024; Roberts et al., 2013) and were found exclusively in the *B. cereus* sensu lato group.

2.4.7. Virulence gene repertoire and distribution within the genus *Bacillus*

Analysis of virulence-associated genes revealed 115 distinct loci distributed across the genus (Figure 7, Table S16). According to VFDB, these genes were classified into several functional categories, with exotoxins being the most prominent. Exotoxin genes were largely restricted to the *B. cereus* sensu lato group and included *alo*, which encodes a cholesterol-dependent cytolysin in *B. anthracis*. This toxin binds to host membranes, induces structural rearrangements, forms pores, and ultimately causes cytolysis (Mosser & Rest, 2006). Additional exotoxin genes included *hblA*, *hblC*, and *hblD*, which are related with hemolytic and cytotoxic activities (Ramm et al., 2021). Likewise, *nheA*, *nheB*, *nheC,* and *cytK*, encoding non-hemolytic enterotoxins and cytotoxins, were also detected. Together, these genes are associated with diarrheal and food poisoning caused by *B. cereus* (Lindbäck et al., 2004; Mostafa et al., 2024; Ramm et al., 2021). Exotoxin determinants were detected in multiple species, including *B. albus, B. anthracis, B. pacificus, B. paranthracis*, and *B. cereus*.

Exoenzymes were represented by *inhA* and its homolog *BAS_RS06430*, encoding zinc-dependent metalloproteases with cytotoxic and exocytic activity. These proteins promote bacterial multiplication, immune evasion, and persistence, contributing to gastrointestinal and non-gastrointestinal infections caused by *B. cereus* (Guillemet et al., 2010; Ramarao & Lereclus, 2005). These genes were consistently present in *B.*

cereus sensu lato species, including *B. cereus, B. albus, B. anthracis, B. sanguinis, B. paranthracis*, and *B. tropicus*.

The Effector Delivery System category was defined by the type VII secretion system (T7SS), which secretes small, signal peptide–independent proteins (~100 amino acids). Key components include *EssC, EsxB*, and *EsxL*. EssC encodes a membrane-associated ATPase required for substrate recognition and secretion, while *EsxB* and *EsxL* represent WXG100 family proteins typically secreted as heterodimers. In Bacillus and related Bacillota, T7SSs are primarily associated with interbacterial antagonism and niche competition, whereas in *Mycobacterium tuberculosis*, homologous systems contribute to immune evasion by suppressing CIITA/MHC-II expression and impairing macrophage activation (Bowran et al., 2023; Fan et al., 2015; Jäger et al., 2018; Sengupta et al., 2017). The T7SS was identified in species such as *B. paramycoides, B. nitratireducens, B. mycoides, B. hominis, B. wiedmannii*, and *B. basilensis*.

Finally, nutritional/metabolic virulence factors were represented by the *asb* operon (*asbA-F*), responsible for the biosynthesis of the siderophore petrobactin. Petrobactin contributes to iron acquisition, oxidative stress protection, and sporulation, thereby facilitating transmission in *B. anthracis* (Cendrowski et al., 2004; Hagan et al., 2018; Nusca et al., 2012). In addition to *B. anthracis*, we detected the operon in *B. toyonensis, B. thuringiensis, B. cereus, B. sanguinis, B. wiedmannii,* and *B. paramobilis*. The *ilsA* gene in *B. cereus* encodes a cell-surface protein containing NEAT (Near-Iron Transporter), LRR (Leucine-Rich Repeat), and SLH (Surface Layer Homology) domains, which facilitate iron acquisition under iron-limited conditions during host infection (Abi-Khalil et al., 2015; Koehler, 2009).

Collectively, these findings show that virulence-associated genes are unevenly distributed across *Bacillus*, with the majority concentrated in the *B. cereus* sensu lato group, where they likely underpin both pathogenic potential and ecological success.

Figure 6. Distribution of antibiotic resistance mechanisms across *Bacillus* species based on CARD database annotations. Six distinct resistance mechanisms were identified: antibiotic efflux (green, 27 genes), antibiotic inactivation (orange, 62 genes), antibiotic target alteration (blue, 28 genes), antibiotic target protection (purple, 13 genes), antibiotic target replacement (brown, 4 genes) and reduced permeability to antibiotic (red, 1 gene).

Figure 7. Functional categories of virulence factors identified across *Bacillus* species, based on VFDB annotations. A total of 13 categories were detected: Adherence (blue, 10 genes), Antimicrobial activity/Competitive advantage (dark orange, 1 gene), Effector delivery system (turquoise, 5 genes), Exoenzyme (violet, 2 genes), Exotoxin (red, 23 genes), Immune modulation (green, 34 genes), Invasion (light blue, 1 gene), Motility (lime green, 4 genes), Nutritional/Metabolic factor (coral, 23 genes), Others (fuchsia, 1 gene), Post-translational modification (brown, 1 gene), Regulation (lilac, 3 genes) and Stress survival (yellow, 7 genes). The Exotoxin and Exoenzyme categories were largely restricted to the *B. cereus* sensu lato group, reflecting its pathogenic potential.

2.4.8. Genetic basis of biofertilization potential in *Bacillus*

Genes associated with plant growth-promoting traits were analyzed using the PLaBAse database, with a focus on biofertilization. Figure 8 and Table S17 summarize the detected genes, which were classified into five major functional groups.

Iron acquisition was the most broadly represented category and included genes responsible for siderophore-mediated iron chelation, such as those encoding bacillibactin. These genes are critical not only for plant growth promotion but also for antifungal activity, as they deprive pathogens of essential iron (Dimopoulou et al., 2021; Ollinger et al., 2006). Genes in this category were found in nearly all *Bacillus* species analyzed, including *B. licheniformis, B. paralicheniformis, B. subtilis, B. spizizenii*, and *B. velezensis*. However, these genes were absent from genomes of several strains across different *Bacillus* species, including, including *B.* spp20*, B.* spp18*, B. oleivorans, B. litorisediminis, B. thermotolerans, B. smithi, B. coahuilensis, B. spp10, B.* spp22*, B.* spp21*, B.* spp15*, B. infantis, B. methanolicus, B. canaveralius, B. aquiflavi, B.* spp13*, B. mesophilus, B.* spp16*, B. gobiensis, B.* spp17*, B. xiamenensis*, and *B. horti*.

Nitrogen acquisition included key genes for ammonium uptake and regulation (*amtB*, *glnA*, *glnR*, *gltB*), nitrate and nitrite assimilation (*nasA*), urease activity (*ureA*, *ureB*), and associated regulators such as *trnA* (He et al., 2023; Nakano et al., 1995). This potential was observed in most *Bacillus* species, including *B. subtilis, B. stercoris, B. velezensis, B. siamensis*, and B*. spizizenii*, but was less frequent in the *B. cereus* sensu lato clade. Phosphate solubilization encompassed multiple phosphorus acquisition strategies. Acidolysis-related genes (*gdh*, *lpd*, *aceE*, *aceF*, *maeB*, *dlp*), genes encoding phosphatases and polyphosphate-related enzymes (*phoA*, *phoD*, *ppx*, *ppa*, *ppk*, *phnW*, *phnX*). In addition, detected transport and regulatory genes (*phoU*, *phoR*, *phoE pstS*, *pstC*, *pstA*, *pstB*, *phnG*, *phnH*, *phnI*, *phnJ*, *phnM*, *phnP*) are associated with the high-affinity phosphate-specific transport (Pst) and phosphonate (Phn) systems, which facilitate phosphate uptake, recycling, and homeostasis under phosphorus limitation (Pan & Cai, 2023; F. Pang et al., 2024). These genes were nearly universal across species, although phosphorus recycling determinants were generally less abundant.

Potassium solubilization was represented by *mdh* and *ackA*, both involved in potassium mobilization and detected in all species examined (Chen et al., 2022). Finally, sulfur assimilation included genes for sulfur uptake and reduction (e.g., *cysE*, *cysK*, *cysC*, *cysH*, *ssuB*, *ssuC*, *ssuA*, *ssuD*) (Albanesi et al., 2005; Ishikawa et al., 2025), which were also widely distributed across the genus. Overall, these results show that the genetic potential for biofertilization is highly conserved in *Bacillus*, with near-universal

representation of phosphate, potassium, and sulfur metabolism genes, while siderophore-mediated iron acquisition and nitrogen assimilation exhibited more variable distributions.

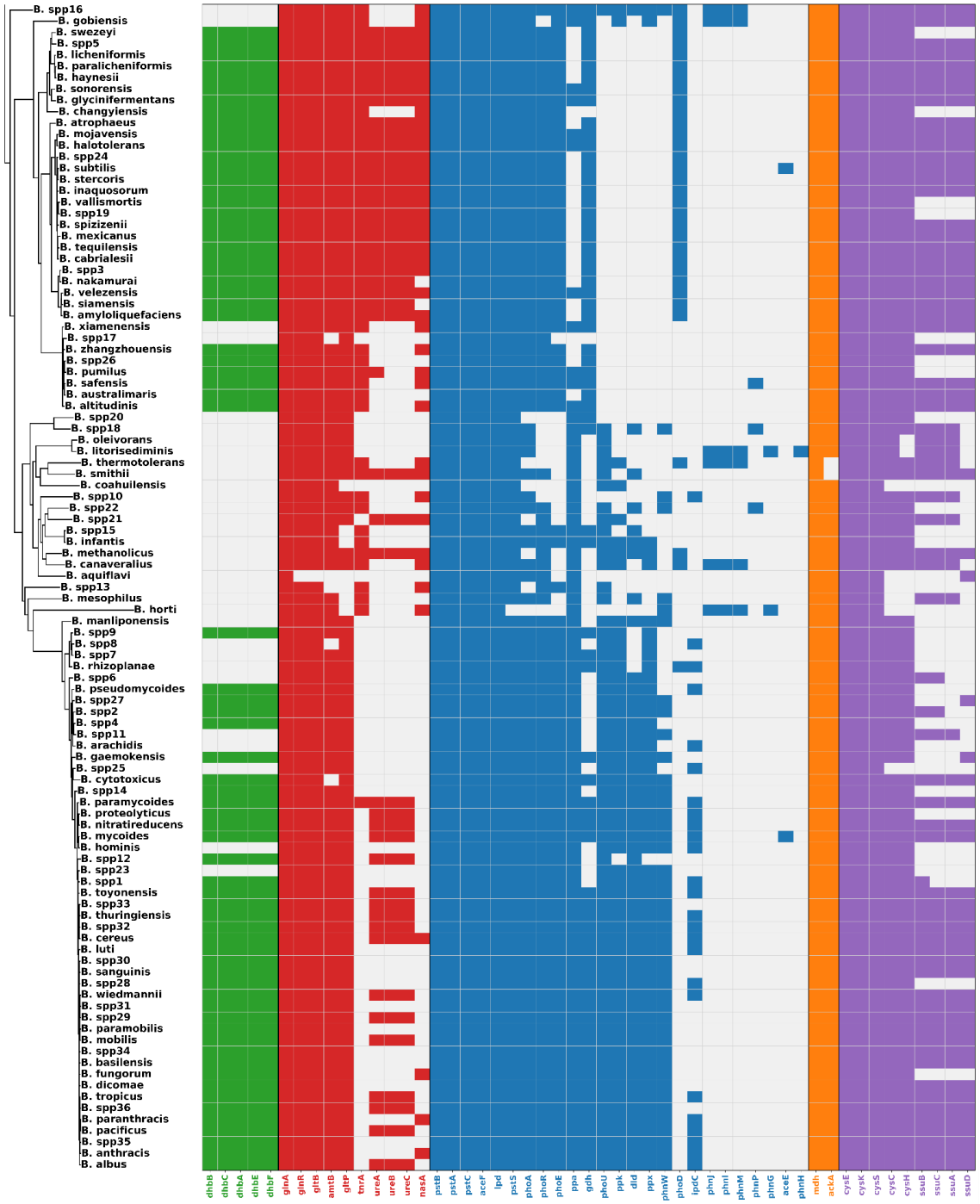

Figure 8. Representative genes associated with biofertilization functions in *Bacillus* species. Genes are grouped into five functional categories: iron acquisition (green, 5 genes), nitrogen acquisition (red, 10), phosphate solubilization (blue, 25 genes), potassium solubilization (orange, 2 genes), and sulfur assimilation (purple, 9 genes).

Collectively, these genes underscore the genetic capacity of *Bacillus* to promote plant growth by enhancing nutrient availability, particularly in the rhizosphere.

2.4.9. Promising *Bacillus* lineages for biofungicide development

Between the 103 communities identified, the most promising species were *B. velezensis, B. siamensis, B. amyloliquefaciens, B. spizizenii, B. subtilis*, and *B. atrophaeus*, which showed the highest antifungal potential. These species stand out not only for the presence of genes associated with the production of chitinases, chitosanase, glucanases, and secondary metabolites, but also for lacking virulence determinants in key categories such as exoenzymes, exotoxins, and the T7SS, features that reinforce their biotechnological safety profile.

Regarding antibiotic resistance, *tet(L)* genes were detected in members of the *B. amyloliquefaciens* group; however, their occurrence likely reflects intrinsic genomic features rather than horizontal transfer. A similar pattern may apply to other antibiotic resistance genes, underscoring the need for in vivo studies to evaluate their expression and functional relevance in other agronomically important *Bacillus* lineages, such as *B. pumilus* (Fu et al., 2021)*, B. subtilis*, and *B. licheniformis* (Caulier et al., 2019).

Notably, these promising species have already been experimentally demonstrated to be effective against a wide range of plant pathogens. *B. velezensis* exhibits a broad antifungal spectrum, acting against multiple phytopathogenic genera such as *Aspergillus, Penicillium, Talaromyces, Cryptococcus, Cryphonectria, Helicobasidium*, and *Cylindrocladium*. In addition, lipopeptide compounds produced by *B. velezensis*—for example, bacillomycin D—have shown potent activity against the anthracnose pathogen *C. gloeosporioides*, surpassing the efficacy of conventional chemical fungicides (Devi et al., 2019; Jin et al., 2020; Xu et al., 2016). Similarly, *B. siamensis* has also demonstrated remarkable antifungal efficacy. A strain isolated from the wheat rhizosphere (*B. siamensis* Sh420) produced lipopeptides that strongly inhibited *Fusarium graminearum*, the causative agent of wheat head blight (Hussain et al., 2024). Another isolate (*B. siamensis* AMU03) was effective against *Macrophomina phaseolina*, which causes wilting in various crops, exhibiting antifungal activity through disruption of the fungal cell membrane (Hussain et al., 2024; Hussain & Khan, 2020).

As for *B. amyloliquefaciens* (e.g., strain SQR9), it produces a wide range of antifungal compounds capable of suppressing various soil-borne pathogens. Extracts from *B. amyloliquefaciens* SQR9 inhibited the growth of *Verticillium dahliae, F.*

*oxysporum, F. solan*i, and *Phytophthora parasitica*. These findings indicate that *B. amyloliquefaciens* exerts its biocontrol activity through the production of lipopeptides (iturins, fengicins, surfactins) and siderophores, which interfere with spore germination and compromise the cell integrity of pathogenic fungi (Li et al., 2014).

In addition, *B. spizizenii* (syn: *B. subtilis* subsp. *spizizenii*) has demonstrated significant antifungal effects. For instance, strain BL-59 (identified as *B. subtilis* subsp. *spizizenii*) markedly inhibited mycelial growth of *Colletotrichum sp*. and *Pestalotiopsis sp*. isolated from infected fruits, with reductions of approximately 49.31% and 42.55%, respectively (Duangkaew & Monkhung, 2021). Similarly, *B. subtilis* subsp. *spizizenii* strain MM19 reduced the mycelial growth of *Alternaria alternata,* the causal agent of leaf blight in marigolds, by up to 83% under laboratory conditions (Priyanka et al., 2018). Together, these findings underscore the potential of *B. spizizenii* as a biocontrol agent against phytopathogenic fungi.

B. subtilis is one of the most extensively studied species for biocontrol applications, exhibiting broad-spectrum inhibition against a wide range of phytopathogens (Akinsemolu et al., 2024). It has shown activity against *Alternaria solani, F. oxysporum, Rhizoctonia solani*, and *Clarireedia jacksonii*, the causal agent of dollar spot disease (Chen et al., 2023; Kaur et al., 2023; Ramesh et al., 2024; Tuyen et al., 2023). This antagonistic activity is largely attributed to the production of diverse antifungal metabolites, including lipopeptides and hydrolytic enzymes, that act synergistically to inhibit the growth and development of multiple plant pathogens. Finally, *B. atrophaeus* stands out for its broad antifungal spectrum and stability. Recent studies have shown that *B. atrophaeus* isolates associated with wild apple trees exert strong antagonism against canker-causing fungi, including *Cytospora mali* and *Cryphonectria parasitica* (Bozorov et al., 2024). Moreover, Pisheh et al. (2024) identified iturine A from *B. atrophaeus* strain HNSQJYH170, which displayed potent fungicidal activity against multiple pathogenic fungi, inhibiting more than 80% of the growth of *F. oxysporum, Aspergillus niger, Penicillium chrysogenum,* and *Mucor hiemalis*. Overall, *B. atrophaeus* exhibits a remarkably broad antifungal profile, effective against both soil-borne and and woody-tissue pathogens. These findings highlight the importance of prioritizing certain *Bacillus* species and strains for the development of next-generation biofungicides. In particular, *B. spizizenii* and *B. atrophaeus* emerge as promising new candidates, as species such as *B. velezensis, B. siamensis, B. amyloliquefaciens,* and *B. subtilis* are already established in agricultural applications. The combination of a broad antifungal spectrum, multiple modes of action (including lipopeptides, enzymes, and volatile organic

compounds), and genetically safe profiles makes these strains ideal reference models for future biotechnological applications. In contrast, members of the *B. cereus* sensu lato clade require careful consideration before being proposed for agricultural applications. This group includes several opportunistic or pathogenic species capable of producing exoenzyme, exotoxin toxins and other virulence determinants that may pose health risk (Bhandari et al., 2013; Liu et al., 2020). Consequently, despite the presence of genes associated with antifungal activity, strains belonging to this clade should undergo rigorous biosafety evaluation before being considered for use as biofertilizers or biocontrol agents.

2.5. CONCLUSIONS

In conclusion, the integrative framework combining genomic distance metrics (Mash/ANI), network analyses, and label propagation provided a robust strategy for delimiting 103 communities within the genus *Bacillus*, enabling the identification of taxonomically coherent clusters as well as potentially undescribed lineages. Pangenome analysis revealed contrasting patterns of openness and genomic fluidity, with a significant inverse relationship ($\varphi = 0.4499 - 0.4242\alpha$). This trend reflects lifestyle-driven adaptive strategies: generalist and opportunistic species maintain open pangenomes with high gene content variability, whereas more specialized taxa exhibit closed pangenomes, reducing genomic flexibility while enhancing niche adaptation. From an applied perspective, groups such as *B. amyloliquefaciens*, *B. pumilus, B. subtilis*, and *B. licheniformis,* harbour extensive repertoires of hydrolytic enzymes, NRPS/PKS clusters, and other secondary metabolite biosynthetic clusters, positioning them as priority candidates for biofertilizer and biocontrol development. Conversely, the coexistence of resistance determinants and virulence factors within the *B. cereus* sensu lato complex underscores the necessity for stringent biosafety assessments and risk evaluation before considering its application in agricultural settings.

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2.8. SUPLEMENTARY FILE

Arquivos complementares:
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3. CAPÍTULO 3. PERSPECTIVAS FUTURAS

O presente estudo constitui a análise genômica mais ampla e sistemática do gênero *Bacillus* realizada até o momento, integrando 10.276 genomas e permitindo a delimitação de 103 comunidades operacionais coerentes. A abordagem metodológica, baseada em métricas de distância genômica, análise de redes e algoritmos de propagação de rótulos, possibilitou enfrentar de forma eficaz uma lacuna crítica do conhecimento: a limitada resolução da taxonomia tradicional fundamentada no gene do rRNA 16S, particularmente evidente na discriminação de espécies filogeneticamente próximas, como aquelas pertencentes ao complexo *B. cereus* sensu lato. Nesse sentido, os resultados obtidos contribuem para uma reorganização mais precisa do gênero, alinhada aos padrões atuais da taxonomia genômica.

Sob uma perspectiva funcional, a mineração genômica revelou um expressivo potencial antifúngico e biofertilizante em espécies como *B. velezensis*, *B. siamensis*, *B. amyloliquefaciens*, *B. spizizenii*, *B. subtilis* e *B. atrophaeus*, caracterizadas por repertórios enriquecidos de enzimas hidrolíticas, clusters biossintéticos de metabólitos secundários (NRPs/PKS) e genes associados à promoção do crescimento vegetal. No entanto, uma linha prioritária de investigação futura consiste na validação experimental desses determinantes genéticos por meio de estratégias de expressão heteróloga, ensaios bioquímicos e bioensaios frente a fungos fitopatogênicos de relevância agrícola. Paralelamente, estudos de regulação e modulação genética poderão contribuir para a otimização da síntese de metabólitos antifúngicos com potencial aplicação biotecnológica.

Por outro lado, a detecção de genes de resistência a antibióticos e de fatores de virulência concentrados principalmente no grupo *B. cereus* sensu lato ressalta a importância da incorporação de avaliações rigorosas de biossegurança em qualquer iniciativa de transferência tecnológica ou aplicação agrícola. Nesse contexto, investigações futuras deverão integrar abordagens multi-ômicas, incluindo proteômica, metabolômica e análises de redes regulatórias, com o objetivo de elucidar os mecanismos moleculares subjacentes à adaptação ecológica, à patogenicidade e às interações com outros microrganismos.

Por fim, a validação do potencial biofertilizante e de biocontrole previsto requer a realização de ensaios em sistemas controlados *in vitro* e estudos em campo *in vivo* (planta), que permitam avaliar o impacto real sobre o crescimento vegetal, a supressão de patógenos e a estabilidade ecológica. Em conjunto, essas perspectivas consolidam as bases para o desenvolvimento racional de bioinsumos agrícolas seguros, eficazes e sustentáveis, posicionando o gênero *Bacillus* como um componente-chave das futuras estratégias biotecnológicas voltadas para uma agricultura mais resiliente e ambientalmente responsável.

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